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The Type Three Secretion System of *Bordetella pertussis*

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A thesis submitted to the University of Dublin
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For the Degree
of
Doctor of Philosophy

Supervised by: Prof. Kingston Mills and Dr. Aoife Boyd

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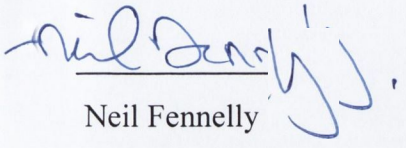

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Abstract

Despite the description of whooping cough as a vaccine preventable disease, and the implementation of extensive vaccination programs, *Bordetella pertussis* remains a significant cause of childhood morbidity and mortality worldwide. The recent observation that increasing numbers of adults are being diagnosed with *B. pertussis*, and may be transmitting the bacteria to infants, has called into question the level of protection provided by current pertussis vaccines, and highlighted the need for a better understanding of the molecular mechanisms underlying *B. pertussis* pathogenesis. The identification of novel *B. pertussis* surface molecules that assist in the design of improved pertussis vaccines and the further characterisation of *B. pertussis* virulence factors are therefore important goals in the field of pertussis research.

Type three secretions systems (T3SS) of animal pathogens are complex multi-protein organelles assembled at multiple sites on the bacterial cell surface, used to deliver bacterial proteins, known as effectors, directly into the eukaryotic cytosol of the host cell. The goal of T3 secretion is the disruption of host cell communication and function, thereby favouring bacterial survival. The highly conserved gene loci encoding a T3SS have been identified in the *B. pertussis* genome. The T3SS of the closely related animal pathogen, *B. bronchiseptica*, has been shown to be indispensable for this pathogen in infection of the host.

The aim of this study was to investigate the possible role of the T3SS of *B. pertussis*, in comparison with *B. bronchiseptica*, during *in vitro* and *in vivo* infection. The findings demonstrate that the T3SS of well characterised *B. pertussis* strains, such as Tohama I and Wellcome 28, is functionally inactive. Furthermore, the induction of T3SS activity by *B. pertussis* Tohama I could not be induced *in vitro* by modulating growth conditions. In contrast, the T3SS of a significant proportion (~70%) of clinical isolates of *B. pertussis* which had not been extensively passaged *in vitro* was found to be functionally active. T3SS deficient *B. pertussis* 12743 and *B. bronchiseptica* RB50 were generated and three known T3SS effectors, Bsp22, BopN and BopD were identified for the first time as substrates of the *B. pertussis* 12743 T3SS. It was also shown that the T3SS of *B. pertussis* 12743, in contrast to *B. bronchiseptica*, did not induce host cell cytotoxicity or modulate phagocytic uptake by macrophage *in vitro*, but may function to increase adherence to host cells or tissues, during *in vitro* and *in vivo* infection.

Using a murine aerosol infection model, the present study has demonstrated that the T3SS of *B. pertussis* 12743 may have a distinct role from that of *B. bronchiseptica*, in that it does not mediate long-term bacterial persistence, but functions to enhance bacterial colonisation of the lung in the early stages of infection. Furthermore, the data also suggested that the presence of a functional T3SS inhibited production of IL-1β, IL-12p40, TNFα, MIP-1 and MIP-2, innate pro-inflammatory responses associated with rapid and potent tissue inflammation and neutrophil recruitment, in the lungs following respiratory infection of mice. In addition, the inhibition of lower immediate pro-inflammatory signalling correlated with a significant reduction in antigen-specific IL-17, IFNγ and IgG production later in infection.

The results presented in this thesis have demonstrated that the T3SS is a functionally active and important virulence factor of *B. pertussis* during murine respiratory infection. The study has identified for the first time two surface associated proteins in *B. pertussis*, Bsp22 and BopN, and has revealed a role for the *B. pertussis* T3SS in promoting host colonisation which appears to involve host tissue binding and inhibition of early pro-inflammatory responses. Furthermore, the high level of T3SS activity among the range of clinical isolates of *B. pertussis* suggests that the T3SS may be a functionally important virulence factor for *B. pertussis* spp. during infection in humans.

Abbreviations

ACT	Adenylate cyclase toxin
Ap	Ampicillin
APC	Antigen presenting cell
ATCC	American type culture collection
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BCR	B cell receptor
BG	Bordet Gengou
BipA	*Bordetella* intermediate protein A
BLAST	Basic local alignment search tool
BMDC	Bone marrow dendritic cell
Bop	Bordetella outer protein
Bp	Base pair
BrkA	*Bordetella* serum resistance protein
BSA	Bovine serum albumin
Bsc	*Bordetella* secretion protein
Bsp22	*Bordetella* secreted protein 22
BteA	*Bordetella* type three effector A
Bvg	*Bordetella* virulence gene
cAMP	Cyclic adenosine monophosphate
CD	Clusters of differentiation
CFU	Colony forming units
CR	Complement receptor
CTLA	Cytotoxic T lymphocyte antigen
Cx	Cephalexin
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase

FHA	Filamentous hemagglutinin
Fim	Fimbriae
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead/winged helix P3
Gm	Gentamicin
GM-CSF	Granulocyte-macrophage colony stimulating factor
HRP	Horseradish peroxidase
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IKK	Ikappa B kinase
IL	Interleukin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IS	Insertion sequence
Jak	Janus kinase
Kn	Kanamycin
LB	Luria Bertani
LBP	LPS-binding protein
LCR	Low calcium response
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompatability complex
MIP	Macrophage inflammatory protein
Mxi	Membrane expression of antigen
NF-κB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern

PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PMN	Polymorphonuclear
PRN	Pertactin
PRR	Pattern recognition receptor
PT	Pertussis toxin
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SCV	*Salmonella* containing vacuole
Sm	Streptomycin
Spa	Surface presentation of antigen
SPI	*Salmonella* pathogenicity island
S+S	Stainer and Scholte
STAT	Signal transducer and activator of transcription
Suc	Sucrose
T3	Type 3
T3SS	Type 3 secretion system
TcfA	Tracheal colonisation factor
TCR	T cell receptor
TCT	Tracheal cytotoxin
TGFβ	Transforming growth factor β
TLR	Toll like receptor
TNF	Tumor necrosis factor
Vag	Virulence activated gene
Vrg	Virulence repressed gene
Yop	*Yersinia* outer protein
Ysc	*Yersinia* secretion protein

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Chapter 1

Introduction

I. GENERAL INTRODUCTION

1.1 The *Bordetellae*

The genus *Bordetella* contains nine species of bacteria. *B. pertussis* is the agent responsible for human pertussis, also known as whooping cough. *B. parapertussis* causes a mild pertussis-like disease in humans and sheep. *B. bronchiseptica* rarely infects humans but does infect a wide range of mammals (Gerlach *et al.* 2001) . It is the agent responsible for kennel cough in dogs, atrophic rhinitis in pigs and snuffles in rabbits (Preston *et al.* 2004). *B. avium* causes respiratory disease in birds and reptiles (Gerlach *et al.* 2001) and particularly rhinotracheitis or coryza in turkeys (Fry *et al.* 2005). *B. hinzii* is a commensal of the respiratory tract of fowl, but has been isolated from an immuno-compromised cystic fibrosis patient in Switzerland (Funke *et al.* 1996). It was responsible for a case of bacteraemia in an AIDS patient (Cookson *et al.* 1994), and a fatal case of septicaemia in an immuno-competent and otherwise healthy adult male in the United States (Kattar *et al.* 2000). Like *B. pertussis*, *B. trematum* and *B. holmseii* appear to be exclusively human pathogens, causing either ear and wound infections, septicaemia or endocarditis in young adults. ((Weyant *et al.* 1995; Vandamme *et al.* 1996). In 2001, a novel species, *B. petrii,* was first isolated from river sediment in an anaerobic bioreactor in Germany (von Wintzingerode *et al.* 2001), and though initially presumed to be an environmental bacteria has recently been isolated from a 67-year old man with mandibular osteomyelitis in London (Fry *et al.* 2005). The latest addition to the genus, *B ansorpii*, was isolated from the purulent exudate of an epidermal cyst, from a 19-year old female chemotherapy patient in South Korea (Ko *et al.* 2005). Thus, with the exception of *B. avium*, all *Bordetella* spp. described to date have the ability to infect humans to some degree or other.

Of these nine *Bordetella* species, only *B. pertussis, B. bronchiseptica* and *B. parapertussis* have been studied in any great detail. These three species have been commonly referred to as the "classical" *Bordetella* spp. and are thought to be closely related on the basis of several criteria, including morphological, physiological and antigenic properties, as well as phage typing and genome content (Parkhill *et al.* 2003). All three species are gram-negative coccobacilli, are strictly aerobic and have an optimum growth temperature between 35 and 37^{0}C, similar to that of their mammalian hosts. In addition, all three species cause upper respiratory disease, involving binding to ciliated epithelial cells and subsequent ciliostasis.

B. pertussis is the causative agent of whooping cough, an acute and debilitating respiratory disease that mainly affects infants. However, recent evidence suggests that it also causes significant numbers of sub-clinical infections in adolescents and adults. The bacterium, initially classified as *Haemophilus pertussis* (because of its requirement for blood in laboratory media), was later reclassified in its own genus, *Bordetella*, in 1952, named after Jules Bordet, who first isolated the bacterium from a child displaying symptoms of whooping cough in 1906 (Gerlach *et al.* 2001). Indeed the symptoms of whooping cough disease were described long before the identification of the responsible organism, with the first clear description emerging in 1640 from the French physician de Baillou, who reported an epidemic with the characteristic symptoms in Paris in 1578 (Gerlach *et al.* 2001). Today, despite extensive vaccination programs, whooping cough is still endemic in many countries. In the 1990's there were an estimated 20 to 40 million cases of *B. pertussis* worldwide and 200,000 to 400,000 deaths per year (Tozzi *et al.* 2005). In the past decade, an unusual increase in pertussis epidemics has been noticed in countries with high levels of vaccine coverage, such as theUnited States, Canada, France and the Netherlands (Bass and Wittler 1994; De Serres *et al.* 1995; de Melker *et al.* 1997; Weber *et al.* 2001).

The initial symptoms of whooping cough resemble those of the common cold (nasal congestion, runny nose, sneezing, red and watery eyes, mild fever, and a dry cough), with disease progressing to a wet cough that usually brings up thick, stringy mucus. Difficulty in expelling this mucus leads to severe and lengthy coughing fits, which can, in some instances, cause the patient to become cyanotic from lack of oxygen. A long inspiratory effort at the end of such coughing fits is usually accompanied by a characteristic high-pitched "whoop" sound (The medical library of the American medical association. 2003). These paroxysmal coughing fits are usually interspersed with bouts of vomiting, and together with absolute lymphocytosis, these represent the more specific or typical symptoms of pertussis infection, often facilitating initial disease diagnosis. Other symptoms include bulging eyes, protrusion of the tongue, salivation, weight loss due to loss of appetite and frequent vomiting, and distension of the neck veins (Mattoo and Cherry 2005). Illness usually lasts 6-8 weeks and neurological responses such as fever, convulsions, seizures and encephalitis (most likely due to cerebral hypoxia), brain damage and death have been reported (Mills 2001). Pertussis-induced instances of pneumonia, often due to secondary infections, are common. One recent study conducted in the Netherlands concluded that as many as 30% of whooping cough patients showed secondary infections, with para-influenza, respiratory synctial virus, mycoplasma

Figure 1.1 Colored scanning electron micrograph of a section of human tracheal epithelium showing the rod-shaped bacteria *Bordetella pertussis* (green), lodged at the base & between the cilia at center. The micrograph shows a region of epithelium in the foreground where the cilia have been flattened by the bacteria.

(http://instruct1.cit.cornell.edu/courses/biog105/images/106slides/unit4slides/pages/slide%2018.html)

pneumoniae, adenovirus, influenza A and influenza B being the most common pathogens (Versteegh *et al.* 2006). Most deaths due to *B. pertussis* infection occur in infants, and it is generally recognised that the source of infection in infants is frequently an adolescent or adult family member (von Konig *et al.* 2002). The disease does not present as severely in adults and as a result is often misdiagnosed as bronchitis or some other upper respiratory tract infection (Mattoo and Cherry 2005).

B. parapertussis infects both humans and sheep, though it is worth noting that to date no evidence exists for the transmission of *B. parapertussis* to humans from sheep. On the contrary, genetic analysis suggests that human and sheep strains constitute distinct clonal populations (Parton 1999). Early symptoms of *B. parapertussis* infection are very similar to those of *B. pertussis* infection, particularly the frequency of paroxysms and post-tussive vomiting, but the duration of disease is typically much shorter in *B. parapertussis* infected patients, and other symptoms such as whooping, fever and luekocytosis are much less frequent and severe. Overall *B. parapertussis* causes a milder form of whooping cough than that seen during *B. pertussis* infection and it is often mistaken for bronchitis.

B. bronchiseptica rarely infects humans but does infect a wide range of mammals (the most well documented being mice, rats, dogs, cats, rabbits, foxes, pigs, sheep and horses) usually producing a chronic, yet asymptomatic, infection that can persist for the life of the animal. In some cases, for example kennel cough in dogs, paroxysmal cough, post-tussive vomiting and pneumonia-induced death can occur (Mattoo and Cherry 2005). In most cases human *B. bronchiseptica* infection is associated with close animal contact (Gueirard *et al.* 1995; Mattoo and Cherry 2005) or severely immuno-compromised individuals (Woolfrey and Moody 1991), though *B. bronchiseptica* infections of immuno-competent children and adults have been reported (Lo Re *et al.* 2001; Fingermann *et al.* 2006).

A representative strain of each of these three species (*B. pertussis* Tohama I, *B. parapertussis* 12822 and *B. bronchiseptica* RB50) was recently sequenced at the Sanger Pathogen Sequencing Unit, Cambridge, UK by Duncan Maskell and colleagues (Parkhill *et al.* 2003). Tohama I, originally isolated in the 1950s, was chosen largely because it is the most widely studied of the pertussis strains and physical maps of its chromosome were available (Stibitz and Garletts 1992). Likewise, *B. bronchiseptica* RB50 is a well characterized clinical isolate and like B. *parapertussis* 12822, original stocks of the bacteria were available (Preston *et al.* 2004). The sequences of these three closely related pathogens revealed differences in the relative size of their genomes. *B. bronchiseptica* has the highest number of genes, 5007, compared to 4404 and 3816 for *B. parapertussis* and *B.*

pertussis respectively (Parkhill *et al.* 2003). Many of the genes unique to *B. bronchiseptica* encode proteins involved in small molecule transport and metabolism, such as surface proteins and autotransporters, which could contribute to the ability of *B. bronchiseptica* to bind to tissues of a diverse range of hosts and utilize a wide range of nutrients, consistent with the wider host range of this species when compared to *B. pertussis* and *B. parapertussis*. Insertion sequence elements (IS elements) are mobile genetic elements that only carry coding information for a transposase enzyme that directs replication and subsequent expansion of the IS element within the host chromosome. Thus, extensive expansion of identical IS elements, such as that seen in *B. pertussis*, enables them to act as foci of homologous recombination, resulting in chromosomal rearrangements and deletions. Indeed, massive rearrangement of the genome has occurred in *B. pertussis* when compared to either *B. bronchiseptica* or *B. parapertussis*, consistent with the presence of over 200 identical copies of IS481 (Parkhill *et al.* 2003). Analysis of pseudogenes (remnants of once active genes that have been silenced by mutation, usually by deletion or by the transposition of an IS element into the coding sequence) and IS elements in *Bordetella* spp., suggests that *B. pertussis* and *B. parapertussis* both evolved separately, but quite recently (only thousands of years ago), from a *B. bronchiseptica*-like ancestor. Maskell and colleagues concluded that the narrow host-restriction of *B. pertussis* and *B. parapertussis* is due to large scale gene loss and inactivation as opposed to acquisition of foreign DNA or gain of function mutations on the part of *B. bronchiseptica.* Thus, it would appear that *B. pertussis* and *B. parapertussis* have only recently (700,000 – 350,000 years ago) evolved from *B. bronchiseptica* and possess a very high degree of genome plasticity, which has important implications for antigenic modulation and recent observations of waning vaccine-induced immunity.

1.2 Immunity and Pathogenesis of *B. pertussis, B. parapertussis* and *B. bronchiseptica*

1.2.1 Bacterial colonization and the innate immune response

Bordetella spp. are highly contagious, gram-negative coccobacilli that are spread by aerial transmission. Upon their arrival in the upper respiratory tract of a susceptible host, the bacteria colonize the respiratory mucosa by binding to ciliated epithelial cells and multiplying extracellularly (Mills 2001), though a number of studies have shown that all three pathogens can to some degree invade and survive within cultured epithelial cells (Lee *et al.* 1990; Schipper *et al.* 1994; Bassinet *et al.* 2000; Ishibashi and Nishikawa 2002; Gueirard *et al.* 2005). This binding leads to paralysis of the cilia, a hair-like brush border lining the epithelium. Synchronous movement of cilia functions to expel invading micro-organisms trapped in viscous mucous secreted from epithelial cells from the respiratory tract. The critical importance of adherence to the respiratory mucosa of these primarily extracellular pathogens is reflected in the apparent redundancy of the various protein adhesins believed to be involved in the attachment process. The virulence factors implicated in facilitating attachment to date include; filamentous hemagglutinin (FHA), fimbriae, pertussis toxin (PT), pertactin (PRN), lipopolysaccharide (LPS), tracheal colonisation factor (TcfA), and the serum resistance locus (BrkA) (Mattoo and Cherry 2005). Also of note is the apparent redundancy in gene content of the two primary adhesins FHA and fimbriae (Discussed in sections 1.3.2.1 and 1.3.2.2 respectively) (Parkhill *et al.* 2003).

Specific patterns on the bacterial cell, known as pathogen associated molecular patterns (PAMPs), are recognised by host cells through membrane bound pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). In addition, nucleotide-binding oligomerisation domain molecules (Nod1, Nod2) are present in the cytosol of epithelial and immune cells (Lavelle 2005). Nod proteins are thought to serve as intracellular receptors of bacterial peptidoglycan, directly activating NF-κB transcription complexes. A common PAMP associated with gram-negative bacteria such as *Bordetella* spp. is the endotoxin or LPS. Epithelial cells have been shown to express membrane bound TLR4, a PRR involved in the recognition of LPS, in the lung and respiratory mucosa (Jiang *et al.* 2005). Hence, PRR recognition of LPS results in activation of epithelial and immune cells, and represents one of the first activations of host immune defences against *Bordetella* infection (Higgins *et al.* 2003; Mann *et al.* 2005). In addition to membrane-

bound PRRs, respiratory epithelial cells have been shown to produce the acute phase protein, LPS-binding protein (LBP) (Dentener *et al.* 2000), which facilitates binding of LPS to both the membrane-bound and soluble CD14 (sCD14) receptor. sCD14-LPS complexes are thought to be involved in initiating responses in cells lacking membrane bound CD14, by complexing with TLR4 to initiate intracellular signalling, and a role for LBP and sCD14 in the activation of human epithelial cells has been demonstrated (Pugin *et al.* 1993). Thus both cellular (TLRs) and humoral (LBP) methods of pathogen recognition are employed by the respiratory epithelium. Structural, immunological and gene coding differences between the LPS of the classical *Bordetella* spp. have been reported (discussed in section 1.3.2.8) (Amano *et al.* 1990; Watanabe *et al.* 1990; Gerlach *et al.* 2001), and hence these differences may be important in terms of different immune activation patterns to each pathogen in the lung.

In addition to their role as a physical remover of microbes through ciliary clearance and secretion of antimicrobial mucus (the latter being increased upon activation in response to gram-negative LPS and gram-positive bacteria (Dohrman *et al.* 1998)), activated epithelial cells also release a range of chemical messengers, including cytokines, which contribute to the acute phase response or inflammation. Colony stimulating factors secreted from epithelial cells stimulate the growth of specific immune cells such as neutrophils and monocytes (Smith *et al.* 1990). For example, Interleukin-6 (IL-6) from epithelial cells enhances the differentiation of myelomonocytic cell lines (Mitani *et al.* 2000), while IL-8 stimulates the migration of monocytes and neutrophils to the site of infection, and induces contraction of airway smooth muscle cells. IL-8 also stimulates the release of histamine from other immune cells, including basophils (Nakamura *et al.* 1991), which induces localized vasodilation, aiding both the traffic and adherence of phagocytic immune cells to the infection site. In addition, epithelial cells upregulate the surface expression of intracellular adhesion molecule (ICAM-1), which has been demonstrated during *B. pertussis* infection (Ishibashi and Nishikawa 2002), and other adhesion molecules, which serve to increase neutrophil adhesion to the pathogen invaded epithelium and subsequent diapedesis, the process by which the leukocytes cross the vascular barrier.

Myeloid cells of the innate immune system consist of mononuclear phagocytes, such as macrophages and the closely related dendritic cells (DC) (both originate from monocytes), which also function as antigen presenting cells (APC), and polymorphonucleocytes (PMN), including neutrophils, basophils and eosinophils. These cells are the so called "sentinels" of the immune system, circulating in the blood and tissue of major organs and constantly sampling their environment. They phagocytose any foreign

particles, including bacteria, and destroy them internally. They also function to drive certain arms of the adaptive immune response and activate other immune cells by cytokine release and antigen presentation.

LPS, and/or any other bacterial components that can bind and activate phagocytes, results in the subsequent release of potent inflammatory mediators, such as IL-1, tumour necrosis factor (TNFα) and IL-12 (Majewska and Szczepanik 2006). These cytokines result in systemic activation of phagocytes, including the resident alveolar macrophage of the lung. IL-1 and TNF-α further activate epithelial cells causing increased mucus release and expression of adhesion molecules, such as integrins, resulting in increased adherence and eventual diapedesis of leukocytes migrating to the site of infection (Fischer *et al.* 1999) (Tonnesen 1989) (Svanborg *et al.* 1999). In addition, epithelial cells upregulate tissue thromboplastin, which in combination with increased release of platelet activating factor (PAF) from neutrophils (as a result of IL-1 and TNF-α stimulation) promotes platelet aggregation and fibrin deposition. This results in further recruitment of infiltrating immune cell adherence to the site of infection, the respiratory epithelium. Epithelial cells also release platelet derived growth factor (PDGF) which causes smooth muscle contraction, again increasing vascular permeability and increasing immune cell to passage to the inflamed site of infection.

TNF-α, IL-1 and IL-12 release from macrophages and DC also activate another group of immune cells, natural killer (NK) cells, to secrete interferon-γ (IFNγ) (Biron *et al.* 1999). IFNγ is also released by T-helper 1 cells (Th1) (Mosmann *et al.* 1986), which differentiate from naïve T cells in the peripheral lymphoid tissues in response to cytokines, including IL-12, which is released from macrophage and DC (Moser and Murphy 2000), and IFNγ, released from NK cells (Biron et al. 1999). IFNγ activates macrophage phagocytosis and stimulates intracellular killing of bacteria (Stout and Bottomly 1989) and secretion of IL-1, TNF-α and IL-12, and this cycle of cell activation continues. NK cells destroy infected host cells and toxins such as tracheal cytotoxin (TCT), dermonectrotic toxin (DNT) and LPS released from the bacterium can damage host epithelial cells (Mattoo *et al.* 2001). For example, TCT causes epithelial cell damage by stimulating the release of IL-1α (Heiss *et al.* 1993) and nitric oxide (NO) (Heiss *et al.* 1993). The damaged respiratory epithelium may then be more easily colonized by other respiratory pathogens and possibly accounts for the chronic pneumonia sometimes seen with *B. pertussis* infection (Preston *et al.* 2004).

This rapid cellular infiltration and production of inflammatory cytokines at the site of infection is known as inflammation and is responsible for the pain, heat, redness and swelling experienced during the early stages of infection or injury. It represents the innate arm of the immune response. Inflammation associated with *B. pertussis* infection of infants generally results in a severe hyperplasia, a swelling of the lung tissue, with increased secretion of mucous from the respiratory epithelium, making breathing difficult and triggering severe coughing fits. Therefore the innate immune response is a central player in the symptoms seen with *B. pertussis* infections.

Macrophages and neutrophils accumulating at the site of infection begin to engulf bacteria. Once internalised, bactericidal enzymes, along with reactive oxygen intermediates (ROIs), and reactive nitrogen intermediates (RNIs), produced by a phenomenon known as the respiratory burst, destroy the bacterial cell and the waste is exocytosed out of the cell (Bogdan *et al.* 2000). This is mainly done by neutrophils, which have a longer respiratory burst than macrophages, the principle weapons being hydroxyl radicals and hypochlorous acid- a compound equivalent to household bleach (Beutler 2004). Neutrophils are extremely important cells in the clearance of bacterial pathogens and a prominent feature of *B. pertussis* infection is a marked increase in the number of circulating neutrophils in the blood, termed leukocytosis. Notably, this increase is absent from *B. parapertussis* and *B. bronchiseptica* infection.

In addition to phagocytosis, macrophages have another very important function, antigen presentation. Like DC, they degrade engulfed bacteria or bacteria-related proteins to peptides (antigen), which are bound by major histocompatability complex molecules (MHC), which are then displayed on the cell surface. These cells then carry the antigen to peripheral lymphoid organs, lymph nodes in the case of tissue antigens and the spleen in the case of blood antigens, where they are presented to lymphocytes known as T cells, which then proliferate in response to the antigen presented (Steinman *et al.* 1999). This was first demonstrated for alveolar macrophages in a bacterial infection by Weinberg and Unanue, who measured the proliferative response of T- cells specific to *Listeria monocytogenes* (Weinberg and Unanue 1981) and subsequently a role for intracellular processing and MHC restriction was demonstrated in this process (Ziegler and Unanue 1981). Antigen is not presented to antibody-producing B lymphocytes via MHC molecules. For B cell stimulation, antigen binds directly to a receptor on the cell surface, a membrane bound immunoglobulin molecule known as the B cell receptor (BCR).

T and B cells represent arms of the adaptive immune response known as cell-mediated and humoral immunity, respectively. While most organisms survive through

innate immunity alone, the adaptive immune response is believed to have evolved in invertebrates in order to facilitate the detection of pathogens residing within host cells, something the PRRs of the innate immune system cannot do. This detection of intracellular pathogens is based upon antigen presentation by a correctly functioning innate immune system and it has been known for some years that macrophage are absolutely crucial for an adaptive immune response to occur (Hoffmann and Dutton 1971). Thus, mononuclear phagocytes or APC represent a critical bridge between innate and adaptive immunity, and the rapidly-acting innate immune system is of crucial importance to the host, not only in terms of delaying pathogen spread and replication, but also in terms of initiating adaptive immunity and subsequent pathogen clearance.

