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The Role of β-Lactamase in Low-Level Cephalosporin-Resistant *Serratia marcescens*

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The Role of β-Lactamase in Low-Level Cephalosporin-Resistant *Serratia marcescens*

by

Celine M. Herra

A thesis submitted for the award of Doctor of Philosophy,
in the Department of Clinical Microbiology,
Faculty of Health Sciences,
University of Dublin, Trinity College.

January, 2002

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Detail from Raphael's 'The Miracle of the Mass at Bolsena'. Fresco, c.1512-13, Stanza d'Eliodoro, Vatican Museum, Rome. From: Raphael, Roger Jones and Nicholas Penny, Yale University Press, New Haven and London, 1983.

Summary

In enterobacteria, high-level cephalosporin resistance is commonly mediated by constitutive over-production or derepression of chromosomal AmpC β-lactamase. This derepression is frequently associated with mutations in the *ampD* regulatory gene but altered AmpC expression may also result from mutation in the *ampR* regulatory gene or in the structural *ampC* gene.

Serratia marcescens has emerged in Dublin hospitals as a serious nosocomial pathogen expressing low-level resistance to cephalosporins. The present study was undertaken to confirm that this resistance was mediated by AmpC β-lactamase and to explore the mechanism of low-level AmpC expression in *S. marcescens* by investigation of the putative AmpC regulatory genes.

During the study period, the increasing prevalence of *S. marcescens* in Dublin hospitals was compounded by an increase in the rate of cephalosporin resistance, rising from 45% of isolates in 1995 to over 60% in 1998. Microbroth dilution and disc induction assays indicated that this resistance was mediated by over-production of AmpC β-lactamase. Susceptibility test methods demonstrated that more than 90% of 'cephalosporin-resistant' isolates expressed low-level resistance to some β-lactam agents (cefotaxime MIC 8–16 mg/l) and were susceptible to other β-lactams. In the remaining 'cephalosporin-resistant' isolates the level of resistance to some β-lactams was increased (cefotaxime MIC~32 mg/l) but susceptibility to other agents such as ceftazidime was only marginally reduced.

B-lactam hydrolysis studies demonstrated that isolates with low-level β-lactam resistance expressed correspondingly low levels of AmpC over-production and were associated with a partially derepressed phenotype. In isolates with increased levels of β-lactam resistance, true AmpC derepression was indicated but the level of β-lactamase over-production was reduced compared to other derepressed enterobacteria. Low-level AmpC-mediated cephalosporin resistance in *S. marcescens* appeared to reflect a reduction in the level of AmpC over-production.

The possible contribution of plasmid-encoded β-lactamases was excluded using the disc synergy test and the cefpodoxime combination disc test. Genotypic resistance detection

methods including plasmid DNA analysis and TEM- and SHV-specific DNA amplification studies were also used to confirm the absence of ESBL-mediated resistance.

The putative *ampD* and *ampR* regulatory genes and the structural *ampC* gene (including the *ampC-ampR* intergenic region) in *S. marcescens* were investigated using DNA hybridisation assays and DNA amplification studies. The *ampD* gene was identified on an open reading frame (ORF) of 564 bp. The predicted 187 amino acid AmpD protein shared significant identity (>90%) with AmpD in other enterobacteria, suggesting a common role in AmpC expression. The partial AmpR sequence displayed considerable diversity (>50%) compared to AmpR in common AmpC-inducible enterobacteria. The *ampC* gene was present on a 1136 bp ORF and the deduced 355 amino acid sequence contained the three signature motifs of a class C β-lactamase. The *ampC-ampR* intergenic region also demonstrated overlapping *ampC-ampR* promoter regions typical of divergent transcription in AmpC-inducible enterobacteria but the DNA sequence of the intergenic region was significantly different compared to the sequence in other enterobacteria.

Sequence analysis indicated that the genetic alterations leading to partially-derepressed or derepressed AmpC phenotypes in the *S. marcescens* population were not linked to mutations in *ampD, ampR* or *ampC.* Transformation studies with plasmids encoding wild-type *ampD* also suggested that derepression in *S. marcescens* isolates was independent of the *ampD* locus. The sequence diversity of the *ampC-ampR* genetic assembly in *S. marcescens* and the absence of *ampD, ampR* or *ampC* mutations in isolates with low-level cephalosporin resistance may indicate a novel mechanism for AmpC β-lactamase regulation.

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Acknowledgements

I wish to express my gratitude to my supervisor, Professor Conor Keane for giving me the opportunity to undertake this project and for his unflagging support and guidance throughout the six years of the study. I also wish to thank to Mr. Liam English, who has assisted and encouraged me every step of the way. I am forever indebted to my 'oracle', Dr. Angela Rossney for her tireless advice on all laboratory aspects of this study and for her invaluable assistance in the preparation of this manuscript.

I would also like to thank Professor David Coleman and Dr. Derek Sullivan from the School of Dental Science, University of Dublin, Trinity College, for introducing me to the world of molecular biology. I am especially grateful to Dr. Nancy Hanson, Director of Molecular Biology for the Center for Research in Anti-Infectives and Biotechnology (CRAB) and Assistant Professor in the Department of Medical Microbiology and Immunology at Creighton University School of Medicine, Nebraska, for giving so freely of her time and knowledge. Nancy's contribution as both scientist and friend has been invaluable in the completion of this study. I also wish to thank the β-lactamase research team in CRAB at Creighton University School of Medicine for giving me the opportunity to visit, to engage in collaborative work and to avail of their expertise. A special word of thanks goes to Dr. Steve Mahlen in the Molecular Biology Laboratory in CRAB at Creighton University School of Medicine for assistance in performing β-lactamase hydrolysis studies.

The continued support of my colleagues in the Department of Microbiology, St James's Hospital is truly appreciated. Special thanks are due to Anne Walsh, Brenda Moloney, Sarah Lalor, Mary Kelleher and Noel Gibbons for their endless concern and encouragement. I am also indebted to my fellow workers in the Department of Clinical Microbiology, University of Dublin, Trinity College, especially Dr. Frederick Falkiner, Dr. Niamh O'Sullivan and Dr. Quanjiang Dong, for their assistance and helpful discussion. I would also like to pay special tribute to Dr. Alison Byrne who worked on the clinical aspects *S. marcescens* infection. Sadly, Alison died suddenly in July, 2000.

Thanks also go to my colleagues in the Sir Patrick Dun (SPD) Research Laboratory for sharing their expertise and for understanding the 'highs and lows' of the world of

research. Most especially, I wish to express my thanks to Dr. Frances Hogan who has been a source of great inspiration throughout my time in the SPD Research Laboratory.

A special word of thanks is also due to my neighbours Alan and Michelle Carr for their 'around the clock' IT consultancy and counselling service. And finally my sincere thanks to my family and friends who have stuck with me from Leaving Certificate to Diploma through Masters and now to Doctorate- I promise this is definitely the last one!

Abbreviations

A:	adenine
AAC:	aminoglycoside acetyltransferase
ANT:	aminoglycoside adenyltransferase
AP:	alkaline phosphatase
API:	Analytical Profile Index
ATCC:	American Type Culture Collection
bp:	base pair (s)
C:	cytosine
CBA:	Columbia blood agar
cfu:	colony forming units
CRAB:	Center for Research in Anti-Infectives and Biotechnology (Creighton University)
CSPD®:	25mM disodium3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
DAT:	disc approximation test
ddNTPs:	dideoxyribonucleotide triphosphates
DIG:	digoxigenin
DNA:	deoxyribonucleic acid
DNase:	deoxyribonuclease
dNTPs:	deoxyribonucleotide triphosphates
dpi:	dots per inch
DST:	disc synergy test
EBI:	European Bioinformatics Institute
EDTA:	ethylene diamine tetraacetic acid, disodium salt
EMBL:	European Molecular Biology Laboratory
ERIC:	enterobacterial repetitive intergenic consensus
ESBLs:	extended-spectrum β-lactamases
G:	guanine
GlcNAc:	N-acetylglucosamine
GNB:	Gram-negative bacilli
ICU:	intensive care unit
IEF:	isoelectric focusing
IS:	insertion sequence

Definitions

Prevalence of S. marcescens

The total number of patients carrying *S. marcescens* at a particular period of time.

Wild-type isolate

A bacterial isolate which carries the antimicrobial susceptibility genotype characteristic of its species without evidence of acquisition of or mutation to a resistant phenotype.

Induction

A temporary phenotypic response to an environmental change where the synthesis of β-lactamase enzyme is transiently increased in direct response to induction by a substrate (inducer) e.g. β-lactam antibiotics.

Derepression/Constitutive β-lactamase over-production

A permanent cellular feature resulting from stable genetic change where highly elevated amounts of β-lactamase enzyme are produced constitutively, independent of the presence or absence of inducers.

Partial derepression

A permanent cellular feature resulting from stable genetic change where the level of uninduced β-lactamase production is considerably higher than β-lactamase production in wild-type isolates but where notable inducibility is retained.

Hyper-inducible

A permanent cellular feature resulting from stable genetic change where a marginal increase in the level of β-lactamase production occurs in the absence of inducers but where induction occurs at a lower concentration of inducer compared to wild-type cells.

Basal-production

Low-level constitutive β-lactamase production.

Similarity

Percentage similarity among DNA base pair sequences.

Chapter I

Serratia marcescens: saprophyte to pathogen

Chapter I

Serratia marcescens: saprophyte to pathogen

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1.1. FROM SACRED HOST TO HUMAN HOST

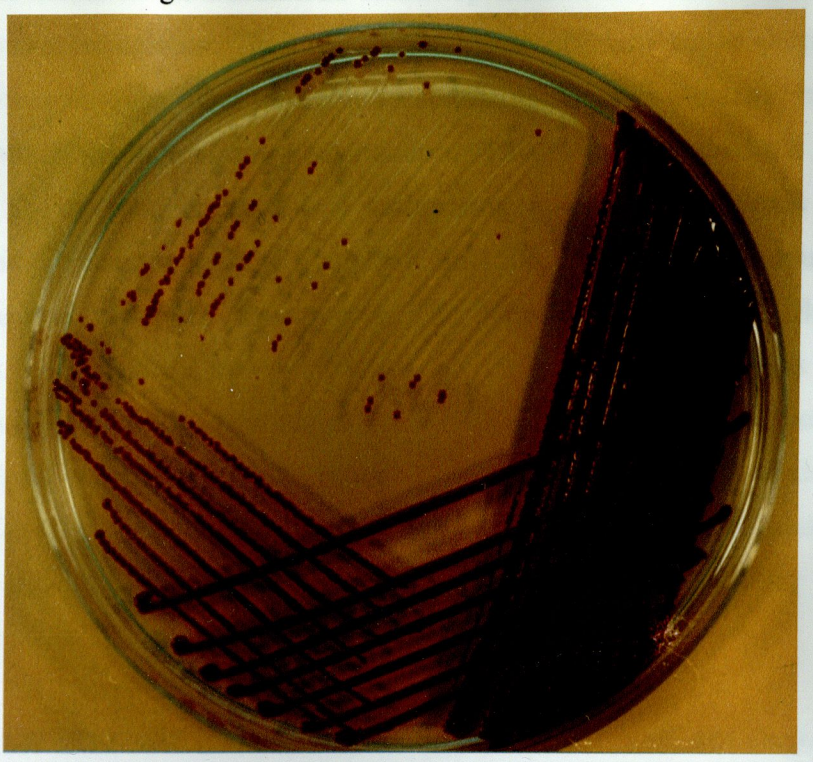
Once considered a harmless saprophyte, *Serratia marcescens* is a now recognised as an important nosocomial pathogen. This organism is credited with a long fanatical history dating back to antiquity, when, because of its production of a red pigment it was considered to have masqueraded as blood (Gaughran, 1969). Early in this century, this distinctive red pigmentation, combined with an apparent low level of virulence, led to the use of *S. marcescens* as a biological marker of infection. From 1960 onwards non-pigmented *S. marcescens* isolates became increasingly associated with human infection. Since then this organism has been implicated as the etiological agent in every type of human infection, especially in the immunocompromised host. In the last two decades *S. marcescens* has emerged as a serious cause of nosocomial infection and a frequent source of outbreaks of hospital infection.

1.1.1. Masquerader of blood

In early Greek and Roman history, an organism producing a red pigment (subsequently considered to be *S. marcescens*) gained notoriety from its predilection for growth on starchy foodstuffs and its ability to produce red pigmented colonies which were easily mistaken for drops of blood (Figure 1.1). The remarkable appearance of these 'bloody' episodes was viewed as manifestations of divine destiny. As early as the sixth century B.C., Pythagoras noted the appearance of a bloody coloration on foodstuffs (Gaughran, 1969). In 332 B.C., the first account of the appearance of blood on foodstuffs was recorded at the siege of Tyre in Phoenicia (modern Lebanon). Classical historians record how the sight of drops of blood trickling from soldiers' bread rallied the dispirited Macedonian army of Alexander the Great, to glorious victory (Curtius, 1962).

Throughout the Middle Ages the miraculous appearance of blood-like spots on bread coincided with the development of church doctrine regarding the Holy Sacrament and had a far more sinister effect (Gaughran, 1969). The starchy Eucharistic bread incubated in the damp environment of medieval churches provided an ideal substrate for the growth of *S. marcescens*. Between 1300 A.D. and 1500 A.D., there were many accounts of the appearance of blood on Eucharistic wafers. However, since consecration of the Host symbolises its conversion into the Body of Christ, the miraculous

Panel A. Pigmented *S. marcescens*



Panel B. Non-pigmented *S. marcescens*



Figure 1.1. Pigmented (panel A) and non-pigmented (panel B) isolates of *S. marcescens* after 24 h growth on Serratia ATCC medium 181 (Atlas & Parks, 1993).

appearance of blood on the Eucharistic bread represented a dramatic testament to the doctrine of transubstantiation. In 1264, historians record how blood dropped from a Eucharistic Host onto the robes of a priest celebrating Mass in Bolsena, in Italy, who allegedly doubted the truth of transubstantiation. This event prompted the Pope of the day, Urban IV, to issue a papal bull authorising a feast day, Corpus Christi, to commemorate the institution of the Eucharist. In 1508, Pope Julius II commissioned a fresco by Raphael, to further commemorate 'The Miracle of the Mass at Bolsena' (see frontispiece). Unfortunately, many of these supposedly miraculous episodes often led to the persecution of Jews who were accused of stabbing the Eucharistic Host. In many cases the religious fanaticism and intolerance of the day led to widespread slaughter and burning of thousands of people. As a result, early scientists concluded that this saprophytic organism may have accounted for more deaths than many pathogenic bacteria (Scheurlen, 1896).

It was not until 1823 that the source of this miraculous blood was attributed to the growth of a micro-organism. Bartolemeo Bizio, a young Italian pharmacist, demonstrated that the 'blood' was caused by a living organism. He named this organism *Serratia marcescens*. Serratia was selected to pay tribute to an Italian physicist, Serafino Serrati who, at that time in Italy was regarded as the inventor of the steamboat. Marcescens is derived from the Latin word 'to decay' because Bizio observed that the pigment deteriorated quickly. In 1848, Ehrenberg renamed the organism *Monas prodigiosus*, or the 'miracle bacterium'. The term 'prodigiosa' was subsequently used in many of the suggested name changes for the organism e.g. *Micrococcus prodigiosus*, *Bacillus prodigiosus*, *Bacterium prodigiosum* and *Erythrobacilllus prodigiosus*. By the 1920's, revision of bacterial taxonomy led to the adoption of the original name, *S. marcescens* (Gaughran, 1969).

The first clinical recognition of *S. marcescens* was also a result of its pigment production. In 1913, the production of 'blood-tinged' sputum was described in a patient suffering from bronchiectasis. Microscopic examination of the patient's sputum showed large numbers of Gram-negative bacteria, which were identified as *S. marcescens* (Dodson, 1968). Similar cases of brochiectasis were subsequently reported. In 1958, the occurrence of 'red diaper syndrome' was reported in an infant with a suspected inborn error of metabolism. When the child's stool was cultured, *S. marcescens* was

isolated in abundance. An exhaustive survey of the attending hospital personnel and of the child's family failed to identify a carrier of the organism. However, it was subsequently discovered that an identical pigmented strain of *S. marcescens* was being used in a nearby biochemistry laboratory in a study of aerosol techniques. The infant remained asymptomatic, but despite therapy, his diapers continued to show red colouration for almost a year (Farmer *et al.*, 1977).

1.1.2. **Biological marker of infection**

In the 20th century, the apparent benign nature of *S. marcescens* and its distinctive red pigment resulted in this organism being considered a suitable candidate for use as a biological indicator. As a biological marker, *S. marcescens* was used in a number of classic experiments which led to improved understanding of the pathogenesis of infection.

In a study in 1906 commissioned to investigate the atmospheric hygiene of the British House of Commons, the investigator recited passages from Shakespeare to an 'audience' of agar plates, after gargling with a liquid culture of *S. marcescens*. In 1920, the throats, mouths and lips of American soldiers were sprayed with a suspension of *S. marcescens* to test the theory that respiratory-tract infections could be transmitted by hand. In studies to investigate the likelihood of bacteraemia following dental extraction, *S. marcescens* was painted on the gum of the tooth to be extracted, before screening for the presence of the organism in subsequent blood cultures (Yu, 1979). The entry of organisms into urinary tracts via indwelling catheters was documented by applying *S. marcescens* to the periurethral epithelium of patients with bladder catheters (Yu, 1979). In 1961, *S. marcescens* was applied to the mouth and pharynx of patients with respiratory-tract infections as a marker of contamination of sputum by mouth flora. In the 1970s, microbiology students in laboratory practicals were instructed to rub their hands with suspensions of the organism to demonstrate bacterial spread by hand shaking. Live suspensions of *S. marcescens* were even sprayed into hospital wards to facilitate study of bacterial dispersal (Yu, 1979).

The most controversial use of *S. marcescens* as a biological marker of infection occurred in the early 1950s when the United States (US) Army used the organism in studies

investigating vulnerability to germ warfare. In these experiments, *S. marcescens* was released from US ships into the ocean, where the organism became aerosolized by ocean waves and was blown inland to San Francisco. In 1976 it emerged that a hospital in San Francisco had experienced an outbreak of *S. marcescens* infections coinciding with the aerosolization experiments. As a result of this disclosure, public hearings were conducted by the US Senate Subcommittee on Health and Scientific Research, where the production of biological warfare agents was condemned. Investigations by the US Centre for Disease Control in Atlanta, Georgia, have subsequently questioned the infectious nature of the aerosolization experiments. In a study documenting 100 nosocomial outbreaks caused by *S. marcescens* in US hospitals, none were caused by strains of *Serratia* spp. with the same serotype or biotype as those used by the US Army (Farmer *et al.*, 1977). Nevertheless, by the time this came to light, the dye had been cast, public opinion was formed and once again, *S. marcescens* had left another mark on history.

1.2. NOSOCOMIAL PATHOGEN

1.2.1. Human infection with non-pigmented isolates

From 1960 onwards, non-pigmented isolates of *S. marcescens* predominated over pigmented strains in the clinical setting and were increasingly implicated in hospital-acquired infection (Elston & Magnuson, 1965; Dodson, 1968). Over time, as hospitals adapted to the needs of growing numbers of 'compromised' patients, non-pigmented *S. marcescens* became established as an important nosocomial pathogen. Today the organism is implicated in a wide range of serious infections including pneumonia and lower respiratory tract infection (Meltz & Grieco, 1973; Balikian *et al.*, 1980), urinary tract infection (Maki *et al.*, 1973), septicaemia, (Crowder *et al.*, 1971; Manfredi *et al.*, 2000), wound infection (Lewis *et al.*, 1989), meningitis (Lewis *et al.*, 1989) and bacterial endocarditis (Williams & Johnson, 1970).

Patients most at risk include those with debilitating or immuno-compromising disorders, those treated with broad-spectrum antibiotics and patients in intensive care units (ICU) who are subjected to invasive instrumentation. In accordance with its opportunistic nature, recent reports indicate that *S. marcescens* is emerging as a serious pathogen in HIV-infected patients (Manfredi *et al.*, 2000). Common infections include respiratory tract infection in intubated patients, urinary tract infection in patients with indwelling catheters and septicaemia in post-surgical patients, especially in those with intravenous catheters. Like all opportunistic pathogens, the pathological manifestations and eventual outcome of infection with *S. marcescens* is largely dependent on the clinical status of the host. The hospital setting and particularly ICUs, provide frequent opportunities for transmission of the organism. The prevalence of certain serotypes in individual hospitals, over specific time periods, suggests that infection with *S. marcescens* probably results from cross-infection.

It is currently estimated that in Europe, *S. marcescens* accounts for approximately 3.5% of nosocomial pneumonia, 1.5% of bacteraemia, 1.5% of skin and soft tissue infection and 1.0% of urinary tract infection, with an overall ranking of tenth in the list of genera most likely to cause nosocomial infection (Fluit *et al.*, 2001). Similar figures have been reported in the US, where *S. marcescens* accounts for 4.6% of nosocomial respiratory

infection, 2% of blood-stream infections, 2.4% of skin and soft tissue infections and 1.9% of urinary tract infection (Jones *et al.*, 1997; Gales *et al.*, 2000). Current reports from the Communicable Disease Surveillance Centre (CDSC) in England and Wales also indicate that *S. marcescens* is responsible for 1.0% of reported bacteraemia and bacterial meningitis, with a ranking of ninth among Gram-negative bacteria (CDSC, 2001a). In an extended review of the *Enterobacteriaceae* most frequently associated with nosocomial infection (*Klebsiella* spp., *Enterobacter* spp., *Serratia* spp. and *Citrobacter* spp.), which covered reports from the CDSC in the calendar years 1999 and 2000, *Serratia* spp. were also reported to account for 10% of bacteraemia (CDSC, 2001b). Between 1990 and 1998, the prevalence of *S. marcescens*-associated bacteraemia also showed a significant upward trend, with a reported annual increase of 15% (Reacher *et al.*, 2000). Furthermore, in 2001 a report detailing a strategy for the control of antimicrobial resistance in Ireland highlighted *S. marcescens* as a significant nosocomial pathogen (SARI, 2001).

1.2.2. **Nosocomial outbreaks and transmission of infection**

By the early 1970's, *S. marcescens* was well established as a cause of outbreaks of hospital infection in the US. It was another ten years before similar outbreaks were reported in England (Black & Hodgson, 1971; Brooks *et al.*, 1979) and Ireland (Coleman *et al.*, 1984). Since then *S. marcescens* has been implicated with increasing frequency in serious nosocomial outbreaks of infection. In many cases it is responsible for repeated clusters of cross-infection and outbreaks within the same hospital (Traub, 2000).

Outbreaks of *S. marcescens* have been described in a variety of clinical settings but particularly in neonatal units (Campbell *et al.*, 1998; Jones *et al.*, 2000) and ICUs (Herra *et al.*, 1998; O'Connell & Humphreys, 2000). Many different environmental sources have been identified, such as antiseptics (Bosi *et al.*, 1996; Vigeant *et al.*, 1998), liquid soaps (Sartor *et al.*, 2000), water from nebulizers (Ringrose *et al.*, 1968; Chaudhuri & Booth, 1992), intravenous solutions and even hand lotions. Medical equipment such as respirators, bronchoscopes (Peltroche-Llacsahuanga *et al.*, 1999), intravenous catheters, blood-pressure monitors (Rogers & Gittens, 1974) and electrocardiogram bulbs (Sokalski *et al.*, 1992) have also been implicated in the dispersal of *S. marcescens*. The

organism has been isolated from the sinks and floors of rooms occupied by infected or colonised patients and even from dust particles within these rooms (Peltroche-Llacsahuanga *et al.*, 1999). *Serratia marcescens* has also been incriminated in outbreaks of transfusion-associated septicaemia. In 1991, a number of episodes of transfusion-transmitted *S. marcescens* septicaemia occurred in Denmark and Sweden, involving six patients and one fatality (Gong *et al.*, 1993). Subsequent investigations revealed the interior of the blood bags were contaminated from the outer transport packaging (Szewzyk *et al.*, 1993).

The ability of *S. marcescens* to cause nosocomial outbreaks of infection has been attributed to a number of factors, the most important being its ability to survive in the hospital environment and to exhibit resistance to multiple antimicrobial agents. Outbreaks involving *S. marcescens* are thus frequently characterised by epidemic spread and resistance to many antibiotics. (Knowles *et al.*, 2000; Pagani *et al.*, 1994). One of the most dramatic outbreaks involving *S. marcescens* occurred in Nashville in 1973, where an epidemic strain resistant to all commercially-available antibiotics, infected 210 patients at four separate hospitals (Schaberg *et al.*, 1976). Over a 21-month period, 21 patients became bacteraemic and eight died. Interhospital transmission occurred via passive carriage on the hands of staff. Subsequently one of the hospitals experienced an outbreak involving a strain of *Klebsiella pneumoniae* with the same antimicrobial resistance profile as the epidemic *S. marcescens* strain. *In vitro* mating studies revealed that this second wave of infection may have resulted from the *in vivo* transfer of resistance determinants from the epidemic *S. marcescens* strain (Thomas *et al.*, 1977).

In some outbreaks of *S. marcescens*, common sources of transmission or cross contamination have been implicated. In a recent report describing two temporally overlapping outbreaks of *S. marcescens*, two clonal types of *S. marcescens* were isolated from separate environmental sources. A contaminated hexetidine mouthwash solution was implicated in the spread of the first outbreak, while contaminated bronchoscopes were involved in the second outbreak (Peltroche-Llacsahuanga *et al.*, 1999). In another episode, a jar of contaminated exfoliating cream was traced to the house of a surgery nurse who wore artificial nails and this was implicated as a source for an outbreak involving seven cardiovascular surgery patients (Passaro *et al.*, 1997). In the majority of outbreaks, however, common sources are not found, despite extensive envionmental

surveillance (Yu, 1979). In these circumstances it is felt that patients colonised with *S. marcescens* may be the source of infection. Success in recovering an environmental source will, however, depend on the sensitivity of the screening protocol used. Even in the absence of an outbreak, environmental contamination has been found when suitable culture methods were used (Byrne *et al.*, 2001). Perhaps, the most sensitive method to exclude an environmental source would be to use a molecular DNA amplification technique which would allow detection of viable bacteria which fail to grow on culture.

In adults, the urinary tract is an important reservoir for *S. marcescens* infection, where indwelling urinary catheters have been identified as major risk factors (Schaberg *et al.*, 1976). The respiratory tract has also been recognised as a major portal of entry, with *S. marcescens* being isolated from the respiratory tract of up to 80% of post-operative patients developing *S. marcescens* bacteraemia (Yu, 1984). In a recent study investigating the role of *S. marcescens* in hospital-acquired lower respiratory tract infection, the throat and nose were identified as common sites of *S. marcescens* carriage, being found in 59% and 31% of colonised patients, respectively (Byrne *et al.*, 2000). In children, colonisation of the gastrointestinal tract is considered a primary reservoir of infection. In almost all reports of *S. marcescens* outbreaks in neonatal ICUs, infants with gastrointestinal colonisation were considered the most likely source of the organism (Campbell *et al.*, 1998; Jones *et al.*, 2000). In some of these studies, persistent rectal colonisation was found in most if not all of the babies involved in the outbreaks (Lewis *et al.*, 1983; van Ogtrop *et al.*, 1997).

Colonisation of the adult gastrointestinal tract with *S. marcescens* has long been disputed. Studies have demonstrated that rates of adult gastrointestinal carriage are largely dependent on the population screened (Byrne *et al.*, 2001). In the healthy host and non-infected immuno-competent patient, carriage rates remain low (<1% and 3%, respectively). However, in non-infected patients in affected wards, carriage rates of 21% have been reported while over 30% of patients infected with *S. marcescens* carry the organism in the gut (Grimont & Grimont, 1978; Byrne *et al.*, 2001). High rates of carriage have also been found in immuno-compromised patients (Young *et al.*, 1980). Furthermore, it is known that once established in adults, gut carriage is persistent (Byrne *et al.*, 2001). Similar to neonates, gastrointestinal carriage in adults may also represent a significant reservoir of infection.

Regardless of the source of *S. marcescens* infection, the predominant mode of spread in all outbreaks is thought to be hand-to-hand transmission by hospital personnel. The recovery of epidemic strains of *S. marcescens* from hand cultures of hospital staff has been reported in many studies (Maki *et al.*, 1973; Schaberg *et al.*, 1976; Lewis *et al.*, 1983). In one hospital, almost 50% of hand cultures from staff were positive at the end of their working shift (van Ogtrop *et al.*, 1997). Factors such as debilitating clinical condition, length of ward-stay and frequent exposure to medical interventions, which predispose patients to infection, most likely act by necessitating increased frequency and intensity of direct contact with staff hands (van der Sar-van der Brugge *et al.*, 1999).

1.2.3. **Epidemiological typing**

The emergence of *S. marcescens*, both as an endemic pathogen causing sporadic infection and as an epidemic pathogen causing serious outbreaks of infection, has stimulated the quest for a suitable epidemiological typing method. Numerous phenotypic techniques have been described, including bacteriocin typing, bacteriophage typing and biotyping. None of these methods have been found to be satisfactory because they lack sufficient discriminatory power or exhibit poor reproducibility (Alonso *et al.*, 1993a).

Serological typing based on lipopolysaccharide (O) and capsular polysaccharide (K) antigens, has been used extensively for typing *S. marcescens* (Pitt, 1982). Up to 26 different O antigens and 14 K antigens are recognised (Aucken *et al.*, 1997). The system has been used successfully in the recognition of epidemic strains in many common-source outbreaks and in distinguishing between clinical and environmental isolates. Studies indicate that the distribution of serotypes varies significantly between clinical and environmental isolates. Serotypes O14:K14 and O27:K14 predominate among clinical isolates but are not found in the environment. Serotypes O6:K14, O8:K14 and O28:K28 are common in the natural environment, but are rare in hospitals (Aucken & Pitt, 1998). Although useful, serotyping is not an ideal epidemiological typing method for *S. marcescens*. Despite revision of the scheme, some nontypable strains still exist. Furthermore, since no commercial sources of the agglutinating sera are available, serological typing can only be performed in a reference laboratory.

More recent approaches to the epidemiological typing of *S. marcescens* have employed genotypic typing methods. Ribotyping has proved to be a highly discriminatory and reproducible typing method for epidemiological investigation of *S. marcescens* (Bingen *et al.*, 1992; Alonso *et al.*, 1993a). Other studies report success using polymerase chain reaction (PCR)-based methods, such as random amplification of polymorphic DNA (RAPD-PCR) (Bosi *et al.*, 1996; Hejazi *et al.*, 1997) and enterobacterial repetitive intergenic consensus (ERIC)-PCR (Liu *et al.*, 1994; van Ogtrop *et al.*, 1997). Nevertheless, despite the application of these rapid PCR-based typing techniques, DNA macrorestriction analysis by pulsed field gel electrophoresis (PFGE) remains the typing method of choice for *S. marcescens* (Aucken *et al.*, 2000; Dorsey *et al.*, 2000). Although tedious and time-consuming, this method is highly discriminatory and reproducible (Miranda *et al.*, 1996; Shi *et al.*, 1997). The availability of interpretive criteria for PFGE banding patterns also helps to standardise decisions on genetic relatedness and clonal relationships (Peltroche-Llacsahuanga *et al.*, 1999).

1.3. LABORATORY IDENTIFICATION AND PATHOGENICITY

1.3.1. The genus *Serratia*

Serratia marcescens is a member of the genus *Serratia,* which is part of the family *Enterobacteriaceae.* Eight species of *Serratia* are recognised within the genus; *S. marcescens*, *S. liquefaciens*, *S. proteamaculans*, *S. grimesii*, *S. plymuthica*, *S. marinorubra* (synonym *S. rubidaea*), *S. odorifera* and *S. ficara* (Grimont *et al.*, 1981). Of all *Serratia* species, *S. marcescens* is the most common clinical isolate and the most important human pathogen.

Serratia is a ubiquitous environmental organism that is widely distributed in nature. Although members of the *S. liquefaciens* group are the most common *Serratia* species associated with the natural environment, both pigmented and some nonpigmented strains of *S. marcescens* have been found in soil, plants and salad vegetables (Grimont *et al.*, 1981). Whilst nonpigmented environmental strains of *S. marcescens* may be acquired by hospitalised patients, perhaps following consumption of vegetables (Grimont *et al.*, 1981), the majority of *S. marcescens* strains implicated in nosocomial infection are associated with the hospital setting and are absent from the natural environment.

1.3.2. Laboratory identification

Serratia spp. are aerobic, motile Gram-negative rods. Phenotypically *Serratia* is one of the easiest genera to differentiate from other genera in the *Enterobacteriaceae* family. Unlike other enterobacteria, strains of *Serratia* usually produce extracellular deoxyribonuclease (DNase), gelatinase and lipase and are resistant to the antibiotics colistin and cephalothin. In the laboratory *Serratia* species are frequently identified using the Analytical Profile Index (API) 20E (bio-Mérieux SA). Studies have shown, however, that in some centres only 85% of *S. marcescens* are correctly identified using the commercially available API system (Hejazi & Falkiner, 1997). This is largely due to the fact that although *S. marcescens* are characteristically distinguished by their inability to ferment arabinose, in the API system they can oxidise the arabinose in the cupule, producing a false positive reaction (Craven *et al.*, 1977). As a result *S. marcescens* are frequently misidentified as *S. liquefaciens*. Identification of *S. marcescens* should

therefore be confirmed using supplementary tests including a negative reaction in phenol red arabinose peptone water sugars.

1.3.3. **Pathogenic properties**

Serratia marcescens is not a primary pathogen. It is rarely associated with primary invasive infection or even a specific condition. It behaves as a true opportunist agent producing infection whenever it gains access to a suitably compromised host. The organism is thus considered to have a low intrinsic pathogenicity. Almost all isolates produce extracellular products such as DNase, chitinase, lecithinase, lipase, gelatinase and siderophores, which are known to act as virulence determinants in other organisms. However, it seems that in *S. marcescens*, these products do not act as potent virulence factors (Aucken & Pitt, 1998).

Recent studies have demonstrated that *S. marcescens* may produce other pathogenicity factors associated with host cell adherence and tissue penetration. The marked difference in the distribution of serotypes found in the hospital and the natural environment indicates that some strains of *S. marcescens* may be more suited for survival in the clinical setting. In a study investigating the association between pathogenic properties and serotype, there was a significant correlation between haemagglutination and the expression of fimbriae with serotypes such as O14:K14, which represent over a third of all hospital isolates (Franczek *et al.*, 1986; Aucken & Pitt, 1998). This indicates that the adherence capability of *S. marcescens* may play a role in the colonisation of patients. In addition, other studies indicate that almost all isolates of *S. marcescens* secrete a cytotoxic haemolysin which mediates irreversible vacuolation and cell lysis (Konig *et al.*, 1987; Hertle *et al.*, 1999). This cytolytic activity may enable the organism to penetrate tissue layers and invade the host.

Apart from these pathogenic properties, clinical isolates of *S. marcescens* also demonstrate a strong adaptive potential and a remarkable capacity to utilise a wide range of nutrients for growth. The organism has been shown to survive and grow under extreme conditions including growth in antiseptics, disinfectants and even double-distilled water (Szewzyk *et al.*, 1993). For opportunistic agents such as *S. marcescens* which exhibit a limited number of virulence factors, the ability to survive in adverse environmental conditions confers a strong selective advantage.

1.4. ANTIMICROBIAL RESISTANCE

Complementary to its capacity for survival, *S. marcescens* also demonstrates a strong potential to express antimicrobial resistance. Both environmental and clinical isolates of *S. marcescens* are invariably resistant to a number of antibiotics including ampicillin, cefuroxime, tetracycline and polymyxin (Grimont & Grimont, 1978; Aucken & Pitt, 1998). In addition, clinical isolates of *S. marcescens* display a selective advantage through their extraordinary ability and readiness to acquire and express many other antimicrobial resistance determinants. The pattern of resistance in clinical isolates is likely to reflect patterns of antimicrobial use. Many hospital isolates now demonstrate multiple antimicrobial resistance to the agents most frequently used in the treatment of nosocomial infection such as cephalosporins, aminoglycosides and fluoroquinolones (Aucken & Pitt, 1998; Knowles *et al.*, 2000) (Table 1.1).

1.4.1. Resistance to β-lactam agents

In accordance with the wide scale use of broad-spectrum cephalosporins in clinical practice, over 35% of clinical isolates of *S. marcescens* currently demonstrate resistance to these agents (Aucken & Pitt, 1998). This resistance normally results from over-production of an inducible chromosomal β-lactamase enzyme and is commonly associated with resistance to third-generation cephalosporins (Follath *et al.*, 1987). B-lactamase-mediated cephalosporin resistance may also result from the acquisition of plasmid-encoded extended-spectrum β-lactamases (ESBLs) (Pagani *et al.*, 1994; Luzzaro *et al.*, 1998). Alternatively, resistance may result from a decrease in the permeability of the outer membrane via porin mutations. In *S. marcescens*, reduced permeability may be combined with β-lactamase production to achieve high-level cephalosporin resistance (Weindorf *et al.*, 1998). Reduced permeability has also been shown to operate independently of β-lactamase production (Nikaido, 1989) and in these cases resistance to β-lactam agents is often combined with resistance to other antibiotics such as aminoglycosides and quinolones (Sanders & Watanakunakorn, 1986; Traub & Bauer, 1987). Resistance to carbapenem β-lactam agents is also emerging in clinical isolates of *S. marcescens* (Livermore, 1995). This carbapenem resistance is mediated by either chromosomally- or plasmid-encoded β-lactamases. Resistance to β-lactam agents in *S. marcescens* is discussed in more detail in chapter two.

Table 1.1. Mechanisms of resistance to common antibiotics in *S. marcescens*.

Antimicrobial class	Resistance mechanism	Gene location
B-lactams	Inactivation by β-lactamases	Chromosomal & plasmid
	Reduced membrane permeability	Chromosomal
Aminoglycosides	Inactivation by acetylases, adenylases, or phosphorylases	Chromosomal & plasmid
	Reduced membrane permeability	Chromosomal
Quinolones	Alteration of target DNA gyrase	Chromosomal
	Reduced membrane permeability	Chromosomal

1.4.2. **Aminoglycoside resistance**

In the 1980's, aminoglycoside resistance was widely reported in *S. marcescens*, being present in up to 50% of clinical isolates. In more recent years, the increased use of agents such as third-generation cephalosporins and quinolones has led to a reduction in the use of aminoglycosides. Consequently, current figures for the rate of aminoglycoside resistance amongst *S. marcescens* isolates has decreased to 20% (Amyes & Gemmell, 1992; Aucken & Pitt, 1998). In *S. marcescens,* aminoglycoside resistance is attributed either to diminished uptake of antibiotic which confers low-level resistance to all aminoglycosides, or more frequently to the presence of aminoglycoside-modifying enzymes, which generally confer higher levels of resistance to one or more aminoglycosides (Sleigh, 1983). In *S. marcescens* the most frequently occurring aminoglycoside-modifying enzymes include *N*-acetyltransferases AAC(6')-I and AAC(3)-I and *O*-adenyltransferase ANT(2")-I.

The geographic distribution of different aminoglycoside-modifying enzymes varies considerably depending on regional aminoglycoside usage. AAC(6')-I, which mediates resistance to tobramycin, netilmicin, amikacin and dibekacin and ANT(2")-I, which confers resistance to gentamicin, tobramycin and dibekacin, are frequently found in the US and the Far East (Shimizu *et al.*, 1985; Shaw *et al.*, 1992; Coria-Jimenez & Ortiz-Torres, 1994). AAC(3)-I which confers resistance to gentamicin and fortimycin is found predominately in the UK (Bullock *et al.*, 1982; Sleigh, 1983).

1.4.3. **Fluoroquinolone resistance**

In recent years, the marked increase in use of fluoroquinolones in the hospital environment has been mirrored by a dramatic increase in the numbers of fluoroquinolone-resistant nosocomial pathogens. This is especially true in the case of *S. marcescens*. Reports indicate that over a twelve year period from 1984 to 1995, the percentage of fluoroquinolone-resistant *S. marcescens* isolates rose from 14% to 70% compared to increases from 5% to 15% for other hospital-acquired enterobacteria such as *Enterobacter cloacae* and *Citrobacter freundii* (Acar & Goldstein, 1997). The emergence of resistance in *S. marcescens* in response to increased fluoroquinolone use is also reflected in the highly divergent rates of resistance between community-acquired isolates (13%) (Acar & Goldstein, 1997) and nosocomial isolates (70%) (Malvone,

1991). Studies investigating the rate of acquired fluoroquinolone resistance have also indicated that selection of resistant mutants in *S. marcescens* occurs at a significantly higher frequency than in other enterobacteria (Watanabe *et al.*, 1990). The minimum inhibitory concentration (MIC) of fluoroquinolones in *S. marcescens* is also 50 times greater than MIC values in other enterobacteria (Korner *et al.*, 1994). Although some studies have indicated that resistance to fluoroquinolones in *S. marcescens* is attributable to reduced permeability (Traub, 1985), the main mechanism for resistance involves mutations in the *gyrA* gene which codes for the A subunit of the target enzyme, DNA gyrase (Fujii *et al.*, 1988; Fujimaki *et al.*, 1989).

1.5. CONCLUSIONS

During the last thirty years, *S. marcescens* has emerged as a serious nosocomial pathogen renowned for its ability to exhibit multiple antimicrobial resistance. Evidence has indicated, however, that hospitals vary greatly in their experience with *S. marcescens*. Many hospitals have few, if any, problems and see very small numbers of isolates in a given time period. When isolates are recovered they are invariably susceptible to commonly used antibiotics. In other hospitals, however, the reverse is true. As a result of cross-infection, many isolates of the same strain are recovered. The strains become endemic within the hospital population and usually develop multiple antimicrobial resistance. Outbreaks of infection are likely to coincide with any breakdown in infection control procedures. Then as the pathogen continues to become established within the hospital, antibiotic usage continues to select for the antibiotic-resistant population.

Chapter II

Resistance to β-lactam antibiotics in Gram-negative bacilli

Chapter II

Resistance to β-lactam antibiotics in Gram-negative bacilli

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2.1. B-LACTAM ANTIBIOTICS

It is over 50 years since the antibiotic era began with the development of penicillin G in 1945. Fifteen years later saw the advent of penicillinase-resistant semisynthetic penicillins and first-generation cephalosporins. Over the next 20 years a great variety of cephalosporins and other β-lactam agents were introduced into clinical practice offering a wider spectrum of antimicrobial action as well as improved clinical efficacy. Today, the β-lactam agents represent the safest, most commonly prescribed antibiotics used in hospitals, accounting for approximately 50% of total global antibiotic consumption.

2.1.1. Action of β-lactam antibiotics

Murein or peptidoglycan is an essential component of the bacterial cell wall that protects the cell from osmotic lysis and determines its shape. The murein is a heteropolymer consisting of glycan chains of alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The glycan chains are linked to each other by short peptides. The murein is a highly cross-linked structure but exists in a dynamic state and is continuously remodeled as the bacterium grows and divides. The integrity of the cell wall rests on a dynamic balance between synthesis and degradation that must be strictly controlled to allow cell growth while avoiding lysis.

B-lactam antibiotics inhibit bacterial growth by inactivation of the penicillin-binding proteins (PBPs). PBPs are trans- and carboxy-peptidases that catalyse the cross-linking of the cell wall murein. The PBPs are located on the outer surface of the cytoplasmic membrane and extend into the periplasmic space. The natural substrate of PBPs is a cell wall polymer terminating in D-alanyl-D-alanine. The tertiary conformation of this polymer is structurally similar to that of the β-lactam bond. By commandeering the active site of the PBP, β-lactams prevent formation of the peptide bridges, causing production of a weakened cell wall and activation of cell-wall degrading enzymes called autolysins. Ultimately the bacteria cannot withstand osmotic forces and lyse.

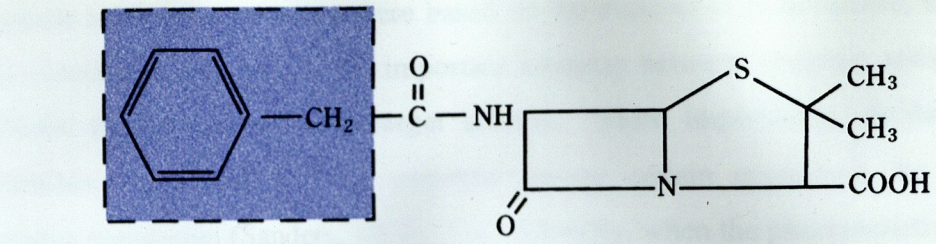
Bacteria can sense perturbations in their cell wall metabolism and translate the resulting stress into signals that induce defensive responses. Antimicrobial resistance thus arises if the PBPs can be protected from the action of the β-lactam agents. Microbial

resistance is most commonly achieved by the production of β-lactam inactivating enzymes– β-lactamases. Some bacterial species have refined this defense mechanism by making β-lactamase synthesis inducible in the presence of β-lactam antibiotics. In addition, microbes may protect the PBPs through the action of permeability barriers or may alter the structure of the target PBPs to prevent β-lactam binding (Livermore, 1991b). Alternatively, PBP targets may be protected by the active removal of the β-lactam drug from the bacterial cell (Li *et al.*, 1994; Poole, 1994).

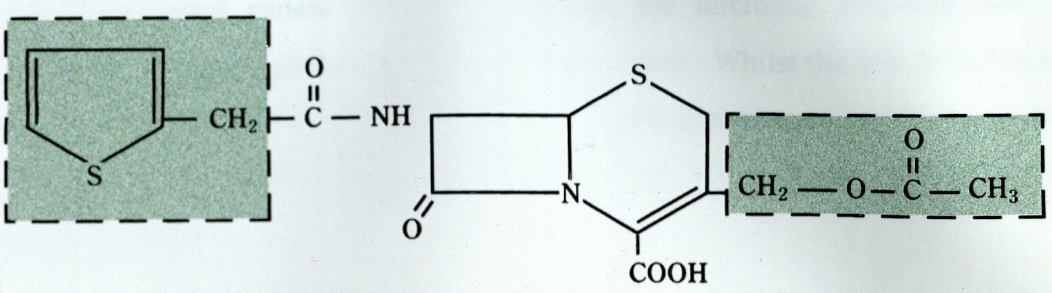
2.1.2. **Emergence of β-lactamase-mediated β-lactam resistance**

When first introduced, penicillin was active against 95% of *Staphylococcus aureus* isolates with the remaining 5% demonstrating resistance due to the production of β-lactamases. Within five years the proportion of β-lactamase-producing isolates had grown to 50% as a result of gene transfer and strain selection (Livermore, 1998). The advent of semisynthetic β-lactamase-resistant penicillins some fifteen years later was, therefore, instrumental in preventing a worldwide pandemic of nosocomial penicillin-resistant staphylococcal infections. Shortly after the introduction of semisynthetic penicillins, broad-spectrum penicillins and first-generation cephalosporins came into use. These agents were active against the Gram-negative organisms that were beginning to increase in clinical importance. These newer β-lactams remained the first line defense against microbes for over 20 years as the number of different β-lactamases produced by Gram-negative bacteria remained limited (Medeiros, 1997). When β-lactamase-mediated resistance emerged in Gram-negative bacteria, the pharmaceutical industry responded by attempting to develop β-lactamase-stable agents or by combining β-lactamase-susceptible agents with β-lactamase inhibitors.

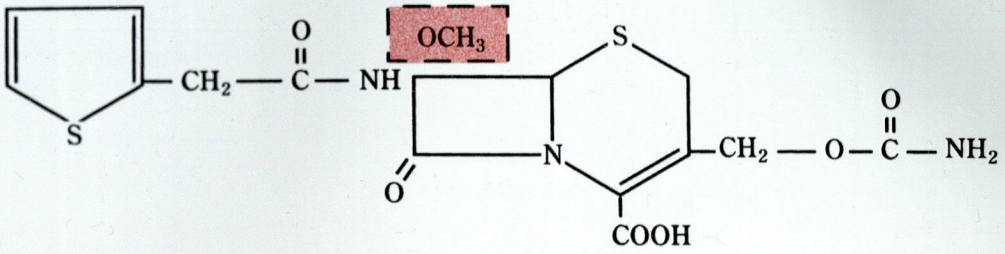
Stability in penicillins and cephalosporins was achieved by attaching bulky substituents to the β-lactam ring that hinder access of the active serine site or that displace water from the active site preventing completion of hydrolysis (Figure 2.1). B-lactam antibiotic classes such as clavams and penicillanic acid sulphones were developed specifically as β-lactamase inhibitors (Livermore, 1998). Within seven or eight years, six novel classes of β-lactam agents including cephamycins, oxyiminocephalosporins, carbapenems, monobatams and clavam and penicillanic acid sulfone β-lactamase



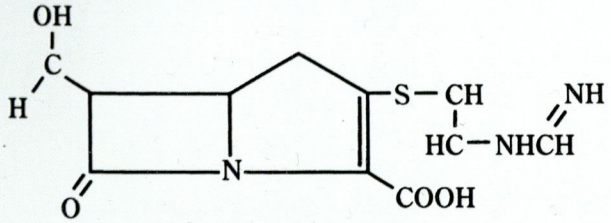
PENICILLIN G (penicillin)



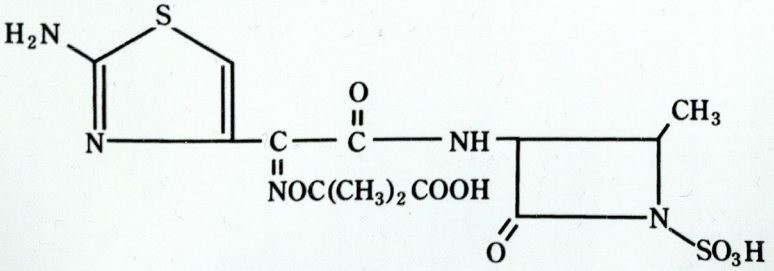
CEPHALOTHIN (cephalosporin)



CEFOXITIN (cephamycin)



IMIPENEM (carbapenem)



AZTREONAM (monobactam)

Figure 2.1. Biochemical structure of a typical example of each of the major classes of β-lactam antibiotics. The highlighted areas represent the side-chain modifications made during the development of these families of antibiotics.

inhibitors were developed (Table 2.1). However, since both stearic hindrance and β-lactamase inhibitor approaches were based on the evasion of β-lactamase, they did not fully consider factors such as the important interplay between enzymic activity, rate of antibiotic permeation and drug/target affinity. More importantly, neither of these approaches recognised the vast potential among certain organisms for altered β-lactamase expression (Sanders, 1992). Consequently, when the pharmaceutical industry unleashed this great variety of novel β-lactams, the microbial response was the production of an equally great array of novel β-lactamases. Whilst the new β-lactamase-stable agents were effective against many of the existing resistance mechanisms, their use resulted in the selection and proliferation of many new forms of β-lactamase-mediated resistance.

Table 2.1. The commonly used β-lactam antibiotics and their clinically useful antibacterial spectra.

B-lactam antibiotic	Staphylococci	Streptococci	Neisseria	Haemophilus	Enterobacteria	Pseudomonas	Anaerobes
Penicillins							
Narrow-spectrum penicillins:							
*penicillin G and V	Penase (–)	+	+	…	…	…	+ (*B. fragilis*)[§]
*penicillinase-resistant penicillins e.g. methicillin	Penase (+)	…	…	…	…	…	…
*ampicillin, amoxycillin	…	Enterococci	+	+	Some	…	…
carbenicillin, ticarcillin	…	…	…	…	Some	Many	some *B. fragilis*
Broad-spectrum penicillins: mezlocillin, piperacillin	Penase (–)	+	+	+	+	Many	+
Penicillin-β-lactam inhibitors: amoxycillin-clavulanate piperacillin-tazobactam	Penase (+)	…	…	β-lactamase (+)	β-lactamase (+)	β-lactamase (+)[†]	some *B. fragilis*
Cephalosporins							
*First-generation: cefaclor, cephalothin, cephradine, cephaloridine	+	+	…	…	Some	…	…
Second-generation: cefixime, cefuroxime,	+[‡]	+	…	+	Many	…	…
Third-generation: cefotaxime, ceftazidime	+	…	Penase (+)	Some	Most	Some	…
Cephamycins							
cefoxitin, cefotetan	…	…	…	…	Many	…	many *B. fragilis*
Nonclassical β-lactams							
imipenem, aztreonam	…	…	…	…	+[✧]	+	…

+, indicates drug would be a reasonable choice for the organism listed; …, indicates drug would not be a reasonable choice; penase (+), penicillinase production; penase (-), penicillinase negative; β-lactamase (+), β-lactamase production; *, a single 'class drug' is used to determine susceptibility; §, except *B. fragilis*; † piperacillin-tazobactam only; [‡], not for cefixime; [✧], for isolates resistant to the other β-lactam antibiotics.

2.2. B-LACTAMASE ENZYMES

B-lactamases are a large and diverse group of approximately 200 enzymes that catalyse the hydrolysis of the β-lactam ring and so destroy antibiotic activity (Bush *et al.*, 1995). Most β-lactamases function by a mechanism of serine ester hydrolysis (Figure 2.2), although a few use zinc ions to attack the β-lactam ring (Livermore, 1998). B-lactamases share several highly conserved amino acid sequences with PBPs and together these form a superfamily of penicillin-recognising enzymes.

2.2.1. Classification of β-lactamase enzymes

Until recently β-lactamases were classified by their hydrolytic spectra, their susceptibility to inhibitors and whether they were chromosomally- or plasmid-encoded. The first scheme to achieve wide acceptance classified β-lactamases into five major groups and was proposed by Richmond & Skyes in 1973 (Richmond & Sykes, 1973). A major revision and expansion of this functional classification was proposed by Bush in 1989 (Bush, 1989a; Bush, 1989b; Bush, 1989c) and updated in 1995 (Bush *et al.*, 1995).

The revised Bush scheme classifies β-lactamases by their substrate preference among penicillin, oxacillin, carbenicillin, cephaloradine, expanded-spectrum cephalosporins and imipenem and by their susceptibility to inhibitors (Bush *et al.*, 1995). This scheme recognises four major β-lactamase groups designated 1–4 (Table 2.2) (Livermore, 1998). Group 1 includes 32 cephalosporinases that are poorly inhibited by clavulanate and in many cases, are prototypical of the chromosomal β-lactamases. Group 2 is the largest category consisting of 138 enzymes that are largely plasmid-borne and that are inhibited by clavulanate. Group 3 are metallo-β-lactamases that hydrolyse a broad spectrum of substrates including carbapenems. Most are chromosomal but some are plasmid-encoded. Group 4 includes a small number of chromosomally-mediated penicillinases that are not inhibited by clavulanate. This functional classification scheme provides a very useful compilation of β-lactamase characteristics. As a phenotypic classification system it presents the problem that point mutations can alter the substrate and inhibitor specificity and change the assigned grouping. Furthermore,

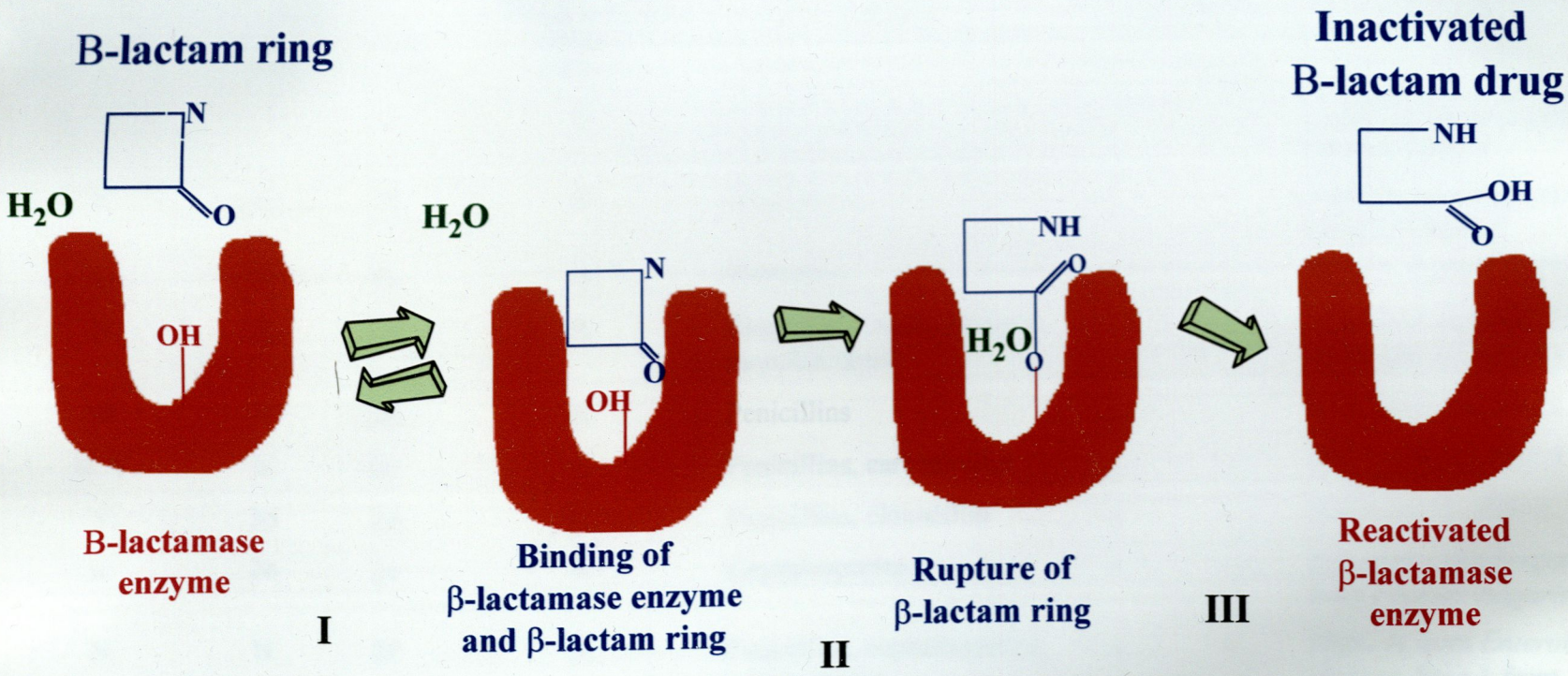


Figure 2.2. Action of class A, C and D β-lactamase enzymes on β-lactam antibiotics. The -OH group shown in the enzyme structure is on the side chain of the active-serine. Phases of the reaction are: (I) reversible non-covalent binding of the β-lactamase and the β-lactam ring; (II) rupture of the β-lactam ring, which becomes covalently acylated on to the active site serine; and (III) hydrolysis of the acyl enzyme to reactivate the β-lactamase and liberate the inactivated drug molecule. Adapted from Livermore, 1998.

Table 2.2. Molecular and functional classification of β-lactamases.

Functional group			Molecular class	Preferred substrate	Inhibited by		Representative enzymes
Richmond-Skyes (1973)	Bush (1989)	Bush (1995)	Ambler (1980)		CA	EDTA	
Ia, Ib, Id	1	1	C	Cephalosporins	–	–	AmpC in Gram-negative bacteria, MIR-1
N	2a	2a	A	Penicillin	+	–	Penicillinases from Gram-positive bacteria
III	2b	2b	A	Penicillins	+	–	TEM-1, TEM-2, SHV-1
N	2b	2be	A	Penicillins, cephalosporins, monobactams	+	–	TEM-3 to-26, SHV-2 to-6, *Klebsiella oxytoca* K1
N*	N	2br	A	Penicillins	±	–	TEM-30 to-36, TRC1
II, V	2c	2c	A	Penicillins, carbenicillin	+	–	PSE-1, PSE-3, PSE-4
V	2d	2d	D	Penicillins, cloxacillin	±	–	OXA-1to-11, PSE-2
Ic	2e	2e	A	Cephalosporins	+	–	Inducible cephalosporinase from *Proteus vulgaris*
N	N	2f	A	Penicillins, cephalosporins, carbapenems	+	–	NMC-A from *Enterobacter cloacae*, Sme-1 from *Serratia marcescens*
N	3	3	B	Most β-lactams including carbapenems	–	+	L1 from *Stenotrophomonas maltophilia*
N	4	4	ND	Penicillins	–	–	Penicillinase from *Pseudomonas cepacia*

CA, clavulanic acid; EDTA, ethylene diamine tetraacetic acid disodium salt; N, not included; N*, not included except K1 in class IV; ND, not determined; –, not inhibited; +, inhibited. Adapted from Bush *et al.*, 1995.

the nomenclature of β-lactamases is often confusing because of the numerous codes used.

In the early 1980s, Ambler proposed an alternative method of β-lactamase classification based on molecular structure instead of activity (Ambler, 1980). This structure-based scheme (Table 2.2) divides β-lactamases into four categories, A to D. Unlike phenotypic classifications, groupings are not distorted by mutations that change activity. The majority of the clinically important β-lactamases belong to Ambler class A and C. Class A (Bush group 2) includes the chromosomal β-lactamases of *Klebsiella* spp., *Citrobacter diversus*, *Proteus vulgaris* and most *Bacteroides* spp. as well as virtually all the common plasmid-encoded β-lactamases. Class C comprises the chromosomal AmpC (Bush group 1) cephalosporinases. Class D (Bush group 2) includes the OXA enzymes which are widely disseminated in *Enterobacteriaceae* as plasmid-encoded β-lactamases and class B (Bush group 3) contains enzymes with zinc-based activity.

2.2.2. **Kinetic properties of β-lactamase enzymes**

For many antibiotics, hydrolysis parameters can be used to explain the microbiological activity observed in organisms producing inactivating enzymes. The hydrolytic capacity of a β-lactamase enzyme can be estimated by considering its kinetic properties, i.e. the maximal hydrolysis rate (V_{max}) and the affinity for the substrate (K_m) (Livermore, 1995). The V_{max} reflects the amount of enzyme present and the number of drug molecules hydrolysed (K_{cat}). The magnitude of the V_{max}/K_m ratio is the critical determinant of the hydrolytic efficiency of the β-lactamase. The role of the permeability barrier can also be included by estimation of the relative rate of drug entry into the cell (Fick's law of diffusion). Resistance is likely to arise when the V_{max}/K_m ratio is high, i.e. when the drug is hydrolysed rapidly (high V_{max}) and the enzyme has a high affinity for the compound (low K_m) and/or when drug entry is slow. Conversely, slow hydrolysis (low V_{max}), low affinity (high K_m) and/or rapid penetration of the antibiotic, reduces the inactivating ability of the β-lactamase enzyme (Livermore, 1995).

2.3. B-LACTAMASE-MEDIATED β-LACTAM RESISTANCE IN GRAM-NEGATIVE BACILLI

In Gram-negative bacilli (GNB), β-lactamase production is the principal and most widespread mechanism of resistance to β-lactam antibiotics. Virtually all GNB produce chromosomally-mediated β-lactamases. In addition, secondary β-lactamases, encoded by plasmids or transposons, are now widespread in Gram-negative species (Livermore, 1998). In Gram-positive organisms, β-lactamase production is extracellular but in Gram-negative bacteria β-lactamases are located in the periplasm. Whether or not the periplasmic β-lactamases of GNB confer resistance depends, not only on the amount of enzyme produced and its ability to hydrolyse the antibiotic in question, but also on the interplay with the permeability barrier provided by the outer membrane (Sanders, 1992).

2.3.1. Chromosomal β-lactamases

The chromosomal β-lactamases of GNB are species-specific but can be grouped into a few broad classes (Table 2.3) (Livermore, 1995). The most important are the class C β-lactamases (Bush group 1) (Bush *et al.*, 1995). These AmpC β-lactamases occur in *Pseudomonas aeruginosa* and most enterobacteria except *Salmonella* spp. and some *Klebsiella* spp. The enzymes from the different species in the group resemble each other closely but the mode of expression varies between species and critically determines their potential to cause resistance (Livermore, 1995). *Escherichia coli, Proteus mirabilis* and the shigellae produce basal amounts of enzyme regardless of whether β-lactam antibiotics are present (Sykes & Matthew, 1976). This small amount of enzyme is insufficient to protect the bacteria against β-lactam drugs. High-level β-lactamase production in these species can arise through mutation. However, since two simultaneous mutations involving an increase in the efficiency of a promoter and inactivation of an attenuator are required for high-level production, the selection of such mutants is rare.

Table 2.3. Chromosomal β-lactamases and their expression in Gram-negative bacilli.

Organism	Name	Molecular Class (1980)	Bush group (1995)	Mode of expression[§]			
					Constitutive*		
				Inducible	Minimal	Moderate	High
Escherichia coli	AmpC	C	1	–	●	–	○
Shigellae	AmpC	C	1	–	●	–	○
Enterobacter spp.	AmpC	C	1	●	○	–	⊙
Citrobacter freundii	AmpC	C	1	●	○	–	⊙
Morganella morganii	AmpC	C	1	●	–	–	⊙
Providencia spp.	AmpC	C	1	●	○	–	○
Serratia spp.	AmpC	C	1	●	–	–	⊙
Klebsiella pneumoniae	SHV-1	A	2b	–	–	●	○[†]
Klebsiella oxytoca	K1	A	2be	–	–	●	⊙
Citrobacter diversus	….	A[‡]	2e	●	○	–	○
Proteus vulgaris	Cefuroximase	A	2e	●	–	–	○

*, minimal production denotes that enzyme is detectable but causes no significant resistance; moderate denotes that the enzyme confers resistance; high denotes high levels of enzymes that confer resistance even to weak substrates; §, ●, normal mode of production; ⊙, frequent but variable, seen in 10-50% of isolates; ○, rare seen in less than 10% of isolates; _, unknown;[†], frequent when SHV-1 is plasmid mediated; [‡], production of class C enzymes seen in a few isolates.
Adapted from Livermore, 1995.

2.3.1.1. **Inducible AmpC β-lactamase expression**

Other species including *P. aeruginosa, Enterobacter* spp., *C. freundii*, *S. marcescens*, *Providencia stuartii* and *Morganella morganii* exhibit inducible expression of class C β-lactamase and show a much greater potential for resistance (Table 2.4). In wild-type isolates trace amounts of AmpC β-lactamase enzyme are produced. In the presence of β-lactam agents or other stimuli, however, expression of AmpC is inducible and bacteria produce a transient increase in β-lactamase production which returns to low-level when the inducer is removed (Livermore, 1995). AmpC induction is linked to perturbations in cell wall synthesis and recycling of cell wall components. Expression of the inducible *ampC* structural gene is regulated by the interplay of at least three genes *ampR*, *ampD* and *ampG*. The protein products of these genes are associated with the cell wall recycling pathway (Hanson & Sanders, 1999). AmpR is a transcriptional regulator which normally acts as a repressor of AmpC (Jacobs *et al.*, 1997). AmpD is a cytosolic amidase which participates in intracellular recycling of muropeptides (Holtje *et al.*, 1994; Jacobs *et al.*, 1995; Dietz *et al.*, 1996). AmpG encodes a transmembrane protein thought to function as a permease for the induction signal (Korfmann & Sanders, 1989; Dietz *et al.*, 1996).

2.3.1.2. **Mechanism of AmpC induction and derepression**

Current models for the mechanism of AmpC induction describe a system which operates as a cell wall sensing device that is controlled by the cytoplasmic concentrations of biosynthetic and degradative intermediates of murein metabolism. Following cell wall breakdown, murein is degraded by cell wall hydrolases to yield GlcNAc-anhydro-MurNAc-tripeptide. This muropeptide is transported into the cytoplasm by the AmpG permease where it is cleaved by AmpD. The resulting tripeptide is incorporated into the peptidoglycan precursor, UDP-MurNAc-pentapeptide, and is shuttled back into the cell surface for re-entry into the growing peptidoglycan chain (Jacobs *et al.*, 1994) (Figure 2.3). Perturbations in the recycling pathway may alter the pool of peptide products available and may thus alter the expression of AmpC β-lactamase.

During normal cell growth in the absence of β-lactam inducers, the AmpR regulator expressed in wild-type cells is maintained in an inactive form by UDP-MurNAc-pentapeptide. This negative effect probably occurs by direct binding of the pentapeptide

Table 2.4. Chromosomal β-lactamase expression and resistance phenotypes in common Gram-negative pathogens.

Organism and β-lactamases	Antibiotic susceptibility pattern for commonly used β-lactam antibiotics*												
	Amp	Amox + clav	Tic	Tic+ clav	Pip	Pip+ tazo	Cxm	Ctx/ Ctr	Caz	Cpm	Fox	Azt	Mer
Normal β-lactamase expression													
E. coli, P. mirablis, shigellae (trace chromosomal enzyme)	○	○	○	○	○	○	○	○	○	○	○	○	○
Enterobacter spp., *C. freundii*, *Serratia* spp., *M. morganii* (inducible AmpC)	●	●	○	□	○	○	○	○	○	○	●	○	○
P. aeruginosa (inducible AmpC)	●	●	○	○	○	○	■	■	○	○	■	○	○
K. pneumoniae, *K. oxytoca* (moderate level of SHV or K1)	●	○	●	○	◆	○	○	○	○	○	○	○	○
P. vulgaris (inducible Class A)	●	○	○	○	○	○	●	○	○	○	○	○	○
C. diversus (inducible Class A)	●	○	○	○	○	○	○	○	○	○	○	○	○
Hyperproduction of β-lactamase													
Enterobacter spp., *C. freundii* (hyperproducing AmpC)	●	●	●	●	●	●	●	●	●	□	●	●	○
M. morganii, Serratia spp. (hyperproducing AmpC)	●	●	●	●	●	□	●	□	□	○	□	□	○
P. aeruginosa (hyperproducing AmpC)	●	●	□	●	●	●	●	●	●	□	●	●	○
K. oxytoca (hyperproducing K1)	●	●	●	●	●	●	●	◆	○	○	○	●	○

*, β-lactam antibiotics abbreviations are as follows: Amp, ampicillin; Amox+clav, amoxycillin-plus-clavulanate; Tic, ticarcillin; Tic+clav, ticarcillin-plus-clavulanate; Pip, piperacillin; Pip+tazo, piperacillin-plus-tazobactam; Cxm, cefuroxime; Ctx, cefotaxime; Caz, ceftazidime; Cpm, cefepime; Fox, cefoxitin; Azt, aztreonam; Mer, meropenem. Resistance phenotypes are as follows: ●, resistant; ■, normal phenotype resistant for reasons other than β-lactamase production; ◆, may appear susceptible if high breakpoints are used, but likely to be clinically resistant; ○, susceptible as normal phenotype; □, susceptibility reduced compared to normal phenotype. Adapted from Livermore, 1998.

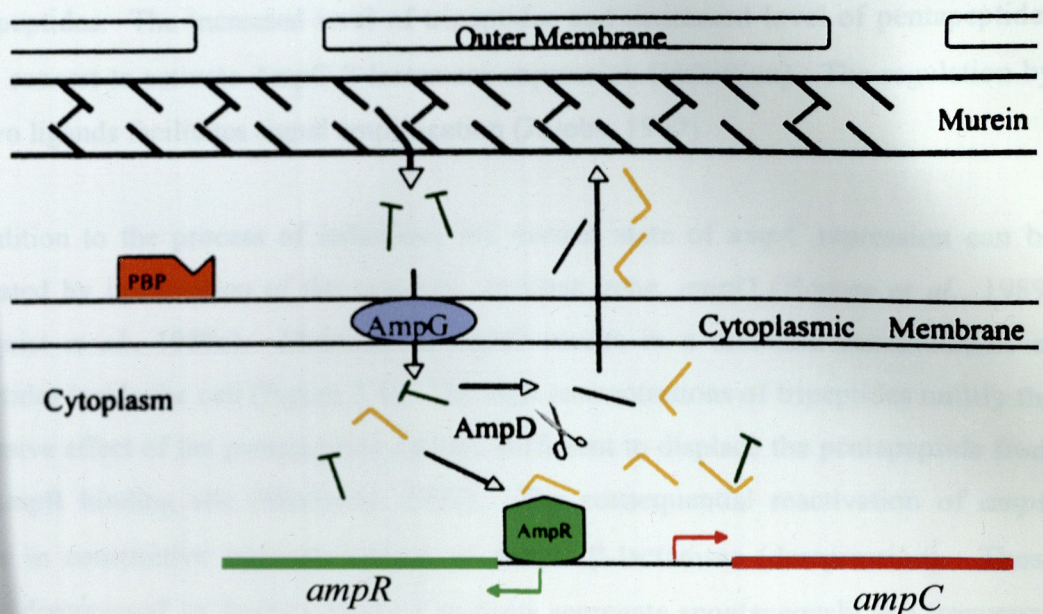


Figure 2.3. A model for peptidoglycan recycling in enterobacteria. Cell wall murein is degraded into muramyl tripeptides (⟩) which enter the cytoplasm via the AmpG permease. In the cytoplasm the tripeptides are hydrolysed by the AmpD amidase to release stem tripetides (/). The stem tripeptides are converted to pentapeptides (⟩) which move into the periplasm for recycling into new peptidoglycan. Under normal growth conditions the pentapeptides also suppress the AmpR transcriptional regulator, probably by binding to it. Adapted from Hanson & Sanders, 1999.

to the AmpR regulator. In this inactive form, AmpR binds to its operator site between the *ampC* and *ampR* genes leading to repression of *ampR* transcription and absence of activation of the *ampC* promotor (resulting in repression of *ampC* expression). This repression of AmpR production can be alleviated in the presence of β-lactam inducers (Figure 2.4). The addition of β-lactam agents causes inactivation of the PBPs on the cytoplasmic membrane. The resulting inhibition of cell wall synthesis brings about an increase in the intracellular levels of tripeptides and a decrease in the pool of pentapeptides. The increased level of tripeptides and decreased level of pentapeptides act in concert to activate AmpC β-lactamase expression (induction). The regulation by the two ligands facilitates signal amplification (Jacobs, 1997).

In addition to the process of induction, the normal state of *ampC* repression can be alleviated by inactivation of the cytosolic amidase gene, *ampD* (Honore *et al.*, 1989; Lindquist *et al.*, 1989a). Mutation in *ampD* results in a dramatic accumulation of tripeptides inside the cell (Figure 2.4). The high concentrations of tripeptides nullify the repressive effect of the pentapeptide and are sufficient to displace the pentapeptide from the AmpR binding site (Medeiros, 1997). The consequential reactivation of *ampR* results in constitutive hyperproduction of AmpC β-lactamase (derepression). These 'stably-derepressed' or 'hyperproducing' mutants segregate spontaneously at a frequency of 10^{-5}–10^{-8} cells within the normal inducible population (Livermore, 1987). Since this constitutive high-level production occurs independent of the presence of inducers, derepressed mutants demonstrate cross-resistance to most β-lactam agents, including the 'β-lactamase-stable' broad-spectrum cephalosporins such as cephamycins, oxyiminocephalosporins, monobactams and clavulanic and penicillanic acid sulfone inhibitors (Table 2.4).

There is debate in the literature as to the identification of the muropeptides involved in the AmpC induction process. In the model presented here, Jacobs *et al.* propose that tripeptides act as the endogenous signal for transcriptional induction of β-lactamase production and the pentapeptides serve as an *ampR* repressor (Jacobs *et al.*, 1997). However, other groups noted that the pentapeptides showed the largest increase in concentration following treatment with β-lactam inducers and therefore propose that induction of *ampC* expression is primarily due to the binding of pentapeptides to AmpR

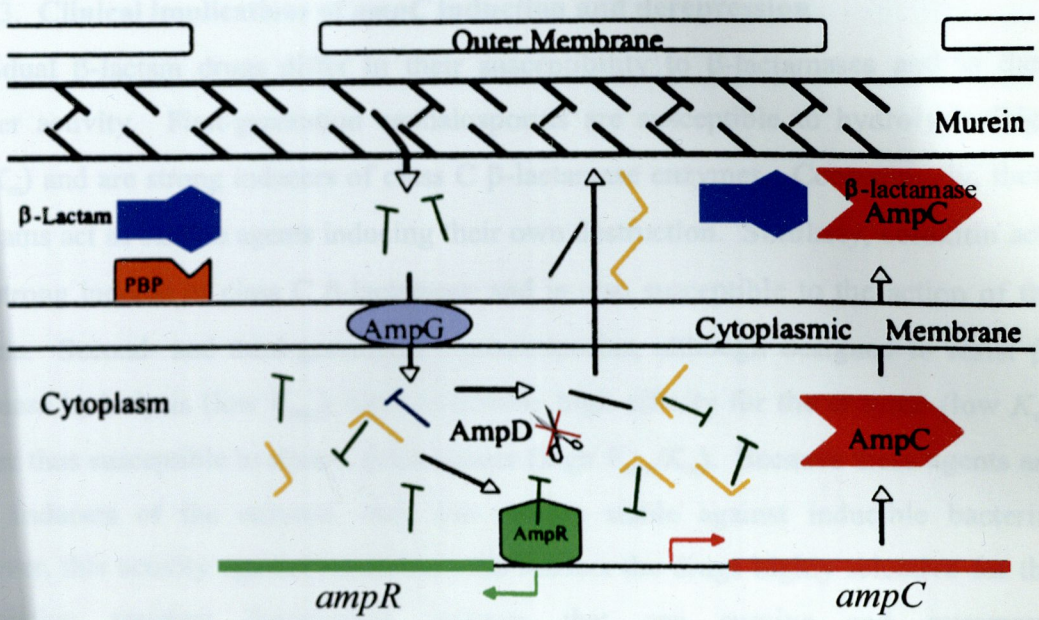


Figure 2.4. A model for β-lactamase induction and derepression in enterobacteria. When large amounts of muramyl tripeptides ( ) are present, either as a result of the binding of β-lactam antibiotics ( ) to penicillin binding proteins ( ) with increased cell wall murein breakdown or as a result of mutation in *ampD* ( ) which renders the amidase inactive, the suppressive effect of the penta-peptides ( ) on the AmpR is nullified. Unprocessed muramyl tripeptides ( ) activate the *ampR* regulator and induce hyperproduction of AmpC β-lactamase from the *ampC* structural gene. Adapted from Hanson & Sanders, 1999.

(Dietz *et al.*, 1997). Induction relies on perturbations in cell wall synthesis and presumably a delicate balance between cytoplasmic pools of cell-wall precursors. Since these interactions are highly dependent on the physiological state of the cell, discrepancy between the models probably results from differences in experimental conditions. Based on available data in the literature, a 'one peptide fits all' model is difficult to accept (Hanson & Sanders, 1999).

2.3.1.3. **Clinical implications of *ampC* induction and derepression**

Individual β-lactam drugs differ in their susceptibility to β-lactamases and in their inducer activity. First-generation cephalosporins are susceptible to hydrolysis (high V_{max}/K_m) and are strong inducers of class C β-lactamase enzymes. Consequently, these β-lactams act as suicide agents inducing their own destruction. Similarly, cefoxitin acts as a strong inducer of class C β-lactamase and is also susceptible to the action of the enzyme. Second- and third-generation cephalosporins, although designed to resist β-lactamase hydrolysis (low V_{max}), have extremely high affinity for the enzyme (low K_m) and are thus susceptible to class C β-lactamase (high V_{max}/K_m). Because these agents are weak inducers of the enzyme, they can remain stable against inducible bacteria. However, this activity against inducible cells renders the drugs highly selective for the pre-existing resistant derepressed mutants that can survive and overgrow. Consequently, the clinical importance of inducible β-lactamases and derepressed mutants has increased dramatically since the introduction of third-generation cephalosporins. Since the selective process occurs within days of treatment with these broad-spectrum agents, it is associated with a high rate of therapeutic failure (Sanders, 1983; Dworzack *et al.*, 1987). With the combination of repeated selection and strain spread, the proportion of derepressed mutants has risen to 15–40% of the inducible bacterial population in major hospitals in North America and Western Europe (Livermore, 1995) and up to 70% in Athens (Tzelepi *et al.*, 1992). Once selected, these mutants are stable, can be transferred from patient to patient and may in time, constitute a major proportion of the isolates of that species in a particular hospital.

The likelihood of selection depends on the infection site, the bacterial population density and the level of available drug. In *Enterobacter* spp. bacteraemia, selection is estimated to occur in approximately 20% of cases treated with third-generation cephalosporins and

is probably higher in similarly treated pneumonias (Chow *et al.*, 1991). The probability of derepressed mutants predominating in an inducible population *in vivo* also varies with the status of the patient, being most likely to occur in chronic infections or where the host is immuno-compromised and thus dependent on the antibiotic for eradication of the bacteria.

Monotherapy with third-generation cephalosporins therefore seems inadvisable for serious infections caused by species where there is a strong likelihood of stably-derepressed variant selection. Since the proportion of stably-derepresssed variants is a function of the organism and not the antibiotic, there is little reason to suppose that one inducer will be more selective than another. Minor variations in selectivity may arise between those antibiotics that kill bacteria rapidly thereby reducing the period for mutant segregation and those that are more slowly bactericidal. In the past, therapeutic approaches to the treatment of derepressed mutants used a double-β-lactam combination or a β-lactam drug in combination with a second non-β-lactam agent such as an aminoglycoside, to inhibit the small population of mutants. However, the literature is now replete with reports of the failure of these combinations to prevent the emergence of multiple resistance to the β-lactam components of these regimens (Sanders & Sanders, 1988; Chow *et al.*, 1991). Consequently, the treatment of choice for organisms exhibiting derepression has moved to fourth-generation cephalosporins or carbapenems.

Unlike their predecessors, fourth-generation cephalosporins, such as cefepime and cefpirome, combine β-lactamase stability and low affinity for the chromosomal enzymes (low V_{max}/K_m) with rapid cell penetration (Jones *et al.*, 1997). These agents can reach the PBP targets in the presence of β-lactamase over-production. Similarly, carbapenems such as imipenem, although strong inducers of class C enzymes, are highly stable to the activity of the enzyme and penetrate into the periplasmic space rapidly thus remaining active against bacteria expressing high levels of β-lactamase (Livermore, 1995).

2.3.1.4. **Extended AmpC β-lactamase activity**

Although AmpC β-lactamase has high affinity for the extended-spectrum cephalosporins, the enzyme has a very low rate of hydrolysis. Thus, cephalosporin resistance will only arise in derepression where the constitutive over-production and

accumulation of enzyme can compensate for this low rate of hydrolysis (Livermore, 1985; Hiraoka *et al.*, 1988). In recent years, however, AmpC β-lactamases with extended cephalosporin activity have been found in isolates of *E. cloacae* and *S. marcescens* (Nukaga *et al.*, 1995; Matsumura *et al.*, 1998). The first report of a clinical isolate expressing an extended-spectrum class C β-lactamase capable of hydrolysing broad-spectrum cephalosporins occurred in *E. cloacae* in Japan in 1992 (Nukaga *et al.*, 1995). The *E. cloacae* isolate was found to produce an AmpC β-lactamase with extended substrate specificity and demonstrated cephalosporin MIC values 8–64 times higher than the *E. cloacae* isolate in which AmpC β-lactamase was originally characterised (i.e. *E. cloacae* P99) (Lindberg & Normark, 1987). A comparison of the amino acid sequence of the extended-spectrum β-lactamase with AmpC from *E. cloacae* P99 revealed a three amino acid insertion in the active site of the enzyme which was shown to confer the extended β-lactamase profile. More recently, an extended-spectrum AmpC-type β-lactamase with activity against third- and fourth-generation cephalosporins was also produced in *E. cloacae* following *in vitro* antibiotic selection (Morosini *et al.*, 1998). Similarly, extension of the substrate specificity of AmpC β-lactamase by an amino acid substitution was also described in a laboratory-generated derivative of *C. freundii* (Tsukamoto *et al.*, 1990). The evolution of extended-spectrum AmpC β-lactamase indicates the potential of all AmpC-bearing species (including non-inducible isolates) to exhibit cephalosporin resistance in the absence of mutation to high-level β-lactamase over-production.

2.3.2. **Plasmid-encoded β-lactamases**

2.3.2.1. **Classical β-lactamases**

Prior to the 1980's, the most common cause of β-lactam resistance in GNBs was mediated by classical plasmid-encoded β-lactamases such as TEM-1, TEM-2, SHV-1, OXA-1, PSE-1 and PSE-4. These enzymes are most commonly produced by the *Enterobacteriaceae* family, but are occasionally reported in other GNBs such as *P. aeruginosa* (Chen *et al.*, 1995). The most prevalent of these enzymes is TEM-1. First described in *E. coli* in 1965 (Matthew, 1979), TEM-1 has spread to 20-60% of enterobacteria. The widespread dissemination of TEM-1 is largely attributed to the fact that the gene encoding the β-lactamase is frequently located on a promiscuous

transposable element, Tn*3* (Du Bois *et al.*, 1995). A few other classical plasmid-encoded β-lactamases such as TEM-2, SHV-1 and OXA-1 have also become widespread in enterobacteria but TEM-1 remains ten times more prevalent than these (Livermore, 1995). The classical plasmid-encoded β-lactamases confer resistance to all anti-Gram-negative penicillins, except temocillin and to the earlier cephalosporins, but have minimal activity against the majority of broad-spectrum β-lactam agents and are intrinsically susceptible to the common β-lactamase inhibitors (Table 2.5). Hyperproduction of these β-lactamases can, however, overwhelm the action of the inhibitor and permit hydrolysis of the β-lactam combination.

2.3.2.2. **Extended-spectrum β-lactamases**

For the first 20 years after their discovery, the classical plasmid-encoded β-lactamases showed rapid dissemination to many enterobacterial species in the absence of any change in kinetics. In the early 1980's, resistance to the newer 'β-lactamase-stable' cephalosporins such as cefotaxime, ceftazidime and ceftriaxone began to appear in clinical isolates of *K. pneumoniae* and *E. coli* (Knothe *et al.*, 1983). This resistance was caused by mutations in the common TEM and SHV β-lactamases which extended the hydrolytic spectrum of the enzymes to include broad-spectrum agents (Table 2.5). Over 75 TEM and 20 SHV extended-spectrum β-lactamases (ESBLs) are now recognised and the number continues to grow. In Europe, recent surveys estimate that in general, ESBL production in enterobacteria has risen from 0.9% in 1991 (Sirot *et al.*, 1992) to 3.2% in 1998, with figures increasing to 7% in units with specialised care such as ICU (De Champs *et al.*, 2000). ESBLs are also emerging in large medical centers in America (Rice *et al.*, 1990; Burwen *et al.*, 1994). Whilst ESBL production continues to predominate in *K. pneumoniae*, occurring in 10% of all hospital isolates and 25% of isolates from specialised units (De Champs *et al.*, 2000), ESBLs are also reported with increasing frequency in other enterobacteria including *E. coli*, *E. cloacae*, *Enterobacter aerogenes*, *P. mirabilis*, *M. morganii*, *Shigella dysenteriae*, *Salmonella* spp. and *S. marcescens* (Jacoby & Medeiros, 1991; Marchandin *et al.*, 1999; De Champs *et al.*, 2000).

Table 2.5. Plasmid-encoded β-lactamase expression and resistance phenotypes in common Gram-negative pathogens.

Organism and β-lactamases	Antibiotic susceptibility pattern for commonly used β-lactam antibiotics*												
	Amp	Amox + clav	Tic	Tic+ clav	Pip	Pip+ tazo	Cxm	Ctx/ Ctr	Caz	Cpm	Fox	Azt	Mer
Resistance conferred by plasmid encoded β-lactamases													
TEM-1, TEM-2, SHV-1 (low-level)	●	□	●	□	◆	○	○	○	○	○	○	○	○
TEM-1, TEM-2, SHV-1 (high-level)	●	●	●	●	●	□	○	○	○	○	○	○	○
TEM/SHV-derived ESBLs (broad-spectrum§)	●	□	●	□	●	□	●	●	●	◆	○	●	○
TEM/SHV-derived ESBLs (ceftazidimase†)	●	□	●	□	●	□	◆	◆	●	◆	○	◆	○
TEM/SHV-derived ESBLs (marginal‡)	●	□	●	□	●	□	◆	◆	◆	◆	○	◆	○
OXA-1	●	●	●	●	◆	□	○	○	○	○	○	○	○
PSE-1, PSE-4	●	□	●	□	●	□	○	○	○	○	○	○	○
PER-1	●	○	●	○	□	□	●	●	●	●	○	●	○
IMP-1	●	●	●	●	–	–	●	●	●	●	●	○	●
Sme-1, Imi-1, NMC-A	●	○	●	○	●	●	–	□	□	–	○	●	◆
Plasmid encoded AmpC	●	●	●	●	●	●	●	●	●	□	●	●	○

*, β-lactam antibiotics abbreviations are as follows: Amp, ampicillin; Amox+clav, amoxycillin-plus-clavulanate; Tic, ticarcillin; Tic+clav, ticarcillin-plus-clavulanate; Pip, piperacillin; Pip+tazo, piperacillin-plus-tazobactam; Cxm, cefuroxime; Ctx, cefotaxime; Caz, ceftazidime; Cpm, cefepime; Fox, cefoxitin; Azt, aztreonam; Mer, meropenem. Resistance phenotypes are as follows: ●, resistant; ◆, may appear susceptible if high breakpoints are used, but likely to be clinically resistant; ○, susceptible as normal phenotype; □, susceptibility reduced compared to normal phenotype;–, no data available.

§, broad-spectrum mutants of TEM and SHV include: TEM-3, -4, -8, -9, -14, -15, -19, -24, SHV-2, -3, -4, -5, -6; †, ceftazidimase mutants of TEM and SHV include: TEM-5, -6,-10, -16, -26; ‡, TEM and SHV mutants with marginal activity include: TEM-7, -11, -12. Adapted from Livermore, 1998.

2.3.2.3. Clinical implications of ESBL production

Many ESBL-producing isolates have been involved in serious outbreaks of infection (Johnson *et al.*, 1992; Meyer *et al.*, 1993). Epidemiological studies have suggested that in some cases, outbreak organisms appeared to have spread between hospitals (Arlet *et al.*, 1994) and that resistance-determinants were transmitted *in vivo* to other bacteria (Rice *et al.*, 1990; Marchandin *et al.*, 1999). ESBL-mediated resistance is, however, more likely to evolve at a local level rather than as a result of bacterial spread. Most ESBL outbreaks in institutions are associated with selective pressure following heavy use of extended-spectrum cephalosporins (Meyer *et al.*, 1993) and lapses in infection control measures (Philippon *et al.*, 1989; French *et al.*, 1996). Analysis of ESBL-producing isolates demonstrates the *de novo* nature of this resistance. In 1988, a hospital in California adopted ceftazidime as the single drug of choice for treating febrile neutropenic children. However, ceftazidime-resistant isolates of *K. pneumoniae* and *E. coli* that produced a TEM-26 ESBL were soon isolated (Naumovski *et al.*, 1992). Some time later, the same prescribing policy was employed in the paediatric oncology unit of a hospital in the UK and again many patients became infected with ceftazidime-resistant *K. pneumoniae* producing a TEM-26 ESBL. Molecular analysis of the ESBL-producing isolates from both hospitals showed significant amino acid differences in the enzymes. The fact that these two phenotypically indistinguishable enzymes appear to have evolved via different routes indicates convergent evolution of ESBL-mediated resistance in response to similar selective antimicrobial pressure (Hibbert-Rogers *et al.*, 1994).

Although most ESBLs remain highly susceptible to β-lactamase inhibitors, other extended-spectrum derivatives are now emerging with low-level inhibitor-resistance (Perilli *et al.*, 1997; Sirot *et al.*, 1997). Other TEM and SHV mutants which lack extended cephalosporin activity have also been found to mediate high-level β-lactamase-inhibitor resistance (Zhou *et al.*, 1994). Inhibitor-resistant derivatives of TEM-1 have been detected in isolates of *Klebsiella* spp. (Sirot *et al.*, 1998) and *P. mirabilis* but have been found most commonly in *E. coli.* In one French hospital study, 5% of all urinary *E. coli* isolates were reported to produce inhibitor-resistant TEM β-lactamases (Henquell *et al.*, 1994).

2.3.2.4. **Non-TEM, non-SHV ESBL derivatives**

Many non-TEM, non-SHV, plasmid-encoded ESBL-derivatives have been observed in recent years. Two groups of class A ESBLs, CTX-M and PER-type, which are distantly related to chromosomal-borne enzymes, have emerged. CTX-M-type enzymes have been reported in clinical isolates of *E. coli*, *K. pneumoniae*, *P. mirabilis*, *C. freundii* and *S. marcescens* from many diverse locations. PER-type β-lactamases which confer resistance to all cephalosporins, aztreonam and penicillins have been found predominantly in clinical isolates of *P. aeruginosa* (Nordmann *et al.*, 1993) but also in rare isolates of *S. typhimurium* (Vahaboglu *et al.*, 1995). Other non-TEM, non-SHV, class D ESBLs detected in *P. aeruginosa* include the OXA-derivatives (Chen *et al.*, 1995; Mugnier *et al.*, 1998). Although the OXA ESBLs confer reduced susceptibility to a wide range of β-lactam agents, ceftazidime resistance seems to be the most reliable phenotypic marker. This enzyme has also been reported in French and Italian isolates of *Acinetobacter baumanii* demonstrating that it has the potential for spread (Vahaboglu *et al.*, 1998). The presence of these enzymes is probably underestimated because of the low awareness of ESBLs in organisms other than *Enterobacteriaceae* and also because ceftazidime resistance in *P. aeruginosa* is usually associated with other resistance mechanisms.

2.3.2.5. **Plasmid-encoded AmpC β-lactamase enzymes**

Perhaps more worrying than the spread of TEM and SHV mutants is the emergence of plasmid-mediated AmpC β-lactamases such as BIL-1 (Payne *et al.*, 1992b), LAT-1 (Tzouvelekis *et al.*, 1993) and MIR-1 (Papanicolaou *et al.*, 1990) (Table 2.6). These are AmpC enzymes encoded by genes that have escaped from the chromosome of *Enterobacter* and *Citrobacter* spp.. As plasmid-mediated AmpC β-lactamases, these enzymes have achieved worldwide distribution and have the ability to demonstrate highly resistant profiles resembling those of derepressed *Enterobacter* isolates (Table 2.5). These enzymes are usually encoded on large plasmids, which also contain genes encoding resistance to other classes of antimicrobials (Pornull *et al.*, 1994) and have been associated with resistance to all antibiotics except carbapenems, fourth-generation cephalosporins and fluoroquinolones. Since the movement of *ampC* genes from chromosome to plasmid has increased distribution of multiple antimicrobial resistance to common nosocomial pathogens such as *E. coli* and *Klebsiella* spp. (Livermore, 1995)

Table 2.6. Plasmid-mediated AmpC β-lactamases.

Family	AmpC origin	β-lactamase	MIC (mg/l) *				First detected
			Ctx	Caz	Fox	Atm	
LAT	*C. freundii*	LAT-1	128	64	64	32	Greece, 1993
		LAT-2	4	8	32	4	Greece, 1995
		LAT-3	128	128	256	64	Greece, 1997
		LAT-4	4	8	64	8	Greece, 1997
		CMY-2	32	128	256	64	Greece, Turkey, Germany, US, 1990
		CMY-3/4	128	64	256	32	France, Tunisia, 1996
		BIL-1	16	64	ND	ND	Pakistan, 1989
FOX	*P. aeruginosa*	FOX-1	4	8	128	0.5	Argentina, 1989
		FOX-2	16	128	256	64	Guatemala, 1993
		FOX-3	2	16	64	2	Italy, 1994
		CMY-1	4	16	64	ND	Korea, 1989
		MOX-1	512	16	ND	16	Japan, 1991
Entb	*E. cloacae*	MIR-1	64	128	512	128	US, 1988
		ACT-1	2	4	256	2	US, 1994
Morg	*M. morganii*	DHA-1	128	ND	512	4	Saudia Arabia, 1992
Hafnia	*H. alvei*	ACC-1	ND	ND	ND	ND	Germany, 1997

*, mean MIC values for various bacterial hosts; ND, not determined.
B-lactam antibiotics are abbreviated as follows: Ctx, cefotaxime; Caz, ceftazidime; Fox, cefoxitin; Atm, aztreonam.

that do not themselves produce inducible AmpC chromosomal β-lactamase, this form of resistance is a true cause for concern.

2.3.3. Carbapenem-hydrolysing β-lactamases

The carbapenem-hydrolysing enzymes are the most diverse of all β-lactamases. In the past, there were relatively few of these enzymes. However, as carbapenem usage increased so did the appearance of carbapenem-hydrolysing β-lactamases. To date, five class A carbapenem-hydrolyzing β-lactamases have been described, Sme-1 to 3, NMC-A and Imi-1 (Table 2.7). These enzymes are chromosomally-encoded and have been detected in small numbers of clinical isolates of *S. marcescens* (Yang *et al.*, 1990) and *E. cloacae* (Rasmussen *et al.*, 1996). Although these carbapenemases can attack penicillins and carbapenems they are largely ineffective against cephalosporins (Table 2.5).

Although rare, class B metallo-carbapenem β-lactamases are of increasing clinical significance, especially in Japan. When produced at high levels, these β-lactamases can produce resistance to all carbapenems, penicillins and cephalosporins. Several species of *Aeromonas* and *Bacillus* and *Stenotrophomonas maltophilia* have been shown to express inducible chromosomally-mediated metallo-β-lactamase that hydrolyses imipenem (Livermore, 1997) (Table 2.7). A plasmid-encoded metallo-β-lactamase, IMP-1, was originally reported in isolates of *P. aeruginosa* and *S. marcescens* (Osano *et al.*, 1994). Subsequently, IMP-1 has also dispersed into other enterobacteria, including *Acinetobacter* spp., *Enterobacter* spp., *E. coli*, *P. vulgaris* and *K. pneumoniae* (Kurokawa *et al.*, 1999). To date these isolates are mostly recovered from patients in Japanese hospitals, but other reports of IMP-1-producing isolates from Singapore and Italy are evidence of a wider distribution. More recent descriptions of similar plasmid-mediated β-lactamases such as MET-1 in isolates of *Shigella flexneri* (O'Hara *et al.*, 1998) and *P. aeruginosa* (Cardosa *et al.*, 1999) clearly indicate that the initial spread of mobile metallo-β-lactamases has commenced.

Table 2.7. Carbapenem-hydrolysing β-lactamases.

Type of β-lactamase	Molecular class Ambler (1980)	Functional group Bush (1995)	Carbapenemases
Serine β-lactamase	A	2f	Sme-1 to Sme-3, MNC-A and Imi-1
	C	1	AmpC (marginal activity when produced at high-level)
	D	2d	ARI-1 (OXA-23), ARI-2
Metallo-β-lactamase	B	3	Chromosomal enzymes of *B. fragilis*, *Aeromonas* spp. and *S. maltophilia*
	B	3	Plasmid-encoded enzymes: IMP-1, MET-1, VIM-1

2.4. NON-B-LACTAMASE-MEDIATED B-LACTAM RESISTANCE IN GNB

2.4.1. PBP modification

PBP-mediated resistance may arise through mutations which alter existing PBPs so that they lose their affinity for β-lactams. Alternatively, bacteria may acquire altered PBPs that are absent from susceptible strains (Livermore, 1991b). This mechanism of resistance is more important in Gram-positive cocci and fastidious Gram-negative bacteria such as *Haemophilus influenzae* and *Neisseria gonorrhoeae*. It is rare in nosocomial GNBs but has been found in *P. aeruginosa* (Godfrey *et al.*, 1981) and in isolated cases of *S. marcescens* (Gutmann & Chabbert, 1984). In these instances, there is evidence to suggest that a reduction in the permeation of β-lactam antibiotics was also required to produce clinically significant levels of resistance (Malouin & Bryan, 1986).

2.4.2. Permeability

It has long been recognised that the ability of β-lactamase enzymes to mediate resistance in GNB is intrinsically linked to the rate of β-lactam permeation through the outer membrane (Richmond, 1978). The rate of permeation depends on the hydrophobicity, the molecular weight and overall charge of the antibiotic. Penicillins and cephalosporins have strong carboxylic acid groups. At neutral pH these compounds will exist as carboxylate ions which are unlikely to cross the lipid bilayer domains of the outer membrane. Thus for most β-lactam antibiotics which have relatively hydrophilic side-chains, diffusion across the outer membrane is predicted to occur through the aqueous channels or pores which are composed of 'porin' proteins (Nikaido, 2000) (Figure 2.5). Two major porins of *E. coli*, OmpC and OmpF, and presumably analogous structures in other enterobacteria, are usually associated with altered permeability to antimicrobial agents. Mutational loss of these porins in GNB is associated with increased resistance to slow penetrating penicillins and cephalosporins (Yoshimura & Nikaido, 1985). In the absence of β-lactamase, however, a decrease in the permeability of the outer membrane via porin mutation has little effect on the overall susceptibility to β-lactam drugs because the amount of drug entering the cell is still sufficient to saturate the PBP targets (Nikaido, 1989). A few clinical isolates of these porin-mutants have

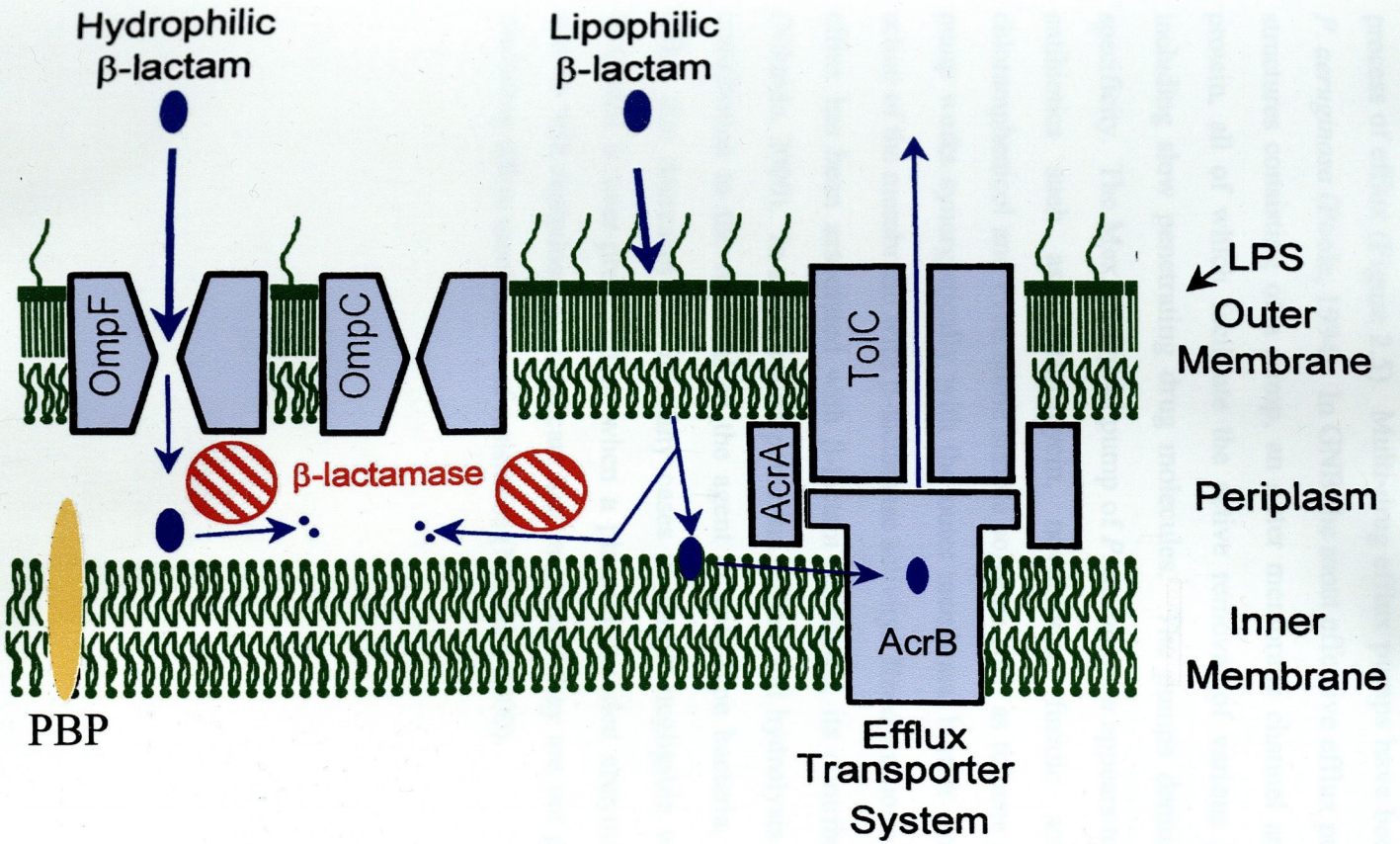


Figure 2.5. Entry of β-lactam molecules into the periplasm of *E. coli*. Hydrophilic compounds cross the outer membrane via porin channels. Lipophilic compounds cross the outer membrane mainly through the lipid bilayer domains. Compounds of both types may then be hydrolysed by the periplasmic β-lactamases. Lipophilic compounds may also be pumped out by the AcrAB-TolC multidrug efflux machinery. The steady-state concentration of drugs in the periplasm is determined by the balance among all these processes. Adapted from Nikaido, 2000.

been reported in some enterobacteria (Nikaido, 1989) but in general, this type of resistance is more often seen in clinical isolates of *P. aeruginosa* (Chen *et al.*, 1995).

Evidence now suggests that permeability-based resistance may also operate through a process of efflux (Figure 2.5). Multi-drug efflux pumps have been found in *E. coli* and *P. aeruginosa* (Poole, 1994). In GNB the most effective efflux pumps are multi-subunit structures consisting of a pump, an outer membrane channel and a membrane fusion protein, all of which facilitate the active removal of various secondary metabolites including slow penetrating drug molecules. The pumps demonstrate wide substrate specificity. The MexAB-OprM pump of *P. aeruginosa* appears to catalyse the efflux of antibiotics such as erythromycin, novobiocin, fusidic acid, tetracycline and chloramphenicol and even some simple solvents such as toluene (Nikaido, 2000). The pump works synergistically with the outer membrane barrier similar to the combined action of the membrane with β-lactamase activity. Recent reports indicate that although efflux has been associated with β-lactam resistance, its contribution may be limited (Nikaido, 2000). In studies with penicillin, efflux and hydrolysis appear to make equal contribution to the removal of the agent in wild-type bacteria. The contribution of efflux also decreases and in many cases is almost negligible when chromosomal β-lactamase is over-produced or when a plasmid-encoded enzyme is present. Similar studies with cephalosporins indicate that in general they are not good substrates for the multidrug efflux mechanism of resistance (Nikaido, 2000).

2.5. B-LACTAM RESISTANCE IN *S. MARCESCENS*

In the past two decades, GNB have developed many mechanisms of resistance to combat the widespread use of broad-spectrum β-lactam agents. They have not only evolved to allow for increased production of existing β-lactamases but also for the production of modified enzymes with extended substrate profiles and decreased susceptibility to β-lactamase inhibitors. One of the most striking examples of the potential of GNB to demonstrate resistance to β-lactam agents is *S. marcescens*. This organism demonstrates most, if not all, common modes of β-lactam resistance.

2.5.1. Chromosomal β-lactamase expression in *S. marcescens*

Similar to other enterobacteria such as *E. cloacae* and *C. freundii*, *S. marcescens* also expresses class C inducible β-lactamase which is capable of undergoing derepression or constitutive over-production (Tajima *et al.*, 1981; Nomura & Yoshida, 1990). The selection of derepressed isolates in *S. marcescens* is thus associated with β-lactam resistance and clinical failure (Follath *et al.*, 1987; Hechler *et al.*, 1989). However, compared to other enterobacteria with inducible AmpC β-lactamase production, derepression in *S. marcescens* appears to occur at a lower level.

Enterobacteria which express inducible AmpC β-lactamase can be divided into two groups on the basis of the degree of resistance conferred by derepression (Stapleton *et al.*, 1995a). The first group consists of *E. cloacae* and *C. freundii* where derepression confers high-level resistance to all third-generation cephalosporins, including cefotaxime and ceftazidime. In the second group which includes *S. marcescens*, *M. morganii* and *P. stuartii*, lower levels of resistance are conferred by derepression (Curtis *et al.*, 1986; Sanders & Sanders, 1986). The heterogeneity of AmpC β-lactamase activity is also evident from differences in the induction profile of organisms with inducible β-lactamase. In *C. freundii* and *E. cloacae*, cefoxitin acts as a strong labile inducer of β-lactamase expression. In *S. marcescens*, *M. morganii* and *P. stuartii*, however, cefoxitin appears more stable to the action of the β-lactamase, with inducible and derepressed isolates demonstrating only moderate levels of resistance. The *in vivo* selection of derepressed mutants also occurs more rarely in clinical isolates of *S.*

marcescens compared to other enterobacteria with inducible AmpC β-lactamase (Sanders & Sanders, 1986).

Evidence thus suggests that in *S. marcescens*, derepression is not associated with the same high-level β-lactam resistance seen in other AmpC-inducible enterobacteria. It is likely that differences in the level of resistance demonstrated by *S. marcescens* relate to differences in the level of expression of the AmpC β-lactamase. However, as yet, the genetic apparatus responsible for the control of inducible AmpC β-lactamase expression in *S. marcescens* has not been identified.

Recent reports also indicate that in *S. marcescens*, AmpC may mediate broad-spectrum cephalosporin resistance not only through β-lactamase over-production but also by extension of its substrate spectrum. Although rare, clinical isolates of *S. marcescens* have been reported where extended-spectrum cephalosporin resistance has arisen through mutations which affect the active site of the enzyme (Matsumura *et al.*, 1998; Raimondi *et al.*, 2001). Moreover, laboratory-derived mutants of *S. marcescens* have been shown to exhibit constitutive over-production of these altered β-lactamase enzymes and thus achieve combined high-level extended-spectrum β-lactam resistance. The production of extended-spectrum chromosomal β-lactamase in *S. marcescens* creates a further opportunity for this organism to exhibit broad-spectrum cephalosporin resistance in addition to mutation to constitutive β-lactamase over-production.

2.5.2. **ESBL expression in *S. marcescens***

In addition to chromosomal β-lactamase expression, *S. marcescens* is also host to a variety of plasmid-encoded β-lactamases. Like most other enterobacteria, *S. marcescens* frequently express classical plasmid-encoded β-lactamases such as TEM-1, which mediate resistance to penicillins and narrow-spectrum cephalosporins. This organism is also associated with the expression of ESBLs. Along with *K. pneumoniae*, *S. marcescens* was implicated in the first report of ESBL-mediated resistance (Knothe *et al.*, 1983). Subsequently, ESBL-producing *S. marcescens* have been involved in both individual episodes and nosocomial outbreaks of infection. In 1987, characterisation of a ceftazidime-resistant clinical isolate of *S. marcescens* in Belgium revealed production

of a novel TEM-derived ESBL, designated TEM-E4 (Payne *et al.*, 1992a). In 1994, other TEM-derived ESBLs were also found in clinical isolates of *S. marcescens* involved in a serious outbreak of infection affecting 43 patients in an ICU in Italy (Pagani *et al.*, 1994). In the latter case, the ESBL designated 'TEM-AQ', mediated high-level resistance to both ceftazidime and aztreonam and low-level resistance to β-lactam inhibitors (Perilli *et al.*, 1997) and was considered a contributing factor in the epidemic spread of the organism. Furthermore, when *S. marcescens* was implicated in another serious outbreak of infection in the same unit less than a year later, high-level broad-spectrum β-lactam resistance was again attributed to ESBL production, but this time resulting from expression of an SHV derivative (Luzzaro *et al.*, 1998).

Dissemination of a large self-transmissible plasmid encoding an SHV-derived ESBL with broad-spectrum cephalosporin and monobactam activity has also been reported in isolates of *S. marcescens* from five different hospitals in Greece (Gianneli *et al.*, 1994). More recent reports indicate the production of a non-TEM, non-SHV, CTX-M-like ESBL from a clinical isolate of *S. marcescens* in Brazil, characterised by high-level resistance to cefotaxime and aztreonam (Bonnet *et al.*, 2000). *Serratia marcescens* may thus acquire and express many different types of plasmid-mediated β-lactamases, which can confer extended anti-β-lactam activity and inhibitor resistance.

2.5.3. **Carbapenemase expression in *S. marcescens***

Although carbapenem resistance is rare in most enterobacteria, *S. marcescens* has been found to express two separate classes of carbapenemases. Sme-1, a class A chromosomal β-lactamase with activity against carbapenems and monobactams, was first described in a clinical isolate of *S. marcescens* in London in 1982 (Naas *et al.*, 1994; Naas *et al.*, 1995). Since then three separate reports of this carbapenemase production have appeared. The *S. marcescens* isolates involved were recovered from different regions in the US, suggesting that convergent evolution of this enzyme may have occurred following selection with increased clinical use of imipenem (Queenan *et al.*, 2000). The increased use of carbapenems in Japan is also associated with the expression of a class B plasmid-encoded metallo-carbapenemase in *S. marcescens* (Ito *et al.*, 1995). This IMP-1 carbapenemase, mediates high-level cross-resistance to

cephalosporins and carbapenems and is now found in up to 4.4% of *S. marcescens* isolates in Japan (Arakawa *et al.*, 2000).

2.5.4. **Permeability**

Although there are many reports of the isolation of β-lactam-resistant porin-deficient mutants in the laboratory, in enterobacteria porin-deficient mutants are rarely seen clinically. This is probably because these permeability mutants have difficulty accumulating nutrients or because their altered surface prevents adhesion to human cells. However, unlike other enterobacteria, *S. marcescens* is an exception to this rule. At least three separate reports of porin-deficient mutants have been published involving clinical isolates of *S. marcescens*. Goldstein *et al.* reported the appearance of an amikacin- and cefotaxime-resistant mutant of *S. marcescens* where the expression of the 41 kDa outer membrane protein was decreased (Goldstein *et al.*, 1983). Sanders *et al.* found that decreased levels of a 41 and 38 kDa outer membrane protein in *S. marcescens* corresponded to the acquisition of β-lactam- and aminoglycoside resistance (Sanders & Watanakunakorn, 1986). Traub *et al.* described a further clinical case involving the emergence of β-lactam- and aminoglycoside-resistant *S. marcescens* permeability mutants with decreased expression of three outer membrane proteins (Traub & Bauer, 1987).

The fact that permeability mutants are readily selected in *S. marcescens* suggests that this organism cannot survive attack by broad-spectrum cephalosporins solely through over-production of chromosomal β-lactamase (Nikaido, 1989). Studies investigating the contribution of over-produced chromosomal β-lactamase in *S. marcescens* indicate the secondary involvement of porin defects in high-level cephalosporin cross-resistance (Hechler *et al.*, 1989; Weindorf *et al.*, 1998).

2.6. CONCLUSIONS

The ability of *S. marcescens* to demonstrate multiple mechanisms of β-lactam resistance is well documented. Nevertheless, little is understood concerning the control of β-lactamase expression in this organism. This is especially true in the case of inducible AmpC chromosomally-encoded β-lactamase production. Studies have confirmed that the production of AmpC β-lactamase and the level of resistance in derepressed *S. marcescens* is significantly less than in other enterobacteria. However, whilst variations in the level of enzyme production and resistance have been reported, little information exists to explain the differences in the level of expression or even to confirm the clinical significance of chromosomal AmpC β-lactamase production in *S. marcescens*.

Chapter III

Serratia marcescens in Dublin hospitals: clinical background & design of study

Chapter III

Serratia marcescens in Dublin hospitals: clinical background & design of study

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3.1. THE HISTORY OF *S. MARCESCENS* IN DUBLIN HOSPITALS

3.1.1. 1982–1984

In Ireland, *S. marcescens* was first reported as a cause of nosocomial infection in the early 1980's in a group of three closely associated adult general hospitals in Dublin. This group of hospitals consisted of one large tertiary-referral teaching hospital (Hospital 1) and two other smaller acute teaching hospitals (Hospitals 2 and 3) (which formed part of the Federated Dublin Voluntary Hospital Group). The first outbreak was reported in Hospital 2 in 1982. In this instance, *S. marcescens* was implicated as the causative agent in two simultaneous outbreaks involving seven wards including the ICU (Coleman *et al.*, 1984). The outbreak involved 30 patients who were either on peritoneal dialysis or presented with bacteriuria. Two strains of *S. marcescens* were recovered during the outbreak, both exhibiting the O14 serotype. One strain demonstrated a multi-resistant phenotype, with antimicrobial resistance determinants encoded on a large transmissible plasmid. The majority of isolates of the second strain were largely susceptible to antimicrobials. No common source was identified for the outbreak.

3.1.2. 1984–1995

In the three years following this outbreak, *S. marcescens* was occasionally isolated from patients in the hospital group. From 1987 onwards, however, the prevalence in these hospitals increased, with *S. marcescens* being isolated from more than 60 patients per year. This increase was especially marked in Hospital 1 where more than 70% of the total number of affected patients were located. In 1990, the problem was exacerbated by a further outbreak of *S. marcescens* infection in Hospital 1 (Hejazi, 1997). The 1990 outbreak primarily affected patients in the ICU and persisted for three years during which time the annual isolation rate of *S. marcescens* from patients in the hospital increased from 54 in 1989 to 119 in 1991 (Figure 3.1). Extensive surveillance screening cultures failed to yield a common source for the outbreak but in 1992, the annual number of affected patients in Hospital 1 decreased to 73 following the opening of a new ICU. Epidemiological typing revealed that one strain of *S. marcescens* was predominant throughout the outbreak and was isolated from 70% of affected patients. The strain exhibited the O14:K14 serotype but was distinct from either of the previous

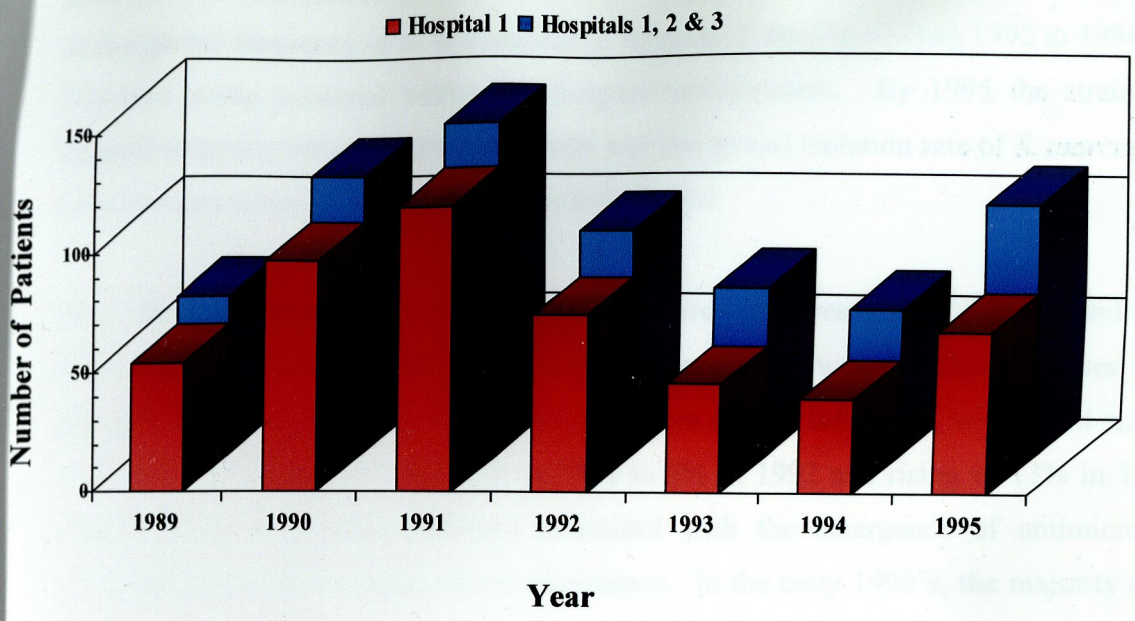

Figure 3.1. The number of patients from whom *S. marcescens* isolates were recovered in Hospital 1 and in Hospitals 1, 2 and 3 from 1989 to 1995.

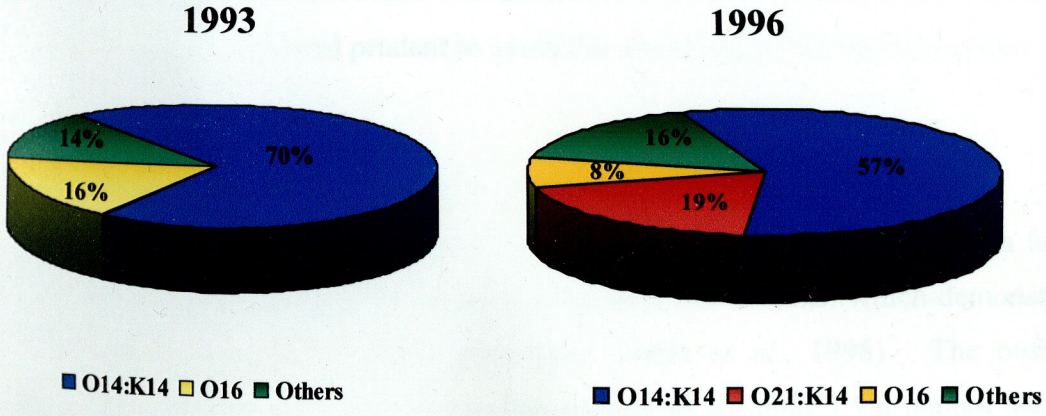

Figure 3.2. Distribution of serotypes of *S. marcescens* isolates recovered from Hospitals 1, 2 and 3 in 1993 and 1996.

outbreak strains isolated in Hospital 2. A second strain of *S. marcescens* with an O16 serotype was also identified in 1992 but was found in only 13% of affected patients. Although the frequency of *S. marcescens* in Hospital 1 decreased from 1992 to 1994, the O14:K14 strain persisted within the hospital environment. By 1995 the strain had become endemic within all three hospitals and the annual isolation rate of *S. marcescens* from patients in the hospital group increased to 104.

Over 50% of *S. marcescens* isolates were recovered from respiratory samples and most of the remainder were isolated from urine specimens and wound cultures. From 1991 onwards, *S. marcescens* was associated with more serious infections, with blood culture isolation rates increasing from 1% in 1990 to 5% in 1992 and rising to 13% in 1995. This increase in serious infection coincided with the emergence of antimicrobial resistance within this *S. marcescens* population. In the early 1990's, the majority of *S. marcescens* isolates recovered from patients in the hospital group remained susceptible to the antimicrobials commonly used in the treatment of nosocomial infection. By 1995, however, there was a notable increase in resistance to antimicrobial agents such as cephalosporins and fluoroquinolones (Hejazi, 1997). With increasing levels of antimicrobial resistance, therapeutic options became limited. This was especially true in the case of resistance to broad-spectrum agents such as cefotaxime. Since resistance to β-lactam drugs such as cefotaxime was attributed to AmpC chromosomal β-lactamase production, it was considered prudent to avoid the use of any of the cephalosporins.

3.1.3. **1996–2000**

Emerging antimicrobial resistance became a bigger problem in 1996 following a further outbreak of infection involving an unusual strain of *S. marcescens,* which demonstrated a multiple antimicrobial resistance phenotype (Herra *et al.*, 1998). The outbreak originated in the ICU of Hospital 2 but later spread to Hospital 3 via patient transfer. The outbreak strain expressed low-level resistance to cefotaxime, ciprofloxacin and gentamicin, antimicrobials commonly used in first line therapy. Patients thus required treatment with meropenem, an agent normally kept in reserve. Epidemiological typing revealed that all isolates implicated in the outbreak exhibited an uncommon serotype, O21:K14. Using PFGE, DNA macrorestriction profiles (following *Xba*I restriction endonuclease digestion of total cellular DNA) showed that the isolates were closely

related, clustering at 90% similarity. A contaminated bed-pan macerator in the ICU of Hospital 2 was identified as a possible source of infection and continued spread of the organism probably occurred via hand transmission by hospital personnel. Although the outbreak was brought under control following implementation of improved infection control measures, a significant number of O21 isolates continued to be recovered from Hospitals 2 and 3 and also from Hospital 1. By 1996, 19% of all *S. marcescens* isolates recovered from the hospital group exhibited the O21:K14 serotype (Figure 3.2). At this time O21:K14 *S. marcescens* isolates were also recovered from other hospitals in the Dublin region and caused an outbreak in one of these unrelated Dublin hospitals. The DNA profiles of the outbreak strain were similar to the patterns obtained for the O21:K14 isolates recovered from Hospitals 2 and 3. Thus it appeared that a second *S. marcescens* strain had become endemic in Dublin hospitals. The emergence of this unusual multi-resistant strain of *S. marcescens,* superimposed on a well-established and increasingly resistant serratia population, exacerbated an already intractable problem (Herra *et al.*, 1998).

A further outbreak of the multi-resistant O21:K14 strain in Hospital 1 in 1998 reaffirmed the role of *S. marcescens* as a persistent nosocomial pathogen (Knowles *et al.*, 2000). The outbreak involved 24 patients in the Bone Marrow Transplantation and Oncology Unit. Many of the patients involved in the outbreak demonstrated prolonged colonisation which frequently led to invasive infection during neutropenic episodes. The high rate of colonisation provided a reservoir for cross-infection and spread. The multi-resistant profile of the O21:K14 outbreak strain required that infected patients be treated with a combination of antimicrobials including meropenem. The antimicrobial resistance of the strain also necessitated inclusion of meropenem in first-line therapy in the Unit for the duration of the outbreak. Concerns regarding the use of meropenem as empiric treatment for prolonged periods of time were compounded by reports of emerging carbapenem resistance in *S. marcescens* (Naas *et al.*, 1994; Ito *et al.*, 1995).

In late 1998, Hospitals 2 and 3 were closed and their services were relocated to a new facility in Dublin. In the new hospital the prevalence of *S. marcescens* was less than it had been in the former hospitals, but the organism continued to be isolated infrequently. In contrast, the prevalence of *S. marcescens* in Hospital 1 continued to rise. Recent studies in Hospital 1 have indicated that patients with *S. marcescens* infection are likely

to experience prolonged carriage of the organism, with 65% of patients demonstrating carriage at more than one site and 43% of patients demonstrating carriage at multiple sites (Byrne *et al.*, 2001). Similarly a significant level of carriage was found in non-infected patients in units where the organism was prevalent. The high rate of colonised patients within the hospital thus provides a constant reservoir for re-infection. As the rate of infection continues to rise in the hospital so too do levels of antimicrobial resistance. From 1998 onwards, the rates of resistance to commonly used agents such as ciprofloxacin and cefotaxime have shown marked increases in Hospital 1.

3.2. CLINICAL BACKROUND OF STUDY

By 1995, the clinical importance of GNBs expressing inducible chromosomal AmpC β-lactamases had been recognised. The potential of organisms such as *E. cloacae* and *C. freundii* to yield stably derepressed mutants with the ability to express high-level β-lactam resistance was well documented (Bennett & Chopra, 1993; Ehrhardt & Sanders, 1993). Resistance to all broad-spectrum β-lactams had emerged in up to 44% of *E. cloacae* and *C. freundii* recovered from infections treated with third-generation cephalosporins (Sanders & Sanders, 1988). Multiply-resistant derepressed mutants of these organisms were reported to have disseminated widely and had accumulated within the environment of many hospitals (Anonymous, 1987).

At this time, the mechanism of AmpC induction had been investigated in organisms such *E. cloacae* and *C. freundii*. The two major genes involved in control of AmpC expression in both these species, *ampR* (Honore *et al.*, 1986; Lindquist *et al.*, 1989b) and *ampD* (Lindberg *et al.*, 1987; Korfmann *et al.*, 1991), had been characterised. Derepression of AmpC β-lactamase had been linked to mutations in the *ampD* gene (Stapleton *et al.*, 1995b). Mutations in the regulatory *ampR* gene had also been shown to alter AmpC expression (Lindberg & Normark, 1987; Bartowsky & Normark, 1991). The first evidence of *ampC*-mutants with extended-spectrum AmpC β-lactamase activity had also been described in clinical isolates of *E. cloacae* (Nukaga *et al.*, 1995). However, in other enterobacteria such as *S. marcescens* and *M. morganii* little was known concerning the clinical significance of inducible β-lactamase production let alone the genetic apparatus involved in the control of AmpC induction or the mutations leading to altered AmpC expression.

In 1995, *S. marcescens* had emerged as a serious nosocomial pathogen in the Dublin hospital group. A retrospective review of *S. marcescens* in these hospitals indicated that in the eight years since the organism was originally recognised as a frequent source of infection, there was an increase in the number of isolates demonstrating antimicrobial resistance (Hejazi, 1997). This increase was most evident in the case of β-lactam agents with high numbers of isolates demonstrating low-level resistance to many of the

commonly used cephalosporins. The true extent and nature of this cephalosporin resistance was, however, unknown.

In enterobacteria, such as *S. marcescens* which express inducible AmpC β-lactamase, cross-resistance to cephalosporins is normally associated with over-production or derepression of the β-lactamase. It thus appeared likely that the cephalosporin resistance shown in *S. marcescens* isolates from the Dublin hospital group resulted from derepression of AmpC β-lactamase. In other enterobacteria, derepression confers high-level cross-resistance to all third-generation cephalosporins. In the *S. marcescens* isolates under review, however, only low-level resistance was demonstrated to agents such as cefotaxime and susceptibility was maintained to other third-generation agents such as ceftazidime. If derepression was occurring in these isolates, the resultant AmpC β-lactamase over-production was associated with lower levels of cephalosporin resistance compared to other enterobacteria. It was also possible, however, that the cephalosporin resistance demonstrated by the *S. marcescens* isolates might have resulted from other β-lactam resistance mechanisms such as ESBLs or reduced permeability.

3.3. STUDY DESIGN

The present study was undertaken to determine whether the cephalosporin resistance in *S. marcescens* isolates from the hospital group was mediated by AmpC β-lactamase and if so, to investigate the mechanism whereby AmpC over-production conferred low-level resistance in these isolates.

The first part of the study was designed to:

- **determine the rate of cephalosporin resistance in the serratia population**

  The prevalence of *S. marcescens* and the rate of cephalosporin resistance in the hospital group was determined using laboratory records gathered during the six years of the study period.

- **investigate phenotypic expression of the cephalosporin resistance**

  Phenotypic expression of resistance was analysed to investigate whether the cephalosporin resistance was due to AmpC β-lactamase production. The pattern of resistance was investigated by qualitative susceptibility testing. The level of cephalosporin resistance was determined using quantitative susceptibility testing. The production of AmpC β-lactamase was characterised using isoelectric focusing. Expression of AmpC β-lactamase was determined using microbroth dilution induction techniques, disc induction methods and β-lactam hydrolysis assays. The presence of plasmid-encoded ESBLs was investigated using the disc synergy test and cephalosporin combination disc susceptibility methods.

- **confirm the involvement of AmpC β-lactamase**

  To provide additional evidence that the cephalosporin resistance was mediated by AmpC β-lactamase production, other likely sources of β-lactamase-mediated resistance were excluded using genotypic resistance detection methods. Plasmid analysis was performed to investigate the presence of ESBLs. Isolates were also screened for the presence of TEM and SHV-derived ESBLs using TEM and SHV-specific PCR and direct PCR product sequencing.

The second part of the study was designed to elucidate the mechanism of low-level AmpC-mediated cephalosporin resistance in *S. marcescens* by:

- **characterising the putative *ampD* and *ampR* regulatory genes and the *ampC* structural gene**

  The *ampD* gene in *S. marcescens* was localised using DNA hybridisation analysis and suspect *ampD*-like fragments were cloned and sequenced. International DNA sequence databases were interrogated to investigate whether this putative *ampD* sequence showed significant identity with known *ampD* sequences from other enterobacteria. The presence of the *ampD* gene in additional clinical isolates of *S. marcescens* was investigated by DNA sequence analysis of PCR products amplified using *S. marcescens*-specific *ampD* primers. The presence of the *ampC* and *ampR* genes, including the *ampC-ampR* intergenic region, was determined following DNA amplification and PCR product sequencing.

- **analysing the sequence and role of the *ampD* gene**

  The *ampD* gene sequences from cephalosporin-resistant and cephalosporin-susceptible *S. marcescens* isolates were analysed to determine if *ampD* was implicated in AmpC-mediated cephalosporin resistance. The DNA sequences were aligned and examined to determine if mutations within *ampD* similar to those described in other enterobacteria were also associated with derepressed phenotypes in *S. marcescens*. The role of the *ampD* gene in altered AmpC expression was also investigated using transformation studies with a recombinant plasmid encoding *ampD* from wild-type *E. cloacae*.

- **analysing the sequence of the *ampR* and *ampC* genes**

  The *ampC* and *ampR* gene sequences and the *ampC-ampR* intergenic region from cephalosporin-resistant and cephalosporin-susceptible *S. marcescens* isolates were analysed to investigate their role in altered AmpC expression. DNA sequences of the genes were aligned and examined for evidence of genetic alteration.

The prevalence studies and the phenotypic and genotypic investigations were performed using the patient populations and *S. marcescens* isolates outlined in Table 3.1.

Table 3.1. Details of patient populations and *S. marcescens* isolates used in the present study.

Investigation	Collection	No. of isolates	Time period	Selection criteria
Prevalence				**Patients**
Hospital 1	....	526	1995–2000	Patients from whom isolates were recovered*
Hospital 1, 2 & 3	....	361	1995–1997	Patients from whom isolates were recovered*
Cephalosporin resistance rate				
Hospital 1	....	319	1995–2000	Patients with cefotaxime-resistant isolates* †
Phenotypic studies				**Isolates**
Pilot study	Pilot§	43	1995§	AmpC-inducible GNBs
Pattern of cephalosporin resistance	Pattern‡	351	1995-1998	Resistance to cefotaxime†
Level of cephalosporin resistance	A	64	1995-1998	Difference in cefotaxime susceptibility category
B-lactam hydrolysis assays	D	9	1995-1998	Difference in AmpC phenotype
Genotypic studies				
Plasmid DNA analysis	A	64	1995-1998	Difference in cefotaxime susceptibility category
PCR for TEM- and SHV- derived ESBLs	B	21	1995-1998	Difference in AmpC phenotype
PCR for identification of *ampD* & *ampR*	B	21	1995-1998	Difference in AmpC phenotype
Hybridisation assays for *ampD* & *ampR*	C	10	1995-1998	Difference in AmpC phenotype
Sequence analysis of *ampD*, *ampR* & *ampC*	D	9	1995-1998	Difference in AmpC phenotype

...., no isolates were collected, data were generated from patient reports in the routine diagnostic microbiology laboratory;
*, repeat isolates recovered from patients during the same episode of infection or during a period of extended colonisation were excluded from these collections;
§, pilot collection included all AmpC-inducible GNBs recovered from sputum, blood and wound specimens (July to September, 1995);
†, isolates exhibiting resistance and moderate resistance using modified Stokes' disc diffusion method (Stokes & Ridgway, 1987);
‡, cephalosporin resistance patterns were determined prospectively against cefotaxime, ceftazidime, piperacillin and piperacillin-plus-tazobactam.

Chapter IV

Phenotypic expression of β-lactam resistance in *Serratia marcescens* recovered from three Dublin Hospitals

Chapter IV

Phenotypic expression of β-lactam resistance in *Serratia marcescens* recovered from three Dublin hospitals

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4.1. INTRODUCTION

This study was designed to determine the rate of cephalosporin resistance in *S. marcescens* isolates recovered from the hospital group and to investigate if the resistance was due to AmpC β-lactamase production. Laboratory records were used to determine the overall prevalence of *S. marcescens*. Using cefotaxime as an indicator β-lactam agent, the rate of cephalosporin resistance in the serratia population was calculated from computer records of cefotaxime-resistant isolates. In addition, the rates of resistance to ciprofloxacin and gentamicin were also determined.

To investigate if the cephalosporin resistance recorded was mediated by AmpC β-lactamase production, the phenotypic expression of resistance was analysed. These studies were conducted in four stages. In the first year of the investigation, a three-month pilot study was set up to investigate the presence of AmpC-mediated cephalosporin resistance in *S. marcescens* isolates and in other inducible AmpC-producing GNBs recovered in the hospital group. B-lactam resistance was analysed using disc diffusion susceptibility testing and microdilution broth minimum inhibitory concentration (MIC) techniques. MIC induction assays and disc induction methods were used to investigate the expression of inducible β-lactamase. Disc synergy tests were used to screen for the production of plasmid-mediated ESBLs in *S. marcescens* isolates. The value of isoelectric focusing in the characterisation of AmpC β-lactamase production was also assessed for *S. marcescens* isolates.

The second stage of the study investigating phenotypic expression of resistance was designed to analyse the pattern of β-lactam resistance in the serratia population. From 1995 to 1998, all *S. marcescens* isolates exhibiting resistance to cefotaxime were tested against a panel of four indicator broad-spectrum β-lactam agents using disc diffusion susceptibility testing. The pattern of β-lactam resistance in *S. marcescens* isolates from the hospital group was compared with resistance in *S. marcescens* isolates recovered from unrelated hospitals in Ireland. The presence of inducible β-lactamase was determined on all cefotaxime-resistant isolates using disc induction tests.

The third stage of the study assessed the level of β-lactam resistance in the serratia population. A selection of cefotaxime-susceptible and cefotaxime-resistant *S. marcescens* isolates collected each year from 1995 to 1998 was investigated. Quantitative susceptibility testing was performed against a panel of five indicator β-lactam agents using the Etest (AB Biodisk) MIC method. Expression of AmpC β-lactamase was determined using disc induction tests. The isolates were screened retrospectively for ESBL production using the cefpodoxime combination disc susceptibility method (Oxoid Ltd.) that became available in 2000 (Carter *et al.*, 2000).

In the final stage of the phenotypic studies, the expression of AmpC β-lactamase was characterised using β-lactam hydrolysis assays. The specific activity of the β-lactamase expressed in cefotaxime-susceptible and cefotaxime-resistant *S. marcescens* isolates was determined by measuring the rate of cephalothin hydrolysis in the presence and in the absence of a strong inducer.

4.1.1. B-lactam susceptibility test methods

Previous studies have shown the interpretive value of antibiograms in predicting β-lactamase activity (Liu *et al.*, 1992). In the present study, β-lactam susceptibility testing was performed using a panel of β-lactam antibiotics chosen to facilitate recognition of resistance patterns associated with particular β-lactamase types. The panel of β-lactam agents included antibiotics in common use e.g. cefotaxime, antibiotics not usually used therapeutically e.g. ticarcillin and antibiotics used alone and in combination with β-lactam inhibitors e.g. piperacillin and piperacillin plus tazobactam. Susceptibility testing was performed using qualitative disc diffusion methods and quantitative MIC techniques including the MIC microdilution broth assay (NCCLS, 1993) and the Etest method. In the latter technique, MICs are determined following diffusion of a preformed antibiotic gradient from an inert plastic strip. In the pilot study, MIC values were determined using microdilution broth assays. Previous reports have shown agreement between microdilution broth MIC and Etest MIC values for organisms with inducible β-lactamase production (Knapp & Washington, 1992). Since Etests are quick and easy to perform, they were used during the remainder of the study.

4.1.2. Investigation of AmpC β-lactamase expression

Inducible AmpC β-lactamase expression is mediated by a chromosomally-located resistance determinant. With the exception of a very small number of isolates that can only produce minimal amounts of enzyme regardless of the presence of inducers (basal producers), nearly all isolates of GNBs known to express inducible AmpC are capable of producing the β-lactamase enzyme (see Section 2.3.1). Routine testing of clinical isolates of these organisms for production of AmpC is seldom necessary. Identification of the organism should be sufficient in most instances to alert the clinician that the isolate has the potential to develop multiple cephalosporin resistance (Sanders & Sanders, 1988).

To characterise expression of inducible AmpC β-lactamase, it is necessary to investigate if production of the β-lactamase is in the inducible state or if it has mutated to a level of constitutive over-production. Traditionally, expression of AmpC is investigated by measuring the enzymatic activity of the β-lactamase, in the presence and in the absence of a strong inducing agent. B-lactamase activity is determined using hydrolysis assays which monitor the rate of hydrolysis of a β-lactam substrate over time. A significant increase in the level of β-lactamase activity in the presence of the inducer is indicative of the presence of inducible β-lactamase. Conversely, assuming the level of activity is sufficiently high to exclude basal production, failure to exhibit increased β-lactamase activity in the presence of the inducer implies constitutive over-production.

The expression of AmpC β-lactamase may also be characterised using MIC induction assays, where the MIC values of indicator β-lactam agents are determined in the presence and in the absence of a strong inducer (Tausk *et al.*, 1985; Chandler & Nolte, 1987). As with hydrolysis assays, a significant (three-fold) increase in the MIC of the indicator β-lactam in the presence of the inducer suggests the presence of inducible β-lactamase production. Constitutive high-level production is inferred in the absence of elevated MICs (assuming MIC values are sufficiently high to outrule basal production).

In the diagnostic laboratory, AmpC expression is more commonly investigated using disc induction methods such as the disc approximation test (DAT) where the inhibitory zone of a β-lactam agent is truncated in the presence of a strong inducing agent. In the

DAT originally proposed by Sanders *et al.* in 1979 (Sanders & Sanders, 1979), a cefoxitin disc was used as the strong inducer and was placed adjacent to cefamandole, the indicator β-lactam. If the zone of inhibition of the test β-lactam adjacent to the inducer disc was flattened by ≥4 mm, the presence of inducible β-lactamase was inferred. In the absence of truncation of the inhibition zone, constitutive high-level production was implied. The DAT was later modified to include cefotaxime as the indicator β-lactam (Thore *et al.*, 1989). Over time, other modifications of the original DAT method have been suggested. Some authors reported that a breakpoint of 4 mm was artificial and proposed that any demonstrable truncation of the cefotaxime zone edge should be considered an indicator of antagonism (Chandler & Nolte, 1987). Other workers used imipenem instead of cefoxitin as the strong inducer (Tausk *et al.*, 1985). In the present study, optimum antimicrobial/inducer disc combinations and inhibition zone breakpoint criteria were determined and the results were correlated with those obtained using microdilution broth MIC induction assays (Tausk *et al.*, 1985). A preliminary investigation in the laboratory had shown good agreement between the DAT and the microdilution broth MIC induction assay when a ≥2 mm reduction in the inhibition zone was accepted as the criterion for a positive DAT.

4.1.3. **Detection of ESBLs**

Although cephalosporin resistance in *S. marcescens* is characteristically associated with the production of AmpC β-lactamase, the contribution of ESBL-mediated resistance must also be considered. To date both TEM and SHV-derived ESBLs have been found to mediate broad-spectrum cephalosporin-resistance in *S. marcescens* e.g. TEM-E4 (Payne *et al.*, 1992a), TEM-AQ (Perilli *et al.*, 1997) and SHV-like (Gianneli *et al.*, 1994; Luzzaro *et al.*, 1998) (see Section 2.5.2). In addition, *S. marcescens* is also host to more unusual non-TEM, non-SHV, CTX-M-like ESBLs (see Section 2.5.2, paragraph two), which also confer resistance to β-lactams such as cefotaxime and aztreonam (Bonnet *et al.*, 2000). It was therefore necessary to investigate the possible involvement of ESBL-mediated resistance in the cephalosporin-resistant *S. marcescens* isolates in this study.

The classical TEM and SHV β-lactamases have activity against penicillins and narrow-spectrum cephalosporins. The degree of resistance conferred on substrates depends on

the amount of enzyme, which can vary up to 150-fold among isolates. This reflects gene dosage and promoter efficiency. Even at low levels, these TEM and SHV enzymes confer unequivocal resistance for penicillins and narrow-spectrum cephalosporins. Most ESBLs are mutants of TEM-1, TEM-2 and SHV-1 with no more than one to four amino-acid sequence substitutions. These changes, which amount to less than 2% of the protein sequence, are sufficient to remodel the enzyme active site to allow activity against almost all cephalosporins. However, although ESBLs are generally considered to have extended activity against broad-spectrum cephalosporins, mutations that broaden the specificity of these enzymes tend to lower their hydrolytic efficiency and often result in enhanced but low-level resistance.

Some ESBLs such as TEM-3 and SHV-2 mediate clearcut resistance to all extended-spectrum cephalosporins and to aztreonam. In other ESBLs such as TEM-12 a single point mutation at the active site of TEM-1 results in high-level resistance to cefuroxime but only minimal resistance to larger cephalosporin molecules such as ceftazidime. A second mutation occurring in the TEM-1 active site e.g. TEM-26 would facilitate efficient hydrolysis of ceftazidime but only raise the MIC of other cephalosporins marginally (Liu *et al.*, 1992; Jacoby, 1994). Thus, ESBLs may produce *in vivo* resistance and yet demonstrate *in vitro* susceptibility to many cephalosporins with MIC values below the breakpoints (Rice *et al.*, 1991). In Europe, it is estimated that up to 35% of ESBL-producing organisms are incorrectly reported as susceptible to cephalosporins such as cefotaxime and ceftriaxone (Carter *et al.*, 2000). The failure to detect ESBL producers not only puts infected patients at risk of receiving inappropriate therapy but also makes it difficult for hospitals to design and implement effective antibiotic policies. In one report, an outbreak involving ESBL-producing *K. pneumoniae* escaped detection for over a year because isolates appeared susceptible by disc diffusion susceptibility testing (Meyer *et al.*, 1993).

In the past, many laboratories screened for the presence of ESBL-producing *K. pneumoniae* and *E. coli* by monitoring slight decreases in susceptibility zones to indicator drugs (NCCLS, 1997). The rationale for this is derived from the fact that although some ESBL producers fall into the susceptible category for agents such as cefotaxime and ceftazidime, they are less susceptible than isolates producing the classical plasmid-mediated β-lactamases. This method has been shown to lack both

sensitivity and specificity because slight decreases in susceptibility are not readily recognised. In a recent study, less than 80% of ESBL-producing *K. pneumoniae* and *E. coli* were correctly identified using disc susceptibility testing (Coudron *et al.*, 1997). Similarly MIC dilution techniques have also been shown to be unreliable for the detection of ESBLs. Using an agar dilution MIC technique, Thomson *et al.* noted that ESBL-producing *K. pneumoniae* and *E. coli* were detected at rates of 43%, 79%, 57% and 93% in tests with cefotaxime, aztreonam, ceftriaxone and ceftazidime, respectively (Thomson & Sanders, 1997).

Since ESBL-mediated resistance may not be readily detected using routine antimicrobial susceptibility testing, many clinical laboratories have adopted more specialised methods. The majority of these methods are based on the principle of the disc synergy test (DST), where a β-lactamase inhibitor is used to potentiate the activity of the indicator drug against an ESBL-producing isolate by enhancing the zone of inhibition (Jarlier *et al.*, 1988; Thomson & Sanders, 1992; Katsanis *et al.*, 1994). Such plate synergy tests often require repeated performance to determine optimium disk positioning (Thomson *et al.*, 1991). Disc synergy methods are also unreliable for screening organisms such as *S. marcescens* which produce chromosomal AmpC β-lactamase that is resistant to the inhibitor and may mask ESBL detection (Bush *et al.*, 1991; Thomson & Sanders, 1992) and which are also known to produce inhibitor-resistant ESBLs (Perilli *et al.*, 1997) (see Section 2.5.2).

In 1999, new approaches to ESBL detection focused on the use of standardised susceptibility test methods in the presence and in the absence of inhibitors. Originally these methods employed standard disc diffusion protocols with single indicator cephalosporin agents such as ceftazidime (NCCLS, 2000). More recent studies expanded this approach and recommended the use of additional indicator cephalosporins such as cefpodoxime for improved detection of ESBLs (Carter *et al.*, 2000). Nevertheless, despite evidence of increased sensitivity, the application of these combination disc susceptibility screening methods remains limited in isolates that produce AmpC β-lactamase and/or inhibitor-resistant ESBLs.

In the present study, the DST was used to screen *S. marcescens* isolates collected in 1995 for ESBL production. A selection of *S. marcescens* isolates collected between

1995 and 1998 was also screened retrospectively for ESBL production using the cefpodoxime combination disc susceptibility method.

4.1.4. **Isoelectric focusing**

For the last 20 years, thin layer polyacrylamide gel isoelectric focusing (IEF) has played a major role in the identification and characterisation of β-lactamases. The method offers a high degree of resolution and can distinguish enzymes that focus only 0.05 pI apart. In the focusing system, β-lactamase enzymes are concentrated and separated in polyacrylamide gels and are subsequently visualised using a chromogenic cephalosporin substrate, nitrocefin, which changes to a red colour following degradation by the enzyme (Matthew *et al.*, 1975). This technique of separation and specific staining of β-lactamases allows demonstration of low levels of activity and permits visual comparison of patterns of β-lactamase bands produced by enzymes from different organisms. The use of supplementary β-lactamase inhibitor and substrate hydrolysis overlay procedures facilitates differentiation between chromosomal and plasmid-mediated β-lactamases (Sanders *et al.*, 1986).

As a consequence of the exponential increase in the numbers of β-lactamases recognised in the last 10 years, IEF no longer provides sufficient resolution to differentiate the majority of β-lactamases. Hence, IEF was considered inappropriate for ESBL screening in the present study. Nevertheless, because the pI value is regarded as an essential determinant for characterisation of β-lactamase enzymes, the value of IEF in the investigation of AmpC β-lactamase expression in *S. marcescens* was assessed.

4.2. MATERIALS AND METHODS

4.2.1. Determination of the extent of cephalosporin resistance

For the first three years of the study, from 1995 to 1997, Hospitals 1, 2 and 3 were located in close proximity to each other and were served by the same centralised diagnostic microbiology laboratory, located in Hospital 1. All *S. marcescens* isolates and patient data from the hospital group were available for these three years. At the end of 1998, Hospitals 2 and 3 were relocated to a new facility in the Dublin region with separate laboratory services. From 1999 onwards, *S. marcescens* isolates and patient data were only available from Hospital 1.

4.2.1.1. Determination of the prevalence of *S. marcescens*

The annual prevalence of *S. marcescens* was determined from computer lists of the total number of *S. marcescens* isolates recovered from Hospitals 1, 2 and 3 from 1995 to 1997 and from Hospital 1 alone from 1995 to 2000. In both cases, repeat isolates recovered from patients during the same episode of infection i.e. within a three-week period, or during a period of extended colonisation were excluded. Computer generated lists of the number of *S. marcescens* isolates were an accurate reflection of the total number of isolates recovered because standard protocol in the diagnostic laboratory required identification of all clinically significant GNBs from all sites.

4.2.1.2. Determination of the rate of cephalosporin resistance

Using cefotaxime as an indicator third-generation cephalosporin, the annual percentage of cephalosporin-resistant isolates was calculated from computer records of all cefotaxime-resistant *S. marcescens* isolates recovered in Hospital 1 from 1995 to 2000. Rates of resistance to ciprofloxacin and gentamicin were also determined. For all three antibiotics, resistance was inferred if isolates were reported as resistant or moderately-resistant by the diagnostic laboratory. Repeat isolates recovered from patients during the same episode of infection or during a period of extended colonisation were excluded.

4.2.2. Phenotypic expression of β-lactam resistance

All laboratory studies investigating the phenotypic expression of resistance were performed using *S. marcescens* isolates recovered in the diagnostic microbiology

laboratory from 1995 to 1998 and included isolates from all three hospitals. These isolates were routinely identified using the API 20E identification system (bio-Mérieux SA, Marcy L'Etoile, France) and arabinose and raffinose peptone water sugars. Susceptibility of isolates to a range of commonly used antibiotics was also tested in this laboratory using a modified Stokes' disc diffusion method (Stokes & Ridgway, 1987).

4.2.2.1. **Pilot study**

Bacterial isolates

From July to September, 1995, a pilot study was performed on all *S. marcescens* isolates and on all other inducible AmpC β-lactamase-producing GNBs recovered from sputum, wound and blood specimens in the diagnostic microbiology laboratory. All GNBs identified by the API 20E as *S. marcescens* (n=43), *E. cloacae* (n=58), *C. freundii* (n=3) and *M. morganii* (n=8), or by the API 20NE system (bio-Mérieux) as *P. aeruginosa* (n=91), were collected. No isolate of *Providencia* spp. was recovered during this time. Isolates were subcultured for purity on Columbia agar (Lab M, Bury, UK) supplemented with 7% (w/v) horse blood and frozen at –70 °C on Protect beads (Technical Service Consultants Ltd., Lancashire, UK). Isolates were removed from –70°C and subcultured on Columbia blood agar (CBA) on the day prior to testing.

Disc diffusion susceptibility testing

Susceptibility testing was performed using a modified Stokes' disc diffusion method (Stokes & Ridgway, 1987) on diagnostic sensitivity test agar (Oxoid Ltd., Basingstoke, UK), with either *E. coli* NCTC 10418 or *P. aeruginosa* NCTC 10662 as control isolates, as appropriate. The antibiotic disks (Oxoid) used were ampicillin (10 μg), augmentin (20 μg amoxycillin-plus-10 μg clavulanic acid), cefuroxime (30 μg), cefotaxime (30 μg) and ceftazidime (30 μg).

MIC microdilution broth assays

MIC values for cefotaxime (Sigma-Aldrich Ireland Ltd., Tallaght, Dublin, Ireland) were determined on all isolates, according to NCCLS recommendations for the microdilution broth method (NCCLS, 1993). Serial dilutions of the antibiotic were prepared and dispensed into 96 well U-shaped bottom microtitre trays (Sigma-Aldrich), to give a range of concentrations from 0.03 to 256 mg/l. Inocula were prepared by direct

suspension of bacterial colonies (from an 18 h culture on CBA) in Mueller-Hinton II broth (MHB) (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md., US) to achieve a density equivalent to a McFarland 0.5 turbidity standard and diluted to give a final bacterial density of 5×10^5 cfu/ml. Two reference strains, *E. coli* NCTC 10418 and *P. aeruginosa* NCTC 10662 were included as control isolates with each batch of tests. Microtitre trays were incubated at 37°C for 18 h. MICs were determined in duplicate and wells without antimicrobials were included as growth controls. The MIC was recorded as the lowest concentration of antibiotic at which no visible growth was observed by the unaided eye and the value was interpreted according to NCCLS performance standards for antimicrobial susceptibility testing (NCCLS, 1994).

MIC microdilution broth induction assays

MIC dilution induction assays were performed on all isolates using two β-lactam/inducer combinations, cefotaxime/cefoxitin and cefotaxime/imipenem. The MICs of the inducing agents, cefoxitin and imipenem were determined according to NCCLS recommendations (NCCLS, 1993). Cefoxitin and imipenem powder was kindly donated by Merck, Sharp & Dohme, Rahway, N.J., US. Serial dilutions of the antibiotics were prepared and dispensed into 96 well microtitre trays to give a range of concentrations from 0.12 to 128 mg/l for cefoxitin and from 0.03 to 32 mg/l for imipenem. Microtitre trays were inoculated, incubated and read as outlined in the cefotaxime MIC microdilution broth assay described above.

The MIC of the indicator β-lactam, cefotaxime, was determined in the presence and in the absence of sub-inhibitory concentrations of the inducing agents according to NCCLS recommendations (NCCLS, 1993). The sub-inhibitory inducer concentration was defined as one quarter the lowest MIC value of the inducing agent. Serial dilutions of cefotaxime covering a range of concentrations from 0.03 to 256 mg/l were prepared in the presence of a constant concentration of cefoxitin (4 mg/l for *S. marcescens* and *M. morganii* isolates and 16 mg/l for other GNBs) and in the absence of cefoxitin. Serial dilutions of cefotaxime were also prepared with imipenem (at a constant concentration of 0.05 mg/l) and without imipenem. Microtitre trays were inoculated in parallel, incubated for 18 h at 37°C and read as described above. An isolate was considered to express inducible β-lactamase when a three-fold or greater increase in the cefotaxime MIC was noted in the presence of an inducing agent.

Disc approximation test

The DAT was performed using cefotaxime/cefoxitin and cefotaxime/imipenem disc (Oxoid) combinations. Bacterial colonies were suspended in tryptone water (Oxoid) to a turbidity equivalent to a 0.5 McFarland standard and swabbed onto diagnostic sensitivity test agar. A cefoxitin disc (30 μg) was positioned next to a cefotaxime disc (30 μg) at a distance equal to the radius of the zone of inhibition produced by cefotaxime when tested alone– 15 mm for enterobacteria and 10 mm for *P. aeruginosa*. Similarly, an imipenem disc (10 μg) was positioned next to a cefotaxime disc on a separate agar plate. The plates were incubated at 37°C for 18 h. The degree of truncation was measured by comparing the radius of the zone of inhibition (measured from the disk centre) around the cefotaxime disc at the edge adjacent to the inducer with the radius of the zone of inhibition on the far side of the cefotaxime disc. An isolate was considered to express inducible β-lactamase when the radius around the cefotaxime disc on the side adjacent to the inducer was reduced by ≥2 mm (see Section 4.1.2, paragraph four).

Disc synergy test

All *S. marcescens* isolates (n=43) were screened for the presence of ESBLs using the double disk test described by Jarlier *et al.* (Jarlier *et al.*, 1988). Bacterial colonies were suspended in tryptone water (Oxoid) to a turbidity equivalent to a 0.5 McFarland standard and swabbed onto diagnostic sensitivity test agar. Two β-lactam indicator discs (Oxoid), cefotaxime (30 μg) and ceftazidime (30 μg) were placed 15 mm (edge to edge) from an augmentin disk (20 μg amoxycillin-plus-10 μg, clavulanic acid). Inoculated plates were incubated at 37°C for 18 h. An enhanced zone of inhibition between either of the β-lactam indicator agents and the augmentin disc was indicative of synergistic activity with clavulanic acid, i.e. the test organism was considered to express an ESBL. ESBL-producing *K. pneumoniae* isolates expressing either TEM-12 or SHV-4 were included as positive controls.

Isoelectric focusing

IEF was performed on all *S. marcescens* isolates (n=43) using a modification of the method described by Matthew *et al.* (Matthew *et al.*, 1975). The preparation of reagents

used in IEF is detailed in Appendix AI.1. The Coomassie Brilliant Blue method used for staining the IEF protein standards is described in Appendix AI.2.

Preparation of crude β-lactamase extracts

The *S. marcescens* isolates were inoculated into 10 ml brain heart infusion broth (Oxoid) and incubated for 14–16 h at 37°C with shaking (100 rpm). Bacterial cells were harvested by centrifugation at 2,500 x *g* for 10 min at 4°C in a Beckman J-6B centrifuge (Beckman Coulter, Fullerton, CA, US), washed and resuspended in 1 ml chilled 0.1 M phosphate buffer, pH 7.0. Cell suspensions were immersed in an ice water bath and disrupted by sonication using a Branson Ultrasonicator (Branson Ultrasonics, Hayes, Middlesex, UK) for three cycles of 30 s on and 30 s off. Cellular debris was removed by centrifugation at 17,400 x *g* for 30 min at 4°C using an Eppendorf 5417 microcentrifuge (Eppendorf GmbH, Hamburg, Germany). Aliquots of supernatant containing crude β-lactamase were stored at –70°C.

Isoelectric focusing

B-lactamase enzymes were analysed by isoelectric focusing on precast polyacrylamide gels (Ampholine PAGplate; Amersham Biosciences AB, Uppsala, Sweden) containing Pharmalyte in the pH range 3.5–9.5. Samples of β-lactamase extracts were thawed and diluted in 0.1 M phosphate buffer, pH 7.0, to a concentration such that a red colour developed within 1-2 min following dilution (1/2) in nitrocefin solution (50 μg/ml, Oxoid). Diluted samples (5 μl) were applied directly to polyacrylamide gels using an applicator strip. The gels were focused at 4°C for 90 min at 1500 V and 15 W in an LKB Multiphor II electrophoresis tank (LKB Instruments, Inc., Rockville, US) using 1 M H_3PO_4 and 1 M NaOH as anode and cathode solutions, respectively. *E. coli* isolates expressing either TEM-1 β-lactamase of pI 5.4 or SHV-1 β-lactamase of pI 7.6, were included as β-lactamase standards. Low (pH 2.5–6.5) and high (pH 5.0–10.5) pI calibration kit markers (IEF Calibration Kit; Amersham Biosciences) were included as protein standards.

Calculation of β-lactamase pI values

After focusing, the gel lanes containing the pI calibration markers were cut off and stained with Coomassie Brilliant Blue. The remainder of the gel was overlaid with 40

ml molten Bacto-Agar [3.8% (w/v), Difco Laboratories, Detroit, MI, US] containing 50 μg/ml nitrocefin. Hydrolysis of the nitrocefin was allowed to continue until the β-lactamase bands were visible (i.e. until red bands developed). The development time of the β-lactamase bands was noted and the migration distance of resolved β-lactamase bands from the cathodic edge of the gel was measured. The pI values of the resolved β-lactamases were calculated by reference to the standard calibration curve constructed by plotting the migration distance of the marker proteins and the standard β-lactamases (Y axis) against their corresponding pI values (X axis). The gel was photographed using a Polaroid MP-4 land camera [Polaroid (Europa) B.V., Enschede, The Netherlands] using a Tiffen 58 green filter (Tiffen Europe Ltd., Oxford, Oxfordshire, UK) and Polaroid type 51 high-contrast film (Sigma-Aldrich).

4.2.2.2. **Pattern of β-lactam resistance**

Bacterial isolates

The pattern of β-lactam resistance was determined on all 'cefotaxime-resistant' *S. marcescens* isolates (n=351) recovered from the hospital group from 1995 to 1998. The criterion for selection was all isolates reported as resistant or moderately-resistant to cefotaxime by the diagnostic laboratory. The pattern of resistance was also determined on 'cefotaxime-resistant' isolates from collections of *S. marcescens* recovered from three unrelated hospitals in Ireland. The isolates were donated by two Dublin hospitals (Hospital 4, n=9 and Hospital 5, n=15) and one hospital outside Dublin (Hospital 6, n=4). On receipt, isolates were tested for identity (using the API 20E system and arabinose and raffinose peptone water sugars), cultured for purity on CBA, tested for resistance to cefotaxime and frozen at –70°C on Protect beads. All isolates were removed from –70°C and subcultured on CBA on the day prior to testing.

Disc diffusion susceptibility testing and Disc approximation test

Susceptibility testing against cefotaxime (30 μg, Oxoid), ceftazidime (30 μg, Oxoid) piperacillin (75 μg, Oxoid) and piperacillin-plus-tazobactam (75-plus-10 μg, Oxoid) was performed on all 'cefotaxime-resistant' isolates using the modified Stokes' disc diffusion method (outlined in Section 4.2.2.1). The DAT test was performed on all isolates using the cefotaxime/imipenem disc combination method described in Section 4.2.2.1.

4.2.2.3. Level of β-lactam resistance

Bacterial isolates

Quantitative susceptibility testing was performed on 64 *S. marcescens* isolates recovered between 1995 and 1998 (Collection A). Sixteen isolates were collected each year. These comprised eight isolates that were cefotaxime-resistant or moderately-resistant (isolates used in Section 4.2.2.2) and eight cefotaxime-susceptible isolates. The susceptibility category was determined by routine disc diffusion testing. All isolates collected were checked for identity (using the API 20E system and arabinose and raffinose peptone water sugars), cultured for purity on CBA and frozen at –70°C on Protect beads. Isolates were removed from –70°C and subcultured on CBA on the day prior to testing.

Etest MIC determination

Etest (AB Biodisk, Solna, Sweden) MICs were performed on all *S. marcescens* isolates in Collection A using a panel of five indicator β-lactam agents including cefotaxime (0.016–256 mg/l), ceftazidime (0.016–256 mg/l), ticarcillin (0.016–256 mg/l), piperacillin (0.016–256 mg/l) and piperacillin-plus-tazobactam (0.016–256-plus-4 mg/l), in accordance with the manufacturer's instructions. Inocula were prepared by suspension of colonies in MHB to achieve a density equivalent to a 0.5 McFarland turbidity standard and swabbed onto Mueller-Hinton II agar plates (BBL). Etest strips were applied and plates were incubated for 16-18 h at 37°C. The MIC was read directly from the printed scale on the strip at the point where the elliptical zone of inhibition intersected the strip and the MIC value was interpreted according to NCCLS performance standards for antimicrobial susceptibility testing (NCCLS, 1994). Isolates exhibiting MIC values falling on susceptibility breakpoints were considered to demonstrate low-level resistance. Colonies growing within the elliptical zone were subcultured and re-tested. Two reference strains, *E. coli* NCTC 10418 and *P. aeruginosa* NCTC 10662 were included as controls in each batch of tests.

Disc approximation test

The DAT test was performed on the 64 *S. marcescens* isolates in Collection A using the cefotaxime/imipenem disc combination method described in Section 4.2.2.1.

Cefpodoxime combination disc susceptibility method

The 32 cefotaxime-resistant isolates in Collection A were screened for the presence of ESBLs using the Oxoid combination disc method (Carter *et al.*, 2000). Disc (Oxoid) susceptibility to cefpodoxime (10 μg) and cefpodoxime-plus-clavulanic acid (10 μg cefpodoxime-plus-1 μg clavulanic acid), was performed on Mueller Hinton agar in accordance with NCCLS recommendations (NCCLS, 2000). Following overnight incubation, zone diameters of the cefpodoxime disc and the cefpodoxime-plus-clavulanic acid combination disc were measured. The presence of an ESBL was inferred if the zone size of the combination disc was increased by ⩾5 mm. ESBL-producing *K. pneumoniae* isolates expressing either TEM-12 or SHV-4 were included as positive controls.

4.2.2.4. B-lactam hydrolysis assays

B-lactam hydrolysis assays were performed during a working visit to the Center for Research in Anti-Infectives and Biotechnology (CRAB), Creighton University School of Medicine, Nebraska, under the guidance of Dr. Nancy Hanson, Director of Molecular Biology for CRAB and Assistant Professor in the Department of Medical Microbiology and Immunology. The assays were later confirmed by Dr. Steve Mahlen in the Molecular Biology Laboratory in CRAB at Creighton University School of Medicine (MCRAB, Creighton University).

Prior to analysis, cefoxitin MICs were determined on the nine *S. marcescens* test isolates described below, using the Etest method (see Section 4.2.2.3). The hydrolysis assays were performed in the presence and in the absence of sub-inhibitory concentrations of cefoxitin i.e. at a concentration that was one quarter the lowest value obtained when cefoxitin MICs were performed on the nine test isolates. The preparation of reagents used in the hydrolysis assays is described in Appendix AI.3. The method for calculation of β-lactamase hydrolytic activity is detailed in Appendix AI.4. The assays were performed on three separate occasions.

Bacterial isolates

B-lactam hydrolysis assays were performed on nine isolates of *S. marcescens* from Collection A recovered between 1995 and 1998. These isolates comprised, three

cefotaxime susceptible isolates with cefotaxime MICs ≤0.25 mg/l, three cefotaxime low-level resistant isolates with cefotaxime MICs 8–16 mg/l and three cefotaxime resistant isolates with cefotaxime MICs 64 mg/l. These *S. marcescens* isolates had been inoculated onto nutrient agar slopes (Oxoid) and dispatched to MCRAB, Creighton University, prior to visiting that laboratory. Upon arrival in the laboratory, the isolates were subcultured onto CBA and frozen at –70°C. A *S. marcescens* reference isolate, Serr1, from MCRAB, Creighton University, was included as a wild-type control. The Serr1 strain had been characterised as wild-type, using β-lactam susceptibility testing and β-lactam hydrolysis assays (Morrow, 1999). All isolates were removed from –70°C and subcultured on CBA before testing.

Preparation of crude β-lactamase extracts

Isolates were inoculated into two 5 ml volumes of MHB and grown overnight at 37°C with shaking (100 rpm). Each overnight culture was diluted 1/20 in fresh MHB (5 ml culture plus 95 ml MHB broth) and incubated at 37°C with shaking (100 rpm) until the cultures reached a cell density which yielded an absorbance of 0.45 at 600 nm. One ml of inducing agent (cefoxitin 400 mg/l, Sigma Chemical Co., St. Louis, MO, US) was added to one of the cultures (induced culture) and 1 ml of sterile MHB broth was added to the other (uninduced) culture. Both cultures were incubated for a further 120 min. After incubation, induction was terminated by the addition of 1 ml of a 1 mM 8-hydroxyquinolone solution to the induced culture and to the uninduced control. The cultures were pelleted by centrifugation at 5,900 x *g* for 20 min at 4°C using a Sorvall RC2B centrifuge (Sorvall Inc. Newtown, CT, US). The supernatants were decanted, the pellets were washed with 10 ml 0.1 M phosphate buffer, pH 7.0 and re-centrifuged at 5,900 x *g* for 20 min at 4°C. Having decanted the supernatants the pellets were frozen overnight at –20°C. The pellets were thawed, resuspended in 4 ml phosphate buffer, pH 7.0, immersed in an ice water bath and disrupted by sonication using a Gallenkamp Soniprobe 150 sonicator (MSE Scientific Instruments, Sussex, UK) for 10 cycles of 10 s on and 10 s off. The sonicates were centrifuged at 5,900 x *g* for 60 min at 4°C in 15 ml conical-bottomed centrifuge tubes. The supernatants were divided into 1 ml aliquots and stored at –70°C. One aliquot of supernatant containing crude β-lactamase extract was diluted 1/10 in phosphate buffer, pH 7.0 and measured for total protein concentration using the Bio-Rad Protein Assay Kit 1 (Bio-Rad Laboratories, Hercules,

CA, US), which is based on the Bradford dye-binding protein quantification method (Bradford, 1976).

Hydrolysis assay

Hydrolysis assays were performed using a Coulter DU-7 spectrophotometer (Beckman Coulter) with a UV lamp and a water bath circulator to maintain the cuvette block at 37°C. Cephalothin solution (0.1 M) (Eli Lilly and Company, Indianapolis, Ind, US) was used as the β-lactam substrate for induced and uninduced β-lactamase extracts. The spectrophotometer was blanked with phosphate buffer, pH 7.0. A quartz cuvette containing 3 ml of cephalothin substrate solution was incubated at 37°C in the spectrophotometer for approximately 5 min. B-lactamase extract (100 µl) was added to the warmed cephalothin solution, the cuvette contents were mixed rapidly by inversion, replaced in the spectrophotometer and the rate of hydrolysis was monitored for 5 min. Absorbance was read at 265 nm. The rate of hydrolysis was calculated on a range of dilutions (1/100 to 1/1000) of induced and uninduced β-lactamase extracts. Hydrolysis activity was expressed as nmoles of cephalothin hydrolysed per min per mg of protein (Δnmoles/min/mg), in the linear phase of the reaction at 37°C.

4.3. RESULTS

4.3.1. Determination of the extent of cephalosporin resistance

4.3.1.1. Prevalence of *S. marcescens*

The annual prevalence of *S. marcescens* in the hospital group between 1995–1997 and in Hospital 1 between 1995–2000, is shown in Figure 4.1. Between 1995 and 1997, there was a sharp increase in the prevalence of *S. marcescens* in the hospital group involving more than 130 patients per annum. During these years, the annual isolation rate for Hospital 1 remained constant involving approximately 60 patients per annum. However, between 1997 and 2000 the isolation rate for Hospital 1 more than doubled.

4.3.1.2. Rate of cephalosporin (cefotaxime), ciprofloxacin and gentamicin resistance

The proportion of *S. marcescens* isolates recovered in Hospital 1 from 1995 to 2000 demonstrating resistance to cefotaxime (taken as an indicator of cephalosporin resistance) is shown in Figure 4.2. In 1995, cephalosporin resistance was present in 45% of *S. marcescens* isolates and by 1998 this figure had increased to over 60%. Figure 4.2 also shows the percentage of *S. marcescens* demonstrating resistance to ciprofloxacin and gentamicin. Between 1995 and 1998, the rate of resistance to ciprofloxacin increased from 17% to 50% of isolates. In the same years, the percentage of isolates demonstrating resistance to gentamicin also showed a marked increase from 0% in 1995 to 30% in 1998, although this rate subsequently fell to 10% in 2000.

4.3.2. Phenotypic expression of β-lactam resistance

4.3.2.1. Pilot study

During the pilot study, a total of 203 GNB isolates (*S. marcescens*, n=43; *P. aeruginosa*, n=91; *E. cloacae*, n=58; *C. freundii*, n=3 and *M. morganii*, n=8) were investigated. The results of disc diffusion susceptibility testing, MIC determination, MIC induction and disc induction tests are detailed in Table 4.1.