1.2.2 Bacterial clearance and the adaptive immune response

Human DC are bone-marrow derived leukocytes, which migrate as immature precursors to sites of potential pathogen entry. Two main types of DC have been described to date, so called "conventional" or $CD11c^{HI}$ myeloid DC (Banchereau and Steinman 1998), such as $CD14^{+}$ blood monocyte derived DC, which differentiate into $CD14^{-}$ DC from monocytes *in vitro* in response to GM-CSF and IL-4 (Romani *et al.* 1994; Sallusto and Lanzavecchia 1994), and Langerhans cells (LC) and dermal DC, which localize to the epithelial surfaces of the skin and mucosa (Schuler and Steinman 1985). The second type are $CD11c^{Lo}$ plasmacytoid DC, which circulate in the blood, and originate from hematopoietic stem cells in response to Flt3L (Maraskovsky *et al.* 2000). Myeloid DC have been shown to express a range of TLR (TLR1-6 and TLR8) depending on the subset and activation state, while plasmacytoid DC selectively express TLR7 and TLR9 (Jarrossay *et al.* 2001). As discussed in section 1.2.1, TLR ligation by PAMPs leads to DC activation, resulting in maturation, migration and cytokine release.

While there are notable exceptions to the rule, it is generally thought that immature DC have a reduced capacity for naïve T-cell activation, and therefore are primarily involved in antigen capture and the induction of tolerance to self antigen by anergising auto-reactive T-cells. In contrast, mature DC have a much poorer capacity for antigen capture and are primarily involved in the induction of T-cell mediated immunity to foreign antigens (Reis e Sousa 2006). DC are termed "professional" APCs, due to the fact that, once they have matured, they constitutively express high levels of MHC and co-stimulatory molecules, and can efficiently present a wide range of antigens, such as

peptides, viral antigens and allergens (Reis e Sousa 2006). In contrast, high level MHC and co-stimulatory molecule expression on macrophage is induced by ingestion of bacteria and recognition of their foreign molecular patterns by PRRs. Studies conducted in the 1980s demonstrated that only DC, in contrast to resting B cells and IFNγ-treated Macrophage, were initially capable of inducing significant T-cell proliferation (Inaba and Steinman 1984). Only following initial DC stimulation, were the other APC found to be capable of promoting proliferation. Thus, while the sole function of mature DC, antigen presentation represents a secondary function of macrophage and B cells that can be induced by microbial interaction, and probably serves to amplify the initial DC-mediated T-cell stimulation.

DC take up antigen such as pathogens and dead or dying cells by a variety of methods involving phagocytosis, pinocytosis, endocytosis and the use of specific receptors (Rossi and Young 2005). In general, antigens acquired from the extracellular environment are usually presented via MHC II, while antigens processed via the cytosolic compartment (intracellular pathogens) are presented via MHC I (Rossi and Young 2005). MHC molecules are upregulated and exported to the cell surface upon DC maturation, along with co-stimulatory molecules and CCR7, a chemokine receptor involved in the migration of matured DC from the periphery to the T-cell rich areas of the draining lymph nodes (Geissmann *et al.* 2002) (Jarrossay et al. 2001).

Upon antigen presentation by an APC, either in peripheral lymphoid organs or circulating in the blood, each antigen-activated lymphocyte proliferates to produce lots of identical progeny. This proliferation, termed "clonal expansion" is induced by TCR ligation with peptide antigen/MHC complex and is driven by IL-2 (Ho and Glimcher 2002). TCR ligation alone, however, is not sufficient for the induction of cell mediated immunity. A second signal, involving ligation of CD28 by co-stimulatory molecules of the B7 family, such as CD80 and CD86, is necessary for complete T-cell activation (Kapsenberg 2003). Co-stimulatory molecule expression on DC is upregulated upon TLR induced maturation, and it has been reported that in the absence of this co-stimulatory signal Th-cells become anergic (Kapsenberg 2003). A third signal, believed to consist of the balance of T-cell polarizing cytokines in the milieu, is believed to be responsible for the direction of antigenic specific responses into cell mediated (Th1), humoral (Th2) or regulatory (Treg) T cell responses (Kalinski *et al.* 1999). Most T-cell polarizing cytokines are produced only weakly or transiently upon DC maturation, and sustained production requires a cross-talk between the DC and T-cell. TCR ligation and co-stimulation cause rapid T-cell expression of CD40 ligand, which binds to CD40 on DC and greatly amplifies

the polarizing cytokine output, through sustained NF-κB activation (Krug *et al.* 2001; O'Sullivan and Thomas 2002). Thus, the type of adaptive immunity elicited depends largely upon bioactive pathogen derived compounds that influence the maturation and cytokine output of DC, and the general cytokine balance in the milieu. This pivotal role in control over T-cell responses places DC as master regulators of the immune response to pathogens. The individual T-cell polarizing factors that influence the induction of T-cell responses are discussed in the following sections.

1.2.2.1 Th1

Cell mediated immunity is mediated by two distinct sets of effector T cells, namely $CD8^+$ and $CD4^+$ T-cells. Intracellular pathogens that replicate in the cytosol, such as viruses and some bacteria, tend to provoke a $CD8^+$ T-cell response, which ultimately results in the killing of infected cells. In contrast, pathogens that tend to accumulate in vesicles inside macrophage and DC, either by phagocytosis or pathogen invasion, tend to promote the induction of $CD4^+$ Th1 cells, which activate macrophage killing and stimulate B cell antibody production. Th1 cells produce a range of cytokines and chemokines with important functions for pathogen clearance: IL-2 which stimulates the growth of T-cells and NK cells (Farrar *et al.* 1978; Watson *et al.* 1979; Farrar *et al.* 1980; Mosmann *et al.* 1986); monocyte chemoattractant protein 1 (MCP-1) which is chemoattractant for macrophage (Bradley *et al.* 1999); lymphotoxin, which assists cytotoxic T lymphocyte function; TNFα which activates and induces nitric oxide (NO) production in neutrophils (Shalaby *et al.* 1985) and macrophage (Ding *et al.* 1988), as well as activating epithelial cells to increase phagocyte binding (Fischer *et al.* 1999); and GM-CSF which increases production of granulocytes, DC and macrophage from hematopoietic precursors (Mosmann et al. 1986). Th1 cells also have an important role in resolving pathogen-mediated immune subversion by releasing Fas ligand to kill macrophage containing pathogens that are resistant to microbicidal activity (Ramsdell *et al.* 1994; Suda *et al.* 1995; Zhang *et al.* 1997). In addition, DC maturation is important for optimal T-cell stimulation, and semi-mature DC or DC infected with pathogen may not express optimal levels of co-stimulatory molecules to activate T-cells. Upregulation of CD40L expression on DC by Th1 cells, through binding to CD80/CD86 on DC via CD28, can provide an additional signal resulting in optimal T-cell activation (Fallarino *et al.* 2002) (Straw *et al.* 2003).

IFNγ has been termed the signature cytokine of a Th1 response. IFNγ has a range of functions including the activation of macrophage (Pace *et al.* 1985), NK cells(Djeu *et al.*

1979) and neutrophils (Shalaby *et al.* 1985). It also increases MHC expression on macrophage and DC (Rosa *et al.* 1983), induces B-cell differentiation (Sidman *et al.* 1984) and IgG2a production (Snapper and Paul 1987) and inhibits Th2 responses (Gajewski and Fitch 1988). IFNγ is produced mainly by NK cells and activated T-cell subsets such as NK T-cells, $CD8^+$ T-cells, Th1 cells, as well as APCs (DC, macrophage and B-cells) (Trinchieri 2003). T-cell polarizing cytokines produced by APC upon MHC-TCR ligation have a large impact on the type of T-cell response elicited. IFNγ, IL-18, IFNα/β, TNF and ICAM1 have all been implicated in directing Th1 cell responses (Kapsenberg 2003).

The IL-12 group of cytokines are instrumental in the direction of Th1 responses through induction of IFNγ gamma (Kobayashi *et al.* 1989) (Hsieh *et al.* 1993) (Manetti *et al.* 1993). IL-12 which is mainly produced by phagocytes and DC, consists of a heterodimer of IL-12p35 and IL-12p40 (Kobayashi *et al.* 1989), which binds directly to the IL-12R, primarily expressed on activated T-cells, NK cells, B-cells and DC (Presky *et al.* 1996) (Grohmann *et al.* 1998) (Airoldi *et al.* 2000).Ligation of IFNα, IFNγ, TNFα and CD28 enhances expression of IL-12R on T cells (Trinchieri 2003). Ligation of IL-12R activates the Jak-STAT signalling cascade leading to STAT4 activation, which in turn induces Th1 specific transcription factors, the most important of which is T-bet (Zhang *et al.* 2001). T-bet is thought to induce chromatin remodelling of the IFN locus, resulting in a transcriptionally competent formation, upregulation of IL-12R β2 subunit and subsequent IFNγ expression (Murphy and Reiner 2002). In addition, T-bet has been shown to be capable of redirecting polarized Th2 cells into a Th1 phenotype, by a mechanism involving IFNγ production and repression of IL-4 and IL-5 (Szabo *et al.* 2000). Production of IL-12 initially occurs in response to bacterial products, such as LPS, and this production is greatly upregulated via CD40 ligation upon antigen presentation. In phagocytes, IL-12 production usually requires other cytokines, such as IFNγ (Hayes *et al.* 1998). Thus IFNγ forms a positive feedback loop during inflammatory and Th1 responses, regulating its own production. The release of IL-12 from NK cells and T-cells is synergistically enhanced by another cytokine, IL-18, a member of the IL-1 family (Bazan *et al.* 1996). IL-12 also upregulates expression of IL-18R, and conversely, IL-12R β2 expression is upregulated by IL-18 on resting T-cells (Nakahira *et al.* 2002). In addition to IFNγ, IL-12 causes NK cells and T-cells to release GM-CSF, TNF and increase the expression of adhesion molecules, perforin and granzymes, thereby augmenting NK and CD8+ T-cell killing (Kobayashi *et al.* 1989) (Trinchieri 1998). IL-12 also has a proliferative effect on pre-activated T-cells and NK cells (Perussia *et al.* 1992).

IL-23 and IL-27, other members of the IL-12 family of cytokines, have also been implicated in directing Th1 responses during DC-T-cell interactions. IL-27 consists of a heterodimer composed of EBI3 and a p28 subunit that bears homology to the p35 subunit of IL-12 (Devergne *et al.* 1997). Produced by LPS activated monocytes and monocyte derived DC (Pflanz *et al.* 2002), IL-27 binds to the IL-27R expressed on a range of lymphocytes, such as resting NK and NKT cells, Treg cells, effector T-cells and memory T-cells (Villarino *et al.* 2005). Like IL-12R, ligation of IL-27R leads to the induction of T-bet, with subsequent increases in IL-12R β2 expression and IFNγ production (Hibbert *et al.* 2003; Lucas *et al.* 2003). Thus, IL-27 augments IL-12 driven Th1 responses and IFNγ production, and the presence of IL-27 during TCR ligation has been shown to result in the proliferation and differentiation of Th1 cells that secrete IFNγ (Pflanz *et al.* 2002).

IL-23 is a heterdimer of the p40 subunit of IL-12 and a novel p19 subunit produced by macrophage, DC, T-cells and endothelial cells in response to microbial products, (Belladonna *et al.* 2002). Its receptor, IL-23R, is expressed on monocytes, DC, NK cells and T-cells (Parham *et al.* 2002). Originally thought to augment IL-12 function by the induction of IFNγ in response to specific pathogens such as *B. pertussis* (Fedele *et al.* 2005), it has recently been shown that IL-23 stimulates the differentiation of a distinct population of IL-17 producing (Th17) T-cells, implicated in chronic inflammation and autoimmunity (Aggarwal *et al.* 2003; Langrish *et al.* 2005). Th17 cells secrete IL-17 but not IFNγ (Langrish *et al.* 2005), and mediate inflammatory responses by activating endothelial cells and monocytes to produce key inflammatory mediators, such as IL-8, IL-1, IL-6, TNFα and GM-CSF (McKenzie *et al.* 2006). These cytokines cause rapid neutrophil recruitment and further activate epithelial cells at the site of infection. Thus IL-23 driven IL-17 production and IL-12 driven IFNγ production are somewhat analogous in mediating similar, yet distinct, pro-inflammatory responses. The currrent understanding is that IL-23, acting on tissue-resident T-cells directs rapid IL-17 mediated neutrophil recruitment to help to contain the infection, until a subsequent IFNγ mediated Th1 response can be generated by the host, which takes several days to develop. Indeed, Th1 responses have been suggested, once generated, to be dominant over an IL-23-Th17 response (Murphy *et al.* 2003). Thus, the balance between IL-12 and IL-23 driven responses may be a key component in the regulation of inflammation. This more potent and rapid IL-23-driven inflammatory response could be required in tissues of vital organs, such as the lung, where significant pathogen invasion can be life threatening. Of interest is the observation that two respiratory pathogens, *Klebsiella pneumoniae* and *Bordetella pertussis*, appear to selectively and rapidly induce IL-23 expression (Happel *et al.* 2003;

Fedele *et al.* 2005). The resultant activation of alveolar phagocytes and resident T-cells and rapid neutrophil recruitment may account for the chronic lung and airway inflammation during *B. pertussis* infection. A notable hallmark of *B. pertussis,* but not *B. bronchiseptica* or *B. parapertussis* infection is severe and chronic lung-tissue inflammation, termed hyperplasia, and a profound lymphocytosis, often presenting long after the bacterium can be successfully recovered from the patient. It has been hypothesised that the exacerbated lung pathology associated with *B. pertussis* infection, which triggers the characteristic paroxymsms, facilitates rapid host transmission, and thereby enabled *B. pertussis* to efficiently move from the broad mammalian host range to the human niche. Implicated in this pathology is the *B. pertussis*-specific expression of PT, which causes increases in target cell cAMP production (discussed in section 1.3.2.3). Interestingly, other cAMP elevating molecules, such as PGE_2, can enhance IL-23 production (Schnurr *et al.* 2005), suggesting that PT may have a similar effect.

As will be discussed in detail in section 1.3.2.8, *B. pertussis* "rough" LPS differs from that of *B. parapertussis* and *B. bronchiseptica* "smooth" LPS by the notable absence of O-antigen expression. Since strains of *B. bronchiseptica* selectively lose expression of O-antigen during transition from an animal to human host (Le Blay *et al.* 1997) (Gueirard *et al.* 1998), it would appear that so called rough LPS is of significant benefit during infection of humans. In addition, recent data from this laboratory has shown that *B. pertussis* derived LPS promoted production of high levels of IL-23, as well as IL-12, from DC (Higgins *et al*; Journal of Immunology; In press). Thus, whether increased IL-23 expression results from the actions of PT, LPS or other mechanisms, it is likely that the severe lung inflammation mediated by IL-23 driven Th17 cells facilitates the rapid transmission of *B. pertussis* from host to host.

Since *Bordetella spp.* are capable of surviving within a range of host cells, including macrophage, PMNs and respiratory epithelial cells (Bassinet *et al.* 2000) (Hazenbos *et al.* 1994) (Hellwig *et al.* 1999) (Saukkonen *et al.* 1991) (Steed *et al.* 1992) (Banemann and Gross 1997) (Guzman *et al.* 1994), cell mediated immunity is crucial for clearance of the bacteria from the respiratory tract. Studies using mice deficient for IFNγ or T-cells (athymic) showed that they failed to clear the bacteria following respiratory challenge (Mahon *et al.* 1997) (Mills *et al.* 1993). Furthermore, T-cells isolated from *B. pertussis* infected children and adults stimulated with *B. pertussis* antigens have been shown to produce cytokine profiles characteristic of Th1 cells (Ryan *et al.* 1997).

1.2.2.2 Th2

As discussed above, Th1 cell mediated responses function to eradicate pathogens intracellularly in phagocytes or infected cells. In contrast, Th2 cells and humoral immune responses function against extracellular pathogens such as worms, parasites and extracellular bacteria. Since almost all pathogens have some extracellular *in vivo* phase to their infectious cycle, the immune response elicited *in vivo* most often consists of a mixture of Th1 and Th2 responses. A humoral immune response is characterised by the production of antigen specific antibodies which, when generated during bacterial infection, play a number of important roles. They bind to spreading factors which bacteria use to break down connective tissue such as fibrin, thereby limiting bacterial spread. They bind to receptors on the cell surface that bacteria use to transport useful materials into the cell, resulting in slower bacterial growth. They bind to the bacterial capsule or fimbriae, resulting in immobilisation and preventing attachment to the epithelium (Thankavel *et al.* 1997) (Weiss *et al.* 1988). In addition, they also bind and neutralise toxins such as LPS or PT (Paton *et al.* 1998; van den Berg *et al.* 1999).

Perhaps the most important role of antibodies in anti-bacterial immunity is that of opsonization and the activation of the complement system by two antibody subtypes, IgG and IgM. Soluble antibody bound to free bacteria can interact with Fc receptors on the cell surface of macrophages and neutrophils, resulting in phagocytosis (Zuckerman and Douglas 1978). Fc receptors can also activate NK cells to kill antibody coated target cells (Herberman *et al.* 1977). In addition, a complex system of plasma proteins activated by antibody-antigen interactions (Cooper 1985), known collectively as complement, is capable of lysing bacterial cells (Allen and Scott 1981). The complement protein, C3b, binds non-specifically to bacteria and antigens near the site of activation, triggering a complement cascade that leads to binding by macrophages resulting in phagocytosis (Ehlenberger and Nussenzweig 1977).

B cells encountering microbial pathogens can be activated without T-cell help for constitutive antibody secretion in response to conserved bacterial antigens, such as LPS (Betts *et al.* 1993), and the binding of these secreted antibodies subsequently facilitates phagocytosis. In contrast, in the case of protein antigens such as toxins or adhesins, the humoral response is initiated by accessory signals, such as CD40 ligation provided by Th2 cells(Lane *et al.* 1992; Noelle *et al.* 1992). B-cells migrating via the lymphatics to the regional lymph nodes or spleen with captured antigen must first cross the T-cell rich area to reach the primary follicle or red pulp (the B-cell area of the lymph node or spleen, respectively) (Garside *et al.* 1998). Here, antigen loaded B-cells are trapped and

subsequently activated by Th2 cells recognising the same antigen. Engagement of the BCR and subsequent CD40 ligation results in increased expression of B7 co-stimualtory molecules on the B-cell and the highly polarized release of IL-4 and IL-5 by the bound T_H2 cell (Valle *et al.* 1989). This leads to proliferation and terminal differentiation of B cells to antibody secreting plasma cells and memory B-cells upon subsequent trafficking to tissues. Terminally differentiated plasma cells no longer express MHC class II or B7 co-stimulatory molecules, and hence lose their responsiveness to antigen and Th2 cells (Shapiro-Shelef and Calame 2005). The majority of B-cells residing in tissues are memory B-cells. In the case of the lung the vast majority of lymphocytes present are memory B and T-cells, suggesting the lung is primed to respond to antigenic challenge at a moments notice (Saltini *et al.* 1990).

As mentioned above, before antibodies are generated against *Bordetella* protein antigens such as PT or FHA, antigen must first be presented by APC to $CD4^+$ T-cells in the presence of Th2 polarizing signals in the regional lymph nodes. Unlike the situation discussed above for Th1 cells, the APC derived signals driving Th2 cell differentiation from naïve T-cells are less clear, leading to speculation that Th2 activation represents a default pathway of adaptive immunity that is skewed towards a Th1 response only by the induction of IL-12 by microbial products (Moser and Murphy 2000). However, several innate immune cell-derived molecules necessary for Th2 cell development have been identified. For example, the co-stimulatory molecule CD86 has been shown to selectively stimulate the production of IL-4 from naïve T-cells (Freeman *et al.* 1995). In addition, OX40L (Ohshima *et al.* 1998; So *et al.* 2006) and MCP-1 (Kapsenberg 2003) have been implicated as polarizing signals in the development of Th2 responses. A number of secreted tissue factors have also been implicated in driving Th2 responses including histamine (Caron *et al.* 2001), TSLP (Soumelis *et al.* 2002), IL-25 (Tato *et al.* 2006), IL-13 (Defrance *et al.* 1994), IL-9 (Schmitt *et al.* 1990), IL-6 (Rincon *et al.* 1997), IL-5 (DeKruyff *et al.* 1990) and IL-4 (Reynolds *et al.* 1987).

Analogous to IFNγ and Th1 responses, IL-4 is the predominant cytokine involved in the induction of Th2 cell responses (Le Gros *et al.* 1990; Seder *et al.* 1992). IL-4, produced by Th2 cells, NK T-cells, basophils, eosinophils and mast cells, binds to IL-4R expressed on B-cells (Ohara and Paul 1987), T-cells (Armitage *et al.* 1990), mast cells (Ohara and Paul 1987) and monocytes and macrophage (Ohara and Paul 1987; Bonder *et al.* 1998). Ligation of IL-4R activates the Jak-STAT signalling cascade leading to STAT6 activation, which in turn induces Th2 specific transcription factors, such as c-Maf and GATA3 (Murphy and Reiner 2002). c-Maf is a Th2 specific transcription factor that

regulates IL-4 production through trans-activation of its promoter, thereby skewing naïve T-cells towards the Th2 cell pathway (Ho *et al.* 1996). GATA3, analogous in function to T-bet, has been implicated in the re-modelling of the IL-4 locus, leading to maximal IL-4 expression in Th2 cells (Ouyang *et al.* 1998). Similar to the effect of T-bet on Th2 cell differentiation, GATA3 can inhibit the generation of Th2 cell responses (Ouyang *et al.* 1998). It can also trans-activate the IL-5 promoter (Zhang *et al.* 1997), leading to expression of IL-5, a cytokine released from Th2 cells that drives B-cell proliferation (Yoshizaki *et al.* 1982). In addition to IL-4 and IL-5, GATA3 has a role in activating expression of IL-13 (Zheng and Flavell 1997), another Th2 cytokine involved in B-cell proliferation, differentiation and isotype switching to IgE (Cocks *et al.* 1993).

Since *Bordetella* spp. are primarily extracellular pathogens, the generation of an effective antibody response is crucial, not only to prevent re-infection but also for bacterial clearance. Like athymic or IFNγ-deficient mice, Ig deficient mice also fail to clear *B. pertussis* following respiratory challenge (Mahon *et al.* 1997). Passive immunization experiments with mice have indicated that antibodies directed against the major virulence factors, such as PT and PRN, can confer reasonable levels of protection against respiratory challenge (Mills *et al.* 1998). Further, passive immunization with *B. pertussis*-specific anti-sera has been shown to reduce the severity of disease in infected children (Granstrom *et al.* 1991).

1.2.2.3 Treg

The constant environment sampling and sentinel activities of DC results not only in the generation of pro-inflammatory Th1 and Th2 responses but also in the generation of so called autoreactive or self-reactive T-cells. Tolerance to self antigen is primarily achieved by a combination of thymic deletion of autoreactive T-cells (Sakaguchi 2000) and peripheral T-cell anergy of those that escape the thymus (Schwartz 1990). However, complete control over pathogenic T-cells and the termination of th1 and Th2 cell responses, and thus the prevention of immunopathology, involves a distinct population of T-cells known as "suppressor" or Treg cells (Battaglia *et al.* 2006). A breakdown in the mechanisms employed by Treg cells in controlling self-reactive T-cell, Th1 and Th2 responses is believed to lead to autoimmune inflammatory (Th1) and allergic (Th2) diseases. Treg cells consist of naturally occurring $CD4^+$ $CD25^+$ T-reg cells that constitutively express the Treg cell associated transcription factor FoxP3 (Khattri *et al.* 2003); Tr1 cells, which are $CD4^+$, secrete high concentrations of IL-10, exhibit variable

CD25 expression and only express FoxP3 upon activation (Levings *et al.* 2005); and so called $CD4^+$ $CD25^+$ Th3 cells, which like Th2 cells secrete IL-10 and IL-4 but also secrete high levels of TGFβ (Battaglia *et al.* 2006).

Naturally occurring T-reg cells develop in the thymus and form part (~5-10%) of the peripheral $CD4^+$ T-cell population, functioning mainly in the suppression of autoimmunity by repressing the activation of self-reactive T-cells (Mills 2004). The mechanism behind this repression is not yet fully understood, though is believed to be contact dependant, possibly involving surface associated anti-inflammatory cytokines such as TGFβ (Nakamura *et al.* 2001), and negation of CD28 co-stimulation by interaction between CD80/CD86 and a competitive ligand, CTLA4 (Read *et al.* 2000).

Inducible Tr1 and Th3 cells originate from uncommitted naïve T-cells in the periphery and arise from APC activation in the presence of polarizing signals distinct from those that promote Th1 or Th2 cells (Battaglia *et al.* 2006). The nature of these signals has been proposed to include a semi-mature or intermediate DC phenotype, characterised by the normal high level expression of B.7 and MHC II molecules, coupled with low level expression of CD40 and ICAM1 (McGuirk and Mills 2002). As discussed in section 1.2.2.1, ICAM1 has been implicated in the generation of Th1 responses during TCR ligation, while CD40 is necessary for complete T-cell activation. Thus a reduced activation state in the absence of Th1-specific polarizing factors, such as ICAM1, may result in Treg cell induction.

Inducible Treg cells are thought to function primarily in the control of excessive pathogen-induced immunopathology during infection (Mills 2004). As mentioned above, Th3 cells differ from Th2 cells by the production of high levels of TGFβ. Tr1 cells differ from Th2 cells by the absence of IL-4 production, in combination with a higher and lower level of IL-10 and IL-2 production respectively. Tr1 or Th3 mediated immunosuppression can be reversed by antibodies neutralizing IL-10 and TGFβ, suggesting these cells exert their repression in a contact independent fashion, (Mills 2004). Both these anti-inflammatory cytokines have a range of inhibitory effects on bystander cells mediating cell-mediated or innate inflammatory responses. For example, TGFβ has been shown to inhibit T-bet expression, a crucial Th1-related transcription factor involved in IFNγ production and Th2 response inhibition (see section 1.2.2.1) (Gorelik *et al.* 2002). IL-10 can inhibit activation, and inflammatory cytokine production by Th1 cells (Fiorentino *et al.* 1991) (Moore *et al.* 1990). It decreases MHC II (de Waal Malefyt *et al.* 1991), costimulatory molecule and adhesion molecule expression on monocytes (Willems *et al.*

1994) and inhibits the release of pro-inflammatory cytokines from activated macrophage (Fiorentino *et al.* 1991), thereby modulating the stimulatory capacity of APC. Thus, natural and inducible Treg cells represent a mechanism whereby the immune system can suppress not only antigen-specific Th1 and Th2 cells, but also innate cells and naïve (CD4+ CD25-) T-cell activation.

An example of suppression of a pro-inflammatory Th1 response and excessive lung pathology by the action of IL-10 secreting Tr1 cells was recently demonstrated by Higgins *et al* (2003). This study showed that LPS-driven innate IL-10 from DC drives the induction of Tr1 cells during *B. pertussis* respiratory challenge (Higgins *et al.* 2003). However, the absence of this innate IL-10 in TLR4 defective mice, and the attenuation of the subsequent *B. pertussis* antigen specific Tr1 response, resulted in increased cellular infiltrate and pathology in the lungs. The authors also noted enhanced antibody responses in the sera of TLR4-defective mice (Higgins *et al.* 2003). Thus the reduction of innate IL-10 (and the subsequent Tr1 response provoked) had a more severe and damaging effect on lung inflammation and pathology than the reduction of innate IL-12 (and the subsequent Th1 response provoked) in these mice. This demonstrates the dominant suppressive effect of Tr1 cells on cell-mediated and humoral immunity, and their critical function in limiting pathogen induced immunopathology in the mucosal tissue of vitally important organs, such as the lung.