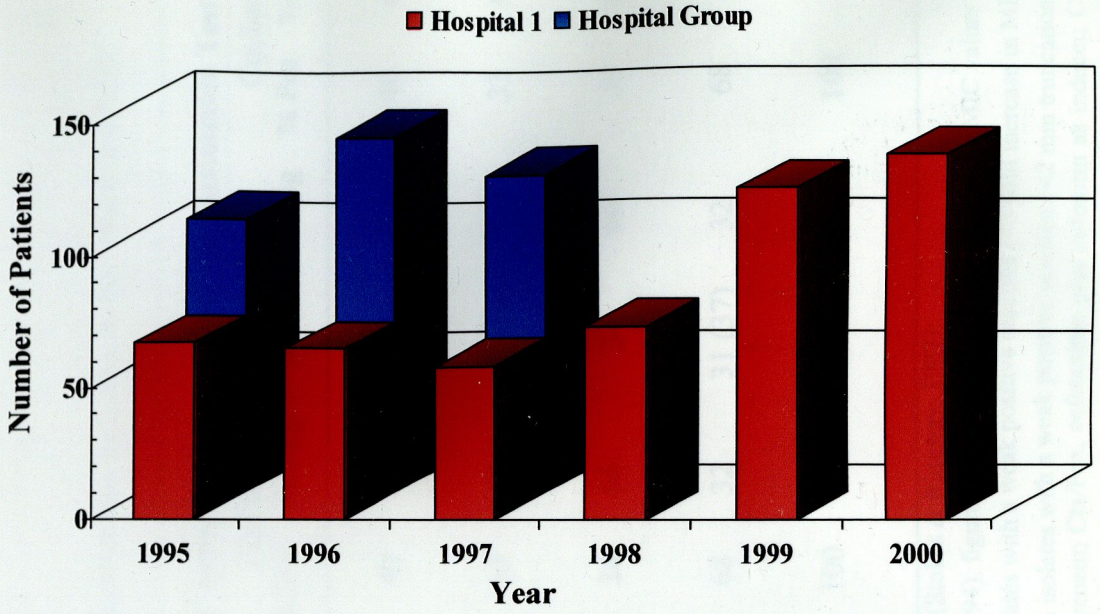


Figure 4.1. The number of patients from whom *S. marcescens* isolates were recovered in Hospital 1 from 1995 to 2000 and in the Hospital Group (Hospitals 1, 2 & 3) from 1995 to 1997. Hospitals 2 and 3 were closed in 1998 (see Section 4.2.1).

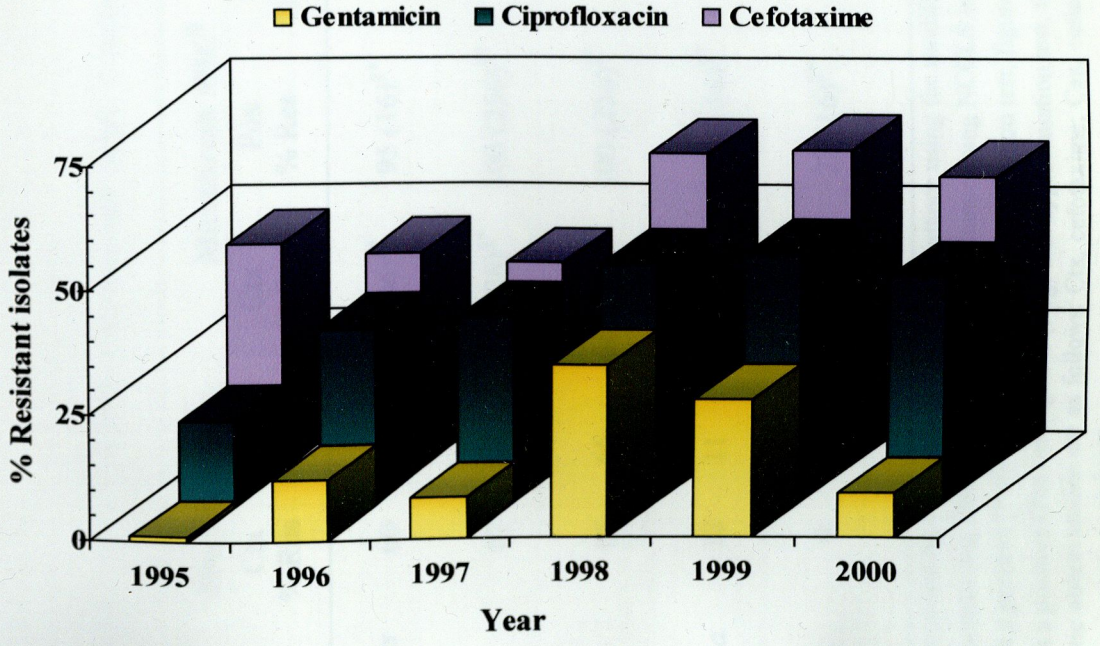


Figure 4.2. The percentage of *S. marcescens* isolates recovered from Hospital 1 demonstrating resistance to cefotaxime, ciprofloxacin and gentamicin from 1995 to 2000.

Table 4.1. Comparison of β-lactam resistance and AmpC β-lactamase expression among *S. marcescens* and other AmpC-inducible GNB.

Species	Disc Diffusion*		Microbroth MIC§			Microbroth MIC induction†				Disc Approximation Test‡			
						Ctx/Fox		Ctx/Imi		Ctx/Fox		Ctx/Imi	
	Ctx % Res	Caz % Res	Ctx % Res	Fox % Res	Imi % Res	% Pos	% Neg	% Pos	% Neg	% Pos	% Neg	% Pos	% Neg
S. marcescens	60	0	60 (16)§*	95 (16)§*	0	32 (8)	60	40	60	37 (3)	60	40	60
E. cloacae	67	67	67 (128)§*	100 (256)§*	0	29 (4)	67	33	67	22 (11)	67	33	67
C. freundii	66	66	66 (128)§*	100 (256)§*	0	34	66	34	66	34	66	34	66
P. aeruginosa	32	11	32 (128)§*	100 (256)§*	0	40 (28)	32	68	32	31 (37)	32	68	32
M. morganii	0	0	0	28 (16)§*	0	100	0	100	0	100	0	100	0

*, % isolates demonstrating resistance or moderate-resistance using the modified Stokes'disc diffusion method (Stokes & Ridgway, 1987);

§, % isolates demonstrating resistance or moderate-resistance using NCCLS interpretive standards (NCCLS, 1994), figures in parentheses ( )§* are the mean MIC values (mg/l);

†, % isolates with a positive (Pos) or negative (Neg) MIC induction test, figures in parentheses represent % isolates with a weak positive reaction (<3 fold increase in MIC);

‡, % isolates with a positive (Pos) or negative (Neg) disc approximation test, figures in parentheses represent % isolates with a weak positive reaction (<2 mm truncation).

B-lactam antibiotic abbreviations are as follows: Ctx, cefotaxime; Caz, ceftazidime; Fox, cefoxitin; Imi, imipenem; Ctx/Fox, cefotaxime plus cefoxitin as inducer; Ctx/imi, cefotaxime plus imipenem as inducer.

Disc diffusion susceptibility testing

All isolates demonstrated resistance to ampicillin, augmentin and cefuroxime indicating the presence of inhibitor-resistant β-lactamase. The rate and pattern of resistance to third-generation cephalosporins varied among the species investigated but was consistent with the production of broad-spectrum β-lactamases. In *E. cloacae* and *C. freundii*, over 60% of isolates demonstrated cross-resistance to cefotaxime and ceftazidime. Thirty-two percent of *P. aeruginosa* isolates were resistant to cefotaxime but only 11% of isolates demonstrated cross-resistance to ceftazidime. In *S. marcescens*, 60% of isolates demonstrated resistance to cefotaxime but all isolates remained susceptible to ceftazidime. All isolates of *M. morganii* tested were susceptible to cefotaxime and ceftazidime.

MIC microdilution broth assays

Using MIC microdilution broth assays the rates of cefotaxime resistance for AmpC-inducible species were the same as those recorded with disc diffusion susceptibility testing i.e. approximately 60% of *E. cloacae, C. freundii* and *S. marcescens* and 30% of *P. aeruginosa* demonstrated resistance to cefotaxime. The level of cefotaxime resistance varied among species. High-level cefotaxime resistance was found in *E. cloacae*, *C. freundii* and *P. aeruginosa*, with MIC values averaging 128 mg/l (Table 4.1). In *S. marcescens*, cefotaxime resistance was low-level (moderate) with the majority of 'resistant' isolates demonstrating an MIC of 8–16 mg/l.

MIC microdilution broth induction assays

The susceptibility profile of all species for both inducing agents was consistent with AmpC β-lactamase production. As a representative of the carbapenem class of antibiotics, imipenem acted as stable β-lactamase inducer with all species remaining highly susceptible to the antibiotic (average MIC <0.5 mg/l). As a representative of the second-generation cephalosporins, cefoxitin acted as a susceptible β-lactamase inducer, with most species demonstrating resistance to the agent. However, as with other cephalosporins, the level of resistance varied among the species tested (Table 4.1). In *E. cloacae*, *C. freundii* and *P. aeruginosa*, 100% of isolates demonstrated high-level resistance to cefoxitin (MIC >256 mg/l). In *S. marcescens*, resistance was also demonstrated by most isolates but at a much lower level with MIC values averaging 16 mg/l. Among *M. morganii* isolates, the rate and level of cefoxitin resistance was

typically low with only 28% of isolates demonstrating low-level resistance (MIC 16 mg/l).

The presence of inducible AmpC β-lactamase was demonstrated in all species using MIC induction assays. All cefotaxime-susceptible isolates of all species showed evidence of inducible β-lactamase production in MIC induction tests with both imipenem and cefoxitin. Since all cefotaxime-resistant isolates were negative in MIC induction tests, resistance among all species was attributed to high-level constitutive AmpC β-lactamase production.

In general, MIC induction studies performed in the presence of imipenem showed higher induction than those performed in the presence of cefoxitin (Table 4.1). Using imipenem, all cefotaxime-susceptible isolates demonstrated strong positive induction reactions yielding four-fold or greater increases in MIC values. With cefoxitin, weak positive MIC induction reactions (two-fold increase in MIC) were produced by cefotaxime-susceptible isolates of some species. These weak positive induction reactions were observed among small numbers of isolates of *S. marcescens* and *E. cloacae* but were more frequent among *P. aeruginosa* where they occurred in 28% of isolates tested (Table 4.1).

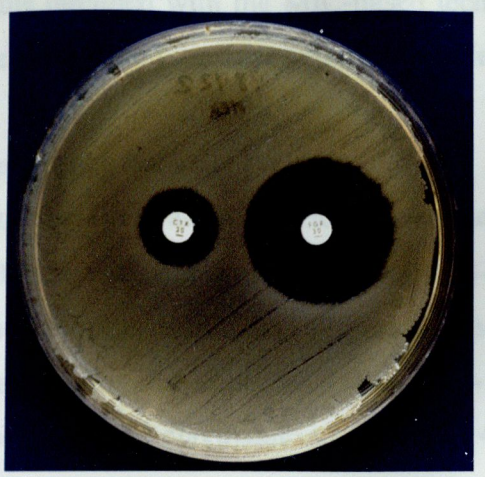
Disc approximation test

In disc induction tests, all cefotaxime-susceptible isolates of all species showed evidence of inducible AmpC β-lactamase production with both imipenem and cefoxitin (Figure 4.3, panel A). The degree of truncation produced in the presence of cefoxitin was reduced compared to results obtained with imipenem and was frequently associated with weak positive reactions in cefotaxime-susceptible isolates (<2 mm truncation in cefotaxime inhibitory zone). As with the MIC induction studies, these weak reactions occurred at a higher frequency in *P. aeruginosa* (Table 4.1). All cefotaxime-resistant isolates failed to display a truncated cefotaxime inhibitory zone in the presence of either inducing agent (Figure 4.3, panel A).

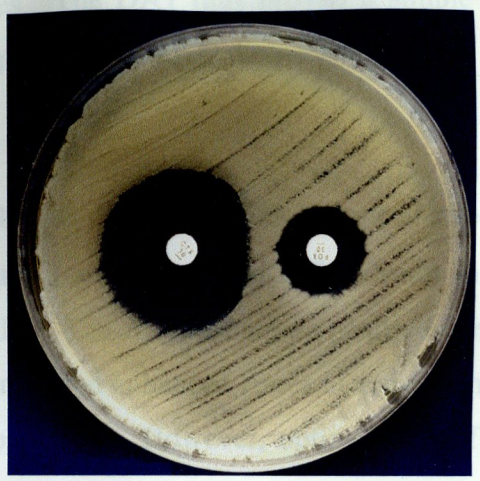
When the DAT was performed using the cefotaxime/imipenem disc combination and a 2 mm truncation breakpoint was adopted, there was 100% agreement with the induction

Panel A. **Disc Approximation Test**

Negative, *S. marcescens*



Positive, *S. marcescens*

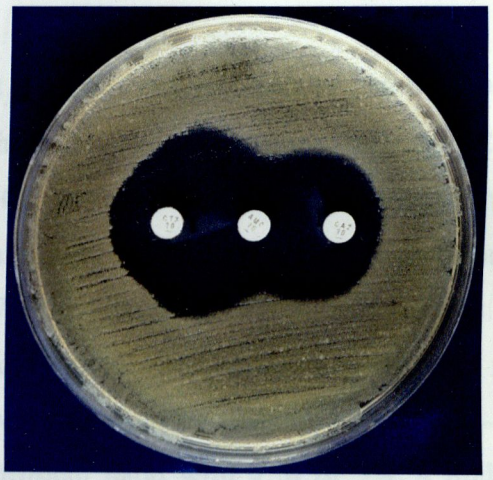


Panel B. **Disc Synergy Test**

Negative, *S. marcescens*



Positive, *K. pneumoniae*-SHV-4 control



Panel C. **Cefpodoxime Combination Disc Test**

Negative, *S. marcescens*



Positive, *K. pneumoniae*-TEM-12 control

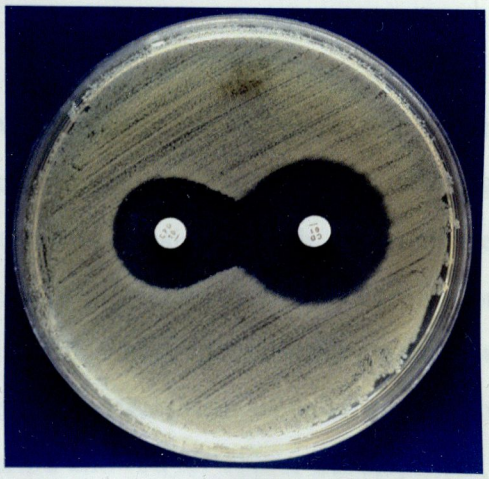


Figure 4.3. Demonstration of AmpC β-lactamase production using the disc approximation test (panel A), ESBL production using the disc synergy test (panel B) and ESBL production using the cefpodoxime combination disc test (panel C). Antibiotics (μg/disc) are: panel A, cefotaxime (30) and cefoxitin (30); panel B, cefotaxime (30), amoxicillin-clavulanic acid (20-10) and ceftazidime (30); panel C, cefpodoxime (10) and cefpodoxime-clavulanic acid (10-1).

results obtained with the MIC-based techniques. The DAT thus proved a simple and sensitive method for detection of inducible AmpC expression in all species.

Disc synergy test

Using the DST, all *S. marcescens* isolates failed to demonstrate an enhanced zone of inhibition with either of the indicator β-lactams (cefotaxime and ceftazidime) in the presence of clavulanic acid (Figure 4.3, panel B). The *K. pneumoniae* positive control isolates expressing either SHV-4 or TEM-12, produced enhanced zones of activity in the presence of the clavulanic acid (Figure 4.3, panel B). However, optimisation of the enhanced zone required precise positioning of the β-lactam indicator discs.

Isoelectric focusing

IEF of crude β-lactamase extracts revealed that all *S. marcescens* isolates had a major β-lactamase band with a pI >9.0 which is characteristic of chromosomal AmpC β-lactamase (Tajima *et al.*, 1981; Joris *et al.*, 1986). In cefotaxime-resistant isolates, these β-lactamase bands developed in less than 1 min and readily diffused upwards through the polyacylamide gel (Figure 4.4, lanes 10–17). In cefotaxime-susceptible isolates the corresponding chromosomal β-lactamase bands were slower to develop and did not diffuse as readily as bands from cefotaxime-resistant isolates (Figure 4.4, lanes 3–9). Four of the isolates analysed produced an additional band with a pI of 5.4, a finding consistent with the production of a classical plasmid-encoded TEM-1 β-lactamase (Figure 4.4, lanes 8 and 16).

In general, there was good agreement between results of IEF and the observed antimicrobial resistance and β-lactamase induction patterns. The presence of a highly basic (pI >9.0) β-lactamase band in all isolates was consistent with the presence of AmpC β-lactamase. The speed of resolution and the intensity of the chromosomal bands also tended to be greatest for those isolates in which the antibiogram suggested high-level β-lactamase production. Nevertheless, the assessment of band intensity was very subjective and largely dependent on the period allowed for colour development, the amount of enzyme loaded and the specific activity of the cell extract. The rapid rate of diffusion of the large chromosomal bands made it difficult to record results accurately

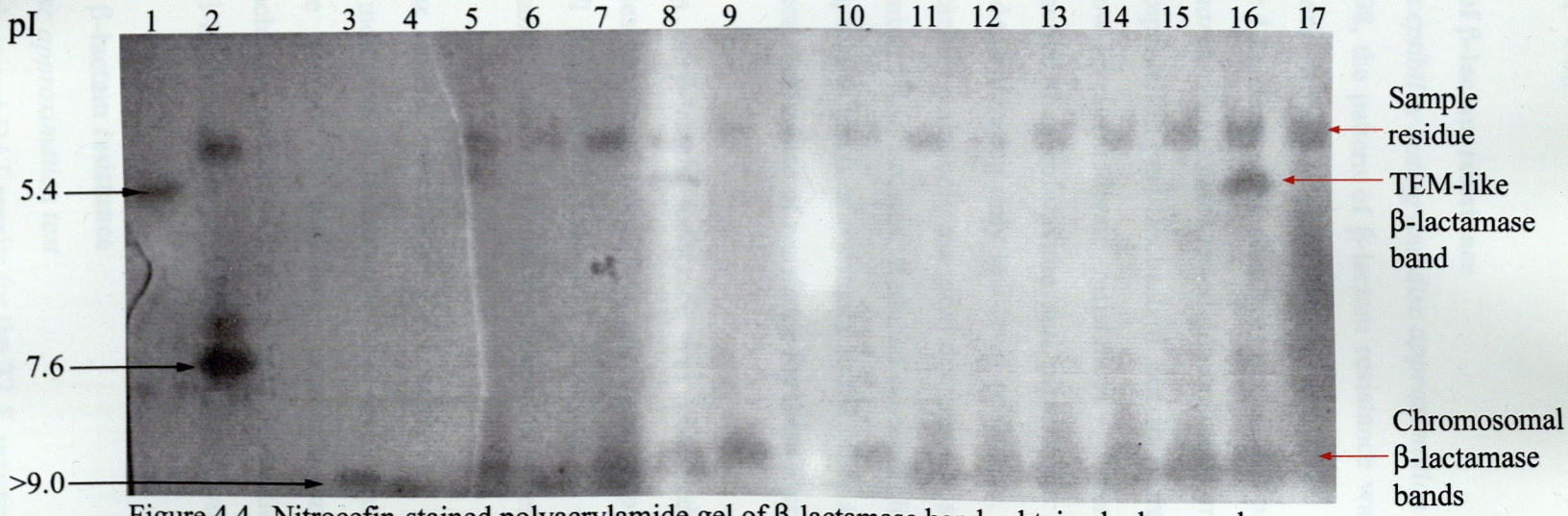


Figure 4.4. Nitrocefin-stained polyacrylamide gel of β-lactamase bands obtained when crude β-lactamase extracts from *S. marcescens* isolates were analysed using isoelectric focusing. Lane contents are as follows: 1, β-lactamase extract from *E. coli* expressing TEM-1 β-lactamase pI 5.4; 2, β-lactamase extract from *E. coli* expressing SHV-1 β-lactamase pI 7.6; 3–9, β-lactamase extracts from cefotaxime-susceptible *S. marcescens* isolates (MIC < 0.5 mg/l); 10–17, β-lactamase extracts from cefotaxime-resistant *S. marcescens* isolates (MIC >16.0 mg/l).

and may have masked the presence of other basic β-lactamase bands such as SHV-derived ESBLs (pI >7.6).

4.3.2.2. **Pattern of β-lactam resistance**

Disc diffusion susceptibility testing and disc approximation test

From 1995 to 1998, the pattern of β-lactam resistance was determined with a total of 351 *S. marcescens* isolates demonstrating resistance or moderate-resistance to cefotaxime (Table 4.2). During the four-year period, the pattern of resistance remained constant. All *S. marcescens* isolates tested demonstrated cross-resistance to cefotaxime, piperacillin and piperacillin-plus-tazobactam but in more than 90% of isolates this resistance was low-level (moderate resistance). Piperacillin-plus-tazobactam was somewhat more active than cefotaxime and piperacillin against *S. marcescens* with 100% of isolates demonstrating only low-level resistance. Over 97% of isolates were susceptible to ceftazidime and only marginal decreases in susceptibility zone sizes were noted in the remaining 3% of isolates. Reduced susceptibility to ceftazidime occurred in isolates expressing higher levels of resistance to the other agents tested. These findings suggested a common mechanism of resistance for these four β-lactams.

The same pattern of β-lactam resistance was observed in cefotaxime-resistant *S. marcescens* isolates recovered from three unrelated hospitals in Ireland. Of the 28 isolates tested, all demonstrated low-level resistance to cefotaxime, piperacillin and piperacillin-plus-tazobactam but remained susceptible to ceftazidime.

All *S. marcescens* isolates tested were DAT negative. This failure to demonstrate inducibility was indicative of constitutive high-level AmpC β-lactamase production. However, in the absence of high-level cross-resistance to all third-generation cephalosporins including ceftazidime, the AmpC production was not characteristic of the high-level of β-lactamase over-production associated with stable derepression.

4.3.2.3. **Level of β-lactam resistance**

Etest MIC and disc approximation test

The Etest MIC values and DAT results for the 32 *S. marcescens* cefotaxime-susceptible isolates are shown in Table 4.3. With the exception of ticarcillin, all isolates were fully

Table 4.2. B-lactam resistance pattern of cefotaxime-resistant and -moderately-resistant *S. marcescens* isolates recovered between 1995 and 1998.

Year	Number of resistant isolates	Percentage resistant and moderately-resistant isolates*							
		Cefotaxime		Piperacillin		Piperacillin/tazobactam		Ceftazidime	
		Moderate	Resistant	Moderate	Resistant	Moderate	Resistant	Moderate	Resistant
1995	60	97	3	97	3	100	0	2	0
1996	71	96	4	96	4	100	0	2	0
1997	78	91	9	91	9	100	0	3	0
1998	142	98	2	98	2	100	0	1	0

*, % isolates demonstrating resistance and moderate resistance using modified Stokes' disc diffusion method (Stokes & Ridgway, 1987).

Table 4.3. Level and expression of β-lactam resistance among cefotaxime-susceptible *S. marcescens* isolates recovered between 1995 and 1998.

Year	Isolate number	DAT	MIC (mg/l)*				
			Cefotaxime	Piperacillin	Ticarcillin	Pip/taz§	Ceftazidime
1995	95.S1	+	0.06	1.0	2.0	0.5	0.06
	95.S2	+	0.06	1.0	2.0	1.0	0.06
	95.S3	+	0.12	2.0	2.0	0.5	0.12
	95.S4	+	0.12	2.0	256**(R)**	1.0	0.12
	95.S5	+	0.25	2.0	2.0	1.0	0.5
	95.S6	+	0.25	2.0	2.0	2.0	0.12
	95.S7	+	0.5	2.0	256**(R)**	2.0	0.25
	95.S8	+	0.5	2.0	4.0	2.0	0.12
1996	96.S1	+	0.06	2.0	1.0	0.5	0.06
	96.S2	+	0.06	1.0	1.0	0.5	0.12
	96.S3	+	0.06	1.0	1.0	0.25	0.12
	96.S4	+	0.12	1.0	1.0	1.0	0.12
	96.S5	+	0.25	2.0	2.0	2.0	0.12
	96.S6	+	0.5	4.0	2.0	2.0	0.25
	96.S7	+	1.0	8.0	8.0	4.0	0.25
	96.S8	w+	2.0	8.0	16	4.0	0.5
1997	97.S1	+	0.12	1.0	1.0	1.0	0.12
	97.S2	+	0.25	1.0	1.0	1.0	0.12
	97.S3	+	0.12	1.0	2.0	1.0	0.12
	97.S4	+	0.5	4.0	2.0	2.0	0.25
	97.S5	+	0.5	2.0	2.0	2.0	0.25
	97.S6	+	1.0	8.0	8.0	4.0	0.25
	97.S7	w+	2.0	8.0	8.0	4.0	0.5
	97.S8	w+	2.0	8.0	256**(R)**	4.0	0.5
1998	98.S1	+	0.06	1.0	2.0	1.0	0.12
	98.S2	+	0.06	1.0	2.0	1.0	0.12
	98.S3	+	0.25	1.0	2.0	1.0	0.12
	98.S4	+	0.25	2.0	2.0	1.0	0.25
	98.S5	+	0.5	2.0	4.0	1.0	0.25
	98.S6	+	0.5	2.0	4.0	1.0	0.5
	98.S7	+	1.0	8.0	8.0	4.0	0.25
	96.S8	w+	2.0	8.0	8.0	4.0	0.5

DAT, disc approximation test; +, positive disc approximation test; w+, weak positive disc approximation test (<2 mm truncation in inhibition zone); §, piperacillin-plus-tazobactam.
*, NCCLS MIC (mg/l) susceptibility breakpoints are as follows: cefotaxime ≤8; piperacillin ≤16; ticarcillin ≤16; piperacillin-plus-tazobactam ≤16-plus-4; ceftazidime ≤8 (NCCLS, 1994).
(R), isolate defined as resistant according to NCCLS MIC interpretive criteria (ticarcillin ≥128 mg/l).

susceptible to the panel of β-lactam antibiotics tested. With third-generation cephalosporins such as cefotaxime and ceftazidime, MIC values remained well below the susceptibility breakpoint (≤8 mg/l) although some isolates showed significantly higher MIC values to cefotaxime (2.0 mg/l) than to ceftazidime (0.5 mg/l). MICs were at least four-fold higher for the extended-spectrum penicillins such as ticarcillin and piperacillin and for β-lactam-inhibitor combinations (piperacillin-plus-tazobactam) although values for combination agents were generally lower than those for the β-lactam agents alone. High-level resistance to ticarcillin (MIC >256 mg/l) was seen in three isolates of *S. marcescens* which were fully susceptible to the other β-lactam agents.

All cefotaxime-susceptible isolates demonstrated expression of inducible AmpC β-lactamase (DAT positive). In isolates with increased cefotaxime MICs (2.0 mg/l), the level of inducibility was reduced (weak DAT positive) (Table 4.3). Cefotaxime MIC values above 2 mg/l appeared to be associated with constitutive high-level production (DAT negative).

Compared to susceptible isolates, 'cefotaxime-resistant' *S. marcescens* isolates demonstrated significantly higher MIC values to cefotaxime, piperacillin, ticarcillin, and piperacillin-plus-tazobactam (Table 4.4). However, in over 90% of isolates resistance was low-level. Amongst these low-level resistant isolates, resistance to cefotaxime and piperacillin-plus-tazobactam was markedly low with 34% of cefotaxime MICs and 100% of piperacillin-plus-tazobactam MIC values falling on susceptibility breakpoints. Although these low-level resistant isolates demonstrated ceftazidime MICs that were approximately four-fold higher than values observed in susceptible isolates, the MICs (≤2 mg/l) still remained well below the susceptibility breakpoint value (8 mg/l). In the small number of isolates (95.R7, 97.R8 and 98.R7) which demonstrated higher levels of resistance to cefotaxime, piperacillin and ticarcillin, MIC values for piperacillin-plus-tazobactam remained low-level resistant (MIC equivalent to susceptibility breakpoint) and MIC values for ceftazidime (≤4 mg/l) were still below susceptibility breakpoint criteria (Table 4.4). Four isolates demonstrated high-level ticarcillin resistance (MIC >256 mg/l) in the absence of a proportional increase in resistance to the other β-lactam agents. The DAT was negative for all 'cefotaxime-resistant' isolates.

Table 4.4. Level and expression of β-lactam resistance among cefotaxime-resistant and -moderately resistant *S. marcescens* isolates recovered between 1995 and 1998.

Year	Isolate number	DAT	MIC (mg/l) *				
			Cefotaxime	Piperacillin	Ticarcillin	Pip/taz§	Ceftazidime
1995	95.R1	–	8.0	16	32	16	0.5
	95.R2	–	16	32	32	16	1.0
	95.R3	–	16	32	32	16	1.0
	95.R4	–	16	32	32	16	1.0
	95.R5	–	16	32	32	16	1.0
	95.R6	–	32	32	32	16	2.0
	95.R7	–	64**(R)**	64	64	16	4.0
	95.R8	–	32	32	32	16	2.0
1996	96.R1	–	8.0	32	256**(R)**	16	0.5
	96.R2	–	8.0	32	32	16	0.5
	96.R3	–	8.0	32	32	16	1.0
	96.R4	–	8.0	32	32	16	1.0
	96.R5	–	16	32	32	16	1.0
	96.R6	–	32	32	64	16	2.0
	96.R7	–	32	32	32	16	2.0
	96.R8	–	32	32	32	16	2.0
1997	97.R1	–	8.0	32	256**(R)**	16	0.5
	97.R2	–	8.0	16	16	16	0.5
	97.R3	–	16	32	32	16	1.0
	97.R4	–	16	32	32	16	1.0
	97.R5	–	16	32	32	16	1.0
	97.R6	–	16	32	32	16	1.0
	97.R7	–	32	64	64	16	1.0
	97.R8	–	64**(R)**	64	64	16	2.0
1998	98.R1	–	8.0	16	16	16	0.5
	98.R2	–	8.0	64	256**(R)**	16	0.5
	98.R3	–	8.0	64	32	16	1.0
	98.R4	–	16	32	64	16	2.0
	98.R5	–	16	128**(R)**	256**(R)**	16	2.0
	98.R6	–	16	32	64	16	1.0
	98.R7	–	64**(R)**	128**(R)**	256**(R)**	16	4.0
	98.R8	–	32	64	64	16	2.0

DAT, disc approximation test; –, negative disc approximation test; §, piperacillin-plus-tazobactam.
*, NCCLS MIC (mg/l) interpretive standards are as follows: cefotaxime susceptible ≤8, moderate =16–32, resistant ≥64; piperacillin susceptible ≤16, moderate =32–64, resistant ≥128; ticarcillin susceptible ≤16, moderate =32–64, resistant ≥128; piperacillin-plus-tazobactam susceptible ≤16-plus-4, moderate =32–64-plus-4, resistant ≥128-plus-4; ceftazidime susceptible ≤8, moderate =16–32, resistant ≥64 (NCCLS, 1994). (R), isolate defined as resistant according to NCCLS MIC interpretive criteria. *S. marcescens* isolates with cefotaxime MICs falling on susceptibility breakpoints (8 mg/l) were categorised as low-level resistant.

Cefpodoxime combination disc method

Using the cefpodoxime combination disc method, all cefotaxime-resistant *S. marcescens* isolates were negative for the presence of ESBLs (Figure 4.3, panel C). The *K. pneumoniae* positive control isolates expressing SHV-4 or TEM-12, displayed a significant increase in zone size (>5 mm) when tested with the cefpodoxime combination disc compared with inhibition zones obtained when the cefpodoxime was tested alone.

4.3.2.4 B-lactam hydrolysis assays

The results of the cephalothin hydrolysis assays are shown in Table 4.5. Similar to the wild-type control, Serr1, cefotaxime-susceptible *S. marcescens* isolates exhibited minimal activity in the uninduced state but in the presence of cefoxitin, β-lactamase activity demonstrated a 50–100-fold induction. *Serratia marcescens* isolates with low-level cefotaxime resistance (MIC 8–16 mg/l) expressed higher levels of β-lactamase activity in the uninduced state but retained marginal inducibility in the presence of cefoxitin (β-lactamase activity increased 1.5–2.5-fold when induced with cefoxitin). The two isolates with a cefotaxime MIC of 8 mg/l demonstrated a level of uninduced β-lactamase activity approximately 25-fold higher than susceptible isolates and a 2.5-fold higher rate when induced with cefoxitin. The isolate with a cefotaxime MIC of 16 mg/l expressed a level of uninduced β-lactamase activity approximately 100-fold higher than susceptible isolates but only a 1.3-fold higher rate when induced with cefoxitin. In cefotaxime-resistant isolates (MIC 64 mg/l), the level of β-lactamase activity was approximately 150-fold higher than uninduced susceptible isolates, regardless of the presence or absence of cefoxitin.

Table 4.5. Specific activity of β-lactamase produced in cefotaxime-susceptible and cefotaxime-resistant isolates of *S. marcescens* determined by cephalothin hydrolysis assays in the presence and in the absence of cefoxitin.

Isolate	**Cefotaxime susceptibility***	**DAT**	**Cephalothin hydrolysis** (nmoles/min/mg protein)			**Predicted Phenotype**
			Cefoxitin absent§	Cefoxitin present†	X-fold induction‡	
Serr1	Susceptible (<2.0)	+	32	1514	47	Wild-type inducible
98. S1	Susceptible (0.06)	+	17	850	50	Wild-type inducible
97.S3	Susceptible (0.12)	+	29	1899	66	Wild-type inducible
96.S5	Susceptible (0.25)	+	30	2936	98	Wild -type inducible
96.R1	Low-level Resistant (8.0)	–	808	1901	2.4	Partially-derepressed
97.R2	Low-level Resistant (8.0)	–	737	1784	2.5	Partially-derepressed
98.R5	Low-level Resistant (16)	–	2894	3704	1.3	Partially-derepressed
95.R7	Resistant (64)	–	4786	4697	0	Derepressed
97.R8	Resistant (64)	–	4254	4613	0	Derepressed
98.R7	Resistant (64)	–	4892	4958	0	Derepressed

*, cefotaxime susceptibility was interpreted from Etest MIC values (see table 4.3 and 4.4), figures in parentheses are the MIC values (mg/l) ;
DAT, disc approximation test; +, positive disc approximation test; –, negative disc approximation test;
§, Specific activity determined in the absence of cefoxitin, results obtained from the mean of three assays;
†, Specific activity determined in the presence of sub-inhibitory concentrations of cefoxitin, (¼ cefoxitin MIC), results obtained from the mean of three assays;
‡, X is the factor by which the specific activity is increased when assays were performed in the presence of cefoxitin;
Serr1 was characterised as wild-type in MCRAB, Creighton University, using β-lactam susceptibility testing and β-lactam hydrolysis assays (Morrow, 1999).

4.4. DISCUSSION

Prior to this study there was evidence of increased cephalosporin resistance within the *S. marcescens* population of the Dublin hospital group (Hejazi, 1997). This resistance was postulated to result from the over-production of inducible AmpC chromosomal β-lactamase. However, since the β-lactam resistance phenotype of the *S. marcescens* isolates did not appear to correlate with the high-level cephalosporin cross-resistance associated with stable derepression in other enterobacteria (Livermore, 1995), this resistance required further study. The first step was to determine the rate of cephalosporin resistance in the *S. marcescens* isolates recovered from the hospital group and to investigate the phenotypic expression of this resistance.

4.4.1. Extent of cephalosporin resistance

Prevalence rates indicate that by 1995, *S. marcescens* was already well established within the hospital group. During the following six years, increases in prevalence closely mirrored outbreaks of infection. The marked increase in prevalence in the hospital group between 1995 and 1997 reflected the first reported outbreak of a multi-resistant O21:K14 *S. marcescens* serotype in Hospitals 2 and 3 (Herra *et al.*, 1998). Similarly, the sharp increase in the isolation rate for Hospital 1 between 1997 and 2000 coincided with the second outbreak of the O21:K14 strain (Knowles *et al.*, 2000).

The problem of increasing prevalence within the hospital group was compounded by an increase in the extent of antimicrobial resistance within this serratia population. The high rate of antimicrobial resistance was particularly evident with cephalosporins such as cefotaxime where rates of resistance in isolates recovered in Hospital 1 increased from 45% in 1995 to more than 60% in 1998. Increased rates of resistance to other commonly used antibiotics such as ciprofloxacin and gentamicin were also observed. As with prevalence, increased rates in antimicrobial resistance coincided with the outbreaks of multi-resistant O21:K14 isolates (Herra *et al.*, 1998; Knowles *et al.*, 2000). However, although control of the second outbreak in 1999 was mirrored by a decrease in the rate of gentamicin resistance, rates of resistance to cefotaxime and ciprofloxacin remained at 60% and 50%, respectively, throughout 1999 and into 2000. This indicates that resistance to cefotaxime and ciprofloxacin became established within the *S.*

marcescens population regardless of the presence or absence of outbreaks of infection with multi-resistant strains.

4.4.2. Phenotypic expression of β-lactam resistance

4.4.2.1. Pilot study

In the pilot study, AmpC β-lactamase production was demonstrated in all AmpC-inducible species tested. With the exception of *M. morganii*, AmpC-mediated cephalosporin-resistance was also observed in all AmpC-inducible species although the expression of the AmpC β-lactamase varied from species to species. In *E. cloacae* and *C. freundii*, cephalosporin-resistance was present in more than 60% of isolates and was associated with a stable fully-derepressed phenotype i.e. isolates displayed high-level constitutive β-lactamase over-production (negative in induction assays) and demonstrated high-level cross-resistance to third-generation cephalosporins such as cefotaxime and ceftazidime.

In *P. aeruginosa*, cephalosporin resistance occurred in 32% of isolates. Although all resistant *P. aeruginosa* isolates demonstrated high-level cefotaxime resistance and high-level constitutive AmpC β-lactamase expression and were thus associated with a derepressed phenotype, only 11% of isolates demonstrated a resistance profile consistent with full derepression i.e. cross-resistant to both cefotaxime and ceftazidime.

Cephalosporin resistance was present in 60% of *S. marcescens* isolates. However, despite evidence of derepression i.e. constitutive β-lactamase over-production, none of these isolates demonstrated a resistance profile characteristic of a fully-derepressed phenotype. The majority of 'resistant' *S. marcescens* isolates demonstrated low-level resistance to cefotaxime and all isolates remained fully susceptible to ceftazidime.

Previous studies have demonstrated that the frequency of derepression and associated cephalosporin resistance varies according to species, site of infection, geographical location and the antibiotic selective pressure in the hospital (Livermore, 1995; Jones *et al.*, 1997). In one report, high-level constitutive β-lactamase production was present in only 16% of consecutive enterobacter isolates in a veterans hospital but was found in 59% of enterobacters recovered from serious infections and treatment failures among

patients in a general hospital (Ehrhardt & Sanders, 1993). In most American and European hospitals, derepression is seen in approximately 15% to 50% of *E. cloacae* and *C. freundii* isolates but lower rates are seen among *M. morganii*, *P. stuartii* and *S. marcescens* (Chen *et al.*, 1993; Livermore, 1995). In *P. aeruginosa* the reported rate of derepression varies from 7% of isolates from ICU patients to 3% of isolates from patients in general wards and 1% of isolates from outpatients (Chen *et al.*, 1995).

The high rates of derepression recorded in the pilot study (60%) may reflect the fact that these test isolates were recovered from infection sites where derepression is likely to occur i.e. respiratory tract, blood and wound infections. At these sites the combination of high bacterial population density and poor antibiotic bioavailability creates a micro-environment conducive to the selection of organisms exhibiting derepression. In the same year (1995), the cephalosporin resistance rate among isolates recovered from all sites was 45% but the latter included *S. marcescens* isolates from the urinary tract where the high level of drug bioavailability makes derepression unlikely. In addition, the high rate of derepression recorded among *S. marcescens* isolates in this study may reflect the high prevalence of the organism in the hospital group and may represent clonal spread of β-lactamase-mediated cephalosporin-resistant *S. marcescens* isolates.

The pilot study demonstrated differences in constitutive β-lactamase expression among AmpC-inducible species and the heterogeneity of their interactions with inducing agents. Cefoxitin acts as a potent inducer of AmpC β-lactamase but is also susceptible to the action of the enzyme (Livermore, 1991b). Accordingly, all isolates of *E. cloacae*, *C. freundii* and *P. aeruginosa* demonstrated high-level resistance to the agent. A much lower level of cefoxitin resistance (MIC 16 mg/l) was found in *S. marcescens* and *M. morganii*. Other studies have also noted that cefoxitin maintains moderate activity against both inducible and derepressed isolates of *S. marcescens* and *M. morganii* (Curtis *et al.*, 1986; Livermore, 1995). This low-level cefoxitin resistance may be related to the lower levels of cephalosporin resistance found in these species.

The results of IEF demonstrated the presence of AmpC β-lactamase in all *S. marcescens* isolates tested and suggested the involvement of AmpC β-lactamase production in cephalosporin resistance. However, in the absence of additional substrate hydrolysis or β-lactamase-inhibitor overlay procedures (Thomson *et al.*, 1991), IEF did not permit

discrimination of inducible and constitutive β-lactamase production or distinction between chromosomal and plasmid-encoded β-lactamases. The expense (£150 per gel) and laborious nature of the method also made IEF an unsuitable choice for this study. MIC induction assays and simple disc induction methods, which readily facilitated characterisation of AmpC β-lactamase expression, were used as a practical alternative to IEF.

Disc synergy methods used in the pilot study helped outrule the possibility that cephalosporin resistance in *S. marcescens* isolates may have resulted from β-lactamase sources other than AmpC, such as plasmid-encoded ESBLs. Since extensive optimisation of the disc synergy method was required to display the presence of ESBLs in the positive control isolates and since the demonstration of ESBLs is frequently masked in AmpC- and inhibitor-resistant ESBL-producing species such as *S. marcescens* (Thomson & Sanders, 1992), additional screening methods were employed. Using the cefpodoxime combination disc screening method, cephalosporin-resistant *S. marcescens* isolates were negative for ESBL production, although again the possibility that the presence of inhibitor-resistant β-lactamases may invalidate these results must be taken into account. The expression of classical plasmid-encoded β-lactamases such as TEM-1 was indicated in cefotaxime-susceptible and -low-level resistant isolates which demonstrated high-level resistance to ticarcillin. Since classical β-lactamases such as TEM-1 characteristically confer high-level resistance to extended-spectrum penicillins such as ticarcillin and are generally ineffective against broad-spectrum β-lactam agents, they were considered an unlikely source of cephalosporin resistance in *S. marcescens* isolates.

4.4.2.2. **Pattern and level of β-lactam expression**

Phenotypic studies investigating the pattern and level of β-lactam resistance also indicated the involvement of AmpC β-lactamase in cephalosporin resistance in *S. marcescens* isolates. The pattern of β-lactam resistance was suggestive of the presence of AmpC-mediated resistance and in the absence of inducibility, this resistance was attributed to constitutive high-level β-lactamase production. However, the β-lactam resistance profile was not consistent with a fully-derepressed phenotype. Over 90% of 'cephalosporin-resistant' *S. marcescens* isolates demonstrated low-level resistance to

agents such as cefotaxime (MIC 8–16 mg/l) and were fully susceptible to other third-generation cephalosporin agents such as ceftazidime. In the remaining isolates that demonstrated significant levels of cefotaxime resistance, decreases in susceptibility to piperacillin-plus-tazobactam and particularly to ceftazidime were marginal. Furthermore, since the isolates with significant levels of cefotaxime resistance frequently demonstrated cross-resistance to other classes of antibiotic such as aminoglycosides, the involvement of a co-resistance factor such as impermeability must also be considered. The fact that the β-lactam resistance profile for *S. marcescens* isolates remained constant during four years of the study period indicates that this resistance phenotype was constant among the test isolates. Similarly, the fact that the same resistance profile was demonstrated by isolates outside this hospital group suggests that this resistance model may be common in other Irish *S. marcescens* isolates.

A review of the literature indicates that many other studies have also demonstrated significant reduction in β-lactam MICs of derepressed isolates of *S. marcescens* (and *M. morganii*) compared to other GNBs with inducible AmpC expression. In the earlier studies, β-lactam MICs for derepressed isolates of *S. marcescens* were consistently lower compared to values for other organisms such as *E. cloacae, C. freundii* and *P. aeruginosa* (Curtis *et al.*, 1986; Sanders & Sanders, 1986; Yang & Livermore, 1989b) (Table 4.6). The difference in the level of AmpC-mediated resistance was particularly evident with ceftazidime, where MICs for derepressed isolates of *S. marcescens* were approximately six-fold lower than values obtained with other inducible GNBs. In more recent reports, the β-lactamase expression of organisms such as *Serratia* and *Morganella* has been classified as derepressed, although the cephalosporin MIC values of these organisms were below susceptibility breakpoints. In these isolates the levels of cephalosporin resistance were approximately 10-fold below those in other derepressed *Enterobacteriaceae* (Chen & Livermore, 1994; Livermore, 1995; Greenhalgh & Edwards, 1997).

In contrast to the substantial body of evidence indicative of low-level AmpC-mediated resistance in *S. marcescens*, only two reports were found where constitutive β-lactamase over-production was associated with high-level cephalosporin resistance (Gianneli *et al.*, 1994; Weindorf *et al.*, 1998). Moreover, in one of these studies the contribution of

Table 4.6. Studies on β-lactam susceptibility of derepressed mutants in organisms with inducible class C β-lactamase reported in the literature.

B-lactam agent	MIC* (mg/l) of derepressed mutants:					Study
	E. cloacae	*C. freundii*	*P. aeruginosa*	*S. marcescens*	*M. morganii*	
Piperacillin	32	128	128	32	32	Curtis *et al.*, 1986
	128	128	>128	32	64	Sanders & Sanders, 1986
	128	64	NT	32	16	Chen & Livermore, 1994
	256	64	>128	32	32	Livermore, 1995
	>128	128	32	128	16	Greenhalgh & Edwards, 1997
	NT	NT	NT	32	NT	This study
Pip/taz§	64	64	>128	32	2.0	Livermore, 1995
	128	64	16	16	4.0	Greenhalgh & Edwards, 1997
	NT	NT	NT	32	NT	This study
Cefotaxime	32	32	>128	4.0	4.0	Curtis *et al.*, 1986
	64	64	>128	8.0	8.0	Sanders & Sanders, 1986
	256	16	512	4.0	2.0	Yang & Livermore, 1989b
	64	32	NT	16	4.0	Chen & Livermore, 1994
	256	32	NT	8.0	4.0	Livermore, 1995
	128	32	>128	4.0	1.0	Greenhalgh & Edwards, 1997
	128	128	128	16	NT	This study
Ceftazidime	64	128	32	0.5	8.0	Curtis *et al.*, 1986
	64	128	>128	0.5	16	Sanders & Sanders, 1986
	64	64	NT	2.0	8.0	Chen & Livermore, 1994
	64	32	>64	1.0	8.0	Livermore, 1995
	64	64	8.0	0.2	NT	Greenhalgh & Edwards, 1997
	NT	NT	NT	1.0	NT	This study

*, MIC or mean MIC value quoted in study; NT, not tested in study; §, piperacillin-plus-tazobactam.

additional β-lactam resistance mechanisms such as reduced permeability was indicated by the demonstration of porin defects in high-level cephalosporin resistant isolates (Hechler *et al.*, 1989; Weindorf *et al.*, 1998).

4.4.2.3. **B-lactam hydrolysis assays**

In the present study, the results of β-lactam hydrolysis assays also indicated that cephalosporin resistance in *S. marcescens* isolates was mediated by AmpC β-lactamase. The hydrolysis pattern of cephalosporin-susceptible isolates (cefotaxime MIC ≤2 mg/l) was characteristic of inducible AmpC β-lactamase expression with isolates demonstrating 50–100-fold increases in β-lactamase activity after induction. These findings are typical of the induction ratios previously recorded in wild-type *S. marcescens* isolates exposed to sub-inhibitory concentrations of cefoxitin (Sanders & Sanders, 1986). In isolates with very low-level cephalosporin resistance (cefotaxime MIC 8 mg/l), β-lactamase expression was characteristic of a partially-derepressed phenotype where the level of uninduced β-lactamase was considerably higher than levels in wild-type isolates but low-level inducibility (2.5-fold) was retained. Previous studies have observed the presence of inducibility in derepressed isolates of *S. marcescens* with low-level cephalosporin resistance (Sanders & Sanders, 1986). This form of partial derepression has also been observed in other inducible species such as *C. freundii* (Stapleton *et al.*, 1995b) and *P. aeruginosa* (Livermore, 1995). *Serratia marcescens* isolates with marginal increases in cephalosporin resistance (cefotaxime MIC 16 mg/l) demonstrated a phenotype suggestive of true derepression where the level of constitutive β-lactamase activity was increased and the degree of inducibility was diminished. The degree of resistance demonstrated by partially-derepressed *S. marcescens* isolates appeared to reflect the amount of enzyme produced in the uninduced state.

In isolates with more significant levels of cephalosporin resistance (cefotaxime MIC 64 mg/l), the expression of AmpC β-lactamase was indicative of true derepression with isolates demonstrating notable increases in levels of β-lactamase production. Nevertheless, the levels of constitutive β-lactamase produced by these derepressed isolates were, at most, 160-fold higher than levels in wild-type isolates. In other enterobacteria such as *E. cloacae* and *C. freundii*, fully-derepressed isolates are frequently associated with a 500–1000-fold increase in β-lactamase production. Thus it

would appear that even in states of true derepression, the overall rate of β-lactamase over-production in *S. marcescens* may occur at a lower level compared to other inducible species. Other workers have also observed this reduced level of constitutive production. Derepressed isolates of *S. marcescens* (and *M. morganii*) have been recorded with levels of β-lactamase six to ten times lower than levels in derepressed isolates of *E. cloacae* and *C. freundii* (Curtis *et al.*, 1986; Yang & Livermore, 1988a; Yang & Livermore, 1988b; Livermore, 1995).

The results of β-lactam hydrolysis indicate that, in *S. marcescens*, high-level constitutive β-lactamase production do not compare with the level of β-lactamase hyperproduction associated with derepression in other AmpC-inducible enterobacteria. In this study, over 90% of 'cephalosporin-resistant' *S. marcescens* isolates demonstrated low-level cefotaxime resistance which was associated with partial derepression. In the remaining 'cephalosporin-resistant' isolates which demonstrated increased levels of cefotaxime resistance, results were indicative of a fully derepressed phenotype, albeit occurring at a somewhat lower level than has been observed in other AmpC-inducible enterobacteria. The lower level of cephalosporin resistance recorded in derepressed isolates of *S. marcescens* may reflect the relative low level of constitutive β-lactamase over-production.

Although previous studies have indicated that AmpC β-lactamase enzymes from species with inducible β-lactamase production share the same kinetic properties for β-lactam drugs (Yang & Livermore, 1988b; Yang & Livermore, 1989a; Chen *et al.*, 1995), more recent reports suggest that in *S. marcescens,* AmpC has lower affinity for some third-generation cephalosporins (Raimondi *et al.*, 2001). For agents such as cefotaxime, the reduced level of AmpC affinity is marginal. However, for other β-lactams such as ceftazidime, AmpC affinity is markedly reduced and appears to mediate a significant reduction in the hydrolytic capacity of the β-lactamase enzyme. This reduced level of AmpC activity may act in concert with the relative reduction in constitutive β-lactamase over-production observed in derepressed isolates of *S. marcescens* to generate a significant reduction in β-lactam MIC values. With β-lactam agents such as ceftazidime which demonstrate very slow rates of hydrolysis (approximately 20 times slower than cefotaxime), the compounding effect of reduced substrate affinity would presumably

mean that any reduction in the level of β-lactamase production would result in a pronounced reduction in the level of resistance.

In this study, the majority of 'cephalosporin-resistant' *S. marcescens* isolates were associated with a partially-derepressed phenotype. This process of derepression resembles AmpC mutational events in *P. aeruginosa*. In studies investigating the mechanisms of β-lactam resistance in *P. aeruginosa*, 75% of derepressed isolates remained inducible by cefoxitin indicating that derepression was partial (Chen *et al.*, 1995). B-lactam induced mutational experiments have also demonstrated that whilst isolates of *E. cloacae* segregate totally derepressed mutants at frequencies of 10^{-5} to 10^{-7}, *P. aeruginosa* isolates segregate partially-derepressed mutants at rates of approximately 10^{-7} but full derepression only occurs at frequencies less than 10^{-9} (Livermore, 1995). Similarly, many studies have indicated that *in vivo* selection of derepressed mutants occurs more rarely in *S. marcescens* compared to other AmpC-inducible enterobacteria (Liu *et al.*, 1992). In addition, *in vitro* selection studies have indicated that derepressed mutants occurred at rates 10^{-6} to 10^{-8} in all β-lactamase inducible enterobacteria except *S. marcescens*. In *S. marcescens*, mutants were obtained at frequencies less than 10^{-9} and these rates were only achieved after mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine treatment (Yang & Livermore, 1988a).

4.4.3. **Conclusion**

During the study period, *S. marcescens* was implicated with increasing frequency as a serious cause of nosocomial infection in the hospital group. Within this serratia population, the rate of cephalosporin resistance increased from 45% to 60% of isolates. The results of phenotypic expression studies indicated that this cephalosporin resistance resulted from constitutive AmpC β-lactamase over-production. Results of phenotypic resistance detection methods also suggested that ESBLs could be excluded as likely sources of cephalosporin resistance.

In other AmpC-inducible enterobacteria such as *E. cloacae* and *C. freundii*, constitutive β-lactamase over-production was characterised by mutation to a fully-derepressed state where hyperproduction of AmpC conferred high-level cross-resistance to all third-generation cephalosporins tested. The β-lactam resistance profile of *S. marcescens* isolates did not appear to correlate with full derepression of AmpC β-lactamase production. The majority of 'cephalosporin-resistant' isolates demonstrated only low-level resistance to some third-generation cephalosporins and remained fully-susceptible to the remainder. These isolates were associated with a partially-derepressed phenotype. In the small percentage of *S. marcescens* isolates where true AmpC derepression was indicated, significant levels of resistance were expressed against some third-generation cephalosporins, but decreases in susceptibility to other agents such as piperacillin-plus-tazobactam and ceftazidime were marginal. Notwithstanding the possible role of reduced AmpC affinity for cephalosporins such as ceftazidime, the low rates of β-lactam resistance recorded in *S. marcescens* isolates appeared to reflect, at least in part, the relative low level of constitutive AmpC β-lactamase over-production.

Chapter V

Molecular investigation of plasmid-mediated β-lactam resistance in *Serratia marcescens*

Chapter V

Molecular investigation of plasmid-mediated β-lactam resistance in *Serratia marcescens*

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5.1. INTRODUCTION

Phenotypic expression studies have indicated the involvement of constitutive AmpC β-lactamase production in cephalosporin resistant *S. marcescens* isolates. The contribution of other likely sources of β-lactamase-mediated cephalosporin resistance such as plasmid-encoded ESBLs was excluded using phenotypic detection methods. The sensitivity of phenotypic screening methods is, however, limited by the substrate specificity and the low level of cephalosporin resistance associated with many ESBLs (see Section 4.1.3, paragraph three). In addition, ESBL detection methods based on the demonstration of synergistic activity of β-lactam inhibitors are unreliable for screening organisms such as *S. marcescens* which may produce inhibitor-resistant ESBLs and which intrinsically express inhibitor-resistant chromosomal AmpC β-lactamase that may mask ESBL detection. In contrast, molecular-based screening methods offer considerable potential for sensitive and specific detection of ESBLs in all organisms. This study was designed to apply genotypic detection methods to investigate the possible involvement of ESBLs in cephalosporin-resistant *S. marcescens* isolates.

5.1.1. Plasmid analysis

ESBLs are plasmid-encoded resistance determinants. The extraction of plasmid DNA from cephalosporin-resistant isolates is a simple cost-effective method that can be used as a preliminary step to screen for the presence of ESBLs (Pitout *et al.*, 1998). To confirm the presence of ESBL-mediated resistance, Southern analysis can be performed using probes derived from DNA sequences that encode ESBL enzymes (Petit *et al.*, 1990). Thus, the demonstration of plasmids of similar size in clinical isolates expressing the same β-lactam resistance profile has been used to infer a common mechanism of ESBL-mediated resistance (Naumovski *et al.*, 1992; Luzzaro *et al.*, 1998). Likewise, the demonstration of similar sized plasmids in epidemiologically related isolates can be useful in the investigation of a putative outbreak caused by dissemination of an ESBL-producing nosocomial pathogen (Johnson *et al.*, 1992; Pagani *et al.*, 1994).

ESBLs tend to be encoded on large multi-resistant plasmids (80 kb or more in size) which are usually present in low copy number (Jacoby & Medeiros, 1991; Livermore,

1995). In one study investigating the properties of 15 different plasmids encoding various ESBLs, all plasmids were large, ranging in size from 80 to 300 kb and encoded a multiplicity of resistance determinants (Jacoby & Sutton, 1991). Previous studies investigating ESBL-mediated resistance in *S. marcescens* have also demonstrated the presence of SHV- and TEM-derived determinants encoded on large plasmids approximately 60-130 kb in size (Payne *et al.*, 1992a; Gianneli *et al.*, 1994; Pagani *et al.*, 1994). Plasmids of this size are, however, difficult to isolate and frequently undergo shearing during extraction procedures.

In the last twenty years, most methods developed for plasmid DNA extraction have been based on alkaline lysis extraction procedures. This technique exploits the different denaturation and renaturation characteristics of covalently closed circular plasmid DNA and chromosomal DNA. In the original method, both chromosomal and plasmid DNA are denatured under alkaline conditions (Birnboim & Doly, 1979). Rapid neutralisation with a high salt buffer causes the chromosomal DNA to renature and form an insoluble aggregate that precipitates out of solution. The covalently closed nature of the plasmid DNA allows it to stay in solution. Plasmid DNA is separated from the insoluble material by centrifugation and recovered by ethanol precipitation. Using this method, plasmids in the size range of 90–120 kb can be isolated. Modifications of this procedure have been designed which avoid the selective precipitation of denatured chromosomal DNA and facilitate isolation of larger plasmids. In these techniques chromosomal DNA is denatured by alkaline lysis and the denatured DNA is eliminated by incubation at elevated temperatures. Plasmids are separated from proteins and cell debris by phenol-chloroform extraction (Kado & Liu, 1981).

Commercial kits have become available which offer a simple, rapid approach to plasmid DNA purification. These kits utilise the principle of alkaline lysis but eliminate the need for phenol-chloroform extraction and differential centrifugation by employing a resin-based matrix to bind plasmid DNA in cleared lysate preparations. Many of these kits use silica-based resin minicolumns, which selectively adsorb plasmid DNA in the presence of high salt concentrations. Following removal of contaminants and reduction of the salt concentration in the resin, plasmid DNA is eluted from the column by centrifugation. The resulting DNA is suitable for applications such as restriction endonuclease digestion and DNA sequencing without further manipulation. Kits of this

design e.g. Wizard™ Miniprep (Promega Corporation) can be used to isolate plasmids of any size but work most efficiently with small plasmids (≤20 kb). The Wizard™ Miniprep system has been successfully used for plasmid analysis of various nosocomial pathogens such as *K. pneumoniae* and *E. cloacae* (Reed *et al.*, 1993).

Other commercial plasmid DNA purification methods use an anion-exchange resin purification minicolumn to selectively separate nucleic acids from other substances such as proteins and carbohydrates. In these systems, plasmid DNA is purified and eluted by gravity flow through the resin column and recovered by isopropanol precipitation. Using this system, plasmids up to 150 kb in size can be extracted with a purity equivalent of two rounds of cesium chloride density gradient centrifugation. One such system, the QIAGEN™ Plasmid Mini Kit (QIAGEN GmbH), has been used in previous studies investigating ESBL-mediated resistance (Pornull *et al.*, 1994).

In the present study, two commercial plasmid DNA purification methods, Wizard™ Miniprep plasmid kit and QIAGEN™ Plasmid Mini Kit were evaluated to determine if they were suitable for screening for plasmids encoding ESBL-mediated cephalosporin resistance in *S. marcescens*. Although the size limitation of the Wizard™ Miniprep plasmid extraction kit was known, the advantages of this kit in terms of cost and convenience warranted its inclusion in the evaluation. Following this evaluation, the QIAGEN™ Plasmid purification system was used to screen a selection of cefotaxime-susceptible and cefotaxime-resistant *S. marcescens* isolates for evidence of plasmid-encoded ESBLs.

5.1.2. **DNA amplification**

The use of DNA probing techniques in the investigation of ESBL-mediated resistance is well established. Traditionally, TEM and SHV-type ESBLs have been detected by Southern analysis using probes derived from intragenic sequences of TEM and SHV-resistant determinants (Petit *et al.*, 1990; Rice *et al.*, 1990; Gianneli *et al.*, 1994). The development of oligotyping colony hybridisation techniques which employ an array of oligonucleotide probes for the detection of specific point mutations has also facilitated the discrimination of known ESBL determinants (Mabilat & Courvalin, 1990; Zhou *et al.*, 1994). Despite these advances, DNA probing techniques are complicated by the

plethora of newly recognised β-lactamase enzymes that differ from each other by only a few amino acids. Consequently, recent genotypic resistance detection methods have focussed on the detection of ESBL-encoding genes using DNA amplification strategies such as polymerase chain reaction (PCR).

PCR is an *in vitro* method for enzymatically synthesising defined sequences of DNA. The reaction uses two oligonucleotide primers that hybridise to opposite strands and flank the target DNA to be amplified. The elongation of the primers is catalysed by a heat-stable DNA polymerase such as *Taq* polymerase. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by *Taq* DNA polymerase, results in exponential accumulation of a specific DNA fragment. The sensitivity of the method has resulted in the extensive use of PCR for the detection and analysis of many resistance genes including those encoding ESBLs (Pitout *et al.*, 1998; De Champs *et al.*, 2000). As a detection method, PCR offers the ability to detect novel β-lactamase genes and the potential to detect multiple β-lactamases expressed in an individual isolate (Sirot *et al.*, 1997). In one recent study, a PCR-based approach was used to identify TEM-1, multiple SHVs, OXA-9 and a plasmid-mediated AmpC β-lactamase in a single multiply-resistant isolate of *K. pneumoniae* (Hanson *et al.*, 1999). In *S. marcescens*, PCR has been widely used to detect both TEM- and SHV-type ESBLs (Luzzaro *et al.*, 1998).

Since most common ESBLs are TEM- or SHV-derived, amplification strategies for ESBL detection are normally based on the use of primers from conserved regions of the 5' and 3' portion of TEM and SHV structural genes. Following amplification, the resulting TEM and SHV-type PCR products can be analysed by techniques such as restriction fragment length polymorphism (Arlet *et al.*, 1995; Hanson *et al.*, 1999) or single strand conformational polymorphism (Leflon-Guibout *et al.*, 2000). However, the definitive confirmation and characterisation of amplified products require that the DNA nucleotide sequence of the amplimers be determined.

5.1.3. **DNA sequencing**

The nucleotide sequence of DNA can be established using chemical degradation or enzymatic methods (Maxam & Gilbert, 1977; Sanger *et al.*, 1977). The enzymatic or dideoxy method of sequencing is based on the ability of DNA polymerase to synthesise a complementary copy of single-stranded DNA template. The DNA polymerase acts by chain elongation at the 3' end of a primer that is annealed to the template DNA until a chain-terminating nucleotide is incorporated. Each sequence determination is carried out as a set of four separate reactions each of which contains all four deoxyribonucleotide triphosphates (dNTPs) (one of which is radiolabeled) plus a different dideoxyribonucleotide (ddNTP) which is present in a limited amount. Since ddNTPs lack the 3'-OH group necessary for chain elongation, the growing nucleotide is terminated selectively at guanine (G), adenine (A), thymine (T) or cytosine (C), depending on the respective dideoxy analogue in the reaction. The resulting fragments, each with a common origin but ending in a different nucleotide, are separated according to size by high-resolution denaturing polyacrylamide gel electrophoresis.

In recent years, dideoxy sequencing has been simplified by the application of PCR methodology in a process known as cycle sequencing. Thermal cycle sequencing offers several advantages over conventional sequencing strategies. Cycle sequencing protocols yield linear amplification of the template DNA and thus reduce the amount of DNA required to obtain a detectable sequence ladder. The methods also employ high annealing temperatures which increase the stringency of hybridisation. In addition, high polymerisation temperatures are used which decrease secondary structure formation in the DNA templates and permit a higher percentage of polymerization through highly structured regions such as GC-rich and palindrome-rich templates. More recently, automated cycle sequencing analysers have been developed which employ multicolour fluorescence-based sequencing chemistries. Using these analysers, the sequence ladder is resolved by high-speed electrophoresis using ultra-thin gels or capillary arrays which increase reproducibility and sample throughput. In automated sequencing systems, fluorescence labeling can be accomplished by direct incorporation of a labeled ddNTP into the growing chain (dye terminators) or by extension of an end-labeled primer. The dye terminator labeling offers the advantage of flexibility and simplicity (four sequencing reactions are performed in one tube).

The ready availability of DNA sequence data has resulted in the development of many sequence software analytical systems. DNA sequences are characterised using bioinformatics protocols such as 'database searching' and multiple sequence alignment. Database search protocols are used to search databases for sequences similar to the candidate sequence (similar sequences are likely to have similar structure and function). There are several different algorithms for implementing database searches but the standard protocol is based on the Blast family of software programs e.g. with Blast N protocols, DNA sequence database searches are run using the European Molecular Biology Laboratory (EMBL) or Genbank databases (Altschul *et al.*, 1997). In this system, sequences producing significant alignments are sorted by scoring for matches and mismatches between all nucleotides using matrix such as Blosum or PAM so that 'best' matches are presented first and all significant matches are formally aligned to show the homologous regions. Multiple sequence alignment (simultaneous alignment of many nucleotide or amino acid sequences) is used to detect or demonstrate similarity between new sequences and existing families of sequences, to find diagnostic patterns to characterise protein families and to suggest oligonucleotide primers for PCR. Pair-wise alignments are performed using dynamic programming that guarantees optimal alignment using a table of scoring matrix (e.g. Blosum62, Pam250) with penalties for insertions or deletions of different lengths. Multiple alignments are then built 'progressively' by a series of pairwise alignments following a branching order in a phylogenetic tree (Feng & Doolittle, 1987). Clustal W is a widely used, general purpose multiple alignment program for DNA or proteins (Thompson *et al.*, 1994).

The recent advances in sequencing technology and bioinformatics have meant that resistance genes can be readily characterized by PCR-based sequencing strategies. The identification of many known ESBLs has been confirmed and characterisation of many novel ESBL-derivatives has been attained by sequence analysis of amplified products (Naumovski *et al.*, 1992; Mugnier *et al.*, 1998). The application of direct PCR product sequencing strategies which circumvent the need for cloning protocols have further increased the ease with which sequencing data of known and novel ESBLs can be attained (Sirot *et al.*, 1998; De Champs *et al.*, 2000). In *S. marcescens*, characterisation of a new TEM-derived β-lactamase by direct PCR sequencing demonstrated that the nucleotide sequence showed all the conserved structural elements typical of a molecular

class A β-lactamase and a high degree of sequence similarity with TEM-1 β-lactamases (Perilli *et al.*, 1997).

In the present study, *S. marcescens* isolates were screened for TEM and SHV-derived ESBLs using TEM-specific and SHV-specific PCR. PCR products were identified by direct PCR cycle sequencing.

5.2. MATERIALS AND METHODS

5.2.1. Plasmid screening

5.2.1.1. Evaluation of commercial plasmid purification kits

Bacterial isolates

The Wizard™ Miniprep Plasmid kit (Promega Corporation, Madison, WI, US) and the QIAGEN™ Plasmid Mini Kit (QIAGEN GmbH, Hilden, Germany) were evaluated using eleven *S. marcescens* isolates (recovered in 1995) which demonstrated a range of cefotaxime MICs. Two reference strains carrying multiple plasmids, *E. coli* NCTC 50192 (Threlfall *et al.*, 1986) and *E. coli* NCTC 50193, were included with each batch of plasmid DNA extractions. These reference strains harboured plasmids in the following size range: *E. coli* NCTC 50192 (strain 39R861), 150, 64, 36, 7.0 kb (98, 42, 23.9, 4.6 MDa); *E. coli* NCTC 50193 (strain V517), 49, 7.9, 5.0, 4.6, 3.3, 2.6, 2.3, 1.8 kb (32, 5.2, 3.5, 3.0, 2.2, 1.7, 1.5, 1.2 MDa). All test and control isolates were removed from –70°C and subcultured on CBA on the day prior to testing.

Wizard ™Miniprep Plasmid kit

A single colony of bacterial culture was inoculated into 5 ml LB broth (Difco) and grown overnight for 14 h at 37°C with shaking (150 rpm). Plasmid minipreps were prepared using 3 ml of this overnight culture, according to the manufacturer's instructions for plasmid purification using a vacuum manifold (Promega's Vac-Man™, Promega). During the procedure all reagents used were supplied in the kit with the exception of the final elution buffer. Plasmid DNA was eluted from the minicolumn using 50 µl of TE buffer pH 8 (Appendix AII.2), which was preheated to 80°C (as recommended for the isolation of large plasmids) and applied to the minicolumn prior to centrifugation at 17,400 x *g* for 20 s in an Eppendorf 5417 microcentrifuge (Eppendorf). Plasmid DNA (10 µl volumes) was electrophoresed in 0.6% (w/v) agarose gels at 70 V in 0.5X Tris-Borate-EDTA (TBE) buffer (Appendix AII.2). Details of agarose gel electrophoresis, ethidium bromide staining and gel photography are outlined in Appendix AIII.1.

QIAGEN™ Plasmid Mini kit

Plasmid minipreps were performed using the 'QIAGEN-tip 20' and a modification of the procedure outlined for the purification of cosmids and low-copy-number plasmids. All reagents used in the extraction procedure were supplied in the kit with the exception of the TE buffer (Appendix AII.2) used to dissolve the final DNA pellet.

A single bacterial colony of bacterial culture was inoculated into 10 ml LB broth and grown overnight for 14 h at 37°C with shaking (150 rpm). Bacterial cells were pelleted at 2,500 x *g* for 10 min at 4°C in a Beckman J-6B centrifuge (Beckman Coulter), resuspended in 1 ml of Buffer P1 and 330 µl was dispensed into each of three tubes. Buffer P2 (330 µl) was added to each tube and mixed gently by inversion. After incubation at RT for 5 min, 330 µl of chilled Buffer P3 was added and mixed immediately. Following incubation on ice for 5 min, the cells were centrifuged at 17,400 x *g* for 10 min using an Eppendorf 5417 microcentrifuge (Eppendorf) and the supernatants were immediately transferred to fresh tubes and recentrifuged for a further 10 min. The cleared supernatant from each set of three tubes was pooled and promptly loaded into the equilibrated 'QIAGEN-tip 20'. Thereafter the plasmid extraction procedure was performed in accordance with the manufacturer's instructions. Plasmid DNA (10 µl volumes) was electrophoresed in 0.6% (w/v) agarose gels at 70 V in 0.5X TBE buffer (Appendix AII.2). Details of agarose gel electrophoresis, ethidium bromide staining and gel photography are outlined in Appendix AIII.1.

5.2.1.2. **Plasmid analysis**

Bacterial isolates

Plasmid analysis was performed on the 64 *S. marcescens* isolates from Collection A which comprised a selection of cefotaxime-susceptible and cefotaxime-resistant isolates recovered between 1995 and 1998 (see Section 4.2.2.3). The two NCTC control isolates harbouring reference plasmids, *E. coli* NCTC 50192 (Threlfall *et al.*, 1986) and *E. coli* NCTC 50193, were included with each batch of tests. An *E. coli* isolate harbouring a TEM-1-encoding plasmid and another harbouring a SHV-1-encoding plasmid were also included with each batch of tests. All test and control isolates were removed from –70°C and subcultured on CBA on the day prior to testing.

QIAGEN™ Plasmid Mini kit

QIAGEN™ plasmid minipreps were performed on all isolates in Collection A according to the method outlined in Section 5.2.1.1.

5.2.2. PCR amplification of TEM- and SHV-derived ESBLs

DNA amplification for TEM-encoding and SHV-encoding genes was performed during a working visit to MCRAB, Creighton University, under the guidance of Dr. Nancy Hanson, Director of Molecular Biology for CRAB and Assistant Professor in the Department of Medical Microbiology and Immunology.

5.2.2.1. Amplification of TEM-encoding and SHV-encoding genes

Bacterial isolates

DNA amplification for TEM-encoding and SHV-encoding genes was performed on 21 *S. marcescens* isolates from Collection A. These isolates (Collection B) comprised representative isolates demonstrating a range of cefotaxime MICs (cefotaxime-susceptible, n=11; cefotaxime-resistant, n=6 and cefotaxime low-level resistant, n=4), recovered between 1995 and 1998 (1995, n=6; 1996, n=4; 1997, n=4 and 1998, n=7). These *S. marcescens* isolates had been transported to MCRAB, Creighton University and frozen at –70°C, as previously described (see Section 4.2.2.4). An *E. coli* containing plasmid pBR322 which encodes a TEM-1 β-lactamase and *K. pneumoniae* 225 containing a 90 kb plasmid encoding TEM-1 and SHV-type β-lactamases (Hanson *et al.*, 1999) were obtained from MCRAB, Creighton University and used as positive amplification controls in each batch of isolates tested.

Primer design

Oligonucleotide primer sets specific for the TEM- and SHV-gene families (including classical and ESBL-derived β-lactamases) were designed in MCRAB, Creighton University (Hanson *et al.*, 1999). Details of the primer sets are included in Table 5.1.

Table 5.1. Details of PCR primer sets specific for TEM and SHV-gene families.

Primer set	Primer name	Primer sequence (5' to 3')	Melting temperature (°C)	Nucleotide position	Amplimer size (bp)
TEM-specific	TEM-prime 1 Forward	AGA TCA GTT GGG TGC ACG AG	70	313-332*	769
	TEM-prime End Reverse	CTT GGT CTG ACA GTT ACC	63	1082-1065*	
SHV-specific	SHV-prime 2 Forward	GGG AAA CGG AAC TGA ATG AG	65	606-625§	151
	SHV-prime 3 Reverse	ATC GTC CAC CAT CCA CTG CA	66	757-738§	

*, numbering according to Sutcliffe, 1978, where the ATG start codon is located at nucleotide position 209;
§, numbering according to Mercier & Levesque, 1990, where the ATG start codon is located at nucleotide position 125.

Preparation of DNA template for PCR

By the time TEM- and SHV-specific DNA amplification was undertaken, the problems associated with nuclease activity in *S. marcescens* isolates had been recognised (see Section 5.4.2). Template DNA was therefore prepared fresh for each DNA amplification reaction using a modification of a previously described method which included the addition of proteinase K (Pitout *et al.*, 1998). Since *S. marcescens* demonstrate maximal expression of the nuclease during early stationary phase, the bacteria were incubated for a shortened time period prior to DNA extraction. All isolates were removed from –70°C and subcultured on CBA on the day prior to DNA extraction.

Bacterial isolates were inoculated into 5 ml volume LB broths (Difco) and incubated for 3 h at 37°C with shaking (150 rpm). Bacterial cultures were placed on ice and 1.5 ml volumes were centrifuged at 17,300 x *g* for 5 min using a Hermle Labnet centrifuge (Hermle Laboratechnik GmbH, Wehingen, Germany). After centrifugation, the pellets were placed on ice, resuspended in 500 µl sterile deionised water and 10 µl of proteinase K (20 mg/ml, Sigma Chemical Co., Appendix AII.3) was added. The cells were lysed by heating to 95°C for 10 min and cellular debris was removed by centrifugation at 17,300 x *g* for 5 min. The supernatants containing template DNA were transferred to fresh microcentrifuge tubes and were used for DNA amplification within 24 h without further purification.

DNA amplification reaction

PCR assays for TEM and SHV-encoding genes were performed at the same time as DNA amplification assays for the structural gene for chromosomal AmpC β-lactamase production in *S. marcescens*. The demonstration of *ampC* PCR product in test *S. marcescens* isolates provided an internal control for the quality of template DNA. The details of the *S. marcescens ampC*-specific primers and amplification reaction are described in Section 6.2.3.2.

The TEM- and SHV- PCR reaction mixture was as follows: 0.2 mM each of dATP, dCTP, dGTP and dTTP (New England BioLabs Inc., Beverly, MA, US), 1.5 mM magnesium chloride, 1X PCR buffer (20 mM Tris-HCL, pH 8.4, 50 mM KCL) 1.25 units *Taq* DNA polymerase (Gibco BRL®/Life Technologies, Gaithersburg, MD, US)

and 0.5 μM of TEM (TEMprime1F, TEMprimeEndR) and SHV (SHVprime2F, SHVprime3R) primers (Gibco BRL®/Life Technologies) in a total volume of 49 μl. A master-mix of reagents was prepared for each batch of tests. The mix was aliquoted into individual tubes and 1 μl of DNA template was added. A negative amplification control containing 1 μl of sterile water was included in each batch of tests. The tubes were centrifuged briefly and 50 μl of sterile mineral oil was layered on the surface.

Amplification was carried out in a Perkin Elmer 480 thermocycler (Perkin-Elmer Corporation, Norwalk, CT, US). The PCR program consisted of an initial denaturation step at 96°C for 30 s followed by 24 cycles of DNA denaturation at 96°C for 30 s, primer annealing at 50°C for 15 s and primer extension at 72°C for 2 min. After the last cycle, the amplified products were stored at 4°C. Amplified DNA (5μl) was electrophoresed in a 1.5% (w/v) agarose gel using 1X TAE buffer (Appendix AII.2) at 70 V for 2 h. Details of ethidium bromide gel staining and photography are outlined in Appendix AIII.1. Reference size DNA fragments were included in each electrophoresis run (100 bp DNA ladder, Gibco BRL®/Life Technologies).

Purification of PCR products

TEM-like PCR products in the expected size range (700-800 bp) were electrophoresed in two separate lanes (20 μl/lane). Suspect bands were excised from the gel and the DNA was extracted and purified using the Qiaquick™ PCR product purification kit (QIAGEN Inc., Chatsworth, CA, US). This kit employs a spin-column containing a silica-gel membrane for the selective adsorption of DNA. TEM-specific PCR products were isolated in accordance with the manufacturer's instructions for Qiaquick™ gel extraction. The DNA from each lane was eluted in 30 μl of sterile deionised water. Eluates from individual PCR products were pooled (60 μl) and concentrated to approximately 30 μl using a speed vacuum centrifuge (DNA SpeedVac Concentrator, Savant Instruments, Inc., Holbrook, NY, US). Prior to sequencing, electrophoresis was repeated using 2 μl of concentrated PCR products to check the quality of the amplimers.

5.2.2.2. **DNA sequencing of TEM-PCR products**

Direct PCR cycle sequencing

TEM-like PCR products were sequenced by automated cycle sequencing with dye-terminator chemistry using a DNA stretch sequencer (Applied Biosystems, Foster City, CA, US). The sequencing was performed by the University of Nebraska Medical Center Core Facility, Nebraska, US.

DNA sequence analysis

Nucleotide sequence analysis was performed using the Blast N software (version 2.2.1) available on the Internet at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1997). Consensus sequences were generated for each PCR product by overlap of the forward and reverse nucleotide sequence (http://www.ncbi.nlm.nih.gov/blast/ bl2seq/wblast2.cgi) and run in 'database search' protocols (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignments were performed using Clustal W software (version 1.81) available on the Internet at the European Bioinformatics Institute (EBI) site (http://www.ebi.ac.uk). The DNA and amino acid sequence of four TEM-like PCR products from *S. marcescens* isolates and the published sequence of TEM-1 (Sutcliffe, 1978; Genbank accession number, GI: 208958) and TEM-1D (Leflon-Guibout *et al.*, 2000; Genbank accession number, GI: 6007800) β-lactamase from *E. coli* were aligned using a Blosum weight matrix (for similar sequences) and default settings for gap (10) and gap extension (0.05) penalties.

5.3. RESULTS

5.3.1. Plasmid screening

5.3.1.1. Evaluation of commercial plasmid purification kits

Ethidium bromide-stained agarose gels containing plasmid DNA extracted with the Wizard™ Miniprep Plasmid kit (panel A) and the QIAGEN™ Plasmid Mini kit (panel B) are shown in Figure 5.1. Plasmids were absent in the cefotaxime-resistant and cefotaxime-susceptible *S. marcescens* isolates selected using both plasmid extraction methods. The amount of residual chromosomal DNA was considerably reduced using the QIAGEN™ miniprep system and the reference plasmid bands in the control strains demonstrated improved resolution in the QIAGEN™ minipreps compared to Wizard minipreps. The Wizard™ Miniprep Plasmid kit failed to yield the 150 kb (98 MDa) reference plasmid in the NCTC 50192 control strain despite many repeat plasmid extractions (Figure 5.1, panel A, lane 1).

5.3.1.2. Plasmid analysis

Among the 64 *S. marcescens* isolates analysed (32 cefotaxime-resistant isolates and 32 cefotaxime-susceptible isolates recovered between 1995 and 1998) eight isolates demonstrated the presence of plasmid DNA (Figure 5.2, panels A and B). Of these eight isolates only three demonstrated cephalosporin resistance. The number and size of plasmids varied among the eight cefotaxime-resistant and cefotaxime-susceptible isolates (Table 5.2). In general the plasmids were of low molecular weight (≤49 kb). Three of the eight isolates (one cefotaxime-resistant, and two cefotaxime-susceptible) demonstrated plasmid bands with sizes similar to the TEM-1 and SHV-1 reference plasmids in the control isolates (Figure 5.2, panel A, lane 7; panel B, lanes 16 and 19) although their corresponding β-lactam resistance profile was not indicative of the presence of classical β-lactamases (ticarcillin MIC ≤32mg/l). None of the seven *S. marcescens* isolates demonstrating evidence of the presence of classical β-lactamases (ticarcillin MIC >256mg/l, see Section 4.3.2.3, paragraphs one and three) yielded plasmids in the size range of the reference TEM-1 or SHV-1 plasmids.

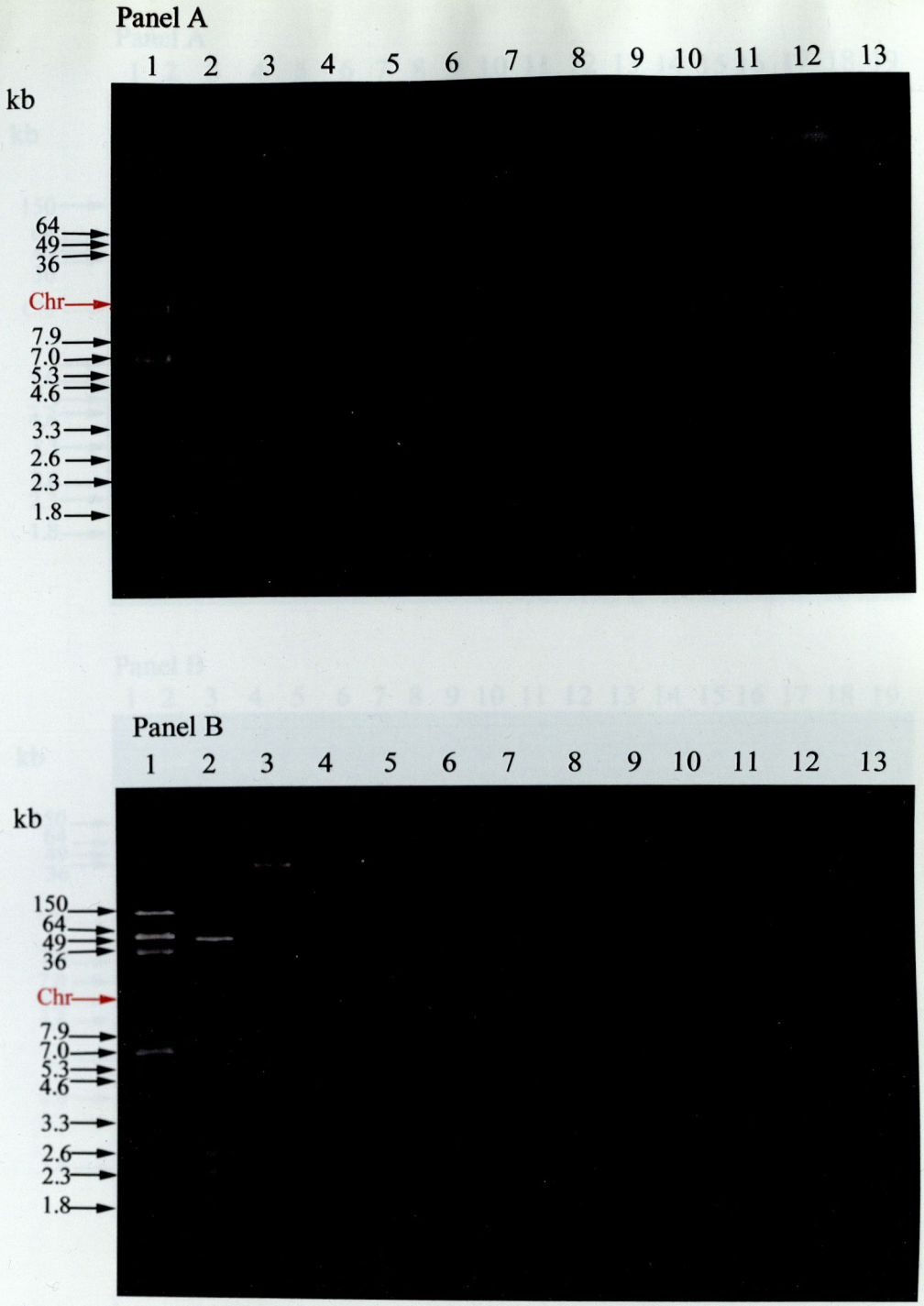

Figure 5.1. Ethidium bromide-stained agarose gels showing plasmid DNA prepared by the Wizard™ Miniprep Plasmid kit (panel A) and the QIAGEN™ Plasmid Mini kit (panel B). Plasmid DNA from control isolates is located in lanes 1 and 2 as follows: 1, *E. coli* NCTC 50192 (strain 39R861) with 150, 64, 36 and 7.0 kb (98, 42, 23.9, 4.6 MDa) plasmids; 2, *E. coli* NCTC 50193 (strain V517) with 49, 7.9, 5.3, 4.6, 3.3, 2.6, 2.3 and 1.8 kb (32, 5.2, 3.5, 3.0, 2.2, 1.7, 1.5, 1.2 MDa) plasmids. Plasmid DNA from test isolates is located in lanes 3–13 as follows: 3–8, cefotaxime-resistant *S. marcescens* isolates; 9–13, cefotaxime-susceptible *S. marcescens* isolates. Chr, chromosomal DNA.

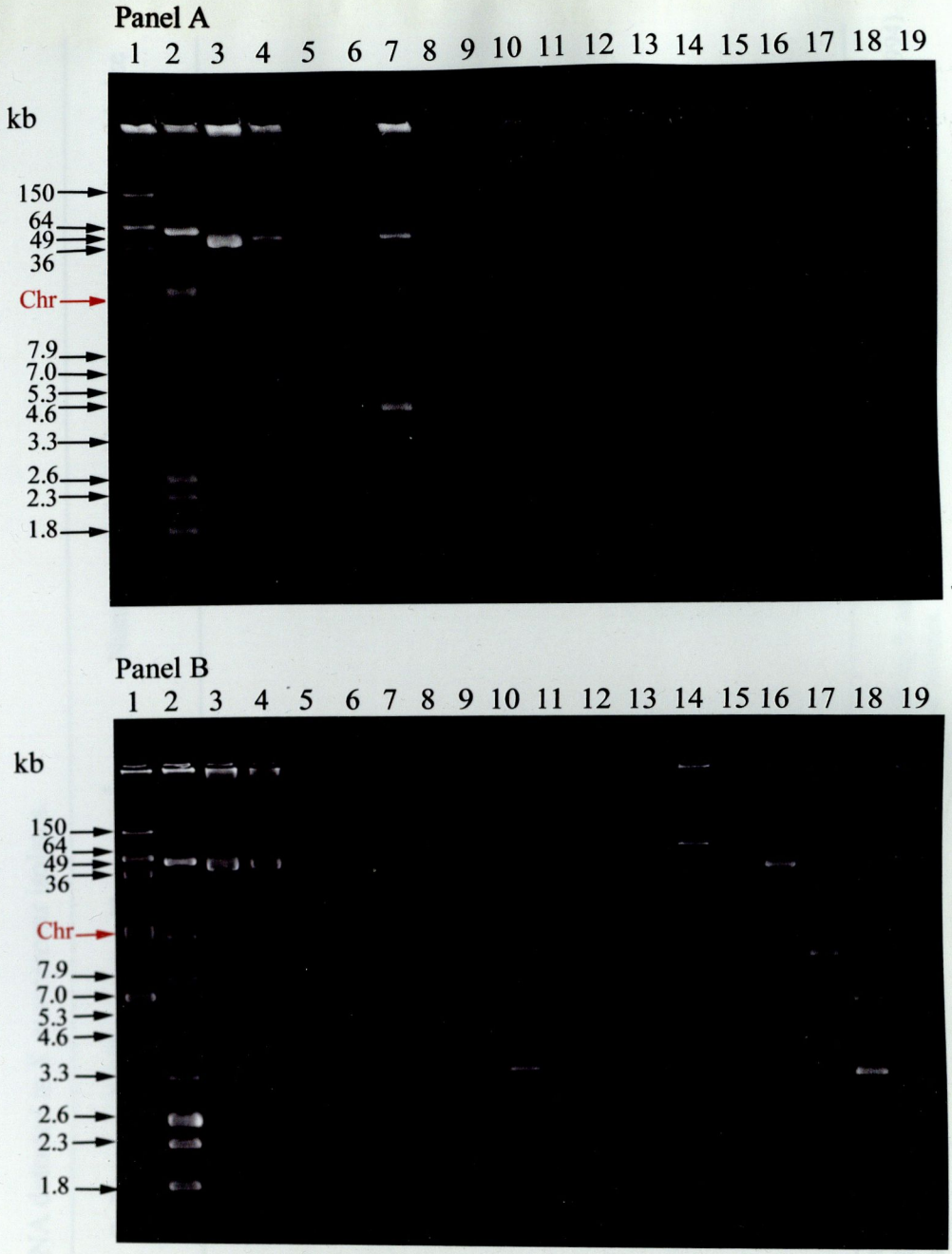

Figure 5.2. Ethidium bromide-stained agarose gels showing plasmid DNA prepared from *S. marcescens* isolates recovered in 1995 (panel A) and between 1996 and 1998 (panel B) using the QIAGEN™ Plasmid Mini kit.

Panel A. Plasmid DNA from control and test isolates is located in lanes as follows: 1, *E. coli* NCTC 50192 with 150, 64, 36 and 7.0 kb plasmids; 2, *E. coli* NCTC 50193 with 49, 7.9, 5.3, 4.6, 3.3, 2.6, 2.3 and 1.8 kb plasmids; 3, *E. coli* control with TEM-1-encoding plasmid; 4, *E. coli* control with SHV-1-encoding plasmid; 5–12, cefotaxime-resistant *S. marcescens* isolates; 13–19, cefotaxime-susceptible *S. marcescens* isolates.

Panel B. Plasmid DNA from control and test isolates is located in lanes as follows: 1–4, as in panel A; 5–13, cefotaxime-susceptible isolates recovered in 1996; 14–17, plasmid bearing isolates recovered in 1997; 18 & 19, plasmid bearing isolates recovered in 1998. Chr, chromosomal DNA.

Table 5.2. Analysis of plasmid DNA detected in *S. marcescens* isolates.

Isolate year	Isolate number	Cefotaxime MIC (mg/l)*	Phenotype§	Number of plasmids	Approximate plasmid size (kb)†
1995	95.R2	16	Low-level Resistant	2	5.0, 49
1996	96.S6	0.5	Susceptible	2	8.0, 3.0
1997	97.S1	0.12	Susceptible	1	>64 <150
	97.S3	0.12	Susceptible	1	>64 <150
	97.S4	0.5	Susceptible	1	49
	97.R4	16	Low-level Resistant	2	>8.0 <35, >8.0 <35
1998	98.R1	8.0	Low-level Resistant	2	6.0, 3.0
	98.S3	0.25	Susceptible	1	49

*, cefotaxime MICs (mg/l) were determined using the Etest method.

§, phenotype was determined using NCCLS interpretive standards for cefotaxime (susceptible, ≤8 mg/l; moderate, 16–32 mg/l; resistant, ≥64 mg/l) (NCCLS, 1994).

†, molecular weight of plasmids was estimated by comparison with reference plasmids in control isolates.

5.3.2. DNA amplification for TEM and SHV-derived ESBLs

5.3.2.1. Detection of TEM and SHV-encoding genes

In the DNA template control amplification reactions for *ampC* β-lactamase, a PCR product of the expected size (1140 bp) was demonstrated in all *S. marcescens* isolates tested, with each batch of DNA amplified (Figure 5.3, panel A). No *ampC* PCR products were found in the *E. coli* and *K. pneumoniae* TEM- and SHV-DNA amplification control isolates using the *S. marcescens ampC*-specific primer pair.

Using SHV-specific primers, a PCR product of the expected size (151 bp) was amplified from the positive control isolate, *K. pneumoniae* 225 (Figure 5.3, panel B, lane 25). No SHV primer-specific products were amplified from cefotaxime-resistant or cefotaxime-susceptible *S. marcescens* isolates. One cefotaxime-susceptible *S. marcescens* isolate demonstrated a non-specific PCR product with SHV-primers which was disregarded due to its large size (~500 bp) (Figure 5.3, panel B, lane 21).

When amplified with TEM-specific primers, both PCR positive control isolates (*E. coli* containing pBR322 and *K. pneumoniae* 225) demonstrated an amplimer of the expected size (769 bp). One cefotaxime-susceptible and five-cefotaxime-resistant (including three with low-level resistance) *S. marcescens* isolates yielded a TEM-like PCR product of the expected size (700–800 bp) (Figure 5.3, panel C). The TEM-like PCR products of the cefotaxime-susceptible isolate and three of the cefotaxime-resistant isolates were sequenced (Figure 5.3, panel D). All the *S. marcescens* isolates demonstrating β-lactam resistance profiles indicative of the presence of classical β-lactamases (ticarcillin MIC>256 mg/l) which were processed for PCR (n=4) yielded a TEM-like PCR product in the expected size range. For both SHV- and TEM- specific primers, PCR products were absent in the negative amplification controls.

5.3.2.2. DNA sequencing of TEM-PCR products

Nucleotide sequence analysis demonstrated that the four TEM-like *S. marcescens* PCR products shared 100% similarity with variant TEM-1D ($bla_{\text{TEM-1D}}$) β-lactamase in *E. coli* (Leflon-Guibout *et al.*, 2000). With Clustal W multiple alignment software, the nucleotide sequence of the four TEM-like PCR products from *S. marcescens* and TEM-1D β-lactamase in *E. coli* demonstrated 100% similarity with each other and 99%

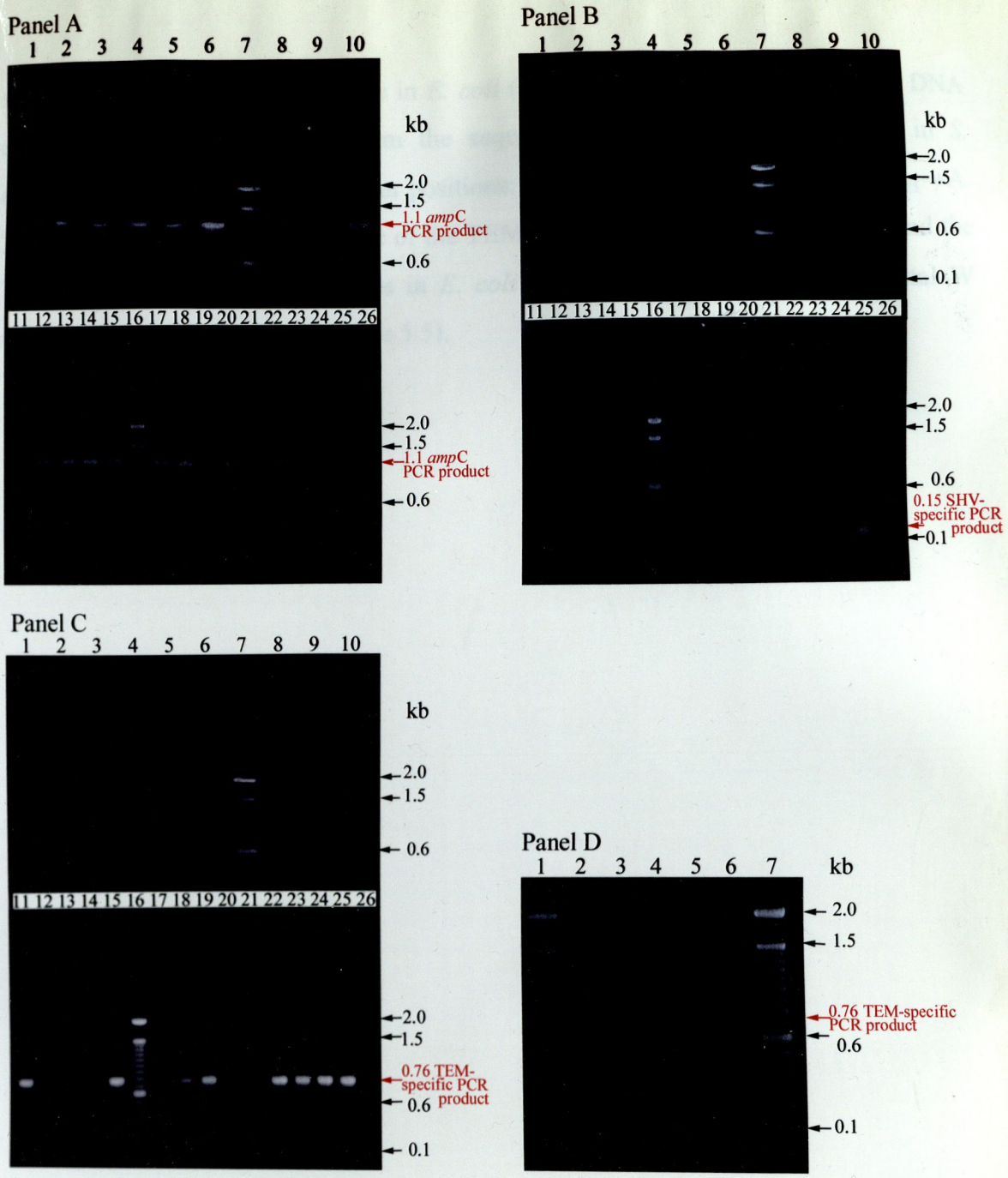

Figure 5.3. Ethidium bromide-stained agarose gels showing PCR products obtained when target DNA was amplified with *ampC*-specific primers (panel A), SHV-specific primers (panel B) and TEM-specific primers (panel C), and purified TEM-like PCR products for sequence analysis (panel D).

Panels A, B, C. Amplimers generated with template DNA from test and control isolates are located in lanes as follows: 1–6, *S. marcescens* isolates recovered in 1995; 8–11, *S. marcescens* isolates recovered in 1996; 12–15, *S. marcescens* isolates recovered in 1997; 17–23, *S. marcescens* isolates recovered in 1998; 24, TEM-specific PCR-positive control (*E. coli* containing pBr322); 25, TEM and SHV-specific PCR-positive control (*K. pneumoniae* 225). Lanes 7 & 16 contain standard size reference markers. Lane 26 contains the PCR negative control (water).

Panel D. Lane contents are as follows;1 & 7, standard size reference markers; 2, PCR negative control (water); 3–6, TEM-like PCR products generated with template DNA from *S. marcescens* isolates.

similarity with TEM-1 β-lactamase in *E. coli* (Sutcliffe, 1978) (Figure 5.4). The DNA sequence of TEM-1 differed from the sequence of the TEM-PCR product in *S. marcescens* and the TEM-1D at four positions: $A_{346}\rightarrow G$, $C_{436}\rightarrow T$, $T_{682}\rightarrow C$ and $G_{925}\rightarrow A$. The predicted amino acid sequence of the TEM-PCR products in *S. marcescens* and the TEM-1D and TEM-1 β-lactamases in *E. coli* shared 100% identity using Clustal W multiple alignment software (Figure 5.5).

CLUSTAL W (1.81) multiple nucleotide sequence alignment for TEM β-lactamases

```
TEM-1   ATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGC 234
TEM-ID  ATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGC 240
96.R1   ------------------------------------------------------------
97.S8   ------------------------------------------------------------
98.R2   ------------------------------------------------------------
97.S8   ------------------------------------------------------------

TEM-1   CCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGT 294
TEM-ID  CCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGT 300
96.R1   ------------------------------------------------------------
97.S8   ------------------------------------------------------------
98.R2   ------------------------------------------------------------
98.R8   ------------------------------------------------------------

                                                        A→G
TEM-1   GAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCT 354
TEM-ID  GAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAGCTGGATCT 360
96.R1   ------------------------------------TGGGTTACATCGAGCTGGATCT  22
97.S8   ------------------------------------TGGGTTACATCGAGCTGGATCT  22
98.R2   ------------------------------------TGGGTTACATCGAGCTGGATCT  22
98.R8   ------------------------------------TGGGTTACATCGAGCTGGATCT  22
                                            ************ ********

TEM-1   CAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCAC 414
TEM-ID  CAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCAC 420
96.R1   CAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCAC  82
97.S8   CAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCAC  82
98.R2   CAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCAC  82
98.R8   CAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCAC  82
        ************************************************************

                                C→T
TEM-1   TTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACT 474
TEM-ID  TTTTAAAGTTCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACT 480
96.R1   TTTTAAAGTTCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACT 142
97.S8   TTTTAAAGTTCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACT 142
98.R2   TTTTAAAGTTCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACT 142
98.R8   TTTTAAAGTTCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACT 142
        ********************* ************************************

TEM-1   CGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAA 534
TEM-ID  CGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAA 540
96.R1   CGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAA 202
97.S8   CGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAA 202
98.R2   CGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAA 202
98.R8   CGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAA 202
        ************************************************************

TEM-1    GCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA 594
TEM-ID   GCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA 600
96.R1    GCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA 262
97.S8    GCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA 262
98.R2    GCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA 262
28.R8    GCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA 262
         ************************************************************

TEM-1   TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTT 654
TEM-ID  TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTT 660
96.R1   TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTT 322
97.S8   TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTT 322
98.R2   TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTT 322
98.R8   TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTT 322
        ************************************************************
```

CLUSTAL W (1.81) multiple nucleotide sequence alignment for TEM β-lactamases cont.

```
                                        T→C
TEM-1   TTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGA 714
TEM-ID  TTTGCACAACATGGGGGATCATGTAACCCGCCTTGATCGTTGGGAACCGGAGCTGAATGA 720
96.R1   TTTGCACAACATGGGGGATCATGTAACCCGCCTTGATCGTTGGGAACCGGAGCTGAATGA 382
97.S8   TTTGCACAACATGGGGGATCATGTAACCCGCCTTGATCGTTGGGAACCGGAGCTGAATGA 382
98.R2   TTTGCACAACATGGGGGATCATGTAACCCGCCTTGATCGTTGGGAACCGGAGCTGAATGA 382
98.R8   TTTGCACAACATGGGGGATCATGTAACCCGCCTTGATCGTTGGGAACCGGAGCTGAATGA 382
        ************************** ******************************

TEM-1   AGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCG 774
TEM-ID  AGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCG 780
96.R1   AGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCG 442
97.S8   AGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCG 442
98.R2   AGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCG 442
98.R8   AGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCG 442
        ************************************************************

TEM-1   CAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGAT 834
TEM-1D  CAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGAT 840
96.R1   CAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGAT 502
97.S8   CAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGAT 502
98.R2   CAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGAT 502
98.R8   CAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGAT 502
        ************************************************************

TEM-1   GGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTAT 894
TEM-ID  GGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTAT 900
96.R1   GGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTAT 562
97.S8   GGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTAT 562
98.R2   GGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTAT 562
98.R8   GGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTAT 562
        ************************************************************
                                        G→A
TEM-1   TGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCC 954
TEM-ID  TGCTGATAAATCTGGAGCCGGTGAGCGTGGATCTCGCGGTATCATTGCAGCACTGGGGCC 960
96.R1   TGCTGATAAATCTGGAGCCGGTGAGCGTGGATCTCGCGGTATCATTGCAGCACTGGGGCC 622
97.S8   TGCTGATAAATCTGGAGCCGGTGAGCGTGGATCTCGCGGTATCATTGCAGCACTGGGGCC 622
98.R2   TGCTGATAAATCTGGAGCCGGTGAGCGTGGATCTCGCGGTATCATTGCAGCACTGGGGCC 622
98.R8   TGCTGATAAATCTGGAGCCGGTGAGCGTGGATCTCGCGGTATCATTGCAGCACTGGGGCC 622
        ************************************************************

TEM-1   AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGA 1014
TEM-ID  AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGA 1020
96.R1   AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGA 682
97.S8   AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGA 682
98.R2   AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGA 682
98.R8   AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGA 682
        ************************************************************

TEM-1   TGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTC 1074
TEM-ID  TGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTC 1080
96.R1   TGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTC 742
97.S8   TGAACGAAATAGACAGA---------------------------------------------- 699
98.R2   TGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTC 742
98.R8   TGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTC 742
        ****************
```

Figure 5.4. Multiple alignment of the nucleotide sequence of four TEM-like PCR products from *S. marcescens* (96.R1, 97.S8, 98.R2, 98.R8) with classical TEM-1D (Genbank accesssion 208958) and TEM-1 β-lactamase (Genbank accession 6007800) from *E. coli*. Nucleotides are numbered according to Sutcliffe, 1978. Translation start and stop codons are in boldface type. The location of the forward and reverse TEM-specific PCR primers is underlined. The four silent nucleotide substitutions in TEM-1D and *S. marcescens* test isolates are highlighted in yellow. *, denotes identical nucleotides

CLUSTAL W (1.81) multiple amino acid sequence alignment for TEM β-lactamases

```
TEM1   MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
TEM1D  MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
9663   ------------------------------------------GYIELDLNSGKILESFRP 18
97177  ------------------------------------------GYIELDLNSGKILESFRP 18
9828   ------------------------------------------GYIELDLNSGKILESFRP 18
268-1  ------------------------------------------GYIELDLNSGKILESFRP 18
                                                    ******************

TEM-1  EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
TEM-1D EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
96.R1  EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 78
97.S8  EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 78
98.R2  EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 78
98.R8  EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 78
       ************************************************************

TEM-1  CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
TEM-1D CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
96.R1  CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 138
97.S8  CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 138
98.R2  CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 138
98.R8  CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 138
       ************************************************************

TEM-1  PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
TEM-1D PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
96.R1  PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 198
97.S8  PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 198
98.R2  PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 198
98.R8  PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 198
       ************************************************************

TEM-1  RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW---- 286
TEM-1D RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW---- 286
96.R1  RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW---- 248
97.S8  RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQ---------------- 232
98.R2  RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW---- 246
98.R8  RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW---- 246
       ********************************
```

Figure 5.5. Multiple alignment of the amino acid sequence of four TEM-like PCR products from *S. marcescens* (96.R1, 97.S8, 98.R2, 98.R8) with classical TEM-1D (Genbank accession 208958) and TEM-1 β-lactamase (Genbank accession 6007800) from *E. coli*. The amino acid sequence of the *S. marcescens* isolates, TEM-1D and TEM-1 share 100% identity. Amino acids are numbered according to Sutcliffe, 1978. *, denotes identical sequences.

5.4. DISCUSSION

With the emergence of many ESBL derivatives and the increasing tendency of organisms to display multiple types of β-lactamases, the use of standard clinical susceptibility testing and phenotypic screening methods may no longer detect all types of β-lactamase-mediated resistance. The role of genotypic-based screening methods for the detection of β-lactamase-mediated resistance has been recognised. *Serratia marcescens* is known to play host to a number of ESBLs including TEM and SHV-derivatives and non-TEM, non-SHV ESBL enzymes which mediate a range of cephalosporin-resistance profiles (see Section 2.5.2). In this study a combination of molecular-based techniques was employed to investigate the presence of ESBL-mediated cephalosporin resistance.

5.4.1. Plasmid analysis

Plasmid analysis was used as a simple low-cost screening method to investigate the possible involvement of ESBL-mediated resistance. The availability of commercial plasmid purification systems enhanced the simplicity of this approach. However, since plasmid purification kits are primarily designed for the isolation of small recombinant DNA constructs following propagation in host organisms in molecular cloning experiments, the suitability of these commercial methods for plasmid screening in bacterial pathogens required assessment. The Wizard™ Miniprep plasmid kit is a simple, rapid (approximately 90 min) technique for the isolation of plasmid DNA but this kit failed to extract plasmids larger than 64 kb in the NCTC reference strain 50192. The use of high-speed spin minicolumns for the elution of plasmid DNA may have damaged the larger plasmids. The QIAGEN™ Plasmid mini kit also proved a simple and rapid plasmid isolation technique requiring approximately 180 min. The use of a gravitational flow minicolumn in the QIAGEN™ system not only improved the purity of the miniprep but also appeared to protect large plasmids from mechanical shearing. The availability of protocols designed for the isolation of low-copy number plasmids also suggested that this system was suitable for use in the screening of bacterial isolates for the presence of plasmid-encoded ESBLs.

From the 32 cephalosporin-resistant isolates analysed with the QIAGEN™ plasmid purification system, only three demonstrated plasmid DNA, with plasmid number and size varying for each isolate. Failure to demonstrate a plasmid of similar size in cephalosporin-resistant *S. marcescens* isolates was suggestive of the absence of a common mechanism of ESBL-mediated resistance. Nevertheless, the difficulty associated with the isolation of large ESBL-encoding plasmids prohibits the use of plasmid extraction as a definitive negative screening method. The possibility of the transfer of ESBLs to other mobile genetic elements such as transposons must also be taken into account. Currently there is no direct evidence to indicate transposition of ESBLs, although most TEM-type ESBL plasmids have been shown to hybridise with a DNA probe specific for the *tnpR* gene of Tn*3*, suggesting that at least one component of transposon machinery is present (Jacoby & Sutton, 1991). Failure to demonstrate a mobile genetic element in ESBLs is also surprising given that ESBLs are derived from classical β-lactamases which are normally resident on transposons (Heritage *et al.*, 1992). Indeed the frequency of transposition of classical β-lactamases may explain why the seven *S. marcescens* isolates in the present study demonstrating phenotypic evidence of classical β-lactamase-mediated resistance failed to show the presence of TEM/SHV-like plasmids. Furthermore, the likelihood that plasmids could be lost due to inappropriate storage of bacterial isolates must also be considered, although in the present study this possibility was minimised by freezing isolates at –70°C following initial isolation and by re-testing for cephalosporin resistance throughout the study period.

5.4.2. **Amplification for TEM and SHV-encoding ESBLs**

Since *S. marcescens* produces extracellular Dnase, extracted chromosomal DNA is liable to auto-digestion (Neal *et al.*, 1999). Previous reports have indicated that the addition of proteinase K into DNA extraction procedures can eliminate the auto-degradation of chromosomal DNA from *S. marcescens* isolates (Alonso *et al.*, 1993b). In the present DNA amplification studies, DNA extraction procedures were modified to include the addition of proteinase K and bacterial incubation periods were reduced to minimise endogenous nuclease production. To validate PCR procedures, DNA template control amplification reactions were also performed. The demonstration of an *ampC*-

specific PCR products in all test *S. marcescens* isolates verified that target nucleic acid was of acceptable quality for TEM and SHV DNA amplification reactions.

The negative results obtained in SHV-specific PCR confirmed the absence of SHV-type ESBLs in cephalosporin-resistant *S. marcescens* isolates. The failure of all test *S. marcescens* isolates (cefotaxime-susceptible and cefotaxime-resistant) to demonstrate the presence of SHV-specific PCR products also confirmed the absence of classical SHV-1 β-lactamase. The presence of TEM-type β-lactamases was indicated in one cefotaxime-susceptible and five cefotaxime-resistant *S. marcescens* isolates demonstrating TEM-like PCR products. PCR product sequencing was therefore required to distinguish between classical- and ESBL-derived TEM β-lactamases.

5.4.3. **Sequence analysis of TEM-specific PCR products**

Nucleotide sequence analysis demonstrated that TEM-like PCR products from *S. marcescens* shared 100% similarity with TEM-1D and 99% similarity with classical TEM-1 β-lactamases in *E. coli*. TEM-1D is a recently described variant of the classical TEM-1 β-lactamase which exhibits four silent nucleotide substitutions (Leflon-Guibout *et al.*, 2000). The translated amino acid sequence of TEM-type PCR products in *S. marcescens* shared 100% identity with classical TEM-1 β-lactamase. The results of TEM-specific PCR thus demonstrated the presence of TEM-1 β-lactamases and the absence of TEM-derived ESBLs in *S. marcescens* isolates. Previous studies have also identified TEM-1 β-lactamase in a significant proportion of *S. marcescens* isolates (Mabilat & Courvalin, 1990). Since the expression of classical β-lactamases is not associated with resistance to broad-spectrum β-lactam agents, the presence of TEM-1 β-lactamase in *S. marcescens* was not considered a probable source of cephalosporin resistance.

The results of PCR thus confirmed the absence of SHV- and TEM-derived ESBLs in cephalosporin-resistant *S. marcescens* isolates. Nevertheless, the presence of non-TEM non-SHV-derived ESBLs remained a possibility. Although rare, non-TEM, non-SHV CTX-M-like ESBLs have been described in *S. marcescens* (Bonnet *et al.*, 2000) (see Section 2.5.2, paragraph two). However, since the demonstration of these CTX-M-like ESBLs in *S. marcescens* is associated with combined high-level resistance to cefotaxime

and aztreonam, which is absent from isolates in this study, the presence of these ESBL derivatives seemed unlikely.

Unlike the plasmid analysis performed in this study, the results of β-lactamase PCR correlated well with the β-lactam resistance profile of the *S. marcescens* isolates. All isolates with resistance profiles suggestive of classical β-lactamase production, which were processed for PCR, demonstrated the presence of TEM-1 β-lactamase PCR products. The value of PCR and direct product sequencing was also evident in the characterisation and differentiation of classical TEM-1 β-lactamases and closely related variant enzymes.

5.4.4. **Conclusion**

The results of the genotypic resistance detection methods used in this study validated the results of the phenotypic screening procedures previously employed. Plasmid analysis and TEM- and SHV-specific PCR confirmed the absence of ESBL-mediated resistance in *S. marcescens* isolates. Having excluded other likely sources of β-lactamases, cephalosporin resistance in *S. marcescens* isolates was attributed to AmpC chromosomal β-lactamase production. The mechanism of low-level AmpC-mediated cephalosporin-resistance in *S. marcescens* isolates thus required investigation.

Chapter VI

Molecular investigation of chromosomal AmpC β-lactamase-mediated resistance in *Serratia marcescens*: *ampC*, the structural gene; *ampR*, the bifunctional regulator; *ampD*, the cytosolic amidase

Chapter VI

Molecular investigation of chromosomal AmpC β-lactamase-mediated resistance in *Serratia marcescens*: *ampC*, the structural gene, *ampR*, the bifunctional regulator and *ampD*, the cytosolic amidase

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6.1. INTRODUCTION

The molecular mechanism of inducible AmpC β-lactamase expression has been characterised in *E. cloacae* and *C. freundii*. Expression of AmpC is linked to perturbation in cell wall synthesis and the interplay of several gene products associated with cell wall recycling (Jacobs *et al.*, 1997) (see Section 2.3.1.2). The two most important genes involved in the control of AmpC expression, *ampR* (Honore *et al.*, 1986; Lindquist *et al.*, 1989b) and *ampD* (Lindberg *et al.*, 1987; Korfmann *et al.*, 1991), have been sequenced and the putative roles of their regulatory proteins have been described. Constitutive over-production or derepression of AmpC β-lactamase is frequently mapped to mutations in the *ampD* locus (Stapleton *et al.*, 1995b; Ehrhardt *et al.*, 1996). This stable derepression of AmpC production confers high-level resistance to all third-generation cephalosporins.

In the present investigation, the results of phenotypic studies demonstrated that cephalosporin resistance in *S. marcescens* was due to constitutive AmpC β-lactamase over-production. In the vast majority of cephalosporin resistant isolates AmpC β-lactamase expression appeared to be associated with a state of partial-derepression where the low level of constitutive β-lactamase over-production conferred only low-level resistance to cephalosporins. In the small percentage of isolates (<5%) where β-lactamase production was suggestive of a truly derepressed phenotype, the level of constitutive β-lactamase over-production appeared reduced compared to other fully-derepressed enterobacteria such as *E. cloacae*. This was reflected in the β-lactam resistance profile with *S. marcescens* isolates expressing only marginal reduction in susceptibility to some β-lactam agents such as piperacillin-plus-tazobactam and remaining fully susceptible to other agents such as ceftazidime. The relative low levels of β-lactamase over-production in *S. marcescens* may mirror variation in the expression of the β-lactamase enzyme but since the genetic machinery involved in the control of the structural *ampC* gene is unknown in this organism, this possibility has not been explored.

6.1.1. *ampC*, the structural gene

AmpC β-lactamase is encoded by the *ampC* structural gene. In *S. marcescens* (SR50) the *ampC* gene has been sequenced and found to encode a 355 amino acid precursor with a 21-residue signal peptide (Nomura & Yoshida, 1990). The mature protein has a predicted molecular mass of 39 kDa and a basic pI value of 9.5 which is typical of chromosomal class C β-lactamase enzymes (Tajima *et al.*, 1981). Comparative analysis of *ampC* β-lactamase genes from clinical isolates of *S. marcescens* demonstrates that within the species, *ampC* genes share greater than 92% amino acid similarity (Matsumura *et al.*, 1998). This high-level of intra-species *ampC* identity is also present in other AmpC-inducible organisms, such as *E. cloacae* and *P. aeruginosa*, with *ampC* genes sharing greater than 98% amino acid similarity within each species (Galleni *et al.*, 1988; Girlich *et al.*, 2000b).

Although AmpC-inducible enterobacteria such as *E. cloacae* and *C. freundii* also share a high level (greater than 70%) of inter-species AmpC identity, this is not evident in *S. marcescens* (Table 6.1). The amino acid sequence of AmpC β-lactamase in *S. marcescens* shares less than 40% identity with AmpC sequences from other organisms with inducible β-lactamase expression (Joris *et al.*, 1986). Nevertheless, a high percentage identity exists between the AmpC amino acid sequence of all AmpC-inducible organisms, including *S. marcescens,* in the vicinity of the active site of the AmpC enzyme (Nomura & Yoshida, 1990). Hence, the mature AmpC protein in *S. marcescens* has been shown to contain the three signature motifs involved in the formation and stabilisation of the active site of class C β-lactamases (Matsumura *et al.*, 1998). These three motifs, serine-X-X-lysine (SXXK) (where X is any amino acid), tyrosine-serine-asparagine (YSN), and lysine-threonine-glycine (KTG) are characteristic of all class C β-lactamases (Lobkovsky *et al.*, 1993; Medeiros, 1997).

Since the *ampC* gene in *S. marcescens* is similar to the *ampC* gene in other AmpC-inducible enterobacteria and has been shown to demonstrate inducible β-lactamase production, it seems likely that the expression of this gene would be controlled in a manner similar to other AmpC-inducible enterobacteria. The regulation of AmpC β-lactamase expression in *S. marcescens* is thus assumed to result from the interactions of specific cofactors within an *ampC-ampR* complex analogous to the *ampC-ampR* systems

Table 6.1. Percent identity of the amino acid sequence of AmpC β-lactamase from *S. marcescens* (SR50) with other chromosomal AmpC β-lactamases.

Bacterial strain	*S. marcescens* SR50	*E. cloacae* P99	*C. freundii* OS60	*M. morganii* SLM01	*P. aeruginosa* PAO1	*E. coli* K12	Study
S. marcescens SR50	100	39	38	39	43	39	Nomura & Yoshida, 1990
E. cloacae P99	....	100	73	54	42	69	Lindberg & Normark, 1987
C. freundii OS60	....	....	100	55	40	76	Lindberg & Normark, 1986
M. morganii SLM01	....	....	....	100	45	57	Barnaud *et al.*, 1997
P. aeruginosa PAO1	....	....	....	....	100	41	Lodge *et al.*, 1990
E. coli K12	....	....	....	....	....	100	Jaurin & Grundstrom, 1981

described in other enterobacteria with inducible *ampC* genes (Naas *et al.*, 1995). Similarly, a common role for *ampD* in the regulation of *ampC* expression is indicated (Weindorf *et al.*, 1998). Identification and characterisation of the *ampR* and *ampD* genes in *S. marcescens* may thus confirm the regulatory mechanism for AmpC expression and may explain the variation in the observed level of constitutive AmpC β-lactamase production.

Recent evidence suggests that *S. marcescens*, like other AmpC-inducible enterobacteria, have developed the ability to express AmpC-mediated cephalosporin resistance, not only through constitutive β-lactamase over-production, but also by extension of the β-lactamase hydrolysis profile (Nukaga *et al.*, 1995; Morosini *et al.*, 1998) (see Section 2.5.1, paragraph four). Cephalosporin-resistant *S. marcescens* isolates have been reported where extended AmpC activity is attributed to mutations which affect the active site of the enzyme. In the first of these reports, extension of the AmpC hydrolytic profile of clinical isolates of *S. marcescens* was shown to result from mutation in the DAES motif within the active site of the AmpC β-lactamase enzyme (Matsumura *et al.*, 1998). In more recent studies, extended-spectrum β-lactamase activity was also reported in laboratory derived mutants of *S. marcescens* demonstrating constitutive AmpC β-lactamase production. In this case cephalosporin resistance was attributed to the combined effect of β-lactamase over-production and mutation in the Thr64 residue in the vicinity of the active site of the β-lactamase enzyme (Raimondi *et al.*, 2001). Any investigation of AmpC β-lactamase-mediated cephalosporin resistance in *S. marcescens* must thus involve examination of the *ampC* structural gene in addition to characterisation of the putative regulatory genes.

6.1.2. *ampR*, the transcriptional regulator

The *ampR* gene is a bifunctional transcriptional regulator of inducible AmpC β-lactamase (Lindberg *et al.*, 1985; Lindberg & Normark, 1987). The gene encodes a 32 kDa protein that is a member of the LysR family of transcriptional regulators. The *ampR* gene is located upstream from *ampC* and is transcribed divergently from a common overlapping intercistronic region (Honore *et al.*, 1986). The inducibility of *ampC* is dependent upon the activation of the AmpR DNA-binding protein. In *E. coli*, *ampC* is constitutively expressed at low levels and is uninducible due to the absence of

ampR (Honore *et al.*, 1986). The AmpR protein functions as a repressor of *ampC* expression during normal cell growth and as an anti-repressor in the presence of β-lactam inducers (Bennett & Chopra, 1993; Jacobs, 1997) (see Section 2.3.1.2, Figure 2.3, Figure 2.4). DNase footprinting experiments have demonstrated that AmpR interacts by binding to a 38 bp region covering the *ampR* promoter which lies immediately upstream of the *ampC* promoter (Lindquist *et al.*, 1989b).

With the exception of *S. marcescens*, the sequence of the *ampR* gene and the *ampC-ampR* intercistronic region have been described in all AmpC-inducible enterobacteria (Honore *et al.*, 1986; Lindquist *et al.*, 1989b; Poirel *et al.*, 1999). Comparative analysis of the amino acid sequence of *ampR* genes reveals considerable inter-species divergence. Although the *ampC-ampR* intercistronic regions in *E. cloacae* and *C. freundii* have a common location (downstream of the fumarate operon, *frdABCD*) and are highly conserved, the *ampR* genes of these species share less than 80% similarity (Lindquist *et al.*, 1989b). The *ampR* genes in *E. cloacae* and *C. freundii* have also been shown to demonstrate marked quantitative differences in AmpC β-lactamase induction levels during transcomplementation experiments (Lindberg & Normark, 1987). In *M. morganii*, the *ampR* gene demonstrates only 60% similarity with *ampR* genes in other AmpC-inducible species. The *ampC-ampR* promoter overlap in *M. morganii* is also significantly shorter than in other enterobacterial species and has a different downstream location, being surrounded by the *hybF* and *orf*-1 genes (Poirel *et al.*, 1999). Among the AmpC-inducible species, significant amino acid identity has been detected in the N terminus of AmpR which contains the putative helix-turn-helix (HTH) motif required for binding to the *ampC-ampR* intercistronic region (Lindquist *et al.*, 1989b).

Although alterations in AmpC β-lactamase expression are most commonly attributed to mutations in AmpD, specific mutations in AmpR associated with noninducible, high-level constitutive and hyperinducible AmpC phenotypes, have also been described. Following site-directed mutagenesis in *C. freundii*, a single amino acid substitution (serine to phenylalanine at position 35) in the DNA binding motif, was shown to prevent AmpR binding in the *ampC-ampR* intergenic region and resulted in a 2.5-fold increase in *ampC* expression compared to wild-type isolates (Bartowsky & Normark, 1991). Similarly, a single amino acid substitution from glycine to glutamic acid at position 102 was shown to give rise to constitutive high-level *ampC* expression in the absence of β-

lactam inducers (Bartowsky & Normark, 1991). *In vitro* selection studies in *E. cloacae* also indicate that single point mutations in *ampR* at asparagine-135 and arginine-86 result in 75–450-fold increases in AmpC β-lactamase activity regardless of the presence or absence of inducers (Kuga *et al.*, 2000). The ease of selection of AmpR mutants in these studies (approximate frequency of 10^{-6} cells) also suggests that these constitutive phenotypes could emerge in the clinical setting. Evidence thus indicates that in addition to the recognised mutations in the *ampD* locus, mutations in the *ampR* gene may also be responsible for the different AmpC phenotypes described in AmpC-inducible enterobacteria (Hanson & Sanders, 1999).

6.1.3. *ampD*, the cytosolic amidase

AmpD is a 20.5 kDa cytosolic amidase involved in the intracellular recycling of cell wall fragments (Kopp *et al.*, 1993). By modifying the AmpC β-lactamase induction signal, AmpD functions as a negative regulator of AmpC expression (Jacobs *et al.*, 1995) (Figure 2.3). The involvement of AmpD in cell wall metabolism indicates that the *ampD* gene is probably conserved among Gram-negative organisms regardless of AmpC inducibility (Jacobs *et al.*, 1994). In *E. coli*, the *ampD* gene has been mapped between the *nadC-aroP* region at minute 2.4 on the chromosome (Lindberg *et al.*, 1985).

The nucleotide sequence of *ampD* genes from isolates of *E. cloacae*, *C. freundii* and *E. coli* has been published (Honore *et al.*, 1989; Kopp *et al.*, 1993). The wild-type *ampD* nucleotide sequence in these species demonstrates 74–83% similarity (Kopp *et al.*, 1993). Divergence occurs in the carboxy termini with the addition of four amino acids in *E. cloacae* and *C. freundii* compared to *E. coli* (Kopp *et al.*, 1993). All sequence data indicates the importance of the carboxy portion of the AmpD protein for the inducible AmpC phenotype.

Constitutive over-production of AmpC β-lactamase is most commonly associated with mutations in *ampD* (Lindberg *et al.*, 1987; Weindorf *et al.*, 1998). In enterobacteria, mutations in *ampD* have been associated with three different phenotypes of derepressed AmpC β-lactamase expression; full and semi-constitutive derepression (high-basal-level and constitutive β-lactamase production), hyperinducible derepression (higher-basal-

level and high-level induction in the presence of low levels of inducing agents) and temperature-sensitive derepression (loss of inducibility at non-permisssive temperatures) (Korfmann *et al.*, 1991; Ehrhardt *et al.*, 1996) (Table 6.2). Mutations that result in alteration of the AmpD protein produce a hyperinducible phenotype e.g. transition from aspartic acid to glycine at position 121 and position 127 in *E. cloacae* is associated with the hyperinducible phenotype (Kopp *et al.*, 1993; Ehrhardt *et al.*, 1996). Mutations that result in complete inactivation of AmpD produce a fully-derepressed phenotype e.g. substitution of tryptophan to termination codon at position 95 in *C. freundii* (Stapleton *et al.*, 1995b). The variation in the site and type of *ampD* mutations documented in the literature indicates that no one mutation or set of mutations can be correlated with a specific AmpC phenotype (Hanson & Sanders, 1999).

The role of AmpD in the regulation of AmpC β-lactamase expression in *S. marcescens* has been inferred from transformation studies with wild-type *ampD*. In these studies, evidence of the restoration of β-lactam susceptibility in cephalosporin-resistant isolates of *S. marcescens* following transformation with plasmids coding for wild-type *ampD*, was used to suggest the involvement of mutated AmpD in derepressed phenotypes (Gianneli *et al.*, 1994; Weindorf *et al.*, 1998). Nevertheless, the presence of *ampD* and its putative role in altered AmpC β-lactamase expression have not as yet been confirmed for *S. marcescens*.

6.1.4. **Design of present study**

In contrast to the extensive investigations undertaken in other enterobacteria with inducible AmpC β-lactamase production, very little work describing the molecular mechanism of AmpC expression has been reported in *S. marcescens*. The present study was designed to identify the *ampC* structural gene and the regulatory *ampD* and *ampR* genes implicated in AmpC expression in *S. marcescens* and to investigate if the low-level AmpC-mediated cephalosporin resistance observed in this study is linked to mutations in these genetic loci. In previous studies involving enterobacteria with inducible AmpC β-lactamase, AmpC phenotypes have been directly linked to the discovery of mutations in one of the three genes, *ampD*, *ampR* or *ampC* in the absence of sequence data on the other two genes (Korfmann *et al.*, 1991; Stapleton *et al.*, 1995b). In addition, many studies investigating altered AmpC expression have characterised

Table 6.2. AmpD mutants and associated AmpC phenotypes reported in the literature.

AmpC phenotype	AmpD mutant	Host	Genotype	Amino acid substitution	Study
Fully derepressed	*ampD*04	*E. cloacae*	...	16 carboxy-terminal residues deleted	Kopp *et al.*, 1993
	*ampD*02	*E. cloacae*	tandem duplication	frameshift after codon 98	Kopp *et al.*, 1993
	*ampD*029	*E. cloacae*	bp deletion at 275	truncation at position 133	Ehrhardt *et al.*, 1996
	CBN1	*C. freundii*	T98G transversion	Valine-33 to Glycine	Stapleton *et al.*, 1995b
	31A12	*C. freundii*	G284A transition	Tryptophan-95 to termination	Stapleton *et al.*, 1995b
	31F11	*C. freundii*	T492G transversion	Aspartic acid-164 to Glutamic acid	Stapleton *et al.*, 1995b
	*ampD*11	*E. coli*	T to G transversion	Tryptophan-7 to Glycline	Honore *et al.*, 1989
(semi-constitutive)*	*ampD*2	*E. coli*	IS*1* insertion	insertion after codon 121	Lindberg & Normark, 1987
Hyperinducible	*ampD*05	*E. cloacae*	A to G transition	Aspartic acid-121 to Glycine	Kopp *et al.*, 1993
	*ampD*1194	*E. claocae*	A to G transition	Aspartic acid-127 to Glycine	Ehrhardt *et al.*, 1996
	CBN3	*C. freundii*	C473A transversion	Alanine-158 to Glutamic acid	Stapleton *et al.*, 1995b
	31E3	*C. freundii*	T283A transversion	Tryptophan-95 to Arginine	Stapleton *et al.*, 1995b
	CBN2	*C. freundii*	T304G transversion	Tryptophan-12 to Aspartic acid	Stapleton *et al.*, 1995b
	ampD1	*E. coli*	52-base frameshift	Deletion after codon 23	Lindberg & Normark, 1987
Temperature sensitive	*ampD*029-5	*E. cloacae*	...	Tryptophan-171 to Cysteine	Ehrhardt *et al.*, 1996

*, isolate with derepressed phenotype showing residual inducibility; bp, base pair. Adapted from Hanson & Sanders, 1999.

genetic mutations derived by *in vitro* antibiotic selection which may not reflect the *in vivo* selection process (Stapleton *et al.*, 1995b; Kuga *et al.*, 2000). In the present study, the presence and sequence of all three genes, *ampD*, *ampR* and *ampC* were investigated in clinical isolates of *S. marcescens* expressing wild-type, partially-derepressed and derepressed AmpC phenotypes.

At the start of this study, numerous attempts were made to detect the *ampD* and *ampR* genes in *S. marcescens* using a PCR-based amplification strategy employing PCR primers derived from conserved regions of *ampD* and *ampR* genes from other enterobacteria with inducible AmpC expression. Subsequently, DNA hybridisation assays were employed to identify the putative regulatory genes using *ampD*- and *ampR*-specific probes derived from wild-type isolates of *E. cloacae* and *C. freundii*. Using this technique, the *ampD* gene was located in *S. marcescens* and suspect *ampD*-like DNA fragments were cloned and sequenced. The *ampD* sequence was confirmed by sequencing cloned PCR products amplified using *S. marcescens*-specific *ampD* primers.

Following attempts to locate *ampR* using hybridisation techniques, the gene was identified and sequenced by PCR amplification using unpublished sequence data of *ampR* in *S. marcescens,* made available by MCRAB, Creighton University. The *ampC* gene (including the *ampC-ampR* intercistronic region) was characterised by sequencing cloned amplimers using PCR primers derived from the known *ampC* sequence in *S. marcescens* (Nomura & Yoshida, 1990). To investigate the genetic locus responsible for constitutive AmpC over-expression in low-level cephalosporin-resistant isolates, the gene sequences of *ampD*, *ampR* and *ampC* in wild-type, partially-derepressed and derepressed *S. marcescens* isolates were aligned and examined for evidence of mutation. The role of *ampD* in altered AmpC expression was also investigated using transformation studies with plasmids encoding wild-type *ampD* from *E. cloacae*.

6.2. MATERIALS AND METHODS

6.2.1. Identification and characterisation of *ampD* in *S. marcescens*

6.2.1.1. PCR amplification for identification of *ampD* using primers derived from *ampD* sequences in *E. cloacae*, *C. freundii* and *E. coli*

Bacterial isolates

DNA amplification to identify *ampD*-encoding genes was performed on 21 *S. marcescens* isolates in Collection B (see Section 5.2.2.1). These isolates comprised representative isolates expressing a range of cefotaxime MICs (cefotaxime-susceptible, n=11; cefotaxime-resistant, n=6 and cefotaxime low-level resistant, n=4), recovered between 1995 and 1998 (1995, n=6; 1996, n=4; 1997, n=4 and 1998, n=7). An *E. coli* isolate (SN03/pNU305) carrying plasmid pNU305 encoding the *ampR* and *ampC* genes from *C. freundii* OS60 (Lindberg *et al.*, 1985) and another *E. coli* isolate (JRG582/pNU405) carrying plasmid pNU405 encoding *ampD* from *C. freundii* OS60 (Lindberg *et al.*, 1987) were kindly donated by Dr Susanne Lindquist, Department of Pediatrics, University of Umeå, Sweden (Table 6.3). These plasmids were used as positive (pNU405) and negative (pNU305) amplification controls in each batch of isolates tested. Wild-type isolates of *E. cloacae*, *C. freundii* and *E. coli* isolated from Hospital 1 were also processed as positive DNA amplification controls with each batch of tests (Table 6.3). All test and control isolates were removed from –70°C and subcultured on CBA on the day prior to DNA extraction.

Preparation of template DNA

Template DNA was prepared using the Wizard™ Genomic DNA Purification Kit (Promega). This kit was originally designed for the rapid extraction of double-stranded genomic DNA from white blood cells but was later modified for DNA extraction from bacterial cells. In the extraction protocol for Gram-negative bacteria, bacterial cells are lysed and treated with RNase. Cellular proteins are removed by salt precipitation which removes proteins but leaves the high molecular weight genomic DNA in solution. Genomic DNA is concentrated and desalted by isopropanol precipitation. The DNA extraction protocol used is described in Appendix AIII.2.1. Plasmids pNU305 and pNU405 were extracted using the QIAGEN™ Plasmid Mini kit as outlined in Section 5.2.1.1.

Table 6.3. Bacterial isolates and plasmids used in the investigation of AmpC β-lactamase expression in *S. marcescens*.

Bacterial isolates/plasmids	**Bacterial source/plasmid host**	**Relevant genotype**	**Study**
Bacterial isolates	Bacterial source		
Wild-type *E. cloacae*	Hospital 1, Dublin	*ampC+, ampR+, ampD+*	This study
Wild-type *C. freundii*	Hospital 1, Dublin	*ampC+, ampR+, ampD+*	This study
Wild type *E. coli*	Hospital 1, Dublin	*ampC+, ampR-, ampD+*	This study
Wild-type *S. marcescens*, Serr1*	MCRAB, Creighton University	*ampC+, ampR+, ampD+*	Morrow, 1999
S. marcescens NCTC 11935	NCTC	*ampC+, ampR+, ampD+*	....
Plasmids	Plasmid host		
pNU305	*E. coli,* SN03/pNU305	*ampC+, ampR+, ampD-*	Lindberg *et al.*, 1985
pNU405	*E. coli,* JRG582/pNU405	*ampC-, ampR-, ampD+*	Lindberg *et al.*, 1987
pNH5	*E. coli,* JRG582/pNH5	*ampC-, ampR-, ampD+*	Honore *et al.*, 1989

MCRAB, Molecular Biology Laboratory at the Center for Research in Anti-Infectives and Biotechnology, Creighton University School of Medicine, Nebraska, United States.
NCTC, National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, United Kingdom.
*, Serr1 was characterised as wild-type in MCRAB, Creighton University, using β-lactam susceptibility testing and β-lactam hydrolysis assays.

DNA was quantified using the agarose gel electrophoresis quantification method outlined in Appendix AIII.3. Prior to amplification, genomic DNA was diluted 1/100 to yield an approximate DNA concentration of 3 ng/µl and plasmid DNA was diluted 1/200 to yield an approximate DNA concentration of 1 ng/µl, for use in amplification reactions.

Primer design

Oligonucleotide primer sets for *ampD* were designed from conserved intergenic regions of the DNA sequence of known *ampD* genes. The DNA sequence of *ampD* from *E. cloacae* (*E. cloacae* 14, Genbank accession number Z14003) (Kopp *et al.*, 1993), *C. freundii* (Cit. OS60, Genbank accession number Z14002) (Kopp *et al.*, 1993) and *E. coli* (Genbank accession number X15398) (Honore *et al.*, 1986) were aligned using Clustal W software available on the Internet (http://www.ebi.ac.uk) and examined for suitable PCR primer sites (Figure 6.1). Two sets of *ampD*-specific primer pairs were designed, *ampD* Set 1 and *ampD* Set 2, with both sets using a common reverse primer. When base-pair mismatches occurred in the selected primer regions, the *E. cloacae ampD* sequence was used as the template for primer design. Primers were custom-made on request by Genosys Biotechnologies Ltd., Cambridgeshire, UK. Details of the primer sets are shown in Table 6.4.

DNA amplification reactions

DNA amplification for *ampD* was performed in conjunction with DNA amplification of the *ampC* structural gene. The demonstration of *ampC* PCR product in the *S. marcescens* test isolates provided an internal control for the quality of template DNA. The details of the *S. marcescens ampC*-specific primers and amplification reactions are described in Section 6.2.3.1.

The *ampD* PCR reaction mixture was as follows: 0.2 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Bell Lane, East Sussex, UK), 1.5 mM magnesium chloride, 1X High Fidelity PCR buffer (Boehringer Mannheim), 2.6 units High Fidelity *Taq* DNA polymerase and *Pwo* DNA polymerase mix (Boehringer Mannheim) and 1 µM of *ampD* primer Set 1 or *ampD* primer Set 2 (Genosys Biotechnologies Ltd.) in a total reaction volume of 45 µl. A master-mix of reagents was prepared for each batch of tests. The mix was aliquoted into individual tubes and 5 µl of DNA template (15 ng

CLUSTAL W (1.81) nucleotide sequence alignment of *ampD* gene for PCR primer selection



Figure 6.1. Multiple alignment of the nucleotide sequence of *ampD* genes from *E. cloacae* (*E. cloacae* 14, Genbank accession number Z14003) (Kopp *et al.*, 1993), *C. freundii* (Cit. OS60, Genbank accession number Z14002) (Kopp *et al.*, 1993) and *E. coli* (Genbank accession number X15398) (Honore *et al.*, 1986). Also shown is the upstream intergenic region between *ampD* and the adjacent *nadC* gene and the beginning of the *nadC* sequence. *, denotes identical sequences. Translation start and stop codons are in boldface type. The location of the five sets of *ampD* primers are underlined: Set 1, ——→; Set 2, - - - -→; Set 3, ——→; Set 4 - - - -→; Set 5, ——→.

Table 6.4. Details of *ampD*-specific PCR primer sets.

Primer set	Primer name	Primer sequence (5' to 3')	Melting temperature (°C)	Nucleotide position*	Amplimer size (bp)
ampD Set 1§	*ampD*1-id-Forward	TGG CTG GCG GAC GCG CG	62	172–189	374
	*ampD*1-id-Reverse	CTG ATA TTG CGC ATC GG	52	546–530	
ampD Set 2§	*ampD*2-id-Forward	TCC GTG GAT CGA TGC GT	54	288–304	258
	*ampD*1-id Reverse	CTG ATA TTG CGC ATC GG	52	546–530	
ampD Set 3†	*ampD*3-280-conf-Forward	CCG CCC GGA GGA TGA	68	216–228	250
	*ampD*3-280-conf-Reverse	GCC CCT GAT ACA TCG ACA CG	68	466–447	
ampD Set 4†	*ampD*4-520-conf-Forward	ATG CCG GCG TGT CGA T	66	440–455	250
	*ampD*4-520-conf-Reverse	GGT AAG CAT GGC GTG AAA CC	66	690–672	
ampD Set 5‡	*ampD*5-flank-conf-Forward	TAA TAC GTT CCA GAA GCG CGT CA	69	9–31✧	708
	*ampD*5-end-conf-Reverse	TCA TGT CAT CTC CTT GTG TGA CGA	69	717–694	

*, numbering according Kopp *et al.*, 1993 (Genbank accession number Z14003), where the ATG start codon is located at nucleotide position 154;
§, PCR primer sets derived from conserved intergenic regions of *ampD* in *E. cloacae* (Kopp *et al.*, 1993), *C. freundii* (Kopp *et al.*, 1993) and *E. coli* (Honore *et al.*, 1986);
†, PCR primer sets derived from intergenic region of cloned *ampD* in *S. marcescens* (this study);
‡, published PCR primer set derived from *ampD* gene sequence in *E. cloacae* (Stapleton *et al.*, 1995b);
✧, forward flanking primer located in the adjacent *nadC* gene (Stapleton *et al.*, 1995b).

genomic DNA/PCR reaction) was added. A negative amplification control containing 5 µl of sterile water was included with each batch of tests.

DNA was amplified in a Perkin Elmer GenAmp 9600 thermocycler (Perkin-Elmer). The amplification protocol used followed the manufacturer's recommendations for use of the Expand™ High Fidelity PCR System for PCR products up to 750 bp. The PCR programme consisted of an initial denaturation step at 94°C for 120 s, 10 cycles of DNA denaturation at 94°C for 15 s, primer annealing at 55°C for 30 s and primer extension at 72°C for 45 sec. This was followed by a further 15 cycles of DNA denaturation at 94°C for 15 s, primer annealing at 55°C for 30 s and primer extension at 72°C for 45 s with the primer extension time being increased by 20 s increments in each cycle. The final step consisted of prolonged extension at 72°C for 4 min. After the last cycle, the amplified products were stored at 4°C. Amplified DNA (5µl) was electrophoresed in a 2.0% (w/v) agarose gel using 0.5X TBE buffer (Appendix AII.2) at 70 V for 2 h. Details of ethidium bromide gel staining and photography are outlined in Appendix AIII.1. Reference size DNA fragments were included in each electrophoresis run (100 bp ladder, DNA molecular weight marker XIV, Boehringer Mannheim).

With each primer set, PCR protocols were optimised for annealing temperature and magnesium ion concentration. A wide range of annealing temperatures covering a gradient from 10°C below to 5°C above the primer melting temperature (Tm) was tested to enhance DNA amplification of PCR products in *S. marcescens* isolates. The magnesium ion titration curve covered the range of 0.5–2.0 mM.

6.2.1.2. **Identification of *ampD* by Southern hybridisation of genomic DNA**

Bacterial isolates

Restriction endonuclease digestion and Southern hybridisation was performed on 10 of the 21 *S. marcescens* isolates in Collection B. These isolates (Collection C) comprised representative isolates expressing a range of cefotaxime MICs (cefotaxime-susceptible, n=3; cefotaxime-resistant, n=4 and cefotaxime low-level resistant, n=3) recovered between 1995 and 1998 (1995, n=2; 1996, n=2; 1997, n=3 and 1998, n=3). A wild-type reference strain *S. marcescens* NCTC 11935 was included with each batch of hybridisation reactions. Two wild-type isolates of *E. cloacae* and *C. freundii* and a

single wild-type isolate of *E. coli* isolated in Hospital 1 were processed for restriction endonuclease digestion and used as positive hybridisation controls with each batch of tests (Table 6.3). Undigested plasmid DNA preparations of pNU305 encoding the *ampR* and *ampC* genes from *C. freundii* OS60 (Lindberg *et al.*, 1985) and pNU405 encoding *ampD* from *C. freundii* OS60 (Lindberg *et al.*, 1987) were included as positive (pNU405) and negative (pNU305) hybridisation controls (Table 6.3). All test and control isolates were removed from –70°C and subcultured on CBA on the day prior to DNA extraction.

Preparation of genomic DNA

In the initial attempts to extract genomic DNA from *S. marcescens* for restriction endonuclease digestion, DNA was extracted using the Wizard™ Genomic DNA Purification Kit (Promega) in accordance with the manufacturer's instructions (unmodified protocol outlined in Appendix AIII.2.1). This protocol was later modified to include a proteinase K step to prevent non-specific degradation of DNA following incubation with restriction endonuclease enzymes. This modified protocol is outlined in Appendix AIII.2.2. Plasmids pNU305 and pNU405 were extracted using the QIAGEN™ Plasmid Mini kit outlined in Section 5.2.1.1. Following extraction, DNA was quantified using the agarose gel electrophoresis quantification procedure (Appendix AIII.3).

Restriction endonuclease digestion of genomic DNA

*Eco*R1 digestion was performed on DNA from 16 isolates (*S. marcescens* test isolates, n =10; *S. marcescens* NCTC 11935 reference strain, n=1; *E. cloacae*, n=2; *C. freundii*, n=2 and *E. coli*, n=1). Uncut lambda DNA (250 µg/ml, Boehringer Mannheim) was included in each batch of tests as a restriction endonuclease digestion control.

The *Eco*R1 digest reaction mixture was as follows: 1X restriction endonuclease buffer (Promega) (90 mM Tris HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) and 10 units of *Eco*R1 (Promega) in a total volume of 17 µl. A master-mix of reagents was prepared for each batch of tests. The master-mix was aliquoted into individual tubes and 3 µl of undiluted genomic DNA (~400 ng/µl) was added to the reaction mixture. The tubes were mixed gently by inversion and incubated for 90 min at 37°C. The incubation time

was minimised to reduce risk of DNA degradation. The digestion reaction was stopped by the addition of 5 µl gel loading buffer (Appendix AII.2). The *Eco*R1-digested DNA samples (25 µl) and the undigested pNU305 and pNU405 plasmid DNA samples were separated by electrophoresis in a 0.6% (w/v) agarose gel (20 x 15 cm) at 60 V for 16 h using chilled recirculating 0.5X TBE (Appendix AIII.1.1). Digoxigenin (DIG)-labeled reference size DNA fragments were included in each electrophoresis run (DNA Molecular Weight Marker III, Boehringer Mannheim). The gel was pre-stained in ethidium bromide (0.5 µg/ml) and photographed as outlined in Appendix AIII.1.

Southern transfer of restriction endonuclease digested genomic DNA

DNA fragments were transferred to positively-charged nylon membranes (Boehringer Mannheim) using the Southern capillary transfer method outlined in Sambrook, Fritsch & Maniatis, 1989 (Sambrook *et al.*, 1989). Since the location of *ampD* was unknown, the optional depurination step recommended for transfer of fragments larger than 10 kb, was included in the protocol. DNA was transferred to nylon membranes using 20X saline-sodium citrate (SSC) transfer buffer (Appendix AIV.1). These membranes were handled in accordance with manufacturer's instructions and were 'pre-wetted' by soaking in 2X SSC (Appendix AIV.1) for 15 min prior to use. The transfer was allowed to proceed for 20 h after which time the DNA was fixed to the membrane by baking at 80°C for 2 h. The membranes were wrapped loosely in aluminum foil and stored at 4°C for future use. All reagents used in the transfer procedure are detailed in Appendix AIV.1.

Generation of ampD-like PCR products for use as ampD probes

AmpD probes were generated from the *ampD*-like PCR products obtained during the amplification procedures designed for identification of the *ampD* gene (see Section 6.3.1.1). Five *ampD* probes were generated from the *ampD*-like PCR products of plasmid pNU405 (*ampD*+) and the wild-type *E. cloacae*, *C. freundii* and *E. coli* amplification control isolates.

Plasmid DNA from pNU405 was amplified using *ampD* primer Set 1 and Set 2 and genomic DNA from wild-type *E. cloacae*, *C. freundii* and *E. coli* was amplified using *ampD* primer Set 2, according to the amplification procedure outlined in Section 6.2.1.1. The final extension step in the PCR protocol was extended to 30 min at 72°C to ensure

that all PCR products were full length and 3'-adenylated. Following amplification, the PCR products were analysed by agarose gel electrophoresis and examined for the presence of a single *ampD*-like band in the expected size range.

Cloning of ampD-like PCR products for use as ampD probes

Suspect *ampD*-like PCR products were cloned using the TOPO™ TA Cloning® Kit, version E (Invitrogen BV, CH Groningen, The Netherlands). TOPO™ TA Cloning® provides a one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. The TOPO plasmid vector, pCR® II TOPO is supplied linearised with single 3'-thymine (T) overhangs for thymine-adenine (TA) cloning and a covalently bound topoisomerase. A map of the 3.9 kb pCR® II TOPO vector featuring the cloning site, the restriction endonuclease sites and the encoded antibiotic resistance determinants is detailed in Appendix AV.4. Ligation of the linearised vector with a PCR product containing 3'-adenine (A) overhangs occurs spontaneously within five minutes at RT. The TOPO™ TA plasmid vector containing the cloned PCR-product insert is then transformed into the chemically competent TOP 10 One Shot™ bacterial cells provided in the cloning kit. Positive transformants are selected using blue/white screening. In this screening system cloning vectors are constructed that produce β-galactosidase by α-complementation following transformation into bacterial cells. Negative transformants will thus appear as blue colonies in the presence of the substrate 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal). In positive transformants the insertion of foreign DNA into the cloning vector disrupts the *lacZ* gene and prevents α-complementation. Positive tranformants are thus recognised as white colonies.

For each batch of competent TOP 10 cells, a transformation control experiment was performed to confirm transformation efficiency. The TOP 10 cells were transformed with supercoiled pUC18 plasmid DNA (10 pg) provided in the kit. Ten µl of the cell suspension was inoculated onto LB agar plates (Sigma-Aldrich) containing 50 µg/ml ampicillin (Sigma-Aldrich, Appendix AV.1) in accordance with the manufacturer's instructions. A colony count >200 cfu was indicative of a suitable transformation efficiency i.e. 10^9 cfu/µg DNA (Appendix AV.2).

Fresh *ampD*-like PCR products for the five *ampD* probes were cloned and transformed according to manufacturer's instructions. For blue/white screening, 40 µl of X-gal (50 mg/ml, Promega) was spread over the surface of LB agar plates containing 50 µg/ml kanamycin (LB kanamycin agar) (Sigma-Aldrich, Appendix AV.1) and incubated for 30 min at 37°C prior to use. To select for transformants, 20 µl of the transformation reaction mixture was spread over the surface of the LB plates. Following overnight incubation at 37°C, 10 white colonies from each reaction were selected for analysis.

Analysis of ampD transformants

Transformants were screened by extraction of plasmid DNA and the clone of interest was identified by agarose gel electrophoresis of restriction endonuclease digested plasmid DNA. Plasmid DNA was extracted using the Wizard® *Plus* SV miniprep DNA purification system (Promega). This system is an improved version of the Wizard™ Miniprep system (Promega) previously described (see Section 5.1.1, paragraph four). In the Wizard® *Plus* SV system, the plasmid DNA extraction protocol is modified to include an alkaline protease treatment step which inactivates endonucleases and other proteins released during bacterial cell lysis. These proteins can adversely affect the quality of the extracted DNA. The Wizard® *Plus* SV also includes ready-prepared DNA purification spin columns which simplify the extraction procedure by avoiding the use of liquid purification resin and syringe columns. In this experiment, the size limitation of the Wizard purification systems (limited to plasmids <20 kb) was not a problem due to the small size of the cloning vector (3.9 kb). Following extraction with the Wizard® *Plus* SV system, plasmid DNA can be used for applications such as restriction endonuclease digestion and automated DNA sequencing, without further preparation.

The 10 white colonies selected from each transformation reaction were inoculated into 5 ml LB broth containing 50 µg/ml kanamycin (LB kanamycin broth) (Sigma-Aldrich, Appendix AV.1) and incubated for 14 h at 37°C with shaking (150 rpm). Plasmid DNA extraction was performed on 2.5 ml aliquots of these cultures in accordance with the centrifugation protocol outlined in the manufacturer's instructions.

Plasmid DNA was digested using *Eco*R1 which cut at *Eco*R1 restriction sites located outside the pCR® II TOPO vector cloning region (*Eco*R1 restriction sites were absent

from the *ampD*-like PCR insert). Restriction endonuclease digestion of the plasmid DNA was performed using the protocol outlined for *Eco*R1 digestion of genomic DNA previously outlined in this section. In the digestion reaction, 4 µl of plasmid DNA was used in a total reaction volume of 20 µl. The *Eco*R1-digested plasmid DNA preparations were analysed by electrophoresis in 1.0% (w/v) agarose gels. Reference size DNA fragments were included in each electrophoresis run (1 kb DNA ladder, Low DNA Mass Ladder™, Gibco BRL®/Life Technologies). Suitable clones were selected, based on the presence of two DNA bands corresponding to the expected size range of the linearised vector and the *ampD*-like PCR inserts.

The five selected clones were re-inoculated into 5 ml LB kanamycin broths and incubated for 14 h at 37°C with shaking (150 rpm). At the same time, the five selected clones were also inoculated onto LB kanamycin agar plates and incubated overnight at 37°C. Each 5 ml broth culture was aliquoted into two 2.5 ml volumes and both samples were processed for plasmid DNA extraction. The two plasmid DNA preparations from each clone were pooled and 4 µl was removed and re-tested for *Eco*R1 digestion. The remaining plasmid preparation (196 µl) was precipitated by the addition of 18 µl 7.5 M ammonium acetate (Appendix AII.3) plus 500 µl ethanol (BDH Laboratory Supplies, Poole, Dorset, UK) at RT. The preparation was mixed by inversion and centrifuged at 17,400 x *g* for 45 min at RT in an Eppendorf 5417 microcentrifuge (Eppendorf) Following centrifugation, the location of the plasmid DNA pellet was marked on the microcentrifuge tube before washing the pellet in 70% (v/v) ethanol. The ethanol was removed gently by manual pipetting and the plasmid pellet was allowed to air dry for 5 min. Plasmid DNA pellets were stored at –20°C before being sent for DNA sequence analysis. LB agar cultures corresponding to the selected *ampD*-like clones were used to re-inoculate a further set of LB kanamycin broths. These broths were used to prepare glycerol stocks of the selected clones. Overnight bacterial cultures (0.85 ml) were mixed with 1.5 ml of sterile glycerol (Sigma-Aldrich) and were frozen at –70°C.

Sequence analysis of ampD-like PCR products for use as ampD probes

Plasmid DNA precipitates of the five *ampD* clones were sent to MWG Biotech UK Ltd., Wolverton Mill South, Milton Keynes, UK, for sequencing. The *ampD*-like PCR inserts were sequenced by automated cycle-sequencing with dye-terminator chemistry using an

ABI 377 sequencer (Applied Biosystems). Cycle sequencing was performed using the M13 universal primer sites on the pCR® II TOPO vector.

Nucleotide sequence analysis of the *ampD* inserts was performed using the Blast N software available on the Internet at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1997). Vector sequences were removed and consensus sequences were generated for each PCR product by overlap of the forward and reverse nucleotide sequence (http://www.ncbi.nlm.nih.gov/blast/ bl2seq/wblast2.cgi). Edited DNA sequences were run in 'database search' protocols for identity with known *ampD* sequences in *E. cloacae*, *C. freundii* and *E. coli*.

DIG-labeling of ampD probes

Following confirmation of the *ampD* sequence, PCR inserts from the five *ampD* clones were labeled using digoxigenin-11-dUTP (DIG-11-dUTP) and used as *ampD*-specific probes. The Digoxigenin (DIG) System (Boehringer Mannheim) is a non-radioactive nucleic acid labeling and detection system that can be used for single-copy gene detection on Southern blots, Northern blots and colony screening. In this protocol, hybridisation of DIG-labeled probes is performed according to standard protocols. Following hybridisation, an anti-digoxigenin antibody-alkaline phosphatase (AP) conjugate is allowed to bind to the probe. The signal is then detected with a colourimetric substrate (where the signal develops directly on the membrane) or with a chemiluminescent alkaline phosphatase substrate (where the signal is detected on X-ray film).

To optimise the DIG-labeling reaction, *ampD*-PCR inserts must be separated from the vector sequences and purified by phenol:chloroform extraction. Plasmid extraction was performed on the stored *ampD* clones using the Wizard® *Plus* SV miniprep DNA purification system (Promega) previously described in this section. The *ampD* insert was separated from the vector by *Eco*R1 digestion of the plasmid DNA. In the restriction endonuclease digestion reaction, a plasmid DNA sample volume of 28 µl was used in a total reaction volume of 140 µl. The *Eco*R1-digested plasmid DNA preparation from each of the *ampD* clones was loaded into six adjacent wells (30 µl/well) in a 1% (w/v) agarose gel. Following electrophoresis at 70 V for 2 h, the

agarose gels were stained in ethidium bromide and viewed on a transilluminator (Appendix AIII.1.3). The *ampD* PCR inserts were excised from the agarose using a sterile scalpel and the DNA was extracted from the agarose using GenElute™ Minus EtBr Spin Columns (Supelco, Bellefonte, PA, US). The six agarose gel slices obtained for each *ampD* clone were placed in separate GenElute™ spin columns and processed in accordance with the manufacturer's instructions. Following elution, the DNA extracted from the six columns was pooled to give an approximate volume of 500 µl for each *ampD* clone. The pooled DNA samples were purified by phenol:chloroform extraction using a modification of the method outlined in Sambrook, Fritsch & Maniatis, 1989, (Sambrook *et al.*, 1989) (Appendix AIII.4). The resulting DNA pellets were dissolved in 25 µl sterile water and the concentration of DNA was estimated using the agarose gel electrophoresis quantification method outlined in Appendix AIII.3. Approximately 800 ng of each *ampD* insert was present following the purification procedure.

The purified *ampD* PCR inserts were DIG-labeled by the random primed method using the DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim). In each DNA labeling reaction, 16 µl of neat template DNA was mixed with 4 µl of DIG-High Prime and incubated for 20 h at 37°C. The yield of DIG-labeled DNA was estimated using DIG quantification test strips included in the starter kit. In this system, dilutions of labeled DNA are spotted onto the membrane of the quantification test strips. The strips are treated with anti-digoxigenin-AP conjugate and the signal is detected with a colourimetric substrate, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution. The DIG labeling efficiency can be determined by comparing the signal intensity of the spots to a control test strip containing quantified amounts of DIG-labeled DNA.

The anti-digoxigenin-AP and the NBT/BCIP solution used in the DIG quantification procedure were provided in the starter kit. The additional reagents used in the detection reaction i.e. wash buffer {0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween 20}, blocking buffer {1% (w/v) blocking reagent in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5} and the detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) were prepared using a DIG Wash and Block Buffer Set (Boehringer Mannheim). Using the DIG quantification procedure outlined by the manufacturer, the yield of labeled *ampD* probes

was estimated at 12.5 μg/ml. The DIG-labeled probes were stored at –20°C ready for use.

Hybridisation with DIG-labeled ampD probes

Hybridisation was performed using a hybridisation oven (Maxi 16, Hybaid Ltd., Teddington, Middlesex, UK), large (300 mm x 35 mm) hybridisation roller bottles (Hybaid) and membrane mesh support sheets (23 cm x 23 cm, Hybaid). All hybridisation procedures, including rolling and insertion of the blots in the hybridisation bottles, were performed in accordance with the manufacturer's instructions for use of the hybridisation equipment.

Southern blots of *Eco*R1-digested genomic DNA were hybridised with the five DIG-labeled *ampD* probes using the method described in the DIG High Prime DNA Labeling and Detection Starter Kit II. In a pilot study, hybridisations were carried out on clean nylon membranes to determine the optimal probe concentration and to avoid high background staining. For DIG-labeled *ampD*-probes, the probe concentration was optimised at 25 ng/ml. Hybridisation was performed under conditions of low stringency (low-temperature, high-salt post-hybridisation washes) to facilitate the binding of *ampD*-probes to non-identical *ampD*-like fragments. Hybridisation solutions were prepared by diluting the denatured *ampD*-probes in 15 ml pre-warmed DIG Easy Hyb solution (DIG Easy Hyb Granules, Boehringer Mannheim). The hybridisation reactions were carried out at 37°C for 16 h. The first post-hybridisation wash was performed at RT for 10 min using 4X SSC buffer and 0.1% (v/v) sodium dodecyl sulphate (SDS) (Appendix AIV.3). After this wash, the membranes were transferred from the hybridisation bottles to large shallow Pyrex™ dishes containing 2X SSC buffer and 0.1% (v/v) SDS (Appendix AIV.3). The dishes were incubated on the shaking platform of the hybridisation oven at 50°C for 30 min.

Chemiluminescent detection was carried out in accordance with the manufacturer's instructions, using the buffers in the DIG Wash and Block Buffer Set (Boehringer Mannheim) and the anti-digoxigenin-AP conjugate and chemiluminescent substrate, 25 mM disodium3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo{3.3.1.1^{3,7}}decan -4-yl) phenyl phosphate (CSPD®) provided in the DIG High Prime DNA Labeling and Detection Starter Kit II. The anti-digoxigenin-AP conjugate was diluted 1:10,000 in 50

ml blocking buffer for use with the CSPD® substrate. The hybridised membranes were placed in an envelope made by sealing the edges of two A4 plastic transparency sheets (Rank Xerox) prior to application of the CSPD® substrate. Following application of the substrate and incubation at 37°C for 15 min the acetate envelope was exposed to 18 x 24 cm Lumi Film (Boehringer Mannheim) using a 18 x 24 cm Fuji EC-A-Cassette [Fuji Photo Film (UK) Ltd., Bedford, UK]. Following 20 min exposure, the Lumi film was developed using an AGFA CURIX 60 automated X-ray film processor (Agfa-Gevaert AG, Munich, Germany).

As recommended by the manufacturer, initial DIG detection experiments were performed using CSPD® as the chemiluminescent substrate. After experience was gained with the hybridisation technique, the CSPD® was replaced with CSPD *Star*™ which gives improved detection sensitivity and reduces exposure times to 1 min or less. When CSPD *Star*™ was used, the concentration of the probe was reduced to 20 ng/ml and the anti-digoxigenin-Ap conjugate was diluted 1:20,000 for use.

Following detection, the membranes were stripped for reprobing using the manufacturer's recommended procedure. Membranes were incubated in alkaline-probe-stripping solution (Appendix AIV.3) and rinsed in 2X SSC. After stripping, the membranes were re-used immediately or stored wet in 2X SSC in sealed hybridisation bags (Boehringer Mannheim).

Localisation of the ampD-like gene on a low-molecular weight DNA fragment

To achieve localisation of the suspect *ampD* gene on a low-molecular weight DNA fragment, genomic DNA was digested using a range of restriction endonuclease enzymes. DNA from three representative *S. marcescens* isolates from Collection C and one wild-type *E. cloacae* control was used. The range of restriction endonucleases (Promega), the concentrations used and the recommended reaction buffers were as follows: *Cla*I (10 units), 10 mM Tris HCl pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT); *Hind*III (10 units), 6 mM Tris HCl pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$ and 1 mM DTT; *Pst*I (10 units), 90 mM Tris HCl pH 7.5, 50 mM NaCl and 10 mM $MgCl_2$ and *Pvu*II (10 units), 6 mM Tris HCl pH 7.5, 50 mM NaCl, 6 mM $MgCl_2$ and 1 mM DTT. In each test, the restriction endonuclease digestion reaction mix was prepared in a volume of 17 µl. DNA was added (3 µl) to the reaction preparation and

the samples were incubated at 37°C for 90 min. The digestion reaction was stopped by the addition of 5 µl gel loading buffer (Appendix AII.2) and the digested samples were electrophoresed in a 0.6% (w/v) agarose gel (20 x 15 cm) at 60 V for 16 h (Appendix AIII.1). DIG-labeled, high- and low-molecular weight reference size DNA fragments {DNA High Molecular Weight Marker III, DIG-labeled; DNA Low Molecular Weight Marker VI, DIG-labeled (Boehringer Mannheim)} were included in each electrophoresis run. Following electrophoresis, the DNA was transferred to positively charged nylon membranes (Boehringer Mannheim) and hybridised with the DIG-labeled *ampD* probe found to produce the most intense hybridisation signal, using the methods outlined for *Eco*R1-digested genomic DNA. The resulting autoradiographs were examined for evidence of the presence of the suspect *ampD* band on a low-molecular DNA fragment (in the size range of 1 kb).

Following analysis with each of the restriction endonucleases, *Pst*1 digestion was deemed the most suitable for *S. marcescens* isolates. *Pst*1 digestion was performed on the genomic DNA from the 10 *S. marcescens* isolates in Collection C and the reference strain *S. marcescens* NCTC 11935. Since DNA from the wild-type control isolates of *E. cloacae*, *C. freundii* and *E. coli* demonstrated poorly resolved DNA banding patterns following *Pst*1 digestion, genomic DNA samples from these isolates were digested with *Eco*R1. The restriction endonuclease-digested samples were electrophoresed and DNA blots were prepared and hybridised with the selected *ampD* probe, as previously described. The size of the suspect *ampD* band was estimated using the DIG-labeled reference size DNA fragments.

6.2.1.3. **Cloning and selection of suspect *ampD* fragments**

Isolation and purification of suspect ampD fragments

Following localisation of the suspect *ampD* fragment, two cefotaxime-resistant *S. marcescens* isolates from Collection C which produced a hybridisation signal were selected for further analysis. Genomic DNA from each isolate was subjected to *Pst*1 digestion (using a reaction volume of 120 µl per test), loaded into 4 adjacent wells (30 µl per well) in a 0.6% (w/v) agarose gel and electrophoresed (as outlined in Section 6.2.1.2). Following electrophoresis, DNA bands located at the position corresponding to the *ampD* hybridisation signal in *Pst*1-digested DNA from *S. marcescens* isolates, were excised from the ethidium bromide stained gel using minimum UV exposure. The

suspect *ampD* fragments from each isolate were extracted from the agarose, pooled, purified and quantified using the method described for preparation of *ampD*-PCR inserts for DIG-labeling (see Section 6.2.1.2).

Cloning of suspect ampD fragments

Following purification, the suspect *ampD* fragments from the two cefotaxime-resistant *S. marcescens* isolates were cloned using the Zero Background™/Kan Cloning Kit, version G (Invitrogen BV). This kit was designed to clone cohesive- or blunt-ended DNA fragments with a low background of non-recombinants. The cloning vector provided in the kit, pZErO™-2 contains the lethal *ccdB* gene fused to the C-terminus of LacZα. The protein product of the *ccdB* gene acts by poisoning DNA-gyrase which results in DNA breakage and cell death. Insertion of a DNA fragment disrupts expression of the *lacZα-ccdB* gene fusion and prevents production of the lethal gene product thereby permitting direct selection of positive recombinants. Cells that contain non-recombinant vector do not survive on subculture.

Using the map of the pZErO™-2 vector (Appendix AV.5), a cloning strategy for insertion of the *ampD* fragment was determined using the *Pst*1 restriction endonuclease sites in the multiple cloning region. Cloning reactions were performed according to the manufacturer's instructions. The pZErO™-2 vector (1 µg) was digested with *Pst*1 at 37°C for 35 min in a total reaction volume of 10 µl. *Pst*1 was removed by phenol:chloroform extraction. Following ethanol precipitation, the DNA pellet was resuspended in 50 µl of water to give an approximate vector concentration of 10 ng/µl. The ligation reaction was performed using the linearised *Pst*1-digested vector and the purified *Pst*1-digested suspect *ampD* fragment. In the initial cloning experiments, the ligation reactions were set up using the recommended insert:vector molar ratio of 2:1 (Appendix AV.3) for cohesive-end ligation. This insert:vector molar ratio was later modified to 10:1 to improve ligation efficiency. With each set of ligation reactions, the recommended control reactions for 'test inserts' (blunt-ended ΦX174 DNA) and 'linearised vector only' were performed according to the manufacturer's instructions.

Transformation was performed using Top 10 One Shot™ (Invitrogen) competent cells. For each transformation reaction (300 µl), 50 µl of transformed cells were spread over

the surface of six LB kanamycin agar plates (Appendix AV.1) and incubated overnight at 37°C. The total transformation reaction volume was inoculated to increase the likelihood of *ampD* selection.

Selection of suspect ampD transformants by colony hybridisation

To select for *ampD* transformants, transformed colonies were subjected to colony blot hybridisation with the selected *ampD* probe. Transformed TOP 10 *E. coli* cells encode an *ampD* gene. Hence, a background hybridisation signal was expected from all transformed colonies. However, since the pZErO™-2 vector is present in high-copy number, it was expected that this background signal could be subtracted from the strong hybridisation signal of colonies carrying the cloned suspect *ampD* fragment.

DNA blots of the transformed colonies were prepared using the method outlined in Sambrook, Fritsch & Maniatis, 1989, for transferring small numbers of bacterial colonies to nylon membranes (Sambrook *et al.*, 1989). Transformed colonies (from the six LB kanamycin plates) were inoculated onto a master LB kanamycin agar plate and onto a colony hybridisation nylon membrane (Boehringer Mannheim) laid on the surface of a replica LB kanamycin agar plate, in a grid pattern. Wild-type *E. cloacae* and *C. freundii* controls were also inoculated on both plates. Following incubation at 37°C for 6–8 h, the master plate was sealed and stored at 4°C whilst the nylon membrane was removed from the surface of the replica plate and processed to allow denaturation and neutralisation of the transferred DNA (Appendix IV.2). The denatured DNA was immobilised on the membrane by baking at 80°C for 120 min.

Prior to hybridisation, the colony blots were subjected to proteinase K treatment to allow digestion of interfering proteins. In this process, individual blots were placed in empty Petri dishes and proteinase K (2 mg/ml, Appendix IV.2) was distributed over the surface of the membrane. Following incubation at 37°C for 60 min, the membranes were blotted between two sheets of 3MM filter paper (Whatman International Ltd., Maidstone, UK) moistened with sterile water to remove cellular and agar debris.