The actions of Treg cells in suppressing adaptive immune responses can be of substantial benefit to the pathogen as well as the host. It has been hypothesised that *B. pertussis* deliberately enhances innate IL-10 production, to recruit Treg cells, and thus reduce the impact of a protective Th1 response in the lung (Mills 2004). In particular, studies from our laboratory have demonstrated that, in addition to LPS, another *B. pertussis* virulence factor, FHA (discussed in section 1.3.2.1), can synergise with LPS to directly and significantly enhance innate IL-10 production from APC, with the resultant recruitment of Treg cells, effecting bacterial persistence (McGuirk *et al.* 2002). Interestingly, other bacterial virulence factors that can synchronise these effects also result in the activation of antigen specific Treg cells. It has also been demonstrated that *B. pertussis* adenylate cyclase toxin (ACT) (discussed in section 1.3.2.4) and cholera toxin are capable of inhibiting CD40 and IL-12 expression, while upregulating IL-10 production by APC (Lavelle *et al.* 2003; Ross *et al.* 2004) and they promote Tr1 as well as Th2 cells. Thus, in addition to the absence of high level CD40 expression and Th1 polarizing factors, such as ICAM1, the presence of IL-10 upon TCR ligation appears to have a role in Treg cell induction.

In addition, the T3SS of *B. bronchiseptica* has been implicated in the modulation of DC maturation by increasing surface expression of MHC II, CD80 and CD86 (Skinner *et al.* 2004). This maturation status, in combination with the inhibition of CD40 and IL-12 by ACT, leads to Treg cell induction. T3SS-mediated migration of these tolerogenic DC to the draining lymph nodes of the respiratory tract (Skinner *et al.* 2005), resulted in the generation of IL-10 producing splenocytes that where subsequently shown to exert an inhibitory effect on IFNγ producing splenocytes, and delayed clearance of the bacterium from the lungs (Pilione and Harvill 2006). Interestingly, a virulence factor from an unrelated bacterium and the first described T3SS effector, the V antigen of *Yersinsia pestis* (Bacon and Burrows 1956), has also been shown to induce IL-10 from macrophage (Sing *et al.* 2002). Thus APC modulation for the purpose of exploitation of the immunosuppressive effects of Treg cells appears to be a specific strategy employed by pathogens such as *Bordetella* spp., to facilitate immune evasion and persistence.

1.2.3 Immunity to *B. pertussis*

Natural respiratory infection with *B. pertussis* usually leads to the development of long-lived protection against subsequent infection (Mills *et al.* 1999), and recovery from natural *B. pertussis* infection in children is associated with the generation of cell mediated immunity (Ryan *et al.* 1997; Hafler and Pohl-Koppe 1998). Further, the signature Th1 cytokine, IFNγ, has been detected in the bronchoalveolar lavage (BAL) fluid of *B. pertussis* infected mice (Torre *et al.* 1993). Whole-cell *pertussis* vaccines (Pw), developed in the 1930's in response to the severity of whooping cough disease and its high rate of transmission, entered routine immunisation regimes for children in the U.S post world war II and resulted in dramatic reduction of *B. pertussis* induced illness (Mattoo and Cherry 2005). Studies with Pw in mice have demonstrated the induction of a clear Th1 response (Redhead *et al.* 1993), while studies in children have demonstrated a similar Th1 cytokine response (Ausiello *et al.* 1997), characterised by moderate to high levels of IFNγ, but undetectable IL-5 (Mills *et al.* 1999).

The association of Pw with severe, albeit rare, neurological complications such as encephalopathy, spasms and sudden infant death syndrome (Mattoo and Cherry 2005) prompted the development of acellular vaccines (Pa), utilising purified, detoxified derivatives of *B. pertussis*. The most common constituents of these vaccines have been combinations of adhesion-related factors, such as fimbriae, FHA, PRN and PT (Olin *et al.* 1997). Studies conducted with Pa in mice and children suggest increasing protection

correlates with increasing numbers of protective antigens (Mills *et al.* 1998) (Olin *et al.* 1997). In contrast to the strong Th1 response induced in children immunized with Pw, the acellular vaccines (3 component and five component) have been shown to induce a more mixed Th1/Th2 response, as evidenced by the presence of both high-level IFNγ and IL-5 secreting T cells (Mills *et al.* 1999). While in mice, Pa appears to induce a strong Th2 response, characterised by high levels of IL-5 and IL-4, but undetectable IFNγ (Mills *et al.* 1999). Unfortunately, the cost of the safer and reduced reactogenicity associated with Pa appears to be a reduction in vaccine efficacy, with only the current 3 (PT, FHA, PRN) and 5 (PT, FHA, PRN, Fim1, Fim2) component acellular vaccines providing around 85% protection against severe disease compared with over 90% for Pw (Olin *et al.* 1997). These observations, in conjunction with observations of waning immunity in countries with a high level of vaccine coverage(Bass and Wittler 1994; De Serres *et al.* 1995; de Melker *et al.* 1997; Weber *et al.* 2001), have prompted the suggestion that additional surface antigens could be included in Pa, to improve efficacy. Suggestions to date have included autotransporters such as BrkA, Vag8, SphB1 and the *Bordetell*a T3SS effector, Bsp22. This protein has been described as an ideal vaccine antigen on account of its abundant production, high-level conservation between the classical *Bordetella* spp. and surface location (Mattoo and Cherry 2005). In addition it is unique to *Bordetella* spp. and has to date been shown to be highly antigenic in mouse, rat and rabbit models (Yuk *et al.* 1998; Yuk *et al.* 2000; Mattoo *et al.* 2004). However, as discussed in section 1.4.6, although the T3SS gene loci are intact and transcribed in *B. pertussis*, to date no secretion of this protein has been demonstrated by *B. pertussis in vitro*.

1.3 Determinants of *Bordetella* pathogenesis

The goal of any bacterial pathogen is to colonize and multiply by exploiting its environment to the fullest extent. Many pathogens have evolved complex mechanisms to subvert and evade the actions of the host's immune system. *Bordetella* spp. contain a complex array of toxins and adhesins, collectively referred to as virulence factors, which are expressed during both *in vitro* and *in vivo* growth. The expression of all these factors (with the exception of TCT) is co-ordinately regulated at the transcriptional level by a two-component signal transduction system, known as the *Bordetella* virulence gene locus or BvgAS regulon. The role of this system, as well as the individual virulence factors it regulates, are discussed in the following sections.

1.3.1 *Bordetella* Virulence gene regulation

The term "antigenic modulation" in *B. pertussis* was first coined in 1960 by Lacey, in response to the observation that the composition of the growth medium had a profound influence on the immunological and pathophysiological activities of *B. pertussis* (Lacey 1960). Specifically, he noted that FHA and mouse virulence were lost when the bacterium was cultured in the presence of $MgSO_4$. It was subsequently shown that this antigenic modulation was rapidly occurring and easily reversible *in vitro* (Idigbe *et al.* 1981), and thus suggested that *B. pertussis* was an environmentally responsive organism, capable of tailoring its protein production to the environmental changes encountered by the bacterium as it enters its human host. Random transposon mutagenesis later identified a locus, originally called *vir*, necessary for the activation of genes encoding known virulence factors, including FHA, PT, ACT and DNT (Weiss *et al.* 1983), and sequence analysis of this locus revealed the presence of 2 genes, named *Bordetella* virulence gene (Bvg) A and S, which collectively constitute the BvgAS virulence regulon in the classical *Bordetella* spp. (Arico *et al.* 1989; Stibitz and Yang 1991). This virulence regulon is part of a signal transduction superfamily, present in a wide range of prokaryotes, known as two-component signal transduction systems (Parkinson and Kofoid 1992). These systems typically consist of a membrane bound sensor (BvgS) and a cytosolic transcriptional activator (BvgA).

BvgS is a sensor kinase protein containing a transmembrane sensor domain and cytosolic transmitter, receiver and output domains. Signal inputs detected by the sensor

domain lead to autophosphorylation of a highly conserved histidine kinase residue in the transmitter domain, and by a series of intramolecular phosphorylation events, the phosphoryl group is eventually transferred by the output domain to BvgA (Smith *et al.* 2001). The phosphorelay can be halted by the transfer of the phosphoryl group to water (to form inorganic phosphate) instead of the output domain, by the receiver domain, thus preventing BvgA activation (Mattoo and Cherry 2005). Activated BvgA directly promotes the transcription of Bvg^+ phase specific genes or virulence activated genes (vag-genes) by binding to cis-acting sequences in their promoter regions, and indirectly by activating other transcriptional activators that control virulence systems. For example, BvgA activates transcription of BtrS, a sigma-70 transcription factor, which in turns activates transcription of the T3SS in *Bordetella* spp. (Mattoo *et al.* 2004). BvgA also activates another protein located downstream of the BvgAS locus, known as BvgR, which has been implicated in the repression of another set of genes, termed virulence repressed genes (vrg-genes) (Merkel and Stibitz 1995).

Phosphorylated BvgA is believed to have a much greater affinity for virulence factor promoter BvgA binding sites, which typically consist of direct or inverted repeats of the heptameric BvgA consensus binding sequence, TTTCCTA, located upstream of various Bvg-regulated genes (Smith *et al.* 2001). This sequence, initially identified in the promoters of *fhaB* and *bvgA* itself, (Roy and Falkow 1991), or sequences homologous to it, are present at varying frequencies and distances from the -35 TATA (transcription initiation / RNA polymerase recognition sequence) region. A model has emerged whereby BvgA initially binds high affinity sites far upstream of the TATA box, forming multimers along the promoter templates and progressively binding weaker sites until the transcription initiation site is reached. Thus the frequency of BvgA binding sequence repeats in a given promoter, and their relative distance from the TATA box, allows BvgA to exert a temporal regulation over individual virulence factor expression. This has been demonstrated with so called "early" BvgA activated promoters such as *fhaB* and *bvgA* itself, in contrast to "late" BvgA promoters such as *pt, cyaA* and the *bsc* locus (Cummings *et al.* 2006). For example, phosphorlyated BvgA was shown to initially bind bp -96 to -77 of the *fhaB* promoter, with this bound region extending as far as the -30 bp (TATA box) with increasing BvgA concentration (Zu *et al.* 1996). This is in contrast to the situation for PT, with BvgA initially binding at position -168, and significantly higher (10-fold) concentrations of BvgA are required for extension to the TATA region, for subsequent RNA polymerase interaction and gene expression (Zu et al. 1996). Thus, early promoters are activated within minutes of BvgA phosphorylation, while the late promoters require sufficient accumulation

of intracellular BvgA, and thus are activated several hours later (Scarlato *et al.* 1990) (Scarlato *et al.* 1991).

It is now widely recognised that *Bordetella* spp. contain two distinct phases of growth, termed Bvg^+ and Bvg^-. As discussed above, the Bvg^+ phase is characterised by the expression of a wide array of virulence factors involved in various mechanisms of bacterial adherence, toxicity and immune evasion. The Bvg- phase of growth can be induced *in vitro* by growing the bacteria under modulating conditions, such as growth at 25°C or in the presence of certain concentrations of SO_4 ions or nicotinic acid (Melton and Weiss 1993). In this phase of growth, the BvgA and BvgR proteins are not activated and thus the repression exerted by BvgR on vrg-genes is removed, resulting in the expression of a number of proteins involved in motility, such as flagellin, and survival under nutrient limiting conditions in *B. bronchiseptica,* suggesting a role in environmental survival (Akerley *et al.* 1992; Cotter and Miller 1997). In contrast, the number of Bvg^- phase proteins in human adapted *Bordetella* is much smaller, and these species are not motile (Cummings *et al.* 2006), suggesting a different function for the Bvg^- phase or the loss of expression of genes that facilitate survival *ex vivo*, possibly as a function of host restriction to humans. Recently an additional phase, known as Bvg^i, so called because it possesses phenotypes intermediate between the Bvg^+ and Bvg^- phases, has been characterised in depth. Originally described in Lacey's landmark study of 1960 (Lacey 1960), it is characterised by reduced virulence potential (due to the loss of expression from late BvgA activated promoters such as PT and ACT) and increased resistance to starvation, along with the expression of a bvg^i-phase specific protein, BipA (Cotter and Miller 1997). Expressed by all of the classic *Bordetella* spp., BipA bears some homology to the bacterial adhesins intimin of *E. coli* and invasin of *Yersinia* spp. and its expression can be induced *in vitro* by growth at 30°C (Fuchslocher *et al.* 2003), suggesting a role for BipA in facilitating aerosol transmission (the temperature in the human nasal cavity typically ranges from 30.3 to 32.3°C) from host to host.

1.3.2 Virulence factors of *Bordetella* spp.

1.3.2.1 Filamentous hemagglutintin (FHA)

FHA, a surface associated and secreted 220 kDa protein, is considered to be the major attachment factor for virulent *Bordetella* spp adherence to ciliated epithelial cells. It contains an arginine-glycine-aspartic acid (RGD) sequence which promotes binding to the very late antigen 5 (VLA5) of bronchial epithelial cells (Ishibashi *et al.* 2001), and the β_2-integrin CR3 (CD11b/CD18) expressed on macrophages, PMNs and other cell types (Mills 2001). Binding to CD11b/CD18 on macrophages induces phagocytosis, but *B. pertussis* has also been shown to prevent intracellular killing in macrophages, presumably by the action of bacterial toxins, therefore FHA mediated binding of *B. pertussis* to CR3 possibly facilitates bacterial persistence in the lungs (Mills 2001). FHA also has a carbohydrate recognition domain that allows it to bind to cilia, and a lectin-like binding domain that allows it to adhere to sulphated carbohydrates and heparin, found at the surface of the respiratory tract and the extracellular matrix (Preston *et al.* 2004), which may facilitate binding to non-ciliated epithelial cells (Smith *et al.* 2001). The binding of FHA to phagocytes confers obvious advantages for the bacterium in terms of immune evasion. Occupation of a vital receptor site, such as VLA5 or CR3 may disrupt phagocyte communication and thereby lessen the impact of the immune response. Also, the tight binding of the pathogen to these cells may offer advantages in terms of more economical killing of phagocytes by the action of bacterial toxins, due to the increased proximity of the host and bacterial cells. In addition, ligation of CR3 has been shown to suppress IL-12 production by macrophages via an IL-10 dependant mechanism, thereby downregulating pro-inflammatory Th1 responses, an important effector mechanism in bacterial clearance (McGuirk and Mills 2000), and FHA has been implicated in inhibition of T cell proliferation to exogenous antigens *in vitro* (Boschwitz *et al.* 1997). In addition, FHA has been shown to induce the production of pathogen specific Treg cells, through its interaction with DC (McGuirk *et al.* 2002). Thus, FHA through its adhesive and immunomodulatory properties is of critical importance to the bacterium in terms of initiating the pathogenic cycle.

The mature 220 kDa FHA protein derives from a 367 kDa precursor, FhaB. The protein is extensively cleaved at both the N and C terminus on translocation across the cytoplasmic membrane and again at the cell surface (Mattoo and Cherry 2005). Correct folding of FHA and transport across the bacterial membranes requires an accessory protein, FhaC (Jacob-Dubuisson *et al.* 1999). Release of FHA from the cell surface

requires the action of an autotransporter, SphB1 (Coutte *et al.* 2001), and SphB1 mutants of *B. pertussis*, in which all FHA remains bound to the cell surface, exhibit defective lung colonization and persistence in mice (Coutte *et al.* 2003).The extreme importance of FHA to *Bordetella* spp. is reflected in its genetic organisation and regulation. FHA is encoded by the *fhaB* gene, and is highly conserved in the three sequenced strains of *Bordetella* spp. In addition, two other genes, *fhaL* and *fhaS* have been found in *Bordetella* spp. Both bear significant sequence homology to *fhaB* and contain similar binding motifs and domains, though they also contain internal sequence differences (Preston *et al.* 2004). This apparent redundancy in gene content may have arisen to prevent inactivation of FHA production through spontaneous mutation. The *fhaB* and *fhaC* genes are separated by three genes involved in fimbrial production, suggesting a possible co-regulation of these adhesins with FHA (Locht 1999). The *fhaB* gene lies immediately upstream of the BvgAS locus, and under virulent growth conditions, FHA is one of the first proteins produced, detected in a matter of minutes (Stibitz 1994). Also, in addition to gene content redundancy, the *Bordetellae* also exhibit redundancy in the *fhaB* promoter sequence, with the presence of P_{fha}*, an additional promoter, located immediately downstream of P_{fha}, which permits constitutive low-level gene transcription of the *fhaB* gene, demonstrating the critical importance of this protein for the bacterium (Smith *et al.* 2001).

1.3.2.2 Fimbriae

Fimbriae, also known as agglutinogens, are long filamentous structures which extend from the bacterial surface. They bind to sulphated sugars that are ubiquitous on cell surfaces in the respiratory tract (Geuijen *et al.* 1996) and also to the very late antigen 5 (VLA5) of monocytes and/or macrophages, which in turn activate expression of CR3 to promote FHA-mediated bacterial adherence and internalisation (Hazenbos *et al.* 1995). Thus they may contribute to the immunomodulatory effect of FHA described above.

The two major subunits produced by the classical *Bordetella* spp. are Fim2 and 3, which form the fimbrial strand, and the minor subunit FimD is thought to be positioned at the tip of this strand (Smith *et al.* 2001). Fim2 and Fim3 are highly antigenic, and serum antibody from whooping cough patients recognizes both proteins (Williamson and Matthews 1996). Several different fimbrial genes exist and are present in the three sequenced strains of *Bordetella* spp., representing the importance of adherence to the host respiratory epithelium for these pathogens. However, there appears to be differences in their expression. For example, the *fimA* gene is intact and expressed at low levels in *B. bronchiseptica* but is inactivated by a deletion in *B. pertussis* and, by a frame-shift

mutation, in *B. parapertussis.* The fact that two different mutations have inactivated the same gene in both human-restricted species suggests that FimA has been selected against for colonization of humans. Another fimbrial gene, *fimN,* is present in both *B. bronchiseptica* and *B. parapertussis* but has been deleted in *B. pertussis.* Other differences exist, but overall *B. bronchiseptica* expresses more fimbrial genes than the other two species, suggesting a role for fimbrial binding in determining host specificity (Preston *et al.* 2004).

1.3.2.3 Pertussis Toxin (PT)

PT, originally termed lymphocytosis promoting factor or histamine sensitizing factor, is a multi-subunit protein exotoxin which is readily expressed *in vitro* by *B. pertussis*, but not by *B. bronchiseptica* or *B. parapertussis* (Arico and Rappuoli 1987; Hausman *et al.* 1996). Like other bacterial AB toxins such as cholera toxin or *E. coli* labile toxin, PT consists of an A subunit that carries the enzymatic activity and a B subunit that mediates binding to surface glycoproteins on a variety of mammalian cells, including ciliated epithelial cells, macrophages and lymphocytes (Locht 1999). Thus the B subunit functions not only to deliver the A subunit in the cytosol of the target cell, but also as an adhesin in the respiratory tract, and isogenic PT mutants of *B. pertussis* exhibit as much as 50% less binding to ciliated epithelial cells in *in vitro* binding assays, when compared to the parental bacteria (Relman *et al.* 1989). The primary function of the A subunit is to disrupt signal transduction processes between intracellular G proteins, resulting in their inactivation, leading to the continual conversion of ATP to cAMP. Elevated cAMP levels then disrupt normal cellular processes in immune cells, thereby contributing to immune dysfunction. It has also been shown to inhibit macrophage, neutrophil and lymphocyte chemotaxis by altering intracellular Ca^{2+} levels (Lad *et al.* 1985) (Lad *et al.* 1985). Systemic effects of the toxin also include profound lymphocytosis, histamine sensitization and hypoglycaemia (through sensitization of the insulin-secretory islets of the pancreas) (Pittman 1984) (Mattoo and Cherry 2005).

Genes p*txA-E* of the *ptx/ptl* operon encode the five subunits (S1-S5) that make up PT. The operon also encodes, directly 3' to the *ptx* genes, a type IV secretion system to export the toxin, composed of the PT liberation genes, *ptl*a-i. (Farizo *et al.* 1996) The operon is intact in all three sequenced species, yet is transcriptionally silent in *B. bronchiseptica* and *B. parapertussis,* due to mutations in the promoter region. In addition, the *ptx*B gene has been inactivated in *B. parapertussis* by the presence of a psuedogene

(Parkhill *et al.* 2003). Therefore, secretion is only seen *in vitro* with *B. pertussis* (Preston *et al.* 2004) (Arico and Rappuoli 1987).

Sequence analysis of the promoter region of the *ptx* operon has revealed that mutations in this region are highly conserved between *B. bronchiseptica* and *B. parapertussis* (Preston *et al.* 2004). This would indicate that the *ptx* promoter of these species is closer to the ancestral promoter than that of *B. pertussis* and not the other way round as previously thought. The genome sequence has revealed that a series of mutations in the –10 to –35 (TATA box) region of the *B. pertussis ptx* promoter have occurred at the BvgA binding site (Preston *et al.* 2004). BvgA is a two-component transcriptional activator that exerts a positive control on the expression of a significant number of virulence factors in *Bordetella* spp., including PT (discussed in section 1.3.1). These mutations function to improve the affinity of BvgA for the *B. pertussis ptx* promoter. Thus it appears *B. pertussis* has evolved to increase expression of PT and this recent evolution is presumably in response to its adaptation to the human host. This raises the question of whether or not the *ptx* operon was functional in the *B. pertussis* ancestor. In addition Hausman and colleagues (1996) have shown that the PT genes in both *B. bronchiseptica* and *B. parapertussis* can indeed express PT from a functional promoter (Hausman *et al.* 1996). It is possible that the *ptx* promoters of *B. bronchiseptica* and *B. pertussis* could function *in vivo* through the action of an additional regulatory protein, either not present or not expressed *in vitro*, and, in support of this hypothesis, modest antibody titres to PT have been observed in children infected with *B. parapertussis* (Hausman *et al.* 1996). The mutation of the *ptx* operon in *B. pertussis,* which results in dramatically increased BvgA binding, may have allowed this species to overcome the need for this additional regulatory factor *in vivo*. However, *B. parapertussis* and *B. pertussis* can, on occasion, be isolated from the same patient, possibly explaining the source of PT antibodies in the above study.

The lack of evident expression of PT by *B. bronchiseptica* and *B. parapertussis* has led researchers to conclude that PT is responsible for much of the pathology associated with whooping cough, particularly when compared to the milder infection with *B. parapertussis* and the asymptomatic infection associated with *B. bronchiseptica* (Pittman 1984). However, with the exception of lymphocytosis, all other features of disease produced by *B. pertussis* and *B. parapertussis* are very similar, though milder in the case of *B. parapertussis*, and it is not believed to express PT (Arico and Rappuoli 1987) (Wirsing von Konig and Finger 1994). It has been hypothesised that the acquisition of virulence factors involved in increased epithelial cell binding and inflammation, such as PT and TcfA, has enabled *B. pertussis* to efficiently move from the broad mammalian to the

human niche. Thus, while the exact role of PT in the exacerbated infection seen with *B. pertussis* patients remains enigmatic, there can be no doubt about its importance to the bacterium. To date PT expression remains the only gain of function mutation observed with *B. pertussis* in its transition from its *B. bronchiseptica*-like ancestor, and thus, is a large part of what makes *B. pertussis* a distinct species, as apposed to merely a deletion mutant of *B. bronchiseptica.*

1.3.2.4 Adenylate cyclase toxin (ACT)

ACT is a 200 kDa extracytoplasmic enzyme that is primarily surface associated, but also released into the surrounding medium during exponential growth of *B. pertussis, B. bronchiseptica* and *B. parapertussis* (Bellalou *et al.* 1990). A role for FHA in this surface localisation has been established (Zaretzky *et al.* 2002), once again highlighting the role of adhesins in positioning the bacteria for toxin delivery. Bioinformatic BLAST analysis reveals the protein is 98% identical between all 3 species at the amino acid level. The protein possesses a calmodulin-sensitive N-terminal catalytic domain and a C-terminus hemolysin domain (Glaser *et al.* 1988; Glaser *et al.* 1988). The genes encoding ACT and its accessory proteins CyaB, C, D and E bear homology to other calcium dependant, pore forming toxins of the RTX family, such as the alpha-hemolysin of *E. coli* (Mattoo and Cherry 2005). CyaB, D and E are necessary for secretion of the toxin (Glaser et al. 1988). Palmitoylation of the ACT protein by the product of *cyaC* facilitates insertion and transmembrane delivery into target cells, where the cytoplasmic eukaryotic cell factor calmodulin activates the toxin resulting in the production of supraphysiological levels of intracellular cAMP, disrupting host cellular functions (Harvill *et al.* 1999).

In vivo studies have shown that *B. pertussis* isogenic ACT mutants colonize the respiratory tract of infected mice, to a much lower degree than wild type bacteria (Weiss *et al.* 1984). It has been demonstrated *in vitro* that ACT has many toxic effects on phagocytes such as, inhibition of chemotaxis, induction of apoptosis in macrophages, inhibition of phagocytosis, superoxide generation and bacterial killing by neutrophils, thus facilitating intracellular persistence in the host by disruption of bacterial killing and antigen presentation (Khelef *et al.* 1993; Harvill *et al.* 1999; Mills 2001; Mattoo and Cherry 2005). Studies from our laboratory have shown that ACT can synergise with LPS to modulate APC function. Specifically, ACT significantly augmented LPS-induced IL-6 and IL-10 and inhibited LPS-driven TNFα and IL-12p70 production from bone marrow-derived DC and

macrophages, thus skewing a pro-inflammatory Th1 response to a more Treg cell response, which has implications for bacterial persistence (Ross *et al.* 2004) (Boyd *et al.* 2005).

1.3.2.5 Tracheal Cytotoxin (TCT)

TCT is a low molecular weight disaccharide-tetrapeptide that has been found to be secreted during log phase of *in vitro* growth by representatives of *B. bronchiseptica, B. pertussis,* and *B. parapertussis* (Cookson *et al.* 1989). The toxin is derived from the peptidoglycan of the bacterial cell envelope and is released from the bacteria as they break down and rebuild their cell wall during growth. As such, TCT is the only virulence factor to be constitutively expressed in large amounts, and is independent of BvgAS regulation. TCT is responsible for much of the epithelial cytopathology in the lung during *B. pertussis* infection (Goldman and Herwaldt 1985; Heiss *et al.* 1993). The toxin paralyses the cilia, disrupts tight junctions and induces specific extrusion of ciliated cells from the respiratory epithelium (Goldman *et al.* 1982). Thus TCT mediated epithelial cell destruction may be, in part, responsible for the violent coughing episodes associated with *B. pertussis* infection, as this is the only way to clear the airways in the absence of functioning cilia. Like other virulence factors, such as LPS or PT, TCT induces IL-1 and NO production by respiratory epithelial cells, contributing to local pathology in the lung (Heiss *et al.* 1993; Heiss *et al.* 1993). Released NO then diffuses to neighbouring ciliated epithelial cells causing damage (Flak and Goldman 1996). It has been reported that TCT inhibits DNA synthesis of hamster tracheal epithelial cells *in vitro* (Cookson *et al.* 1989) and it is possible that this may delay the normal replacement of destroyed epithelial cells *in vivo*, by blocking division and differentiation of the underlying regenerative basal cell population, thereby contributing to disease persistence. Furthermore, it has been demonstrated that TCT is toxic for human neutrophils (Cundell *et al.* 1994), and therefore may directly contribute to the survival of *B. pertussis in vivo*.