The colony blots were hybridised with the selected *ampD* probe and hybridisation was detected using the DIG system outlined for Southern blots (see Section 6.2.1.2). Three

membranes were processed in each hybridisation bottle. The hybridisation reaction was performed using CSPD *Star*™ substrate.

Following detection, autoradiographs of the hybridised blots were examined for the presence of colonies producing strong hybridisation signals. The corresponding colonies from the master LB plate were inoculated onto fresh LB kanamycin agar plates and into 5 ml LB kanamycin broths, which were incubated overnight at 37°C. Each 5 ml broth culture was aliquoted into two 2.5 ml volumes and plasmid DNA was extracted using the Wizard® *Plus* SV system previously described (see Section 6.2.1.2). The two plasmid DNA preparations from each colony were pooled and a 3 µl aliquot was digested with *Pst*1 to ensure the selected clone contained an insert in the correct size range. The remaining plasmid DNA was precipitated and prepared for sequence analysis as described for the cloned *ampD*-PCR inserts (see Section 6.2.1.2). The corresponding LB agar cultures of the selected *ampD* clones were inoculated into fresh 5 ml LB kanamycin broths which were incubated overnight at 37°C. These LB broths were used to prepare glycerol stocks which were stored at –70°C.

Sequencing suspect ampD fragments

The cloned *ampD* fragments were sequenced (MWG Biotech UK Ltd.) by automated cycle sequencing with dye-primer chemistry using a LiCor 4200 infra red DNA sequencer (LI-COR Inc.). Bi-directional, long-read (up to 1.5 kb) cycle sequencing was performed using the M13 universal primer sites on the pZErO™-2 vector. Nucleotide sequence analysis was performed as outlined for *ampD*-PCR products (see Section 6.2.1.2). The DNA sequence from the two *ampD* inserts were aligned using Clustal W software (version 1.81) available on the Internet at the European Bioinformatics Institute (EBI) site (http://www.ebi.ac.uk).

Following sequence confirmation, the cloned *ampD* insert from one of the two *S. marcescens* isolates was separated from the pZErO™-2 vector using *Pst*1 digestion and was used to generate a DIG-labeled *S. marcescens*-specific *ampD* probe (as described for DIG-labeling of cloned *ampD*-PCR inserts, Section 6.2.1.2). The original Southern blots of *Eco*R1-digested DNA from the *S. marcescens* isolates in Collection C were re-hybridised with this *S. marcescens*-specific *ampD* probe.

6.2.1.4. PCR amplification for confirmation of *ampD* using primers derived from *ampD* sequences in *S. marcescens* and *E. cloacae*

Bacterial isolates

DNA amplification for confirmation of *ampD*-encoding genes was performed on template DNA from nine of the 10 *S. marcescens* isolates in Collection C. This collection of isolates (Collection D) comprised three cefotaxime susceptible isolates, three cefotaxime low-level resistant isolates and three cefotaxime resistant isolates, recovered between 1995 and 1998 (1995, n=1; 1996, n=2; 1997, n=3 and 1998, n=3). The AmpC β-lactamase expression of the nine isolates was previously characterised using β-lactam hydrolysis assays (Table 4.5). The three cefotaxime susceptible isolates, 98.S1, 97.S3 and 96.S5, with cefotaxime MICs ≤0.25 mg/l, were categorised as wild-type inducible. The three cefotaxime low-level resistant isolates, 96.R1, 97.R2 and 98.R5, with cefotaxime MIC values of 8–16 mg/l, were considered partially derepressed. The three cefotaxime-resistant isolates, 95.R7, 97.R8 and 98.R7 with cefotaxime MICs of 64 mg/l were associated with a derepressed phenotype.

Template DNA from a wild-type reference strain *S. marcescens* NCTC 11935 was included with each batch of amplification reactions. Template DNA from one of the *S. marcescens ampD*-clones (generated in Section 6.2.1.3) and one of the *E. cloacae* control isolates were processed as amplification controls. Test and control isolates were removed from –70°C and subcultured on CBA on the day prior to DNA extraction.

Preparation of template DNA

Genomic DNA was prepared using the Wizard™ Genomic DNA Purification Kit (Promega). The DNA was extracted using the proteinase K modified protocol outlined in Appendix AIII.2.2. Plasmid DNA was extracted from the *S. marcescens ampD*-clone using the Wizard® *Plus* SV system described in Section 6.2.1.2.

Extracted DNA was quantified using the agarose gel electrophoresis method (Appendix AIII.3). Prior to amplification, fresh dilutions of DNA were prepared in sterile water. For genomic DNA extracted from *S. marcescens* isolates, a 1/5 dilution was prepared to yield an approximate DNA concentration of 60 ng/µl. For genomic DNA extracted from *E. cloacae*, a 1/100 dilution was prepared to yield an approximate DNA

concentration of 4 ng/µl. Plasmid DNA was diluted 1/50 to yield an approximate DNA concentration of 1 ng/µl.

Primer design

Three sets of oligonucleotide primers were used for confirmation of the *ampD* gene. Two sets of primer pairs, *ampD* Set 3 and *ampD* Set 4 were designed from the intergenic region of the cloned *ampD*-fragment from *S. marcescens*. These primer sets were designed to generate two 250 bp overlapping amplimers (Figure 6.1). An additional set of published primers derived from the *ampD* sequence in *E. cloacae* (Genbank accession number Z14002) were also used for the confirmation of *ampD* in *S. marcescens* (Stapleton *et al.*, 1995b). This primer pair (*ampD* Set 5) was designed to generate a 708 bp amplimer extending from the *nadC* gene (which lies upstream of the *ampD* gene) to the 3' end of the *ampD* gene (Figure 6.1). Primers were custom-made by Genosys Biotechnologies Ltd. Details of the primer sets are included in Table 6.4.

DNA amplification reaction

The *ampD* PCR reaction mixture (*Taq* PCR Core Kit, QIAGEN GmbH) for *ampD* primer Set 3 and *ampD* primer Set 4 was as follows: 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1X PCR buffer {Tris-HCl, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, pH 7.5}, 1X Q-solution, 2.5 units *Taq* DNA polymerase and 1 µM of *ampD* primer Set 3 or *ampD* primer Set 4 (Genosys Biotechnologies Ltd.) in a total reaction volume of 45 µl. A similar reaction mixture was prepared for *ampD* primer Set 5, but the primer concentration was 5 µM as detailed in the authors protocol (Stapleton *et al.*, 1995b). For the three *ampD* primers sets, a master-mix of reagents was prepared for each batch of tests. The mix was aliquoted into individual tubes and 5 µl of diluted DNA template (~300 ng genomic DNA for *S. marcescens*, ~20 ng genomic DNA for *E. cloacae*) was added. A negative amplification control containing 5 µl of sterile water was included with each batch of tests.

In accordance with the manufacturer's recommendations for use of *Taq* PCR Core Kit, parallel DNA amplification reactions were performed for all primer sets in the presence and in the absence of Q-solution. In addition, optimum magnesium ion concentrations and annealing temperatures were determined for each primer set. The latter was

established using the gradient block facility on the PCR Gene Express™ thermocyler (Hybaid) to obtain temperature gradient curves covering a temperature range from 10°C below to 5°C above the Tm with the temperature being increased by 1.25°C incremental steps.

An additional study was designed to investigate the combined effects of the quantity and quality of template DNA on PCR product yield. Using *ampD* primer Set 5, a series of amplification reactions were performed on template DNA from one *S. marcescens* isolate from Collection D, using 'fresh' DNA extracted with proteinase K on the day prior to amplification, 'old' DNA extracted with proteinase K three months prior to amplification (stored a –20C) and 'fresh' DNA extracted without the addition of proteinase K. In each case, the DNA samples were tested at dilutions of 1/100 (15 ng DNA/PCR reaction), 1/10 (150 ng DNA/PCR reaction) and 1/2 (750 ng DNA/PCR reaction), in the presence and in the absence of Q-solution.

DNA was amplified in a PCR Gene Express™ thermocyler (Hybaid). For *ampD* primer Set 3, the amplification program consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 61°C for 30 s and primer extension at 72°C for 1 min and a final extension at 72°C for 10 min. For primer Set 4 and primer Set 5, similar amplification protocols were used but in these protocols, a 'touchdown' PCR strategy was employed. 'Touchdown' PCR was utilised to enhance the specificity of the initial primer-template duplex formation and hence increase the specificity of the overall amplification reaction. In the 'touchdown' protocol, the annealing temperature for the first five amplification cycles was set at the estimated Tm of the primer set (Tm for *ampD* Set 4 was 66°C and the Tm for *ampD* Set 5 was 69°C). In the following five amplification cycles, the annealing temperature was decreased to 2°C below the Tm. For the remaining 25 cycles, the annealing temperature was decreased by a further 2°C.

Amplified DNA (5 µl) was electrophoresed in a 2.0% (w/v) agarose gel using 0.5X TBE buffer at 70 V for 2 h. Details of ethidium bromide gel staining and photography are outlined in Appendix AIII.1. Reference size DNA fragments were included in each

electrophoresis run (100 bp ladder, DNA molecular weight marker XIV, Boehringer Mannheim).

6.2.1.5. **Sequence analysis for confirmation of *ampD***

Unlike the 250 bp amplimers obtained with *ampD* primer Set 3 and 4, amplification with *ampD* primer Set 5 was designed to yield an amplified product (708 bp) that would correspond to the full sequence of the *ampD* gene. The *ampD*-like PCR products obtained with primer Set 5 were thus selected for confirmation of the *ampD* sequence in *S. marcescens*.

Purification of ampD PCR products

The *ampD*-like PCR products (obtained with primer Set 5) from the nine *S. marcescens* isolates in Collection D and from the reference strain *S. marcescens* NCTC 11935 were purified using the Qiaquick™ PCR product purification kit (QIAGEN GmbH). The kit was used in accordance with the manufacturer's instructions for direct purification of PCR products from amplification reactions.

Cloning of ampD PCR products

Following purification, the *ampD*-like PCR products were cloned using the TOPO TA Cloning Kit for Sequencing, version E2 (Invitrogen BV). Similar to the TOPO TA cloning kit previously described (see Section 6.2.1.2), the TOPO Cloning Kit for Sequencing is also based on a single-step cloning strategy for direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. With this kit the PCR products are inserted into the pCR®4-TOPO® vector (Appendix AV.6) which encodes the lethal *ccd*B gene fused to the C-terminus of the *lacZ*α fragment. Ligation of a PCR product disrupts expression of the *lacZ*α-*ccd*B gene fusion and thus permits growth of positive transformants. Since cells containing non-recombinant vector do not survive on subculture, blue/white screening to select recombinants is not necessary.

The TOPO® cloning and One Shot® transformation reactions were set up according to the manufacturer's instructions. To select for transformants, 20 µl of the transformation reaction was inoculated onto LB kanamycin agar plates (Appendix AV.1) and the plates were incubated overnight at 37°C.

Analysis of ampD transformants

Ten colonies from each transformation reaction were selected, inoculated into 5 ml LB kanamycin broths (Appendix AV.1) and incubated for 14 h at 37°C with shaking (150 rpm). Plasmid DNA extraction was performed on 2.5 ml of this culture using the Wizard® *Plus* SV system. The resulting plasmid DNA preparations were analysed by *Eco*R1 digestion and agarose gel analysis as outlined in Section 6.2.1.2. Suitable clones were selected by examining for the presence of two DNA bands corresponding to the expected size range of the linearised vector (3.9 kb) and the *ampD*-like PCR inserts (~700 bp). Plasmid DNA precipitates from the selected clones were prepared for sequence analysis using the methods described in 6.2.1.2. LB kanamycin agar cultures of the selected *ampD* clones were inoculated into fresh 5 ml LB kanamycin broths which were incubated overnight at 37°C. These LB broths were used to prepare glycerol stocks which were stored at –70°C.

Sequence analysis of ampD PCR products

Plasmid DNA precipitates of the *ampD* clones were sent to MWG Biotech UK Ltd., for sequence analysis. The *ampD* PCR inserts were sequenced by automated cycle sequencing with dye-terminator chemistry using an ABI 377 sequencer (Applied Biosystems). Cycle sequencing was performed using a 'Comfort Read' protocol (designed for DNA sequence read lengths up to 700 bp) and the M13 universal primer sites on the pCR®4 TOPO® vector.

Nucleotide sequence analysis of the *ampD* PCR products was performed using 'database search' protocols for identity with known *ampD* sequences, as described in Section 6.2.1.2. Multiple sequence alignments were performed using Clustal W software (version 1.81). The nucleotide sequence of the *ampD*-like PCR products from *S. marcescens* NCTC 11935 and the nine *S. marcescens* isolates from Collection D were aligned using a Blosum weight matrix (for similar sequences) and default settings for gap (10) and gap extension (0.05) penalties. The deduced AmpD amino acid sequence in *S. marcescens* was also aligned with the AmpD sequence from wild-type isolates of *E. cloacae* (Ent. 029, Genbank accession number U40785), *C. freundii* (Cit. 0S60, Genbank accession number Z14002) and *E. coli* (Genbank accession number X15398).

6.2.1.6. **Transformation with plasmid encoding wild-type *ampD* from *E. cloacae***

Transformation studies were performed on cefotaxime-resistant *S. marcescens* isolates with plasmid pNH5 which carries the wild-type *ampD* gene from *E. cloacae* and the kanamycin resistance determinant (Honore *et al.*, 1989) (Table 6.3). An *E. coli* isolate (JRG582/pNH5) carrying plasmid pNH5 was kindly provided by Professor Martin Cormican, Department of Microbiology, University College Hospital, Galway.

Extraction of plasmid DNA

Plasmid pNH5 was extracted using the Promega Wizard® *Plus* SV system (described in Section 6.2.1.2) and quantified using the agarose gel electrophoresis procedure outlined in Appendix AIII.3. Plasmid DNA samples were diluted 1/10 in sterile water to achieve an approximate DNA concentration of 5 ng/µl.

Selection of candidate hosts for transformation

Prior to transformation, candidate host bacterial cells were investigated for inherent kanamycin resistance using a selective agar screening method. On the day prior to screening, the three cefotaxime-resistant *S. marcescens* isolates from Collection D and three isolates of *E. cloacae* demonstrating high-level cross-resistance to cefotaxime and ceftazidime (indicative of AmpC derepression) recovered in the 1995 pilot study (see Section 4.4.2.1, paragraph one) were removed from –70°C storage. The results of β-lactam hydrolysis studies had previously indicated that the three isolates of *S. marcescens* were associated with a derepressed AmpC phenotype (Table 4.5). The six test isolates were subcultured onto CBA and after overnight incubation at 37°C, were inoculated into 100 ml LB broths and incubated at 37°C with shaking (150 rpm) for 4 h. One hundred µl of each of these bacterial cultures was spread over the surface of LB kanamycin agar plates (Appendix AV.1), incubated overnight at 37°C and examined for evidence of bacterial growth.

Preparation and transformation of competent cells

Transformation was performed on one 'derepressed' *S. marcescens* isolate and on one 'derepressed' *E. cloacae* isolate that failed to demonstrate bacterial growth on LB kanamycin agar plates. Prior to transformation, the bacterial cells were rendered 'competent' by chemical treatment using the calcium chloride method (Protocol II) outlined in Sambrook, Fritsch & Maniatis, 1989 (Sambrook *et al.*, 1989). On the day

prior to testing, the two bacterial isolates were removed from –70°C and subcultured onto CBA. A single colony was inoculated into 100 ml LB broth in a 1 l flask. The broths were incubated at 37°C with vigorous shaking (300 rpm) for approximately 3 h until the OD_{600} measured between 0.4–0.5 (GeneQuantpro, Amersham Biosciences). Bacterial cells were aliquoted into 20 ml volumes, harvested by centrifugation at 2,500 x *g* for 10 min at 4°C in a Beckman J-6B centrifuge (Beckman Coulter), pooled into a single sample and treated with ice-cold 0.1 M calcium chloride (Appendix AII.3) as outlined in Protocol II (Sambrook *et al.*, 1989). The cells, dispensed in 200 µl volumes, were transformed directly with 10 µl of pNH5 plasmid DNA (5 ng/µl). Following incubation on ice for 30 min and heat-shock treatment at 42°C for 90 s, 800 µl of SOC medium (Gibco BRL®/Life Technologies) was added and preparations were incubated at 37°C for 45 min. Transformants were selected by spreading 50 µl of the transformation reaction mixture over the surface of two LB kanamycin agar plates. The plates were incubated overnight at 37°C.

Analysis of pNH5 transformants

Ten colonies from each of the transformation reactions were selected for analysis. Quantitative susceptibility testing to cefotaxime and ceftazidime was performed using the Etest (AB Biodisk) method described in Section 4.2.2.3. Plasmid DNA extraction and agarose gel analysis was also performed on the orginal clinical isolates and their respective transformants using the method outlined in Section 6.2.1.2.

6.2.2. Identification and characterisation of *ampR* in *S. marcescens*

6.2.2.1. PCR amplification for identification of *ampR* using primers derived from *ampR* sequences in *E. cloacae* and *C. freundii*

Bacterial isolates

DNA amplification to identify *ampR*-encoding genes was performed on the 21 *S. marcescens* isolates in Collection B (detailed in Section 6.2.1.1). Plasmids pNU305 and pNU405 and wild-type isolates of *E. cloacae*, *C. freundii* and *E. coli* (Table 6.3) were processed as DNA amplification controls with each batch of tests.

Preparation of template DNA

Template DNA was prepared using the unmodified Wizard™ Genomic DNA Purification Kit (Promega) outlined in Appendix AIII.2.1. Plasmids pNU305 and pNU405 were extracted using the QIAGEN™ Plasmid Mini Kit (see Section 5.2.1.1). Extracted DNA was quantified using the agarose gel electrophoresis method (Appendix AIII.3). Prior to amplification, genomic DNA was diluted 1/100 (3 ng/µl) and plasmid DNA was diluted 1/200 (1 ng/µl).

Primer design

Oligonucleotide primer sets for *ampR* were designed from conserved intergenic regions of the DNA sequence of known *ampR* genes from *E. cloacae* MHN1, (Genbank accession number X04730) (Honore *et al.*, 1986) and *C. freundii* OS60 (Genbank accession number M27222) (Lindquist *et al.*, 1989b). Two sets of *ampR*-specific primer pairs were designed, *ampR* Set 1 and *ampR* Set 2, using a common reverse primer. When base-pair mismatches occurred in the selected primer regions, the *E. cloacae ampR* sequence was used as the template for primer design. Details of the primer sets are included in Table 6.5. Primers were custom-made on request by Genosys Biotechnologies Ltd.

DNA amplification reaction

DNA amplification for identification of *ampR* was performed using the Expand™ High Fidelity System (Boehringer Mannheim) and PCR reaction mixture employed with amplification procedures for identification of *ampD* (see Section 6.2.1.1). The PCR reaction was carried out in a Perkin Elmer GenAmp 9600 thermocycler (Perkin-Elmer) using the amplification protocol outlined for identification of *ampD*. For both sets of *ampR* primers, PCR protocols were optimised for annealing temperature and magnesium ion concentration as previously described (see Section 6.2.1.1). Amplification for *ampR* was performed in conjunction with amplification of the *ampC* structural gene. Details of the *ampC* DNA template control amplification reaction are included in Section 6.2.3.1. Amplified DNA (5 µl) was electrophoresed in a 2.0% (w/v) agarose gel using 0.5X TBE buffer at 70 V for 2 h (Appendix AIII.1). Reference size DNA fragments were included with each electrophoresis run (100 bp ladder, DNA molecular weight marker XIV, Boehringer Mannheim).

Table 6.5. Details of *ampR*-specific PCR primer sets.

Primer set	Primer name	Primer sequence (5' to 3')	Melting temperature (°C)	Nucleotide position	Amplimer size (bp)
ampR Set 1	*ampR*1-id-Forward	CGG GCT TTT GAA GCC GC	56	223–239*	580
	*ampR*1-id-Reverse	CCG GCC GCC TGC ATC C	58	803–788	
ampR Set 2	*ampR*2-id-Forward	CCG CGA GAC ATC TCA GC	56	239–255*	564
	*ampR*1-id Reverse	CCG GCC GCC TGC ATC C	58	803–788	
ampR Set 3	*SmampRF*1-Forward	GCT CGC CAC CTG AAC TTC ACC	70	115–135§	650
	*SmampRR*1-Reverse	CCA CGC CAT CGC ACT GGA TCG	77	766–746	

*, numbering according to Honore *et al.*, 1986, where the ATG start codon is located at nucleotide position 191;
§, numbering according to Morrow, 1999, where the ATG start codon is located at nucleotide position 67.

6.2.2.2. **Identification of *ampR* by Southern hybridisation of genomic DNA**

Restriction endonuclease digestion and Southern transfer of genomic DNA

Southern blots of *Eco*R1-digested genomic DNA from the 10 *S. marcescens* isolates in Collection C were prepared using the methods outlined for *ampD* hybridisation studies (see Section 6.2.1.2). *Eco*R1-digested genomic DNA from *S. marcescens* NCTC 11935 and wild-type control isolates of *E. cloacae*, *C. freundii* and *E. coli* (Table 6.3) were included with each batch of tests. Undigested plasmid DNA preparations of pNU305 and pNU405 were also included as hybridisation controls.

Generation and cloning of ampR-like PCR products for use as ampR probes

Three *ampR* probes were generated from the *ampR*-like PCR products of plasmid pNU305 (*ampR*+), wild-type *E. cloacae* and wild-type *C. freundii*. These PCR products were obtained using *ampR* primer Set 2 and the *ampR*-amplification procedure outlined in Section 6.2.2.1. The *ampR*-like PCR products were cloned using the TOPO™ TA Cloning® Kit, version E (Invitrogen BV) and the *ampR* sequence of selected transformants was confirmed by sequencing using the methods outlined in Section 6.2.1.2. Edited DNA sequences were run in 'database search' protocols for identity with known *ampR* sequences in *E. cloacae* and *C. freundii*.

Hybridisation with DIG-labeled ampR probes

Following confirmation of the *ampR* sequence, PCR inserts from the three *ampR* clones were purified and DIG-labeled using the DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim) (see Section 6.2.1.2). Southern blots of *Eco*R1-digested genomic DNA were hybridised with the three DIG-labeled *ampR* probes under the conditions of low stringency used in the *ampD* hybridisation studies. Chemiluminescent detection was carried out in accordance with the manufacturer's instructions for use with CSPD *Star*™.

6.2.2.3. **PCR amplification for identification of *ampR* using primers derived from the *ampR* sequence in *S. marcescens***

DNA amplification studies for *ampR*-encoding genes were re-designed during a working visit to MCRAB, Creighton University, using *S. marcescens ampR* DNA sequence data made available by Dr. Nancy Hanson, Director of Molecular Biology for CRAB.

Bacterial isolates

DNA amplification for *ampR* was performed on the 21 isolates of *S. marcescens* from Collection B detailed in Section 6.2.1.1. These *S. marcescens* isolates had been transported to MCRAB, Creighton University and frozen at –70°C, as previously described (see Section 4.2.2.4). The wild-type *S. marcescens* reference isolate, Serr 1, from which the *S. marcescens ampR* sequence data was derived, was provided by MCRAB, Creighton University and was included as a positive amplification control with each batch of isolates tested.

Preparation of template DNA

Template DNA was prepared according to the modified proteinase K 'boiling' method used for the extraction of DNA for amplification of TEM- and SHV-β-lactamase encoding genes (see Section 5.2.2.1). Template DNA was prepared fresh for each DNA amplification reaction.

Primer design

Oligonucleotide primers (*ampR* Set 3) specific for the intergenic region of *ampR* in *S. marcescens* were designed at MCRAB, Creighton University. Details of the primer set used are included in Table 6.5.

DNA amplification reaction

To provide an internal control for the quality of template DNA, amplification assays for *ampR* was performed at the same time as amplification assays for the structural *ampC* gene. Details of the *S. marcescens ampC*-specific primers and amplification reaction protocol are described in Section 6.2.3.2.

The *ampR* PCR reaction mixture was as follows: 0.2 mM each of dATP, dCTP, dGTP and dTTP (New England BioLabs Inc.), 2.0 mM magnesium chloride, 1X PCR buffer (20mM Tris-HCL, pH 8.4, 50 mM KCL), 1.25 units *Taq* DNA polymerase (Gibco BRL®/Life Technologies) and 0.5 μM of *ampR* primer Set 3 in a total volume of 49 μl. A master-mix of reagents was prepared for each batch of tests and 1 μl of DNA template was added to each aliquot. A negative amplification control containing 1 μl of sterile water was included with each batch of tests.

DNA was amplified in a Perkin Elmer 480 thermocycler (Perkin-Elmer). The PCR program consisted of an initial denaturation step at 96°C for 30 s followed by 24 cycles of DNA denaturation at 96°C for 30 s, primer annealing at 50°C for 15 s and primer extension at 72°C for 2 min. Amplified DNA (5 µl) was electrophoresed in a 1.5% (w/v) agarose gel using 1X TAE buffer at 70 V for 2 h. Reference size DNA fragments were included in each electrophoresis run (100 bp DNA ladder, Gibco BRL®/Life Technologies). Suspect *ampR*-like PCR amplimers (~650 bp in size) from the DNA of the nine *S. marcesces* isolates in Collection D (see Section 6.2.1.4) were purified using the Qiaquick™ PCR product purification kit (QIAGEN Inc.) and concentrated using the method outlined for TEM-like PCR products (see Section 5.2.2.1).

DNA sequencing

Direct cycle sequencing of *ampR*-like PCR products from the nine *S. marcescens* isolates in Collection D was performed by the University of Nebraska Medical Center Core Facility, Omaha, US, using a DNA stretch sequencer (Applied Biosystems).

Nucleotide sequence analysis of the *ampR* PCR products was performed using 'database search' protocols for identity with known *ampR* sequences (see Section 6.2.1.2). Multiple sequence alignments were performed using Clustal W software (version 1.81). The nucleotide sequence of the nine *ampR* PCR products from *S. marcescens* were aligned using a Blosum weight matrix and default settings for gap and gap extension penalties. The amino acid sequence of the deduced AmpR protein from the test *S. marcescens* isolates was aligned with AmpR from wild-type *S. marcescens,* Serr1, (using sequence data made available from MCRAB, Creighton University) and with an additional unpublished *S. marcescens* AmpR sequence which was deposited in the Genbank (Genbank accession number AJ271368) after these *ampR* amplification studies were completed. The AmpR amino acid sequence in *S. marcescens* was also aligned with the analogous AmpR proteins from wild-type isolates of *E. cloacae* (Ent. MHN1, Genbank accession number X04730), *C. freundii* (Cit. OS60 Genbank accession number 27222), *M. morganii* (Mor str. 1, Genbank accession number AF055067), *Hafnia alvei* (*H. alvei* 1, Genbank accession number AF180952) and *P. stuartii* (*P. stuartii* VDG96, Genbank accession number Y17315).

6.2.3. Identification and characterisation of *ampC* in *S. marcescens*

Amplification for the structural *ampC* β-lactamase gene was included as an internal control for the quality of template DNA in all PCR studies performed 'in-house' and in MCRAB, Creighton University. In a separate study, DNA amplification of *ampC*, including the *ampC-ampR* intercistronic region, was undertaken to obtain DNA sequence data of the *ampC* structural gene in *S. marcescens*.

6.2.3.1. 'In-house'-DNA template quality control amplification reaction

DNA amplification studies for *ampC* was performed 'in house' as a control for the quality of template DNA used in all preliminary DNA amplification procedures for the identification of *ampD* (see Section 6.2.1.1.) and *ampR* (see Section 6.2.2.1).

Bacterial isolates

Amplification reactions for *ampC* were performed using the template DNA extracted from the 21 *S. marcescens* isolates in Collection B and the wild-type control isolates tested for amplification of *ampD* (see Section 6.2.1.1) and *ampR* (see Section 6.2.2.1).

Primer design

An oligonucleotide primer set, *ampC* Set 1, specific for the intergenic region of *ampC* was designed from the published DNA sequence of *ampC* in wild-type *S. marcescens* (Nomura & Yoshida, 1990). Details of the primer set are included in Table 6.6. Primers were custom made on request by Genosys Biotechnologies Ltd.

DNA amplification reaction

DNA amplification for *ampC* was performed using the High Fidelity PCR reaction mixture and PCR protocol outlined for the identification of *ampD* (see Section 6.2.1.1). Amplified DNA was electrophoresed in a 2.0% (w/v) agarose gel (Appendix AIII.1) and reference size DNA fragments were included with each electrophoresis run (100 bp ladder, DNA molecular weight marker XIV, Boehringer Mannheim).

DNA sequence analysis

PCR products from one of the cefotaxime-susceptible isolates and one of the cefotaxime-resistant isolates from Collection B were cloned using the TOPO™ TA

Table 6.6. Details of *ampC*-specific PCR primer sets.

Primer set	Primer name	Primer sequence (5' to 3')	Melting temperature (°C)	Nucleotide position	Amplimer size (bp)
ampC Set 1	*ampC*1-id-Forward	ACA GCA GCA GGA TAT CGA CG	65	89–109*	860
	*ampC*1-id-Reverse	CGT GCC GTT CAT GAT CAT CC	68	950–930	
ampC Set 2	SDMC-Forward	CCT GCA ACC TAA GAG CTT CT	66	1–20*	1140
	SDMC-Reverse	CGC GGT GGA TGA TGT GGT AA	62	1140–1121	
ampC Set 3	*ampC*3con-Forward	CGT ACC TGC TGG CTG ATG G	63	19–37§	1507
	*ampC*3con-Reverse	GCA GCA TCG GTG GTA GGG	66	1525–1508	

*, numbering according to Nomura & Yoshida, 1990, where the ATG start codon is located at nucleotide position 24;
§, numbering according to unpublished *S. marcescens ampC* sequence deposited in the Genbank (Genbank accession number AJ271368), where the ATG start codon is located at nucleotide position 347.

Cloning® Kit, version E (Invitrogen BV) and the resultant transformants were sent to MWG Biotech UK for sequence confirmation (see Section 6.2.1.2).

6.2.3.2. **Creighton University-DNA template quality control amplification reaction**

In MCRAB, Creighton University, DNA amplification reactions for *ampC* were performed as a control for the quality of template DNA used in amplification procedures for identification of TEM- and SHV-type β-lactamase encoding genes (see Section 5.2.2.1) and in amplification procedures for the characterisation of *ampR* (see Section 6.2.2.3).

Bacterial isolates

Amplification reactions for *ampC* were performed using the template DNA extacted from the 21 *S. marcescens* isolates in Collection B and the wild-type control isolates tested for amplification of TEM- and SHV-type β-lactamase encoding genes (see Section 5.2.2.1) and for amplification of *ampR* (see Section 6.2.2.3).

Primer design

The *S. marcescens ampC*-specific oligonucleotide primer set, *ampC* Set 2, was designed in MCRAB, Creighton University. Details of the primer set used are included in Table 6.6.

DNA amplification reaction

The *ampC* amplification reaction mixture and PCR protocol were similar to those described for the amplification of *ampR* in Section 6.2.2.3. Amplified DNA (5 µl) was electrophoresed in a 1.5% (w/v) agarose gel using 1X TAE buffer at 70 V for 2 h and reference size DNA fragments were included with each electrophoresis run (100 bp DNA ladder, Gibco BRL®/Life Technologies).

6.2.3.3. **Characterisation of *ampC* and the *ampC-ampR* intercistronic region**

Bacterial isolates

DNA amplification for confirmation of the structural *ampC* gene and the *ampC-ampR* intercistronic region was performed on the nine *S. marcescens* isolates in Collection D (see Section 6.2.1.4). The *S. marcescens* NCTC 11935 control isolate and the wild-type *E. cloacae* control isolate were processed with each batch of tests.

Preparation of template DNA

Template DNA was prepared using the proteinase K modified protocol outlined for the Wizard™ Genomic DNA Purification kit (Appendix AIII.2.2). Extracted DNA was quantified using the agarose gel electrophoresis method (Appendix AIII.3) and fresh dilutions of DNA were prepared in sterile water prior to each amplification reaction. DNA extracted from *S. marcescens* isolates was diluted 1/5 to yield an approximate DNA concentration of 80 ng/µl. DNA extracted from *E. cloacae* was diluted 1/100 to yield an approximate DNA concentration of 4 ng/µl.

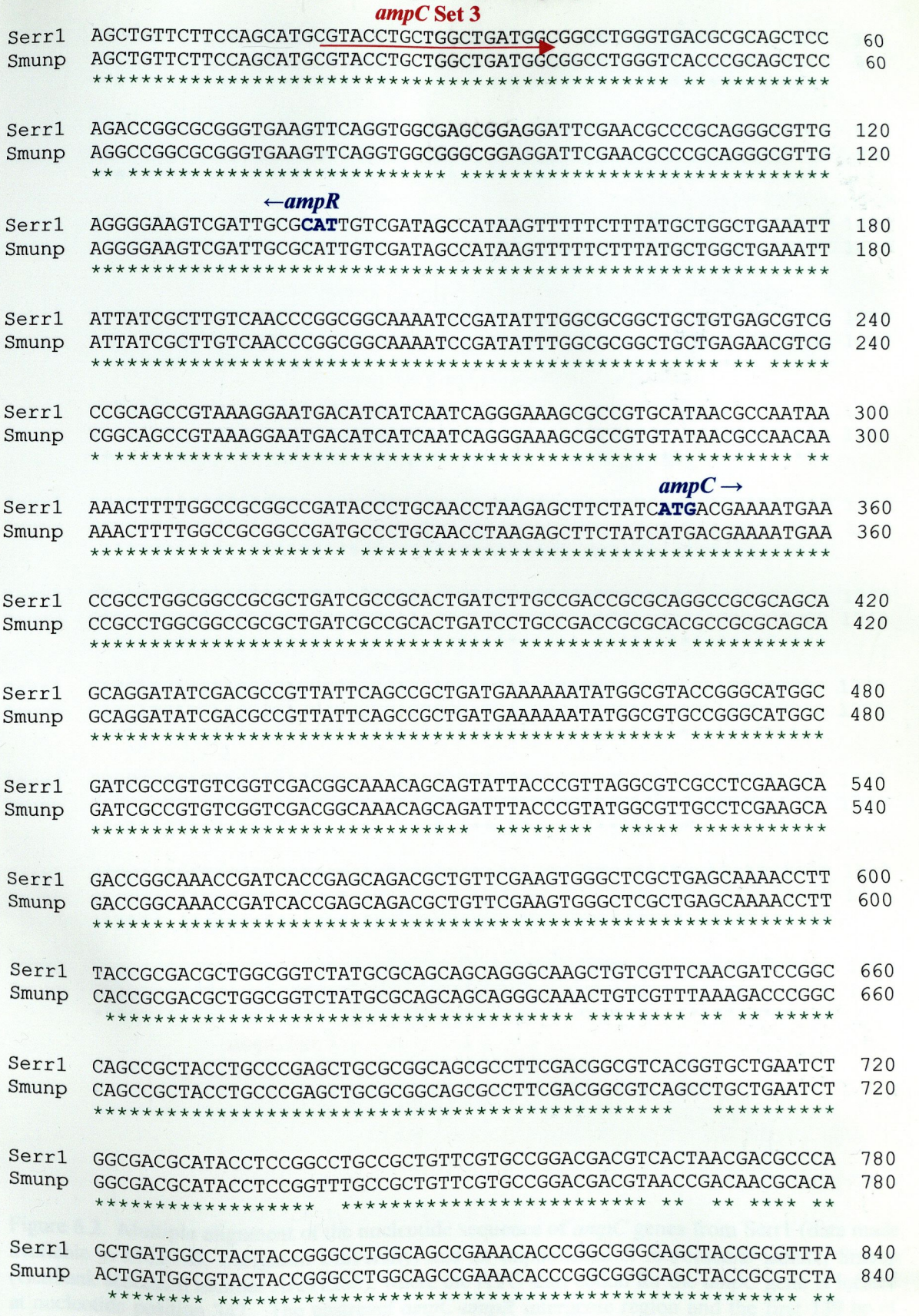
Primer design

Oligonucleotide primers for the *ampC* gene and the upstream intercistronic *ampC-ampR* region were designed using sequence data made available by Dr. Nancy Hanson, Director of Molecular Biology for CRAB and unpublished *S. marcescens ampC-ampR* data deposited in the Genbank (Genbank accession number AJ271368). The primer pair, *ampC*-primer Set 3, were designed to generate a 1.5 kb amplimer covering the sequence of the *ampC* structural gene and extending upstream through the *ampC-ampR* intercistronic region into the divergently transcribed *ampR* gene (Figure 6.2). Primers were custom made on request by Genosys Biotechnologies Ltd. Details of the *ampC* Set 3 primer pair are included in Table 6.6.

DNA amplification reaction

Amplification for *ampC* was performed using the *Taq* PCR Core Kit (QIAGEN GmbH) reaction mixture outlined in amplification procedures for confirmation of *ampD* (see Section 6.2.1.4). Parallel amplification reactions were performed in the presence and in the absence of Q-solution. DNA was amplified in a PCR Gene Express™ thermocycler (Hybaid) using a PCR 'touchdown' protocol. The amplification program consisted of an initial denaturation step at 94°C for 3 min, followed by 5 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 63°C for 30 s and primer extension at 72°C for 1 min. In the following five amplification cycles, the annealing temperature was decreased to 61°C. For the remaining 20 cycles, the annealing temperature was decreased a further 2°C. A final extension step was performed at 72°C for 10 min. Amplified DNA was electrophoresed in a 2.0% (w/v) agarose gel (Appendix AIII.1) and reference size DNA

CLUSTAL W (1.81) nucleotide sequence alignment for *ampC* primer Set 3 selection



CLUSTAL W (1.81) nucleotide sequence alignment for *ampC* primer Set 3 selection contd.

```
Serr1   TTCCAACCTCGGCATCGGCATGCTGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTT  900
Smunp   TTCCAACCTCGGCATCGGCATGCTGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTT  900
        ************************************************************

Serr1   TATCCAGGCGATGGAACAGGGGATGCTGCCGGCGCTGGGCATGAGCCACACCTACGTTCA  960
Smunp   TATCCAGGCGATGGAACAGGGGATGCTGCCGGCGTTGGGCATGAGCCACACCTACGTTCA  960
        ********************************** ************************

Serr1   GGTGCCGGCGGCGCAGATGGCTAACTATGCGCAGGGTTACAGCAAGGACGATAAGCCGGT 1020
Smunp   GGTGCCGGCGGCGCAGATGGCCAACTATGCGCAGGGCTACAGCAAGGACGATAAACCGGT 1020
        ********************** ************** ***************** *****

Serr1   GCGGGTCAACCCCGGCCCGCTGGACGCCGAATCTTACGGCATCAAGTCCAACGCTCGCGA 1080
Smunp   GCGGGTCAATCCCGGCCCGCTGGACGCCGAGTCTTACGGCATCAAGTCCAACGCCCGCGA 1080
        ********* ********************* ********************** *****

Serr1   TCTGATTCGCTATCTGGACGCCAACCTGCAGCAGGTGAAGGTGGCGCAGCCATGGCGCGA 1140
Smunp   TCTGATTCGCTATCTGGACGCCAACCTGCAGCAGGTGAAGGTGGCGCAACCGTGGCGCGA 1140
        ************************************************ ** ********

Serr1   CGCGCTGGCCGCGACGCACGTCGGGTATTACAAGGCGGGCGCGTTCACGCAGGATCTGAT 1200
Smunp   GGCGCTGGCCGCCACGCACGTCGGTTATTACAAGGCGGGTGCGTTCACGCAGGATCTGAT 1200
         *********** *********** ************** ********************

Serr1   GTGGGAGAACTACCCGTATCCAGTGAAACTGTCGCGTTTGATTGAAGGCAACAACGCCGG 1260
Smunp   GTGGGAGAACTACCCGTATCCGGTGAAGCTGTCGCGTTTGGTTGAAGGCAACAACGCCGG 1260
        ********************** ***** ************ ********************

Serr1   GATGATCATGAACGGCACGCCGGCCACCACCATCACGCCGCCGCAGCCGGAATTGCGCGC 1320
Smunp   GATGATCATGAACGGCACGCCGGCCACCGCCATCACGCCGCCGCAGCCGGAATTGCGCGC 1320
        ***************************** ******************************

Serr1   CGGCTGGTATAACAAAACCGGCTCCACCGGCGGCTTCTCCACCTACGCGGTATTTATCCC 1380
Smunp   CGGCTGGTATAACAAAACCGGCTCTACCGGCGGTTTCTCCACCTACGCGGTGTTTATCCC 1380
        ************************ ******** ***************** ********

Serr1   GGCGAAAAATATCGCCGTGGTGATGCTGGCCAACAAGTGGTTCCCGAACGACGATCGCGT 1440
Smunp   GGCGAAGAATATCGCCGTGGTGATGTTGGCCAACAAGTGGTTCCCGAATGACGATCGCGT 1440
        ****** ****************** ********************** ***********

Serr1   CGAGGCGGCTTACCACATCGTCCAGGCGCTGGAGAAGCGCTGACGGCCAGGGGCGCCGAC 1500
Smunp   CGAGGCGGCTTACCACATCATCCAGGCGCTGGAGAAGCACTGACGCTCAGGGGCGCCGGC 1500
        ******************* ****************** ******  *********** *

                ampC Set 3
Serr1   GAGGCGCCCCTACCACCGATGCTGCCGTTCACGGTGCTCGCCGC- 1545
Smunp   GAGGCGCCCCTACCACCGATGCTGCCGTTCACGCCGCTCGC-GCA 1545
        ******************************  ****** **
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Figure 6.2. Multiple alignment of the nucleotide sequence of *ampC* genes from Serr1 (data made available in MCRAB, Creighton University) and an unpublished *S. marcescens* isolate, Smunp (Genbank accession number AJ271368), where the ATG start codon for the *ampC* gene is located at nucleotide position 347. The upstream *ampC-ampR* intergenic region and the first 139 bp of *ampR* are also shown. The *ampR* data shown are negative strand sequence. Translational start and stop codons are in blue type. *, denotes identical sequences. The location of the *ampC* Set 3 primers is underlined ⟵.

fragments were included with each electrophoresis run (100 bp ladder, DNA molecular weight marker XIV, Boehringer Mannheim).

Cloning and sequence analysis

The *ampC*-like PCR products obtained using DNA extracted from four of the *S. marcescens* isolates in Collection D were purified using the Qiaquick™ PCR product purification kit (QIAGEN GmbH) in accordance with the manufacturer's instructions. The purified PCR products were cloned using the TOPO™ TA Cloning Kit for Sequencing, version E2 (Invitrogen BV) and suitable transformants were selected and prepared for sequence analysis using the methods outlined in Section 6.2.1.2.

Plasmid precipitates of two *ampC* clones were sent to MWG Biotech UK, for sequence analysis using an ABI 377 sequencer (Applied Biosystems). Cycle sequencing was performed using a 4X Comfort Read® sequence overlap protocol (designed for DNA sequence read lengths >700 bp) and a combination of custom-made and M13 universal primers.

Multiple sequence alignments were performed using Clustal W software (see Section 6.2.1.2.). The *ampC* sequence of the PCR products from the two *S. marcescens* test isolates were aligned with the *ampC* sequence from Serr1 (Genbank accession number AF384203) and with three other wild-type *S. marcescens ampC* sequences available in the Genbank, *S. marcescens* SR50 (Genbank accession number X52964) (Nomura & Yoshida, 1990), *S. marcescens* SST1 (Genbank accession number AB008455) (Matsumura *et al.*, 1998) and an unpublished *S. marcescens* isolate (Genbank accession number AJ271368). The *ampC* sequence obtained from one of the PCR products amplified using the 'in house' *ampC* DNA template control amplification reactions was also included in the alignment. The sequences of the *ampC-ampR* intercistronic region from the PCR products of the two *S. marcescens* test isolates were also aligned with the *ampC-ampR* intercistronic sequence from Serr1 (unpublished sequence data made available in MCRAB, Creighton University) and from an unpublished *S. marcescens* isolate (Genbank accession number AJ271368).

6.3. RESULTS

6.3.1. Identification and characterisation of *ampD* in *S. marcescens*

6.3.1.1. PCR amplification for identification of *ampD* using primers derived from *ampD* sequences in *E. cloacae*, *C. freundii* and *E. coli*

DNA template control amplification reaction

The *ampC* DNA amplification reactions (*ampC* primer Set 1) performed as a control for the quality of template DNA, yielded the expected PCR product of 860 bp using DNA from all *S. marcescens* isolates (Figure 6.3, panel A). The use of the *S. marcescens-ampC* specific primer pair in the template control amplification reactions failed to yield PCR products using DNA from the wild-type *E. cloacae*, *C. freundii* and *E. coli* control isolates.

DNA amplification for ampD

Using *ampD* primer Set 1 (derived from the *ampD* sequence in *E. cloacae*, *C. freundii* and *E. coli*) a PCR product of the expected size (~370 bp) was amplified from DNA extracted from the wild-type *E. cloacae* control and the *ampD*-encoding pNU405 plasmid (Figure 6.3, panel B, lanes 8 and 9). No *ampD*-primer-specific products were amplified from DNA from the remaining positive controls i.e. wild-type *C. freundii and E. coli*. DNA from all *S. marcescens* isolates from Collection B failed to produce an *ampD*-specific PCR product using *ampD* primer Set 1 (Figure 6.3, panel B, lanes 2–7).

When amplified with *ampD* primer Set 2 (also derived from the *ampD* squence in *E. cloacae*, *C. freundii* and *E. coli*), DNA from all PCR positive control isolates (*E. cloacae*, *C. freundii*, *E. coli* and pNU405) demonstrated an amplimer of the expected size (~250 bp). As with *ampD* primer Set 1, DNA from all *S. marcescens* isolates tested failed to yield an *ampD*-like PCR product when amplified with *ampD* primer Set 2 (Figure 6.3, panel C, lanes 2–7). The PCR negative controls (water and *ampD*- plasmid pNU305) were negative with both sets of *ampD* primers. The *ampD*-PCR products from plasmid pNU405 (obtained with *ampD* primer Set 1 and primer Set 2) and the *ampD*-PCR products from the wild-type *E. cloacae*, *C. freundii* and *E. coli* control isolates (obtained with *ampD* primer Set 2) were selected for use as *ampD*-specific probes in hybridisation studies.

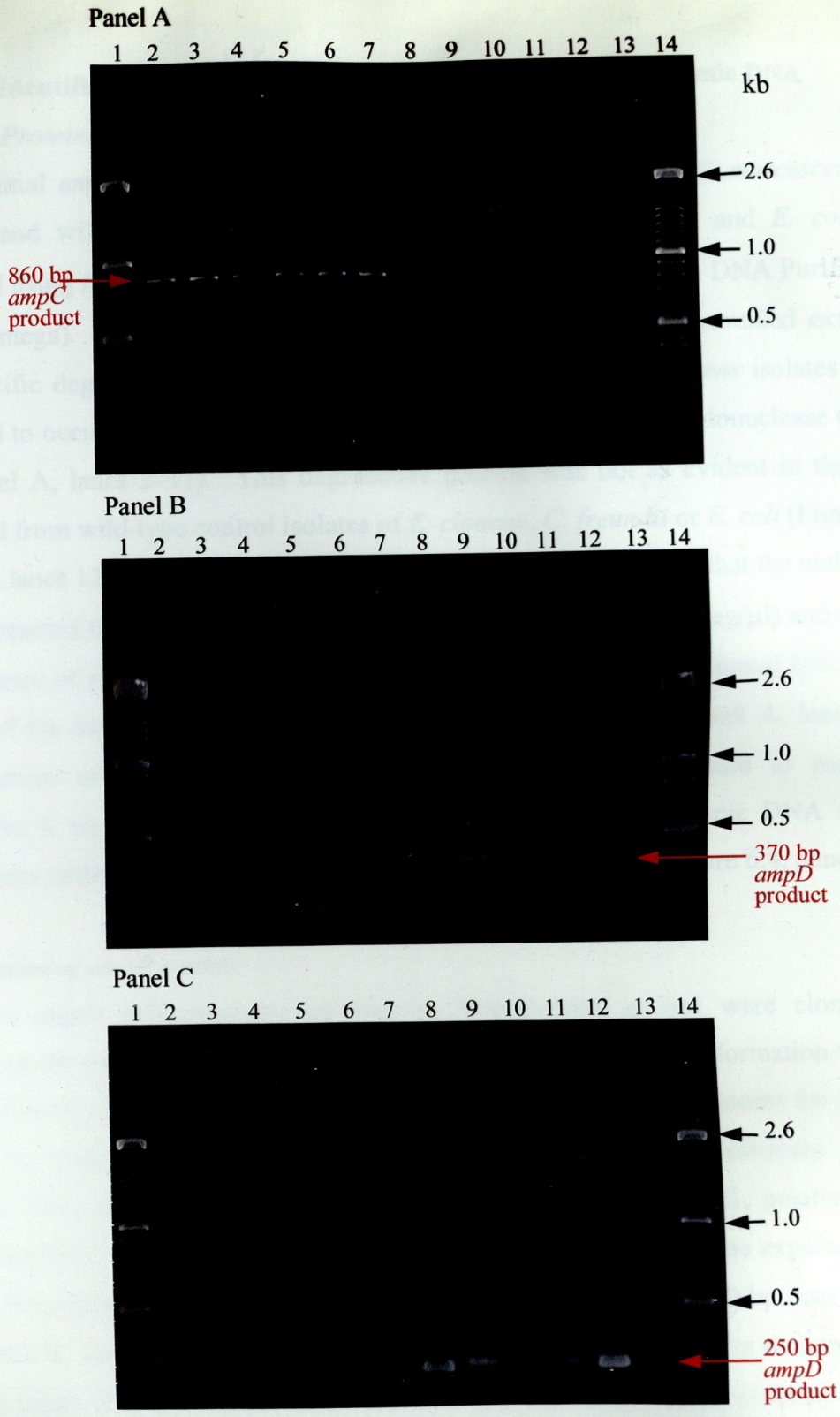


Figure 6.3. Ethidium bromide-stained agarose gels showing PCR products obtained when target DNA was amplified using *ampC* primer Set 1 (panel A), *ampD* primer Set 1 (panel B) and *ampD* primer Set 2 (panel C). Amplimers generated with template DNA from test and control isolates are located in lanes as follows: 2–7, *S. marcescens* isolates from Collection B; 8, wild-type *E. cloacae* (*ampD*+); 11, wild-type *C. freundii* (*ampD*+); 12, wild-type *E. coli* (*ampD*+). Amplimers generated with plasmid template DNA from *E. coli* isolates carrying plasmid pNU405 (*ampD*+) and plasmid pNU305 (*ampD*-) are located in lanes 9 &10, respectively. Lanes 1 & 14 contain 100 bp standard size reference markers. Lane 13 contains the PCR negative control (water).

6.3.1.2. **Identification of *ampD* by Southern hybridisation of genomic DNA**

Value of Proteinase K in the DNA extraction protocol

In the initial *ampD* hybridisation studies, genomic DNA from the *S. marcescens* test isolates and wild-type control isolates of *E. cloacae*, *C. freundii* and *E. coli* was extracted using the recommended protocol for the Wizard™ Genomic DNA Purification Kit (Promega). Following *Eco*R1 digestion, agarose gel analysis revealed extensive non-specific degradation of the DNA extracted from all *S. marcescens* isolates which appeared to occur within 10 min of incubation with the restriction endonuclease (Figure 6.4, panel A, lanes 2–11). This degradative process was not as evident in the DNA extracted from wild-type control isolates of *E. cloacae*, *C. freundii* or *E. coli* (Figure 6.4, panel A, lanes 12–14). Agarose gel quantification analysis indicated that the undigested DNA extracted from *S. marcescens* was of adequate quantity (~400 ng/µl) and showed no evidence of shearing, exhibiting a single discrete band of chromosomal DNA in the region of the lambda DNA concentration standards (Figure 6.5, panel A, lanes 1–6). Modification of the Wizard™ Genomic DNA extraction procedure to include a proteinase K treatment step reduced DNA degradation when genomic DNA from *S. marcescens* isolates was incubated with restriction endonuclease (Figure 6.4, panel B).

Preparation of ampD probes

The five *ampD* PCR products selected as hybridisation probes were cloned and sequenced for confirmation of the presence of the *ampD* gene. Transformation with the TOPO cloned *ampD* PCR products yielded over 200 transformant colonies for each LB kanamycin plate inoculated. When plasmid DNA from 10 transformant colonies selected from each transformation reaction was digested with *Eco*R1, positive clones were identified by the presence of a DNA band in the size range of the expected *ampD* insert. For *ampD* primer Set 1, positive transformants displayed a 370 bp insert (Figure 6.5, panel B, lane 3). For *ampD* primer Set 2, positive transformants demonstrated a 250 bp insert (Figure 6.5, panel B, lanes 5, 7, 9 and 11). Approximately half the colonies selected for analysis contained the expected *ampD* insert.

The nucleotide sequences of the cloned *ampD* PCR products from the *E. cloacae*, *C. freundii* and *E. coli* control isolates demonstrated greater than 98% similarity with the published *ampD* sequences from wild-type *E. cloacae* (*E. cloacae* 14, Genbank accession number Z14003), *C. freundii* (Cit. OS60, Genbank accession number Z14002)

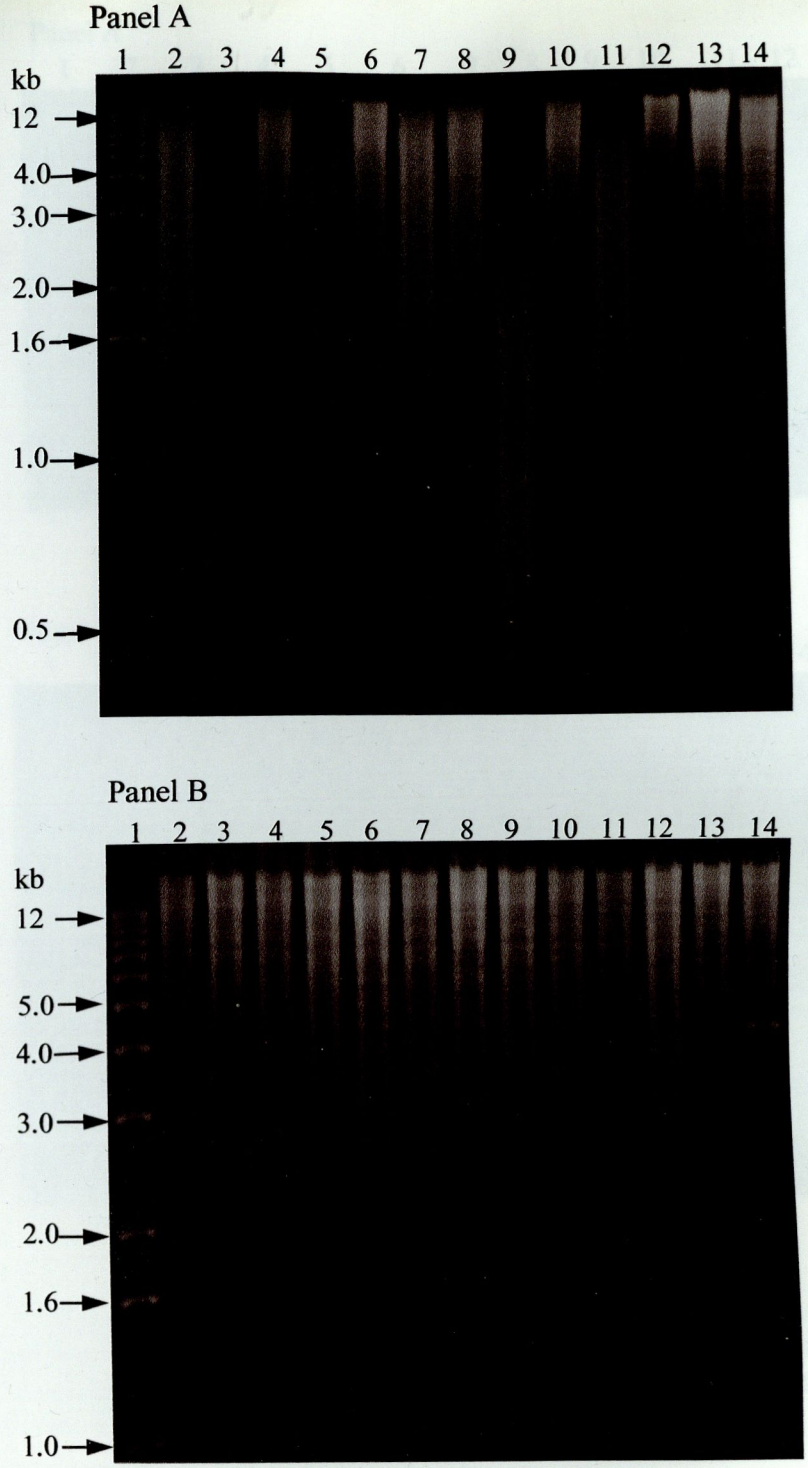


Figure 6.4. Ethidium bromide-stained agarose gels showing *Eco*R1-digested genomic DNA extracted using the Promega Genomic DNA purification kit without proteinase K (panel A) and with proteinase K (panel B). Lane contents are as follows: 1, 1 kb standard reference size markers; 2–11, DNA extracted from *S. marcescens* isolates in Collection C; 12–14, DNA extracted from wild-type *E. cloacae, C. freundii* & *E. coli*, respectively. When DNA from *S. marcescens* isolates was extracted in the absence of proteinase K (panel A), extensive degradation of DNA was observed following *Eco*R1 digestion.

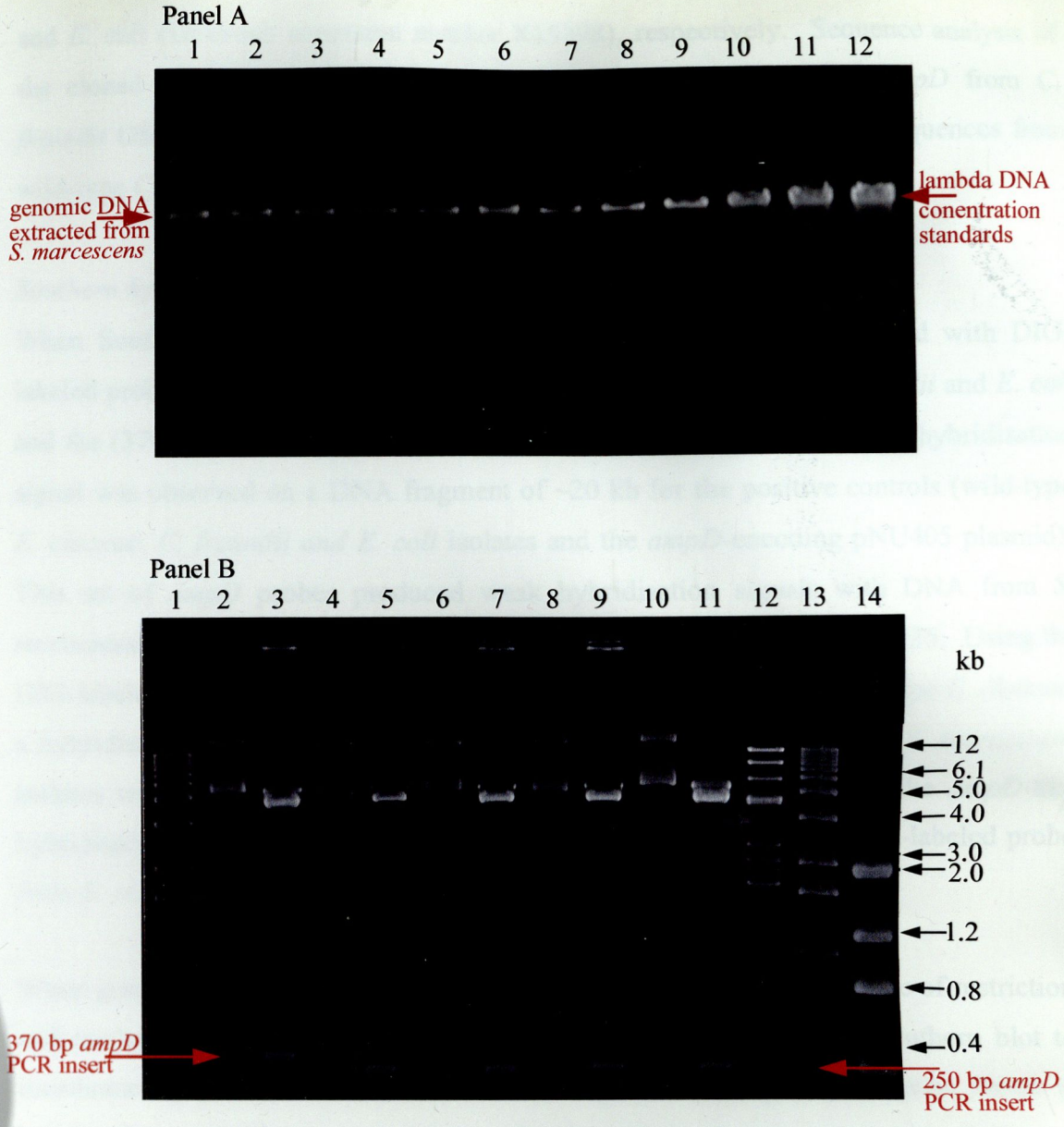


Figure 6.5. Panel A. Quantification of genomic DNA using ethidium bromide-stained agarose gel electrophoresis. Lane contents are as follows: l–6, genomic DNA (1/20 dilution) from *S. marcescens* isolates in Collection B; 7–12, lambda DNA concentration standards equivalent to 25, 50, 75, 100, 150 and 200 ng DNA.

Panel B. Ethidium bromide-stained agarose gel showing plasmid DNA extracted from TOPO TA clones of *ampD* PCR products for use as *ampD* hybridisation probes. Lane contents are as follows: 1 & 13, 1 kb standard size reference markers; 2, 4, 6, 8 & 10, undigested plasmid DNA extracted from clones of *ampD* PCR products generated with template DNA from plasmid pNU405 (amplified using *ampD* primer Set 1) and with template DNA from *E. cloacae, C. freundii, E. coli* and plasmid pNU405 (amplified using *ampD* primer Set 2), respectively; 3, 5, 7, 9 & 11, *Eco*R1-digested plasmid DNA of clones of *ampD* PCR products generated with template DNA from plasmid pNU405 (amplified using *ampD* primer Set 1) and with template DNA from *E. cloacae, C. freundii, E. coli* and pNU405 (amplified using *ampD* primer Set 2), respectively; 12, *Eco*R1-digested lambda control; 14, low DNA mass ladder.

and *E. coli* (Genbank accession number X15398), respectively. Sequence analysis of the cloned *ampD*-PCR products from plasmid pNU405 (encoding *ampD* from *C. freundii* OS60) demonstrated 100% identity with the published *ampD* sequences from wild-type *C. freundii* (Cit. OS60, Genbank accession number Z14002).

Southern hybridisation

When Southern blots of *Eco*R1-digested genomic DNA were hybridised with DIG-labeled probes derived from the (250 bp) *ampD*-PCR products of *C. freundii* and *E. coli* and the (370 bp and 250 bp) *ampD*-PCR products of plasmid pNU405, a hybridisation signal was observed on a DNA fragment of ~20 kb for the positive controls (wild-type *E. cloacae, C. freundii and E. coli* isolates and the *ampD*-encoding pNU405 plasmid). This set of *ampD* probes produced weak hybridisation signals with DNA from *S. marcescens* isolates from Collection C and with *S. marcescens* NCTC 11935. Using the DIG-labeled *ampD* probe derived from the 250 bp PCR product of wild-type *E. cloacae,* a hybridisation signal was evident for the positive controls and for all *S. marcescens* isolates tested (Figure 6.6, panel A). For the *S. marcescens* isolates the *ampD*-like hybridisation band was located on a DNA fragment of ~5 kb. The DIG-labeled probe from *E. cloacae* was selected as the most useful *ampD* probe.

When genomic DNA from *S. marcescens* isolates was treated with a range of restriction endonucleases including *Cla*I, *Hind*III and *Pvu*II and transferred by Southern blot to membrane filters, hybridisation with the selected *E. cloacae-ampD* probe failed to localise the *ampD*-like gene on a low molecular weight fragment (1–2 kb). Following hybridisation of *Pst*1-digested genomic DNA, the *ampD*-like band was localised on a DNA fragment of ~1.2 kb for all *S. marcescens* isolates tested (Figure 6.6, panel B). Negative results were obtained for plasmid pNU305 (*ampD* negative control) in all hybridisation assays.

6.3.1.3. **Cloning, selection and sequencing of putative *ampD* fragments**

In the initial cloning experiments of the *Pst*1-digested 1.2 kb putative *ampD* fragments from two cefotaxime resistant *S. marcescens* isolates (97.R8, 98.R7), ligation reactions were set up using the recommended insert:vector molar ratio of 2:1 for cohesive-end ligation. The transformation efficiency of these cloning reactions was low with less than 10 transformant colonies growing on each LB kanamycin plate inoculated. Following

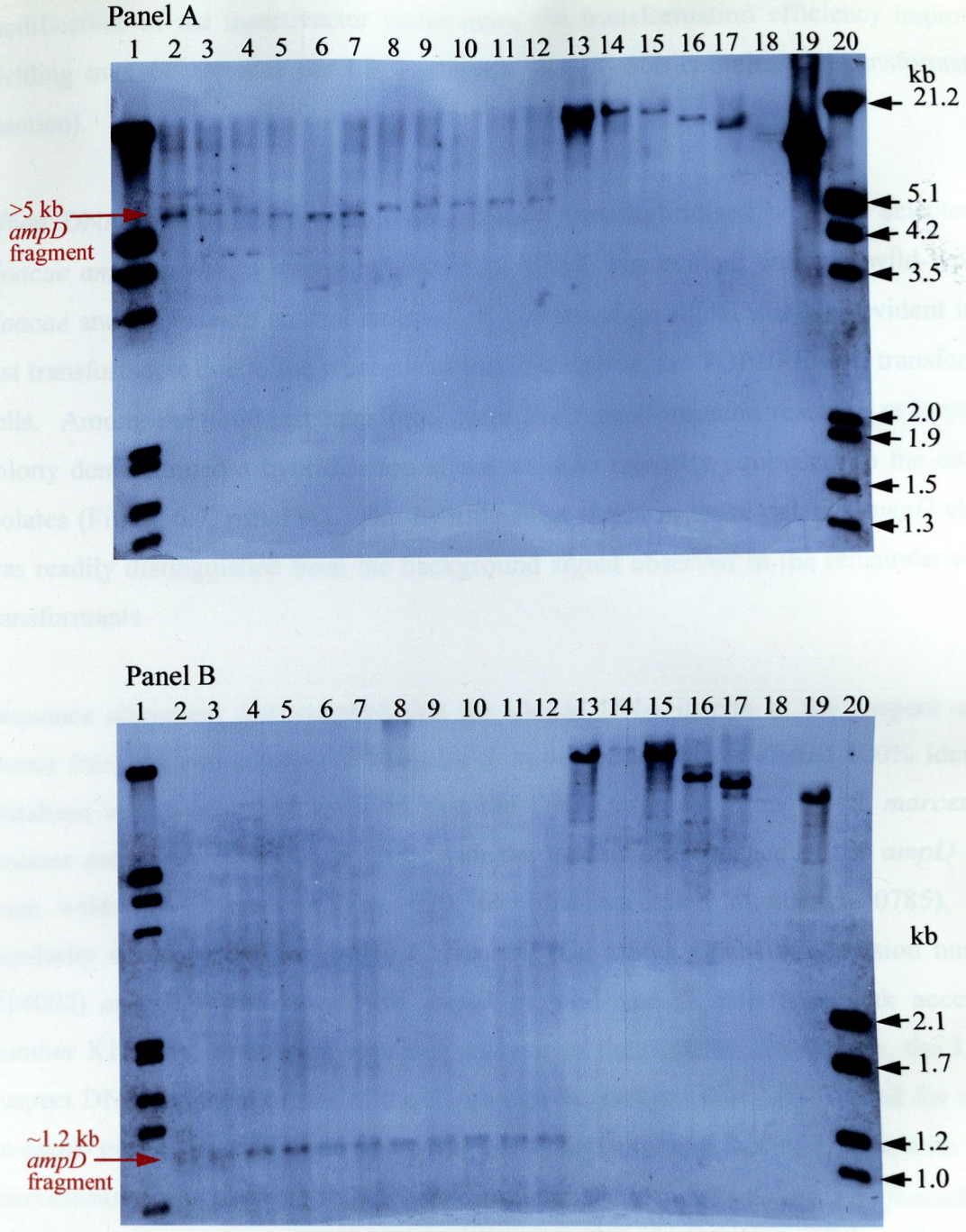


Figure 6.6. Autoradiograph of *Eco*R1-digested (panel A) and *Pst*1-digested (panel B) DNA following Southern transfer and hybridisation using a DIG-labeled 250 bp *ampD* probe derived from the *ampD*-PCR product of wild-type *E. cloacae*. DNA extracted from test and control isolates is located in lanes as follows: 2, *S. marcescens* NCTC 11935; 3–12, *S. marcescens* isolates from Collection C; 13 & 14, wild-type *E. cloacae* positive hybridisation controls; 15 & 16, wild-type *C. freundii* positive hybridisation controls; 17, wild-type *E. coli* positive hybridisation control. Lanes 18 & 19 contain undigested DNA from plasmid pNU305 (negative hybridisation control) and from plasmid pNU405 (positive hybridisation control), respectively. Lanes 1 & 20 contain DIG-labeled standard reference size markers.

modification of the insert:vector molar ratio, the transformation efficiency improved yielding over 60 colonies per LB kanamycin plate (~360 colonies per transformation reaction).

When DNA blots of the transformed colonies were hybridised with the selected *E. cloacae ampD* probe, a strong hybridisation signal was evident with the wild-type *E. cloacae* and *C. freundii* control isolates. A hybridisation signal was also evident in all test transformants due to the presence of native *ampD* in the TOP10 *E. coli* transformed cells. Among the ~360 test transformants in each transformation reaction, at least one colony demonstrated a hybridisation signal with an intensity equivalent to the control isolates (Figure 6.7, panel A). The hybridisation signal in these putative *ampD* clones was readily distinguished from the background signal observed in the remainder of the transformants.

Sequence alignment demonstrated that the cloned DNA inserts in the suspect *ampD* clones from the two cefotaxime-resistant *S. marcescens* isolates shared 100% identity. Database search protocols revealed that the DNA fragment from the *S. marcescens* isolates demonstrated 86% similarity with the nucleotide sequence of the *ampD* gene from wild-type *E. cloacae* (Ent. 029, Genbank accession number U40785), 80% similarity with *ampD* in wild-type *C. freundii* (Cit OS60, Genbank accession number Z14002) and 75% similarity with *ampD* in wild-type *E. coli* (Genbank accession number X15398). Following sequence analysis of the putative *ampD* gene, the 1.2 kb suspect DNA fragment of one of the *S. marcescens* isolates was DIG-labeled for use as an *ampD* probe. Hybridisation of the original *Eco*R1-digested Southern blots with the *S. marcescens-ampD* specific probe produced a signal for the *E. cloacae*, *C. freundii* and pNU405 controls and for all *S. marcescens* isolates tested (Figure 6.7, panel B). The hybridisation band was located on a similar sized DNA fragment (~5 kb) identified using the *E. cloacae*-derived *ampD* probe.

6.3.1.4. PCR amplification to investigate the presence of *ampD* in *S. marcescens* using primers derived from *ampD* sequences in *S. marcescens* and *E. cloacae*

Using *ampD* primer Set 3, a PCR product of the expected size (250 bp) was amplified using DNA from the positive control isolates (*S. marcescens ampD*-clone and wild-type *E. cloacae*) and from all *S. marcescens* isolates tested, including *S. marcescens* NCTC

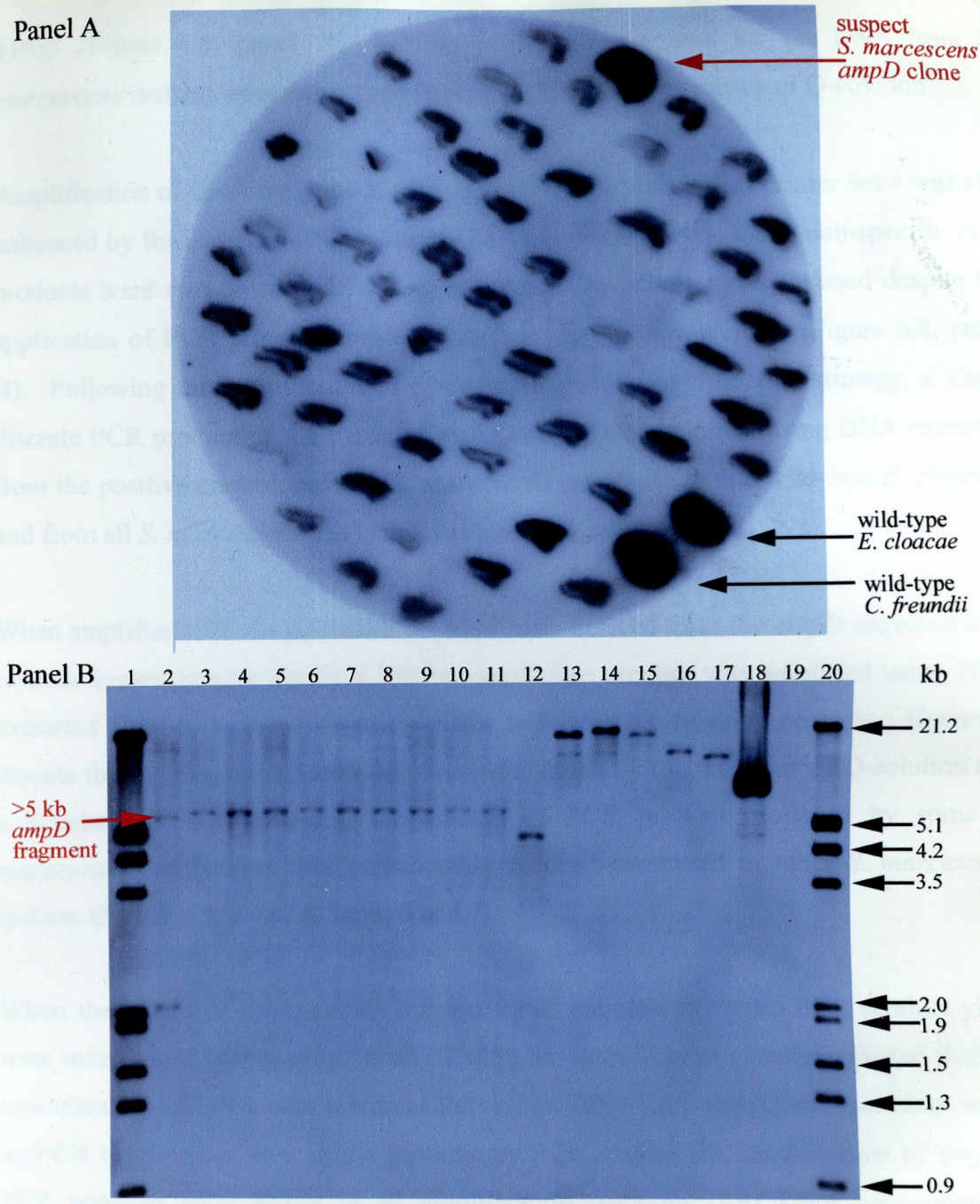


Figure 6.7. Panel A. Autoradiograph of *S. marcescens ampD*-fragment transformant colonies following colony blot hybridisation using a DIG-labeled 250 bp *ampD* probe derived from the *ampD*-PCR product of wild-type *E. cloacae*. The positive hybridisation signal from the putative *ampD* clone is readily distinguishable from the backround signal generated by the native *ampD* gene carried by all transformants.

Panel B. Autoradiograph of *Eco*R1-digested chromosomal DNA, following Southern transfer and hybridisation using a DIG-labeled *S. marcescens* 1.2 kb *ampD*-specific probe. DNA extracted from test and control isolates is located in lanes as follows: 2, *S. marcescens* NCTC 11935; 3–11, *S. marcescens* isolates from Collection D; 13 & 14, wild-type *E. cloacae* positive hybridisation controls; 15 & 16, wild-type *C. freundii* positive hybridisation controls; 17, wild-type *E. coli* positive hybridisation control. Lane 12 contains undigested plasmid DNA from *S. marcescens ampD* clone (positive hybridisation control). Lanes 18 & 19 contain undigested DNA from plasmid pNU405 (positive hybridisation control) and from plasmid pNU305 (negative hybridisation control), respectively. Lanes 1 & 20 contain DIG-labeled standard size reference markers.

11935 (Figure 6.8, panel A). The amplification reactions for the DNA from *S. marcescens* isolates were enhanced when performed in the presence of Q-solution.

Amplification of DNA from the *S. marcescens* isolates with *ampD* primer Set 4 was also enhanced by the addition of Q-solution. In this case, however, many non-specific PCR products were also amplified. These non-specific products were obtained despite the application of PCR protocols with elevated annealing temperatures (Figure 6.8, panel B). Following the introduction of a 'touchdown' PCR amplification strategy, a single discrete PCR product of the expected size (250 bp) was obtained using DNA extracted from the positive control isolates (*S. marcescens ampD*-clone and wild-type *E. cloacae*) and from all *S. marcescens* test isolates (Figure 6.8, panel C).

When amplified with the published set of primers derived from the *ampD* sequence in *E. cloacae* (*ampD* primer Set 5) a 708 bp *ampD*-like product was amplified using DNA extracted from *S. marcescens* test isolates and from the positive controls. However, despite the optimisation of the amplification reaction by the inclusion of Q-solution and a 'touchdown' PCR protocol, the amount of PCR product produced by some *S. marcescens* test isolates was considerably reduced compared to other *S. marcescens* isolates (Figure 6.9, panel A, lanes 6 and 7).

When the effects of the quantity and quality of template DNA on PCR product yield were investigated (using *ampD* primer Set 5), the amplification results indicated that the concentration of DNA was a critical factor. At DNA concentrations equivalent to 15 ng/PCR reaction (as used in the preliminary PCR studies for identification of *ampD*), PCR products were absent in all amplification reactions. At DNA concentrations equivalent to 150 ng/PCR reaction, weak DNA bands were obtained for two of the three DNA preparations. At 750 ng DNA/PCR reaction (twice the DNA concentration used in PCR studies for confirmation of the *ampD*), PCR products were obtained from all DNA samples (Figure 6.9, panel B). Amplification yield was also affected by the quality of template DNA, with non-specific PCR products occurring in amplification reactions performed with DNA extracted in the absence of proteinase K treatment (Figure 6.9, panel B, lanes 18 and 19). The age of template DNA (extracted up to three months prior to amplification) did not affect the amplification yield (following extraction with proteinase K) (Figure 6.9, panel B, lanes 4–7). In all cases, the presence

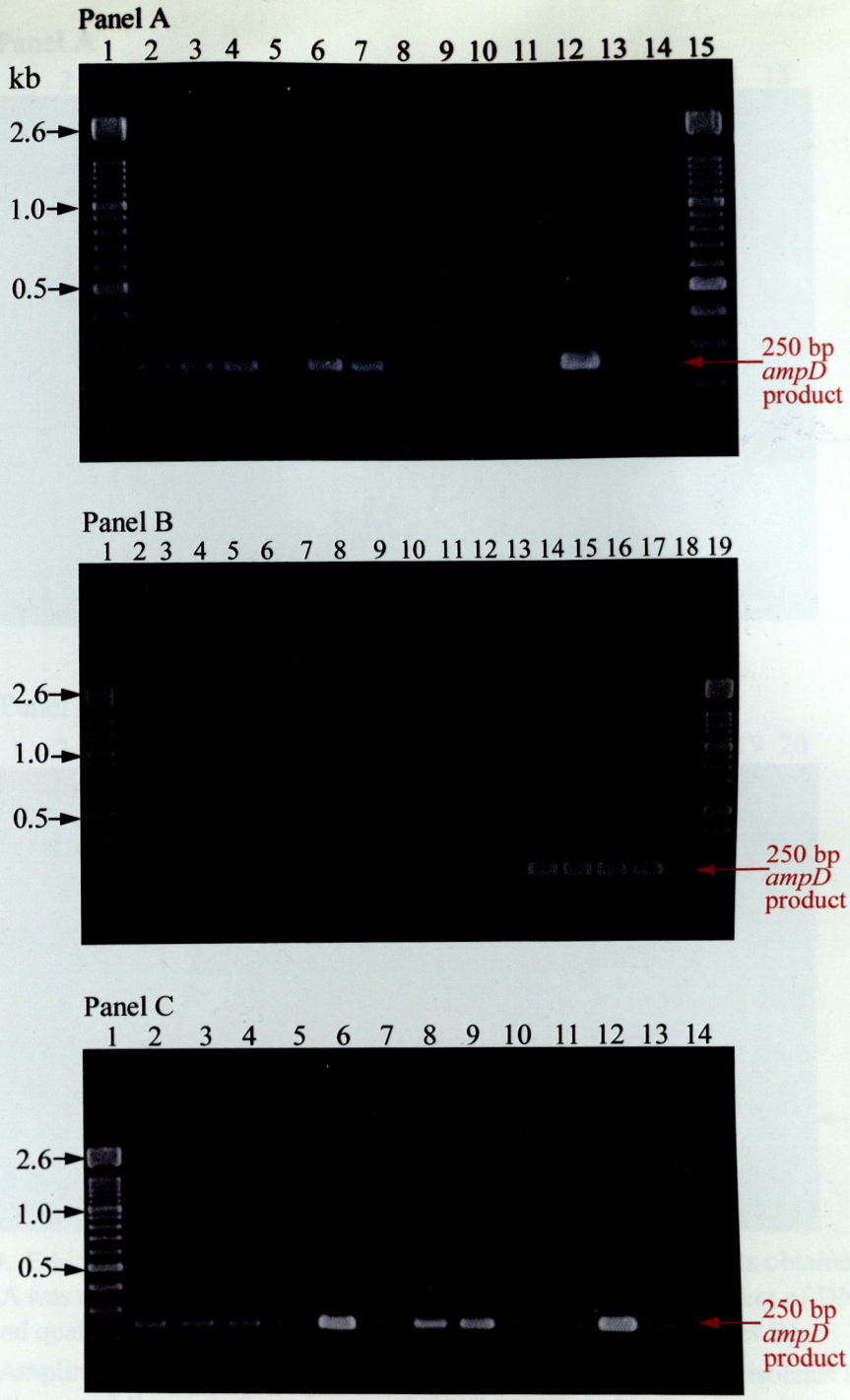


Figure 6.8. Ethidium bromide-stained agarose gels showing PCR products obtained when target DNA was amplified using *ampD* primer Set 3 (panel A) and *ampD* primer Set 4 (panel C), and temperature gradient curve obtained with *ampD* primer Set 4 (panel B).

Panels A, C. Amplimers generated with template DNA extracted from control and test isolates are located in lanes as follows: 2, *S. marcescens* NCTC 11935; 3–11, *S. marcescens* isolates from Collection D; 12, *S. marcescens ampD* clone; 13, wild-type *E. cloacae*. Lanes 1 & 15 contain 100 bp standard size reference markers (lane 15 in panel A only). Lane 14 contains the PCR-negative control (water).

Panel B. Lane contents are as follows: 2–5, 6–9 & 10–13, amplimers generated with template DNA from three *S. marcescens* isolates amplified at annealing temperatures of 57.9°C, 60.5°C, 61.5°C & 64.3°C, respectively; 14–17, amplimers generated with template DNA from *S. marcescens ampD* clone amplified at annealing temperatures of 57.9°C, 60.5°C, 61.5°C & 64.3°C; 1 & 19, 100 bp standard size reference markers; 18, PCR-negative control (water).

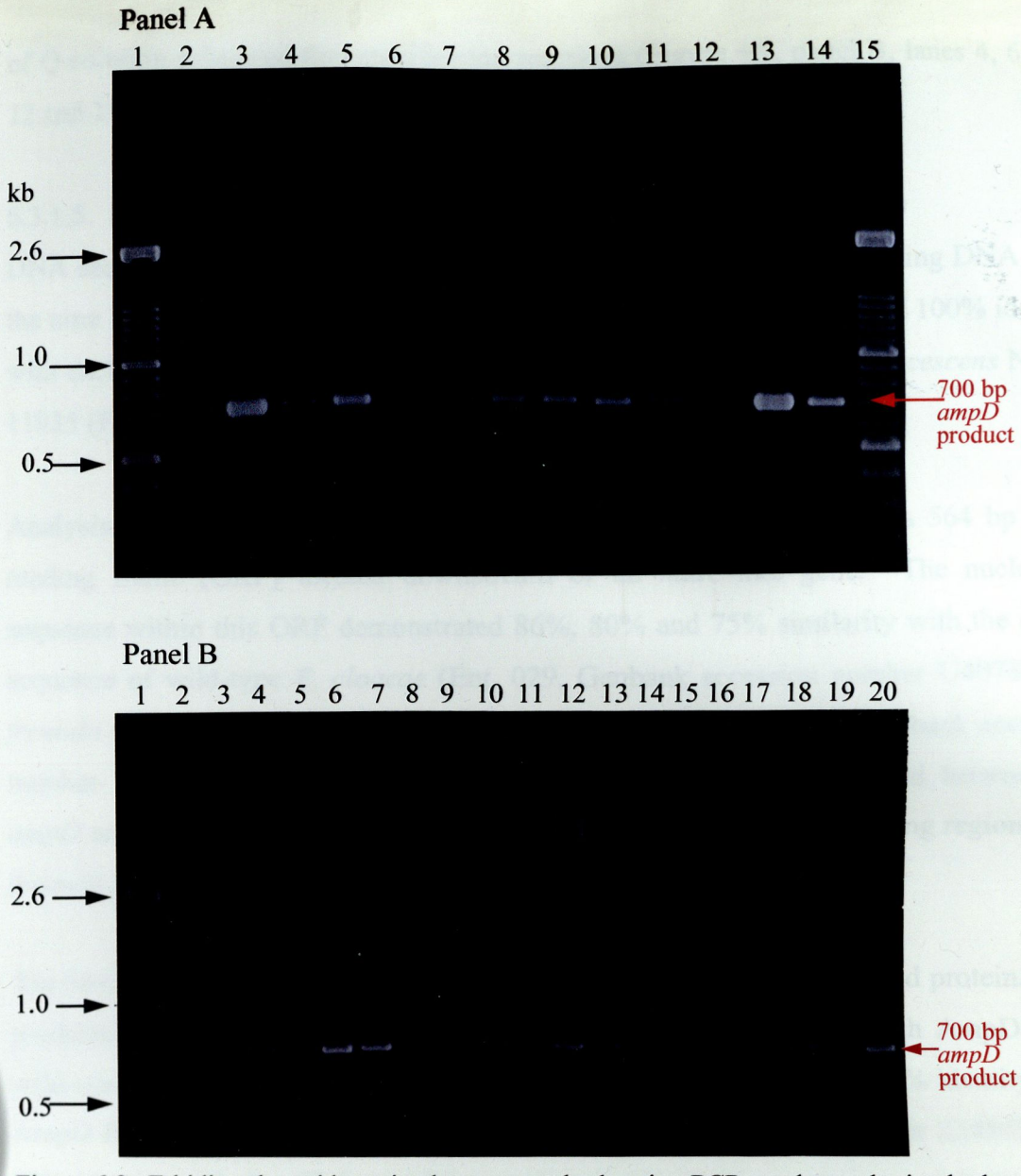


Figure 6.9. Ethidium bromide-stained agarose gels showing PCR products obtained when target DNA was amplified using *ampD* primer Set 5 (panel A) and the effect of DNA quantity and quality on PCR product yield using *ampD* primer Set 5 (panel B).

Panel A. Amplimers generated with template DNA from control and test isolates are located in lanes as follows: 2, *S. marcescens* NCTC 11935; 3–11, *S. marcescens* isolates from Collection D; 13, *S. marcescens ampD* clone; 14, wild-type *E. cloacae*. Lanes 1 & 15 contain 100 bp standard size reference markers. Lane 12 contains the PCR-negative control.

Panel B. Lane contents are as follows: 1, 100 bp standard size reference markers; 2–7, amplimers generated with template DNA from *S. marcescens* 97.R8, using 'old' template DNA (extracted three months prior to amplification with proteinase K) tested at concentrations of 15 ng, 150 ng and 750 ng/PCR reaction, in the presence and absence of Q-solution, respectively; 8–13, amplimers generated with template DNA from *S. marcescens* 97.R8, using 'fresh' template DNA (extracted with proteinase K) tested at concentrations of 15 ng, 150 ng and 750 ng/PCR reaction, in the presence and absence of Q-solution, respectively; 14–19, amplimers generated with template DNA from *S. marcescens* 97.R8, using 'fresh' template DNA (extracted without proteinase K) tested at concentrations of 15 ng, 150 ng and 750 ng/PCR reaction, in the presence and absence of Q-solution, respectively; 20, amplimers generated with template DNA from wild-type *E. cloacae*.

of Q-solution enhanced the amplification reactions (Figure 6.9, panel B, lanes 4, 6, 10, 12 and 18).

6.3.1.5. **Sequence analysis of the putative *ampD* gene in *S. marcescens***

DNA sequence alignment of the 708 bp *ampD* PCR products obtained using DNA from the nine *S. marcescens* isolates in Collection D, indicated that they shared 100% identity with each other and with the *ampD* PCR product from DNA from *S. marcescens* NCTC 11935 (Figure 6.10).

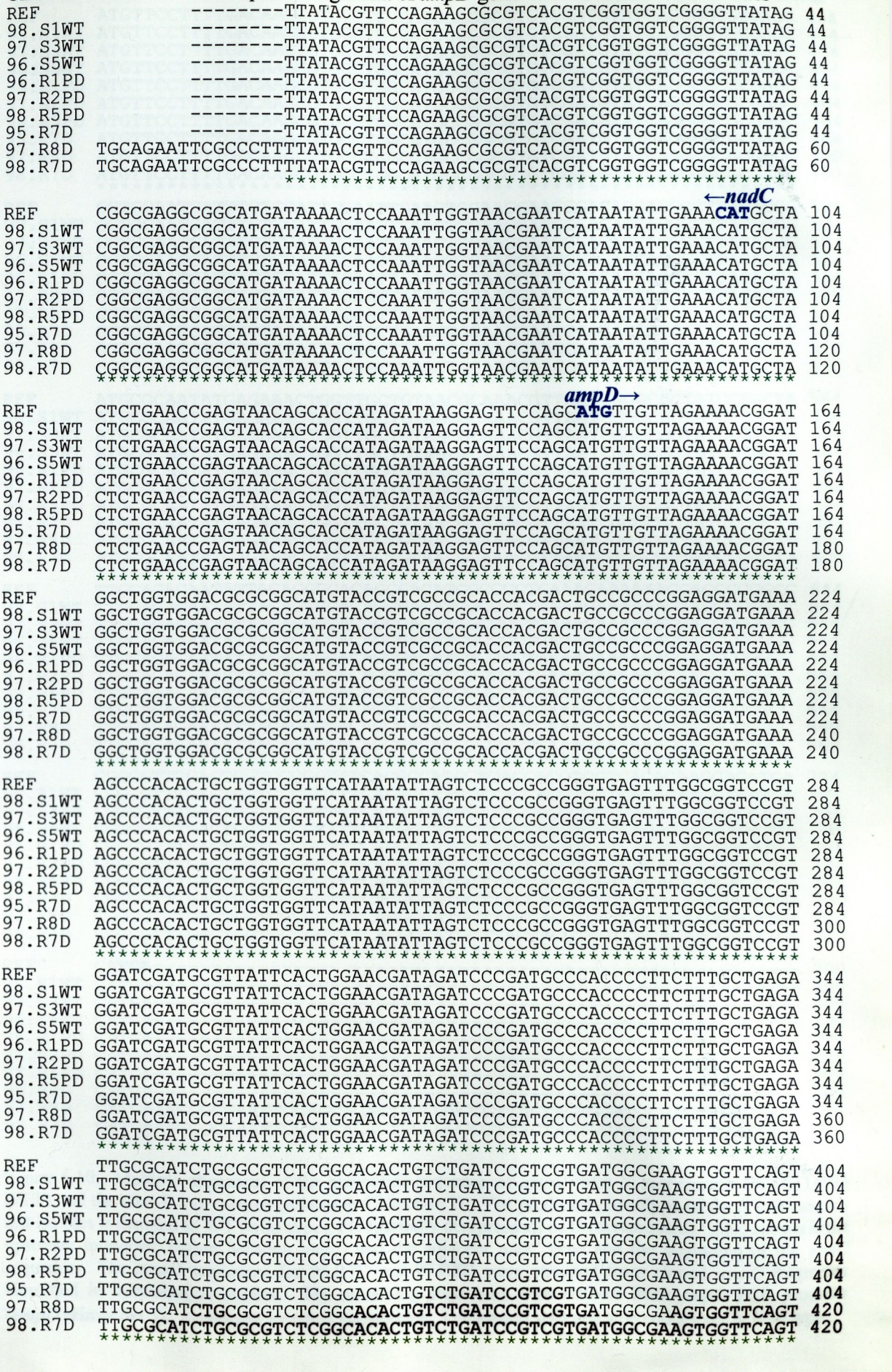
Analysis of the DNA sequence of the *ampD* PCR products revealed a 564 bp open reading frame (ORF) located downstream of an *nadC*-like gene. The nucleotide sequence within this ORF demonstrated 86%, 80% and 75% similarity with the *ampD* sequence of wild-type *E. cloacae* (Ent. 029, Genbank accession number U40785), *C. freundii* (Cit. OS60 Genbank accession number Z14002) and *E. coli* (Genbank accession number X15398), respectively. The 45 bp intergenic sequence located between the *ampD* and *nadC* genes demonstrated 100% identity with the corresponding region in *C. freundii* (Cit. OS60 Genbank accession number Z14002).

The 564 bp ORF from *S. marcescens* encoded a predicted 187 amino acid protein. The predicted *S. marcescens* AmpD protein demonstrated 90% identity with AmpD from wild-type *E. cloacae* (Ent. 029, Genbank accession number U40785), 82% identity with AmpD from wild-type *C. freundii* (Cit. OS60 Genbank accession number Z14002) and 78% identity with AmpD from wild-type *E. coli* (Genbank accession number X15398) (Figure 6.11, panels A and B). None of the published AmpD amino acid substitutions (e.g. aspartic acid to glutamic acid at codon 164 in *C. freundii* 31F11), or truncations (e.g. truncation at codon 133 in *E. cloacae ampD*029) associated with derepressed phenotypes in other enterobacteria were identified in the cefotaxime-resistant (derepressed) or cefotaxime low-level resistant (partially-derepressed) isolates of *S. marcescens* (Figure 6.11, panel B).

6.3.1.6. **Transformation with plasmid encoding wild-type *ampD* from *E. cloacae***

Transformation of derepressed clinical isolates of *S. marcescens* and *E. cloacae* with pNH5 plasmid DNA (encoding wild-type *ampD* from *E. cloacae*) yielded approximately 50 transformant colonies per LB kanamycin plate inoculated. Among the 10

CLUSTAL W nucleotide sequence alignment of *ampD* gene in test *S. marcescens* isolates



CLUSTAL W nucleotide sequence alignment of *ampD* gene in test *S. marcescens* isolates contd.

```
REF      ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 464
98.S1WT  ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 464
97.S3WT  ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 464
96.S5WT  ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 464
96.R1PD  ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 464
97.R2PD  ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 464
98.R5PD  ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 464
95.R7D   ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 464
97.R8D   ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 480
98.R7D   ATGTTCCTTTTGACAAGCGTGCCTGGCATGCCGGCGTGTCGATGTATCAGGGGCGCGAGC 480
         ************************************************************

REF      GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 524
98.S1WT  GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 524
97.S3WT  GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 524
96.S5WT  GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 524
96.R1PD  GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 524
97.R2PD  GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 524
98.R5PD  GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 524
95.R7D   GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 524
97.R8D   GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 540
98.R7D   GGTGCAATGATTTCTCCATTGGAATTGAACTGGAAGGAACGGACACCACGCCTTACACCG 540
         ************************************************************

REF      ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 584
98.S1WT  ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 584
97.S3WT  ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 584
96.S5WT  ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 584
96.R1PD  ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 584
97.R2PD  ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 584
98.R5PD  ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 584
95.R7D   ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 584
97.R8D   ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 600
98.R7D   ATGCGCAATATGAGAAACTGGTTGCTGTAACGCAAACGTTAATCGGGCGCTATCCCGCTA 600
         ************************************************************

REF      TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 644
98.S1WT  TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 644
97.S3WT  TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 644
96.S5WT  TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 644
96.R1PD  TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 644
97.R2PD  TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 644
98.R5PD  TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 644
95.R7D   TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 644
97.R8D   TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 660
98.R7D   TTGCAGACAATATTACAGGGCACAGCGACATCGCCCCTGAAAGAAAAAAACGACCCCGGCC 660
         ************************************************************

REF      CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 704
98.S1WT  CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 704
97.S3WT  CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 704
96.S5WT  CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 704
96.R1PD  CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 704
97.R2PD  CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 704
98.R5PD  CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 704
95.R7D   CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 704
97.R8D   CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 720
98.R7D   CGGCGTTTGACTGGTCCCGGTTTCACGCCATGCTTACCACGTCGTCACACAAGGAGATGA 720
         ************************************************************

REF      CATGA------------------------------------------------------- 709
98.S1WT  CATGA------------------------------------------------------- 709
97.S3WT  CATGA------------------------------------------------------- 709
96.S5WT  CATGA------------------------------------------------------- 709
96.R1PD  CATGA------------------------------------------------------- 709
97.R2PD  CATGA------------------------------------------------------- 709
98.R5PD  CATGA------------------------------------------------------- 709
95.R7D   CATGA------------------------------------------------------- 709
97.R8D   CATGAAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACC 780
98.R7D   CATGAAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACC 780
         *****
```

Figure 6.10. Multiple alignment of the nucleotide sequence of *ampD* from *S. marcescens* NCTC 11935 (REF) and the nine *S. marcescens* isolates in Collection D. The predicted phenotype of the *S. marcescens* test isolates is denoted as WT (wild-type), PD (partially derepressed) or D (derepressed). Also shown is the upstream intergenic region between *ampD* and the adjacent *nadC* gene and the beginning of the *nadC* sequence. The *ampD* sequences for 98.R7D and 97.R8D were obtained from cloned *ampD* fragments identified in hybridisation studies. The remaining sequences were derived from *ampD* PCR products (*ampD* primer Set 5). *, denotes identical nucleotides. Translation start and stop codons are in blue type.

Panel A. Amino acid sequence alignment of AmpD protein from *S. marcescens* and *E. cloacae*

```
98.R7D  MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA 60
Ent029  MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA 60
        ************************************************************

98.R7D  HPFFAEIAHLRVSAHCLIRRDGEVVQYVPFDKRAWHAGVSMYQGRERCNDFSIGIELEGT 120
Ent029  HPFFAEIAHLALSAHCLIRRDGEVVQYVPFDKRAWHAGVSMYHGRERCNDFSIGIELEGT 120
        ********** :******************************:*****************

98.R7D  DTTPYTDAQYEKLVAVTQTLIGRYPAIADNITGHSDIAPERKNDPGPAFDWSRFHAMLTT 180
Ent029  DTTPYTDSQYQQLAALTRTLIGLYPAIADNITGHSDIAPARKTDPGPAFDWPRFPAMLTA 180
        *******:**::*.*:*:**** **************** **.********.** ****:

98.R7D  SSHKEMT 187
Ent029  SSDKEIP 187
        **.**:.
```

Panel B. Amino acid sequence alignment of AmpD in enterobacteria

```
                                    CBN1Valine 33→Glycine
98.R7D  MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA 60
Ent029  MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA 60
CitOS60 MLLDEGWLAEARRVPSPHYDCRPDDENPSLLVVHNISLPPGEFGGPWIDALFTGTIDPNA 60
E.coli  MLLEQGWLVGARRVPSPHYDCRPDDETPTLLVVHNISLPPGEFGGPWIDALFTGTIDPQA 60
        ***::***. **:*****:****:**.*:*****************************:*
  ampD11 tryptophan→Glycine
                                    31A12 termination  ampD02 frameshift
98.R7D  HPFFAEIAHLRVSAHCLIRRDGEVVQYVPFDKRAWHAGVSMYQGRERCNDFSIGIELEGT 120
Ent029  HPFFAEIAHLALSAHCLIRRDGEVVQYVPFDKRAWHAGVSMYHGRERCNDFSIGIELEGT 120
CitOS60 HPYFAGIAHLRVSAHCLIRRDGEIVQYVPFDKRAWHAGVSSYQGRERCNDFSIGIELEGT 120
E.coli  HPFFAEIAHLRVSAHCLIRRDGEIVQYVPFDKRAWHAGVSQYQGRERCNDFSIGIELEGT 120
        **:** **** :***********:**************** *:*****************
          ampD029 truncation                31F11 Aspartic→Glutamic  ampD04termination
98.R7D  DTTPYTDAQYEKLVAVTQTLIGRYPAIADNITGHSDIAPERKNDPGPAFDWSRFHAMLTT 180
Ent029  DTTPYTDSQYQQLAALTRTLIGLYPAIADNITGHSDIAPARKTDPGPAFDWPRFPAMLTA 180
CitOS60 DTLAYTDAQYQQLAAVTNALITRYPAIANNMTGHCNIAPERKTDPGPSFDWARFRALVTP 180
E.coli  DTLAYTDAQYQQLAAVTRALIDCYPDIAKNMTGHCDIAPDRKTDPGPAFDWARFRVLVS- 180
        ** .***:**::*.*:*.:**  ** **.*:***.:*** **.****:***.** .:::
      ampD2 ISI
98.R7D  SSHKEMT 187
Ent029  SSDKEIP 187
CitOS60 SSHKEMT 187
E.coli  ---KETT 187
        **:**:
```

Figure 6.11. Panel A. Alignment of the AmpD amino acid sequence from a representative *S. marcescens* isolate from Collection D (98.R7D) with a wild-type isolate of *E. cloacae* (Ent 029 Genbank accession number U40785). The predicted phenotype of the *S. marcescens* test isolate is denoted as D (derepressed). Amino acid differences are highlighted in yellow.

Panel B. Alignment of the AmpD amino acid sequence from a representative *S. marcescens* isolate from Collection D (98.R7D) with wild-type isolates of *E. cloacae* (Ent. 029, Genbank accession number U40785), *C. freundii* (Cit OS60, Genbank accession number Z14002) and *E. coli* (Genbank accession number X15398). The predicted phenotype of the *S. marcescens* test isolate is denoted as D (derepressed). The published *ampD* mutations which correlate with derepressed phenotypes in *E. cloacae* (▌), *C. freundii* (▌) and *E. coli* (▌) are highlighted.

*, denotes identical amino acids; ':' denotes conserved substitutions and '.' denotes semi-conserved substitutions.

transformants selected from each transformation reaction, approximately five colonies demonstrated evidence of positive transformation. In *S. marcescens,* transformation with wild-type *ampD* produced a marginal increase in β-lactam susceptibility, with MIC values for cefotaxime and ceftazidime decreasing from 64 to 16 mg/l and from 2 to 1 mg/l, respectively, when the original clinical isolate was compared with *ampD* transformants. In the derepressed isolate of *E. cloacae*, transformation with wild-type *ampD* was associated with full restoration of β-lactam susceptibility with MIC values for cefotaxime and ceftazidime decreasing from 128 mg/l in the original host to 0.125 mg/l in *ampD* transformants. Plasmid DNA analysis of the clinical isolates and their respective transformants confirmed the acquisition of a plasmid in the size range typical of pNH5 (9.0 kb).

6.3.2. Identification and characterisation of *ampR* in *S. marcescens*

6.3.2.1. PCR amplification for identification of *ampR* using primers derived from *ampR* sequences in *E. cloacae* and *C. freundii*

DNA template control amplification reaction

The *ampC* DNA amplification reactions (*ampC* primer Set 1) performed as a control for the quality of template DNA, yielded an 860 bp amplimer using DNA from all *S. marcescens* test isolates (Figure 6.12, panel A, lanes 2–7). DNA from wild-type *E. cloacae*, *C. freundii* and *E. coli* control isolates failed to yield PCR products when amplified with the *S. marcescens-ampC* specific primer pair.

DNA amplification for ampR

Using *ampR* primer Set 1 and *ampR* primer Set 2, a PCR product of the expected size (~580 bp) was amplified using DNA from wild-type *E. cloacae* and *C. freundii* control isolates and from the *ampR*-encoding pNU305 plasmid (Figure 6.12, panel B, lanes 8–10). All DNA from *S. marcescens* isolates from Collection B failed to produce an *ampR*-specific PCR product using these primer sets. For both sets of primers, *ampR*-specific PCR products were absent in the negative controls. The *ampR*-PCR products obtained using DNA from plasmid pNU305 and from wild-type *E. cloacae* and *C. freundii* control isolates (amplified with *ampR* primer Set 2) were used to generate *ampR*-specific probes for hybridisation studies.

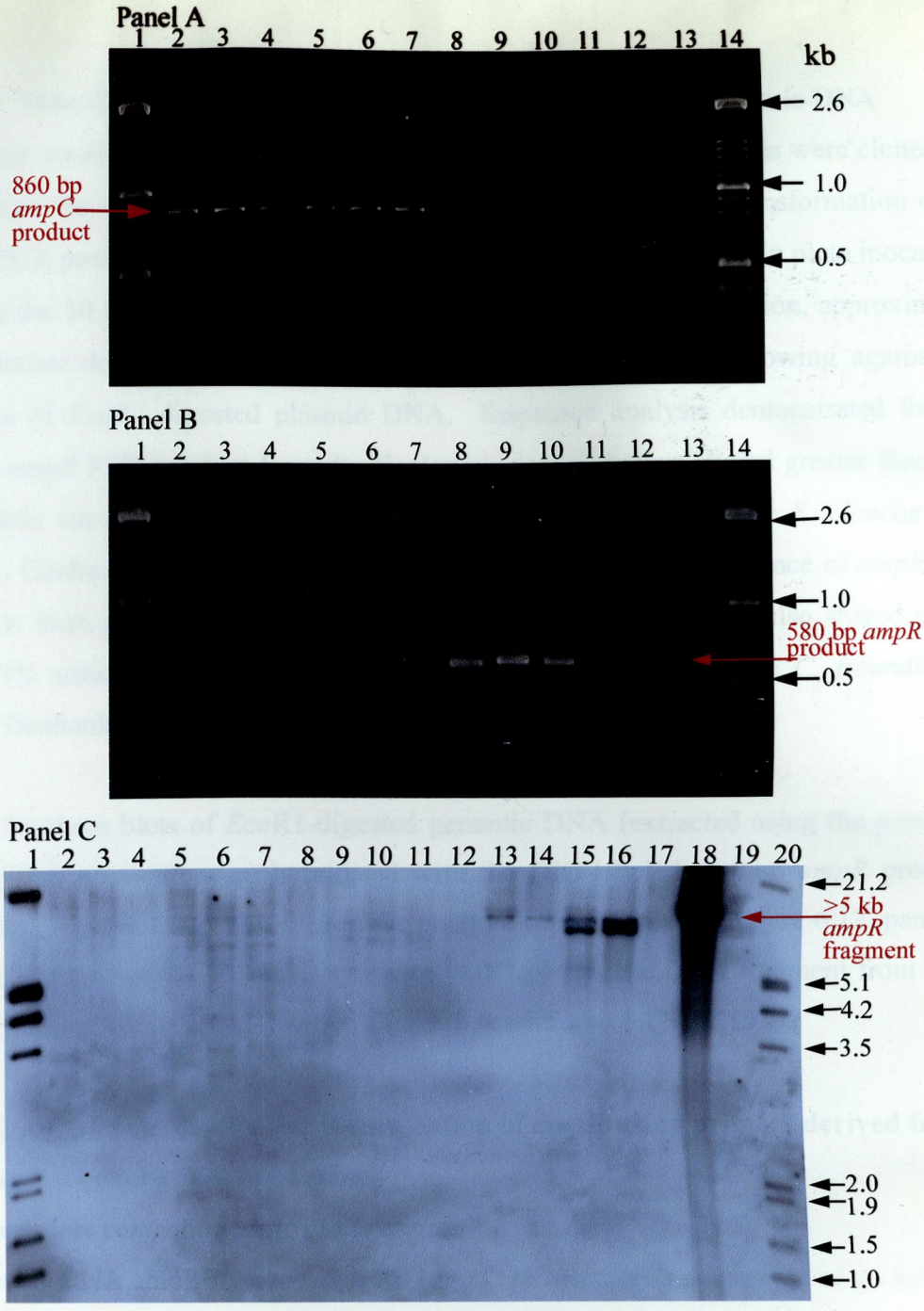


Figure 6.12. Panels A, B. Ethidium bromide-stained agarose gels showing PCR products obtained when target DNA was amplified using *ampC* primer Set 1 (panel A) and *ampR* primer Set 1 (panel B). Amplimers generated with template DNA extracted from control and test isolates are located in lanes as follows: 2–7, *S. marcescens* from Collection C; 8, wild-type *E. cloacae* (*ampR*+); 9, wild-type *C. freundii* (*ampR*+); 11, wild-type *E. coli* (*ampR*-). Amplimers generated with plasmid template DNA from *E. coli* isolates carrying plasmid pNU305 (*ampR*+) and plasmid pNU405 (*ampR*-) are located in lanes 10 and 12, respectively. Lanes 1 & 14 contain 100 bp standard size reference markers. Lane 13 contains the PCR-negative control (water).
Panel C. Autoradiograph of *Eco*R1-digested chromosomal DNA following Southern transfer and hybridisation using a DIG-labeled 564 bp *ampR* probe derived from the *ampR*-PCR product of wild-type *C. freundii.* DNA extracted from test and control isolates is located in lanes as follows: 2, *S. marcescens* NCTC 11935; 3–12, *S. marcescens* isolates from Collection C; 13 & 14, wild-type *E. cloacae*; 15 & 16, wild-type *C. freundii*; 17, wild-type *E. coli.* Lanes 18 & 19 contain undigested DNA from plasmid pNU305 (positive hybridisation control) and from plasmid pNU405 (negative hybridisation control), respectively. **Lanes 1 & 20 contain DIG-labeled standard reference size markers.**

6.3.2.2. **Identification of *ampR* by Southern hybridisation of genomic DNA**

The three *ampR* PCR products selected for use in hybridisation studies were cloned and sequenced for confirmation of the presence of the *ampR* gene. Transformation of the *ampR* PCR products yielded over 200 transformants per LB kanamycin plate inoculated. Among the 10 transformants selected from each transformation reaction, approximately six colonies demonstrated the expected *ampR* insert (560 bp) following agarose gel analysis of *Eco*R1-digested plasmid DNA. Sequence analysis demonstrated that the cloned *ampR* PCR product from the *E. cloacae* control isolate shared greater than 99% nucleotide similarity with the published *ampR* gene from wild-type *E. cloacae* (Ent. MHN1, Genbank accession number X04730). The nucleotide sequence of *ampR* PCR products from plasmid pNU305 and the *C. freundii* control isolate also shared greater than 99% similarity with the published *ampR* gene from wild-type *C. freundii* (Cit. OS60, Genbank accession number M27222).

When Southern blots of *Eco*R1-digested genomic DNA (extracted using the proteinase K modified protocol) were hybridised with the DIG-labeled 564 bp *ampR* probes, a hybridisation signal was observed in the positive control isolates (Figure 6.12, panel C). The three *ampR* probes failed to hybridise with any specific DNA fragment from the *S. marcescens* isolates from Collection C and *S. marcescens* NCTC 11935.

6.3.2.3. **PCR amplification for identification of *ampR* using primers derived from the *ampR* sequence in *S. marcescens***

DNA template control amplification reaction

The *ampC* DNA amplification reactions (*ampC* primer Set 2) performed as a control for the quality of template DNA, yielded an amplimer of the expected size (1.1 kb) using DNA from all *S. marcescens* isolates (Figure 6.13, panel A). An *ampC* PCR product was also obtained using DNA from the wild-type *S. marcescens* reference isolate, Serr1, provided by MCRAB, Creighton University.

DNA amplification for ampR

When amplified with *ampR* primer Set 3, an *ampR*-like PCR product (650 bp) was obtained using DNA from all *S. marcescens* isolates tested (Figure 6.13, panel B). PCR products were absent in the negative control reactions.

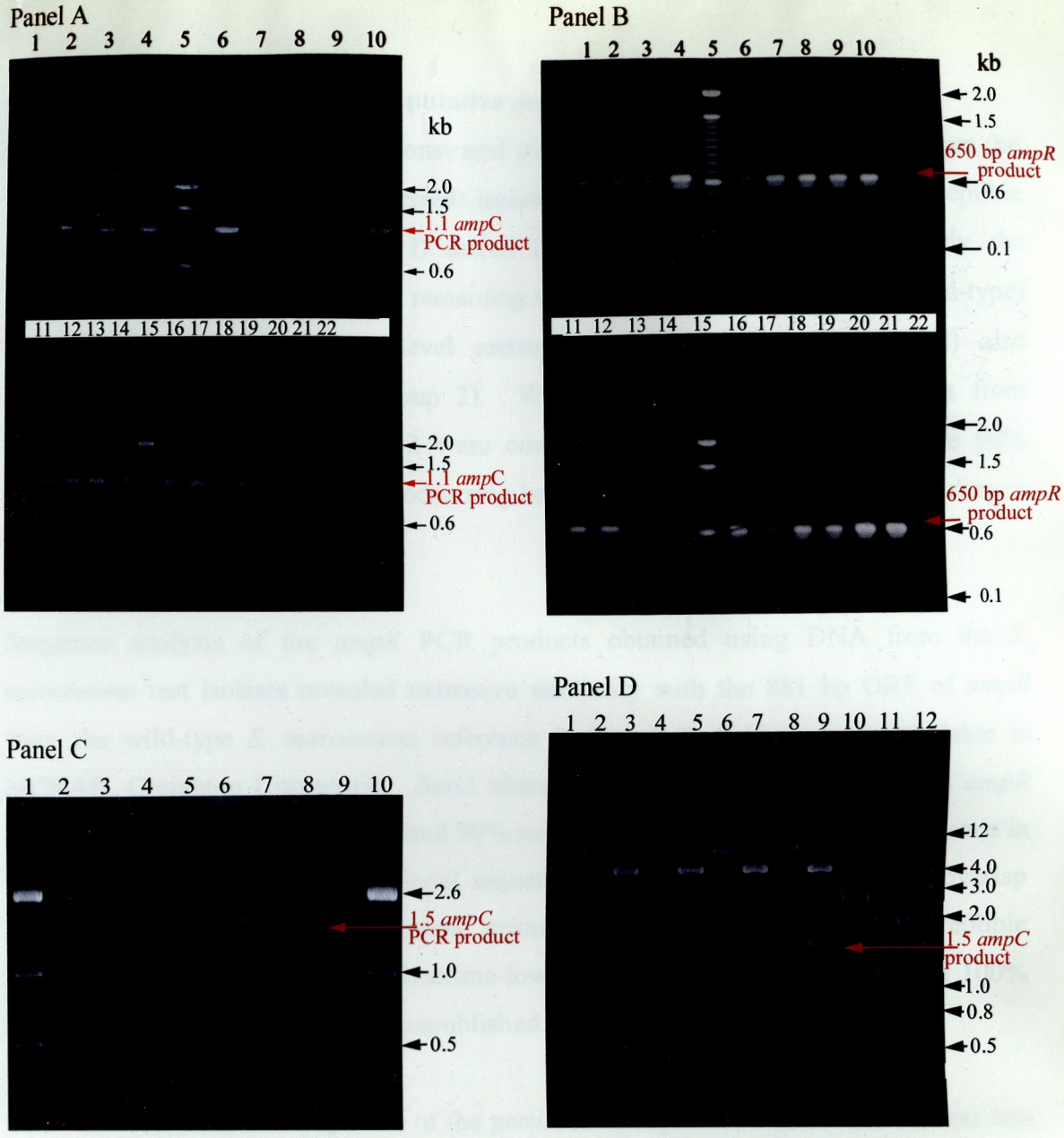


Figure 6.13. Ethidium bromide-stained agarose gels showing PCR products obtained when target DNA was amplified with *ampC* primer Set 2 (panel A), *ampR* primer Set 3 (panel B) and *ampC* primer Set 3 (panel C), and plasmid DNA extracted from TOPO TA clones of *ampC* PCR products for DNA sequence analysis (panel D).

Panels A, B. Amplimers generated with template DNA from test and control isolates are located in lanes as follows: 1–4, 6–14 & 16–20, *S. marcescens* isolates from Collection C; 21, wild-type *S. marcescens*, Serr 1. Lanes 5 & 15 contain 100 bp standard size reference markers. Lane 22 contains the PCR-negative control (water).

Panel C. Amplimers generated with template DNA from test and control isolates are located in lanes as follows: 2, *S. marcescens* NCTC 11935; 3–7, *S. marcescens* isolates from Collection D; 8, wild-type *E. cloacae*. Lanes 1 & 10 contain 100 bp standard reference markers. Lane 9 contains the PCR-negative control (water).

Panel D. Undigested and *Eco*R1-digested plasmid DNA extracted from clones of *ampC* PCR products (generated with template DNA from four *S. marcescens* isolates in Collection D) is located in lanes 2 & 3, 4 & 5, 6 & 7 and 8 & 9, respectively. Lanes 1 & 12 contain 1 kb standard size reference markers. Lanes 10 & 11 contain 100 bp standard size reference markers and a low DNA mass ladder, respectively.

6.3.2.4. **Sequence analysis of the putative *ampR* gene in *S. marcescens***

Nucleotide alignment studies demonstrated that the *ampR*-like PCR products from the three cefotaxime-resistant (derepressed) isolates and two of the cefotaxime-susceptible isolates (wild-type) in Collection D shared 100% identity (Group 1). Similarly, the *ampR*-like PCR products from the remaining cefotaxime-susceptible isolate (wild-type) and the three cefotaxime low-level resistant isolates (partially-derepressed) also demonstrated 100% identity (Group 2). When the *ampR*-like PCR products from isolates in Group 1 and Group 2 were compared, they were found to share 98% similarity, with six bp differences occurring between the 617 bp of the *ampR* amplimers (Figure 6.14).

Sequence analysis of the *ampR* PCR products obtained using DNA from the *S. marcescens* test isolates revealed extensive similarity with the 881 bp ORF of *ampR* from the wild-type *S. marcescens* reference isolate, Serr 1 (data made available in MCRAB, Creighton University). Serr1 shared 98% similarity with the partial *ampR* sequence from isolates in Group 1 and 99% similarity with the partial *ampR* sequence in isolates from Group 2. The additional sequence data obtained from the *ampR* overlap region of the *ampC* PCR product (*ampC* primer Set 3) from one cefotaxime-susceptible *S. marcescens* isolate and one cefotaxime-low-level resistant isolate also shared 100% identity with the first 69 bp of the unpublished *ampR* gene (Figure 6.14).

The deduced amino acid sequence of the partial AmpR protein from *S. marcescens* test isolates in Group 1 and Group 2 shared 99% identity, with one conserved amino acid difference occurring at position 198 (arginine in Group 1 isolates, histidine in Group 2 isolates). Alignment of the predicted partial AmpR amino acid sequence from the *S. marcescens* test isolates demonstrated 93% identity with AmpR from wild-type Serr1 and 97% identity with the AmpR sequence from an unpublished wild-type *S. marcescens* isolate deposited in the Genbank (Genbank accession number AJ271368) (Figure 6.15, panel A). The amino acid diversity observed between the AmpR sequence in isolates from Group 1 and Group 2 was also evident in the wild-type reference isolates with histidine occurring at position 198 in Serr1 and arginine occurring at the same position in the unpublished *S. marcescens* isolate. The AmpR sequence in the *S. marcescens* isolates failed to demonstrate evidence of the *ampR* mutations previously

CLUSTAL W (1.81) nucleotide sequence alignment of *ampR* in *S. marcescens* isolates

```
Serr1    ATGCGCAATCGACTTCCCCTCAACGCCCTGCGGGCGTTCGAATCCTCCGCTCGCCACCTG 60
98.S1WT  ------------------------------------------------------------
97.S3WT  ------------------------------------------------------------
96.S5WT  ATGCGCAATCGACTTCCCCTCAACGCCCTGCGGGCGTTCGAATCCTCCGCTCGCCACCTG 60
96.R1PD  ------------------------------------------------------------
97.R2PD  ATGCGCAATCGACTTCCCCTCAACGCCCTGCGGGCGTTCGAATCCTCCGCTCGCCACCTG 60
98.R5PD  ------------------------------------------------------------
95.R7D   ------------------------------------------------------------
97.R8D   ------------------------------------------------------------
98.R7D   ------------------------------------------------------------

Serr1    AACTTCACCCGCGCCGGTCTGGAGCTGCGCGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 120
98.S1WT  ---------CGCGCCGGTCTGGAGCTGCGCGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 51
97.S3WT  ---------CGCGCCGGTCTGGAGCTGCGTGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 51
96.S5WT  AACTTCACCCGCGCCGGTCTGGAGCTGCGTGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 120
96.R1PD  ---------CGCGCCGGTCTGGAGCTGCGCGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 51
97.R2PD  AACTTCACCCGCGCCGGTCTGGAGCTGCGCGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 120
98.R5PD  ---------CGCGCCGGTCTGGAGCTGCGCGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 51
95.R7D   ---------CGCGCCGGTCTGGAGCTGCGTGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 51
97.R8D   ---------CGCGCCGGTCTGGAGCTGCGTGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 51
98.R7D   ---------CGCGCCGGTCTGGAGCTGCGTGTCACCCAGGCCGCCGTCAGCCAGCAGGTG 51
                  ********** ********** ********** ********** **********

Serr1    CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 180
98.S1WT  CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 111
97.S3WT  CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 111
96.S5WT  CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 180
96.R1PD  CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 111
97.R2PD  CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 180
98.R5PD  CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 111
95.R7D   CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 111
97.R8D   CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 111
98.R7D   CGCATGCTGGAAGAACAGCTGGGCATTCAGCTGTTCCGCCGCCTGCCGCGCGGGCTGGAT 111
         ************************************************************

Serr1    CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAA 240
98.S1WT  CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAA 171
97.S3WT  CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAG 171
96.S5WT  CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAG 240
96.R1PD  CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAA 171
97.R2PD  CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAA 240
98.R5PD  CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAA 171
95.R7D   CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAG 171
97.R8D   CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAG 171
98.R7D   CTGACCGAAGAGGGCCAGGCGCTGCTGCCGGTATTGAGCGACGCCTTCGACCGCATCGAG 171
         ************************************************************

Serr1    GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 300
98.S1WT  GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 231
97.S3WT  GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 231
96.S5WT  GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 300
96.R1PD  GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 231
97.R2PD  GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 300
98.R5PD  GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 231
95.R7D   GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 231
97.R8D   GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 231
98.R7D   GCGGTGCTGCAGCAGTTCGAAGGCGGGCATTTCCACGAGGTGCTGACGGTGGCGGTGGTC 231
         ************************************************************

Serr1    GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 360
98.S1WT  GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 291
97.S3WT  GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 291
96.S5WT  GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 360
96.R1PD  GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 291
97.R2PD  GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 360
98.R5PD  GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 291
95.R7D   GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 291
97.R8D   GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 291
98.R7D   GGCACCTTCGCCGTCGGCTGGCTGATGCCGCGGCTGGCGGCATTCCGCACGGCGCACCCG 291
         ************************************************************
```

CLUSTAL W (1.81) nucleotide sequence alignment of *ampR* in *S. marcescens* isolates

```
Serr1    TTTATCGATCTGCGGGTGCTCACCCACAACAACCCTGTGTCAATTCTGTCGGCCGACGGC 420
98.S1WT  TTTATCGATCTGCGGGTGCTCACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 348
97.S3WT  TTTATCGATCTACGGGTGCTGACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 348
96.S5WT  TTTATCGATCTACGGGTGCTGACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 417
96.R1PD  TTTATCGATCTGCGGGTGCTCACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 348
97.R2PD  TTTATCGATCTGCGGGTGCTCACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 417
98.R5PD  TTTATCGATCTGCGGGTGCTCACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 348
95.R7D   TTTATCGATCTACGGGTGCTGACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 348
97.R8D   TTTATCGATCTACGGGTGCTGACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 348
98.R7D   TTTATCGATCTACGGGTGCTGACCCACAACAACCTGGT--CAAT-CTGTCGGCCGACGGC 348
         ********** ******** ************  **  **** ***************

Serr1    ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCTCCCTGCAATATCAAGCT 480
98.S1WT  ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 407
97.S3WT  ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 407
96.S5WT  ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 476
96.R1PD  ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 407
97.R2PD  ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 476
98.R5PD  ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 407
95.R7D   ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 407
97.R8D   ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 407
98.R7D   ATGGATTTCGCCATCCGCTTCGGCGAAGGCCTCTGGCCCGCCACC-TGCAATATCAAGCT 407
         ************************************** ** ***************

Serr1    GTTCCGACGCGCCCGCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 540
98.S1WT  GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 465
97.S3WT  GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 465
96.S5WT  GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 534
96.R1PD  GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 465
97.R2PD  GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 534
98.R5PD  GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 465
95.R7D   GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 465
97.R8D   GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 465
98.R7D   GTTC-GACGCGCC-GCTGACGGTGCTCTGCCCGCCGGCGGTCGCCGCGCGCCTGCGCACG 465
         **** ******** ************************************************

Serr1    CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAGCAC 600
98.S1WT  CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAGCAC 525
97.S3WT  CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAACGC 525
96.S5WT  CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAACGC 594
96.R1PD  CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAGCAC 525
97.R2PD  CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAGCAC 594
98.R5PD  CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAGCAC 525
95.R7D   CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAACGC 525
97.R8D   CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAACGC 525
98.R7D   CCGCAGGATCTGCAGCACGAGCAGCTGATGCGCACCTACCGCAAGGACGAGTGGGAACGC 525
         ************************************************** * *

Serr1    TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 660
98.S1WT  TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 585
97.S3WT  TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 585
96.S5WT  TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 654
96.R1PD  TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 585
97.R2PD  TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 654
98.R5PD  TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 585
95.R7D   TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 585
97.R8D   TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 585
98.R7D   TGGTTCGCGGCGGCACAGGTGACGCCGTGGCGGATCAACGGCCCGGTGTTCGACTCGTCA 585
         ************************************************************

Serr1    GGCATGATGGTGGAAGGCGCGATCCAGTGCGATGGCGTGGCGCTGGCGCCGGTCAGCATG 720
98.S1WT  AGGCTGATGGTGGAAGGCGCGATCCAGTGCGA---------------------------- 617
97.S3WT  AGGCTGATGGTGGAAGGCGCGATCCAGTGCGA---------------------------- 604
96.S5WT  AGGCTGATGGTGGAAGGCGCGATCCAGTGCGA---------------------------- 670
96.R1PD  AGGCTGATGGTGGAAGGCGCGATCCAGTGCGA---------------------------- 593
97.R2PD  AGGCTGATGGTGGAAGGCGCGATCCAGTGCGA---------------------------- 686
98.R5PD  AGGCTGATGGTGGAAGGCGCGATCCAGTGCGA---------------------------- 617
95.R7D   AGGCTGATGGTGGAAGGCGCCATCCAGTGCGA---------------------------- 617
97.R8D   AGGCTGATGGTGGAAGGCGCGATCCAGTGCGA---------------------------- 617
98.R7D   AGGCTGATGGTGGAAGGCGCGATCCAGTGCGA---------------------------- 617
          *  ****************************
```

Figure 6.14. Multiple alignment of the nucleotide sequence of *ampR* from Serr1 (data made available in MCRAB, Creighton University) with the partial *ampR* sequence from the nine *S. marcescens* isolates in Collection D. The predicted phenotype of the *S. marcescens* test isolates is denoted as WT (wild-type), PD (partially derepressed) or D (derepressed). The full *ampR* sequence is encoded on an 881 bp open reading frame. The sequence data for the first 69 bases of *ampR*, obtained from the *ampR* overlap region of the *ampC* PCR product (*ampC* primer set 3, Section 6.2.3.3), for *S. marcescens* isolates 96.S5WT and 97.R2PD are included in the alignment. Nucleotide differences are highlighted in yellow. *, denotes identical nucleotides. The translational start codon is shown in blue type.

Panel A. CLUSTAL W amino acid sequence alignment of AmpR in *S. marcescens* isolates

```
SmGp1    MRNRLPLNALRAFESSARHLNFTRAGLELRVTQAAVSQQVRMLEEQLGIQLFRRLPRGLD 60
SmGp2    MRNRLPLNALRAFESSARHLNFTRAGLELRVTQAAVSQQVRMLEEQLGIQLFRRLPRGLD 60
Serr1    MRNRLPLNALRAFESSARHLNFTRAGLELRVTQAAVSQQVRMLEEQLGIQLFRRLPRGLD 60
Smunp    MRNRLPLNALRAFESSARHLNFTRAGLELRVTQAAISQQVRMLEEQLGIQLFRRLPRGLD 60
         ************************************:*********************

SmGp1    LTEEGQALLPVLSDAFDRIEAVLQQFEGGHFHEVLTVAVVGTFAVGWLMPRLAAFRTAHP 120
SmGp2    LTEEGQALLPVLSDAFDRIEAVLQQFEGGHFHEVLTVAVVGTFAVGWLMPRLAAFRTAHP 120
Serr1    LTEEGQALLPVLSDAFDRIEAVLQQFEGGHFHEVLTVAVVGTFAVGWLMPRLAAFRTAHP 120
Smunp    LTEEGQALLPVLSDAFDSIEAVLQQFEGGHFHEVLTVAVVGTFAVGWLMPRLAAFRTAHP 120
         ************************************************************

SmGp1    FIDLRVLTHNN-LVNLSADGMDFAIRFGEGLWPATCNIKLFDAP-LTVLCPPAVAARLRT 178
SmGp2    FIDLRVLTHNN-LVNLSADGMDFAIRFGEGLWPATCNIKLFDAP-LTVLCPPAVAARLRT 178
Serr1    FIDLRVLTHNNPVSILSADGMDFAIRFGEGLWPASLQYQAVPTRPLTVLCPPAVAARLRT 180
Smunp    FIDLRVLTHNN-LVNLSADGMDFAIRFGEGLWPATCNIKLFDAP-LTVLCPPTVAARLST 178
         ********** :  ******************: : : .  : *******:*****  *

SmGp1    PQDLQHEQLMRTYRKDEWERWFAAAQVTPWRINGPVFDSSRLMVEGAIQC---------- 228
SmGp2    PQDLQHEQLMRTYRKDEWEHWFAAAQVTPWRINGPVFDSSRLMVEGAIQC---------- 228
Serr1    PQDLQHEQLMRTYRKDEWEHWFAAAQVTPWRINGPVFDSSGMMVEGAIQCDGVALAPVSM 240
Smunp    PQDLQHEQLMRTYRKDEWERWFTAAQVTPWRINGPVFDSSRLMVEGAIQCDGVALAPVSM 238
         ******************:**:***************** :********
```

Panel B. Amino acid sequence alignment of AmpR in AmpC-inducible enterobacteria

```
                                  H       T       H
98.R7D   MRNRLPLNALRAFESSARHLNFTRAGLELRVTQAAVSQQVRMLEEQLGIQLFRRLPRGLD 60
Smunp    MRNRLPLNALRAFESSARHLNFTRAGLELRVTQAAISQQVRMLEEQLGIQLFRRLPRGLD 60
Serr1    MRNRLPLNALRAFESSARHLNFTRAGLELRVTQAAVSQQVRMLEEQLGIQLFRRLPRGLD 60
Haf.al   MRSHLPLNALRAFEASARHLSFTRAGLELSVTQAAVSQQVRMLEAKLGTQLFKRLPRGLE 60
Prov.s   YRHRLPLNALRAFEASARHLSFTRAGLELNVTQAAVSQQVRLLEEQLGLELFIRLPRGLA 60
Morg.m   VRRYLPLNPLRAFEAAARHLSFTRAAIELNVTHAAVSQQVRALEEQLGCVLFTRVSRGLV 60
Ent.cl   TRSYLPLNSLRAFEAAARHLSFTHAAIELNVTHSAISQHVKTLEQHLNCQLFVRVSRGLM 60
Cit.fr   TRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKSLEQQLNCQLFVRGSRGLM 60
          *  :***.*****::****.**:*.:** **:::*:**:*: ** :*.  ** * .***

                                  serine→phenylalanine
98.R7D   LTEEGQALLPVLSDAFDRIEAVLQQFEGGHFHEVLTVAVVGTFAVGWLMPRLAAFRTAHP 120
Smunp    LTEEGQALLPVLSDAFDSIEAVLQQFEGGHFHEVLTVAVVGTFAVGWLMPRLAAFRTAHP 120
Serr1    LTEEGQALLPVLSDAFDRIEAVLQQFEGGHFHEVLTVAVVGTFAVGWLMPRLAAFRTAHP 120
Haf.al   LTDEAHVLLPVLSDAFSQIETVLKQFEGGHFHEVLTVAAVGTFAVGWLMPRLQSFNTEHP 120
Prov.s   LTDEGLALLPVLSRSFDQIESLLQQFEDGHYHEVLSVSVVGTFAVGWLLPRLPAFAALYP 120
Morg.m   LTHEGEGLLPVLNEAFDRIADTLECFSHGQFRERVKVGAVGTFAAGWLLPRLTGFYDSHP 120
Ent.cl   LTTEGENLLPVLNDSFDRIAGMLDRFANHRAQEKLKIGVVGTFATGVLFSQLEDFRRGYP 120
Cit.fr   LTTEGESLLPVLNDSFDRMAGMLDRFATKQTQEKLKIGVVGTFAIGCLFPLLSDFKRSYP 120
         ** *.  *****. :*. :  *  *  : :* :.:.*****  * *:.  *  *   :*

                          arginine→cysteine          glycine →glutamic acid
98.R7D   FIDLRVLTHNN-LVNLSADGMDFAIRFGEGLWPATCNIKLFDAP-LTVLCPPAVAARLRT 178
Smunp    FIDLRVLTHNN-LVNLSADGMDFAIRFGEGLWPATCNIKLFDAP-LTVLCPPTVAARLST 178
Serr1    FIDLRVLTHNNPVSILSADGMDFAIRFGEGLWPASLQYQAVPTRPLTVLCPPAVAARLRT 180
Haf.al   FVELRLLTNNN-LVNLAGEGLDFAIRFGTGMWPTTHNAELFGAP-LSVLCTPSVAQRLQH 178
Prov.s   YIDLRIMTHNN-VVNLAAEGVDFAIRFGEGLWPLAENTALFSAA-HTVLCSEKVANKLTH 178
Morg.m   HIDLHISTHNN-HVDPAAEGHDYTIRFGNGAWHESDAELIFSAP-HAPLCSPAIAEQLQQ 178
Ent.cl   HIDLQLSTHNN-RVDPAAEGLDYTIRYGGGAWHGTEAEFLCHAP-LAPLCTPDIAASLHS 178
Cit.fr   HIDLHISTHNN-RVDPAAEGLDYTIRYGGGAWHDTDAQYLCSAL-MSPLCSPTLASQIQT 178
         .::*:: *:**     :.:* *::**:* * *  :     :  : **.  :*  :

                  aspartic acid→valine
98.R7D   PQDLQHEQLMRTYRKDEWERWFAAAQVTPWRING--PVFDSSRLMVE------------- 223
Smunp    PQDLQHEQLMRTYRKDEWERWFTAAQVTPWRING--PVFDSSRLMVEGAIQCDGVALAPV 236
Serr1    PQDLQHEQLMRTYRKDEWEHWFAAAQVTPWRING--PVFDSSGMMVEGAIQCDGVALAPV 238
Haf.al   PEDLAKEILMRSYRAEEWNSWFLAAGIEPIRVNG--PIFDSSRLMVEGAIQNAGVALAPA 236
Prov.s   PLDLKDHSLMRSYRKDEWEKWQIAAELEPWRVKG--PIFDSSRLMVEAALLTDGVALAPS 236
Morg.m   PDDVHRFTLLRSFRRDEWSRWLDCAGGTPPSPSQPVMVFDTSLAMAEAAQLGAGVAIAPV 238
Ent.cl   PADILRFTLLRSYRRDEWTAWMQAAGEHPPSPTHRVMVFDSSVTMLEAAQAGVGIAIAPV 238
Cit.fr   PADILKFPLLRSYRRDEWALWMQAAGEAPPSPTHNVMVFDSSVTMLEAAQGGMGVAIAPV 238
       * *:    *:*::* :**  *  .*  *   .    :**:*  * *

Smunp    SMFRRELAAGALQRPFATEAALGAYWLTHLKSRDLTPAMKTFIGWIRREAEEEQRRD 293
Serr1    SMFRRDLAAGALQRPFAAEAALGAYWLTHLKSRDLTPAMKTFIG------------- 282
Haf.al   RMFEHELQNGLLVSPFDIEVEVGSYWLTWLKSKPMTPAMQLFHQWLLVQAKD----- 288
Prov.s   CMFEHELSAGTLVQPFDISVTLGGYWLSRLKSRPTTPAMAIFRHWLLAEVQK----- 288
Morg.m   CMFSRLLQSGALVQPFAAEITLGGYWLTRLQSRTETPAMQQFARWLLNTAAA----- 290
Ent.cl   DMFTHLLASERIVQPFATQIELGSYWLTRLQSRAETPAMREFSRWLVEKMKK----- 290
Cit.fr   RMFTHLLSSERIVQPFLTQIDLGSYWITRLQSRPETPAMREFSRWLTGVLHK----- 290
```

Figure 6.15. Panel A. Alignment of the partial AmpR amino acid sequence from representative Group 1 (SmGp1) and Group 2 (SmGp2) *S. marcescens* isolates in Collection D, with AmpR from Serr1 (data available in MCRAB, Creighton University) and *S. marcescens* Smunp (Genbank accession number AJ271368). Amino acid differences are highlighted.

Panel B. Multiple alignment of the partial AmpR sequence from *S. marcescens* 98.R7D, Serr1 and Smunp with AmpR from wild-type isolates of *H. alvei* (*H. alvei* 1, Genbank accession number AF180952), *P. stuartii* (*P. stuartii* VDG96 Genbank accession number Y17315), *M. morganii* (Mor str 1, Genbank accession number AF055067), *E. cloacae* (Ent MHN1, Genbank accession number X04730) and *C. freundii* (Cit OS60, Genbank accession number M27222). The predicted phenotype of the *S. marcescens* test isolate, 98.R7 is denoted as D (derepressed). The predicted helix-turn-helix (HTH) DNA binding motif is shown. *, denotes identical amino acids; ':', denotes conserved substitutions and '.', denotes semi-conserved substitutions. The published AmpR mutations in *E. cloacae* (R) and *C. freundii* (R) are also highlighted.

associated with altered AmpC expression in *C. freundii* and *E. cloacae* (Bartowsky & Normark, 1991; Kuga *et al.*, 2000) (Figure 6.15, panel B).

When compared with other enterobacteria, the partial AmpR sequence from the *S. marcescens* test isolates demonstrated 71% identity with AmpR from wild-type *P. stuartii* (*P. sturatii* VDG96, Genbank accession number Y17315) and less than 50% identity with AmpR from wild-type *E. cloacae* (Ent. MHN1, Genbank accession number X04730), *C. freundii* (Cit. OS60, Genbank accession number M27222) and *M. morganii* (Mor. Str 1, Genbank accession number AF055067). The partial AmpR sequence from the *S. marcescens* test isolates also demonstrated 73% identity with AmpR from wild-type *Hafnia alvei* (*H. alvei* 1, Genbank accession number AF180952). Multiple alignment of the AmpR sequence in all six enterobacteria demonstrated that some interspecies identity occured within the N-terminal portion of the AmpR which contains the predicted HTH DNA binding motif (Figure 6.15, panel B). This was especially true for *S. marcescens* and *H. alvei*, where the AmpR sequence in both species shared 91% identity across the predicted HTH region.

6.3.3. Characterisation of the *ampC* gene and the *ampC-ampR* inter-cistronic region in *S. marcescens*

The *ampC* PCR products from the DNA template control amplification reactions (*ampC* primer Set 1) of one cefotaxime-susceptible and one cefotaxime-resistant *S. marcescens* were found to share 100% identity. The partial *ampC* sequence from the *S. marcescens* test isolates demonstrated 96% similarity with the published *ampC* sequence in *S. marcescens* (SR50, Genbank accession number X52964).

6.3.3.1. PCR amplification for *ampC* and the *ampC-ampR* intercistronic region

In DNA amplification reactions designed to characterise the *ampC* gene and the *ampC-ampR* intercistronic region (*ampC* primer Set 3), a low-yield PCR product of the expected size (1.5 kb) was amplified using DNA from four of the nine *S. marcescens* isolates in Collection D and from *S. marcescens* NCTC 11935 (Figure 6.13, panel C). DNA from the *E. cloacae* control isolate failed to yield an *ampC*-like PCR product when amplified with the *S. marcescens ampC*-specific primer pair. When the *ampC* PCR products obtained using DNA from the four *S. marcescens* test isolates were

concentrated during purification procedures, faint non-specific bands were evident in two of the isolates. Among the four PCR products processed for TOPO cloning and transformation, positive transformants with PCR inserts of the correct size were only obtained from the two isolates that yielded a single specific PCR product of the expected size (Figure 6.13, panel D, lanes 3 and 9). These two *ampC* clones, derived from one cefotaxime-susceptible isolate and one cefotaxime low-level resistant isolate were sequenced.

6.3.3.2. **Sequence analysis of *ampC* and the *ampC-ampR* intercistronic region**

Nucleotide alignment revealed that the *ampC* PCR products from the two *S. marcescens* isolates shared 99% similarity with five base pair differences occurring between the 1.5 kb amplimers. Within the 1.5 kb sequence, an ORF of 1136 bp was identified as the *ampC* gene. This *ampC* sequence demonstrated 99% similarity with the partial *ampC* sequence from a cefotaxime-resistant isolate from Collection D (obtained from an *ampC* DNA template control amplification reaction). The *ampC* sequence in the *S. marcescens* test isolates also demonstrated 96% similarity with the *ampC* from Serr1 (Genbank accession number AF384203), 98% similarity with an unpublished wild-type *S. marcescens ampC* sequence deposited in the Genbank (Genbank accession number AJ271368) and 96% similarity with the *ampC* sequence in *S. marcescens* wild-type isolates SR50 (Genbank accession number X52964) and SST1 (Genbank accession number AB008455) (Figure 6.16).

The predicted 355 amino acid AmpC sequences for the *S. marcescens* test isolates were found to share 100% identity with each other and 98% and 97% identity with Serr 1 and the unpublished *S. marcescens* AmpC sequence in the Genbank, respectively. Similarly high percentage identities were observed with the published AmpC sequences in *S. marcescens* SR50 and SST1 (Figure 6.17). The three conserved amino acid motifs characteristic of class C β-lactamases were identified within the 356 amino acid AmpC sequence of the *S. marcescens* test isolates at the following locations; the serine-X-X-lysine (SXXK) motif at position 58-61, the tyrosine-serine-asparagine (YSN) motif at position 143-145 and the lysine-threonine-glycine (KTG) motif at position 308-310 (Medeiros, 1997). The published amino acid substitutions associated with extended-spectrum AmpC activity in *S. marcescens* were not located in the AmpC sequence of the test isolates (Matsumura *et al.*, 1998; Raimondi *et al.*, 2001) (Figure 6.17).

CLUSTAL W (1.81) nucleotide sequence alignment of *ampC* in *S. marcescens* isolates

```
Serr1    CCTGCAACCTAAGAGCTTCTATCATGACGAAAATGAACCGCCTGGCGGCCGCGCTGATCG 60
Smunp    CCTGCAACCTAAGAGCTTCTATCATGACGAAAATGAACCGCCTGGCGGCCGCGCTGATCG 60
96.S5WT  CCTGCAACCTAAGAGCTTCTATCATGACGAAAATGAACCGCCTGGCGGCCGCGCTGATCG 60
97.R2PD  CCTGCAACCTAAGAGCTTCTATCATGACGAAAATGAACCGCCTGGCGGCCGCGCTGATCG 60
97.R8D   ------------------------------------------------------------
SR50     CCTGCAACCTAAGAGCTTCTATCATGACGAAAATGAACCGCT--GCG-CCGCGCTGATCG 57
SST1     CCTGCAACCGAAGAGCCTCTATCATGACGAAAATGAACCGCCTGGCGGCCGCGCTGATCG 60

Serr1    CCGCACTGATCTTGCCGACCGCGCAGGCCGCGCAGCAGCAGGATATCGACGCCGTTATTC 120
Smunp    CCGCACTGATCCTGCCGACCGCGCACGCCGCGCAGCAGCAGGATATCGACGCCGTTATTC 120
96.S5WT  CCGCACTGATCTTGCCGACCGCGCAGGCCGCGCAGCAGCAGGATATCGACGCCGTTATTC 120
97.R2PD  CCGCACTGATCTTGCCGACCGCGCAGGCCGCGCAGCAGCAGGATATCGACGCCGTTATTC 120
97.R8D   ------------------------------GCAGCAGCAGGATATCGACGCCGTTATTC 29
SR50     CCGCACTGATCCTGCCGACCGCGCACGCCGCACAGCAGCAGGATATCGACGCCGTTATTC 117
SST1     CCGCACTGATCTTGCCGACCGCGCACGCCGCGCAGCAGCAGGATATCGACGCCGTTATTC 120
                                       ****************************

Serr1    AGCCGCTGATGAAAAAATATGGCGTACCGGGCATGGCGATCGCCGTGTCGGTCGACGGCA 180
Smunp    AGCCGCTGATGAAAAAATATGGCGTGCCGGGCATGGCGATCGCCGTGTCGGTCGACGGCA 180
96.S5WT  AGCCGCTGATGAAAAAATATGGCGTACCGGGCATGGCGATCGCCGTGTCGGTCGACGGCA 180
97.R2PD  AGCCGCTGATGAAAAAATATGGCGTACCGGGCATGGCGATCGCCGTGTCGGTCGACGGCA 180
97.R8D   AGCCGCTGATGAAAAAATATGGCGTACCGGGCATGGCGATCGCCGTGTCGGTCGACGGCA 89
SR50     AGCCGCTGATGAAAAAATACGGCGTGCCGGGCATGGCGATCGCCGTGTCGGTCGACGGCA 177
SST1     AGCCGCTGATGAAAAAATATGGCGTGCCGGGCATGGCGATCGCCGTGTCGGTCGACGGCA 180
         ******************* ***** ********************************

Serr1    AACAGCAGTATTACCCGTTAGGCGTCGCCTCGAAGCAGACCGGCAAACCGATCACCGAGC 240
Smunp    AACAGCAGATTTACCCGTATGGCGTTGCCTCGAAGCAGACCGGCAAACCGATCACCGAGC 240
96.S5WT  AACAGCAGATTTACCCGTATGGCGTCGCCTCGAAGCAGACCGGCAAACCGATCACCGAGG 240
97.R2PD  AACAGCAGATTTACCCGTATGGCGTCGCCTCGAAGCAGACCGGCAAACCGATCACCGAGG 240
97.R8D   AACAGCAGATTTACCCGTATGGCGTCGCCTCGAAGCAGACCGGCAAACCGATCACCGAGG 149
SR50     AACAGCAGATTTACCCGTATGGCGTCGCCTCGAAGCAGACCGGTAAACCGATCACCGAGC 237
SST1     AACAGCAGATTTACCCGTATGGCGTCGCCTCGAAGCAGACCGGCAAACCGATCACCGAGC 240
         ******** ******** ***** ***************** ***************

Serr1    AGACGCTGTTCGAAGTGGGCTCGCTGAGCAAAACCTTTACCGCGACGCTGGCGGTCTATG 300
Smunp    AGACGCTGTTCGAAGTGGGCTCGCTGAGCAAAACCTTCACCGCGACGCTGGCGGTCTATG 300
96.S5WT  AGACGCTGTTCGAAGTGGGCTCGCTGAGCAAAACCTTCACCGCGACGCTGGCGGTCTATG 300
97.R2PD  AGACGCTGTTCGAAGTGGGCTCGCTGAGCAAAACCTTCACCGCGACGCTGGCGGTCTATG 300
97.R8D   AGACGCTGTTCGAAGTGGGCTCGCTGAGCAAAACCTTCACCGCGACGCTGGCGGTCTATG 209
SR50     AGACGCTGTTTGAAGTGGGCTCGCTGAGCAAAACCTTCACCGCGACGCTGGCGGTCTATG 297
SST1     AGACGCTGTTCGAAGTGGGCTCGCTGAGCAAAACCTTCACCGCGACGCTGGCGGTCTATG 300
         ********** ************************* ***********************

Serr1    CGCAGCAGCAGGGCAAGCTGTCGTTCAACGATCCGGCCAGCCGCTACCTGCCCGAGCTGC 360
Smunp    CGCAGCAGCAGGGCAAACTGTCGTTTAAAGACCCGGCCAGCCGCTACCTGCCCGAGCTGC 360
96.S5WT  CGCAGCAGCAGGGCAAGCTGTCGTTCAACGATCCGGCCAGCCGCTATCTGCCCGAGCTGC 360
97.R2PD  CGCAGCAGCAGGGCAAGCTGTCGTTCAACGATCCGGCCAGCCGCTACCTGCCCGAGCTGC 360
97.R8D   CGCAGCAGCAGGGCAAGCTGTCGTTCAACGATCCGGCCAGCCGCTATCTGCCCGAGCTGC 269
SR50     CGCAGCAGCAGAGCAAACTGTCGTTTAAAGACCCGGCCAGCCACTATCTGCCCGACGTGC 357
SST1     CGCAGCAGCAGGGCAAGCTGTCGTTCAACGATCCGGCCAGCCGCTACCTGCCCGAGCTGC 360
         *********** **** ******** ** ** ********** *** ********  ***

Serr1    GCGGCAGCGCCTTCGACGGCGTCACGGTGCTGAATCTGGCGACGCATACCTCCGGCCTGC 420
Smunp    GCGGCAGCGCCTTCGACGGCGTCAGCCTGCTGAATCTGGCGACGCATACCTCCGGTTTGC 420
96.S5WT  GCGGCAGCGCCTTCGACGGCGTCAGCCTGCTGAATCTGGCGACGCATACCTCCGGCCTGC 420
97.R2PD  GCGGCAGCGCCTTCGACGGCGTCAGCCTGCTGAATCTGGCGACGCATACCTCCGGCCTGC 420
97.R8D   GCGGCAGCGCCTTCGACGGCGTCAGCCTGCTGAATCTGGCGACGCATACCTCCGGCCTGC 329
SR50     GCGGCAGCGCCTTCGACGGCGTCAGCCTGCTGAATCTGGCGACCCACACCTCCGGCCTGC 417
SST1     GCGGCAGCGCCTTCGACGGCGTCAGCCTGCTGAATCTGGCGACGCATACCTCCGGCCTGC 420
         ************************   ***************** ** ********  ***
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CLUSTAL W (1.81) nucleotide sequence alignment of *ampC* in *S. marcescens* isolates contd.1

```
Serr1     CGCTGTTCGTGCCGGACGACGTCACTAACGACGCCCAGCTGATGGCCTACTACCGGGCCT 480
Smunp     CGCTGTTCGTGCCGGACGACGTAACCGACAACGCACAACTGATGGCGTACTACCGGGCCT 480
96.S5WT   CGCTGTTCGTGCCGGACGACGTCACCAACAACGCCCAGCTGATGGCCTACTACCGGGCCT 480
97.R2PD   CGCTGTTCGTGCCGGACGACGTCACCAACAACGCCCAACTGATGGCCTACTACCGGGCCT 480
97.R8D    CGCTGTTCGTGCCGGACGACGTCACCAACAACGCCCAGCTGATGGCCTACTACCGGGCCT 389
SR50      CGCTGTTTGTGCCGGACGACGTGACCAACAACGCCCAGCTGATGGCCTATTACCGGGCCT 477
SST1      CGCTGTTCGTGCCGGACGACGTGACCGACAACGCCCAACTGATGGCCTACTACCGGGCCT 480
          ******* ************** **  ** **** ** ******** ** **********

Serr1     GGCAGCCGAAACACCCGGCGGGCAGCTACCGCGTTTATTCCAACCTCGGCATCGGCATGC 540
Smunp     GGCAGCCGAAACACCCGGCGGGCAGCTACCGCGTCTATTCCAACCTCGGCATCGGCATGC 540
96.S5WT   GGCAGCCGAAACACCCGGCGGGCAGCTACCGCGTCTATTCCAACCTCGGCATCGGCATGC 540
97.R2PD   GGCAGCCGAAACACCCGGCGGGCAGCTACCGCGTCTATTCCAACCTCGGCATCGGCATGC 540
97.R8D    GGCAGCCGAAACACCCGGCGGGCAGCTACCGCGTCTATTCCAACCTCGGCATCGGCATGC 449
SR50      GGCAGCCGAAACACCCGGCGGGCAGCTACCGCGTCTATTCCAACCTCGGCATCGGCATGC 537
SST1      GGCAGCCGAAACATCCGGCGGGCAGCTACCGCGTCTATTCCAACCTCGGCATCGGCATGC 540
          ************* ***************** ** ************************

Serr1     TGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTTTATCCAGGCGATGGAACAGGGGA 600
Smunp     TGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTTTATCCAGGCGATGGAACAGGGGA 600
96.S5WT   TGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTTTATCCAGGCGATGGAACAGGGGA 600
97.R2PD   TGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTTTATCCAGGCGATGGAACAGGGGA 600
97.R8D    TGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTTTATCCAGGCGATGGAACAGGGGA 509
SR50      TGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTTTATCCAGGCGATGGAACAGGGGA 597
SST1      TGGGCATGATCGCCGCCAAGAGCCTCGACCAGCCGTTTACCCAGGCGATGGAGCAGGGGA 600
          ************************************ ************ *******

Serr1     TGCTGCCGGCGCTGGGCATGAGCCACACCTACGTTCAGGTGCCGGCGGCGCAGATGGCTA 660
Smunp     TGCTGCCGGCGTTGGGCATGAGCCACACCTACGTTCAGGTGCCGGCGGCGCAGATGGCCA 660
96.S5WT   TGCTGCCGGCGCTGGGCATGAGCCACACCTACGTTCAGGTGCCGGCGGCGCAGATGGCTA 660
97.R2PD   TGCTGCCGGCGCTGGGCATGAGCCACACCTACGTTCAGGTGCCGGCGGCGCAGATGGCTA 660
97.R8D    TGCTGCCGGCGCTGGGCATGAGCCACACCTACGTTCAGGTGCCGGCGGCGCAGATGGCTA 569
SR50      TGCTGCCGGCATTGGGCATGAGCCACACCTACGTGCAGGTGCCGGCGGCGCAGATGGCCA 657
SST1      TGCTGCCGGCGTTGGGCATGCGCCACACCTACGTTCAGGTGCCGGCGGCGCAGATGGCCA 660
          **********  ******** ************ ********************** *

Serr1     ACTATGCGCAGGGTTACAGCAAGGACGATAAGCCGGTGCGGGTCAACCCCGGCCCGCTGG 720
Smunp     ACTATGCGCAGGGCTACAGCAAGGACGATAAACCGGTGCGGGTCAATCCCGGCCCGCTGG 720
96.S5WT   ACTATGCGCAGGGTTACAGCAAGGACGATAAGCCGGTGCGGGTCAACCCCGGCCCGCTGG 720
97.R2PD   ACTATGCGCAGGGTTACAGCAAGGACGATAAGCCGGTGCGGGTCAATCCCGGCCCGCTGG 720
97.R8D    ACTATGCGCAGGGTTACAGCAAGGACGATAAGCCGGTGCGGGTCAATCCCGGCCCGCTGG 629
SR50      ACTATGCGCAGGGTTACAGCAAGGACGATAAGCCGGTGCGGGTCAATCCTGGCCCGCTGG 717
SST1      ACTATGCGCAGGGCTACAACAAGGACGATAAGCCGGTGCGGGTCAATCCCGGCCCGCTGG 720
          ************* **** ************ ************** ** **********

Serr1     ACGCCGAATCTTACGGCATCAAGTCCAACGCTCGCGATCTGATTCGCTATCTGGACGCCA 780
Smunp     ACGCCGAGTCTTACGGCATCAAGTCCAACGCCCGCGATCTGATTCGCTATCTGGACGCCA 780
96.S5WT   ACGCCGAGTCTTACGGCATCAAGTCCAACGCTCGCGATCTGATTCGCTATCTGGACGCCA 780
97.R2PD   ACGCCGAATCTTACGGCATCAAATCCAACGCTCGCGATCTGATTCGCTATCTGGACGCCA 780
97.R8D    ACGCCGAATCTTACGGCATCAAATCCAACGCTCGCGATCTGATTCGCTATCTGGACGCCA 689
SR50      ACGCCGAGTCTTACGGCATCAAGTCCAACGCCCGCGATCTGATTCGCTATCTGGACGCCA 777
SST1      ACGCCGAGTCTTACGGCATCAAGTCCAACGCTCGCGATCTGATTCGCTATCTGGACGCCA 780
          ******* ************** ******** ****************************

Serr1     ACCTGCAGCAGGTGAAGGTGGCGCAGCCATGGCGCGACGCGCTGGCCGCGACGCACGTCG 840
Smunp     ACCTGCAGCAGGTGAAGGTGGCGCAACCGTGGCGCGAGGCGCTGGCCGCCACGCACGTCG 840
96.S5WT   ACCTGCAGCAGGTGAAGGTGGCGCAGCCGTGGCGCGACGCGCTGGCCGCGACGCACGTCG 840
97.R2PD   ACCTGCAGCAGGTGAAGGTGGCGCAGCCGTGGCGCGACGCGCTGGCCGCGACGCACGTCG 840
97.R8D    ACCTGCAGCAGGTGAAGGTGGCGCAGCCGTGGCGCGACGCGCTGGCCGCGACGCACGTCG 749
SR50      ACCTGCAGCAGGTGAAGGTAGCG--AGCGTGGCGCGA--CGGTGGCCGCGACGCACGTCG 833
SST1      ACCTGCAGCAGGTGAAGGTCGCGCACGCGTGGCGCGAGGCGCTGACCGCGACGCACGTCG 840
          ******************** ***    * ********  ** ** **** **********
```

CLUSTAL W (1.81) nucleotide sequence alignment of *ampC* in *S. marcescens* isolates contd.2

```
Serr1    GGTATTACAAG-GCGGGCGCGTTCACGCAGGATCTGATGTGGGAGAACTACCCGTATCCA 899
Smunp    GTTATTACAAG-GCGGGTGCGTTCACGCAGGATCTGATGTGGGAGAACTACCCGTATCCG 899
96.S5WT  GTTATTACAAG-GCGGGCGCGTTCACGCAGGATCTGATGTGGGAGAACTACCCGTATCCG 899
97.R2PD  GTTATTACAAG-GCGGGCGCGTTCACGCAGGATCTGATGTGGGAGAACTACCCGTATCCG 899
97.R8D   GTTATTACAAG-GCGGGCGCGTTCACGCAGGATCTGATGTGGGAGAACTACCCGTATCCG 808
SR50     GTTATTACAAGCGCGGGCGCGTTCACGCAGGATCTGATGTGGGAGAACTACCCGTATCCG 893
SST1     GTTATTACAAG-GCGGGCGCGTTCACGCAGGATCTGATGTGGGAGAACTACCCGTACCCG 899
         * ********* ***** ************************************* **

Serr1    GTGAAACTGTCGCGTTTGATTGAAGGCAACAACGCCGGGATGATCATGAACGGCACGCCG 959
Smunp    GTGAAGCTGTCGCGTTTGGTTGAAGGCAACAACGCCGGGATGATCATGAACGGCACGCCG 959
96.S5WT  GTGAAACTGTCGCGTTTGATTGAAGGCAACAACGCCGGGATGATCATGAACGGCACGCCG 959
97.R2PD  GTGAAACTGTCGCGTTTGATTGAAGGCAACAACGCCGGGATGATCATGAACGGCACGCCG 959
97.R8D   GTGAAACTGTCGCGTTTGATTGAAGGCAACAACGCCGGGATGATCATGAACGGCACGC-- 866
SR50     GTGAAACTGTCGCGTTTGATTGAAGGCAACAACGCCGGGATGATCATGAACGGCACGCCG 953
SST1     GTGAAATTGTCTCGTTTGATCGAGGGCAACAACGCCGGGATGATCATGAACGGCACGCCG 959
         *****  **** ****** * ** ********************************

Serr1    GCCACCACCATCACGCCGCCGCAGCCGGAATTGCGCGCCGGCTGGTATAACAAAACCGGC 1019
Smunp    GCCACCGCCATCACGCCGCCGCAGCCGGAATTGCGCGCCGGCTGGTATAACAAAACCGGC 1019
96.S5WT  GCCACCGCCATCACGCCGCCGCAGCCGGAATTGCGCGCCGGCTGGTATAACAAAACCGGC 1019
97.R2PD  GCCACCGCCATCACGCCGCCGCAGCCGGAATTGCGCGCCGGCTGGTATAACAAAACCGGC 1019
97.R8D   ------------------------------------------------------------
SR50     GCCACCGCCATCACGCCGCCGCAGCCGGAATTGCGCGCCGGCTGGTATAACAAAACCGGC 1013
SST1     GCTACCGCCATCACGCCGCCGCAGCCGGAATTGCGCGCCGGCTGGTATAACAAAACCGGC 1019

Serr1    TCCACCGGCGGCTTCTCCACCTACGCGGTATTTATCCCGGCGAAAAATATCGCCGTGGTG 1079
Smunp    TCTACCGGCGGTTTCTCCACCTACGCGGTGTTTATCCCGGCGAAGAATATCGCCGTGGTG 1079
96.S5WT  TCCACCGGCGGCTTCTCCACCTACGCGGTATTTATCCCGGCGAAAAATATCGCCGTGGTG 1079
97.R2PD  TCCACCGGCGGCTTCTCCACCTACGCGGTATTTATCCCGGCGAAAAATATCGCCGTGGTG 1079
97.R8D   ------------------------------------------------------------
SR50     TCCACCGGCGGTTTCTCCACCTACGCGGTATTTATCCCGGCGAAGAATATCGCCGTGGAG 1073
SST1     TCTACCGGCGGTTTCTCCACCTACGCGGTGTTTATCCCGGCGAAGAATATCGCCGTGGTG 1079

Serr1    ATGCTGGCCAACAAGTGGTTCCCGAACGACGATCGCGTCGAGGCGGCTTACCACATCGTC 1139
Smunp    ATGTTGGCCAACAAGTGGTTCCCGAATGACGATCGCGTCGAGGCGGCTTACCACATCATC 1139
96.S5WT  ATGCTGGCCAACAAGTGGTTCCCGAACGACGATCGCGTCGAGGCGGCTTACCACATCGTC 1139
97.R2PD  ATGCTGGCCAACAAGTGGTTCCCGAACGACGATCGCGTCGAGGCGGCTTACCACATCGTC 1139
97.R8D   ------------------------------------------------------------
SR50     ATGCTGGCCAACAAGTGGTTCCCGAACGACGATCGCGTCGAAGCGGCTTACCACATCATC 1133
SST1     ATGCTGGCCAACAAATGGTTCCCGAACGACGATCGGGTCGAGGCGGCTTACCGCATCGTG 1139

Serr1    CAGGCGCTGGAGAAGCGCTGACGGCCAGGGGCGCCGACGAGGCGCCCCTACCAGCGATGC 1199
Smunp    CAGGCGCTGGAGAAGCACTGACGCTCAGGGGCGCCGGCGAGGCGCCCCTACCACCGATGC 1199
96.S5WT  CAGGCGCTGGAGAAGCGCTGACGGCCAGGGGCGCCGACGAGGCGCCCCTACCACCGATGC 1199
97.R2PD  CAGGCGCTGGAGAAGCGCTGACGGCCAGGGGCGCCGACGAGGCGCCCCTACCACCGATGC 1199
97.R8D   ------------------------------------------------------------
SR50     CAGGCGCTGGAGAAGCGCTGA---------------------------------------- 1154
SST1     CAAGCGTTGGATAAGCGCTGA---------------------------------------- 1160
```

Figure 6.16. Multiple alignment of the nucleotide sequence of *ampC* from two *S. marcescens* isolates, 96.S5WT and 97.R2PD, in Collection D with the *ampC* sequence from Serr1 (Genbank accession number AF384203) and from *S. marcescens* wild-type isolates Smunp (Genbank accession number AJ271368), SR50 (Genbank accession number X52964) and SST1 (Genbank accession number AB008455). The partial *ampC* sequence from *S. marcescens* isolate 97.R8D (derived from a 860 bp *ampC*-PCR product) is also included in the alignment. The predicted phenotype of the *S. marcescens* test isolates is denoted as WT (wild-type), PD (partially derepressed) or D (derepressed). The five nucleotide differences in the *ampC* sequence of the *S. marcescens* test isolates are highlighted in yellow. * denotes identical nucleotides. Translational start and stop codons are in blue type.

CLUSTAL W (1.81) amino acid sequence alignment of AmpC in *S. marcescens* isolates

```
                    -22                 +1
Serr1     -------MTKMNRLAAALIAALILPTAQAAQQQDIDAVIQPLMKKYGVPGMAIAVSVDGK 31
Smunp     -------MTKMNRLAAALIAALILPTAHAAQQQDIDAVIQPLMKKYGVPGMAIAVSVDGK 31
97.R2PD   -------MTKMNRLAAALIAALILPTAQAAQQQDIDAVIQPLMKKYGVPGMAIAVSVDGK 31
SR50      -------MTKMNRLAAALIAALILPTAQAAQQQDIDAVIQPLMKKYGVPGMAIAVSVDGK 31
SST1      -------MTKMNRLAAALIAALILPTAHAAQQQDIDAVIQPLMKKYGVPGMAIAVSVDGK 31
          ** ************************:******************************

Serr1     QQYYPLGVASKQTGKPITEQTLFEVGSLSKTFTATLAVYAQQQGKLSFNDPASRYLPELR 91
Smunp     QQIYPYGVASKQTGKPITEQTLFEVGSLSKTFTATLAVYAQQQGKLSFKDPASRYLPELR 91
97.R2PD   QQIYPYGVASKQTGKPITEETLFEVGSLSKTFTATLAVYAQQQGKLSFNDPASRYLPELR 91
SR50      QQIYPYGVASKQTGKPITEETLFEVGSLSKTFTATLAVYAQQQGKLSFNDPASRYLPELR 91
SST1      QQIYPYGVASKQTGKPITEQTLFEVGSLSKTFTATLAVYAQQQGKLSFNDPASRYLPELR 91
          ** ** *************:*****************************:***********
                                              threonine → isoleucine
Serr1     GSAFDGVTVLNLATHTSGLPLFVPDDVTNDAQLMAYYRAWQPKHPAGSYRVYSNLGIGML 151
Smunp     GSAFDGVSLLNLATHTSGLPLFVPDDVTDNAQLMAYYRAWQPKHPAGSYRVYSNLGIGML 151
97.R2PD   GSAFDGVTLLNLATHTSGLPLFVPDDVTNNAQLMAYYRAWQPKHPAGSYRVYSNLGIGML 151
SR50      GSAFDGVTLLNLATHTSGLPLFVPDDVTNNAQLMAYYRAWQPKHPAGSYRVYSNLGIGML 151
SST1      GSAFDGVSLLNLATHTSGLPLFVPDDVTDNAQLMAYYRAWQPKHPAGSYRVYSNLGIGML 151
          *******::*******************::*******************************

Serr1     GMIAAKSLDQPFIQAMEQGMLPALGMSHTYVQVPAAQMANYAQGYSKDDKPVRVNPGPLD 211
Smunp     GMIAAKSLDQPFIQAMEQGMLPALGMSHTYVQVPAAQMANYAQGYSKDDKPVRVNPGPLD 211
97.R2PD   GMIAAKSLDQPFIQAMEQGMLPALGMSHTYVQVPAAQMANYAQGYSKDDKPVRVNPGPLD 211
SR50      GMIAAKSLDQPFIQAMEQGMLPALGMSHTYVQVPAAQMANYAQGYSKDDKPVRVNPGPLD 211
SST1      GMIAAKSLDQPFTQAMEQGMLPALGMRHTYVQVPAAQMANYAQGYNKDDKPVRVNPGPLD 211
          ************ ************* ******************.**************

Serr1     AESYGIKSNARDLIRYLDANLQQVKVAQPWRDALAATHVGYYKAGAFTQDLMWENYPYPV 271
Smunp     AESYGIKSNARDLIRYLDANLQQVKVAQPWREALAATHVGYYKAGAFTQDLMWENYPYPV 271
97.R2PD   AESYGIKSNARDLIRYLDANLQQVKVAQPWRDALAATHVGYYKAGAFTQDLMWENYPYPV 271
SR50      AESYGIKSNARDLIRYLDANLQQVKVAQPWRDALAATHVGYYKAGAFTQDLMWENYPYPV 271
SST1      AESYGIKSNARDLIRYLDANLQQVKVAHAWREALTATHVGYYKAGAFTQDLMWENYPYPV 271
          ****************************:.**:**:*************************
  Glutamic acid→Lysine
Serr1     KLSRLIEGNNAGMIMNGTPATTITPPQPELRAGWYNKTGSTGGFSTYAVFIPAKNIAVVM 331
Smunp     KLSRLVEGNNAGMIMNGTPATAITPPQPELRAGWYNKTGSTGGFSTYAVFIPAKNIAVVM 331
97.R2PD   KLSRLIEGNNAGMIMNGTPATAITPPQPELRAGWYNKTGSTGGFSTYAVFIPAKNIAVVM 331
SR50      KLSRLIEGNNAGMIMNGTPATAITPPQPELRAGWYNKTGSTGGFSTYAVFIPAKNIAVVM 331
SST1      KLSRLIEGNNAGMIMNGTPATAITPPQPELRAGWYNKTGSTGGFSTYAVFIPAKNIAVVM 331
          *****:***************:************************************

Serr1     LANKWFPNDDRVEAAYHIVQALEKRRPGAPTRRPYQRCC 370
Smunp     LANKWFPNDDRVEAAYHIIQALEKHRSGAPARRPYHRCC 370
97.R2PD   LANKWFPNDDRVEAAYHIVQALEKRRPGAPTRRPYHRCC 370
SR50      LANKWFPNDDRVEAAYHIVQALEKRRPGAPTRRPYHRCC 370
SST1      LANKWFPNDDRVEAAYRIVQALDKR---------------- 356
          ****************:*:***:*:
```

Figure 6.17. Multiple alignment of the deduced AmpC amino acid sequence from *S. marcescens* test isolate 97.R2PD with the AmpC sequence from Serr1 (Genbank accession number AF384203) and from *S. marcescens* wild-type isolates Smunp (Genbank accession number AJ271368), SR50 (Genbank accession number X52964) and SST1 (Genbank accession number AB008455). The predicted phenotype of the *S. marcescens* test isolate is denoted as PD (partially derepressed). The three conserved motifs, SLSK, YSN and KTG, characteristic of class C β-lactamases are highlighted in yellow. The published AmpC mutations associated with extended-spectrum activity are also highlighted (▌). The amino acids designated –22 to –1 represent the putative signal peptide. *, denotes identical sequences; ':', denotes conserved substitutions; '.', denotes semi-conserved substitutions.