1.3.2.6 Dermonecrotic toxin (DNT)

DNT, so named because of its ability to cause necrotic skin lesions when injected subcutaneously into mice (Livey and Wardlaw 1984), is one of the least studied virulence factors of *Bordetella* spp. It is unique among *Bordetella* virulence factors due to its lack of secretion, and therefore, primarily cytoplasmic location (Cowell *et al.* 1979). It has been

hypothesised that the toxin may exert its effect upon bacterial cell lysis, or be secreted only at specific anatomic sites *in vivo* (Locht 1999). The primary activity of DNT on host cells has been shown to be the toxin-induced formation of actin stress fibres (Horiguchi *et al.* 1997), but so far an *in vivo* role for DNT has not been demonstrated. Indeed, in a lethality model using intranasal challenge of infant mice, isogenic DNT mutants were unimpaired in their ability to cause lethal infection (Weiss and Goodwin 1989).

1.3.2.7 *Bordetella* autotransporters

Autotransporters are a diverse group of proteins, including lipases, invasins, toxins, adhesins and proteases, united in the ability to direct their own export to the outer membrane (Henderson and Nataro 2001). They can remain surface associated or can be released into the extracellular milieu following cleavage by an endogenous membrane protease (Mattoo and Cherry 2005). Members of this family with predicted virulence functions in *Bordetella* spp. include PRN (Charles *et al.* 1989) (Montaraz *et al.* 1985; Li *et al.* 1991), TcfA (Finn *et al.* 1991), Serum-resistance protein (BrkA) (Fernandez and Weiss 1994),Virulence activated gene 8 (Vag8) (Finn and Amsbaugh 1998) and SphB1 (Coutte *et al.* 2001). As discussed above in section 1.3.2.1, SphB1 is an autotransporter involved in the release of FHA from the bacterial cell surface. Vag8 is an outer membrane protein expressed by *B. bronchiseptica, B. pertussis* and *B. parapertussis* whose C-terminus bears significant homology to that of PRN, TcfA and BrkA, implicating it as an autotransporter (Mattoo and Cherry 2005). The exact role of Vag8 is unclear. However, recently it has been shown to be transcriptionally regulated by BvgA and necessary for export, but not transcription, of T3SS substrates in *B. bronchiseptica* (Mattoo *et al.* 2004).

PRN is a non-fimbrial protein closely associated with the outer membrane of *Bordetella* spp. that may play a role in adherence to monocytes (Hazenbos *et al.* 1994). It is polymorphic between the various *Bordetella* species and even between different strains of the same species (Mooi *et al.* 1998). It has a molecular mass of 69 kDa in *B. pertussis*, 70 kDa *in B. parapertussis* and 68 kDa in *B. bronchiseptica* (Boursaux-Eude and Guiso 2000). In *B. pertussis* it acts as an agglutinogen, promoting attachment to certain eukaryotic cells via an RGD motif. This motif, like in FHA, mediates binding to CR3, and it has been shown that PRN can augment the suppressive effect of FHA on LPS-induced IL-12 production *in vitro* (McGuirk and Mills 2000). It has also been demonstrated that expression of PRN in *Salmonella* and *E. coli* spp. can increase the adherence and invasiveness of these bacteria to cell lines (Everest *et al.* 1996). PRN also contains, at its

C-terminus, proline-rich repeats which are implicated as the major protective epitopes of the protein (Charles *et al.* 1991), and PRN has been shown to be of significant benefit to the host when included in acellular pertussis vaccines (Gustafsson *et al.* 1996).

BrkA is responsible for resistance of *Bordetella* spp. to killing by serum and the action of antimicrobial peptides in *B. pertussis*, but interestingly, is not required for serum resistance in *B. bronchiseptica* (Rambow *et al.* 1998). BrkA also contains RGD binding motifs, and mutants lacking BrkA are impaired in binding to eukaryotic cells *in vitro* and are less virulent than their wild-type counterparts during *in vivo* infection of mice (Weiss and Goodwin 1989). In addition, in a study conducted by Fernandez and Weiss (1998) 27 clinical isolates of *B. pertussis* from a children's hospital where found to be as, or more resistant to serum killing than the wild-type strain, Bp338 (Fernandez and Weiss 1998). Further, over-expression of BrkA in Bp338 in this same study was found to increase survival of the bacteria 2-5 fold. Thus it would appear that BrkA expression is of significant advantage to the bacterium and it is possible that BrkA-mediated resistance to killing by the antibody dependent pathway of complement may enable *B. pertussis* to colonize previously infected or vaccinated individuals. This has important implications in light of studies highlighting waning immunity (see section 1.2.3)

TcfA was originally discovered through transposon mutatgenesis of *vag* genes in *B. pertussis*, the mutants demonstrating as much as a 10-fold reduction in colonization and persistence in the trachea, but interestingly, not the lungs (Finn *et al.* 1991; Finn and Stevens 1995). Like FHA, PRN and BrkA, TcfA contains an RGD binding sequence and several proline rich repeats, and consistent with its role as an autotransporter, is both cell associated and secreted (Locht 1999). TcfA is not produced by *B. parapertussis* or *B. bronchiseptica*, and thus as a *B. pertussis*-specific virulence factor, may play a role in the increased transmission and pathology of *B. pertussis* infection.

1.3.2.8 Lipopolysaccharide (LPS)

LPS is a complex glycolipid and structural component of the bacterial outer membrane produced by most gram negative bacteria. LPS binds TLR4 and CD14 on host cells. In addition to its role in phagocyte activation and subsequent pro-inflammatory cytokine release (TNFα, IL-1 and IL-6), it also has mitogenic and toxic properties (Ayme *et al.* 1980) (Amano *et al.* 1990; Watanabe *et al.* 1990). *B. pertussis* LPS has been shown to synergise with TCT in mediating epithelial cell damage (Flak *et al.* 2000). The structure of LPS differs somewhat between the classical *Bordetella* spp. All 3 species produce LPS

composed of lipid A with a branched chain core oligosaccharide, known as band B (Peppler 1984). In *B. pertussis*, band B can be modified by the addition of a complex trisaccharide to form band A (Peppler 1984). These two distinct forms of LPS are also produced by *B. bronchiseptica*, but not by *B. parapertussis* (Le Blay *et al.* 1994). This is due to a mutation in the *wlb*H gene involved in band A trisaccharide biosyntheseis in *B. parapertussis* (Allen *et al.* 1998). Thus, *B. parapertussis* produces only a smaller version of *B. pertussis* band B LPS.

In addition, *B. bronchiseptica* and *B. parapertussis* contain an additional locus, *wbm*, upstream of the *wlb* genes, implicated in the production of so called O-antigen or side chains. These O-side chains are linked to the core-trisaccharide region of band B (Burns *et al.* 2003), and have been implicated in protection against antimicrobial killing (Mattoo and Cherry 2005). Interestingly, the *wbm* locus has been shown to be necessary for efficient trachea and lung colonization by *B. parapertussis*, but not by *B. bronchiseptica* (Burns *et al.* 2003). LPS expressing these O-side chains has been referred to as "smooth" LPS, as apposed to *B. pertussis* "rough" LPS. It would appear that so called rough LPS is of significant advantage to the bacterium in colonisation of humans, since the *wbm* locus has been deleted from the *B. pertussis* genome, as evidenced by the presence of IS elements flanking the region (Gerlach *et al.* 2001). In addition, it has been reported that *B. bronchiseptica* strains often lose their expression of O-side chains during infection of human hosts (Le Blay *et al.* 1997) (Gueirard *et al.* 1998). The disadvantage to the bacterium of O-side chain expression in infection of humans is currently not clear. It is possible that, prior to the emergence of *B. pertussis*, its *B. bronchiseptica*-like O-side chain expressing ancestor may have been circulating in humans, and *B. pertussis* selected against expression of this recognisable antigen. In addition, O-side chains are large polysaccharides that cover the cell surface, and hence may shield other surface antigens and proteins from the respiratory epithelium, which may have a negative effect on host cell binding and subsequent colonization.

1.3.2.9 Type three secretion system

The recent sequencing of the genomes of *B. pertussis, B. bronchiseptica* and *B. parapertussis* has revealed the presence of a Type three secretion system (T3SS) locus in all three species. T3SS are specialised secretory apparatuses that allow Gram-negative bacterial pathogens, such as *Bordetella* spp., either adhering to the surface of a host cell or localised within phagosomes, to inject proteins, known as effectors, directly into the

eukaryotic cell, plasma membrane or extracellular milieu. These effectors often resemble eukaryotic signalling factors, enabling them to interfere with signal transduction pathways and thereby subvert the normal function/communication of the cell, favouring the survival of the invading bacteria. T3SS of animal pathogens, including the *Bordetella* T3SS, will be discussed in detail in the following section.

1.4 Type three secretion systems of animal pathogens

1.4.1 Overview and distribution of T3SS

T3SS are specialised secretory apparatuses that allow Gram-negative bacterial pathogens, such as *Bordetella* spp., either adhering to the surface of a host cell or localised within phagosomes, to inject proteins, known as effectors, directly into the eukaryotic cell, plasma membrane or extracellular milieu. First discovered in *Yersinsia* spp. in 1994, the T3SS has been shown to be immensely important for *Yersinia* pathogenesis (Cornelis 2002). Since then T3SS have been identified in a diverse range of gram-negative bacteria including *Aeromonas, Bordetella, Bradyrhizobium, Burkholderia, Chlamydia, Chromobacterium, Citrobacter, Edwardsiella, Erwinia, Escherichia, Pantoea, Photorhabdus, Pseudomonas, Ralstonia, Rhizobium, Salmonella, Shigella, Sodalis, Vibrio* and *Xanthomonas* spp. (Hueck 1998; He *et al.* 2004; Zheng *et al.* 2005).

The full secretion apparatus comprises ~25 proteins, the bulk of which combine to form a needle-like structure known as an injectisome (Fig. 1.1), assembled at multiple sites on the cell surface during virulent bacterial growth. The system also contains 2-3 translocators which form a pore in the eukaryotic cell membrane creating a continuous channel from bacterial to eukaryotic cytosol through which effectors pass. Though the basic structure of the needle complex is reasonably well conserved among all species possessing a T3SS, the effector proteins they secrete are not, and may reflect host specificity. In general these effectors bear more homology to eukaryotic signalling factors than they do to bacterial proteins, and their functions are diverse; apoptotic and necrotic cell death, re-organisation of actin cytoskeleton, prevention of phagocytosis, mediating uptake, intracellular survival, disruption of signalling cascades, intestinal epithelial cell attachment and release of nutrients from plant cells (Hueck 1998; Zheng *et al.* 2005). The T3SS is arguably the most complex prokaryote protein secretion system known, and is

unique among secretion systems in that its substrates have no classical signal sequences, no periplasmic intermediates and are exclusively virulence related.

1.4.2 From Virulence plasmids to Yops : Evolution of the injectisome concept

In most species, the identification of T3SS effector proteins preceded the identification of the T3SS complex itself. Detection of substrates of the T3SS, which ultimately led to the identification of the system itself, has been facilitated by the tendency of the genes encoding these systems to be clustered together, be it on large virulence plasmids, as is the case for *Yersinia* and *Shigella* spp., or on so called chromosomally encoded pathogenicity islands, as is the case for *Salmonella, E. coli* and *Pseudomonas* spp. (Hueck 1998). However, genes encoding T3SS substrates can also be located distantly from the secretion apparatus genes, as is the case for the *Bordetella* effector, BteA (Panina *et al.* 2005).

In 1980 Gemski and colleagues reported that a large (~ 70 kb) plasmid present in both *Yersinia enterocolitica* and *Yersinsia pseudotuberculosis* was necessary for these bacteria to confer their full display of virulence on their hosts (in this case Kerato-conjunctivitis in guinea-pigs, as a measure of invasiveness, and fatality in mice) (Gemski *et al.* 1980) (Gemski *et al.* 1980). A similar sized and highly homologous (55% at the nucleotide level) plasmid was later isolated from the human pathogen *Yersisnia pestis*, the causative agent of bubonic plague (Portnoy and Falkow 1981), and shown to be interchangeable for virulence in these species (Wolf-Watz *et al.* 1985). These authors also noted an apparent requirement for Ca^{2+} for growth at 37°C *in vitro*. Similar observations of plasmid encoded virulence occurred with pathogenic *Shigella* spp. These mammalian pathogens invade the epithelial cells of the colon, causing dysentery. It was shown by Sansonetti *et al* that spontaneous avirulent *Shigella* spp. that were unable to invade Hela epithelial cell monolayers had lost a large 140 megadalton plasmid, and that, upon receipt of the plasmid in trans, they regained this invasion ability (Sansonetti *et al.* 1982). With the observation that these plasmids were critical for these species in terms of establishing infection, the identification of the gene products encoded on these plasmids began in earnest.

Since the putative plasmid encoded virulence determinants seemed to mediate epithelial cell invasion, most of the early attempts focused on identifying proteins associated with the outer membrane of the bacteria- the interface between the bacterial cell and its host. The few proteins identified by SDS-PAGE in this manner from wild-type

bacteria were shown to be missing from the outer membrane fractions of their plasmid-cured avirulent counterparts (Bolin *et al.* 1982) (Portnoy *et al.* 1981.; Hale *et al.* 1983) Importantly, it was shown for the first time that the previously observed Ca^{2+} growth dependency of *Yersinia* spp. also applied to the expression of virulence plasmid encoded proteins (hereafter referred to as effectors) from these species., with the observation that increasing [Ca^{2+}] negatively impacted the expression of these newly identified outer membrane proteins, (Bolin *et al.* 1982) and in some cases completely prevented it (Portnoy *et al.* 1981). The connection between Ca^{2+} dependence and virulence was further affirmed by the construction of transposon insertion mutations into the virulence plasmid of *Y. pestis* which simultaneously abolished both Ca^{2+} dependence and virulence in mice (Portnoy *et al.* 1983). This relationship between effector release and [Ca^{2+}] facilitated the further identification of outer membrane proteins. Heesemann and colleagues used magnesium oxalate to precipitate and remove Ca^{2+} from their growth medium and detected greatly elevated levels of 5 outer membrane proteins (Heesemann *et al.* 1984). These proteins were named Yop 1-5 (***Yersinia*** **o**uter membrane **p**rotein) and their corresponding genes *yop*A-E (Bolin *et al.* 1985). A similar approach was used by Bolin and colleagues to show that in Ca^{2+} containing media the proteins were produced at 37°C but not detected in the outer membrane fraction. Further, the proteins were not expressed at 26°C (Bolin *et al.* 1982). This implicated [Ca^{2+}] and temperature as triggers for Yop release and presented a provocative correlation between the external environment and the 37°C maintained, low [Ca^{2+}] environment of the host. Indeed, sera from mice infected with *Y. pestis, Y. enterocolitica* or *Y. pseudotuberculosis* was shown to specifically recognize Yop2-5 by western blot, demonstrating the in vivo expression of these proteins for all three pathogenic *Yersinia* spp. (Bolin *et al.* 1985). Similarly, sera from convalescent humans and monkeys consistently recognized 4 proteins, shown to be surface associated, from several different virulence plasmid containing *Shigella* spp. (Oaks *et al.* 1986). The authors named these proteins Ipa A-D (for **I**nvasion **p**lasmid **a**ntigen), and noted, like the Yops of *Yersinsia*, their temperature dependent detection at 37°C only.

Systematic insertion mutagenesis into the *Yersinia* virulence plasmids led to the identification of genes showing transcriptional regulation by Ca^{2+} and encoded in nearby gene clusters, such as Yop F-J (Straley and Bowmer 1986). A common technique used was transposon mediated insertion of antibiotic resistant cassettes into virulence plasmid loci, with the subsequent isolation of mutants which exhibited both antibiotic resistance and the loss of an effector protein attribute, such as Ca^{2+} dependence at 37°C (Mulder *et al.* 1989), invasive virulence (Sasakawa *et al.* 1988; Hromockyj and Maurelli 1989), or the

absence of the newly discovered effector proteins themselves in the outer membrane fractions (Andrews *et al.* 1991). The isolation of mutants that were deficient for effector release but still capable of producing appreciable intracellular stores of these proteins, led to the identification of the *mxi/spa* loci (**M**embrane e**x**pression of **i**nvasion plasmid antigens/ **S**urface **p**resentation of **a**ntigen) in *Shigella* spp. (Andrews *et al.* 1991) (Venkatesan *et al.* 1992). Several genes in this group, located upstream of the Ipa effectors, were shown to be necessary for Ipa export and localised to the inner membrane, and therefore implicated in the export of the effectors themselves (Andrews *et al.* 1991). One of these proteins, MxiA, showed strong homology (68%) to LcrD (Andrews and Maurelli 1992), a regulator of the Ca^{2+} response in *Yersinia* which, corroborating the role of MxiA in Ipa effector export, was known to be necessary for Yop export (Plano *et al.* 1991)

The eventual isolation of effector proteins in the supernatant as well as the outer bacterial membrane, led to the conclusion that these proteins were most likely secreted proteins, that were co-purified with membrane fractions on account of their predominantly insoluble nature (Michiels *et al.* 1990). Other studies showed that the majority of Yops did not sediment upon high speed centrifugation and had no significant contamination with LPS, while western blot analysis showed the majority of these proteins were found in the supernatant under low [Ca^{2+}] growth conditions (Heesemann *et al.* 1986). More concrete evidence of secretion was shown by Galan and Curtis, who identified a series of T3SS proteins required for invasion of epithelial cell monolayers, the wall of the small intestine and the spleen by *Salmonella* spp. (Galan and Curtiss 1989). The genes encoding these proteins, termed *inv*A-D, were fused to the 5' end of the alkaline phosphatase gene, *pho*A, lacking its internal export signal. Therefore the observation of alkaline phosphatase activity in the supernatants of these mutants implicated the Inv proteins in directing the secretion of PhoA and consequently suggested that the Inv proteins were secreted by the bacteria themselves (Galan and Curtiss 1989). Interestingly, InvA displayed significant homology with MxiA of *Shigella* and LcrD of *Yersinsia* (Galan *et al.* 1992) and was shown to be necessary for *Salmonella* invasion and for generation of the characteristic membrane blebbing associated with *Salmonella* entry (Takeuchi 1967) (Forsberg *et al.* 1987).

Comparisons of gene sequences of cloned effector proteins with deduced N-terminal amino acid sequences of the corresponding proteins purified from supernatants revealed no cleavage of the N-terminus (Michiels *et al.* 1990). N-terminus cleavage is a feature of substrates of type 2 secretion systems which is responsible for targeting of proteins across the inner membrane (Van Gijsegem *et al.* 1993) (Salmond and Reeves 1993). However, the generation of hybrid proteins fused to alkaline phosphatase or β-

galactosidase deprived of their signal sequence, allowed Michiels and Cornelis to demonstrate that the N-terminal 48 aa of YopE, 98 aa of YopH and 76 aa of YopQ (3 *Yersinia* T3SS effectors) were necessary for these proteins to be secreted (Michiels and Cornelis 1991), though no real homology existed between these N-terminal sequences. Thus, the cloning and subsequent analysis of the identified *yop, inv* and *ipa* genes was revealing information about a novel, distinct and seemingly exclusively virulence-related bacterial protein export mechanism.

The continued analysis and random transposon mutagenesis of the virulence plasmid loci of *Yersinia* spp. resulted in several insertions into regions that did not encode Yop proteins but abolished their secretion (Michiels *et al.* 1991). One of these loci, *virC*, contained ~13 ORFs that seemed to be organised in a contiguous operon. These ORFs were named *ysc*A – M (**Y**op **s**ec**c**retion) (Michiels *et al.* 1991). Some of these *ysc* genes, which were transcriptionally regulated in the same manner as the *yop* genes with respect to [Ca^{2+}] and temperature, encoded putative membrane spanning proteins and one (YscC) had homology to a protein known to be involved in protein export, PulD of *Klebsiella pneumoniae* (d'Enfert *et al.* 1989). Furthermore, insertion mutations in three of these genes, *yscD*, *yscJ* and *yscL*, abolished Yop secretion (Michiels *et al.* 1991). The authors concluded that these *ysc* genes constituted a novel membrane spanning secretion machinery that, because none of the substrates contained classical N-terminal signal sequences, exported proteins directly across both membranes in a single step.

The term type three secretion system was first coined by Salmond and Reeves in 1993, and though initially only applied to the *Yersinia ysc* system (Salmond and Reeves 1993), it was noted that homologues of some of the *ysc* genes existed in a plant pathogen, *Pseudomonas solanocearum* (Van Gijsegem *et al.* 1993) (Salmond and Reeves 1993). Homologues of the *ysc* genes were subsequently detected in other pathogens. For example the *Shigella* genes *mxiJ* and *mxiH* were noted to be homologous to *yscJ* and *yscF* in *Y. enterocolitica* (Allaoui *et al.* 1992), and *mxiD* homologous to *yscC* (Allaoui *et al.* 1993). All three of these *mxi* genes, when mutated, abolished secretion of Ipa proteins in *Shigella* spp. In *Salmonella* spp. DNA fragments corresponding to the virulence region of the chromosome were successfully hybridized with virulence plasmid linked sequences of *Shigella* spp., demonstrating significant homology between the DNA sequences (Groisman and Ochman 1993). Analysis of this region, encoded downstream of the *inv* effector genes, revealed the presence of 9 genes homologous to the *spa* gene cluster in *Shigella* spp. The authors went so far as to demonstrate that virulence abolished by mutations in these genes (*spaP* in *Salmonella*) could be restored by provision of the corresponding homolog in trans

(*spa24* from *Shigella*) from the other species (Groisman and Ochman 1993). This apparent functional conservation of the secretion and translocation machinery was further demonstrated later by the elegant work of Rosqvist *et al.* The *Shigella* effector *ipaB* and its chaperone *ipgC* (Menard *et al.* 1994) were cloned into a plasmid under the control of an IPTG-inducible promoter and inserted into *Y. pseudotuberculosis* wild-type and Δ *ysc* bacteria in trans. They found the *Shigella* effector to be efficiently secreted by *Yersinia* after the addition of IPTG, in a low [Ca^{2+}] medium at 37°C, in a type three specific manner (Rosqvist *et al.* 1995). Similarly, they then showed that the *Yersinia* effector YopE could be efficiently secreted by the *Salmonella* T3SS. Thus, these homologous genes belonged to a complex export system that seemed to be interchangeable and functionally conserved between non-related bacterial species.

Transposon mutagenesis of discovered effector proteins consistently showed defects in virulence in live bacterial infection models. For example, YopE from *Yersinia* was shown to be necessary for virulence in mice, transcriptionally regulated by [Ca^{2+}] and temperature, and necessary for inhibition of phagocytosis and cytotoxicity to macrophage (Bolin and Wolf-Watz 1988; Forsberg and Wolf-Watz 1988; Rosqvist *et al.* 1988; Rosqvist *et al.* 1990). YopH was shown to be a powerful protein tyrosine phosphatase (PTPase) (Guan and Dixon 1990) involved in inhibition of complement or Fc-mediated phagocytosis (Cornelis *et al.* 1998). InvE of *Salmonella*, identified by virtue of its proximity to the *inv*A-D locus, was necessary for epithelial cell invasion (Ginocchio *et al.* 1992) and showed significant homology to LcrE/YopN of *Yersinia* (Forsberg *et al.* 1991). Despite the fact that these effectors were secreted and therefore presumed extracellularly located, it appeared that adherence of the pathogen at the cell surface was required for these cytotoxic and anti-phagocytic effects, as demonstrated for YopE from *Yersinsia* in 1990 (Rosqvist *et al.* 1990) and for general invasiveness of *Salmonella* spp. (Lee and Falkow 1990). It was also long known that both *de novo* protein synthesis and bacterial viability were required for these effects (Goguen *et al.* 1986; Finlay *et al.* 1989). Rosqvist *et al* also suggested that the adhesin Yop1A or YadA, a protein located in the *yop* gene cluster (Bolin *et al.* 1985), was involved in this adherence of *Yersinia* to host cells (Rosqvist *et al.* 1990). Thus it appeared effector release and host cell adherence were coupled for the virulence associated functions of this new *ysc* secretion machinery.

The authors followed up this observation by cloning *yopE* into a vector under the control of an IPTG-inducible promoter and purifying the protein from *E. coli.* (Rosqvist *et al.* 1991). When the protein was added to cells, no cytotoxicity was observed. However, when the protein was micro-injected into cells with glass beads, YopE-dependent

cytotoxicity was observed within 1 hour. This provided clear evidence that the target of YopE was intracellular, and indicated that the targets of other Yop proteins could also be located inside the host cell. When microinjected into epithelial cells, supernatent purified Yop proteins from a *yopD* mutant were able to cause cytotoxicity of the target cell in a YopE-dependent manner. However, a *yop*D mutant did not induce cytotoxicity *in vivo* or during *in vitro* infection of epithelial cells, suggesting a role for YopD in the delivery or translocation of YopE across the plasma membrane to the cytosol of the target cell (Rosqvist *et al.* 1991). This hypothesis was subsequently and elegantly proven by two independent methods. First, by fusing *yopE* to the gene encoding the calmodulin-activated adenylate cyclase domain of ACT from *B. pertussis* (Glaser *et al.* 1988), Sory and Cornelis showed infected epithelial cell cAMP accumulation in a *ysc*, *yopD* and *yad*A dependent manner (Sory and Cornelis 1994). Since bacteria do not produce calmodulin, cAMP accumulation demonstrated that the hybrid protein was translocated to the cytosol of the epithelial cell. Second, Rosqvist *et al* used confocal fluorescence microscopy to show that YopE appeared in the cytosol of wild type *Y. pseudotuberculosis* infected epithelial cells, but not in the cytosol of cells infected by a *yopD* mutant (Rosqvist *et al.* 1994), confirming the critical role of YopD in the translocation of YopE. It was also demonstrated that target cell contact could induce polarized transfer of YopE, even under growth conditions that normally repressed Yop secretion, such as Ca^{2+} containing medium, and that another Yop protein, YopN, was involved in the regulation of this mechanism (Rosqvist *et al.* 1994). This was the first observation of eukaryotic cell contact as a trigger for Yop translocation.

These pivotal experiments provided the first real demonstration of the complete T3SS concept, that the Ysc proteins represented a complex system used to inject bacterial effector proteins directly into the cytosol of the host cell. The T3SS was distinct from other bacterial secretions mechanisms in that the substrates lacked typical secretion signals, did not have a periplasmic intermediate and had specific chaperones. Moreover, the T3SS appeared to be exclusively virulence related. Thus, the T3SS injectisome was not merely another secretion system, but represented a complex and sophisticated weapon used by the pathogen to subvert target cell function in order to favour its own survival in the host.

1.4.3 The T3SS injectisome complex

The complex multiprotein T3SS apparatus or injectisome is assembled at multiple sites on the cell surface in the virulent phase of bacterial growth. The archetype of the T3SS injectisome is that of *Yersinia* spp., where the system was first discovered (Michiels *et al.* 1991). It consists of a basal body, which spans the peptidoglycan layer and the two bacterial membranes, topped with a needle like protrusion at the cell surface (Fig 1.1). The internal part of the injectisome, anchored in the bacterial inner membrane, has counterparts in the basal body of the flagellum, suggesting a common evolutionary origin (Aizawa 2001). The genes encoding the basal body proteins YscJLNQRSTUY are very well conserved among T3SS from other species such as *Shigella*, *Salmonella* and *Bordetella* spp. (Table 1) (Cornelis *et al.* 1998; Hueck 1998; Yuk *et al.* 1998; Yuk *et al.* 2000), and through their homology to the flagellar basal body, have a similar predicted function in anchoring the basal body in the bacterial inner membrane. The respective homologs of these proteins from the flagellar system are indicated in Figure 1.1.

At the base of the injectisome is a well conserved cytoplasmic ATPase protein, YscN, (discussed in chapter 4), that is presumed to provide the energy necessary to assemble the apparatus and export the effectors across the membranes and out of the cell (Fig 1.1) (Woestyn *et al.* 1994; Cornelis *et al.* 1998; Hueck 1998). The YscN protein is possibly the most well conserved of all the injectisome components (Table 1), with homologs identified in all T3SS discovered to date (Hueck 1998) (Gophna *et al.* 2003). This ATPase protein has been shown to be crucial for T3SS operation and pathogenesis in *Yersinia* spp. (Woestyn *et al.* 1994), *Salmonella* spp. (Eichelberg *et al.* 1994; McKinney *et al.* 2004), *Shigella* spp. (Tamano *et al.* 2000; Jouihri *et al.* 2003) and *B. bronchiseptica* (Yuk *et al.* 2000). Further, the ATPase activity of YscN has been demonstrated *in vitro* for YscN (Blaylock *et al.* 2006), and for InvC, the YscN homolog in *Salmonella* spp. (Table 1) (Eichelberg *et al.* 1994).

Spanning the bacterial outer membrane is an outer membrane protein, YscC, that bears significant homology to a family of proteins known as secretins (Koster *et al.* 1997) (Fig 1). Koster *et al* have demonstrated that this protein forms a ring-shaped structure with a central pore that is targeted to the bacterial outer membrane (Koster *et al.* 1997). In addition to *Yersinia* (Haddix and Straley 1992), mutation of YscC or its homologs (Table 1) has been shown to abolish T3SS function in *Shigella* (Allaoui *et al.* 1993) and *Salmonella* (Kaniga *et al.* 1994).

Filling the central pore created by the YscC secretin, is the needle component of the injectisome, which protrudes from the bacterial cell surface. The needle component is

formed by the polymerization of a single protein, YscF (Hoiczyk and Blobel 2001). Mutation of YscF in *Yersinia* (Allaoui *et al.* 1995), and its homologs MxiH in *Shigella* (Tamano *et al.* 2000) and PrgI in *Salmonella* (Kimbrough and Miller 2000) (Table 1) completely abolishes effector secretion *in vitro*, and a recent study has demonstrated the effective use of YscF as a protective, immunogenic antigen in mice (Matson *et al.* 2005).

Thus, secretion of effector proteins by the T3SS of animal pathogens requires a complex multi-protein secretion machinery, consisting of a broadly conserved base anchored in both bacterial membranes and an externally protruding needle component. Collectively these components form the T3SS injectisome, a device to deliver bacterial proteins directly from the bacterial cytosol to the external milieu.

1.4.4 The T3SS process and effectors

Once assembled at the cell surface, the T3SS injectisome is able to export effectors into the extracellular milieu, but not the cytosol of a target cell. The delivery of T3SS effectors into host cells, termed translocation, requires another group of proteins known as translocators, called YopB and YopD in *Yersinia* (Table 1) (Fig 1.1). Both YopB (Hakansson *et al.* 1996) (Boland *et al.* 1996) and YopD (Rosqvist *et al.* 1994) (Sory *et al.* 1995) have been shown to be necessary for the translocation of *Yersinia* effector proteins to host cells, but not for their secretion to the external milieu. In addition, homologues of YopB, such as SipB from *Salmonella* (Wood *et al.* 1996) (Collazo and Galan 1997), and IpaB from *Shigella* (High *et al.* 1992) have been shown to be necessary for translocation and T3SS-mediated invasiveness.

YopB and YopD possess coiled coil domains (Cornelis et al. 1998), which are indicative of protein-protein interactions, and it has been demonstrated that YopD has the ability to bind directly to YopB, forming a complex in the bacterial cytoplasm (Neyt and Cornelis 1999). They also possess hydrophobic domains, suggesting the ability to interact with membranes (Hakansson *et al.* 1993). The hydrophobic region of YopB resembles that of the RTX toxins, a family of toxins involved in membrane disruption (Welch 1991) (Hakansson et al. 1993), and YopB and YopD have been shown to induce pore formation in the membranes of infected macrophage (Neyt and Cornelis 1999). Although translocator proteins from different species may have low similarities at the amino acid level (Table 1), they are similar in containing multiple transmembrane domains. Recently, the *Bordetella* YopB and YopD homologues, BopB and BopD (Table 1), were shown to be complexed together in the *Bordetella bronchiseptica* cytoplasm prior to secretion, and to be necessary

for T3SS-mediated cytotoxicity and pore formation in target cells (Kuwae *et al.* 2003) (Nogawa *et al.* 2004). Consequently, it is generally assumed that these translocators are exported to the bacterial surface by the secretion apparatus upon activation following target cell adherence, where they form a pore in the eukaryotic cell membrane, into which the injectisome inserts. Thus, pore formation by the translocators at the tip of the T3SS injectisome facilitates the formation of a continuous channel from bacterial to eukaryotic cytosol, through which effectors pass.

While the T3SS machinery is well conserved across multiple species (Table 1), the effector proteins that these systems secrete are not, and may reflect host specificity. In general, these effectors bear more homology to eukaryotic signalling factors than they do to bacterial proteins. Despite their lack of conservation, some common targets of T3SS effectors have emerged. T3SS effectors target cell signalling proteins in the eukaryotic cell, leading to disruption of cell signalling pathways, and consequently, the effects that they regulate. The model system is that of *Yersinia* spp., where several effectors have been identified with the common effect of host phagocyte paralysis. YopE is a GTPase activating protein which causes accelerated GTP hydrolysis and eventual paralysis of the actin cytoskeleton and target cell cytotoxicity (Black and Bliska 2000). YopT exerts a similar effect on host cell cytoskeletal operation. It is a cysteine protease, which cleaves Rho GTPases close to their C-terminus, resulting in their inactivation by removal from the cell plasma membrane, and subsequent actin depolymerisation (Sorg *et al.* 2001). YopO is a serine/threonine kinase that exerts a negative effect on cytoskeleton dynamics, though its exact mode of action is unclear (Juris *et al.* 2000). YopH is a powerful phosphotyrosine phosphatase (PTPase) that dephosphorylates several eukaryotic proteins including the focal adhesion protein $p130_{cas}$ and disrupts focal adhesions in macrophage, resulting in cytoskeletal paralysis (Hamid *et al.* 1999). YopH has been shown to suppress oxidative burst in macrophages (Bliska and Black 1995), and reduce calcium signalling (responsible for phagocytosis) in PMN's (Andersson *et al.* 1999). The actions of these effectors help invading *Yersinsia* spp. to evade phagocytosis.

Other *Yersinia* effectors function to block the pro-inflammatory response of infected cells. For example, YopP/J disrupts the NF-κB signalling pathway, preventing its translocation to the nucleus, in addition to disrupting the MAPK pathways, by binding to the upstream kinases MAPKKs and IKKβ, and acetylating critical residues involved in the activation of MAPKKs and IKKß, thereby preventing these residues from being phosphorylated (Mukherjee *et al.* 2006). This results in reduced release of TNF-α from macrophages (Boland and Cornelis 1998) and IL-8 from epithelial cells (Schesser *et al.*

1998), and eventual apoptosis of the target cell. It also reduces the presentation of adhesion molecules, such as ICAM1 and E-selectin on the epithelial cell surface (Denecker *et al.* 2002), which leads to reduced phagocyte recruitment at the site of infection. YopH also plays a role in disrupting immune cell signalling. It inhibits the synthesis of MCP1 in infected macrophage (Sauvonnet *et al.* 2002), and disrupts the adaptive immune response by impairing T and B cell activation through their antigen receptors (Yao *et al.* 1999).

In contrast, the primarily intracellular pathogens *Salmonella* and *Shigella* utilise a T3SS to induce uptake into epithelial cells, similarly through the disruption of host cytoskeleton dynamics (Galan and Curtiss 1989) (Sansonetti *et al.* 1982; Menard *et al.* 1993; Hueck 1998). *Shigella* spp. are capable of invading a variety of cells during intestinal infection, including epithelial cells, macrophage, nuetrophils and DC. Several different effectors have been implicated in stimulating the re-organization of the actin cytoskeleton, involved in triggering bacterial uptake to host cells, such as IpaA (Bourdet-Sicard *et al.* 1999), IpaB (Skoudy *et al.* 2000), IpaC (Tran Van Nhieu *et al.* 1999), IpgD (Niebuhr *et al.* 2002), VirA (Yoshida *et al.* 2002) and IpgB1 (Ohya *et al.* 2005). Recently, another effector, OspE2, has been shown to be expressed by intracellular *Shigella*, and functions in the maintenance of cellular morphology necessary for bacterial cell to cell spreading (Miura *et al.* 2006).

In *Salmonella* spp., two completely separate T3SS are expressed at different times and are required for different aspects of bacterial colonization and persistence in the host organism (Hensel *et al.* 1997) (Hueck 1998) (Ochman *et al.* 1996). One T3SS, which exhibits contact-dependent secretion, is encoded in *Salmonella* pathogenicity island 1 (SPI-1) and is involved in bacterial interactions with epithelial cells (Francis *et al.* 1993), while the second T3SS is encoded in SPI-2 and is expressed in intracellular bacteria and translocates proteins across the phagosomal membrane (Hensel *et al.* 1997) (Ochman *et al.* 1996). SPI-1 effectors involved in actin cytoskeleton disruption include SopE (Hardt *et al.* 1998), SopE2 (Bakshi *et al.* 2000) (Stender *et al.* 2000) and SopB (Zhou *et al.* 2001) as well as SptP (Fu and Galan 1999). In excess of 20 *Salmonella* SPI-2 effectors have been identified to date (Waterman and Holden 2003), and the majority function in the protection of the *Salmonella* containing vacuole (SCV) that is established following internalisation of *Salmonella* by phagocytes, which in turn permits intracellular replication and the establishment of an intracellular niche for the bacterium.

The disruption of MAPK signalling is also a target of the *Shigella* and *Salmonella* T3SS. Two *Shigella* effectors, OspF and OsC1, are activators of the ERK1/2 signalling pathways, and have a role in increasing neutrophil transepithelial migration, which

ultimately facilitates bacterial access to the intestinal submucosa and disease progression (Zurawski *et al.* 2006). Both the *Shigella* IpaH9.8 effector, or its homologue, SspH1, in *Salmonella*, have like Yop P/J of *Yersinia* spp, been shown to be inhibitiors of NF-kB activation and IL-8 release from epithelial cells (Haraga and Miller 2003). Furthermore, IpaH9.8 has been shown to significantly reduce MIP2, IL-1β, GM-CSF and RANTES production in the lungs of mice following intra-nasal challenge with *Shigella* spp. (Okuda *et al.* 2005).

Thus the process of T3SS effector protein translocation as outlined above is an essential part of the infectious cycle of T3SS-bearing pathogens, and through the killing of target cells, prevention of phagocytosis or bactericidal mechanisms and disruption of the host immune response by interfering with eukaryotic signalling cascades, facilitates pathogen infection and survival.

1.4.5 Regulation of effector release

As discussed earlier (section 1.4.2), T3SS effectors lack a specific secretion signal, though an N-terminal amphipathic helix has been implicated in directing secretion by Lloyd *et al*, who replaced the first 15 residues of YopE with hydrophobic, acidic or amphipathic residues and found that only recombinant proteins with amphipathic residues were secreted effectively (Lloyd *et al.* 2001). It has also been suggested that a mRNA-encoded secretion signal is recognised as a direction for secretion of the effector protein (Sorg *et al.* 2005). T3SS chaperones have also been implicated in the recognition of effectors by the injectisome. Chaperones are characterized as small, acidic proteins that lack ATPase activity and associate with one or several T3SS secretion substrates within bacterial cells prior to secretion (Page and Parsot 2002). A number of possible functions for T3SS chaperones have been proposed such as stabilization of their effector substrates, the prevention of premature interactions between effectors in the bacterial cytosol and the maintenance of the effectors in an unfolded secretion-competent state prior to secretion (Page and Parsot 2002). It also has been suggested that chaperones may function in guiding effector-chaperone complexes to the injectisome, through interaction with the ATPase located at the base of the secretion apparatus (Fig 1.1) (Gauthier and Finlay 2003). In addition, since some effectors possess chaperones and some do not, it has been proposed that chaperones could constitute an additional secretion signal, and therefore confer a

hierarchy of secretion on T3SS substrates (Boyd *et al.* 2000; Birtalan *et al.* 2002; Wulff-Strobel *et al.* 2002).

Effector secretion is a tightly regulated process and the premature release of effector proteins is prevented in the absence of eukaryotic cell contact. In the most well studied cases, the secretion of T3SS effector proteins has been shown to depend upon the adhesion of the invading bacteria to host cells. In the case of *Yersinia* spp. it has been shown that effector secretion (Rosqvist *et al.* 1994) (Pettersson *et al.* 1996) and indeed transcription (Pettersson *et al.* 1996) is dependent on adherence of the bacterium to a eukaryotic cell. For example, mutation of either the YadA or Inv adhesins in *Yersinia* spp. prevents effector delivery (Cornelis *et al.* 1998), and secretion of the Ipa effector proteins by *S. flexneri* has also been shown to require cell contact (Watarai *et al.* 1995). Thus, cell contact, presumably mediated either by bacterial adhesins or phagocytic receptors, is the trigger for effector release *in vivo*.

In some cases, signals mimicking or replacing this cell contact have been shown to induce *in vitro* secretion of effectors in the absence of host cells. Again the best studied case is that of *Yersinia* spp., where a drop in Ca^{2+} levels has been shown to induce mass secretion of Yop effectors *in vitro* (Michiels *et al.* 1990). In the case of *Salmonella*, a change in pH from acidic to alkaline conditions is sufficient to activate SPI-1 secretion (Daefler 1999), while a switch from high Mg^{2+} concentration to low Mg^{2+} concentration mediates the switch from SPI-1 to SPI-2 gene expression (Francis *et al.* 2002). *In vitro* secretion of T3SS effectors by *Shigella* can be induced by the addition of serum or congo red dye (Bahrani *et al.* 1997).

Prior to adherence of the bacterium to a target cell, a secretion plug is thought to function in keeping the needle tightly shut. A well conserved T3SS protein implicated in this function is a protein known as YopN in *Yersinia* spp. (Forsberg *et al.* 1991). Homologs of YopN exist in other T3SS (Table 1) and though its exact mode of action is unknown, *yopN* mutants transcribe and secrete large amounts of Yop effectors *in vitro*, regardless of eukaryotic cell contact or [Ca2+] (Forsberg *et al.* 1991) (Boland *et al.* 1996). Thus, this protein appears to be a surface-located sensor protein which controls T3SS effector release by facilitating a localised opening of the T3SS injectisome only points of cell contact, or in response to specific chemical signals.

Thus, the release of effector proteins and the activation of T3SS both *in vitro* and *in vivo* is a tightly, and possibly temporally, regulated process, that functions to deliver a multitude of different effectors with individual functions. These effectors then

function in concert to bring about subversion of the host cell to favour bacterial survival and prolong infection.

1.4.6 The *Bordetella* T3SS.

The T3SS locus, present in *B. bronchiseptica, B. pertussis* and *B. parapertussis*, contains approximately 29 genes (www.sanger.ac.uk/projects/b_pertussis/bronchiseptica). The bulk of these genes encode putative proteins that constitute the needle complex, and in accordance with accepted nomenclature, have been designated *bsc* genes. This is partly due to the fact that each of them bears some sequence homology to the corresponding *ysc* genes of *Yersinia* spp. (Table 1). The specific functions of some of these proteins have either been elucidated or can be inferred from sequence homology with known T3SS proteins from other bacteria. For example, the homolog of BscC in *Yersinia*, YscC, has been shown to polymerise to form a ring shaped structure at the distal part of the basal body (Koster *et al.* 1997), with a central pore through which the needle protrudes (Fig 1). A homolog of BscC in *Pseudomonas syringae,* HrcC, was shown by Deng and Huang (1999) to be located in the outer membrane (Deng and Huang 1999). Similarly, YscF, has been shown to polymerise to form the protruding needle (Hoiczyk and Blobel 2001) and *yscF* mutants are unable to secrete Yops (Allaoui *et al.* 1995). *Bordetellae* BscJ shows homology to HrcJ from *Pseudomonas syringae*, a protein shown to span the periplasmic space, connecting the secretion channel to the inner and outer bacterial membranes (Deng and Huang 1999). Other highly conserved features associated with T3SS such as the YscN cytoplasmic ATPase, the YopN sensor/plug protein and the translocators YopB and YopD, are all present and intact in *Bordetella* spp. We have constructed a table of TTSS genes from species from *Bordetella, Yersinia, Pseudomonas, Salmonella* and *Shigella* (Table 1). Thus, due to the relative homology of *bsc* system to that of the *ysc* system and others it is likely *Bordetella* spp. form a similar type of injectisome.

The first evidence of a T3SS in *Bordetella* spp. was reported by Yuk *et al* (Yuk *et al.* 1998), who used arbitrary-primed PCR to detect the presence of genes expressed only during the bvg^+ phase of growth in *B. bronchiseptica* RB50. One of these genes, *bscN*, bears strong homology to the gene encoding the *Yersinia* spp. T3SS ATPase, YscN. The detection of *bscN* in *B. bronchiseptica* RB50 facilitated the detection of homologues of the *yscI, J, K, L* and *O* genes in addition to *bscN* in this study, and therefore strongly suggested the presence of a *ysc*-type T3SS injectisome in *B. bronchiseptica.* These gene loci, *bscI, J, K, L, N* and *O*, were also detected in the genomes of 4 strains of *B. pertussis* (Tohama I,

ATCC 9797 (18323 [NCTC 10739], GMT1 and 17471) and 2 strains of *B. parapertussis* (H1 – ovine and A168- human). However expression of the *bsc* loci was restricted to *B. pertussis* strain ATCC 9797 (18323 [NCTC 10739], hereafter referred to as *B. pertussis* 18323) and *B. parapertussis* H1, suggesting that the expression of the T3SS was selected against in human pathogens (Yuk *et al.* 1998). Interestingly, the first detection of the entire T3SS operon was not in *B. bronchiseptica* RB50 but in *B. pertussis* Tohama I by Kerr and colleagues (Kerr *et al.* 1999). In this study, a 36 kb region of the *B. pertussis* Tohama I chromosome was found to encode 20 ORFs, homologous to the *ysc* encoded T3SS of *Yersinia* spp., which were subsequently named *bscC, U, T, S, R, Q, O, N, L, K, J, I, bcrH2, bopB, bopD, bcrH1, lcrE* (later named *bopN*), *lcrD, bscD and F* (Kerr *et al.* 1999).

In addition to studies on the expression of *bscN* by *Bordetella* spp., Yuk *et al* also demonstrated that BscN was necessary to induce a cytotoxic effect in cultured epithelial cell monolayers, and moreover, that wild-type *B. bronchiseptica* RB50 supernatant alone could not induce this effect (Yuk *et al.* 1998). The *bscN* gene was also shown to be required for the secretion of 5 proteins that were specifically recognised by serum taken from a *B. bronchiseptica*-infected rabbit, suggesting these proteins were secreted during infection *in vivo* (Yuk *et al.* 1998). Three of these proteins were later identified as the *Bordetella* T3SS substrates, Bsp22, BopN and BopD (Yuk *et al.* 2000). Deletion mutants of Bsp22 and BscN in *B. bronchiseptica* RB50 showed defective long-term colonization of the tracheas and significantly higher antibody titres 35 days post infection in mice (Yuk *et al.* 2000). The authors also reported that *bscN* mutant *B. bronchiseptica* bacteria were, in contrast to the wild-type bacteria, unable to induce the aggregation of NF-κB in the cytoplasm of epithelial cells. This cytoplasmic aggregation of NF-κB prevents its nuclear translocation and subsequent gene activation in epithelial cells, and is a hallmark of T3SS activity that has also been observed in *Yersinia* (Orth *et al.* 1999), *Salmonella* (Haraga and Miller 2003) and *Shigella* spp. (Okuda *et al.* 2005). Lastly, using SCID-beige mice, which are deficient in NK, B and T cells, this group reported that in the absence of an adaptive immune response the T3SS of *B. bronchiseptica* confers no advantage on the bacterium, as *bscN* mutant bacteria killed these mice similarly to the wild-type bacteria (Yuk *et al.* 2000). Consistent with reports detailing the lack of *bsc* loci expression by *B. pertussis* Tohama I, Fauconnier *et al* reported no differences between wild-type and BcrD mutant *B. pertussis* Tohama I over a range of phenotypes tested (Fauconnier *et al.* 2001). These included *in vitro* protein secretion, *in vitro* epithelial cell invasion, *in vitro* macrophage cytotoxicity and mouse lung colonization (Fauconnier *et al.* 2001).

The effects of mutating these critical T3SS proteins on bacterial pathogenesis have demonstrated the importance of the T3SS for the pathogenesis of *B. bronchiseptica* RB50, and suggest that the T3SS may be non functional in *B. pertussis* Tohama I. More recent studies have provided further results in agreement with these reports. For example, mutants of the translocator proteins BopB (Kuwae *et al.* 2003) and BopD (Nogawa *et al.* 2004) have been shown to be unable to induce the morphological changes in epithelial cells that are associated with wild type *B. bronchiseptica* infection, namely cytoplasm shrinkage, cell rounding and detachment from the substrata. Neither BopB nor BopD mutants were able to induce cell death in macrophages, nor were they able to induce the T3SS-dependent hemolysis of red blood cells seen with the wild type bacteria (Kuwae *et al.* 2003; Nogawa *et al.* 2004).

A role for the T3SS of *B. bronchiseptica* in the modulation of adaptive immune responses through subverting the APC function of cells of the innate immune system has recently emerged. Skinner *et al* (2004) reported that *B. bronchiseptica* infection of DC enhances cell surface expression of MHC class-II, CD80 and CD86, through the activation of MAPK signalling in a T3SS-dependent manner. This, in combination with the inhibition of CD40 and IL-12, mediated by ACT, results in DC with a so called semi-mature or tolerogenic phenotype (Skinner *et al.* 2004). These DC were subsequently shown to direct the generation of IL-10 secreting T-cells early in *B. bronchiseptica* infection, and to inhibit the production of IFNγ, thereby inducing an immunosuppressive state and promoting bacterial persistence (Skinner *et al.* 2005) (Pilione and Harvill 2006). In addition, Reissinger *et al* have demonstrated that the *B. bronchiseptica* T3SS can downregulate ERK and p38 MAPK signalling in macrophage, resulting in attenuated production of IL-6 and IL-10 (Reissinger *et al.* 2005).

The *Bordetella* effector proteins mediating this range of effects on host cells have so far remained unidentified. Recently, a single *Bordetella* T3SS effector was identified independently in *B. bronchiseptica* by two separate groups and named BteA (Panina *et al.* 2005) or BopC (Kuwae *et al.* 2006). This effector has been shown by both groups to be the cause of the T3SS dependent cytotoxicity by *B. bronchiseptica in vitro*.

Although the T3SS of *B. bronchiseptica* has been shown to mediate multiple effects on host cells, these effects have not been demonstrated for the T3SS of *B. parapertussis* and *B. pertussis* spp. The T3SS of all 3 species are 98% identical at the nucleotide level, which implies a similar role in infection. However, there are still some doubts as to whether these human restricted pathogens express T3SS and there are contradictory reports in the literature. As discussed above, Yuk *et al* (1998) reported no

transcription of essential T3SS components *bscN, bscJ, bscK and bscL* by *B. pertussis* Tohama I *in vitro* (Yuk *et al.* 1998). Fauconnier et al (2001) noted that *bscN* was not expressed by *B. pertussis* Tohama I *in vitro* (Fauconnier *et al.* 2001), in contrast to the significant expression observed by Yuk *et al* (1998) for the same gene in *B. bronchiseptica*, using a similar experimental approach. More recently however, a transcriptional regulator, the ECF sigma factor, *btrS,* located on the T3SS locus, has been shown to be required for transcription of the *bsc* locus in *Bordetella* spp. (Mattoo *et al.* 2004). The authors reported transcription of the *bsc* locus in a *btrS*-dependent manner for *B. bronchiseptica* RB50, *B. pertussis* Tohama I and the *B. parapertussis* FR107 and H1 strains. However, genome sequence analysis conducted by Preston *et al* (2004) has revealed that *btrS* is a non-functional pseudogene in the *B. parapertussis* isolate chosen for sequencing, *B. parapertussis* 12822, suggesting a transcriptional block, though it is intact in *B. pertussis* Tohama I (Preston *et al.* 2004). More recently, Hot *et al* (2003) showed by microarray analysis that *bscJ* was indeed transcriptionally upregulated by *B. pertussis* Tohama I *in vitro* (Hot *et al.* 2003).

The emerging picture suggests that *B. pertussis* and *B. parapertussis* do transcribe the T3SS locus *in vitro* and that differences in expression may be related to strain origin and experimental conditions. However, to date there are no reports of T3SS effector secretion *in vitro* by these two species. Mattoo *et al* (2004) reported a distinct lack of the T3SS effectors Bsp22 and BopD in culture supernatants of *B. pertussis* Tohama I and the human restricted *B. parapertussis* 12822 (Mattoo *et al.* 2004). This was in notable contrast to positive detection of these T3SS effectors in supernatant samples from the ovine *B. parapertussis* FR107 and H1 strains. These data suggest the possibility that while transcription of the *bsc* locus occurs in these species, the signals necessary to activate assembly of the needle complex and subsequent secretion of effector proteins are absent *in vitro*. Further study of the T3SS of both *B. pertussis* and *B. parapertussis* is necessary to determine the use and importance of T3SS by these species.