Sequence analysis of the *ampC* PCR products from the two *S. marcescens* isolates in Collection D also revealed the partial sequence of a divergently transcribed *ampR*-like gene, 206 bp upstream of the *ampC* gene. Across the *ampC-ampR* intercistronic region the DNA sequence from the *ampC* PCR products of the *S. marcescens* isolates shared 100% identity (Figure 6.18). Within the *ampC-ampR* intercistronic region, a 39 bp sequence was observed with significant similarity (>90%) to the putative AmpR binding site identified in *M. morganii* and *H. alvei* (Poirel *et al.*, 1999; Girlich *et al.*, 2000a). When compared with the homologous regulatory regions in Serr1 (unpublished data made available at MCRAB, Creighton University) and in an unpublished wild-type *S. marcescens* isolate deposited in the Genbank (Genbank accession number AJ271368), the *ampC-ampR* region demonstrated 99% and 97% similarity, respectively (Figure 6.18).

Nucleotide sequence alignment of the *ampC-ampR* intercistronic region from *S. marcescens*.

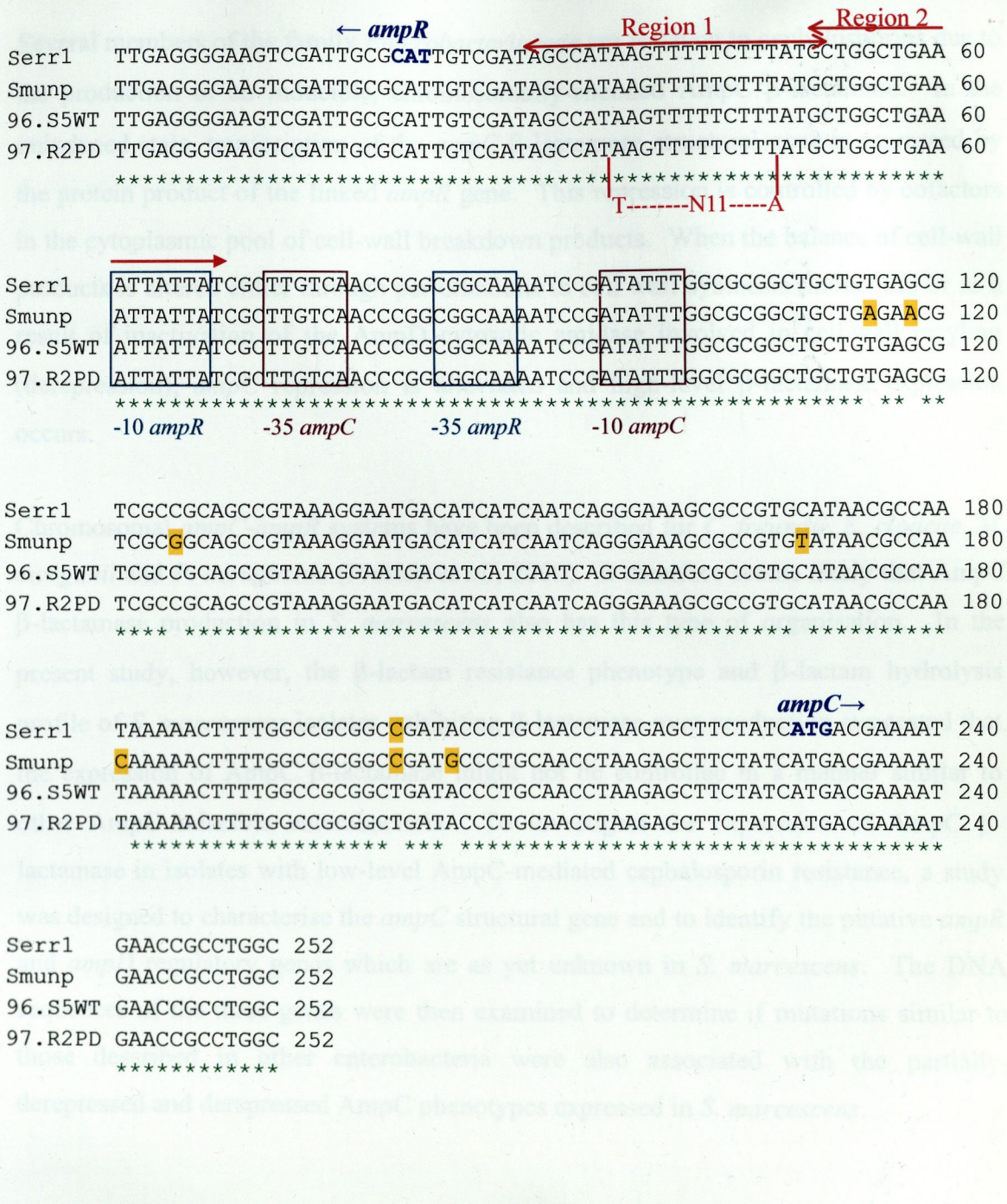

Figure 6.18. Multiple alignment of the nucleotide sequence of the *ampC-ampR* intercistronic region from the *S. marcescens* test isolates 96.S5WT and 97.R2PD with the *ampC-ampR* sequence from Serr1 (data made available at MCRAB, Creighton University) and from *S. marcescens* isolate, Smunp (Genbank accession number AJ271368). The predicted phenotype of the *S. marcescens* test isolates is denoted as WT (wild-type) or PD (partially-derepressed). The *ampR* data shown are negative strand sequence. The two components (Region 1 & Region 2) of the putative AmpR binding site are shown. The putative LysR binding motif (T-N_{11}-A) in Region 1 is also shown. The predicted –10 and –35 sequences for *ampR* and *ampC* are also displayed. Translational start sites are in blue type. The nucleotide differences are highlighted in yellow. *, denotes identical sequences.

6.4. **DISCUSSION**

Several members of the family *Enterobacteriaceae* are resistant to cephalosporins due to the production of an inducible, chromosomally-encoded AmpC β-lactamase. In the uninduced state, transcription of the *ampC* β-lactamase structural gene is repressed by the protein product of the linked *ampR* gene. This repression is controlled by cofactors in the cytoplasmic pool of cell-wall breakdown products. When the balance of cell-wall products is altered either through perturbations in cell wall synthesis (induction) or as a result of inactivation of the AmpD cytosolic amidase involved in cell-wall recyling (derepression), *ampC* repression is alleviated and high-level β-lactamase expression occurs.

Chromosomal *ampC-ampR* systems have been described for *C. freundii*, *E. cloacae*, *M. morganii* and *P. aeruginosa* (Preston *et al.*, 2000). It therefore seems likely that AmpC β-lactamase production in *S. marcescens* also has this type of organisation. In the present study, however, the β-lactam resistance phenotype and β-lactam hydrolysis profile of *S. marcescens* isolates exhibiting β-lactamase over-production suggested that the expression of AmpC β-lactamase might not be controlled in a manner similar to other AmpC-inducible enterobacteria. To investigate the expression of AmpC β-lactamase in isolates with low-level AmpC-mediated cephalosporin resistance, a study was designed to characterise the *ampC* structural gene and to identify the putative *ampR* and *ampD* regulatory genes which are as yet unknown in *S. marcescens*. The DNA sequences of the three genes were then examined to determine if mutations similar to those described in other enterobacteria were also associated with the partially-derepressed and derepressed AmpC phenotypes expressed in *S. marcescens*.

6.4.1. **Identification and characterisation of *ampD* in *S. marcescens***

Preliminary DNA amplification studies for identification of the *ampD* gene were among the first set of molecular investigations performed in this project. At that stage, the problems associated with extracellular DNase activity in *S. marcescens* isolates were not anticipated. Template DNA was extracted using the standard Wizard™ Genomic DNA Purification Kit protocol designed for enterobacteria and assurance of its good quality was indicated by direct visualisation of a discrete band of chromosomal DNA. In

addition, the demonstration of *ampC*-specific PCR products in DNA template control amplification reactions suggested that the target DNA extracted from *S. marcescens* isolates was of suitable quality for *ampD* PCR studies.

The *ampD* gene is present in all *Enterobacteriaceae* tested to date, even in those lacking an inducible AmpC β-lactamase (Jones *et al.*, 1997). The conservation of *ampD* among Gram-negative organisms relates to its involvement in essential cell activities i.e. cell wall recycling. The presence of *ampD* PCR products was thus anticipated in *S. marcescens*. Nevertheless, DNA from all *S. marcescens* test isolates failed to yield *ampD*-specific PCR products in the preliminary amplification studies. Since PCR conditions were validated by the generation of amplimers from positive control isolates and since the quality of template DNA was confirmed in template control amplification reactions, the possibility that amplification failure resulted from *ampD* primer-template mismatch was considered. To compensate for the possible lack of sequence identity, low stringency DNA hybridisation studies were designed for the localisation of *ampD* using DIG-labeled probes derived from *ampD*-PCR products from DNA extracted from the amplification control isolates.

Hybridisation assays were initially performed using DNA extracted with the standard Wizard™ Genomic DNA Purification Kit protocol. In the DNA quantification assay the presence of a single discrete band of DNA indicated that genomic DNA was of good quality. The absence of shearing in the extracted DNA and the subsequent appearance of extensive degradation following treatment with restriction endonuclease indicated that this DNA was susceptible to auto-degradation. This auto-degradation was most likely due to endogenous DNase, since *S. marcescens* is a more active producer of DNase than other enterobacteria (Gross & Holmes, 1984). A review of the literature revealed that this degradative process was previously reported in *S. marcescens* (Alonso *et al.*, 1993b). In that study, non-specific degradation of DNA extracted from *S. marcescens* isolates occurred following as little as 10 min incubation with restriction endonucleases, an effect that was not observed with other Gram-negative species. The authors also demonstrated that when DNA was incubated under the time, temperature and buffer conditions for restriction digestion, but in the absence of the restriction endonuclease enzyme, the DNA remained undegraded. It was thus considered that endogenous DNase activity in addition to restriction endonuclease activity was

responsible for the degradative process observed. The authors suggested that the non-specific digestion of DNA could be eliminated by introducing a proteinase K treatment step in the DNA extraction protocol. In the present study, the use of proteinase K also prevented DNA degradation.

The results of *ampD* hybridisation studies indicated the presence of an *ampD*-like gene in *S. marcescens* test isolates on a 1.2 kb fragment. DNA analysis of the cloned *ampD*-like fragments from two *S. marcescens* isolates confirmed that the suspect *ampD* gene in *S. marcescens* shared substantial identity with *ampD* from wild-type *E. cloacae* and to a lesser extent with the *ampD* from wild-type *C. freundii* and *E. coli*. The identity of the *ampD* gene was also indicated from the results of hybridisation studies where the *ampD*-like fragment from one *S. marcescens* isolate was DIG-labeled for use as an *ampD* probe. Hybridisation with the *S. marcescens-ampD* specific probe produced a hybridisation signal in all *S. marcescens* isolates and this signal was localised at the same position as the hybridisation band produced with the known *ampD* probe from *E. cloacae*.

Following identification of the putative *ampD* gene, it was necessary to confirm the presence of the *ampD* gene sequence in a number of *S. marcescens* isolates and to determine the presence or absence of mutations implicated in derepressed AmpC phenotypes. Additional DNA amplification studies were devised using *S. marcescens-ampD* specific PCR primers designed to overcome the predicted problems of primer-template mismatch. Examination of the *S. marcescens-ampD* sequence data indicated that the degree of primer-template mismatch occurring in the preliminary amplification studies was minimal. Since only one base pair difference was evident in the 5' end of each forward and reverse primer, it seemed unlikely that this level of mismatch would, by itself, account for amplification failure. It was thus possible that additional factors such as the structure of template DNA i.e. secondary structure formation, may have contributed to the lack of amplification. Another likely explanation was that the quality of template DNA (which had been extracted in the absence of proteinase K) used in the preliminary PCR studies was inadequate to allow amplification of *ampD*.

An amplification strategy was designed to circumvent factors thought to contribute to amplification difficulties. Since short DNA target sequences are less likely to contain

difficult secondary structures, *S. marcescens-ampD* specific primers were designed to generate two short (250 bp) overlapping PCR products. A set of validated *ampD* primers derived from the conserved flanking regions of the *ampD* gene in *E. cloacae* was also employed (Stapleton *et al.*, 1995b). In addition, the PCR reaction mixture was modified to allow use of a 25-fold increase in the concentration of template DNA (extracted using the proteinase K modified Wizard™ Genomic DNA extraction protocol) and to include the use of Q-solution (QIAGEN GmbH). Q-solution is a detergent-like reagent which facilitates the amplification of 'difficult' DNA templates by modifying the melting behaviour of the DNA (QIAGEN GmbH, 1996). The possibility that suitable DNA could be extracted by the bacterial cell boiling method used to prepare DNA for amplification of TEM- and SHV-encoding genes (see Section 5.2.2.1, paragraph three) and the *ampR* gene (see Section 6.2.2.3, paragraph three), was considered. However, since the use of this method requires inclusion of a proteinase K treatment step and necessitates extraction of fresh DNA for each amplification assay, the modified Wizard™ Genomic DNA extraction protocol optimised for use in the *ampD* hybridisation studies was selected.

The results of PCR studies for the confirmation of the *ampD* sequence verified the difficulty involved in the amplification of *ampD* from *S. marcescens*. Using the *ampD* primer sets designed for the two 250 bp overlapping amplimers, a high yield of *ampD*-like PCR product was obtained using the DNA from all *S. marcescens* isolates including *S. marcescens* NCTC 11935. However, even with these short amplimers, it was necessary to employ a specialised PCR 'touchdown' protocol to obtain a single specific *ampD*-like product. Furthermore, although an *ampD*-like PCR product was obtained from DNA from all *S. marcescens* isolates using the published set of *ampD* primers (Stapleton *et al.*, 1995b), DNA from many isolates produced a low-yield PCR product despite extensive attempts to optimise the reaction conditions. When a study was performed to identify the critical factors involved in the amplification of the *ampD* gene, the results indicated that both the quantity and quality of template DNA determined the success of the amplification reaction. The poor quality of the DNA extracted in the absence of proteinase K and the low quantity of DNA used in the preliminary *ampD* PCR studies militated against successful amplification of the *ampD* template. Use of improved quality and increased quantity of template DNA together with the modified

reaction conditions used in the *ampD* confirmatory PCR studies, enhanced amplification of *ampD* target DNA.

DNA sequence analysis of the cloned *ampD*-like PCR products obtained using the published set of *ampD* primers confirmed the identification of the previously unknown *ampD* sequence in *S. marcescens*. Similar to *ampD* genes described in other AmpC-inducible enterobacteria, the *ampD* gene in *S. marcescens* consisted of an ORF of 564 bp located downstream of the *nadC* gene. The *ampD* gene in *S. marcescens* demonstrated significant similarity with the *ampD* sequence from wild-type *E. cloacae* and, to a lesser extent, with the wild-type *ampD* sequence in *C. freundii* and *E. coli*. The upstream intergenic region between *nadC* and *ampD* also shared significant similarity with the analogous region in other AmpC-inducible enterobacteria such as *C. freundii*. This high degree of *ampD* similarity was consistent with the level of *ampD* similarity (74-83%) previously recorded for other enterobacteria (Kopp *et al.*, 1993). The predicted 187 amino acid AmpD protein also demonstrated significant identity with the deduced AmpD from other enterobacteria. This identity suggests that, like other species with inducible AmpC, the *S. marcescens* AmpD also acts as a cytosolic amidase involved in the regulation of AmpC β-lactamase expression and murein metabolism.

Alignment of the *ampD* gene and the upstream *nadC-ampD* intergenic region from the nine *S. marcescens* test isolates and *S. marcescens* NCTC 11935, demonstrated that all nucleotide sequences shared 100% identity, regardless of the β-lactam susceptibility profile and the predicted AmpC phenotypes of the isolates. The *ampD* sequence from the *S. marcescens* isolates also failed to demonstrate the presence of mutations previously associated with derepressed phenotypes in other enterobacteria. These results indicated that in the *S. marcescens* test isolates examined, genetic alterations leading to partially-derepressed or derepressed AmpC phenotypes were not linked to mutations in *ampD* (Herra & Keane, 2001).

In enterobacteria, AmpC β-lactamase derepressed phenotypes are most commonly associated with mutations in the *ampD* locus (Jones *et al.*, 1997). Reports of β-lactamase derepression have, however, largely focused on isolates of *E. cloacae* or *C. freundii* where the frequency of *ampD* mutation, the high level of cephalosporin cross-resistance and the extremely high rate of constitutive β-lactamase over-production are

reflective of true AmpC derepressed phenotypes (Kopp *et al.*, 1993; Ehrhardt *et al.*, 1996). In addition, many of the reported *ampD* mutations have arisen in laboratory-derived mutants repeatedly exposed to *in vitro* antibiotic selective pressure under conditions which the organism is unlikely to encounter *in vivo*. In contrast, the *S. marcescens* isolates investigated in the present study were clinical isolates with low-level cephalosporin resistance and the majority exhibited β-lactamase hydrolysis profiles indicative of partial derepression. Moreover, even in the small percentage of *S. marcescens* isolates where the β-lactam hydrolysis pattern was suggestive of true AmpC derepression, failure to demonstrate high-level cross-resistance to all broad-spectrum agents including piperacillin-plus-tazobactam and ceftazidime was not characteristic of a fully-derepressed phenotype. Hence, it is possible that in partially-derepressed and derepressed isolates of *S. marcescens*, where the levels of AmpC β-lactamase-mediated resistance are considerably reduced compared to fully-derepressed isolates in other AmpC-inducible species, the mutational process involved in constitutive AmpC over-expression is different. It is also important to note that previous studies have demonstrated the presence of constitutive AmpC over-production in other AmpC-inducible enterobacteria in the absence of *ampD* mutations. In *C. freundii*, partially-derepressed phenotypes have been reported without evidence of *ampD* mutation (Stapleton *et al.*, 1995b). Moreover, recent reports of AmpC derepression in isolates of *P. aeruginosa* have also demonstrated the involvement of loci outside the *ampD* gene (Langaee *et al.*, 2000).

The results of the *ampD* transformation assays performed in the present study also suggest that constitutive AmpC β-lactamase over-production in *S. marcescens* isolates was not associated with mutations in the *ampD* gene. In the fully-derepressed isolate of *E. cloacae* which demonstrated high-level cephalosporin cross-resistance, the involvement of *ampD* was inferred by demonstrating the elimination of β-lactam resistance following transformation with wild-type *ampD*. However, in the derepressed *S. marcescens* isolate expressing lower levels of cephalosporin resistance, only marginal restoration of β-lactam susceptibility occurred. In contrast to these results, previous studies have reported the elimination of β-lactam resistance in derepressed isolates of *S. marcescens* (Gianneli *et al.*, 1994; Weindorf *et al.*, 1998). Examination of the β-lactam MIC values reported in these studies indicate, however, that resistance was only

eliminated in *S. marcescens* isolates expressing high-level AmpC-mediated cephalosporin cross-resistance (including resistance to ceftazidime). In isolates with lower levels of AmpC-mediated resistance (with β-lactam MIC values similar to values obtained with the *S. marcescens* isolates in the present study), the level of restoration of β-lactam susceptibility was reduced (Weindorf *et al.*, 1998).

The results of these studies indicate that in isolates of *S. marcescens*, where the high level of cephalosporin cross-resistance is truly characteristic of full-derepression, constitutive over-expression of AmpC may arise through mutation in the *ampD* gene. In such cases, the introduction of a functional *ampD* can override the defect in the native *ampD*, eliminate the accumulation of AmpC activating factors and restore full β-lactam susceptibility. However, in the majority of clinical isolates of *S. marcescens* where the levels of β-lactam resistance in partially-derepressed and derepressed phenotpyes are considerably reduced compared to levels of resistance in fully-derepressed phenotypes, constitutive over-expression of AmpC may be generated by an *ampD*-independent process. In the latter case, the introduction of an additional copy of *ampD* during transformation studies may act in combination with the native functional *ampD* to compensate for the imbalance in activating/inactivating factors. As a result, transformation with *ampD* would reduce the level of β-lactamase conferred resistance but not necessarily eliminate it.

6.4.2. **Identification and characterisation of *ampR* in *S. marcescens***

Preliminary amplification procedures for the *ampR* gene were also performed at an early stage of the study before the deleterious effects of *S. marcescens* DNase activity were recognised. Initial amplification reactions for *ampR* were therefore performed using template DNA extracted in the absence of proteinase K. Similar to the results obtained when investigating *ampD*, *S. marcescens* isolates also demonstrated *ampC*-specific amplimers in DNA template control amplification reactions but failed to produce specific products in *ampR* DNA amplification studies. However, unlike the results of the *ampD* hybridisation assays, low stringency hybridisation with DIG-labeled *ampR* PCR products from wild-type amplification control isolates also failed to hybridise with genomic DNA from the *S. marcescens* isolates (when genomic DNA was extracted in the presence of proteinase K). It thus appeared that whilst factors such as DNA quality

or quantity may have contributed to *ampR* amplification failure, it was more likely that lack of sequence identity between the designed *ampR* primers and the *ampR* target prevented the production of *ampR*-like PCR products. The assumption of *ampR* divergence in *S. marcescens* was supported by the degree of *ampR* diversity (20–40%) recorded among other AmpC-inducible enterobacteria (Lindberg & Normark, 1987; Poirel *et al.*, 1999).

Confirmation of *ampR* divergence in *S. marcescens* was obtained from analysis of unpublished *S. marcescens ampR* sequence data made available at MCRAB, Creighton University. Hence, on the basis of these data, DNA amplification studies for *ampR* were re-designed. The research group in MCRAB, Creighton University, had also experienced difficulties with amplification reactions involving *S. marcescens* isolates and had attributed these problems to DNase-induced template DNA damage. In the collaborative *ampR* amplification studies, a DNA extraction method was thus devised which incorporated the use of a shortened bacterial incubation time (DNase production is maximal during stationary phase) and a proteinase K treatment step. Using *S. marcescens ampR*-specific primers and 'DNase-inhibited' template DNA, a high-yield *ampR*-specific PCR product was obtained in all test isolates of *S. marcescens*.

Sequence analysis of the *ampR* PCR products from the nine test isolates revealed identification of the partial *ampR* gene in *S. marcescens*. The nucleotide sequence of the *ampR* PCR product and the deduced partial AmpR protein shared extensive identity (>93%) with the unpublished AmpR sequence data from wild-type *S. marcescens* isolate, Serr 1 (Morrow, 1999). However, although the partial AmpR sequence from *S. marcescens* was shown to demonstrate considerable identity (~70%) with the AmpR sequence from infrequently encountered AmpC-inducible enterobacteria such as *P. stuartii* and *H. alvei*, substantial variation (>50%) was observed when the sequence was compared with AmpR from more prevalent inducible species such as *E. cloacae* and *C. freundii*. These results suggest that although other common AmpC-inducible enterobacteria display considerable inter-species *ampR* diversity, in *S. marcescens* the *ampR* gene appears to demonstrate an even greater level of genetic heterogeneity.

Alignment of the *ampR* sequence from the nine *S. marcescens* test isolates revealed minimal sequence disparity (1%) amongst the isolates. Although such disparity may

arise during DNA amplification if *Taq* DNA polymerase allows base misincorporation, such errors tend to occur at random. The consistency of the nucleotide differences in the *ampR* sequence data presented in this study make this an unlikely explanation. Furthermore, since the observed base pair differences were not confined to a single β-lactam susceptibility-type pattern, they did not appear to be linked to altered AmpC expression. In addition, since the deduced AmpR amino acid sequence from the test *S. marcescens* demonstrated a single semi-conserved amino acid substitution which was also noted in the protein sequences of known wild-type *S. marcescens* isolates, the observed sequence difference was attributed to intra-species *ampR* diversity. The AmpR in *S. marcescens* also failed to demonstrate the presence of mutations previously associated with altered AmpC β-lactamase production in other AmpC-inducible enterobacteria. This *ampR* sequence data, albeit partial, thus indicated that AmpC expression in partially-derepressed and derepressed *S. marcescens* isolates was not linked to mutational events in *ampR*.

6.4.3. Characterisation of *ampC* and the *ampC-ampR* intercistronic region

DNA amplification studies to characterise the *ampC* gene were designed to generate a large 1.5 kb PCR product which would include the *ampC* structural gene and extend upstream through the *ampC-ampR* intercistronic region. During amplification procedures, less than half the *S. marcescens* test isolates generated an *ampC*-specific PCR product. Since the *ampC* primers were designed from known *S. marcescens* *ampC*-sequence data and all test isolates had previously demonstrated the presence of an *ampC* amplimer (≤1 kb) in DNA template control reactions, amplification problems were attributed to the size of the PCR product. In the GC-rich DNA extracted from *S. marcescens* isolates, large target sequences are likely to contain secondary structures such as hairpin loops, which could reduce the PCR yield or cause the PCR to fail. Despite the introduction of optimisation procedures including the use of increased DNA concentration, the inclusion of Q-solution and the application of 'touchdown' PCR protocols, it was not possible to resolve amplification difficulties in all test isolates. In addition, the presence of weak bands, suggestive of non-specific amplimers, in some *ampC* PCR reactions reduced the transformation efficiency of the cloned PCR products. Consequently, of the nine *S. marcescens* isolates processed, only two isolates (one wild-

type and one partially-derepressed) were available for *ampC* sequence analysis. Thus, to allow comparison of the *ampC* sequences from all AmpC phenotypes observed, it was necessary to include sequence data from a derepressed isolate obtained from *ampC* DNA template control amplification reactions. Whilst this additional data contained the sequence of most of the *ampC* gene including the regions previously implicated in *ampC* mutational events, it did not provide sequence data for the *ampC-ampR* intercistronic region.

Sequence analysis of the *ampC* PCR products confirmed identification of the *ampC* gene. As previously described in *S. marcescens* isolates, the *ampC* gene consisted of an ORF of 1136 bp which encoded a putative protein of 355 amino acids (Nomura & Yoshida, 1990). The deduced amino acid sequence demonstrated the characteristics of a typical AmpC β-lactamase enzyme i.e. the sequence included the three signature motifs involved in the formation of the active site of class C enzymes (Medeiros, 1997). The deduced AmpC sequence from *S. marcescens* test isolates also demonstrated at least 97% identity with the AmpC sequence from wild-type *S. marcescens* isolates (Nomura & Yoshida, 1990; Matsumura *et al.*, 1998). These results confirmed the high-level of AmpC conservation among *S. marcescens*.

When the AmpC sequences from the *S. marcescens* test isolates were compared, all three proteins shared 100% identity. Although limited by the small number of isolates tested, these results suggest that β-lactamase production in partially-derepressed and derepressed *S. marcescens* isolates was not associated with mutations in the structural *ampC* gene. The absence of mutations previously associated with extended AmpC β-lactamase activity in *S. marcescens* also suggests that the *ampC* gene was not implicated in altered AmpC expression in the *S. marcescens* test isolates (Matsumura *et al.*, 1998; Raimondi *et al.*, 2001). Confirmation of these preliminary observations on the role of the *ampC* gene in altered AmpC expression clearly require analysis of *ampC* sequence data from a larger series of *S. marcescens* isolates.

Sequence analysis of the *ampC-ampR* intergenic region revealed that the *S. marcescens* test isolates demonstrated overlapping *ampC-ampR* promoter regions. This overlap is indicative of divergent transcription of the *ampC* and *ampR* genes which is characteristic of inducible *ampC* genes in other enterobacteria (Lindquist *et al.*, 1989b; Poirel *et al.*,

1999). A LysR binding motif, similar to that described in *M. morganii* and *H. alvei*, was also identified in the *ampC-ampR* intercistronic region of the *S. marcescens* isolates (Poirel *et al.*, 1999; Girlich *et al.*, 2000a). Analysis of the *ampC-ampR* intergenic region also demonstrated that this region was highly conserved in *S. marcescens.* Demonstration of *ampC-ampR* similarity between the two *S. marcescens* test isolates suggested that AmpC expression in isolates with low-level cefotaxime resistance (partially-derepressed) did not appear to be associated with mutations in the promoter regions of the *ampC* and *ampR* genes.

Previous studies performed in MCRAB, Creighton University, have indicated substantial disparity in the *ampC-ampR* intergenic sequence of *S. marcescens* compared to other enterobacteria with inducible AmpC (Morrow *et al.*, 1999). Sequence analysis of the *ampC-ampR* region revealed an *ampC* 5' untranslated region (UTR) of 124 nucleotides in *S. marcescens* which was at least 70 bases longer than the *ampC* 5' UTR of *E. cloacae* and *C. freundii.* When the 5' UTR from *S. marcescens* was analysed for secondary structures, a previously unidentified 57 base stem-loop structure was recognised (Morrow *et al.*, 1999). Since the formation of secondary structures such as stem-loops in the 5' UTR of prokaryotes is commonly associated with post-transcriptional regulatory functions, these results may suggest a unique regulation mechanism of AmpC expression in *S. marcescens.* It is interesting to note that additional base pair sequences have been detected in the 5' UTR of the *ampC-ampR* overlap in *H. alvei* (67 bp segment) and *P. stuartii* (28 bp segment) both of which were found to demonstrate significant identity with the HTH DNA binding motif in the N terminus region of AmpR in *S. marcescens* (Girlich *et al.*, 2000a).

6.4.4. **Conclusion**

The results of this study confirm the identification of the structural *ampC* gene and the *ampD* and *ampR* regulatory genes in clinical isolates of *S. marcescens.* The previously unidentified *ampD* gene shared significant identity with the *ampD* gene from other enterobacteria. This level of conservation was expected since *ampD* is known to be involved in fundamental cell wall recycling pathways in Gram-negative bacteria. The *ampR* and *ampC* genes were highly conserved among *S. marcescens* isolates but showed significant divergence from the *ampR* and *ampC* genes in other common enterobacteria

with inducible AmpC expression, such as *E. cloacae* and *C. freundii*. Likewise, although the *ampC-ampR* intergenic region also demonstrated extensive intra-species similarity, significant disparity was observed when the sequence from *S. marcescens* was compared to the *ampC-ampR* region in other AmpC-inducible species. Furthermore, previous studies have indicated that the DNA sequence difference demonstrated in the *ampC-ampR* region of *S. marcescens* may be associated with AmpC regulatory functions (Morrow *et al.*, 1999). These results indicate that although AmpC expression in *S. marcescens* appears to result from the interaction of a genetic assembly analogous to the *ampC-ampR* systems described in other enterobacteria, the sequence diversity displayed within the *S. marcescens ampC-ampR* complex may indicate a novel mechanism for AmpC β-lactamase regulation.

The results of DNA sequence alignment studies and *ampD* transformation assays suggest that the mechanism of AmpC over-production in *S. marcescens* isolates demonstrating low-level AmpC-mediated resistance, may also differ compared to other enterobacteria with inducible β-lactamase production. Although constitutive AmpC over-production in enterobacteria is most commonly associated with mutations in *ampD*, the *ampD* sequence from wild-type, partially-derepressed and derepressed isolates of *S. marcescens* demonstrated full sequence identity. The results of *ampD* transformation studies also indicated that the mechanism of derepression in *S. marcescens* isolates with low-level cephalosporin resistance was independent of the *ampD* locus. Similarly, although altered AmpC expression in enterobacteria has been attributed to mutations in the *ampR* and *ampC* genes, the *S. marcescens* isolates in the present study demonstrated >98% sequence similarity in their respective *ampR* and *ampC* genes, regardless of the AmpC β-lactamase phenotype of the isolates. These results suggest that in *S. marcescens* isolates with low-level AmpC-mediated resistance, genetic alterations leading to partially-derepressed and derepressed AmpC phenotypes were not linked to mutations in *ampD*, *ampR* or *ampC*. The absence of these mutations in *S. marcescens* isolates with low-level AmpC-mediated cephalosporin resistance may also indicate a novel mechanism for AmpC β-lactamase regulation with respect to other AmpC-inducible enterobacteria.

Chapter VII
General Discussion

7.1. GENERAL DISCUSSION

The clinical and epidemiological importance of AmpC-inducible Gram-negative bacteria and their stably derepressed mutants has increased dramatically since the introduction of third-generation cephalosporins. Resistance to broad-spectrum β-lactams has emerged in 16–44% of AmpC-inducible species following treatment with third-generation cephalosporins, even when these antibiotics were used in combination with agents from other antimicrobial classes (Jones *et al.*, 1997). Multiply-resistant, stably-derepressed mutants have spread widely both locally, within hospitals and internationally (Livermore, 1998). These resistant mutants are most often isolated from seriously ill patients such as those in an intensive care setting and pose one of the greatest challenges to contemporary chemotherapy of infections in hospitalised patients.

In the past, the mechanism of AmpC expression has been studied most extensively in prevalent AmpC-inducible species such as *E. cloacae* and *C. freundii*. In these organisms the phenotypic expression and clinical significance of AmpC β-lactamase production has been well documented (Ehrhardt & Sanders, 1993). The structural gene, *ampC*, and the regulatory genes, *ampR* and *ampD*, have been cloned and sequenced and much of the mechanism for control of AmpC expression has been elucidated (Honore *et al.*, 1989; Lindquist *et al.*, 1989b). AmpC β-lactamase expression and *ampC-ampR* regulatory systems have also been described for other AmpC-inducible species such as *P. aeruginosa* and *M. morganii* (Lodge *et al.*, 1993; Poirel *et al.*, 1999). More recently, the mechanism of AmpC β-lactamase production in infrequently encountered AmpC-inducible species such as *P. stuartii* and *H. alvei* has been reported (Girlich *et al.*, 2000a). Nevertheless, despite the increasing prevalence of *S. marcescens* as a serious nosocomial pathogen and despite its increasing association with broad-spectrum β-lactam resistance (Knowles *et al.*, 2000), the expression of AmpC β-lactamase in *S. marcescens* has remained largely unexplained.

Although β-lactam resistance in *S. marcescens* is increasingly associated with the acquisition of plasmid-mediated ESBLs (Perilli *et al.*, 1997; Luzzaro *et al.*, 1998), studies have demonstrated that cephalosporin resistance is most commonly mediated by AmpC β-lactamase production (Hechler *et al.*, 1989; Weindorf *et al.*, 1998). Previous

investigations have suggested that the production of AmpC β-lactamase and the associated level of β-lactam resistance in derepressed isolates of *S. marcescens* appeared reduced compared to other enterobacteria with inducible β-lactamase expression (Curtis *et al.*, 1986; Stapleton *et al.*, 1995a). Other reports have also postulated that constitutive β-lactamase over-production in *S. marcescens* may occur as a result of mutation in *ampD* (Weindorf *et al.*, 1998). More recently, mutations in *ampC* have been linked to extended AmpC β-lactamase activity in *S. marcescens* (Raimondi *et al.*, 2001). However, none of these studies has investigated the molecular mechanism of AmpC expression in *S. marcescens,* nor have they considered the clinical significance of the AmpC resistant phenotypes expressed in this species. Moreover, previous studies have tended to work on individual isolates or small groups of isolates, many of which were derived by laboratory selection. The results of such studies may thus have little application to a clinical population of *S. marcescens* isolates.

Serratia marcescens was first reported as a cause of nosocomial infection in Dublin hospitals in the 1980's (Coleman *et al.*, 1984) and soon became established within the hospital environment (Hejazi *et al.*, 2000). By 1995, there was evidence of emerging resistance to antimicrobial agents such as β-lactams with increasing numbers of isolates demonstrating low-level resistance to commonly used cephalosporins (Hejazi, 1997). This low-level cephalosporin resistance was assumed to arise from constitutive AmpC β-lactamase over-production and as a result, therapy with all third-generation cephalosporins was contra-indicated (van Ogtrop *et al.*, 1997). Moreover, numerous reports of failure of combination therapy with cephalosporins and other β-lactam agents or with cephalosporins and agents from other classes of antibiotics such as aminoglycosides, necessitated the use of agents such as meropenem which are normally kept in reserve (Sanders & Sanders, 1992). Hence, as the prevalence of β-lactam-resistant *S. marcescens* isolates continued to rise in Dublin hospitals and as antimicrobial treatment options became increasingly limited based on the assumption of AmpC derepression, an investigation of this resistance was necessary. Since *S. marcescens* was already established as an endemic pathogen in Dublin hospitals it was necessary to include large numbers of clinical isolates in the study. In this way the prevalence of AmpC β-lactamase-mediated resistance and the mechanism of AmpC β-lactamase expression in *S. marcescens* could be investigated in a clinical setting.

Increasing prevalence of cephalosporin resistance had been the impetus for the present investigation and over the six-year study period, the rate of cephalosporin resistance among *S. marcescens* continued to rise from 45% to 60% of isolates. Phenotypic expression studies indicated that the cephalosporin resistance was mediated by over-production of AmpC β-lactamase. The majority of 'cephalosporin resistant' *S. marcescens* isolates expressed low-level resistance to some cephalosporins and remained fully susceptible to other broad-spectrum β-lactam agents. A small percentage of 'cephalosporin resistant' isolates demonstrated increased levels of resistance to β-lactam agents such as cefotaxime, but susceptibility to other β-lactams such as ceftazidime was only marginally reduced. Hence, the β-lactam resistance profile of the *S. marcescens* population was not indicative of a fully-derepressed AmpC phenotype.

B-lactam hydrolysis assays demonstrated that the majority of 'cephalosporin resistant' *S. marcescens* isolates (low-level resistant isolates) were associated with a partially-derepressed phenotype where marginal inducibility was retained and levels of AmpC β-lactamase over-production remained low. In the small number of isolates with increased levels of cephalosporin resistance, the β-lactam hydrolysis pattern was indicative of true derepression but even in these isolates, the level of constitutive β-lactamase over-production appeared reduced compared to other derepressed enterobacteria. The reduced level of β-lactam resistance in partially-derepressed and derepressed isolates of *S. marcescens* appeared to mirror the relative reduction in the level of AmpC β-lactamase over-production. These results indicate that *S. marcescens* demonstrates a propensity to mutate to a state of partial-derepression rather than a fully-derepressed phenotype. This predisposition has not been described in other AmpC-inducible enterobacteria but is prevalent in *P. aeruginosa* (Livermore, 1995). Studies have shown that over 75% of *P. aeruginosa* isolates demonstrating constitutive β-lactamase over-production were associated with a partially derepressed phenotype and similar to *S. marcescens*, the reduced level of β-lactam resistance in these isolates was related to a reduction in β-lactamase over-production (Chen *et al.*, 1995).

Recent studies have suggested that low-levels of β-lactam resistance in derepressed isolates of *S. marcescens* may be related to the reduced affinity of the AmpC β-lactamase for some cephalosporins (Raimondi *et al.*, 2001). Whilst this reduced enzyme

activity may explain the marked reduction in MIC values for third-generation cephalosporins such as ceftazidime which exhibit slow rates of hydrolysis, it does not account for the relative reduction in resistance to other broad-spectrum β-lactams such as piperacillin-plus-tazobactam. Thus it is likely that low levels of constitutive β-lactamase over-production combined with reduced levels of β-lactamase activity are responsible for the significant reduction in β-lactam resistance observed in derepressed isolates of *S. marcescens,* compared to other AmpC-inducible enterobacteria.

To gain further evidence that AmpC β-lactamase production was responsible for cephalosporin resistance in the serratia population, the possible contribution of other likely sources of β-lactamase-mediated resistance was evaluated using a combination of phenotypic and genotypic resistance detection methods. Phenotypic expression studies and plasmid DNA analysis failed to demonstrate the presence of ESBL-mediated resistance in cephalosporin-resistant *S. marcescens* isolates. DNA amplification for TEM and SHV-specific ESBLs revealed the presence of TEM-like PCR products in ~30% of the *S. marcescens* isolates. Sequence analysis of the TEM-like PCR products demonstrated, however, that they shared 100% identity with classical TEM-1 β-lactamase, which has no intrinsic activity against broad-spectrum β-lactam agents. The contribution of TEM and SHV-derived ESBLs was thus excluded as a source of cephalosporin resistance in the *S. marcescens* population.

It thus appeared that the cephalosporin resistance demonstrated by the *S. marcescens* test isolates was mediated by AmpC β-lactamase over-production. However, the indication that AmpC derepression in *S. marcescens* was not associated with the same high level of β-lactamase over-production evident in other derepressed enterobacteria suggested variation in the mechanism of AmpC expression. Hence, since the AmpC-regulatory machinery in *S. marcescens* was unknown, genetic studies were designed to identify the putative *ampD* and *ampR* regulatory genes and the *ampC* structural gene and to examine the molecular mechanism of constitutive AmpC over-production. Previous studies of AmpC expression in other enterobacteria have correlated mutations in individual AmpC-related genes, usually *ampD*, with the demonstration of AmpC phenotypes (Stapleton *et al.*, 1995b). Reports also indicate the involvement of mutations in *ampR* and *ampC* in altered AmpC β-lactamase expression (Kuga *et al.*, 2000; Raimondi *et al.*,

2001). The present investigation was thus designed to investigate all three genetic loci, *ampD*, *ampR* and *ampC* in clinical isolates of *S. marcescens* expressing wild-type, partially-derepressed and derepressed AmpC phenotypes.

The *ampD* gene in *S. marcescens* was identified on a 564 bp ORF located downstream of the *nadC* gene. The ORF encoded a putative protein of 187 amino acids. This is the first report of sequence data describing the *ampD* gene in *S. marcescens*. The predicted AmpD protein in *S. marcescens* shared over 90% identity with AmpD in *E. cloacae* suggesting that AmpD is highly conserved among AmpC-inducible enterobacteria and inferring a common involvement in murein metabolism and AmpC expression. However, in contrast to other enterobacteria, sequence analysis of the *ampD* gene in *S. marcescens* indicated that β-lactamase over-production in partially-derepressed and derepressed isolates was not associated with mutations in *ampD* (Herra & Keane, 2001). Transformation studies with plasmid DNA encoding wild-type *ampD* from *E. cloacae* also suggested that altered AmpC expression in *S. marcescens* test isolates was not linked to *ampD*. Although previous reports of *ampD* transformation studies in *S. marcescens* had implied the involvement of the *ampD* gene in constitutive β-lactamase over-production, these results were confined to rare isolates demonstrating a high-level cephalosporin resistance profile indicative of full derepression (Weindorf *et al.*, 1998).

The results of the present study indicated that constitutive β-lactamase over-production in *S. marcescens* with low-level β-lactam resistance, was independent of the *ampD* locus. Although constitutive β-lactamase over-production in other AmpC-inducible species is most commonly associated with mutations in *ampD*, partially-derepressed isolates of *C. freundii* and derepressed isolates of *P. aeruginosa* have been described in the absence of *ampD* involvement (Stapleton *et al.*, 1995b; Langaee *et al.*, 2000). Studies have also suggested that in *P. aeruginosa*, full derepression may only emerge via a second mutation in bacterial cells that are already partially derepressed (Livermore, 1991a). If a similar mechanism existed in *S. marcescens*, it could be envisaged that partial derepression might arise through mutation at a genetic locus other than *ampD* and that full derepression might then arise following a second mutational event involving the *ampD* gene. More recent reports in *P. aeruginosa* have also demonstrated the presence of an additional ORF downstream of *ampD*, which encodes a protein that exhibits properties of a cytoplasmic protein (Langaee *et al.*, 1998). It is postulated that

this cytoplasmic protein may affect AmpC β-lactamase expression by interacting directly with AmpD or by acting as a regulator of *ampD* expression. The identification of this cytoplasmic protein demonstrates the presence of a genetic locus other than *ampD,* which may be involved in constitutive β-lactamase over-production.

The *ampR* gene sequence in *S. marcescens* is as yet unpublished. In this present study, the partial *ampR* sequence in *S. marcescens* was identified on a 617 bp PCR product. When the deduced AmpR sequence in *S. marcescens* was compared to AmpR in common AmpC-inducible enterobacteria such as *E. cloacae*, considerable diversity (>50%) was displayed. When compared with the AmpR sequence from infrequently encountered AmpC-inducible species such as *P. stuartii* and *H. alvei,* the *S. marcescens* AmpR sequence demonstrated increased identity, particularly within the amino-terminal DNA-binding domain typical of transcriptional activators of the LysR family. These results suggest that in *S. marcescens*, AmpR displays a significant level of genetic divergence. Nevertheless, the AmpR sequence identity shared amongst the *S. marcescens* test isolates indicated that mutations resulting in partially-derepressed and derepressed phenotypes were not associated with the *ampR* gene.

The structural *ampC* gene in *S. marcescens* was identified on an ORF of 1136 bp. The three consensus sites, SXXK, YSN and KTG, characteristic of class C β-lactamases were detected in the deduced AmpC amino acid sequence (Medeiros, 1997). The unpublished *ampC-ampR* intercistronic region in the *S. marcescens* test isolates demonstrated the presence of overlapping *ampC* and *ampR* promoter regions typical of the divergent transcription of *ampR* and *ampC* described in other AmpC-inducible species (Poirel *et al.*, 1999). A LysR binding motif with significant sequence similarity to the binding motif present in the *ampC-ampR* intercistronic region of *M. morganii* and *H. alvei* was also identified in the *ampC-ampR* intercistronic region of *S. marcescens*. Although limited by the small number of sequences analysed, the sequence identity between wild-type and partially-derepressed isolates indicated that altered β-lactamase production in the *S. marcescens* population was not related to mutational events in the *ampC* gene or the *ampC-ampR* intergenic region.

Despite the demonstration of intra-species identity, the *ampC-ampR* intercistronic region in *S. marcescens* displayed substantial diversity when compared to analogous sequences

in common AmpC-inducible enterobacteria. Previous studies in MCRAB, Creighton University, have suggested that this sequence diversity may be associated with the regulation of AmpC expression (Morrow, 1999). Sequence analysis of the *ampC-ampR* region identified the presence of a unique 57 base stem-loop structure in the *ampC* 5' UTR of *S. marcescens* (Morrow *et al.*, 1999). The formation of secondary structures within the 5' UTR of the transcript are commonly associated with post-transcriptional regulation (Hansen *et al.*, 1994). Although it is generally considered that the regulation of gene expression in prokaryotes is controlled at the level of transcription initiation, more recent studies indicate that prokaryotic genes possess more complex control systems such as post-transcriptional regulatory mechanisms (Brumlik & Storey, 1998). The presence of a stem-loop in the *ampC* 5' UTR of *S. marcescens* may thus indicate the presence of a novel regulatory mechanism of AmpC expression.

The sequence diversity of the *ampC-ampR* genetic assembly in *S. marcescens* and the absence of *ampD*, *ampR* and *ampC* mutations in partially derepressed and derepressed isolates with low-level cephalosporin resistance, indicate that the mechanism of AmpC regulation in *S. marcescens* may differ compared to other AmpC-inducible enterobacteria. Further investigation of the molecular mechanism of AmpC β-lactamase expression in *S. marcescens* is thus warranted. To confirm that the genetic alterations leading to partially-derepressed or derepressed AmpC phenotypes in *S. marcescens* were not linked to mutations in *ampD, ampR* or *ampC*, it would be necessary to expand DNA amplification studies to enable sequence analysis of all three genes from a large number of clinical isolates representative of all AmpC phenotypes.

In the *ampC* amplification assays performed in the present study, less than half the *S. marcescens* test isolates produced an *ampC*-specific PCR product. To facilitate amplification of the required 1.5 kb *ampC* amplimer in a larger number of *S. marcescens* isolates, the introduction of an additional amplification strategy should be considered. The application of DNA amplification systems such as Expand™ High Fidelity (Boehringer Mannheim) which combine the 5'–3' polymerase activity of *Taq* DNA polymerase with the 3'–5' proof-reading ability of *Pwo* DNA polymerase and thus offer greater fidelity and higher yield than conventional thermostable polymerases, is one worthwhile approach. When *ampR* DNA amplification assays were undertaken in the present study, the final 20 bp sequence at the 3' end of *ampR* in *S. marcescens* was

unknown. Since then this sequence was deposited in the Genbank by other workers (Barnaud *et al*, Genbank accession number AJ271368). Thus amplification and sequence analysis of the full *ampR* gene is now possible. Alternatively, it may be useful to consider techniques for the examination of genomic clones of *ampR* and *ampC*, similar to the strategy used in the investigation of the *ampD* gene in the present study. The *S. marcescens ampD* transformation studies performed in the current investigation were also limited by low sample number. In further investigations, *ampD* complementation assays could be extended by examining an increased number of *S. marcescens* isolates and by analysing transformants for direct evidence of altered AmpC expression in addition to investigating restoration of β-lactam susceptibility.

Further insight into the mechanism of low-level AmpC β-lactamase over-production in in partially-derepressed and derepressed isolates of *S. marcescens* would be gained by application of Northern hybridisation assays to investigate expression of the *ampC*, *ampD* and *ampR* genes. Preliminary studies performed in MCRAB, Creighton University, indicate that the amount of uninduced *ampC* transcript produced by *S. marcescens* was two-fold less than *ampC* RNA extracted from similar cell numbers of *E. cloacae* and *C. freundii* (Mahlen & Hanson, 2000). These results suggest that even in the wild-type AmpC phenotype, the expression of the *ampC* structural gene in *S. marcescens* is reduced compared to other AmpC-inducible enterobacteria. The application of Western blot assays, for comparison of total AmpC protein production, would also prove valuable in the investigation of AmpC expression in *S. marcescens*.

To further clarify the mechanism of altered AmpC expression and low-level cephalosporin resistance in *S. marcescens,* possible sites of additional genetic loci involved in constitutive AmpC β-lactamase over-production should also be explored. AmpG encodes a transmembrane protein which is thought to function as a permease for the AmpC induction signal and similar to *ampD*, this gene has also been found in all enterobacteria tested to date (Dietz *et al.*, 1996). Previous studies in *E. cloacae* and *C. freundii* have demonstrated that, in the absence of *ampG*, neither induction nor high-level AmpC expression is possible (Korfmann & Sanders, 1989; Lindquist *et al.*, 1993). Identification of *ampG* in *S. marcescens* would allow investigation of its possible role in altered AmpC expression. The investigation of additional AmpC-associated genetic structures similar to the ORF located downstream from *ampD* in *P. aeruginosa* may also

represent a candidate genetic locus with involvement in constitutive β-lactamase over-production in *S. marcescens* (Langaee *et al.*, 1998).

It might also be useful to extend the investigation of AmpC expression to include the possible involvement of other regulatory proteins. Recent reports suggest that the protein product of the *gcvA* gene (which is involved in the regulation of enzymes required in the glycine cleavage system) may act as a transcriptional activator of *ampC* expression in response to glycine (Hanson & Sanders, 1999). The role of bacterial proteins involved in cell wall division and cell wall synthesis should also be considered. The protein product of the *ftsZ* gene is intimately involved in the reaction cascade that mediates the septation-division process (Bennett & Chopra, 1993). Inactivation of the *fts*Z gene could thus trigger AmpC expression due to perturbations in cell wall synthesis (Hanson & Sanders, 1999). Evidence also indicates that PBP's are involved in the regulation of AmpC expression by determining the level of anhydromuramylpeptides in the periplasmic space (Pfeifle *et al.*, 2000). Inactivation of nonessential and essential PBPs results in increased levels of AmpC β-lactamase induction (Pfeifle *et al.*, 2000).

In the present study, although attempts were made to exclude the possible involvement of other likely sources of β-lactamase-mediated resistance, the contribution of non-β-lactamase resistance mechanisms was not considered. Previous studies have described the secondary contribution of reduced β-lactam permeability in *S. marcescens* isolates demonstrating constitutive AmpC β-lactamase over-production (Weindorf *et al.*, 1998). Although evidence of porin defects has been confined to rare high-level β-lactam-resistant isolates of *S. marcescens,* the possible contribution of reduced permeability in low-level β-lactam-resistant isolates also warrants investigation.

Regardless of the many possible mechanisms of resistance (summarised in Table 7.1), the data presented in the current study indicate a unique regulation of AmpC β-lactamase production in *S. marcescens* compared to other enterobacteria. In addition to exploring the mechanism of expression, an unanswered question is 'why would *S. marcescens* employ a different mechanism?' Perhaps the simple answer lies in the fact that *S. marcescens* is in itself 'different' from other enterobacteria. The organism has a 58 mol% G + C content (Grimont & Grimont, 1978). This is the highest G + C content

Table 7.1. Possible mechanisms of resistance in low-level cephalosporin-resistant *S. marcescens* isolates.

Resistance	Possible mechanism	References
AmpC β-lactamase-mediated		
Mutations implicated in AmpC-inducible GNBs		
ampD (Figure 2.4, Table 6.2)	Inactivation of AmpD alleviates *ampC* repression*	Weindorf *et al.*, 1998
ampR (Figure 6.15, panel B)	Inactivation of AmpR prevents *ampC* repression*	Kuga *et al.*, 2000
ampC (Figure 6.17)	Extension of AmpC β-lactamase hydrolysis profile*	Matsumura *et al.*, 1998
Suggested mutations in AmpC-related genes		
ampG	Alteration in cytoplasmic pool of AmpC-induction signals	Lindquist *et al.*, 1993
Genes in addition to *ampD*	Partial-derepression arises by mutation in sites other than *ampD*§	Langaee *et al.*, 1998
Other putative mutational sites		
gcvA	Transcriptional activation of *ampC* in response to glycine	Hanson & Sanders, 1999
ftsZ	Activation of *ampC* expression by perturbation in the cell wall	Hanson & Sanders, 1999
PBPs	Altered *ampC* expression by regulating the muropeptide level	Pfeifle *et al.*, 2000
ESBL-mediated		
TEM- and SHV-ESBLs (Table 2.5)	Extension of substrate profile of classical TEM/SHV enzymes*	Luzzaro *et al.*, 1998
Non-TEM, non-SHV-ESBLs (Table 2.5)	Extension of substrate profile of non-TEM/non-SHV enzymes	Bonnet *et al.*, 2000
Reduced permeability		
Porin mutation (Figure 2.5)	Reduced permeability to antimicrobial agents	Weindorf *et al.*, 1998

*, mechanisms of resistance investigated in the present study;
§, full derepression may subsequently arise by a second mutation in the *ampD* gene.

among enterobacteria. The genome size of *S. marcescens* is also larger than that of *E. coli* by 10^9 daltons (Grimont & Grimont, 1978). Studies on DNA relatedness also show that the genus *Serratia* is distinct from all known genera of the family *Enterobacteriaceae*. *Serratia marcescens* is more distantly related to *E. coli* than are *Shigella* spp., *Citrobacter* spp., *Salmonella* spp., *K. pneumoniae* or *Enterobacter aerogenes*. Moreover, all comparative studies on enzyme properties and regulation show consistent differences between the genus *Serratia* and the rest of the enterobacteria (Grimont & Grimont, 1978). Since *Serratia* spp. clearly appear to have diverged from *E. coli* to a greater extent than have *Enterobacter* spp., *Klebsiella* spp. or *Citrobacter* spp. perhaps it not surprising that the expression of an enzyme dependent on fundamental cell wall recycling properties may also differ. Considering the divergence of *S. marcescens* from enterobacteria and the difference in its natural habitat compared to other enterobacteria (Grimont & Grimont, 1978), it is possible that in *S. marcescens*, AmpC has additional functions in cell wall synthesis and recycling, other than the protective response against β-lactam antibiotics.

Considering the possibility of additional AmpC functions in *S. marcescens*, it is also conceivable that the production of high-level constitutive β-lactamase production seen in other enterobacteria, may represent too high a genetic price for this organism. Studies have shown that the generation time of *S. marcescens* isolates with high-level AmpC production is twice that of isolates with lower levels of resistance and that the former isolates reverted spontaneously to low-level resistance in the absence of selection (Hechler *et al.*, 1989). Similar findings have been described in *Salmonella* spp. which, in contrast to most members of the *Enterobacteriaceae* family, lack the *amp*C structural gene. Studies investigating the absence of *ampC* have suggested that the maintenance and expression of *amp*C may represent an untenably high cost for *Salmonella* spp. in terms of reduction in essential survival factors such as growth rate and invasiveness (Morosini *et al.*, 2000). Following transformation with AmpC-encoding plasmids, isolates of *Salmonella typhimurium* demonstrated an alteration in colony morphology and a dramatic reduction in the growth rate, invasiveness and intracellular replication. However, following acquisition of both *amp*C and the negative regulatory *ampR* gene, the deleterious burden of AmpC expression was removed (Morosini *et al.*, 2000). Thus similar to *S. marcescens*, the biological cost of AmpC expression in *Salmonella* may only become limiting during high-level constitutive β-lactamase production. A possible

mechanism by which *S. marcescens* can overcome this high biological cost and still exhibit high-level β-lactam resistance is to combine lower-level AmpC β-lactamase production with reduced permeability arising from porin defects (Weindorf *et al.*, 1998).

Alternatively, differences in the level of constitutive AmpC over-production and β-lactam resistance levels in *S. marcescens* may reflect differences in the extent to which the organisms have been exposed to β-lactam agents. Although *S. marcescens* has become widely distributed in hospitals in the last two decades, affected patients frequently display colonisation rather than infection and, in many cases, treatment is not required (Aucken & Pitt, 1998). It is therefore possible that *S. marcescens* has not been exposed to the same degree of broad-spectrum β-lactam selective pressure experienced by other AmpC-inducible enterobacteria such as *E. cloacae*. Studies have demonstrated that the pattern of β-lactam resistance among enterobacteria can be correlated with the use of individual agents (Jones *et al.*, 1997). Ceftazidime was introduced in 1983 but has not been widely used except in cases of serious sepsis. Differences in exposure rates may thus explain why 21–40% of *E. cloacae* express resistance to ceftazidime but only 4% of clinical isolates of *S. marcescens* show similar resistance (Jones *et al.*, 1997; Aucken & Pitt, 1998). Statistics from a case control study performed during 1993 and 1994 in one Dublin hospital also suggested that exposure of *S. marcescens* to ceftazidime was considerably less than exposure to other antimicrobial agents. Only 5% of patients with *S. marcescens* infection were treated with ceftazidime compared to 35% and 30% of patients with *S. marcescens* infection, who were treated with cefotaxime and gentamicin, respectively (Hejazi, 1997).

The essential differences in genetic makeup, natural habitat and innate pathogenicity of *S. marcescens* compared to other common enterobacteria, may thus explain why the relative level of AmpC-mediated resistance in *S. marcescens* is reduced. Nevertheless, whilst it may appear that, compared to the resistance levels seen in *E. cloacae* and *C. freundii*, (which clearly preclude the use of third-generation cephalosporins), AmpC mutants in *S. marcescens* might still be within the therapeutic range for a least some β-lactam agents, the risk of selecting fully-derepressed mutants suggests that caution should be exercised (Curtis *et al.*, 1986; Livermore, 1995). The increasing prevalence of *S. marcescens* as a nosocomial pathogen and the propensity of the organism for molecular evolution suggests that it would be prudent to avoid the use of all third-

generation cephalosporins, either alone or in combination with other agents, in clinical settings where AmpC derepression is a possibility.

Although it may appear that *S. marcescens* is unique among AmpC-inducible enterobacteria, it is more likely that the *ampC-ampR* genetic interaction in *S. marcescens* is merely different from the AmpC expression mechanisms described in common enterobacteria. The data presented in the current investigation suggest that *S. marcescens* may share similarity with the *ampC-ampR* genetic assembly recently described in infrequently encountered AmpC inducible species such as *H. alvei* and *P. stuartii* (Girlich *et al.*, 2000a). It may be that the mechanism of AmpC expression is not the same for every organism encoding an inducible *ampC* gene. Each genus and perhaps each species may regulate *ampC* expression by mechanisms unique to the physiological requirements of that organism (Hanson & Sanders, 1999).

As more is learnt about the mechanisms of β-lactamase resistance, it is becoming clear that bacteria are extremely adept at customising resistance mechanisms to overcome the action of β-lactam agents. The β-lactamases of all Gram-negative bacteria including *S. marcescens* are evolving dynamically. In the past twenty years, Gram-negative bacteria have responded to the widescale use of broad-spectrum β-lactam agents not only by increasing the amounts of protective β-lactamase produced but also by modifying the enzymes to generate extended substrate profiles. More recently these bacteria have expanded their opportunities for resistance by demonstrating the ability to simultaneously express multiple types of β-lactamases, some of which are encoded by genes that have moved from chromosomal to plasmid locations and have demonstrated the capability of widespread dissemination (Hanson *et al.*, 1999). This ongoing evolutionary process presents many problems for the accurate detection of β-lactam resistance and selection of appropriate treatment.

The emerging pattern of β-lactamase-mediated antimicrobial resistance clearly demonstrates that β-lactam resistance is a function of time and β-lactam use. In Europe and the US where broad-spectrum β-lactam agents have been used as first line drugs, β-lactamase-producing Gram-negative bacteria have become a clinical threat while carbapenem resistance has remained rare (Jones *et al.*, 1997). In Japan where

carbapenems have been used as first line drugs in every clinical setting for the last 15 years, carbapenem-resistant Gram-negative bacteria are isolated with increasing frequency in clinical settings whilst ESBL-producing bacteria are uncommon (Arakawa *et al.*, 2000). It thus appears that whilst β-lactam resistance is manifest globally, emerging resistance more often originates and develops locally, within individual countries, cities and hospitals, where bacteria are repeatedly exposed to similar antibiotics under similar clinical conditions (Medeiros, 2000). Although β-lactam resistance as a clinical problem was not inevitable, the extensive overuse of broad-spectrum β-lactam agents has made it unavoidable (Levy, 2000).

To preserve the efficacy of current effective β-lactam agents the judicious use of antimicrobials must be promoted. The use of more appropriate β-lactams, shorter treatment courses and cyclic treatment regimens should be considered. That said, whilst there are many recommendations for the prudent use of β-lactams, few studies have defined 'appropriate' use in specific terms of optimium dosages to maximise cure and to minimise selection of resistance genes, or optimium duration of antibiotic therapy for specific infections. Moreover, assuming comprehensive strategies for appropriate antimicrobial use are devised, continual audit must also be undertaken to guarantee successful implementation of these policies.

In the past, it was possible to design new β-lactam agents to overcome antimicrobial resistance. However, the ever-increasing diversity and spread of clinically significant β-lactamase enzymes combined with the increasing tendency for bacteria to express multiple enzymes, undermines this strategy. Fourth-generation agents such as cefpirome and cefepime were developed to meet the challenge of AmpC-mediated resistance but are now faced with the emergence of ESBLs to which they are susceptible. Similarly whilst carbapenems are presently considered an obvious strategy to overcome both AmpC enzymes and ESBLs, the emergence of carbapenemases such as IMP-1 threatens this approach (Ito *et al.*, 1995). In the near future there is no guarantee that the development of new antimicrobials can keep pace with the ability of bacterial pathogens to develop resistance. Since few new β-lactamase-resistant drugs are presently at advanced stages of development, the options for control of β-lactamase-

mediated resistance lie in accurate detection and monitoring of resistance and most importantly in the design and implementation of effective infection control policies.

To facilitate the effective monitoring of β-lactam resistance, clinical laboratories must employ accurate methods for the detection of β-lactamase production. Firstly, it is necessary that laboratories engage in an active programme of bacterial identification. In the absence of accurate speciation of GNB, it is not possible to predict the likely mechansim of resistance nor is it possible to select suitable resistance detection methods. In the clinical microbiology laboratory, the detection of β-lactamase-mediated resistance is largely based on routine qualitative disc diffusion susceptibility test methods. In the case of AmpC β-lactamase, these methods are generally considered adequate since the level of resistance associated with constitutive AmpC over-production, even for low-level AmpC-producers such as *S. marcescens*, are within the sensitivity range of the disc diffusion method. Moreover, since AmpC expression is chromosomally-encoded, all isolates of GNB known to express inducible AmpC are capable of producing the enzyme. Hence, it is generally agreed that identification of the organism is sufficient to indicate the possibility of AmpC-mediated resistance and the requirement to avoid cephalosporin therapy (Sanders & Sanders, 1988).

The specificity and sensitivity of qualitative susceptibility test methods is inadequate for the accurate detection of plasmid-encoded ESBLs in the clinical laboratory (Coudron *et al.*, 1997). In Europe, it is estimated that up to 35% of ESBL-producing organisms are incorrectly reported as susceptible to cephalosporins (Carter *et al.*, 2000). A recent report of a joint working party of the Irish Academy of Medical Laboratory Science and the Irish Society of Clinical Microbiologists has emphasised the need to adopt a standardised susceptibility test protocol to improve the accuracy of susceptibility test methodology and resistance detection (Anonymous, 2001). Regardless of the advantages of improved standardisation, it should be noted that the interpretation of all disc diffusion susceptibility test methods is limited by the design of the test method. In the context of β-lactamases such as ESBLs, which mediate low-level resistance *in vitro*, a susceptible result is little guarantee of clinical success. Since the predictive value of resistance detection is higher, it is advisable that susceptibility test protocols be designed to emphasise accurate detection of resistance.

Clinical laboratories must employ specialised methods for the accurate detection of ESBLs. Traditional disc synergy methods such as the double disc synergy test are less than optimal for the detection of ESBLs (Thomson *et al.*, 1991). The use of Etest ESBL strips which incorporate the use of a β-lactamase inhibitor offer improved sensitivity and convenience, but high expense is a limiting factor (Cormican *et al.*, 1996). The introduction of the Oxoid combination disc method, employing susceptibility testing to both ceftazidime and cefpodoxime indicator β-lactams, in the presence and in the absence of clavulanic acid, is a practical alternative for ESBL detection in the clinical laboratory (Carter *et al.*, 2000). Nevertheless, whilst these newer phenotypic methods offer improved accuracy they are still considered unreliable for ESBL screening in organisms which produce inhibitor-resistant β-lactamases such as AmpC that may mask ESBL detection (Carter *et al.*, 2000). The value of these resistance detection methods is also compromised by the increasing prevalence of organisms that co-produce multiple types of β-lactamases which evade detection by these screening procedures (Carter *et al.*, 2000). Considering the limitations of the current phenotypic methods it is necessary that clinical laboratories review the application of genotypic resistance detection methods, which combine the advantages of sensitivity and specificity (Courvalin, 1995).

The application of DNA amplification-based detection strategies has been widely recognised, not only for ESBL screening but also in the characterisation of unusual β-lactam resistance profiles (Hanson *et al.*, 1999). In the clinical laboratory, the use of such molecular methods in routine resistance detection procedures awaits resolution of issues such as cost and convenience. It may also be necessary to confirm the expression of resistance genes using techniques such as mRNA detection rather than detection of the presence of the resistance gene. Another issue to be addressed is the existence of resistance mechanisms for which encoding genes are as yet unknown. Failure to detect resistance genes that have undergone unknown mutation is another problem that must also be resolved before these methods become commonplace in diagnostic practice. At present, the introduction of quantitative nucleic acid amplification strategies in the clinical laboratory is helping to overcome some of these issues. The application of real-time PCR assays not only facilitates determination of gene expression but also allows characterisation of gene mutation. Real-time PCR assays using flourescence resonance energy transfer (FRET) probes and melting curve mutation detection have already been described as a practical option for the rapid detection of ESBLs (Randegger & Hächler,

2001). In the near future, use of DNA array technology may facilitate the development of combined assays not only to detect and identify bacterial pathogens but also to investigate the presence and expression of resistance genes (Anthony *et al.*, 2001).

One lesson based on the experience of the past 50 years is clear. Early detection of resistance genes through gobal and local surveillance and prompt containment of organisms carrying such genes, are necessary to control the spread of resistance. The problem of antmicrobial resistance has been identified by many countries world-wide as a threat to public health. The World Health Organisation has recognised antimicrobial resistance as a significant cause of prolonged morbidity and increased mortality. The European Union has also prioritised the need to develop an international policy to address the problem of antimicrobial resistance. In Ireland, a recent document 'A Strategy for the Control of Antimicrobial Resistance in Ireland' published by the National Diaease Surveillance Centre has recognised the need to develop a national strategy for the control of the development and the spread of antimicrobial resistance (SARI, 2001). This report has advised the development of a 'three tier' antimicrobial resistance strategy based on local, regional and national settings. In this report the authors suggest that 'local ownership' of antimicrobial resistance should be encouraged and that co-operation with international antimicrobial resistance policies should be promoted. An integral part of each tier in the strategy is the development and strengthening of the existing services for the detection of resistance. Consequently, the report emphasises the need for clinical laboratories to provide reproducible antimicrobial resistance data, to participate in local and national surveillance networks to collate resistance data, to develop genotypic resistance detection methods and to engage in research into antimicrobial resistance mechanisms.

The potential of organisms such as *S. marcescens* to express and acquire multiple mechanisms of β-lactam resistance has long been recognised. The capacity of this organism to adjust and combine these resistance mechanisms to favour its own adaptive response is now evident. In the future, as insight into the 'custom-made' resistance mechanisms of this pathogen is obtained, perhaps antimicrobial resistance detection methodology and antimicrobial treatment policies will be adapted accordingly, so that the medical and pharmaceutical profession can mount an appropriate response.

Appendices

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Appendix I. PHENOTYPIC EXPRESSION OF β-LACTAM RESISTANCE

All reagents used were analar grade. All water used was purified by reverse osmosis, deionisation and UV treatment to attain an 18 MΩ quality (Maxima Four, Elga Vivendi Water Systems, Bucks, UK).

AI.1. IEF reagents

Phosphate buffer (0.1 M), pH 7.0

Add 30.5 ml 0.1 M di-sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_20$, BDH) to 19.5 ml 0.1 M sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4.H_2O$, BDH). Bring to 100 ml volume with water.

Nitrocefin stock solution (500 μg/ml)

Dissolve 1 mg lyophilised nitrocefin (Oxoid, SR112A) in 2 ml rehydration fluid (1.9 ml 0.1 M phosphate buffer, pH 7.0 and 0.1 ml dimethylsulphoxide, Oxoid, SR112A). Store in the dark at 4°C.

Nitrocefin working solution (50 μg/ml)

For β-lactamase sample preparation, dilute nitrocefin stock solution 1/10 in 0.1 M phosphate buffer, pH 7.0.

To resolve β-lactamase bands on polyacrylamide gels, add 4 ml stock solution to 36 ml molten Bacto-agar (3.8% w/v) (Difco Laboratories).

Anode solution, orthophosphoric acid (1 M)

Dilute 34 ml concentrated orthophosphoric acid (H_3PO_4, BDH) in water to a final volume of 500 ml. Store in the dark at RT and use within six months.

Cathode solution, sodium hydroxide (1 M)

Dissolve 4.0 g sodium hydroxide (NaOH, BDH) in water. Bring to 100 ml volume with water. Store at RT and use within one week.

AI.2. Coomassie blue staining for IEF pI calibration markers

Coomassie blue staining solutions

Fixing solution

Dissolve 29 g trichloroacetic acid (BDH) and 8.5 g 5-sulphosalicylic acid (BDH) in water. Bring to 250 ml volume with water. Store at RT and use within three months.

Destaining solution

Add 160 ml glacial acetic acid (BDH) to 500 ml ethanol (BDH). Bring to 2 l volume in water and stir. Store at RT and use within two weeks.

Staining solution

Dissolve 0.5 g Coomassie brilliant blue G (Sigma-Aldrich, B0770) in 400 ml methanol (BDH) and 70 ml glacial acetic acid (BDH). Bring to 1 l volume with water. Heat to 60°C to dissolve. Filter through Whatman filter paper grade 1 (Whatman International) before use. Discard after single use.

Coomassie blue staining method

Fixation:	Immerse gel in fixing solution for 30 min
Wash:	Wash in destaining solution for 5 min
Stain:	Immerse in staining solution for 10 min
Destain:	Immerse in destaining solution overnight.

AI.3. B-lactam hydrolysis assay reagents

Cefoxitin inducing agent

Cefoxitin was used as an inducing agent at a concentration of 4 mg/l which was ¼ the MIC of test isolates (MIC 16 mg/l). The solution was prepared at 100X the required concentration by dissolving 4.8 mg cefoxitin (Sigma Chemical Co.) in 12 ml water (400 mg/l). The 100X solution was diluted 1:100 in bacterial culture to give a final concentration of 4 mg/l.

8-Hydroxyquinolone (1 mM)

Grind 1.5 mg of 8-hydroxyquinolone crystals (Sigma Chemical Co.) in a small volume of water and bring to 10 ml volume in water. Store at RT.

Phosphate buffer (0.1 M), pH 7.0

4.0 M potassium dihydrogen orthophosphate (KH_2PO_4, Sigma Chemical Co.), 13.6 M di-potassium hydrogen orthophosphate (K_2HPO_4, Sigma Chemical Co.).

Cephalothin (0.1 M)

Dissolve 4.56 mg of cephalothin (Eli Lilly) in 100 ml phosphate buffer, pH 7.0.

AI.4. Calculation of β-lactamase specific activity (Δnmoles/min/mg)

ΔAbs/min = Absorbance (Abs) at T_0 – Abs at T_1

where T_0 = time zero
T_1 = 1 minute

$$\Delta\text{nmoles/min/ml} = \frac{(\Delta\text{Abs/min}) \times 10}{K_{Hydrolysis}} \times \text{Dilution factor}$$

where $K_{Hydrolysis} = \dfrac{\Delta\text{Abs}}{\text{Molar concentration}}$

for cephalothin, $K_{Hydrolysis} = 0.01$
Dilution factor = 1

$$\Delta\text{nmoles/min/mg} = \frac{\Delta\text{nmoles/min/ml}}{\text{Protein concentration.}}$$

Appendix II. GENERAL MOLECULAR BIOLOGY REAGENTS

All reagents used were analar grade. All water used was purified by reverse osmosis, deionisation and UV treatment to attain an 18 MΩ quality (Maxima Four).

AII.1. Common stock solutions

The following stock solutions were prepared by standard methods as outlined in Sambrook, Fritsch & Maniatis, 1989 (Sambrook *et al.*, 1989):

0.5 M EDTA, disodium salt (BDH), pH 8.0

Ethidium bromide solution (Sigma-Aldrich), 10 mg/ml

10 N NaOH (BDH)

Tris-acetate-EDTA (TAE) at 50X concentration

Tris-borate-EDTA (TBE) at 5X concentration

1 M Tris (hydroxymethyl methylamine)-hydrochloride (Tris-HCL) (BDH)

AII.2. Common buffers

Working solutions were prepared by diluting stock solutions as required.

Gel loading buffer

0.25% (w/v) bromophenol blue (Sigma-Aldrich) in 50% (v/v) glycerol (Sigma-Aldrich) in 0.5X TBE.

TAE (1X)

40 mM Tris-acetate, 0.1 mM EDTA, pH 8.

TBE (0.5X)

45 mM Tris-HCl; 45 mM boric acid; 1 mM EDTA, pH 8.

TE

10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.

AII.3. **Other solutions**

Ammonium acetate (7.5 M)

Dissolve 57.8 g ammonium acetate (CH_3COONH_4, BDH) in 80 ml water. Bring to 100 ml volume with water and filter sterilise (Millex 0.45 μm syringe filter unit, Millipore Corporation, Bedford, MA, US)

Calcium chloride (1 M)

Dissolve 5.4 g of calcium chloride ($CACl_2.6H_20$, BDH) in 20 ml water and filter sterilise (Millex 0.22 μm syringe filter unit, Millipore Corporation). Aliquot in 1 ml volumes and store at –20°C. When preparing competent cells dilute a 1 ml aliquot in 99 ml water and filter sterilise (Millex 0.45 μm syringe filter unit, Millipore).

Ethidium bromide staining solution (0.5 mg/l)

Dilute ethidium bromide stock solution (10 mg/ml) 1:20,000 in water to give a final concentration of 0.5 mg/l. Store in the dark at RT.

Proteinase K (Sigma-Aldrich, 100 mg)

Dissolve 100 mg proteinase K in 5 ml water (20 mg/ml) and aliquot in 500 μl volumes. Store at –20°C.

RNase (Dnase-free) (Boehringer Mannheim, 500 μg/ml)

RNase in 10 mM Tris-HCl, 5 mM $CaCl_2$, 50% glycerol; pH 7.0, at a concentration of 500 μg/ml was purchased ready for use. This solution was diluted 1/100 in sample DNA to give a final concentration of 5 μg/ml, in accordance with the manufacturer's instructions.

Sodium acetate (3 M)

Dissolve 40.8 g sodium acetate trihydrate ($CH_3COONa.3H_20$, BDH) in 80 ml water. Adjust to pH 5.2 with glacial acetic acid (BDH). Bring to 100 ml volume with water. Sterilise by autoclaving at 121°C for 15 min.

Appendix III. GENERAL MOLECULAR BIOLOGY METHODS

AIII.1. Agarose gel analysis

AIII.1.1. Agarose gel electrophoresis

Gel loading buffer was added to all DNA samples at a ratio of 1:5. Electrophoresis of restriction endonuclease (RE)-digested chromosomal DNA was performed using a Hoefer® HE 100 SuperSub™ horizontal tank (Hoefer Pharmacia Biotech Inc., San Francisco, CA, US) and a chiller unit (Mini Chiller Model 1000, Bio-Rad). All other electrophoresis was performed using Bio-Rad horizontal electrophoresis tanks. Gels were prepared using type II medium EEO agarose (Sigma-Aldrich) and freshly diluted TBE (0.5X). All gels were prepared to a depth of 6 mm and DNA was electrophoresed in fresh TBE (0.5X). Electrophoresis was performed under the following conditions:

Analyte	Agarose concentration % (w/v)	Gel size (cm)	Voltage (V)
PCR products < 800 bp	2	10 x 15	70
PCR products > 800 bp	1	10 x 15	70
Plasmid DNA	0.6	10 x 15	70
Chromosomal DNA	0.8	10 x 15	70
Chromosomal DNA RE* digests	0.7	20 x 15	60

*RE, restriction endonuclease

In MCRAB, Creighton University, agarose gel electrophoresis of PCR products was performed using a 10 x 20 cm horizontal tank (Midwest Scientific, St Louis, MO, US). Agarose gels were poured at a concentration of 1.5% (w/v) using Agarose I (Amresco, Solon, Ohio, US) in freshly diluted TAE (1X) and DNA was electrophoresed using TAE (1X).

AIII.1.2. **Staining of agarose gels**

All 10 x 15 cm and 10 x 20 cm gels were stained in ethidium bromide (0.5 mg/l) for 10 min immediately after electrophoresis. Excess ethidium bromide was removed by washing in water for 10 min. Larger gels (20 x 15 cm) were pre-stained prior to electrophoresis by the addition of ethidium bromide (10 mg/ml) to the cooled molten agarose to a final concentration of 0.5 mg/ml.

AIII.1.3. **Photography**

Gels were viewed on a transilluminator (2011 Macrovue, LKB) under UV light at 312 nm and photographed with an MP4 land camera [Polaroid (Europa) BV] using Polaroid 667 film (Sigma-Aldrich) and a 23A Wratten filter (Sigma-Aldrich). When negatives were required, Polarioid 665 film (Sigma-Aldrich) was used.

AIII.1.4. **Image processing**

Gel photographs were digitised at 720 dots per inch (dpi) and 42 bit/pixel colour depth using an Epson Perfection™ 1240U Colour Image Scanner (Epson UK Ltd., Hemel Hempstead, Herts, UK) connected to a Gateway 450 personal computer (Gateway Europe, Dublin, Ireland). Images saved in Photoshop Document File format were processed with Adobe Photo Shop post-production software (Version 5.0, Adobe System, US).

AIII.2. **Extraction of genomic DNA**

AIII.2.1. **Wizard™ Genomic DNA Purification Kit protocol**

DNA was extracted using the Wizard™ Genomic DNA Purification Kit (Promega). All isolates were removed from –70°C and subcultured on CBA on the day prior to extraction. A single colony of bacterial culture was inoculated into 5 ml LB broth (Difco Laboratories) and grown overnight for 14 h at 37°C with shaking (150 rpm). DNA was prepared using 1 ml of this overnight culture, according to the manufacturer's instructions for isolation of genomic DNA from Gram-negative bacteria. During the procedure, all reagents used were supplied in the kit with the exception of the isopropanol (BDH) and ethanol (BDH) used in the final precipitation step. DNA pellets were rehydrated overnight at 4°C and quantified using the agarose gel method outlined in Appendix AIII.3 before storage at –20°C.

AIII.2.2. **Modified Wizard™ Genomic DNA Purification Kit protocol**

The Wizard™ Genomic DNA Purification Kit protocol was modified to include a proteinase K step to prevent autodegradation of DNA during restriction endonuclease digestion. Previous reports had suggested that proteinase K should be added at the end of the extraction procedure after the DNA is dissolved. However, with this modification, an additional phenol-chloroform purification step is required to remove proteinase K from the final DNA preparation. In the present study, the proteinase K step was integrated into the extraction protocol following consultation with the technical support team in Promega Corporation. In a pilot study, it was observed that the addition of proteinase K following incubation with RNase solution adversely affected the activity of RNase. Hence, an additional RNase digestion step was added to optimise the extraction protocol, as described below.

In the modified protocol, bacterial cultures were prepared as outlined in Appendix AIII.2.1. DNA was extracted following the manufacturer's instructions for the bacterial cell lysis and RNase digestion steps. Following the recommended RNase incubation procedure, 30 µl of proteinase K (20 mg/ml, Sigma-Aldrich, Appendix AII.3) was added to each of the cell lysates. The preparations were mixed by gentle inversion and incubated for 30 min at 55°C. Samples were allowed to cool to RT and the remainder of the extraction procedure was performed according to the manufacturer's protocol. Following overnight rehydration of the DNA pellets in 100 µl TE buffer (Appendix AII.2) at 4°C, 1 µl of DNase-free, RNase (500 µg/ml, Boehringer Mannheim, Appendix AII.3) was added to each of the DNA samples and the samples were incubated at 37°C for 30 min. DNA preparations were quantified using the agarose gel method outlined below (Appendix AIII.3) before storage at –20°C.

AIII.3. **Quantification of Genomic DNA**

The ultraviolet spectrophotometric method of DNA quantification was used initially to determine the concentration (A_{260}) and relative purity ($A_{260/280}$) of extracted DNA, according to the method outlined in Sambrook, Fritsch & Maniatis, 1989 (Sambrook *et al.*, 1989). This procedure was replaced with an agarose gel electrophoresis method which, in addition to accurate quantification, allowed direct visualisation of DNA quality. Using a modification of the method outlined in Sambrook, Fritsch & Maniatis,

1989 (Sambrook *et al.*, 1989), DNA was quantified against a series of DNA concentration standards (unmethylated lambda DNA), as described below.

Lambda DNA (250 µg/ml, Boehringer Mannheim) was diluted 1/10 in gel loading buffer (Appendix AII.2) and varying volumes were loaded into the wells of a 0.8% (w/v) agarose gel to yield a DNA concentration range of 25-200 µg/ml. DNA from test isolates (5 µl of neat DNA and 5 µl of DNA diluted 1/20 in sterile water) were loaded into the agarose gel wells adjacent to the lambda DNA concentration standards. DNA was electrophoresed at 70 V in TBE buffer for 2 h and the gel was stained and photographed as outlined in Appendix AIII.1. The quantity of DNA was estimated by comparison with the lambda DNA concentration standards (Figure 6.5, panel A).

AIII.4. **Phenol:chloroform extraction of DNA**

Following extraction of the *ampD* PCR inserts, the solubilised *ampD* DNA preparations were purified by a modification of the phenol:chloroform extraction method outlined in Sambrook, Fritsch & Maniatis, 1989 (Sambrook *et al.*, 1989). The pooled DNA preparations were treated with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, Sigma-Aldrich), mixed by inversion and centrifuged at 17,400 x *g* for 10 min in an Eppendorf 5417 microcentrifuge (Eppendorf), as outlined in the protocol. The upper aqueous layer was carefully removed and placed in a fresh microcentrifuge tube. An equal volume chloroform:isoamyl alcohol (24:1, Sigma-Aldrich) was added to the aqueous layer. The samples were mixed by inversion, centrifuged at 17,400 x *g* for 10 min and the upper aqueous layer was carefully removed and placed in a fresh microcentrifuge tube. The aqueous layer was mixed with 1/10 volume of 3 M sodium acetate, pH 5.2 (Appendix AII.3) and twice the volume of cold absolute ethanol (BDH). The samples were placed at –20°C for 1 h and centrifuged for 30 min at 4°C at 17,400 x *g*. The DNA pellet was washed twice in 70% (v/v) ethanol and allowed to air dry for 5 min.

Appendix IV. **DNA TRANSFER AND HYBRIDISATION REAGENTS**

All reagents used were analar grade. All water used was purified by reverse osmosis, deionisation and UV treatment to attain an 18 MΩ quality (Maxima Four, Elga).

AIV.1. **Southern transfer**

Depurination solution (*0.25 M HCl*)

Dilute 21.55 ml concentrated HCl (BDH) in water to a final volume of 1 l.

Denaturation solution (*1.5 M NaCl, 0.5 N NaOH*)

Dissolve 87.75 g NaOH (BDH) and 20 g NaCl (BDH) in water. Bring to 1 l volume with water and store at RT.

Neutralisation solution (*0.5 M Tris-HCl pH 7.5, 3 M NaCl*)

Dissolve 63.5 g Tris-HCl (BDH) and 11.8 g Tris-base (BDH) in 800 ml water. Adjust to pH 7.5 by adding approximately 3 ml concentrated HCl (BDH). Add 175.5 g NaCl (BDH). Bring to 1 l volume with water and store at RT.

Transfer buffers

20X SSC (3 M NaCl, 0.3 M sodium citrate)

Dissolve 175.3 g of NaCl (BDH) and 88.2g of sodium citrate (BDH) in 800 ml water. Adjust to pH 7.0 with a few drops of 10 N NaOH. Bring to 1 l volume with water. Sterilise by autoclaving at 121°C for 15 min and store at RT.

2X SSC (0.3 M NaCl, 0.03 M sodium citrate)

Add 50 ml 20X SSC to 400 ml water. Bring to 500 ml volume with water.

AIV.2. **Colony blot transfer**

Denaturation solution (*1.5 M NaCl, 0.5 N NaOH, 0.1% [w/v] SDS*)

Dissolve 87.75 g NaOH (BDH) and 20 g NaCl (BDH) in 800 ml water. Add 10 ml 10% (w/v) sodium dodecyl sulfate (SDS, Sigma-Aldrich). Bring to 1 l volume with water and store at RT.

Neutralisation solution (*1.0 M Tris-HCl pH 7.5, 1.5 M NaCl*)

Dissolve 127 g Tris-HCl (BDH) plus 23.6 g Tris-base (BDH) in 800 ml water. Adjust to pH 7.5 by adding approximately 3.0 ml concentrated HCl (BDH). Add 87.75 g NaCl (BDH). Bring to 1 l volume with water and store at RT.