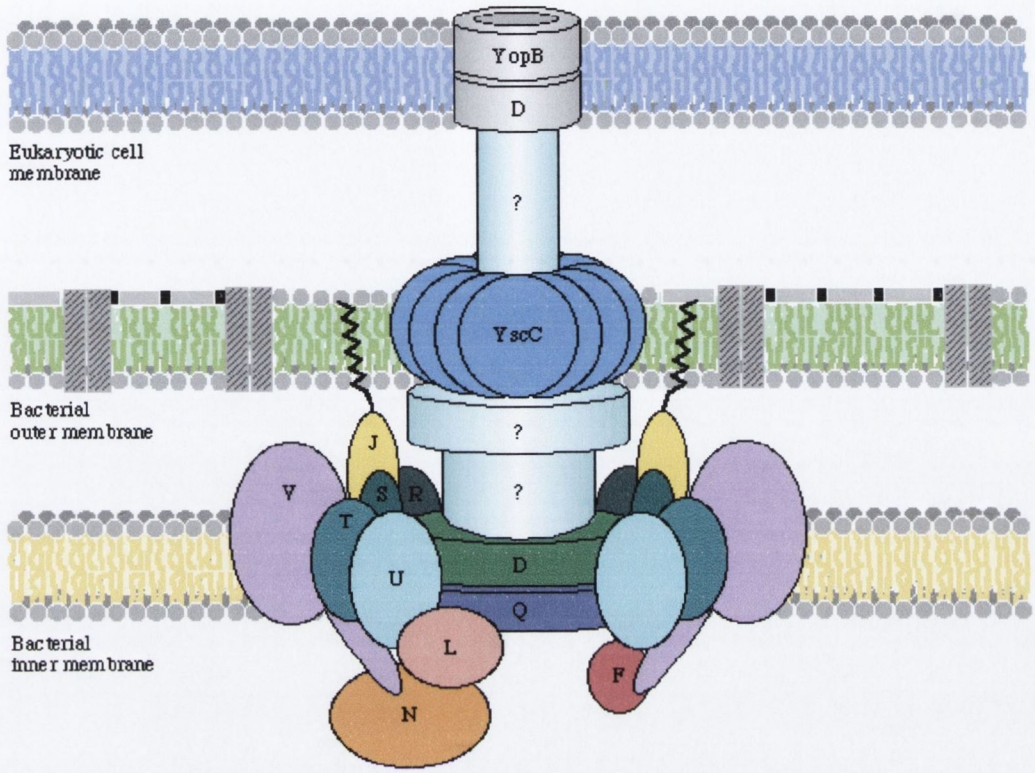
1.5 Aims of this study

The aims of this study were to investigate the activity of the T3SS of *B. pertussis* during *in vitro* culture, to identify novel T3SS effectors in *Bordetella*, and to examine the importance of the *Bordetella* T3SS during *in vitro* infection of innate immune cells, and in *vivo* following aerosol infection of mice.

The specific aims were to:

- Optimise the *in vitro* secretion of effectors by *Bordetella* spp. using anti-sera raised against Bsp22, a T3SS effector shown to be secreted by *B. bronchiseptica.*
- Compare the expression and *in vitro* activation of the T3SS by lab-adapted *B. pertussis* strains versus relatively low-passage or recent clinical isolates of *B. pertussis.*
- Mutate one of the proteins essential for the operation of the T3SS, the putative cytoplasmic ATPase, BscN, in order to generate T3SS-deficient bacteria.
- Compare secretion profiles of mutant *Bordetella* with those of wild type bacteria to identify T3SS-dependent effectors.
- Examine bacterial uptake and survival of, and the induction of T3SS-dependant cytotoxicity by, wild type and Δ*bscN* bacteria following *in vitro* culture with innate immune cells, such as macrophages, DC and epithelial cells.
- Examine production of key inflammatory mediators such as IL-10, IL-12p70, IL-12p40, IL-23, TNFα and IL-1β by DC following *in vitro* culture with wild-type and Δ*bscN* bacteria.
- Examine bacterial counts in the lung, the local innate immune response in the lung (IL-10, IL-12p70, IL-12p40, IL-23, TNFα, IL-1β, IL-6, MIP1 and MIP2), serum antibody responses (total IgG, IgG1 and IgG2a) and antigen specific adaptive immune responses (IL-10, IFNγ and IL-17) following aerosol challenge of BALB/c

mice with wild-type and Δ*bscN* bacteria, in order to determine the importance of the T3SS of *Bordetella* spp. during *in vivo* infection.



Yersinia TTSS	YscC	YscD	YscF	YscJ	YscL	YscN	YscQ	YscR	YscS	YscT	YscU	YscV
Bordetella TTSS	BscC	BscD	BscE	BscJ	BscL	BscN	BscQ	BscR	BscS	BscT	BscU	BscV
Flagellar System				FliF	FliH	FliI	FliY	FliP	FliQ	FliR	FlhB	FlhA

FIGURE 1.2 The T3SS injectisome. A schematic representation of the T3SS injectisome of *Yersinia* spp. showing the localization of the relevant Ysc proteins. The full complement of the Ysc and needle is given below, and homologous proteins from *Bordetella* spp. and a similar system, the bacterial flagellum, are shown where relevant. Figure adapted from http://www.genome.jp/dbget-bin/www_bget?path:bbr03070.

Bordetella		*Yersinia*		*Shigella*	*Salmonella*		*Pseudomonas*	
pertussis	*bronchiseptica*	*enterocolitica*	*pseudotuberculoisis*	*flexneri*	**SPI-1**	**SPI-2**	*syringae*	*aeruginosa*
BscU-349	*BscU-349*	*YscU-354*		*SpaS/40-342*	*SpaS-356*	*SsaU-352*	*HrcU-359*	
BscT-266	*BscT-266*	*YscT-261*	*YscT-261*	*SpaR/29-256*	*SpaR-263*	*SsaT-259*	*HrcT-264*	
BscS-88	*BscS-91*	*YscS-88*	*YscS-99*	*SpaQ/9-86*	*SpaQ-86*	*SsaS-88*	*HrcS-88*	
BscR-223	*BscR-223*	*YscR-217*	*YscR-217*	*SpaP/24-216*	*SpaP-224*	*SsaR-215*	*HrcR-208*	
BscQ-359	*BscQ-359*	*YscQ-307*	*YscQ-307*	*SpaO/33-293*	*SpaO-303*	*SsaQ-216*	*HrcQA-238 HrcQB-133*	
BscP-182	*BscP-182*							
BscO-169	*BscO-169*	*YscO-154*	*YscO-154*	*SpaM/13-112*	*InvI/SpaM-147*	*SsaO-125*	*HrpO-148*	*PscO-141*
BscN-444	*BscN-444*	*YscN-439*	*YscN-439*	*SpaL/47-430*	*InvC-432*	*SsaN-433*	*HrcN-449*	*PscN-440*
BscL-212	*BscL-187*	*YscL-223*	*YscL-221*			*SsaK-314*	*HrpE-193*	*PscL-231*
BscK-220	*BscK-220*	*YscK-209*	*YscK-209*				*HrpD-133*	*PscK-206*
BscJ-274	*BscJ-274*	*YscJ-244*	*YscJ-236*	*MxiJ-242*	*PrgK-252*	*SsaJ-249*	*HrcJ-268*	*PscJ-248*
BscI-135	*BscI-142*	*YscI-115*	*YscI-115*	*MxiI-97*	*PrgJ-101*		*HrpB-124*	*PscI-113*
BscD-426	*BscD-426*	*YscD-418*	*YscD-419*			*SpiB-323*	*HrpQ-330*	*PscD-432*
BscE-99	*BscE-99*							
BscF-88	*BscF-88*	*YscF-87*		*MxiH-83*	*PrgI-80*			*PscF-85*
BscW-132	*BscW-132*							
BscC-600	*BscC-600*	*YscC-607*		*MxiD-566*	*InvG-562*	*SpiA-497*	*HrcC-748*	*PscC-600*
BopB-402	*BopB-400*	*YopB-401*	*YopB-401*					
BopD-313	*BopD-313*							
BopN-365	*BopN-365*	*YopN-293*	*YopN-293*	*Orf15-355*	*InvE-372*		*HrpJ-368*	*PopN-288*
BcrD-699	*BcrD-699*	*LcrD-704*	*LcrD-704*	*MxiA-686*	*InvA-685*	*SsaV-681*	*HrcV-695*	*PcrD-706*
BcrH1-161	*BcrH1-161*	*SycD-168*	*LcrH-168*	*IpgC-155*		*SscA-157*		
BcrH2-153	*BcrH2-153*							
Bcr4-173	*Bcr4-173*							
Bsp22-205	*Bsp22-205*							
Bp2255-91	*BB1618-91*							
Bp2258-125	*BB1615-125*							
Bp2259-136	*BB1614-136*							
Bp2260-122	*BB1613-122*							

TABLE 1. T3SS gene table showing the full T3SS locus of *B. bronchiseptica* RB50 and *B. pertussis* Tohama I. Homologs from other species are included where relevant. Homologies are based on BLAST analysis of amino acid sequences. The numbers after each gene indicate the number of amino acids in their corresponding proteins.

Chapter 2

Materials and Methods

2.1 Bacterial strains

All bacterial strains used in this study are listed in Table 2.1

2.2 Media, buffers and reagents

2.2.1 Ammonium chloride lysis solution

0.87 g Ammonium chloride (NH_4Cl).

Dissolved in 100 ml ddH_2O, pH adjusted to 7.2, and filter sterilized before use

2.2.2 Antibiotics

All antibiotics used in this study were purchased from Sigma (UK). The relevant antibiotics used, and their working concentrations, are listed in Table 2.2.

2.2.3 Bordet Gengou agar base

250 ml ddH_2O

2.5 ml Glycerol

7.5 g Bordet Gengou agar (BD Difco, USA).

The medium was autoclaved at 121°C for 20 mins, allowed to cool and supplemented with 1 ml of cephalexin (10 mg/ml) and 50 ml of pre-warmed sterile defibrinated horse blood.

2.2.4 1% Casein

6 g NaCl

10 g Casamino acids

Dissolved in 1 L of ddH_2O, pH adjusted to 7.2 and autoclaved at 115°C for 20 minutes

2.2.5 Cell culture medium

Roswell park memorial institute (RPMI)-1640 medium and Dulbecco's modified Eagle's medium (DMEM) were supplemented with 8% heat inactivated (56°C for 30 mins) foetal calf serum (FCS), 100 mM L-Glutamine (Gibco), 100 μg/ml streptomycin (Gibco). Complete RPMI was used to culture all cells isolated from murine spleens as well as bone marrow derived DC, and complete DMEM was used to culture the murine J774 macrophage and human HEK 293T epithelial cell lines.

2.2.6 Electrophoresis running buffer

3 g	Tris base
14.4 g	Glycine
1 g	SDS
1L	dH_2O

2.2.7 Electrophoresis sample buffer (3X)

3 ml	Glycerol
1.5 ml	Beta-mercaptoethanol
3 ml	10% SDS
3.8 ml	0.5M Tris (pH 6.0)
10 mg	Bromophenol blue

2.2.8 Electrophoresis transfer buffer

2.73 g	Tris base
12.96g	Glycine
780 ml	dH_20
120 ml	Methanol

2.2.9 Luria Bertani broth (LB)

10 g Tryptone

5 g Yeast extract

10 g NaCl

Dissolved in 1 L of ddH_2O, pH adjusted to 7.2 and autoclaved.

2.2.10 NaCl- LB broth

10 g Tryptone

5 g Yeast extract

Dissolved in 1 L of ddH_2O, pH adjusted to 7.2 and autoclaved.

2.2.11 LB agar base

LB broth was solidified by the addition of agar (BD Difco, USA; 15 g/L) prior to autoclaving.

2.2.12 MacConkey agar base

40 g MacConkey agar (BD Difco, USA)

10 ml Glycerol

Dissolved in 1 L ddH_20 and autoclaved.

.2.2.13 Phosphate buffered saline (PBS)

8 g Sodium chloride (NaCl)

1.16 g Sodium hydrogen phosphate (Na_2HPO_4)

0.2 g Potassium dihydrogen phosphate (KH_2PO_4)

0.2 g Potassium chloride (KCl)

Dissolved in 1 L ddH_2O and pH adjusted to 7.

2.2.14 Stainer and Scholte medium (S+S)

basal medium

10.72 g	L-Glutamic acid (monosodium salts)
0.24 g	L-Proline
2.5 g	NaCl
0.5 g	KH_2PO_4
0.2 g	KCl
0.3 g	Magnesium chloride ($MgCl_2.6H_2O$)
0.4 g	Calcium chloride ($CaCl_2.2H_2O$)
1.525 g	Tris

Dissolved in 1 L of ddH_2O and pH adjusted to 7.2. The medium was then autoclaved at 121°C for 20 mins, allowed to cool and 1 ml of supplement added per 100 ml of basal medium before use.

Supplement (for Stainer and Scholte medium)

0.4 g	L-Cysteine (dissolved in 1 ml of concentrated HCl)
0.5 g	Ferrous sulphate ($FeSO_4.7H_2O$)
0.6 g	Ascorbic acid
0.7 g	Nicotinic acid
1 g	Glutathione

Dissolved in 100 ml of ddH_2O, filter sterilized and stored at 4°C for up to 5 weeks.

2.2.15 S+S (Bvg-)

S+S medium supplemented with additional MgSO4 or nicotinic acid has been shown to prevent BvgAS activation of virulence factors in *Bordetella* spp. To prepare S+S (Bvg-), S+S medium was supplemented with 2.64 g/L Nicotinic acid (20 mM) and autoclaved.

2.2.16 SOB broth

20 g	Tryptone
5 g	Yeast extract
0.5 g	NaCl
0.19 g	KCl

Dissolved in 1 L dH2O and autoclaved.

2.2.17 SOC broth

To prepare SOC, 100 µl 2M glucose and 100 µl Mg^{2+} were filter sterilised and added to 10 ml of autoclaved SOB on the day of use.

2.2.18 Tris Borate EDTA buffer (10X; TBE)

107.8 g	Tris base
50 g	Boric acid
7.44 g	EDTA

Dissolved in 1 L of ddH_2O and pH adjusted to 8.3.

2.3 Molecular biology materials

2.3.1 6X DNA load dye

100 µl	1M Tris-HCl pH 8.0
1 ml	0.5M EDTA pH 8.0
5 g	Sucrose
0.025 g	Bromophenol blue

Dissolved in 10 ml dH_2O.

2.3.2 Plasmids

A description of all plasmids used and generated in this study is provided in Table 2.3

2.3.3 Primers and PCR products

A list of all primers, and relevant PCR protocols and products produced by primer pairs, is provided in Tables 2.4 and 2.5 respectively. Unless otherwise stated all PCR products used in this study were amplified from *B. pertussis* strain 338 genomic DNA, prepared from a mid-log culture using the Aquapure DNA Isolation Kit (Bio-rad, CA, USA) according to the manufacturer's instructions.

2.3.4 Restriction enzymes

All restriction enzymes used in this study where purchased from Invitrogen (UK).

2.4 Animals

BALB/c mice used in this study were obtained from Harlan (UK). All mice were maintained under the guidelines of the Irish Department of Health and were 6-8 weeks old at the initiation of each experiment.

2.5 Antibodies and cytokine standards

The specificities and source of monoclonal and polyclonal antibodies used in this study are listed in Table 2.6. Murine cytokine standards were obtained from commercial sources as detailed in Table 2.7.

2.6 Cell lines

2.6.1 Murine J774 macrophage

The J774 murine macrophage cell line, which was derived from BALB/c mice, was acquired from the European Collection of Cell Cultures (EACC) and was cultured in complete DMEM as required. The cells were passaged every 3-4 days depending on the rate of growth.

2.6.2 Human HEK 293T epithelial cells

The human embryonic kidney HEK 293T cell line was obtained from Dr. Andrew Bowie and cultured in complete DMEM as required. The cells were passaged every 3-4 days depending on the rate of growth.

2.7 Antigens

2.7.1 Heat-killed *Bordetella* spp.

Bordetella spp. harvested in stationary phase growth were concentrated to ~ 2-5 X 10^{10} CFU/ ml in 1% casein. Aliquots were then heated at 65°C for 30 mins and stored at -20°C until use.

2.7.2 Sonicated *B. pertussis* antigen

B. pertussis was harvested in stationary phase of growth and pelleted at 10,000 rpm for 15 minutes. The resulting pellet was resuspended in PBS. Bacteria were sonicated for 2 minutes with a pulse setting using a Branson sonifier 250. The sonicate was centrifuged at

12,000 rpm for 10 minutes and the protein concentration of the supernatant was estimated by BCA assay.

2.7.3 PT

Purified PT antigen was obtained from Kaketsuken (Japan) and inactivated by heat treatment.

2.7.4 FHA

Purified FHA antigen was obtained from Kaketsuken (Japan)

2.8 Cloning and molecular biology techniques

2.8.1 Growth of *Escherichia coli*

Unless otherwise stated, *E. coli* strain XL1-blue were used as the host strain for plasmid construction, replication and recombinant protein production throughout this study. *E.coli* were streaked from glycerol stocks onto LB agar supplemented with the appropriate antibiotics, and incubated at 37°C overnight. Single colonies were then used to inoculate appropriately sized cultures in LB broth supplemented with antibiotics as necessary.

2.8.2 Preparation of heat shock competent *E. coli* XL1-blue

E. coli XL1-blue cells were rendered heat shock competent by the *Inoue* method for preparation and transformation of competent cells (Molecular Cloning, Sambrook and Russell, Cold Spring Harbour Laboratory Press, NY, USA).

2.8.3 Preparation of heat shock competent *E. coli* SM10λpir

E. coli SM10λpir cells were rendered heat shock competent with $CaCl_2$ according to the standard technique described by Sambrook *et al* (Molecular Cloning, Sambrook and Russell, Cold Spring Harbour Laboratory Press, NY, USA).

2.8.4 Glycerol stocks of *E. coli*

Glycerol stocks of *E. coli* strains were prepared and stored at -80°C for long term storage. To prepare, a 6 ml overnight culture of *E. coli* was aliquoted into 4 X 1.5 ml cultures and pelleted at 13,000 rpm for 2 minutes in a benchtop centrifuge. Each aliquot was then washed once with 1.5 ml LB. All four aliquots were then resuspended in a total volume of 1.5 ml LB, to which was added 1.5 ml of sterile 20% v/v glycerol. This suspension was then aliquoted into two cryotubes and frozen at -80°C.

2.8.5 DNA electrophoresis

DNA fragments were visualised alongside molecular weight markers following electrophoresis on a 1% (w/v) agarose gel for 45-60 mins at 100 V unless otherwise stated. The gels were prepared with 30 μg/ml ethidium bromide and visualised under a UV lamp.

2.8.6 Purification of DNA fragments from agarose gels

DNA fragments were excised from agarose gels and purified immediately using the QIAquick gel extraction kit (Qiagen, UK) according to the manufacturer's instructions.

2.8.7 Plasmid construction

Purified PCR products and relevant cloning vectors were digested with appropriate restriction enzymes, as detailed in Chapter 3 for *bsp22* and chapter 4 for *bscN*, for 3 h at 37°C. Linearised cloning vectors were dephosphorylated with 0.05 U calf intestinal alkaline phosphatase (Promega, USA), for 2 X 30 minutes at 37°C. Digested PCR product and cloning vector DNA were then quantified by measuring $A260_{nm}$ before ligation in a 1:3 molar ratio of vector DNA:PCR product with T4 DNA Ligase (Promega, USA).

Ligation reactions were carried out at 16°C for 12 h, before being placed at 4°C until use. For transformation and plasmid production, 3-5 ul of ligation reaction was added to 50 ul of *E. coli* XL1-blue cells rendered heat shock competent by the *Inoue* method for preparation and transformation of competent cells. Cells were heat shocked at 42°C and allowed to recover at 37°C for 1 hour before being plated on selective media. Resulting colonies were inoculated into 3 ml of LB broth, again supplemented with the appropriate antibiotic, and grown overnight at 37°C. Minipreps of plasmid DNA were prepared, by the alkaline lysis method, using miniprep solution 1 (50 mM Tris pH8.0, 10 mM EDTA, 200 μg/ml RNase A, dH_2O) , miniprep solution 2 (0.2M NaOH, 1% (w/v) SDS, dH_20) and miniprep solution 3 (3M Potassium acetate, 5M Glacial acetic acid, dH_20) to resuspend and lyse the cells and precipitate genomic DNA respectively. Plasmid DNA was then precipitated with ethanol, and resuspended in TE buffer (GIBCO, UK). DNA digestion with appropriate restriction enzymes and DNA sequencing was then performed to verify the presence of the correct plasmid.

2.8.8 Midiprep of plasmid DNA

Single colonies of *E. coli* XL1-blue harbouring the appropriate plasmid were used to inoculate a 30 ml culture, which was grown overnight in LB broth supplemented with the appropriate antibiotics for plasmid selection. The following morning, 20 ml of this culture was

then used to prepare midipreps of plasmid DNA using the QIAfilter plasmid midiprep kit (Qiagen, UK) according to the manufacturers instructions.

2.8.9 DNA sequencing

All DNA sequencing reactions of plasmids and PCR products were carried out by MWG biotech, Ebersberg, Germany.

2.8.10 Allelic exchange

Allelic exchange (Fig 2.1) is a process that relies on 2 separate recombination events to replace a chromosomal wild type allele with a plasmid-located mutant allele lacking an internal portion of the wild type gene. Thus, the mutant allele contains 2 separate sites for homologous recombination with the wild type allele on the chromosome. A first recombination event, selected for by means of a positive selection marker, eg. gentamicin (Gm) or kanamycin (Kn), at one of these sites inserts the linearised plasmid DNA into the chromosome at that site, as the plasmid cannot replicate extra chromosomally. First recombinants can then be propagated in the absence of that marker to encourage a second recombination event, which results in the regeneration of the plasmid, leaving either the wild type or the mutant allele on the chromosome (Fig 2.1).

Plasmids carrying the bscN mutant alleles were first transformed by heat shock into *E. coli* SM10λpir. When mated with other bacteria, this strain of *E. coli* transfers large numbers of plasmid to the recipient via transconjugation. *Bordetella* were grown for 3 days, and *E. coli* were grown overnight on agar plates. Colonies of wild type *B. bronchiseptica* RB50 and Sm resistant *B. pertussis* were harvested from the plates and added to 100 µl S+S medium with *E. coli* SM10λpir harbouring the plasmids containing the mutant bscN alleles in a 1:10 ratio.

These cell mixtures were then plated onto BG agar and placed at 37°C for 8 h to allow mobilisation of plasmid from *E. coli* to *Bordetella*. Initially, the bacteria were then streaked for single colonies on BG agar plates supplemented with Gm (for matings involving pNF4) or Kn (for matings involving pNF11) and incubated at 37°C for 3 days to select for first recombinants. Gm or Kn resistance was confirmed by replating on BG agar supplemented with Gm or Kn. These colonies were then inoculated into 1 ml S + S medium and left to grow for overnight, before being centrifuged and resuspended in 150 µl S+S, and plated on BG agar supplemented with Sm (for matings involving pNF4) or 10% (w/v) sucrose (for matings involving pNF11) to select for second recombinants. Sm/sucrose resistant Gm/Kn sensitive colonies (see Fig 2.1) were screened by PCR to check for the presence of either the truncated mutant allele or the wild type allele. Wild type and mutant alleles render PCR products of different sizes and so can be easily differentiated. Each colony was inoculated into 1 ml S+S in a 48-well tissue culture plate, and incubated at 37°C overnight. The plates were spun and the pellets resuspended in 100 µl S+S. 99 µl of this suspension was then added to 900 µl S+S in a new well of a 48-well tissue culture plate and again left overnight to grow. The remaining 1 µl was added to 24 µl of a Taq polymerase master mix (Qiagen, UK) with oligonucleotides PAB20 5'- GCCCTGCGGATCCCGCG - 3' and BscN_test_rev 5' - ACCGCGGCGCCCTTGACCA - 3', and screened by PCR for the presence of the wild-type or mutant allele.

For allelic exchange involving pNF13, the matings were performed as described above, however instead of selecting first recombinants by plating onto BG agar supplemented with Kn, 10 fold dilutions of the bacteria were plated directly onto BG agar supplemented with Gm to select for double recombinants of the bscN::Gm^r allele. All Gm resistant colonies were then restreaked onto both Kn and Gm containing BG agar plates, and Gm resistant, Kn

sensitive colonies were then screened by PCR as described above for the pNF4 and pNF11 matings.

2.9 Recombinant His-Bsp22 protein production

E. coli XL1-blue harbouring pAPB15 was induced to express His-Bsp22 by the addition of 1mM isopropyl-β-thiogalactopyranoside (IPTG, Bioline, UK) to an exponentially growing bacterial culture in LB broth supplemented with ampicillin, with shaking at 37°C. The bacterial culture was centrifuged and the pellet was resuspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0) at 5 ml per gram weight. The resuspended solution was then aliquoted into 1 ml fractions in 1.5 ml eppendorf tubes and stirred on a rotary blood stirrer until cell lysis occurred. Tubes were then spun at 15000g for 20 min to pellet the cellular debris, and the supernatants were loaded onto a QIAexpress Ni-NTA column (Qiagen, UK). The column was then washed with 5 ml buffer B and 5ml buffer C (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6.3) before the protein was eluted with buffer E (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5). 1 ml protein fractions were then analysed by SDS-PAGE (Fig 3.4 A) and the protein concentration determined by Bradford assay. The concentration of the pooled fractions was found to be ~280 μg/ml. This protein was then left stirring in a 10,000 MWCO dialysis membrane (Pierce, USA) overnight in dialysis buffer (1 X PBS, 2 M urea, pH 8.0). The following day the dialysis membrane was placed into Dulbeccos PBS pH 8.0 and placed at 4°C to inhibit protease activity. After dialysis the protein concentration was determined by Bradford assay and was found to be ~230 μg/ml.

2.10 Anti-Bsp22 antibody production

For antibody production, ~230 μg (1 ml) of purified recombinant His-Bsp22 was emulsified with complete Freund's adjuvant (CFA) (Difco, USA) and 1 ml of this mixture was used to inoculate a rabbit subcutaneously (day 1). The rabbit was boosted twice (day 14 and day 28), with 115 μg of His-Bsp22 emulsified with an equal amount of incomplete Freund's adjuvant (IFA)(Difco, USA). A test-bleed was performed and the serum was shown to specifically recognize His-Bsp22 by western blot, demonstrating that the rabbit had raised antibodies specific for Bsp22. On day 40 post immunisiation the rabbit was exsanguinated and 100 ml of harvested blood was centrifuged and ~ 60 ml of serum was generated. This serum was shown to contain antibodies that specifically recognise rHis-Bsp22 (Fig 3.4 C).

2.11 Growth of *Bordetella* spp.

Bordetella spp. were routinely grown on BG agar (BD Difco, USA) supplemented with antibiotics at the concentrations described in Table 2.1. For inoculation of precultures, colonies of *B. pertussis* and *B. bronchiseptica*, grown on BG agar for 3 and 2 days respectively, were transferred to 30 ml S+S using a disposable inoculating loop, and incubated at 37°C with vigourous shaking for 2 days and 1 day respectively. The OD_{600} of precultures were then measured and 100 ml cultures were inoculated at a starting OD_{600} of 0.2 and grown to stationary phase.