Proteinase K solution (2 mg/ml)

To prepare 3 ml proteinase K solution (sufficient for 6 colony blots), dilute 300 µl proteinase K, 20 mg/ml (Appendix AII.3) in 2.7 ml 2X SSC.

AIV.3. **Hybridisation**

Post-hybridisation wash solutions

4X SSC, 0.1% SDS

Add 100 ml 20X SSC and 5 ml 10% (w/v) SDS (Sigma-Aldrich) to 350 ml water. Bring to 500 ml volume in water. Store at RT.

2X SSC, 0.1% SDS

Add 50 ml 20X SSC and 5 ml 10% (w/v) SDS (Sigma-Aldrich) to 400 ml water. Bring to 500 ml volume in water. Store at RT.

Alkaline probe stripping solution (*0.2 M NaOH, 0.1% SDS*)

Dissolve 4 g NaOH (BDH) in 450 ml water. Add 5 ml 10% (w/v) SDS (Sigma-Aldrich). Bring to 500 ml volume with water. Store at RT.

Appendix V. **DNA CLONING TECHNIQUES**

AV.1. **Growth media for transformation reactions**

Kanamycin stock solution (10 mg/ml)

Dissolve 0.637 g kanamcycin monosulphate salt (785 μg/mg potency, Sigma-Aldrich) in water. Bring to 50 ml volume in water and filter sterilise (Millex 0.45 μm syringe filter unit, Millipore). Dispense in 5 ml volumes and store at –20°C.

Ampicillin stock solution (10 mg/ml)

Dissolve 0.501 g ampicillin sodium salt (981 μg/mg potency, Sigma-Aldrich) in 5 ml phosphate buffer, pH 7.0. Bring to 50 ml volume with water and filter sterilise (Millex 0.45 μm syringe filter unit, Millipore). Dispense in 5 ml volumes and store at 4°C.

LB selective agar (containing either 50 μg/ml kanamycin or 50 μg/ml ampicillin)

Dissolve 39 g LB agar (Sigma-Aldrich) in 1 l sterile water. Sterilise by autoclaving at 121°C for 15 min and allow to cool to approximately 55°C. Add 5 ml of kanamycin or ampicillin stock solution (10 mg/ml) and pour aseptically into sterile 9 cm Petri dishes (Sarstedt Ltd., Drinagh, Wexford, Ireland) (approximately 20 ml/plate).

LB selective broth (containing 50 μg/ml kanamcyin)

Dissolve 15.5 g LB broth (Sigma-Aldrich) in 1 l sterile water. Sterilise by autoclaving at 121°C for 15 min and allow to cool to approximately 55°C. Add 5 ml kanamycin stock solution (10 mg/ml) and dispense aseptically in 5 ml volumes into sterile 30 ml Universal containers (Bibby Sterilin Ltd., Stone, Staffs, UK).

AV.2. **Calculation of transformation efficiency in competent cells**

The transformation efficiency in competent cells was calculated from the number of transformants per μg of plasmid DNA (cfu/μg) using the following formula:

$$\text{Transformation efficiency (cfu/μg)} = \frac{\text{Number of colonies}}{\text{pg transformed DNA}} \times \frac{10^6 \text{ pg}}{1 \mu g} \times D$$

where D = final dilution plated.

AV.3. **Calculation of vector:insert molar ratio for cohesive-end ligation**

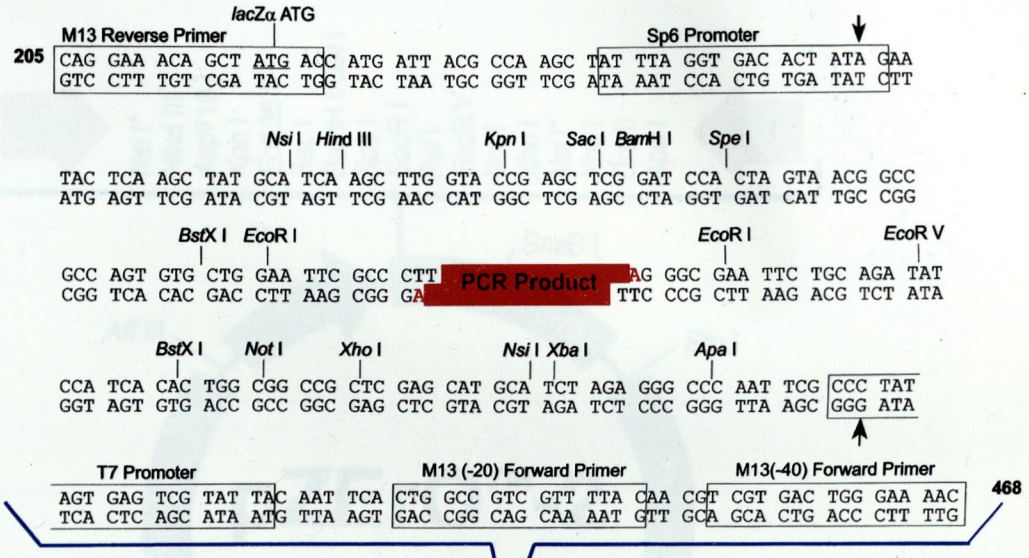
The amount of insert DNA required to generate a molar ratio of 2:1 between insert and linearised pZErO™-2 vector for cohesive-end ligation was calculated using the following formula:

$$\text{x ng insert} = \frac{\text{molar:insert ratio} \times \text{bp size of insert} \times \text{ng of linearised vector}}{\text{bp size of vector}}$$

where molar:insert ratio = 2
pZErO™-2 vector = 3297 bp.

AV.4. Map of pCR II TOPO™ cloning vector

The figure shown below depicts a map of the pCR II TOPO™ cloning vector and the DNA sequence surrounding the TOPO™ cloning site. The restriction endonuclease sites are labeled to indicate the cleavage sites. The start of transcription for Sp6 and T7 are indicated by arrows. The genetic determinants encoded on the vector are listed below.



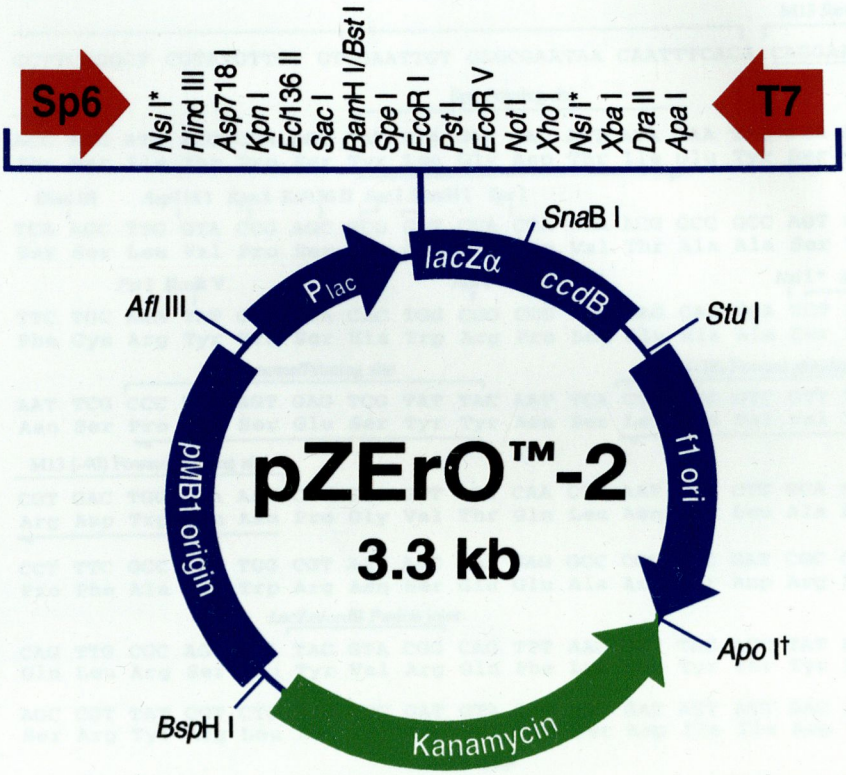
Comments for pCR®II-TOPO®
3950 nucleotides

LacZα gene: bases 1-588
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-399
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
M13 (-40) Forward priming site: bases 453-468
f1 origin: bases 590-1004
Kanamycin resistance ORF: bases 1338-2132
Ampicillin resistance ORF: bases 2150-3010
pUC origin: bases 3155-3828

AV.5. pZErO™-2 cloning vector

AV.5.1. Map of pZErO™-2 cloning vector

The figure shown below depicts a map of the cloning site in the pZErO™-2 vector. Restriction endonuclease sites are labeled to indicate the cleavage sites. The genetic determinants encoded on the vector are listed below.



Comments for pZErO™-2
3297 nucleotides

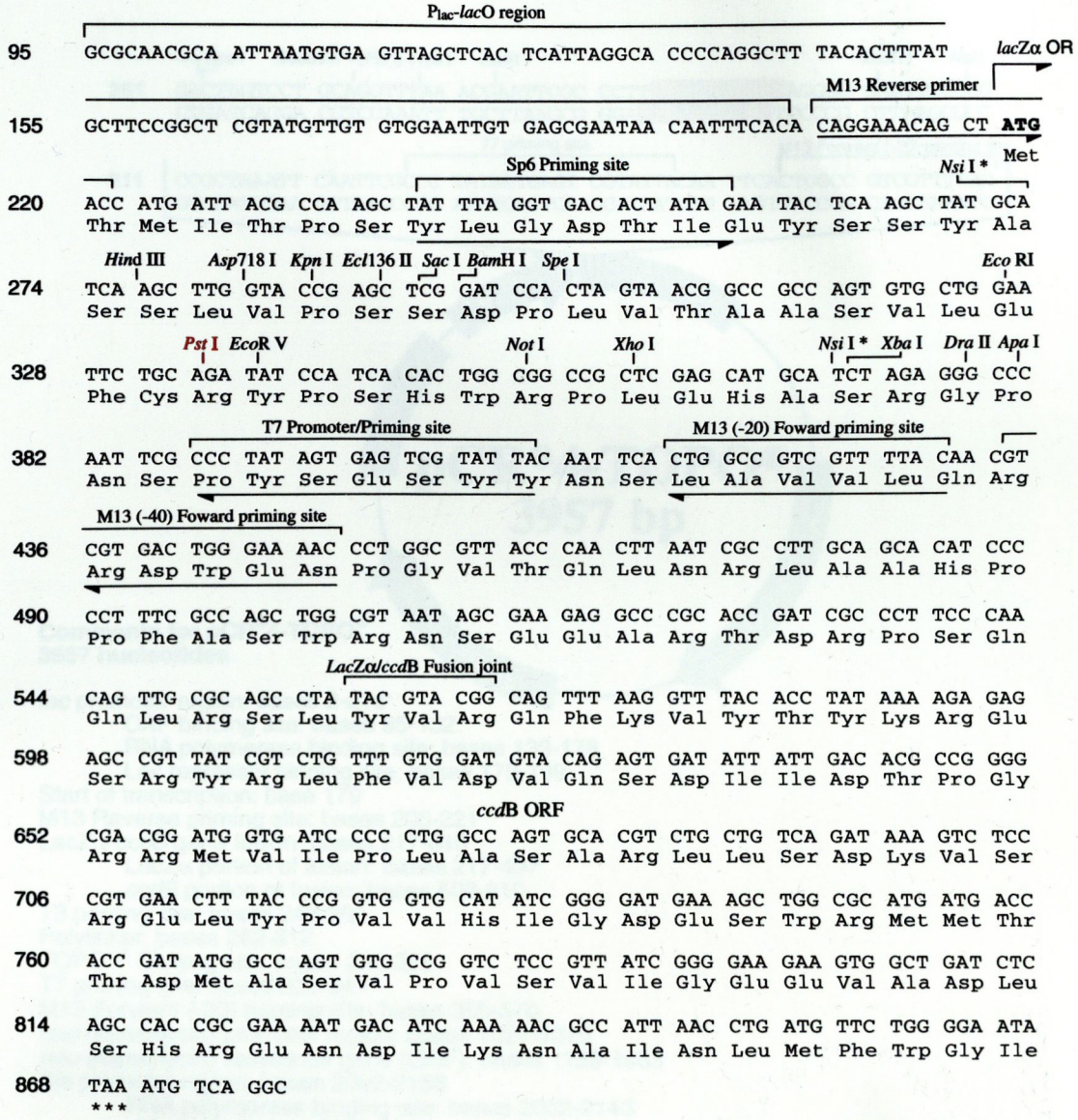
Lac Promoter/Operator Region: bases 95-216
M13 Reverse Priming Site: bases 205-221
LacZα ORF: bases 217-558
Sp6 Promoter/Priming Site: bases 239-256
Multiple Cloning Site: bases 269-381
T7 Promoter/Priming Site: bases 388-407
M13 (-20) Forward Priming Site: bases 415-430
M13 (-40) Forward Priming Site: bases 434-450
Fusion Joint: bases 559-567
*ccd*B Lethal Gene ORF: bases 568-870
f1 origin: bases 895-1307
Kanamycin Resistance ORF: bases 2116-1322
pMB1 origin (pUC-derived): bases 2502-3175

* The two *Nsi* I sites in the MCS are the only sites in the vector.

† There are two tandem *Apo* I sites at this location. *Apo* I also recognizes the *Eco*R I site.

AV.5.2. DNA sequence of the multiple cloning site in pZErO™-2 vector

The figure shown below depicts a map of the DNA sequence of the multiple cloning site and surrounding regions in the pZErO™-2 vector. Restriction endonuclease sites are marked to indicate the cleavage sites.



AV.6. Map of pCR 4-TOPO™ cloning vector

The figure shown below depicts a map of the pCR 4-TOPO™ cloning vector and the DNA sequence surrounding the TOPO™ cloning site. Restriction endonuclease sites are labeled to indicate the cleavage sites. The genetic determinants encoded on the vector are listed below.



Comments for pCR®4-TOPO®
3957 nucleotides

lac promoter region: bases 2-216
    CAP binding site: bases 95-132
    RNA polymerase binding site: bases 133-178
    Lac repressor binding site: bases 179-199
Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
    LacZα portion of fusion: bases 217-497
    ccdB portion of fusion: bases 508-810
T3 priming site: bases 243-262
Polylinker: bases 262-312
TOPO™ Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
neo (kanamycin) promoter region: bases 1021-1070
neo (kanamycin) resistance gene (ORF): bases 1159-1953
bla promoter region: bases 2062-2156
    RNA polymerase binding site: bases 2062-2143
    -35 region: bases 2087-2093
    bla promoter (P3): bases 2116-2122
Start of transcription: base 2122
Ribosome binding site: bases 2145-2149
bla (ampicillin) resistance gene (ORF): bases 2157-3017
pMB1 origin (pUC-derived): bases 3162-3835

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Byrne A.H., Boyle B., Herra C.M., Hone R., Keane C.T. *Serratia marcescens* causing hospital-acquired lower respiratory tract infection. *J Hosp Infect* 2000; **45**: 242-4.

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Journal of Hospital Infection (2000) **45**: 242–251

Letters to the Editor

doi:10.1053/jhin.2000.0761, available online at http://www.idealibrary.com on IDEAL

Serratia marcescens causing hospital-acquired lower respiratory tract infection

Sir,

Serratia marcescens, a Gram-negative rod (GNR) is recognized as a hospital-acquired pathogen and two outbreaks of *S. marcescens* infection have been described in Dublin hospitals.[1] *Serratia marcescens* is the second most frequently encountered GNR in respiratory samples from our hospital. Although pneumonia and lung abscess have occasionally been documented[2,3] there is little published evidence of its role as a respiratory pathogen. Most reports merely record the presence of the organism in respiratory secretions without describing the accompanying clinical findings.

We reviewed the spectrum of respiratory disease associated with the isolation of *S. marcescens* in our hospital. The study was performed in two parts, involving two tertiary referral university hospitals in Dublin, with a total of 1300 beds. A retrospective chart review was conducted over 12 months in both hospitals and a prospective review was performed over six months in one hospital. Only patients with a pure culture of *S. marcescens* isolated from respiratory secretions (sputum and bronchoalveolar lavage) were included. All sputum samples were freshly collected, non-salivary samples. Respiratory specimens were processed in the laboratory according to standard microbiological procedures. Isolates were identified as *s. marcescens* using the API 20E system (BioMérieux). Identification was confirmed using additional tests, including arabinose and raffinose peptone broth sugar fermentation.

Tracheo-bronchitis was defined by the presence of increased shortness of breath and purulent sputum with pyrexia and/or leucocytosis. Pneumonia was defined as the presence of new or progressive chest X-ray changes in addition to the above. Other causes of these abnormalities were excluded. In the absence of symptoms and signs of infection attributable to *S. marcescens*, patients were deemed colonized. Representative isolates were sent for serotyping at the Laboratory of Hospital Infection, Central Public Health Laboratory, Colindale, London.

A total of 52 patients were reviewed. Of 27 retrospectively reviewed patients (nine females), the mean age was 67 years (range 36–91). Twenty-five prospectively reviewed patients (eight females) had a mean age of 72 years (range 49–92). Thirteen patients (25%) had significant infection. Seven of these had pneumonia (four retrospective) and six had tracheobronchitis (four retrospective). Thirty-nine patients were colonized (nineteen retrospective). Seventy percent of infected patients were postoperative and 70% were mechanically ventilated, compared with 23% and 13% of colonized patients, respectively.

Symptoms and signs of infection associated with the isolates are shown in Table I. Blood cultures were positive for *S. marcescens* in six patients (three retrospective), three of these had pneumonia, two had tracheobronchitis and one colonized patient had a transient bacteraemia (pyrexia coincident with time of blood culture which settled without antimicrobial therapy). Four patients (all colonized) had the organism isolated from a site other than the respiratory tract: one arterial line tip; one tracheostomy swab; one burn swab; and one wound swab. There were eight deaths. While *S. marcescens* infection was not considered to be the direct cause of death in any patient, it was deemed to be a contributory factor in two patients with severe underlying disease.

Thirty-one isolates were sent for serotyping and the results are summarized in Table II. Seven

Table I *Clinical findings and therapy of patients with* S. marcescens *isolates*

	Pneumonia $N=7$	Tracheobronchitis $N=6$	Colonization $N=39$
pyrexia	3	6	3
leucocytosis	6	4	11
purulent sputum	7	6	29
radiological signs of pneumonia	7	0	6

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Table II *Distribution of serotypes in different clinical categories*

Pneumonia	No.	Tracheobronchitis	No.	Colonization	No.
O14:K14	6	O14:K14	1	O14:K14	9
O21:K14	1	ONT:K14	1	O21:K14	2
		O21:KNT	1	O21:K3	3
		O8:K14	1	ONT:K14	1
		O2:K2	1	O19:KNT	1
				O21:KNT	1
				ONT:K28	1
				ONT:KNT	1

distinct O and K combinations were identified plus possible O non-typable and K non-typable variants. The most common serotype identified was O14:K14 (52%).

We have identified clinically significant infection in 25% of patients with respiratory isolates of *Serratia marcescens*. The pathogenicity of the organism in the episodes we describe is supported by the absence of other potential pathogens at any other site, the absence of other identifiable causes of the clinical findings and the presence of *S. marcescens* septicaemia in five patients with clinical signs of infection. We feel this rate may accurately reflect the incidence of significant infection in susceptible groups. *Serratia marcescens* infection may have contributed to the deaths of two patients. One previous study noted a high mortality rate for *S. marcescens* pneumonia.[4] While predisposing factors to infection were not identified by this study, ventilation and post-operative state were both associated with infection rather than colonization.

Although the retrospective review was conducted over a longer period than the prospective study, similar patient numbers were reviewed for each. This apparent anomaly may be partly explained by the difficulties we experienced retrieving charts for the retrospective review. Furthermore, the prospective study was based in a hospital where two documented outbreaks of *S. marcescens* infection had occurred and this may have led to increased awareness and consequently increased isolation and identification of the organism in the laboratory. It is also likely that this hospital has a higher overall incidence of *S. marcescens* infection. The actual incidence of *S. marcescens* isolation from the respiratory tract (including mixed cultures) is higher than represented by our figures.

Among our *S. marcescens* isolates, O14:K14 was the commonest serotype, occurring in 58% of infected and 47% of colonized patients. These results are in agreement with a recent study which identified O14:K14 as the commonest clinical serotype.[5] An additional study of virulence characteristics from the same group revealed an association between haemagglutination and serotype O14:K14, suggesting that O14:K14 may be more adherent than other serotypes.[6] The increased adherence associated with the O14:K14 serotype may confer a selective advantage in establishing initial colonization in the host. The subsequent development of infection is likely to depend on individual host factors in addition to virulence characteristics of the organism.

This study is limited by its small size and the fact that not all patients were prospectively reviewed, however, it does indicate that *S. marcescens* is a frequent respiratory isolate in our hospital group. While the majority of these isolates represent colonization, tracheobronchitis and pneumonia with septicaemia can occur. The ability of this hospital-acquired pathogen to cause significant lower respiratory tract infection should be recognized.

Acknowledgement

Our grateful thanks to Dr H. Aucken, Laboratory of Hospital Infection, Central Public Health Laboratory, Colindale, London, who serotyped the isolates.

Research grant from Wyeth-Lederle.

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doi:10.1053/jhin.2000.0764, available online at http://www.idealibrary.com on IDEAL®

Genotypic similarities among the methicillin-resistant *Staphylococcus aureus* isolates recovered from stools, the respiratory tract and other sites in 20 patients

Sir,

Methicillin-resistant *Staphylococcus aureus* (MRSA) is occasionally recovered from stools of patients. Several previous reports indicate that MRSA is frequently recovered from the upper respiratory tract of patients with MRSA enterocolitis.[1,2] Thus, MRSA in the respiratory tract may be the source of MRSA in stool. To clarify whether MRSA in the respiratory tract is indistinguishable from the MRSA isolated from stool, we analysed MRSA isolates from stool and respiratory tract specimens from individual patients using pulsed-field gel electrophoresis (PFGE).

A total of 65 MRSA isolates recovered from 20 patients at Tokyo Women's Medical University Hospital from September 1996 to August 1997 were studied (Table I). Methicillin-resistant *S. aureus* was detected in both the stool and the respiratory tract specimens of these 20 patients who had no definite clinical manifestation of MRSA enterocolitis. The MRSA isolates from other body sites were also available in six patients. All isolates were obtained from primary culture and stocked at −80°C until analysis.

Each MRSA isolate was incubated overnight in Mueller–Hinton (MH) broth at 37°C. A bacterial pellet made by centrifugation was resuspended in 10 μL of 1 M NaCl with 10 mM EDTA (pH 8.0), and embedded in 100 μL of 2% Clean Cut agarose (Bio-Rad Laboratories, Hercules, USA). The plug was incubated at 37°C in 400 μL of the lysis buffer containing 5 mg/mL lysozyme (Sigma, St. Louis, USA), 20 μg/mL lysostaphin (Sigma), 1 M NaCl, 10 mM Tris-HCl (pH 8.0), 0.5% Brij 58, 0.2% deoxycholate, and 0.5% sarkosyl, and then treated at 50°C with 500 μL of the buffer containing 0.1 mg/mL proteinase K (Boehringer Mannheim, Germany) and 0.25 M EDTA (pH 8.0). After washing four times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), chromosomal DNA in the plug was digested with *SmaI* (TaKaRa, Kyoto, Japan) for 20 h at 25°C. Finally, the plug was loaded on to a 1% agarose gel and run for 20 h in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0). Pulsed-field gel electrophoresis (PFGE) was performed using the GenePath strain typing system (Bio-Rad Laboratories). After electrophoresis the gel was stained for 30 min with ethidium bromide and photographed under UV illumination. The DNA restriction patterns of MRSA were compared. Interpretation of the restriction patterns was based on the criteria described by Tenover *et al.*[3] Susceptibility to antibiotics listed in Table I was determined by microdilution using the 'Microscan WalkAway' automatic bacteria identification system (Dade, Tokyo, Japan).

Representative PFGE patterns are shown in Fig. 1. The results of the PFGE analyses and antibiograms are summarized in Table I. In 17 of the 20 patients (patients 1 to 17), the MRSA isolates from stools and respiratory tract specimens showed the same DNA restriction patterns. The results indicate that MRSA isolates from the stool and respiratory tract specimens were genetically indistinguishable in 17 patients. In patients 4, 16 and 17, morphologically different MRSA colonies were noted on primary cultures from stools and/or respiratory tract specimens, but PFGE of individual colonies revealed the same DNA restriction patterns.

In two patients (patients 18 and 19), minor differences in PFGE patterns of the MRSA isolates from stool and respiratory tract specimens were noted (Figure 1). In patient 18, there were two band differences between the MRSA isolates from the nasal swab and from the stool, and these isolates were considered to be closely related strains. In patient 19, there were four band differences between the MRSA isolate from the throat swab and from the stool, and they were considered to be possibly related strains. In one patient (patient 20) seven DNA restriction fragments differed between the MRSA isolates from the stool and the respiratory tract specimen, and they were considered to be genetically different (Figure 1). The MRSA isolates from the exudate from a decubitus ulcer and

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Scand J Infect Dis 33: 822–826, 2001

ORIGINAL ARTICLE

Rate of Carriage of Serratia marcescens in Patients with and without Evidence of Infection

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The epidemiology of Serratia marcescens is poorly understood. We designed a study to investigate carriage sites of the organism, and possible modes of transmission of infection. Using Sorbitol–MacConkey agar with colistin 200 IU/ml and MacConkey agar with a 10 μg colistin disc we performed cultures from various sites in patients already infected with S. marcescens. Over the same period of time we also investigated all patients in the intensive care unit (ICU) for colonization with the agent. Environmental screening was performed in the ICU only. Of 37 infected patients, 65% demonstrated carriage at a second site and 43% at multiple sites. Throat carriage was found in 59%, faecal carriage in 42%, nasal carriage in 31% and urinary carriage in 22%. Carriage over several weeks was found in 22%. Of 40 ICU patients, 10% demonstrated nasal and/or throat carriage. Environmental screening yielded 4 isolates. All ICU patient strains and a strain from the ICU bedpan macerator were O14:K14 with similar random amplified polymorphic DNA types. These results show that patients with S. marcescens infection are likely to carry the organism at multiple sites and that carriage may be prolonged. A significant level of carriage was also found in non-infected patients in a unit where the organism was prevalent.

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INTRODUCTION

Serratia marcescens is an important nosocomial pathogen which is associated with a range of infections, including septicaemia, urinary tract infection, pneumonia and wound infection. As with other opportunistic pathogens, immunocompromised and chronically ill patients (1) are most vulnerable to infection. Furthermore, the organism frequently demonstrates resistance to a wide range of antimicrobials (2, 3) and this may limit therapeutic options.

Over the last 17 y S. marcescens has been a frequent cause of infection in Dublin hospitals (4–6). In our hospital group it has become established as an endemic nosocomial pathogen with a baseline incidence of 1–2 newly infected patients per week. Patients in the intensive care unit (ICU) have been most frequently involved and we have described 2 outbreaks of infection in this setting (4, 5). A further outbreak of S. marcescens has also been described in patients in our bone marrow transplantation unit (6).

Despite the extensive number of studies describing outbreaks of S. marcescens infection (7–10) the epidemiology remains unclear. Environmental sources of outbreaks of infection have been identified, including disinfectants (11) and medical equipment, such as mechanical respirators and fibre-optic bronchoscopes (12). However, in the majority of outbreaks patients are thought to act as reservoirs of infection with spread occurring by staff hand carriage (10, 12). While patients have been shown to be colonized with S. marcescens at numerous sites, the reported rates vary greatly between studies (13, 14). Gastrointestinal tract (GIT) carriage of S. marcescens is generally considered infrequent in adults, although it is recognized as an important source of infection in neonatal outbreaks. There are, however, conflicting reports in the literature regarding adult GIT carriage (15, 16). The differences between carriage rates reported at various sites may relate in part to the different patient populations studied, and also to the different culture techniques used. Accurate identification of S. marcescens carriage sites is, however, necessary for the design of effective control procedures. This was especially relevant in our own hospital group where we have reported 3 outbreaks of S. marcescens infection (4–6).

In this study we investigated the carriage sites of S. marcescens in 2 different patient groups. As S. marcescens is known to be prevalent in the ICU, all patients admitted to this unit were screened during the study period. Environmental screening was also performed in the ICU. In addition, patients on general wards already known to be infected with the organism were screened for additional carriage sites.

MATERIALS AND METHODS

Evaluation of selective media

Two types of media previously described for the selective isolation of S. marcescens, Caprylate–Thallous (CT) agar (17) and Sorbitol–MacConkey agar (Oxoid) with colistin 200 IU/ml (SMAC) (18), were considered for isolation procedures. A third medium, MacConkey agar (Oxoid) with a 10 μg colistin disc (Oxoid) (MAC), which has traditionally been used in this laboratory as the standard medium for the isolation of S. marcescens, was also

* Dr. Alison Byrne died in July 2000.

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DOI: 10.1080/00365540110077385

included in the evaluation. The SMAC and MAC media were prepared in our media department. The CT agar was acquired commercially (Cruinn Diagnostics Limited, Dublin, Ireland) as its complex mode of preparation and the toxicity of thallous sulphate rendered it unsuitable for production in a routine media department.

To determine the most appropriate selective medium for the detection of S. marcescens, the sensitivity of each medium was assessed using spiked specimens according to the method of Starr et al. (17). Bacterial suspensions containing 10^3–10^7 Serratia cells/ml were prepared using a modification of the Miles and Mizra method (19). Bacterial suspension was added in 1 ml aliquots to an equal volume of pooled sputum, urine or stool sample from patients known to be Serratia-free and 0.05 ml samples of the resulting specimens were inoculated onto the test medium. The plates were incubated at 30°C and the colonies counted at 48 h. All spiking was performed in duplicate.

The CT agar was considered unsuitable as S. marcescens was only recovered at bacterial densities $> 10^5$ and contaminating organisms grew in high numbers. Using MAC and SMAC, S. marcescens was present at all concentrations, although with MAC bacterial densities of $< 10^6$ cells/ml proved difficult to recover from within the relatively small zone of inhibition surrounding the colistin disc. The increased inhibitory effect resulting from the addition of colistin to SMAC, combined with the distinctive pink colonial morphology, thus rendered SMAC the selective medium of choice. To ensure maximum recovery of S. marcescens both SMAC and MAC were used for screening.

Screening

Two patient groups were studied for carriage of S. marcescens. Study A included all new patients admitted to the ICU over a 3-month period, with initial samples being taken within 24 h of admission when possible. In study B, all patients in general wards already known to be culture-positive for S. marcescens at any site were screened over a 7-month period. Screening in studies A and B continued throughout the patients' period of hospitalization. All patients were screened using urine samples and nasal and throat swabs (Transwabs, Medical Wire and Equipment Co. Ltd.), taken twice weekly when possible. Stool samples were also collected twice weekly or as often as available. Patient data recorded from the charts included presenting complaint, past medical history, antibiotic history and invasive procedures.

Environmental samples were taken in the ICU at the beginning of the study period and 2 months later. Samples included swabs from all counter tops, window sills, beds, ventilator masks and tubing, dialysis equipment, sinks, drains, taps, bedpan macerators, urine jugs, urine jugs and from the external surface of unused intravenous infusion bags and giving sets. Contact plates were taken from bedclothes and curtains. Food and water testing, air sampling and in-use tests of disinfectants were also performed twice during the study period. Random hand swabs were taken from medical and nursing staff 4 times during the study period.

Bacterial isolation and epidemiological typing

All screening specimens from both patient groups and swabs from the environmental survey were inoculated onto the SMAC and MAC media. Food samples were homogenized in Ringer's solution (20 g in 80 ml) and dilutions of 1 : 10, 1 : 100 and 1 : 1000 were prepared. A 20-µl portion of each dilution and homogenate was inoculated onto both media. A 10-ml portion of homogenate was incubated overnight in an enrichment broth and inoculated (20 µl) onto both media. Water samples were filtered using Millipore filters (0.45 µm pore size) and the filters inoculated onto both media. Air sampling was performed using a Biotest RCS centrifugal air sampler with Biotest Hycon Agar Strips C. In-use tests of disinfectants were performed by making a 1 : 10 dilution of each disinfectant in appropriate diluent (nutrient broth or nutrient broth with Tween 80 3% w/v). Samples of dilute and neat disinfectant (20 µl) were inoculated onto both media. Hand samples were taken using saline-moistened swabs.

All laboratory samples were inoculated within 2 h of being taken and incubated at 30°C for up to 1 week. All oxidase-negative, Gram-negative bacilli were identified and spotted on DNase–toluidine blue agar. Organisms that were DNase-positive were identified using the API (20E) system (bio-Merieux SA, Montalieu Vercieu, France). Identification was confirmed using supplementary arabinose and raffinose peptone broth sugar fermentation to exclude S. liquefaciens (20). Isolates were sent to Colindale Public Health Laboratory for serotyping and randomly amplified polymorphic DNA typing (RAPD-PCR).

RESULTS

Screening

Of 40 ICU patients in Study A, 4 without evidence of S. marcescens infection were positive on screening over a 47-d period: 1 nose, 1 throat and 2 nose and throat swabs grew S. marcescens. A further 2 patients who were negative when screened in the ICU had S. marcescens isolated from routine samples 3 and 4 d, respectively after discharge to the general ward. One patient was negative on screening over a 25-d period despite blood cultures positive for S. marcescens.

In Study B, 37 patients with S. marcescens infection were screened for the duration of their hospital stay, which averaged $\leq$ 2 weeks. The results are summarized in Table I. Carriage at another site was identified in 24 patients (65%). Fifteen patients (43%) demonstrated carriage at multiple sites which tended to persist for the duration of their hospital stay. Of 6 patients with prolonged hospital stays, 4 demonstrated persistent carriage over a 4–8-week period (mean 5 weeks). In addition, 2 patients demon-

Table I. *Carriage sites of 37 patients infected with S. marcescens*

Initial isolation site	Total No. of patients	% of patients positive at each site			
		Nose	Throat	Urine	Faeces
Sputum	21	30 (6/20)	70 (14/20)	25 (4/16)	58 (7/12)
Blood	6	50 (3/6)	33 (2/6)	20 (1/5)	50 (1/2)
Wound and others	10	33 (2/6)	57 (4/7)	17 (1/6)	0 (0/5)
Total	37	31 (11/35)	59 (20/34)	22 (6/27)	42 (8/19)

Table II. *Serotyping results of S. marcescens isolates*

Serotype	Study A	Study B
O14:K14	17	6
O21:K14	6	–
O21:K3	4	–
O8:K14	1	–
ONT:K14	4 [a]	–
ONT:K22	1	–
O19:KNT	1	–
O4:KNT	1	–

[a] Isolates identified as O14:K14 with RAPD typing.

strated persistent carriage on readmission to hospital, 2 and 6 months, respectively after the initial isolation of S. marcescens from clinical specimens.

A total of 136 environmental samples were taken. All food, water, air and disinfectant testing was negative. All swabs from the hands of staff were also negative. Three of 4 sinks sampled in the main ICU area were positive for S. marcescens. The bedpan macerator in the ICU was also positive for S. marcescens. Two of the sinks and the bedpan macerator remained positive on both screening samples, 2 months apart. All other environmental samples were negative.

Epidemiological typing

Serotyping results for the 6 patient isolates from Study A showed that all patients were infected with serotype O14:K14 (Table II). In addition, all 6 strains had similar banding patterns on RAPD-PCR. Thirty-five isolates from 37 patients were sent for serotyping from Study B. Serotype O14:K14 was most frequently identified, being found in 17 patients (49%), and 13 of these isolates had similar RAPD profiles. O21:K14 was found in 6 patients (17%), and these isolates were found to have 2 distinct RAPD profiles. The remaining serotypes identified were 4 O21:K3 (11%), again all with similar RAPD profiles, and 1 O8:K14 (3%). The O antigen was non-typable in 5 (14%) patients, 4 of these isolates being identified as O14:K14 on RAPD. Two strains (6%) had non-typable K -antigen, and these also had unique RAPD profiles. Of the 21 (61%) patients colonized with serotype O14:K14, 20 (95%) demonstrated carriage at other sites. Of 13 patients colonized with non-O14 strains, 4 (31%) had carriage at other sites. Of the 4 environmental isolates, serotype O14:K14 was only exhibited by the isolate recovered from the bedpan macerator. The sink isolates were ONT:KNT and ONT:K22. RAPD-PCR results did not suggest any relationship between the ONT:KNT strains and any of the patient strains.

DISCUSSION

The mode of spread and routes of infection of S. marcescens are not fully understood. Investigations of outbreaks of infection implicate hand-to-hand transmission by staff (8, 10, 12). Patients themselves are often thought to act as reservoirs of infection. We have recently reported a large outbreak of S. marcescens in a bone marrow transplantation unit (6) where persistent faecal carriage was an important factor in prolonging the outbreak. Precise knowledge of carriage sites is essential in order to better understand the transmission and control of infection.

The reported carriage sites of S. marcescens from various studies are summarized in Table III. The majority of these are point prevalence studies (13, 15, 21) conducted during an outbreak. While 2 studies did conduct continuous screening, these investigated limited patient subgroups only (13, 14). In addition, only 1 study was performed on Serratia-infected patients (15). In the present study we carried out extensive screening over a 7-month period, during which there was no evidence of an outbreak of S. marcescens infection. Carriage was investigated in all patients in the ICU, where S. marcescens is know to be prevalent, and also in patients on general wards who were already known to be infected with the organism.

Carriage of S. marcescens was found in 10% of ICU patients. This figure is lower than carriage rates reported in other studies where ongoing screening was also performed on non-infected compromised patients. This difference may, however, result from the sampling difficulties experienced with ICU patients. Patients already known to be infected with S. marcescens (study B) had a carriage rate of 65%. Fifteen (43%) infected patients demonstrated carriage at numerous sites and this carriage tended to persist throughout their hospital stay, which averaged $\approx$ 2 weeks. The throat was the commonest site of carriage, with S. marcescens being isolated from 59% of these patients, and nasal carriage was 31%. Throat carriage was highest in patients who previously had S. marcescens isolated from sputum (70%). The very high rate of carriage reported here, compared to that of a previous study (15), may reflect the prolonged and repeated screening methods used. All the cases in Study B appeared to be examples of sporadic infection as there was no geographical or temporal link between them.

S. marcescens is thought to have a predilection for the urinary tract (13). We found a urinary carriage rate of 22% in infected patients only. None of the ICU patients demonstrated urinary carriage but this may reflect their exposure to broad-spectrum antimicrobials.

The presence of S. marcescens in the adult gut has long been disputed (13, 16, 22). In agreement with the results of previous authors (15) who isolated S. marcescens from the faeces of $\approx$ 30% of infected patients we also found a high rate of faecal carriage (42%) in infected patients. The high rate of faecal carriage obtained in our study may again reflect the extended selective screening techniques employed. Urinary carriage was identified in 37% of patients with faecal carriage, which suggests that autogenous urinary infection may occur in some cases and conflicts with

Table III. *Summary of previous studies investigating carriage sites of S. marcescens*

Reference	Patient population	No. of patients screened	Screening duration	Carriage rates (%)					
				Nose	Throat	Gums	Axilla	Rectum	Urine
(14)	Lymphoma	444	Twice only	22	–	22	14	32	–
	Haematological neoplasm	296	Twice weekly for 51 months	18	–	39	20	43	–
	Solid tumours	574	Twice only	14	–	31	22	17	–
(21)	General medical-surgical patients	69	1 d	–	4	–	–	0	6
(13)	General medical-surgical patients and staff	136	1 d	–	4	–	–	2	9
	Male urology patients	62	Alternate days for 27 d	–	2	–	–	3	16
(15)	Serratia-infected patients	34	1 d	0	3	–	–	11	–
Present study	Serratia-infected patients	37	Twice weekly for 7 months	34	59	–	–	42	22
	Non-Serratia-infected patients	40	Twice weekly for 3 months	8	8	–	–	0	0

the findings of previous authors (10). No faecal carriage was found in ICU patients but this may again reflect exposure to broad-spectrum antimicrobials or the small number of ICU stool specimens collected.

Serotyping demonstrated that the majority of isolates in both groups were of the O14:K14 or O21:K14 serotypes. This reflects the serotypes previously associated with outbreaks in our hospital group (4, 5). RAPD typing was also useful as an alternative and readily accessible epidemiological typing tool. In general, there was good correlation between serotyping and the RAPD types, with the majority of isolates from each serotype demonstrating distinct DNA profiles. However, RAPD typing had the advantages of characterizing serotype non-typable strains and of establishing epidemiological links between patients and environmental sources. The O14:K14 strain isolated on 2 separate occasions from the bedpan macerator in the ICU had a similar RAPD banding pattern to the O14:K14 strains isolated from patients in the ICU. The macerator was implicated as the source of a previous outbreak of S. marcescens infection (5). While no positive stool samples were identified in the ICU, we have demonstrated a significant level of faecal carriage in infected patients. Faecal carriage may therefore provide a previously unrecognized reservoir of infection in adults.

Hand carriage is likely to be important in the transmission of infection. In this study all staff hand swabs were negative. However, the swabs were collected randomly. A more accurate reflection of hand carriage may be achieved through routine swabs at the beginning, during and at the end of a shift. Furthermore, staff awareness of and attention to adequate handwashing may have improved during the study period. Effective infection control policies in the unit may also explain the low number of isolates recovered from the environment. Nevertheless, the fact that any isolates were recovered from the ICU despite adherence to the infection control procedures reflects the capacity of this organism for survival. This survival may also reflect the ability of S. marcescens to withstand the selective pressure exerted by broad-spectrum antimicrobials in this environment (23).

We have demonstrated that patients infected with S. marcescens at 1 site are likely to have multiple carriage sites. Of particular interest, we found a high level of faecal carriage in infected patients. Once established, carriage often persisted for the duration of the hospital stay and clearly represents a potential source of infection to other patients. Significant levels of carriage were also detected in patients with no prior evidence of S. marcescens infection. This probably reflects the high prevalence of the organism in our hospital group. These patients represent a previously unrecognized reservoir of infection. To prevent spread of S. marcescens infection in an area where the organism is frequently isolated, vigilant infection control measures are required when dealing with all patients, whether or not they are known to be infected.

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Submitted February 26, 2001; accepted May 3, 2001

P104. Phase variation of biofilm adhesin production in Staphylococcus epidermidis is unaffected by growth conditions and involves modulation of adhesin gene expression

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The importance of Staphylococcus epidermidis as a major nosocomial pathogen is based on its ability to form biofilms on implanted biomaterials. Biofilm formation is a phase variable phenotype (subject to ON-OFF switching) and is dependent on expression of the ica operon which encodes a polysaccharide adhesin. Although a minority of biofilm negative (OFF) variants contain a disrupted ica operon, the molecular mechanism responsible for production of the majority of phase-OFF variants remains unknown. In addition, nothing is known about environmental conditions, which influence ON-OFF switching frequencies.

Our study had two principal aims:

1. To assess the affect of changing growth conditions on phase variation;
2. To determine the molecular basis for production of phase-OFF variants.

We developed a new assay to measure phase variation frequencies following growth of single colonies under different environmental conditions. None of the parameters tested including high osmolarity, sub-inhibitory tetracycline, anaerobic, low temperature and nutrient-limiting conditions significantly affected switching frequencies. Furthermore, using long range polymerase chain reaction, the majority of biofilm-negative variants examined were found to contain an intact ica operon indicating that altered regulation of ica operon expression was responsible for loss of biofilm forming capacity. We are currently examining the molecular basis for this altered regulation. Taken together, our data suggest that modulation of ica operon expression is the key molecular mechanism employed for both the regulation of biofilm formation and phase variation of polysaccharide adhesin production.

P105. Salmonella Bredeney, an emerging problem

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Salmonella enterica serotype Bredeney has emerged as the third most commonly identified serotype among human clinical isolates referred to the Irish National Salmonella Reference Laboratory in the years 1998-2000. A total of 112 isolates of Salmonella Bredeney from animal, food and human sources from both Ireland and Northern Ireland were studied. Antimicrobial susceptibility testing, pulsed field gel electrophoresis (PGFE), DNA amplification fingerprinting (DAF) and plasmid profiles were performed. Eight different PFGE patterns were obtained, with the majority (n=97) grouping as PFGE pattern A. PFGE group A was predominant in animals, food and humans and within this group antimicrobial resistance was more common in isolates of animal origin than in human clinical isolates. There was good overall concordance between the groups identified by PFGE and DAF. A total of 74% of isolates were susceptible to all antimicrobial agents tested. Resistance to multiple antimicrobial agents, ASSuTTm, was observed in a single isolate. Plasmid profiling was carried out on a subset of the Bredeney strains (31) including many resistant isolates. Seven different profiles, a-g, were obtained, with the majority (18) fitting into profile b. Resistance to SuTM appears to be associated with the 25Kb plasmid.

P106. Investigation of chromosomal β-lactamase expression in clinical isolates of Serratia marcescens

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Serratia marcescens has emerged in Dublin hospitals as a serious nosocomial pathogen exhibiting multiple antimicrobial resistance determinants including cross-resistance to cephalosporins.[1] In most enterobacteria cross-resistance to cephalosporins is associated with over-production or derepression of chromosomal AmpC β-lactamase arising from mutations in a trans-acting gene, ampD.[2] In cephalosporin-resistant S. marcescens, howev-

er, constitutive AmpC production and cross-resistance appear to occur at a lower level. In this study, we investigate if cephalosporin cross-resistance in S. marcescens is also associated with ampD mutations. Following Southern hybridisation of Pst1 digested chromosomal DNA, the ampD gene was located in S. marcescens on a fragment of ~1.1kb, using DIG-labelled polymerase chain reaction (PCR)-derived probes from Enterobacter cloacae. Suspect DNA fragments were cloned and target inserts were sequenced following colony-hybridisation of the transformants. The ampD sequence was confirmed by PCR, using S. marcescens-specific ampD primers. The resulting ampD sequences from nine clinical isolates (of varying cephalosporin susceptibility) and one control strain of S. marcescens were aligned and examined for evidence of mutation. Analysis revealed that cephalosporin-resistance in S. marcescens was not associated with the predicted ampD mutational events,[2] with all isolates tested demonstrating almost 100% similarity with each other. These results suggest that over-production of AmpC β-lactamase in S. marcescens may represent a novel mechanism unlike the stable derepression seen in other enterobacteria.

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P107. Toxin detection in Clostridium difficile-associated diarrhoea: an evaluation

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The study evaluated the performance of diagnostic approaches for Clostridium difficile-associated diarrhoea (CDAD). Consecutive stool specimens (n=200), from 133 patients, for routine investigation of C. difficile infection were examined by cytotoxin assay (CTA), culture and by four enzyme immunoassays (EIA) test systems: Oxoid Toxin A (OTA), Meridian ImmunoCard Toxin A (MICA), TechLab Tox A/B II (TLAB) and Premier Toxins A&B (PRAB).

Based on both the clinical and laboratory criteria the overall incidence rate for CDAD was 27%. Sixty-nine (34.5%) specimens were positive by one or more of the six methods and 16 (8%) were positive by all six methods. When discordant results were resolved by chart review the adjusted sensitivity and specificity were as follows: CTA 94%, 98%; OTA 51%, 98%; MICA 54%, 99%; TLAB 77%, 98%; PRAB 80%, 98%; culture 49%, 100%, respectively. The toxin A specific assays (OTA and MICA) and culture demonstrated poor sensitivity and would be unsuitable as stand-alone diagnostic tests for CDAD. The EIAs detecting both toxins A and B (TLAB and PRAB) performed better with the PRAB kit providing the best EIA performance. The TLAB and PRAB tests are acceptable tests for diagnosis of CDAD but are not equivalent to CTA.

P108. A 12-year review of CNS abscesses; presentation and aetiology

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Pyogenic abscesses within the central nervous system are potentially life-threatening. Early diagnosis and aggressive management can greatly improve outcome.

We are reviewing 170 patients presenting to the National Neurosurgical Centre in Beaumont Hospital between 1988 and 2000 with infections of the central nervous system. Retrospective review to date of 115 males and 46 females revealed that the age range was 1-83 years.

The most common presentation was headache, closely followed by pyrexia and focal neurological signs.

Volume 170
Number 1
January, February, March 2001

Supplement 2

Irish Journal of Medical Science

Offical Organ of
The Royal Academy of Medicine in Ireland,
Established 1832

National Scientific Medical Meeting

6th April 2001

The Royal College of Physicians of Ireland

Journal of Hospital Infection (1998) 38: 135–141

An outbreak of an unusual strain of *Serratia marcescens* in two Dublin hospitals

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Summary: We describe a serious outbreak of infection caused by a strain of *Serratia marcescens* in two Dublin hospitals which occurred over an 11 week period and affected a total of 15 patients. A contaminated bed-pan macerator in the Intensive Care Unit of one hospital was identified as the possible source of infection and spread of the organism probably occurred via hand transmission by hospital personnel and via patient transfer to a second hospital. All isolates of *S. marcescens* involved in the outbreak had the same antimicrobial susceptibility pattern, with reduced susceptibility to gentamicin, cefotaxime and ciprofloxacin. Epidemiological typing revealed that the strains of *S. marcescens* isolated in the outbreak were of an uncommon serotype, O21:K14, and using pulsed-field gel electrophoresis, *Xba*I DNA macrorestriction profiles clustered at 90% similarity. The DNA patterns of the outbreak strain were also highly similar to *S. marcescens* isolates of the same serotype recovered from a separate Dublin hospital during the same time period as the outbreak described here. In addition, the isolates clustered at 82% similarity with strains of the same serotype from a retrospective collection of *S. marcescens* isolates from various hospitals in the Dublin area, indicating that these may be genetic variants of the same strain. Although the outbreak was brought under control following implementation of infection control measures, a significant number of similar O:21 isolates of *S. marcescens* have since been identified in four Dublin hospitals. These results suggest the unique spread of a single strain of *S. marcescens* in Dublin hospitals.

Keywords: *Serratia marcescens*; epidemiological typing.

Introduction

Serratia marcescens is now established as a nosocomial pathogen, causing a range of infections such as pneumonia, urinary tract infection, wound infection and bacteraemia.[1] Outbreaks of *S. marcescens* have been described in a variety of clinical settings where many different environmental sources, such as disinfectants,[2] bronchoscopes[3] and electrocardiogram bulbs[4]

Received 30 July 1997; revised manuscript accepted 14 January 1998.

0195–6701/98/060135+07 $12.00/0

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have been identified. More recently *S. marcescens* has also been associated with transfusion-transmitted septicaemia occurring in neonatal units in Denmark and Sweden.[5]

In Ireland, *S. marcescens* first emerged as a pathogen in the 1980s, when it was reported as the causative agent in two simultaneous outbreaks in a Dublin hospital.[6] Since then, strains of *S. marcescens* with a predominant O14:K14 serotype have become endemic in our hospital group and at present up to three new patients become infected or colonized each week. Whilst many other centres have reported outbreaks of infection involving multiple antimicrobial resistance,[7,8] the majority of our *S. marcescens* isolates have remained, in general, susceptible to most of the frequently used antimicrobials. However, recently an outbreak of an unusual strain of *S. marcescens* occurred in two hospitals within our hospital group which was resistant to cefotaxime and gentamicin and also showed reduced susceptibility to ciprofloxacin. The details of this outbreak are described here. The emergence of this unusual strain in our endemic population and its relationship to other similar strains seen in Dublin hospitals is also examined.

Patients and methods

Description of outbreak

During an 11 week period, from 29 February to 13 May 1996, we observed an outbreak of *S. marcescens* infection in two hospitals (A and B) within our hospital group. Of the 15 patients involved in the outbreak, seven were considered to have clinically significant infection, i.e., patients had signs and symptoms of infection and *S. marcescens* was cultured from the infected sites (Table I). The other eight patients were colonized with *S. marcescens* in the absence of any signs or symptoms of infection. The hospitals in question are located in close proximity and there is cross-over of both staff and patients between them. Hospital A has 250 beds, with a four-bed Intensive Care Unit (ICU) and hospital B has 180 beds with a three-bed ICU.

In the index case, a 33 year old man, *S. marcescens* was first isolated from blood cultures, sputum, and abdominal drain fluid, nine days after admission to ICU in hospital A. As a result of his underlying illness he stayed in ICU for a further three weeks. He remained colonized with *S. marcescens* in the respiratory tract until his discharge from hospital 10 weeks later.

Over the following 11 weeks, *S. marcescens* was isolated from a further 14 patients suspected of being involved in the outbreak. Eleven of these cases were located solely in hospital A, two were transferred from hospital A to hospital B prior to isolation of *S. marcescens* and one case was located solely in hospital B (Figure 1).

Infection control measures

At the start of week nine it became apparent that an outbreak was occurring and infection control measures were implemented. Strict hygiene measures were adopted, and the contaminated macerator and sluice room in hospital A were thoroughly cleaned. The patients involved in the outbreak were isolated if possible, or cohorted together. Staff were educated about *S. marcescens*, particularly modes of transmission; the importance of effective handwashing was emphasized.

Extensive environmental sampling was undertaken in the ICU of hospital A. Samples were taken from a variety of sites, including hand basins, hand disinfectants, urine jugs, the sluice, infusion bags, ventilation equipment and the blood gas analyser, using moistened swabs and transported to the laboratory using sterile Trans tubes containing transport media (Medical wire and Equipment Co. Ltd., Corsham, UK). Although hand swabs are most frequently used for the detection of *S. marcescens* carriage in medical personnel,[9,10] infection control measures including rigorous handwashing had already been implemented in the ward. As an alternative, healthcare workers in the area were therefore screened for possible *S. marcescens* carriage using throat swabs, although previous studies have failed to identify staff carriage using this site.[11]

Table 1 *Details of seven patients with clinically significant* S. marcescens *infection*

Patient number	Age, sex	Hospital	Ward	Diagnosis	No. of days in hospital before first *Serratia* isolation	Site of infection	Treatment	Short-term outcome
1	33, M	A	ICU	Acute pancreatitis	9	Sputum, BC, abdominal fluid	Meropenem	Recovered
4	25, M	A	ICU	Peritonitis	11	Sputum, urine, abdominal fluid	Meropenem	Recovered
5	88, F	B	Ward	Perforated appendix	28	Sputum, urine, abdominal fluid	Meropenem	Recovered
7	90, M	A	ICU	Perforated DU	6	BC	Meropenem	Died
9	84, F	A	ICU	Perforated bowel	8	BC, sputum	Meropenem	Recovered
10	70, M	B	ICU	Pneumonia; ventilated	19	Sputum, BAL	Meropenem	Recovered
13	69, M	A	ICU	Bowel obstruction	3	Sputum, abdominal fluid	Meropenem	Recovered

M, male; F, female; DU, duodenal ulcer; BC, blood culture; BAL, bronchoalveolar lavage.

Bacterial isolates

Over the 11 week period in question, 36 isolates of *S. marcescens* were collected from 12 of the patients involved in the outbreak in hospitals A and B. A further three patients were infected with the outbreak strain, but unfortunately isolates were not available for study. Of the 36 isolates, 10 were recovered from blood cultures, 11 from sputa, one from a bronchoalveolar lavage, 10 from deep abdominal sites, two from central and arterial line tips and two from urine cultures. Two additional isolates were recovered from the bed-pan macerator in the ICU of hospital A. The 38 isolates were cultured for purity on Columbia agar (Lab M, Bury, UK) supplemented with 7% horse blood and frozen at −70°C on Protect beads (Technical Service Consultants Ltd., Lancashire, UK). They were removed from −70°C and subcultured twice on Columbia blood agar immediately before testing.

Bacterial identification

Isolates were identified using the API 20E (Bio-Mérieux SA, Montalieu Vercieu, France) system. Additional identification tests including arabinose and raffinose peptone broth sugar fermentation[12] were used where necessary.

Susceptibility testing

Routine susceptibility testing was performed using a modified Stokes disc diffusion method on Diagnostic Sensitivity Test agar (Oxoid Ltd, Basingstoke, UK), with *Eschericia coli* NCTC 10418, as the control. The antimicrobials used were ampicillin (10 μg), cefotaxime (30 μg), cefuroxime (30 μg), ceftazidime (30 μg), ciprofloxacin (1 μg), gentamicin (10 μg), meropenem (10 μg) and piperacillin/tazobactam (75 μg + 10 μg). To characterize the outbreak strain further and to compare it with previous *S. marcescens* outbreaks in our hospital group[6] an extended antimicrobial susceptibility profile was also performed using chloramphenicol (10 μg), sulphafurazole (100 μg), trimethoprim (1·25 μg), tetracycline (10 μg) and on a wide range of aminoglycosides including amikacin (30 μg), tobramycin (10 μg), kanamycin (30 μg), neomycin (30 μg) and netilmicin (30 μg). Isolates were also tested for susceptibility to phenylmercuric acetate (10 μg) and mercuric ions (10 μg of $HgCl_2$).

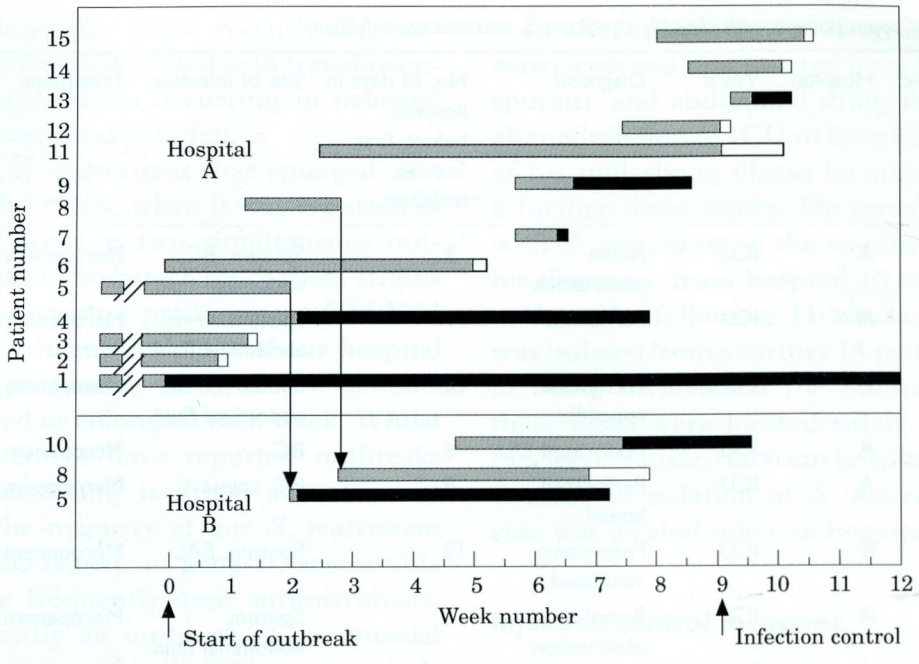

Figure 1 Duration of hospital stay of patients, prior to first isolation of *S. marcescens* (▨), colonized with *S. marcescens* (□) and infected with *S. marcescens* (■), in hospitals A and B. Patients 1, 2, 3 and 5 were in hospital for varying periods of time prior to the start of the outbreak. Patients 5 and 8 were transferred from hospital A to B.

Minimum inhibitory concentrations (MIC) were determined for gentamicin (Sigma, UK), cefotaxime (Sigma, UK) and ciprofloxacin (Bayer, Berkshire, UK) according to the NCCLS recommendations for the microdilution broth method.[13] Serial dilutions of the antimicrobials were prepared and dispensed into 96 well microtitre trays, to give a final concentration of 0·25–256 μg/mL for gentamicin and cefotaxime and 0·06–64 μg/mL for ciprofloxacin. The bacterial inoculum was grown overnight in Mueller–Hinton II broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, USA) and diluted to give a bacterial density of 1×10^5 cfu/mL. Two NCTC reference strains, *E. coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662, were included as controls. Microtitre trays were incubated at 37°C for 18 h. The MIC was recorded as the lowest concentration of antimicrobial agent at which no visible growth was observed.

Epidemiological typing

O and K serotypes and bacteriophage reactions[14] were determined by the Laboratory of Hospital Infection, Central Public Health Laboratory (CPHL), Colindale, UK, on a representative isolate from each patient and from environmental isolates. The isolates were also compared using pulsed-field gel electrophoresis (PFGE) of *Xba*I chromosomal digests.[15] Representative isolates from this study and isolates of *S. marcescens* of the same serotype and similar antimicrobial susceptibility from a separate Dublin hospital, recovered over a time period corresponding to the outbreak described here, were also examined by PFGE. A retrospective survey of serotyping results on *S. marcescens* from various hospitals in the Dublin area, dating back to 1982, also revealed a small number of sporadic isolates in 1989 and 1994, with the same serotype as the outbreak strain. Since this serotype was infrequent, these strains were also compared using PFGE. The banding patterns produced were analysed using GelCompar (Applied Maths, Kortrijk, Belgium) and a dendrogram of percentage similarity, as calculated by the Dice correlation coefficient and represented by UPGMA, was prepared. The PFGE banding patterns and calculated values

for percentage similarity were interpreted using the criteria outlined by Struelens *et al.*, 1992.[16]

Results

A case was defined as any patient, in either hospital A or hospital B, who was infected or colonized with *S. marcescens* of the same type as the index case. Of the 15 patients involved, seven were infected and eight were colonized. Seven of 14 cases in hospital A, including the index case, were located in ICU. No other ward in hospital A had more than two cases isolated and of the three cases in hospital B, two had been transferred from hospital A. One of the patients infected with *S. marcescens*, who had a severe underlying disease, died, but all others recovered from their infection following treatment with meropenem.

During environmental testing, *S. marcescens* was isolated from the internal and external surface of the ICU bed-pan macerator, in hospital A. All other environmental samples were negative. Repeat samples from the internal and external surface of the macerator after the outbreak were also found to be negative for *S. marcescens*. Staff screening for *S. marcescens* was negative.

Susceptibility testing

All isolates recovered from both patient and environmental sources from hospitals A and B gave the same characteristic susceptibility pattern and were resistant to ampicillin, cefuroxime, sulphafurazole, trimethoprim, tetracycline and mercuric ions. Reduced susceptibility was also demonstrated to cefotaxime, gentamicin and ciprofloxacin. Quantitatively, all *S. marcescens* isolates had the same antimicrobial susceptibility, with MIC values of 16 μg/mL for gentamicin, 32 μg/mL for cefotaxime and 1·0 μg/mL for ciprofloxacin.

Epidemiological typing

All representative isolates from the outbreak in hospitals A and B were of the same serotype, O21:K14. None of the isolates demonstrated strong phage reactions but gave a weak but consistent reaction with phage 5, which is uncommon for this serotype (H. Aucken, personal communication). PFGE banding patterns (Figure 2) showed that outbreak isolates differed by up to three bands from each other and clustered at approximately 90% similarity by the Dice coefficient. Isolates from a separate Dublin hospital, recovered over a similar time period as the outbreak, produced banding patterns which clustered with the outbreak strain at a percentage similarity of approximately 93%, which

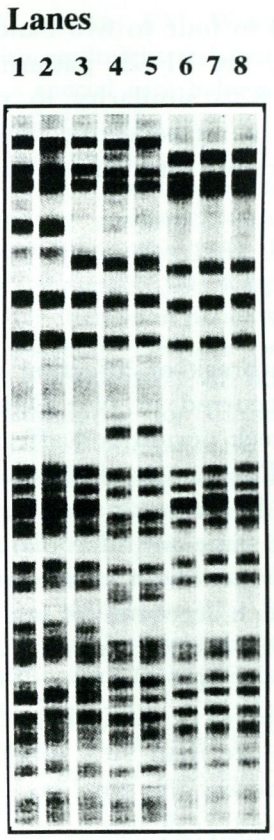

Figure 2 Comparison of pulsed-field gel electrophoresis of whole-cell DNA from *S. marcescens* isolates digested with *Xba*I, using a composite Gel Compar generated image. Lanes 1–2 show the PFGE patterns of isolates from two patients in the outbreak described here. Lanes 3–5 show the PFGE pattern of separate isolates of *S. marcescens* from a retrospective collection of O21:K14 strains from hospitals in the Dublin area and lanes 6–8 show the PFGE patterns of isolates of *S. marcescens* from a separate Dublin hospital, recovered over a time period corresponding to the outbreak described here.

corresponded to four to five band differences. Furthermore, the PFGE pattern of outbreak isolates had 82% similarity to representative strains from a retrospective collection of *S. marcescens* strains from various hospitals in the Dublin area.

Discussion

A serious outbreak of infection caused by *S. marcescens* occurred in two Dublin hospitals over an 11 week period and affected a total of 15 patients. The results of the study show that all strains of *S. marcescens* isolated in the outbreak had the same antimicrobial susceptibility profile and were of an uncommon serotype, O21:K14, which accounts for less than 4% of a collection of 500 distinct strains of *S. marcescens*, compiled at CPHL.[17] The DNA macrorestriction profiles of the isolates from the outbreak were more than 90% similar, indicating that they represent the same genetic lineage or strain.[16] The outbreak strain also demonstrated greater than 90% similarity with *S. marcescens* isolates from another Dublin hospital, with which no epidemiological link could be established. Furthermore, PFGE of DNA from a retrospective collection of *S. marcescens* isolates also indicates that this outbreak strain may have evolved from the O21:K14 strain previously associated with isolated episodes of infection in the Dublin area.

The epidemiological data indicate that the outbreak originated in the ICU in hospital A, and that transmission of infection to hospital B occurred via patient transfer. We identified the bed-pan macerator in ICU in hospital A as a possible source of infection. Whilst staff screening using throat swabs was found to be negative, the predominant mode of spread of *S. marcescens* is thought to be hand transmission by hospital personnel.[18,19] We therefore believe that the spread of the organism to and from the macerator probably occurred via the hands of healthcare workers.

Following implementation of infection control measures and extensive re-education of staff, the outbreak was brought under control. However, it is worthwhile noting that over the 15 month period since the outbreak, ongoing surveillance of *S. marcescens* has identified an additional seven isolates with the same antimicrobial susceptibility and serotype as the outbreak strain. These strains were isolated in both hospitals A and B, from six different wards, with none occurring in the ICU in hospital A. Over this time period, a further three such isolates have also been identified from other hospitals in the Dublin area. In addition, since the time of the outbreak, epidemiological typing has also identified seven O21:KNT *S. marcescens* isolates, with the same antimicrobial susceptibility as the outbreak strain. These isolates are thought to represent acapsular variants of the O21:K14 outbreak strain, which may have lost the K antigens with the passage of time (H. Aucken, personal communication). These results may therefore indicate that, whilst the outbreak was curtailed, the O:21 *S. marcescens* strain appears to have become endemic in some Dublin hospitals.

Nosocomial outbreaks of *S. marcescens* are commonly associated with multiple antimicrobial resistance.[7,20] A worrying aspect of the outbreak described here, however, is that the *S. marcescens* strain demonstrated resistance to the antimicrobials most frequently used in first-line therapy of nosocomial infection, in our hospital group, i.e., gentamicin, cefotaxime and ciprofloxacin. Furthermore, since cephalosporin resistance in *S. marcescens* is largely attributable to class one cephalosporinase,[21] the use of other cephalosporins such as ceftazidime was not indicated. Patients infected with the outbreak strain therefore required treatment with meropenem, an antimicrobial normally kept in reserve. The emergence of a strain of multiresistant *S. marcescens* causing infection in a number of hospitals in a localized geographical area is clearly a cause for concern.

Acknowledgements

The authors wish to thank Dr Hazel Aucken from the Laboratory of Hospital Infection, at

the Central Public Health laboratory in Colindale, for her expert advice during this study. We also acknowledge the kind gift of ciprofloxacin from Bayer plc.

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Bone Marrow Transplantation (2000) 25, 873–877
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An outbreak of multiply resistant *Serratia marcescens*: the importance of persistent carriage

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Summary:

An outbreak of multi-resistant *Serratia marcescens* involving 24 patients occurred in a bone marrow transplant and oncology unit, from September 1998 to June 1999, of whom 14 developed serious infection. This is the first such outbreak described in a BMT unit. All isolates demonstrated the same antimicrobial susceptibility pattern and were the same unusual serotype O21:K14. The antimicrobial susceptibility profile showed reduced susceptibility to ciprofloxacin, gentamicin and piperacillin-tazobactam. As the latter two antimicrobials are part of our empiric therapy for febrile neutropenia, they were substituted with meropenem and amikacin during the outbreak. Investigation revealed breaches in infection control practices. Subsequently, the outbreak was contained following implementation of strict infection control measures. A prominent feature of the outbreak was prolonged carriage in some patients. These patients may have acted as reservoirs for cross-infection. This report also indicates that patients who become colonised with *Serratia marcescens* may subsequently develop invasive infection during neutropenic periods. *Bone Marrow Transplantation* (2000) **25,** 873–877.

Keywords: *Serratia marcescens*; outbreak; persistence; neutropenia

Serratia marcescens is now well established as a nosocomial pathogen, resulting in considerable morbidity and mortality in susceptible patients. It is associated with a range of infections, such as pneumonia, urinary tract infection, wound infection and bacteraemia.[1] Outbreaks have been described in a variety of clinical settings and different environmental sources have been identified as reservoirs for *Serratia marcescens*, including disinfectants,[2,3] pressure transducers[4] and bronchoscopes,[5] amongst others. In addition, *Serratia marcescens* often demonstrates multiple antibiotic resistance,[6–8] necessitating the use of antibiotics usually kept in reserve. We describe an outbreak of *Serratia marcescens* in a haematology/oncology unit involving 24 patients. The outbreak was characterised by long-term persistence of *Serratia marcescens* in the environment and prolonged patient carriage, factors which undoubtedly contributed to the length of the outbreak. Furthermore, patients who were carriers frequently developed invasive infection during periods of neutropenia.

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Received 27 August 1999; accepted 13 November 1999

Materials and methods

Patient data

From September 1998 to June 1999, we observed an outbreak of *Serratia marcescens* involving 24 patients, in a 31-bed haematology-oncology unit, which incorporates the National Bone Marrow Transplant unit. The unit has four six-bedded wards, three single rooms and four HEPA-filtered BMT rooms. Fifteen patients were haematology patients, six of whom were receiving cytotoxic chemotherapy, eight were post BMT and one patient, the index case, was on cytotoxic chemotherapy during his first two episodes of sepsis and in the early post-BMT phase for his third. Nine patients were oncology patients, all of whom were receiving cytotoxic chemotherapy for their underlying disease. An infected case was defined as a patient with symptoms and/or signs of infection and *Serratia marcescens* was cultured from an infected site. A colonised case was defined as a patient in whom *Serratia marcescens* was cultured in the absence of symptoms and/or signs of infection. The number of new cases per month is shown in Figure 1.

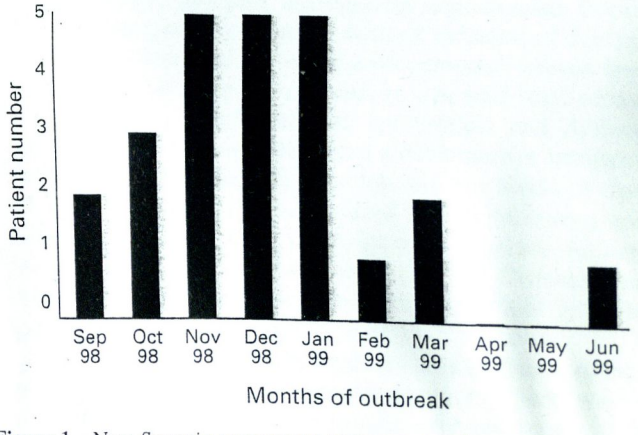

Figure 1 New *Serratia marcescens* cases per month.

Infection control

In November 1998, it became apparent that an outbreak was occurring and infection control measures were reviewed. Patients known to be infected or colonised were isolated in single rooms, where possible. All staff were educated about modes of transmission of *Serratia marcescens* and staff were re-trained in hand-washing using Glo-Germ (Glo-Germ Product Co, Moat, UT, USA). Briefly, the procedure is as follows: fluorescent dye is rubbed on to the hands, which are then washed and placed under a fluorescent lamp. Failure to remove the dye following handwashing is easily observed. A thorough investigation of ward procedures was undertaken, including extensive environmental screening. A variety of sites were sampled, including bathrooms, the sluice room, commodes, urine jugs, the bedpan washer, disinfectants, mouthwash, icemaker, total parenteral nutrition infusates, intravenous pumps and a BMT bed post cleaning and disinfection. In all cases moistened swabs were used and these were transported to the laboratory using sterile Trans tubes, containing transport media (Medical Wire and Equipment Co Ltd, Corsham, UK). Environmental screening was repeated in January 1999. A total of 159 environmental samples were processed. In January, 12 staff members, during their working day, were randomly screened for hand carriage of *Serratia marcescens*, using finger agar plates.

Bacterial isolates and identification

A total of 109 isolates of *Serratia marcescens* were collected from 23 of the patients involved in the outbreak. A further patient was also thought to be involved in the outbreak, but unfortunately isolates were not available for study. The organisms were isolated from different clinical sites including blood, intravenous catheter tips, urine, faeces, sputum, wound swabs, throat and skin. Two *Serratia marcescens* isolates were recovered from environmental sampling.

Isolates were identified using the API 20E (BioMérieux, Montalieu Vercieu, France) system. Arabinose and raffinose peptone broth sugar fermentation were also used to confirm identification.

Susceptibility testing

Susceptibility testing was performed on all isolates using a modified Stokes disc diffusion method on diagnostic sensitivity test agar (Oxoid Ltd, Basingstoke, UK), with *Escherichia coli* NCTC 10418, as the control. The antimicrobials used were ampicillin (10 μg), amoxicillin (20 μg)–clavulanic acid (10 μg), cefuroxime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), piperacillin (75 μg)–tazobactam (10 μg), ciprofloxacin (1 μg), gentamicin (10 μg), tobramycin (10 μg) amikacin (30 μg) and meropenem (10 μg).

The minimum inhibitory concentrations (MIC) for cefotaxime, ceftazidime, pipercillin/tazobactam, gentamicin and ciprofloxacin were determined by the E test (AB Biodisk, Sweden), on one representative isolate from each patient and both environmental isolates. *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662 were tested in conjunction.

Epidemiological typing

O and K serotypes were determined by the Laboratory of Hospital Infection, Central Public Health Laboratory (CPHL), Colindale, UK, on one isolate from each of 23 patients and on the two environmental isolates.[9]

Results

Twenty-four patients were involved in the outbreak, with the highest number of new cases arising in November, December and January (Table 1). The male/female ratio was 13/11, with a median age of 41.5 years (range 20–70). Ten patients were colonised with *Serratia marcescens*, which was isolated from such sites as throats, wounds, sputum and urine in mixed culture. Fourteen patients had 17 episodes of clinically significant infection and required therapy with antibiotics. There were nine episodes of septicaemia, one maxillary sinusitis and pneumonia and seven urinary tract infections (Table 1). No death was directly attributable to infection with *Serratia marcescens*.

In the colonised group, only one of 10 patients was neutropenic (neutrophil count $<0.5 \times 10^9$/l) at the time of initial isolation of *Serratia marcescens*. However, nine patients were neutropenic during 17 episodes of clinical infection. Importantly, seven of nine of these neutropenic patients who developed infection, were previously known to be colonised with *Serratia marcescens* (Figure 2). Eight patients were not neutropenic when they developed invasive infection, but only one patient was known to be colonised with *Serratia marcescens*.

Not all patients in the unit were screened for *Serratia marcescens* colonisation. However, *Serratia marcescens* was screened for in all clinical samples and also in faecal specimens after the outbreak was apparent. Persistence in the routine clinical specimens was a prominent feature. Overall, the average length of carriage was 7 weeks. In the colonised group, the average length of carriage was 2.6 weeks. In the infected group, the average length of carriage was 10.1 weeks, lasting 32 weeks in one patient.

Infection control

Serratia marcescens was isolated from the control buttons of an intravenous infusion pump in November 1998 and in January 1999, *Serratia marcescens* was isolated from a urine jug assigned to a patient without evidence of *Serratia marcescens* carriage. All other environmental screens were negative for *Serratia marcescens*, as was staff hand screening. However, *Staphylococcus epidermidis* and *Acinetobacter* species were isolated from a bone marrow transplant bed after cleaning and disinfection and the hands of three of 12 staff members were positive for the following noncommensal organisms: *Staphylococcus aureus*, *Acinetobacter* species, *Flavobacterium* species and *Chryseomonas* species. In addition, the Glo-Germ procedure indicated inadequate hand washing by some staff members.

Investigation of ward procedures highlighted a number of breaches in infection control practice. There was no cleaner's storage room built in the original design of the

Table 1 Cases of *Serratia marcescens* infection

Patient	*Age*	*Sex*	*Diagnosis*	*Date of infection*	*Neutropenic* ($<0.5 \times 10^9/l$)	*Infection*	*Treatment*	*Comments*
1	22	M	ALL	Sep 1998	N	Septicaemia	Meropenem + amikacin + removal of CVC	
				Nov 1998	Y	Septicaemia Cutaneous septic emboli	Meropenem + amikacin + removal of CVC	
				Feb 1999	Y	Septicaemia	Meropenem + amikacin	
2	56	F	Ovarian carcinoma	Oct 1998	N	UTI	Meropenem + amikacin	
9	64	M	Non-Hodgkin's lymphoma	Nov 1998	N	Septicaemia	Meropenem + amikacin + removal of CVC	
10	31	M	Desmoid tumour	Nov 1998	N	Septicaemia	Meropenem + amikacin + removal of CVC	
7	41	M	AML + GVHD	Nov 1998	N	Pneumonia Maxillary sinusitis	Meropenem + amikacin	Died from disseminated Aspergillus infection
12	55	F	AML	Dec 1998	Y	Septicaemia	Meropenem + amikacin + removal of CVC	
13	59	M	AML	Dec 1998	Y	UTI	Meropenem + amikacin	
19	20	M	Ewing's sarcoma	Jan 1999	Y	Septicaemia	Meropenem + amikacin	
				Apr 1999	Y	UTI	Meropenem + amikacin	
17	35	F	CML	Jan 1999	N	UTI	Meropenem	
16	45	F	AML	Jan 1999	N	UTI	Meropenem	
20	26	F	Ewing's sarcoma	Jan 1999	Y	UTI	Meropenem + amikacin	
6	30	M	AML	Feb 1999	Y	Septicaemia	Meropenem + amikacin	
23	34	M	CML	Mar 1999	N	UTI	Meropenem	
22	20	F	AML	Jun 1999	Y	Septicaemia	Meropenem + amikacin	

M = male; F = female; N = no; Y = yes; BAL = broncho-alveolar lavage; UTI = urinary tract infection; CVC = central venous catheter.

ward. This led to poor cleaning practices in the unit, such as the inappropriate storage of cleaning agents and cloths in the sluice room and the stacking of urine jugs without adequate drying. In addition, attendant staffing levels were sub-optimal. When these control of infection breaches were rectified, only one new case occurred after March 1999.

Susceptibility testing and epidemiological typing

All *Serratia marcescens* isolates tested from both patient and environmental sources, demonstrated the same susceptibility pattern and MIC values. The isolates showed resistance to ampicillin, amoxycillin-clavulanic acid and cefuroxime and reduced susceptibility to cefotaxime (MIC 8.0 μg/ml), pipercillin-tazobactam (MIC 8.0 μg/ml), gentamicin (MIC 16 μg/ml) and ciprofloxacin (MIC 1.0 μg/ml). Isolates remained susceptible to ceftazidime (MIC 0.5 μg/ml), amikacin, tobramycin and meropenem. Epidemiological typing revealed that all representative isolates taken from the outbreak were of the same serotype, O21:K14.

Discussion

There have been many recent publications describing hospital outbreaks of *Serratia marcescens*.[2,3,6,10] This is the first reported outbreak in a BMT and oncology unit. A feature of the outbreak was prolonged patient carriage, which frequently led to invasive infection during neutropenic episodes. Investigation of the outbreak indicated that lapses in infection control policies had occurred and environmental contamination was also evident.

Prolonged carriage was most notable in those patients who developed infection with *Serratia marcescens* (Table 3). However, there are few studies in the literature recording patient carriage. A report in 1976 showed persistence of *Serratia marcescens* for more than 3 months in 12

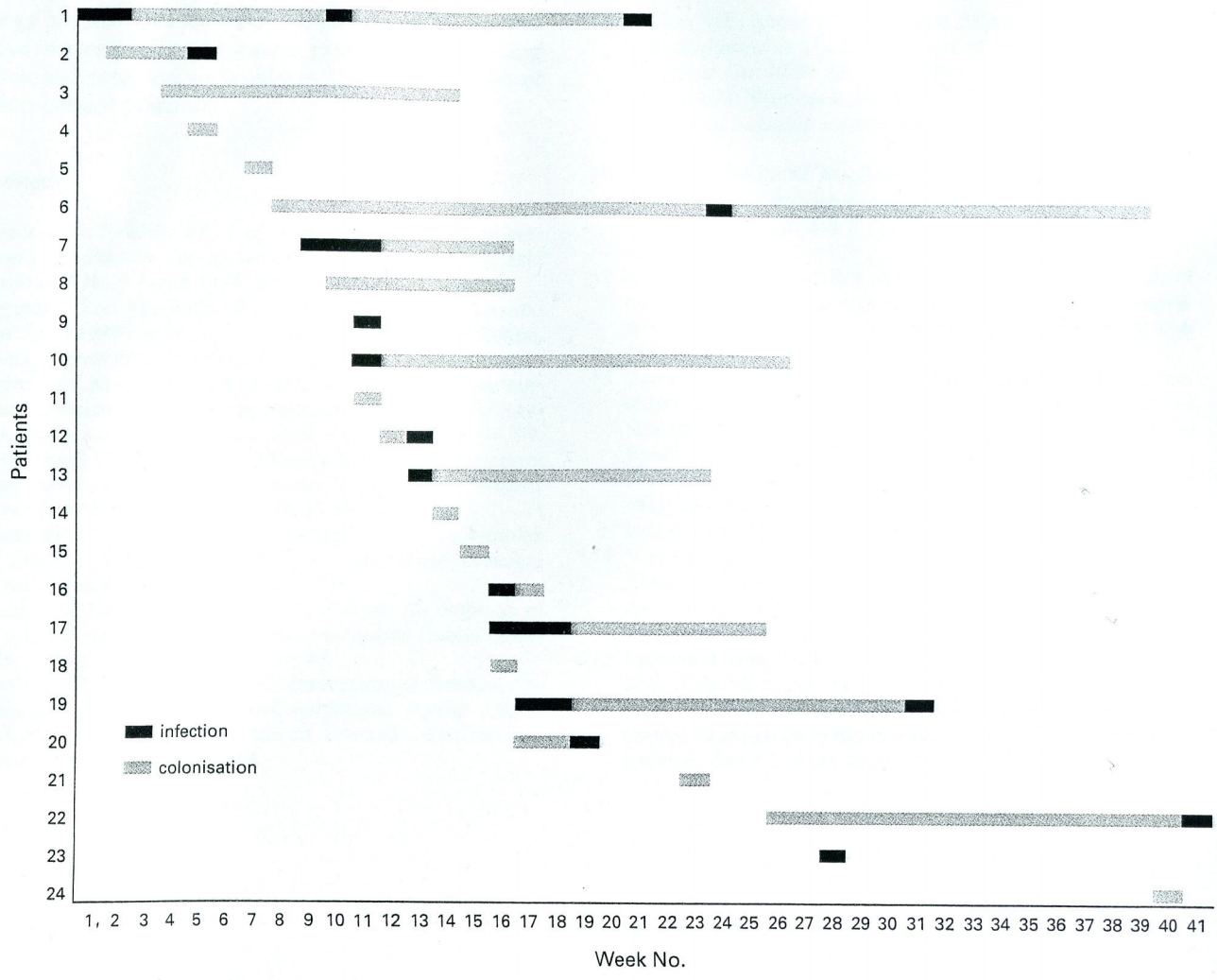

Figure 2 Duration of *Serratia marcescens* carriage.

of 128 patients with clinically significant infection.[11] Another report in 1998 showed persistence of *Serratia marcescens* in the respiratory secretions of a patient 1 year after initial isolation.[10]

It is significant that nine of 17 episodes of infection, as opposed to one of 10 episodes of colonisation, were associated with neutropenia. In the group which developed clinical infection, patients who were not neutropenic usually developed infection concurrently with their initial isolation of *Serratia marcescens*, ie exogenous infection in seven of eight patients. In the group of patients who were neutropenic and developed clinical infection, patients were far more likely to have acquired *Serratia marcescens* prior to the development of infection, ie endogenous infection in seven of nine patients. This emphasises the role of neutropenia in predisposition to infection.

Similar to many other reports, we did not identify any environmental source for the outbreak. The environmental isolates found were probably due to contamination. The role of contamination of the hospital environment and inadequate ward cleaning in the spread of nosocomial infection remains controversial. A previous study suggested a role for a heavily contaminated environment in the spread of *Serratia marcescens*,[12] but this remains unproven. Many outbreaks of *Serratia marcescens* have implicated hand transmission in the spread of this organism.[11,13,14] Despite our negative results, we also believe that hand transmission played a major role in the spread of *Serratia marcescens*, as highlighted by contamination of the control buttons of an intravenous pump, the inadequate hand washing and non-commensal flora on three of 12 staff hands screened. We believe that a combination of a prolonged patient reservoir, sub-optimal cleaning practices and hand transmission were important factors in the outbreak.

The *Serratia marcescens* isolates involved in this outbreak demonstrated reduced susceptibility to the antimicrobials most frequently used as first-line therapy in the unit, ie pipercillin-tazobactam, gentamicin and ciprofloxacin. As *Serratia marcescens* characteristically produces class 1 chromosomal β-lactamase, this precluded the use of cephalosporins,[15] further limiting the range of antibiotics which could be used. This had important therapeutic implications, necessitating the use of meropenem and amikacin as first-line therapy, during the outbreak. Although gram-positive bacteria have now become the dominant cause of infection in neutropenic patients in most haematology/

oncology units,[16] this outbreak demonstrates that gram-negative bacteria can still cause severe, life-threatening infection and pose serious problems in the enforcement of infection control practices.

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