2.12 Protein analysis and western blot

To analyse the secreted proteins of *Bordetella* spp by western blot, cultures were harvested for each bacterial strain at the same timepoint post inoculation, or at the same stage of bacterial growth as determined by OD_{600} measurements. Cultures were pelleted at 3000 rpm

for 20 mins. The supernatant was filtered through a 0.2 μm filter to ensure complete removal of whole cell bacteria and bacterial debris, and total supernatant proteins were precipitated for one hour on ice with 10% (w/v) trichloroacetic acid (TCA). Following precipitation the proteins were pelleted by centrifugation at 3000 rpm for 20 mins and resuspended in SDS sample buffer (10 μl per 1 ml of original bacterial supernatant).

For western blot analysis, 10 μl of sample was run on a 10% (w/v) SDS-PAGE gel (Separating gel; 4 ml dH_2O, 3.3 ml Bis-Acrylamide, 2.5 ml 1.5 M Tris pH 8.8, 100 μl 10% (w/v) SDS, 100 μl Ammonium persulphate and 33 μl TEMED. Stacking gel; 2 ml dH_2O, 430 μl Bis-Acrylamide, 820 μl 0.5 M Tris pH 6.8, 33 μl 10% (w/v) SDS, 33 μl Ammonium persulphate, 20 μl TEMED) for 30-45 mins at 30 mA per gel. For Coomassie staining, gels were first soaked in Coomassie stain (0.5 g Coomassie blue (Biorad), 50 ml acetic acid, 225 ml methanol, 225 ml dH_2O) for 45 minutes or longer if neccessary. Gels that had reached the desired staining intensity were destained in Coomassie destain (35 ml acetic acid, 25 ml ethanol, 440 ml dH_2O). For western blot, gels were soaked in transfer buffer and transferred to nitrocellulose membranes (Sigma, USA) at 225 mA. Membranes were then washed with PBS and blocked with PBS + 0.5% (w/v) Bovine Serum Albumin (BSA; Sigma, USA) for 30 minutes at room temperature or overnight at 4°C. Antibodies were diluted (anti-Bsp22 1:1000, anti-FHA 1:5000, anti-PT 1:10000) in PBS + 0.5% (w/v) BSA and the membranes were incubated in this solution for 1 h. The membranes were washed with PBS + 0.1% (w/v) Tween 20 (Sigma, USA) 5 times for 5 min each and incubated for 1 h in IgG-HRP conjugate (Sigma, USA) diluted 1:10000 in PBS + 0.5% (w/v) BSA. The PBS-Tween washing was repeated before the membranes were incubated for 5 minutes in Supersignal west pico chemiluminescent substrate (Pierce, USA). To visualise bands, membranes were exposed to Hyperfilm ECL (Amersham Biosciences, UK) and developed.

2.13 Preparation of protein samples for MALDI-TOF analysis

For MALDI-TOF analysis of protein samples, supernatants were harvested from *Bordetella* spp. following 24 hours of growth in liquid culture. Filtered supernatants were precipitated with 30% Ammonium chloride and the protein pellets were collected by centrifugation at 3000 rpm for 25 mins. Pellets were frozen and supplied on dry ice to The Laboratory of Mass Spectrometry, The Polish Academy of Sciences, Warsaw, Poland where the samples were analysed.

2.14 J774 phagocytic uptake assay

J774 macrophage were cultured overnight in 24 well plates at 1 X 10^6/well in DMEM supplemented with FCS and 100 mM L-glutamine. The following morning stationary phase cultures of *Bordetella* spp. were harvested by centrifugation at 3000 rpm for 20 minutes, and resuspended in 1% casein. Bacteria were then quantitated by estimating OD_{600} and added to triplicate wells at a MOI of 100. Following incubation at 37°C for 2 hours, the contents of each well was harvested and an additional 1 ml of DMEM (supplemented with FCS and 100 mM L-glutamine) was added. The contents of each well was then pelleted by centrifugation at 1200 rpm for 5 minutes. The pellets were washed in 10 ml medium to remove any unbound bacteria, and resuspended in either in 1 ml of medium supplemented with 100 μg/ml Kn to kill extracellular bacteria or 1 ml medium alone (as a control). The cell suspensions were incubated at 37°C for a further 1 hour, and then were pelleted by centrifugation at 1200 rpm for 5 minutes. The cells were washed in 10 ml media and were resuspended in 1 ml lysis buffer (PBS, 1% (w/v) Triton-X-100) and left at room temperature for 5 mins. 100 μl samples of the appropriate dilutions of the lysed cells were then plated in triplicate onto BG agar plates and incubated at 37°C for 2 days for *B. bronchiseptica* and 3-5 days for *B. pertussis*.

2.15 Cytotoxicity assays

Cytotoxicity of cultured cells following incubation with *Bordetella* spp. was measured using the Cytotox 96 non-radioactive cytotoxicity assay (Promega), which measures lactate dehydrogenase (LDH) release as a measure of cell lysis. *Bordetella* spp. were harvested, resuspended in 1% (w/v) casein, quantitated and added to the cells in 24 well plates in triplicate at the appropriate MOI as described in section 2.13. For LDH measurement, a 50 µl sample of supernatant was removed from each well and added to a 96 well plate, and the LDH assay was performed according to the manufacturers instructions.

2.16 Generation of murine BMDC

Bone marrow-derived dendritic cells (BMDC) were generated from BALB/c mice using a modification of the method described by Lutz *et al.* (Lutz *et al.* 1999) Mice were euthanised and their femurs and tibiae removed and dissected from the surrounding tissue. The bone marrow was flushed out with RPMI-1640 medium using a 27G needle attached to a 10 ml syringe. Cell aggregates were disintegrated using a larger needle to prevent damaging the cells. The resulting cells were pelleted by centrifugation at 1200 rpm for 5 mins and resuspended in 1 ml of ammonium chloride lysis solution for 1 min to lyse red blood cells. Lysis was stopped by the addition of 9 ml of RPMI and cells were centrifuged again and resuspended in 5 ml of fresh RPMI. Cell viability was assessed using trypan blue. Immature BMDC were prepared by culturing cells at 1 x 10^6 cells/ml in a 50 ml tissue culture flask in RPMI medium supplemented with 40 ng/ml GM-CSF, a supernatantt from a GM-CSF-expressing J558 cell line (provided by Nathalie Winter, Pasteur Institute, Paris, France). After 3 days culture, an additional 15-20 ml of fresh RPMI supplemented with 40 ng/ml GM-CSF

was added to each flask of cells. On day 6 cell culture supernatants were carefully removed to eliminate contaminating non-adherent cells from the culture. 10 ml of warm sterile PBS was added to the flask and gently agitated; the cell suspension was then transferred to a sterile 50 ml tube. 15 ml of sterile warmed EDTA (0.02% (w/v)) was then added to each culture flask and incubated for 10 mins to loosen adherent cells. The loosened cells were removed with a pipette and added to the cells in PBS and these were centrifuged at 1200 rpm for 5 mins. The resulting cell pellets were resuspended in 5 ml of RPMI and cell viability was assessed. Cells were then re-cultured at 1 x 10^6 cells/ml in complete RPMI supplemented with 40 ng/ml GM-CSF. After a further 4 days of incubation, 15-20 ml of fresh RPMI containing 40 ng/ml GM-CSF was added to each flask. 2 days later the loosely adherent cells were harvested, viability determined and the resulting immature DC were added to 24 well tissue culture plates (1 X 10^6/ml) overnight in complete RPMI with 10 ng/ml GM-CSF and incubated overnight before use the following day.

2.17 Cytokine production by DC following infection with *Bordetella*

BMDC were generated from BALB/c mice as described in section 2.15. *Bordetella* spp. were harvested, quantified and BMDC were infected in triplicate wells on 24 well plates at the range of MOI shown in the fugure legends. Following 6 hours incubation at 37°C, supernatants were harvested and cytokine concentrations were determined by enzyme-linked immunosorbent assay (ELISA).

2.18 Determination of cytokine concentrations by ELISA

2.18.1 Murine IL-1β, IL-10, IL-17, IL-12p70, MIP-1, MIP-2 and TNF-α ELISA

The concentrations of IL-1β, IL-10, IL-17, IL-12p70, MIP-1, MIP-2 and TNF-α were measured using commercially available ELISA kits (R&D systems). High binding certified 96-well microtitre plates (Greiner Bio-One) were coated overnight at 4°C with 50 µl/well of rat anti-mouse IL-1β (4 µg/ml), IL-10 (2 µg/ml), IL-17 (2 µg/ml), IL-12p70 (4 µg/ml), MIP-1 (0.4 µg/ml), MIP-2 (2 µg/ml) or TNF-α (0.8 µg/ml) capture antibody in PBS. Plates were washed in wash buffer and non-specific binding sites were blocked by adding 200 µl/well of blocking solution (PBS, 1% BSA, 5% Sucrose) and plates were incubated for 2 hours at room temperature. Plates were washed and 50 µl/well of cell supernatant or serially diluted IL-1β standard (0-1000 pg/ml), IL-10 standard (0-2000 pg/ml), IL-17 standard (0-1000 pg/ml), IL-12p70 standard (0-1500 pg/ml), MIP-1 standard (0-500 pg/ml), MIP-2 standard (0-1000 pg/ml) or TNF-α standard (0-150 ng/ml) in reagent diluent (PBS 1% (w/v) BSA solution) were added and the plates were incubated overnight at 4°C. Plates were washed and incubated with 50 µl/well of biotinylated goat anti-mouse IL-1β (100 ng/ml), IL-10 (400 ng/ml), IL-17 (200 ng/ml), IL-12p70 (400 ng/ml), MIP-1 (100 ng/ml), MIP-2 (75 ng/ml) or TNF-α (150 ng/ml) in reagent diluent were added and incubated for 2 hours at room temperature. Plates were washed and IL-1β, IL-10, IL-17, IL-12p70, MIP-1, MIP-2 and TNF-α were detected by incubating plates for 30 minutes with 50 µl/well of HRP conjugated streptavidin (1:200 in reagent diluent). After washing the plates, 50 µl/well of o-phenylendiamine (OPD) substrate in phosphate citrate buffer was added and colour development was stopped with 1 M H_2SO_4. The OD values were determined by measuring absorbance at 492 nm using a microtitre plate reader and cytokine concentrations for test samples were evaluated using a standard curve

prepared using recombinant mouse IL-1β, IL-10, IL-17, IL-12p70, MIP1, MIP2 and TNF-α of known concentration.

2.18.2 Murine IL-12p40, IL-6 and IFNγ

Murine IL-12p40, IL-6 and IFNγ were detected by specific immunoassays using matched pairs of monoclonal antibodies (PharMingen; Table 2.6). High binding certified 96-well microtitre plates (Greiner Bio-One) were coated overnight at 4°C with 50 µl/well of rat anti-mouse IL-12p40 (2 µg/ml), IL-6 (1 µg/ml) or IFNγ (5 µg/ml) in PBS. Plates were washed and non-specific binding sites were blocked by adding 200 µl/well of blocking solution (PBS, 10% (w/v) milk powder for IL-12p40 and IFNγ or PBS, 3% (w/v) BSA for IL-6) for 2 hours at room temperature. Plates were washed and incubated overnight at 4°C with 50 µl/well of cell supernatant or serially diluted IL-12 standard (0-5000 pg/ml), IFNγ standard (0-20 ng/ml) or IL-6 standard (0-5000 pg/ml). After washing the plates in wash buffer, IL-12p40, IFNγ and IL-6 were detected by adding biotinylated anti-IL-12p40 (2 µg/ml in PBS), anti-IFNγ (2.5 µg/ml) or IL-6 (1 µg/ml) antibodies and incubated for 1 hour at room temperature. Plates were washed and IL-12p40, IFNγ and IL-6 were detected by incubating plates for 30 mins with 50 µl/well of streptavidin HRP (PharMingen) (1:1000 in PBS). Plates were washed again and 50 µl/well of OPD substrate in phosphate citrate buffer was added until colour development was detected. Enzymatic colour development was stopped with 25 µl/well 1 M H_2SO_4. Absorbance was read at 492 nm and concentrations of IL-12p40, IFNγ and IL-6 in supernatants were evaluated from a standard curve prepared using recombinant murine IL-12p40, IFNγ or IL-6 of known concentrations.

2.18.3 Murine IL-23

The concentration of murine IL-23 was determined using a commercially available ELISA kit (e-Bioscience). High binding certified 96-well microtitre plates (Greiner Bio-One) were coated overnight at 4°C with 50 µl/well of IL-23 capture antibody (2 µg/ml) in e-Bioscience coating buffer. Plates were washed in wash buffer and non-specific binding sites were blocked by adding 200 µl/well of blocking solution (e-Bioscience assay diluent) and plates were incubated for 2 hours at room temperature. Plates were washed and 50 µl/well of cell supernatant or serially diluted IL-23 standard (0-4000 pg/ml) in e-Bioscience assay diluent was added and the plates were incubated overnight at 4°C. Plates were washed and incubated with 50 µl/well of biotinylated IL-23 detection antibody (1 µg/ml) in e-bioscience assay diluent and incubated for 2 hours at room temperature. Plates were washed and IL-23 was detected by incubating plates for 30 minutes with 50 µl/well of HRP conjugated streptavidin (1:250 in e-Bioscience assay diluent). After washing the plates, 50 µl/well of TMB (e-Bioscience) was added and colour development was stopped with 1 M H_2SO_4. The OD values were determined by measuring absorbance at 450 nm using a microtitre plate reader and cytokine concentrations for test samples were evaluated using a standard curve prepared using recombinant mouse IL-23 of known concentration.

2.19 Aerosol infection of mice

Mice were infected with *Bordetella* spp. using a modified version of the method described by Sato *et al* (Sato *et al.* 1980). *Bordetella* spp. were grown at to mid-log phase at 37°C under agitation conditions in S+S and bacteria was concentrated by centrifugation and

resuspended at 2 x 10^{10}/ml in physiological saline containing 1% (w/v) casein. Mice were exposed for 15 mins to an aerosol of the challenge inoculum using a nebuliser directed into a chamber containing groups of 24 mice. Four mice from each experimental group were sacrificed three hours after aerosol challenge and at 3, 7, 14, 21 and 28 days after challenge to assess the number of viable bacteria in the lungs.

2.20 Enumeration of viable bacteria in the lungs and trachea

Lungs and tracheas were aseptically removed, and homogenised seperately in 1 ml of sterile 1% (w/v) casein on ice. 100 µl of neat or serially diluted homogenate were plated in triplicate onto BG agar plates and incubated at 37°C for 4 days. The numbers of colony forming units (CFU) were estimated. Results are given as the mean number of *B. pertussis* CFU for individual lungs and tracheas from 4 mice per experimental group. The sensitivity of the assay was 0.56 CFU/ml

2.21 Measurement of cytokine concentrations in the lung

Lungs were removed from mice and homogenised as described in section 2.19. Lung homogenates were centrifuged at 13000 rpm for 5 mins, and 50 µl of supernatant from each lung was used to determine the concentrations of IL-10, IL-12p70, IL-12p40, IL-23, IL-6, IL-1β, TNFα, MIP-1 and MIP-2 by ELISA as described in section 2.17.

2.22 Determination of antigen-specific serum total IgG and IgG subclass titres

Serum samples from infected mice were prepared from peripheral blood removed from the thoracic cavity after sacrifice. Blood was allowed to clot for 2 hours at room temperature and then placed at 4°C for 2-4 hours. Blood was centrifuged at 10,000 rpm for 5 mins and the serum was collected and transferred to fresh tubes before storing at -20°C. The titres of antigen-specific total IgG, IgG2a and IgG1 antibodies in sera were determined by ELISA. *B. pertussis* 12743 or *B. bronchiseptica* RB50 sonicate (5 μg/ml) in PBS (50 μl/well), was added to flat bottomed 96 well microtitre plates (Greiner Bio-One) and incubated overnight at 4°C. Plates were washed with wash buffer and blocked with PBS supplemented with 10% w/v dried milk powder (200 μl/well) for 2 hours. Plates were washed and sera were serially diluted 2 fold in PBS on the plates. After overnight incubation, the plates were washed and incubated with 50 μl/well of biotin-conjugated anti-mouse IgG monoclonal antibody (Sigma) diluted 1:1000 in PBS, biotin-conjugated anti-mouse IgG2a (PharMingen) or biotin-conjugated anti-mouse IgG1 (Pharmingen) diluted 1:10,000 in PBS. Plates were washed and incubated for 40 minutes at room temperature with 50 μl/well of HRP-conjugated streptavidin diluted 1:1000 in PBS. Plates were washed and colouration was developed with 50 μl/well OPD in phosphate citrate buffer. The enzymatic reaction was stopped by addition of 50 μl/well 1 M H_2SO_4 and absorbance was measured at 492 nm. Results are expressed as $\log_{10}$ endpoint antibody titres determined by extrapolation of the straight part of the dilution curve to the OD_{492nm} value of the control serum for naïve mice.

2.23 Detection of antigen-specific T cell responses

Spleens were removed aseptically from groups of 4 mice per group 14, 21 and 28 days after aerosol challenge. Each individual spleen was homogenised in 5 ml of complete RPMI medium using the barrel of a sterile 5 ml syringe and filtered through a 70 μm nylon cell strainer (BD Falcon, USA). The filter membrane was rinsed with an additional 5 ml of complete RPMI and the cells were collected by centrifugation. Pellets were resuspended in 1 ml ammonium chloride lysis solution and incubated at room temperature for 3-5 minutes to lyse the red blood cells. Following lysis, an additional 10 ml complete RPMI was added to samples were centrifuged at 100 rpm for 5 minutes to collect the spleen cells. Spleen cells (2 x 10^6 cells/ml) were stimulated with antigen (heat killed *Bordetella*, sonicated *Bordetella*, PT and FHA), PMA/anti-CD3 (positive control) or medium alone (negative control) and incubated at 37°C for 72 hours. Supernatants were removed after 72 hours and IL-10, IFN-γ and IL-17 concentrations were determined by ELISA, as described in section 2.17.

2.24 Flow cytometry analysis

Treg cell surface marker expression was analysed by staining with specific antibodies and flow cytometry analysis was performed using a FACS Caliber flow cytometer (Becton-Dickinson, San Jose, CA). The left lung-lobe from groups of 4 mice from each group infected with *Bordetella* spp. was harvested at variuos timepoints following aerosol challenge. The lung lobes were first rinsed in 5 ml complete RPMI to remove blood clots and chopped up finely using a sterile scalpel blade. The samples were incubated at 37°C with rocking for 1 hour in 1 ml Hanks's balanced salt solution (HBSS) with 100 μl of a 10 mg/ml solution of collagenase D. The resulting cell suspensions were then passed through a 70 μm cell strainer and red blood cells were lysed. Cell suspensions were washed twice with PBSA (PBS

containing 0.1% (w/v) sodium azide) supplemented with 0.1% (w/v) BSA, by centrifugation at 1800 rpm for 5 minutes. Rat anti-mouse CD4-PE (phycoerythrin) and rat anti-mouse CD25 PE-Cy5 (phycoerythrin-Cy5) were added to 1 X 10^6 cells in a 100 µl volume. Cells were incubated at room temperature for 15 minutes in the dark. Cell suspensions were washed once in PBSA and centrifuged at 1800 rpm for 5 minutes. The pellets were resuspended in 100 µl of 2% (w/v) paraformaldehyde and stored at 4°C until use or analysed immediately using a Becton Dickinson FACS calibur and CellQuest software.

For FoxP3 staining, cell suspensions from infected lungs were prepared and stained with the CD4 and CD25 surface markers as described above. Cell suspensions stored in 2% (w/v) paraformaldehyde were first washed once with PBSA as described above and centrifuged at 1800 rpm for 5 minutes. The pellets were resuspended in 1 ml of freshly prepared Fix/Perm solution (e-Biosciences; 1 part Fix/Perm concentrate with 3 parts Fix/Perm diluent) and incubated for 4 hours at 4°C. Cell suspensions were then washed once in PBSA and twice in 2 ml of permeabilisation buffer (e-Biosciences) by centrifuging for 5 minutes at 1800 rpm. Cell pellets were resuspended in 500 µl of permeabilisation buffer and 1 µl of rat anti-mouse FoxP3-FITC (Flourescein isothyocyanate) conjugate and incubated at 4°C for 30 minutes in the dark. Cells were washed with 2 mls permeabilisation buffer by centrifugation for 5 minutes at 1800 rpm. Pellets were resuspended in 100 µl of 2% (w/v) paraformaldehyde and stored at 4°C until use or analysed immediately using a Becton Dickinson FACS calibur and CellQuest software.

2.25 Statistical analysis

Statistical analysis was performed using the computer based mathematical package InStat. Statistical differences in mean antibody or cytokine concentrations and bacterial load were determined by unpaired student's t test.

Table 2.1 Bacterial Strains

Bacterial strain	Source	Genotype / characteristics	Use
E. coli XL1-Blue	Stratagene	General cloning strain	Cloning and transformation
E. coli SM10 λ pir	Miller and Mekalanos 1988	Efficient transconjugation	Transformation
B. pertussis ATCC 9797 (18323 [NCTC 10739])	ATCC	Wild type virulent	Amplification of *bsp22*; Monitoring *in vitro* T3SS activity
B. pertussis Tohama I	ATCC	Wild type virulent	Amplification of *bscN* allele; Monitoring *in vitro* T3SS activity
B. pertussis ATCC 12743 (5375 [3865])	ATCC	Wild type virulent	Investigation of T3SS function
B .pertussis 12743 bscN	This study	Δ*bscN*- T3SS deficient	Investigation of T3SS function
B. pertussis Wellcome 28	Dr. Keith Redhead	Wild-type virulent	Monitoring *in vitro* T3SS activity
B. pertussis ATCC 9340 (5[17921])	ATCC	Wild type virulent	Monitoring *in vitro* T3SS activity
B. pertussis 12742 (5374 [3747])	ATCC	Wild type virulent	Monitoring *in vitro* T3SS activity
B. bronchiseptica RB50	ATCC	Wild-type virulent	Investigation of T3SS function
B. bronchiseptica RB50 bscN	This study	Δ*bscN*- T3SS deficient	Investigation of T3SS function
B. pertussis 0558	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity

B. pertussis 0335	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 0888	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 0546	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 0597	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 0640	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 0780	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 0950	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 1868	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 1926	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 2740	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 2890	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 2913	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 2777	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 2778	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity
B. pertussis 0304	Prof. Frits R Mooi	Wild type; clinical isolate	Monitoring *in vitro* T3SS activity

Table 2.2 Antibiotics

Antibiotic	**Concentration**	**Use**
Cephalexin (Cx)	30 μg/ml	Selection of *Bordetella* on blood agar
Ampicillin (Ap)	150 μg/ml	Maintenance of plasmid expressionin *E. coli*
Gentamicin (Gm)	10 μg/ml	1) Allelic exchange. 2) Maintenance of *bscN*::Gmr mutant allele expression
Streptomycin (Sm)	100 μg/ml	Allelic exchange
Kanamycin (Kn)	1) 25 μg/ml 2) 100 μg/ml	1) Allelic exchange 2) Phagocytic uptake assay

Table 2.3 Plasmids

Plasmid	Description/ Features	Source	Figure
pQE-80	Ampicillin resistance cassette; T5 promoter and Lac repressor for protein expression.	Qiagen (UK)	Fig 3.2; 4.2
pAPB15	650 bp His-*bsp22* in pQE-80; Ampicillin resistance cassette; T5 promoter and Lac repressor for protein expression.	This study	Fig 3.3
pNF2	568 bp *bscN*1 insert in pQE-80; Ampicillin resistance cassette; T5 promoter and Lac Repressor for protein expression.	This study	Fig 4.3
pNF3	1.7 kb *ΔbscN* insert in pQE-80; Ampicillin resistance cassette; T5 promoter and Lac repressor for protein expression	This study	Fig 4.4
pSS1129	Ampicillin resistance cassette; Gentamicin resistance cassette; rpsL cassette (streptomycin sensitivity)	(Stibitz 1994)	Fig 4.5
pNF4	1.7 kb *ΔbscN* insert in pSS1129; Ampicillin resistance cassette; Gentamicin resistance cassette; rpsL cassette (streptomycin sensitivity)	This study	Fig 4.6
pNF7	New 1 kb *ΔbscN* insert in pQE-80; Ampicillin resistance cassette; T5 promoter and Lac repressor for protein expression.	This study	Fig 4.8
pEGBR	Kanamycin resistance cassette; Ampicillin resistance cassette; *SacB* and *SacR* cassettes (sucrose sensitivity)	(Akerley *et al.* 1995)	Fig 4.9
pNF11	1 kb *ΔbscN* insert in pEGBR; Kanamycin resistance cassette; Ampicillin resistance cassette; *SacB* and *SacR* cassettes (sucrose sensitivity)	This study	Fig 4.10
pcDNA3.1/His TOPO	TOPO insertion site; Ampicillin resistance cassette; Neomycin resistance cassette; human cytomegalovirus promoter; SV40 promoter;	Invitrogen (UK)	Fig 4.12
pGMr-TOPO	Plasmid pcDNA3.1/His-TOPO containing 770 bp Gmr cassette and upstream UTR from pNF4	This study	Fig 4.14
pNF12	Derivative of pQE-80 containing the 1795 bp *bscN*::Gmr mutant allele	This study	Fig 4.15
pNF13	Derivative of pEGBR containing the 1795 bp *bscN*::Gmr mutant allele and 138 bp of pNF12 vector DNA	This study	Fig 4.16

Table 2.4 Primers

Primer	Sequence	Primer binding site
PAB37	CGGAAGCTTTTAGCGCATGTTGCTGGTG	Bp 596-615 of *bsp22*; Fig 3.1
PAB38	AGCGGATCCAGCATTGATCTCGGAGTTCAC	Bp 4 – 25 of *bsp22*; Fig 3.1
PAB20	GCCCTGCGGATCCCGCG	Bp 65 – 81 of *bscL* (5' flanking gene to *bscN*); Fig 4.1
NF5	TACTGACGCATGCCCCTATCC	Bp 1 – 12 of *bscN*; Fig 4.1
NF4	GCTGGGCATGCTGGTCAAGGGC	Bp 560 – 582 of *bscN*; Fig 4.1
PAB21	GCCGGCTCGCGATGCATCG	Bp 470 – 489 of *bscO* (3' flanking gene to *bscN*); Fig 4.1
BscN_test_rev	ACCGCGGCGCCCTTGACCA	Bp 550 – 569 of *bscN*; Fig 4.1
Gmr_for_2	ATAGCATGCTGACGCACACCG	Bp 3506 – 3528 of pNF4; Fig 4.6
Gmr_rev	GCATGCTTAGGTGGCGGTAC	Bp 2763 – 2783 of pNF4; Fig 4.6
recombtest_P1	ACGAGATAGCGCTGCGTTGC	*Bordetella* chromosome-164 bp upstream *bscN1* recombination site; Fig 4.18, 4.20
recombtest_P2	CTGGGTTCGTGCCTTCATCC	Bp 21-41 Gmr cassette of *bscN*::Gmr mutant allele; Fig 4.18, 4.20
recombtest_P3	GCATACGGGAAGAAGTGATGC	Bp 737-758 Gmr cassette of *bscN*::Gmr mutant allele; Fig 4.18, 4.20
recombtest_P4	CCACAACGATACGGGCTTCG	*Bordetella* chromosome- 1011 bp downstream *bscN2* recombination site; Fig 4.18, 4.20

Table 2.5 PCR reactions

Template	Primer 1	Primer 2	Annealing Temp	Extension time	Cycles	Product size	Use
B. pertussis genomic DNA	PAB 37	PAB 38	62°C	2 minutes	35	627 bp	Amplification of *bsp22*
B. pertussis genomic DNA	PAB20	NF5	56_oC	2 minutes	35	603 bp	Amplification of *bscN1*
B. pertussis genomic DNA	PAB21	NF4	56°C	2 minutes	35	1269 bp	Amplification of *bscN2*
Live *Bordetella* colonies	PAB20	BscN_test_rev	62°C	5 minutes	35	1364 bp mutant *bscN*::Gm^r allele. 1128 bp wild type allele	Colony screening following allelic exchange
pNF4	Gmr_for_2	Gmr_rev	57°C	2 minutes	35	770 bp	Amplification of Gmr cassette
Live *Bordetella* colonies	recombtest_P1	recombtest_P4	56.5°C	2 minutes	35	3 kb	Investigation of first recombinant genotype
Live *Bordetella* colonies	recombtest_P1	recombtest_P2	56.5°C	2 minutes	35	765 bp	Investigation of first recombinant genotype
Live *Bordetella* colonies	recombtest_P3	recombtest_P4	56.5°C	2 minutes	35	1618 bp	Investigation of first recombinant genotype

Table 2.6 Antibodies

Antibody Specificity	Description	Supplier
Mouse IL-1β	Biotin conjugate	R&D Systems
Mouse IL-6	Biotin conjugate	PharMingen
Mouse IL-10	Biotin conjugate	R&D Systems
Mouse IL-12p40	Biotin conjugate	PharMingen
Mouse IL-12p70	Biotin conjugate	R&D Systems
Mouse TNF-α	Biotin conjugate	R&D Systems
Mouse IFN-γ	Biotin conjugate	PharMingen
Mouse IL-17	Biotin conjugate	R&D Systems
Mouse MIP-1	Biotin conjugate	R&D Systems
Mouse MIP-2	Biotin conjugate	R&D Systems
Mouse IL-23	Biotin conjugate	e-Biosciences
B. pertussis PT	Sheep polyclonal antiserum	NIBSC (UK)
B. pertussis Bsp22	Rabbit polyclonal antiserum	This study
B. pertussis FHA	Rabbit polyclonal antiserum	Dr. Brian Keogh, Opsona therapuetics
Mouse CD4	Phycoerythrin (PE) conjugate	e-Biosciences
Mouse CD25	Phycoerythrin-Cy5 (PE-Cy5) conjugate	e-Biosciences
Mouse FoxP3	Fluorescein isothyocyanante (FITC) conjugate	e-Biosciences

Table 2.7 Cytokine standards

Cytokine	Source
Murine IL-1β	R&D Systems
Murine IL-6	PharMingen
Murine IL-10	R&D Systems
Murine IL-12p40	PharMingen
Murine IL-12p70	R&D Systems
Murine TNFα	R&D Systems
Murine IFNγ	PharMingen
Murine IL-17	R&D Systems
Murine MIP-1	R&D Systems
Murine MIP-2	R&D Systems
Murine IL-23	e-Biosciences

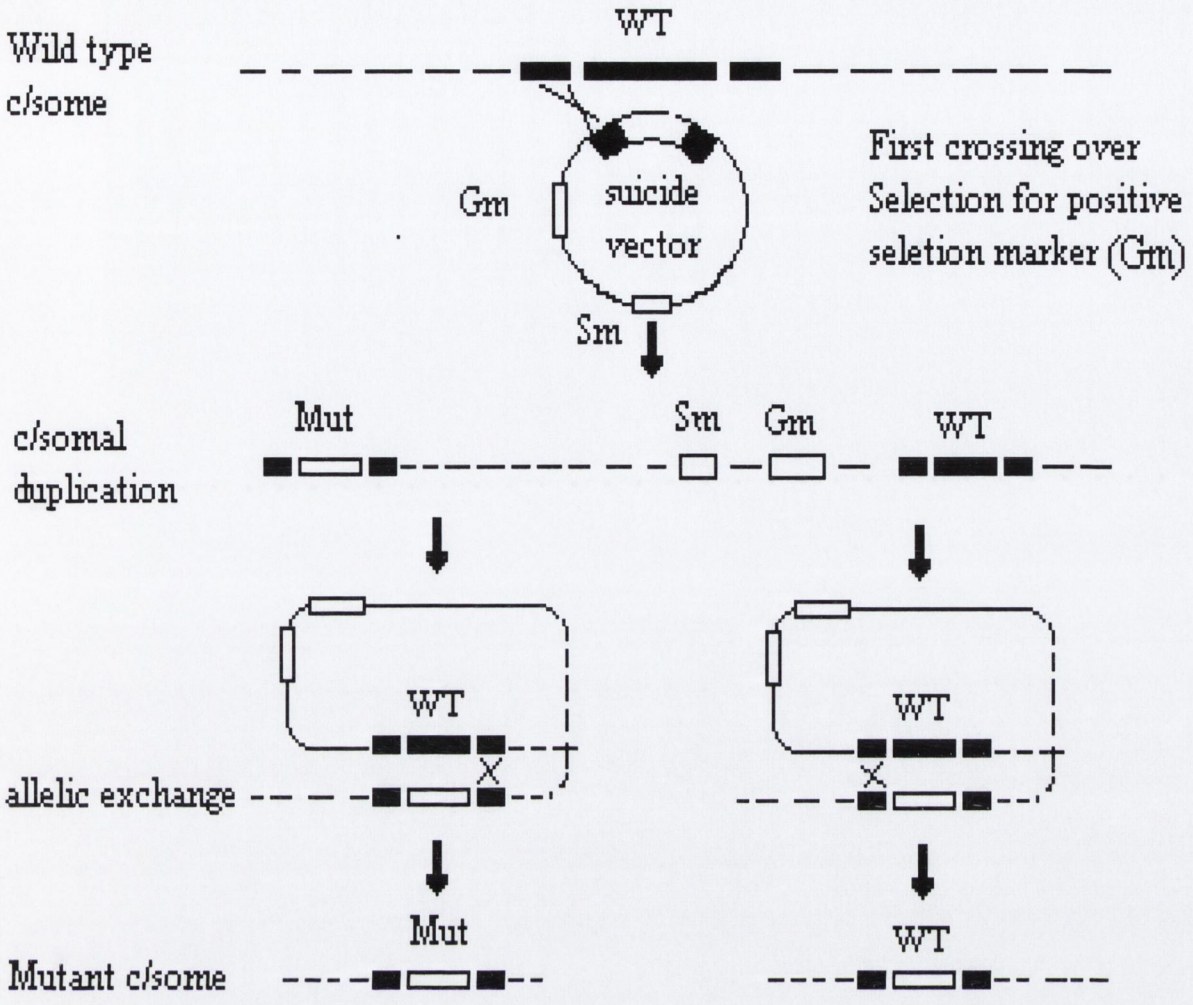


FIGURE 2.1 – Allelic exchange of alleles is mediated by means of a suicide plasmid carrying a positive selection marker (Gm) to select for the integration of the linearised vector into the bacterial chromosome via homologous recombination. Clones expressing this selction marker are then grown to induce a second recombination event, reforming the plasmid and leaving either the wild type or the mutant allele on the chromosome, depending on which site the second recombination event occurs at. Selection for the absence of the plasmid is achieved by using a counterselectable marker (rpsL- encodes target of streptomycin).

Chapter 3

In Vitro Type Three Secretion by *B. pertussis*

3.1 Introduction

The focus of this study was to investigate the importance of the T3SS of *B. pertussis* and *B. bronchiseptica* on the course of infection. The secretion of effector proteins by the bacterial cell to the extracellular milieu or the cytosol of a eukaryotic cell is central to the function of the T3SS. In pathogenic bacteria, these proteins generally interfere with host cell processes and contribute significantly to virulence. The *in vivo* signal for activation of T3 secretion is presumed to be eukaryotic cell contact, which appears to be mediated either by bacterial adhesins or through binding to receptors on target cells, with adhesion of the pathogen shown to be necessary for subsequent effector translocation (Watarai *et al.* 1995; Cornelis *et al.* 1998).

Effectors have been shown to be secreted *in vitro* through the low Ca^{2+} response of *Yersinia* spp. (Michiels *et al.* 1990), the Congo red binding of *Shigella* spp. (Bahrani *et al.* 1997), or the alkaline pH and low Mg^{2+} triggers of the *Salmonella* spp. SPI-1 and SPI-2 T3SS respectively (Daefler 1999) (Francis *et al.* 2002). In the case of *Bordetella* spp. however, the *in vitro* signals required for activation of the T3SS are poorly understood. T3SS-dependent *in vitro* secretion by *Bordetella bronchiseptica* has been demonstrated following growth of the bacterium at 37°C with vigourous aeration in S+S medium (Yuk *et al.* 2000) (Kuwae *et al.* 2003) (Yuk *et al.* 1998). However, in each case secretion was optimal at the mid exponential/early stationary phase of growth. To date, the T3 effector proteins Bsp22, BopB, BopD, BopN and BteA/BopC have been shown to be present in culture supernatants of *B. bronchiseptica* (Yuk *et al.* 2000) (Kuwae *et al.* 2003) (Panina *et al.* 2005). The demonstration of the secretion of effector proteins at 37°C is in agreement with current knowledge of expression of Bvg^+ phase virulence factors (discussed in section 1.3.1). The transcription of T3SS genes of *B. bronchiseptica* RB50, *B. pertussis* 18323, *B. pertussis* Tohama I and 2 strains of *B. parapertussis* has been shown to be regulated by the BvgAS control system (Yuk *et al.* 1998) (Mattoo *et al.* 2004).

Bioinformatic analysis of the T3SS operon in both *B. pertussis* and *B. bronchiseptica* has revealed an extremely high level of similarity between the 2 species (Table 1). The T3SS gene sequences have been downloaded from the Sanger pathogen sequencing unit (http://www.sanger.ac.uk/Projects/B_pertussis/) along with the Artemis

genome viewer available from the same web-page, and this software has been used in this study to compare the relative sizes of *Bordetella* T3SS gene products (Table 1). In addition, BLAST analysis performed on these putative T3SS genes showed that the T3SS genes in *B. pertussis* and *B. bronchiseptica* have high homology with T3SS genes from other species. This analysis has revealed a nucleotide level similarity of 98% across the entire T3SS gene cluster in both *B. pertussis* and *B. bronchiseptica.*

The sequence analysis provides circumstantial evidence that the T3SS functions in a similar manner in both species. However, to date, *in vitro* secretion of effectors by *B. pertussis* has not been reported. On the contrary, recent studies (Mattoo *et al.* 2004) failed to detect *Bordetella* T3SS effectors in culture supernatants of *B. pertussis* Tohama I in contrast to the abundant T3SS effector proteins present in supernatant of cultured *B. bronchiseptica.* This is in agreement with earlier studies which reported that *B. pertussis* Tohama I did not express *B. bronchiseptica* specific T3SS-dependant phenotypes such as effector secretion, *in vitro* cytotoxicity and enhanced mouse lung and trachea colonization observed with *B. bronchiseptica* (Fauconnier *et al.* 2001). This apparent lack of functional T3SS activity in *B. pertussis* is consistent with the current dogma of *Bordetella* spp. biology ; It has been suggested that the recent evolution of *B. pertussis* from *B. bronchiseptica*, in response to the increased concentration and dense clustering of the human host, has sacrificed its long-term host persistence (by losing expression of virulence factors that promote persistence, such as the T3SS) in favour of increased and more rapid host transmission (by acquiring expression of virulence factors implicated in increased host transmission, such as PT and TcfA).

However, as is often the case, genetic studies have suggested that the function of the T3SS in *B. pertussis* is slightly more complicated than originally thought. There appears to be some confusion relating to the expression of T3SS genes in *B. pertussis.* Yuk *et al* (1998) demonstrated that, in contrast to *B. bronchiseptica*, the essential T3SS components *bscN, bscJ, bscK and bscL* were not transcribed by *B. pertussis* Tohama I *in vitro* (Yuk *et al.* 1998). Fauconnier *et al* (2001) also noted that *bscN* was not expressed by *B. pertussis* Tohama I *in vitro* (Fauconnier *et al.* 2001), despite using a similar experimental approach to that used in *B. bronchiseptica.* More recently, Hot *et al* (2003) showed, by microarray analysis, that *bscJ* was transcriptionally upregulated *in vitro* by *B.*

pertussis Tohama I (Hot *et al.* 2003). In addition a regulatory locus 3' to the *bsc* locus encoding genes involved in the transcriptional activation, translation and secretion of the T3SS gene products has been identified in *Bordetella* (Mattoo *et al.* 2004). Moreover, this locus is intact, transcriptionally active and BvgAS activated in *B. pertussis* Tohama I and *B. parapertussis,* suggesting an evolutionary pressure to maintain the T3SS (Mattoo *et al.* 2004). Thus, the emerging picture suggests that while human adapted *Bordetella* transcribe the T3SS during *in vitro* growth, secretion is post-transcriptionally blocked in these strains.

Bsp22 is a 22 kDa hydrophilic polypeptide first detected in culture supernatants of *B. bronchiseptica* cultures by Yuk and colleagues (Yuk *et al.* 2000) and more recently by Kuwae *et al* (2003). It is encoded by a 615 bp ORF located on the T3SS operon in both *B. pertussis* and *B. bronchiseptica* (http://www.sanger.ac.uk/Projects/B_pertussis/ and Table 1). It is a unique protein, showing no apparent homology to any known protein outside of *Bordetella* spp. in the genbank database and is not present in T3SS gene clusters in other bacteria. Bsp22 has been shown to be secreted in a T3SS-dependent manner and important for *B. bronchiseptica* establishing its full range of T3SS-mediated effects, including persistent colonization of the respiratory tracts of mice, down-regulating the production of anti-*Bordetella* antibodies in infected mice and the induction of apoptosis/cytotoxicity in macrophages (Yuk *et al.* 2000). The relative abundance of Bsp22 in culture supernatants makes it suitable for use as a marker for the Bordetella T3SS.

This study examined the *in vitro* secretion of the *Bordetella* T3SS substrate Bsp22 by *B. bronchiseptica* RB50, laboratory adapted *B. pertussis* Tohama I and a range of recent clinical isolates of *B. pertussis* obtained from the ATCC and from nosocomial infections in the Netherlands. The results show that while Bsp22 can readily be detected in the culture supernatants of *B. bronchiseptica* RB50 and a significant proportion of clinical isolates of *B. pertussis* that have not been extensively passaged in the laboratory, it could not be detected in *B. pertussis* Tohama I and Wellcome 28, nor can it be induced in *B. pertussis* Tohama I by modulating growth conditions.

3.2 Results

3.2.1 Cloning of *bsp22*

Due to its relative abundance in the supernatants of wild-type *B. bronchiseptica* cultures, this study set out to clone and express *B. pertussis* Bsp22 in *E. coli* and use purified recombinant Bsp22 as an antigen to generate Bsp22-specific polyclonal antiserum. The aim was to use this antiserum to optimise and monitor the *in vitro* activation of the T3SS of *Bordetella* spp. The *bsp22* gene was amplified from *B. pertussis* strain 18323 genomic DNA by PCR with oligonucleotides (MWG Biotech, Germany) PAB37 5'-CGGAAGCTTTTAGCGCATGTTGCTGGTG-3' which bind bp 596-615 of *bsp22* and PAB38 5'-AGCGGATCCAGCATTGATCTCGGAGTTCAC-3' which bind bp 4-25 of *bsp22* (Fig 3.2 and Table 2.4). The resulting product was digested with *Hin*DIII and *Bam*HI (cleavage sites underlined in primer sequences) (Invitrogen, CA, USA) and inserted into corresponding sites of the His-tagged expression vector pQE-80 (QIAGEN, UK) (Fig 3.3 and Table 2.3). This vector contains the phage T5 promoter for strong expression and 2 *lac* operator sequences to ensure efficient repression of the promoter prior to induction with IPTG, as well as an ampicillin resistance cassette to facilitate selection. The *bsp22* gene was cloned in-frame with the 6X N-terminal His tag located on pQE-80. The ATG start codon of *bsp22* was not included in the PCR product, since this may have resulted in expression of smaller proteins lacking the 6 X His-tag. The sequence and orientation of the cloned gene was confirmed by restriction digestion and sequencing (MWG Biotech). This plasmid was named pAPB15 (Fig 3.4 and Table 2.3) from which recombinant His-tagged Bsp22 could be expressed in *E. coli.*

3.2.2 Expression and purification of recombinant Bsp22 and its use for generating Bsp22 specific polyclonal antiserum

Recombinant His-Bsp22 (rHis-Bsp22) was expressed in *E .coli* strain XL1-Blue bearing pAPB15 by addition of 1 mM IPTG to an exponentially growing bacterial culture. rHis-Bsp22 was purified on an Ni-NTA in denaturing conditions (Nickel

exchange column, QIAGEN) and pure protein fractions of the expected molecular weight were eluted (Fig 3.5 A) according to manufacturers instructions. This protein was detected using a His-tag specific antibody by western blot (Fig. 3.5 B). ~ 2.3 mg of protein was purified per 150 ml culture, and was 80–85 % pure by Coomassie stained SDS. rHis-Bsp22 was dialysed in order to remove salts and urea from the preparation, and emulsified with CFA. The dialysed protein was used to generate Bsp22-specific polyclonal anti-serum from an adult rabbit. A rabbit was immunised with 115μg rHis-Bsp22 emulsified in CFA twice boosted on days 14 and 28 with 115 μg rHis-Bsp22 emulsified with IFA and the serum was collected on day 40. This serum was shown to recognise rHis-Bsp22 by western blotting (Fig.3.5 C).

3.2.3 *B. pertussis* Tohama I, in contrast to *B. bronchiseptica* RB50, does not readily display T3SS activation *in vitro*.

Several reports have demonstrated the relative abundance of *Bordetella* T3SS substrates, such as Bsp22, BopN, BopD (Yuk et al. 2000) (Nogawa *et al.* 2004), BopB (Kuwae *et al.* 2003) and BteA/BopC (Panina et al. 2005; Kuwae *et al.* 2006) in the supernatants of *B. bronchiseptica* RB50 cultures. However to date T3SS substrates have not been found in the supernatants of *B. pertussis* cultures. Further, there have been conflicting reports on the transcription of the *bsc* loci in *B. pertussis* (Yuk *et al.* 1998; Fauconnier *et al.* 2001; Hot *et al.* 2003; Mattoo *et al.* 2004). Therefore, this study examined the production and secretion of Bsp22 by both *B. bronchiseptica* RB50 and *B. pertussis* Tohama I over the course of *in vitro* growth in S+S liquid broth. Bacterial colonies removed from BG agar plates were used to inoculate precultures. Actively growing precultures were then used to inoculate 100 ml cultures at a starting OD_{600} of 0.2, and whole cell lysates (to analyse production) and supernatant protein samples (to analyse secretion) were prepared following precipitation with 10% TCA after 8, 12, 24, 28 and 32 hours of growth. The samples were resolved using 10% SDS gels and then subjected to western blotting with polyclonal anti-Bsp22 antiserum. While slight production of Bsp22 was detected in whole cell lysates prepared following 24, 28 and 32 hours of growth, secreted Bsp22 could not be detected in the supernatant of *B. pertussis*

Tohama I at any timepoint during the course of *in vitro* growth (Fig 3.6 A). In contrast production and secretion of Bsp22 was detected during *in vitro* growth of *B. bronchiseptica* RB50 after (Fig 3.6 B), during the early-log and stationary phases of growth (Fig 3.6 D).

These results suggest that while *B. pertussis* Tohama I might produce low concentrations of Bsp22 during the later phases of bacterial growth, it does not secrete Bsp22, and therefore does not possess T3SS activity *in vitro*.

3.2.4 *In vitro* T3 secretion by *B. pertussis* Tohama I cannot be induced by modulating growth conditions.

In some species, the secretion of T3SS substrates *in vitro* requires a specific signal, such as a drop in $[Ca^{2+}]$ or pH, as has been demonstrated for *Yersinia* and *Salmonella* spp. respectively. It has been demonstrated that the T3SS of *Bordetella* spp. is regulated by the BvgAS regulon, and that the *B. bronchiseptica* RB50 T3SS loci are expressed under Bvg^+ or virulent growth conditions (Yuk *et al.* 1998). However, in contrast to *B. pertussis, B. bronchiseptica* has an excess of 1000 genes that are not expressed or inactivated in *B. pertussis* (Parkhill *et al.* 2003), the majority of which are believed to endow *B. bronchiseptica* with the ability to interact with a wider range of molecules and nutrients than *B. pertussis,* and hence permit a wider host range and an *ex vivo* growth phase (Preston *et al.* 2004). *B. pertussis*, an exclusively human pathogen, does not survive *ex vivo*. Therefore, it is possible, that the signals necessary for T3SS activation in *B. pertussis* Tohama I are not present during normal *in vitro* growth in S+S broth.

Consequently, we examined the effect of modulating growth conditions on the secretion of Bsp22 during *in vitro* growth of *B. pertussis* Tohama I. A preculture of *B. pertussis* Tohama I was grown to stationary phase (OD_{600} ~1.5, Fig 3.6 C) and bacteria were subcultured to a starting OD_{600} ~0.7 (mid-log phase, Fig 3.6 C) in 5 mls of S+S. Following 2 hours of growth at 37°C, the S+S medium was modified by the addition of a) 5% FCS as a source of mammalian proteins, b)1% glycerol as a source of excess

carbohydrate, c)1mM EDTA as a chelating agent for divalent cations, d)1 μg/ml BSA as a source of excess protein to mop up protease activity and e) 100 mM Na_2PO_4 as an additional buffering agent to regulate pH. Supernatants were removed from each culture after 4 hours and subjected to western blotting with anti-Bsp22 antibodies. Secretion of Bsp22 by *B. pertussis* Tohama I could not be detected following the addition of FCS, glycerol, EDTA, BSA or Na_2PO_4 to S+S medium (Fig. 3.7).

These results demonstrate that T3SS activity of *B. pertussis* Tohama I cannot be induced *in vitro* by modulating these growth conditions.

3.2.5 *In Vitro* T3 secretion by low passage *B. pertussis* isolates 12743 and 9340.

B. pertussis Tohama I was first isolated in Japan in 1954 (Kasuga *et al.* 1954) and is the most well studied and well characterised of *B. pertussis* isolates, which is partly why it was chosen for genomic sequencing (Parkhill *et al.* 2003). However, due to its extensive study and characterisation, original stocks of the bacterium are not available. Thus it is likely that most isolates of *B. pertussis* Tohama I in use by researchers today have been through extensive laboratory passage. Since T3SS activity is regulated and activated *in vivo* by eukaryotic cell contact, we investigated the T3SS activity *in vitro* in 3 isolates of *B. pertussis* from the ATCC, which had not been extensively passaged in the laboratory, in comparison to the laboratory-adapted strains, *B. pertussis* Tohama I and *B. pertussis* Wellcome 28.

100 ml cultures of a) *B. pertussis* ATCC 12743 (5375 [3865]), hereafter referred to as *B. pertussis* 12743, b) *B. pertussis* ATCC 12742 (5374 [3747]), hereafter referred to as *B. pertussis* 12742, c) *B. pertussis* ATCC 9340 (5 [17921]), hereafter referred to as *B. pertussis* 9340, d) *B. pertussis* Tohama I and e) *B. pertussis* Wellcome 28 were inoculated at a starting OD_{600} of 0.2, and grown to stationary phase. Protein samples from the supernatants of stationary phase cultures were precipitated with 10% TCA, resolved on 10% SDS gels and analysed by western blotting with anti-Bsp22 antibodies. Significant quantities of secreted Bsp22 was detected in the supernatants of *B. pertussis* 12743 and *B. pertussis* 9340, indicating that these 2 strains of *B. pertussis* possess T3SS

activity *in vitro* (Fig 3.8). However, secreted Bsp22 could not be detected in supernatants from *B. pertussis* 12742, or either of the two laboratory adapted strains *B. pertussis* Tohama I and *B. pertussis* Wellcome 28, suggesting that these strains do not display T3SS activity *in vitro*.

Strain *B. pertussis* 12743 was chosen for further analysis and the production and secretion of Bsp22 by this strain was investigated over the course of *in vitro* growth, in the manner described earlier for *B. bronchiseptica* RB50 and *B. pertussis* Tohama I (Section 3.2.3). Significant quantities of secreted Bsp22 was detected in the supernatant of *B. pertussis* 12743 culture after 12, 24, 28 and 32 hours (Fig 3.9 A), during the late-log and stationary phases of growth (Fig 3.9 B).

These results demonstrate that *B. pertussis* 12743 and *B. pertussis* 9340 possess T3SS activity *in vitro*, and that *B. pertussis* 12743, like *B. bronchiseptica* RB50, secretes Bsp22 over the course of *in vitro* growth.

3.2.6 *B. pertussis* 12743 and 9340 are not slow growing variants of *B. bronchiseptica*.

Since *Bordetella* T3SS activity and Bsp22 secretion is usually a feature only of *B. bronchiseptica*, the phenotype of *B. pertussis* 12743 and *B. pertussis* 9340 was investigated to examine the possibility that they may more closely resemble *B. bronchiseptica* than *B. pertussis*. Several studies have that reported *B. pertussis*, unlike *B. bronchiseptica*, is unable to grow on Macconkey agar base (Mazengia *et al.* 2000) (Vandamme *et al.* 1996) (von Wintzingerode *et al.* 2001). Therefore *B. pertussis* 12743 and *B. pertussis* 9340 were tested for the ability to grow on Macconkey agar base, in comparison to *B. bronchiseptica* and *B. pertussis* Tohama I.

Bacteria were plated from glycerol stocks onto BG agar and then subcultured after 3 days growth directly onto Macconkey agar plates. Figure 3.10 shows photographs of the agar plates following 3 days incubation at 37°C. In contrast to *B. bronchiseptica* RB50 (Fig 3.10 A) no growth of *B. pertussis* Tohama I (Fig 3.10 B), *B. pertussis* 12743

(Fig 3.10 C) or *B. pertussis* 9340 (Fig 3.10 D) was detected on Macconkey agar plates after incubation. These results suggest that, with the exception of T3SS activity, *B. pertussis* 12743 and *B. pertussis* 9340 are phenotypically similar to *B. pertussis* Tohama I and not merely slow growing variants of *B. bronchiseptica*.

3.2.7 *In vitro* T3 secretion by clinical isolates of *B. pertussis*

To further investigate the possibility that T3SS activity by *B. pertussis* spp. is a feature of non-lab adapted isolates, the supernatants from stationary phase cultures of 16 additional clinical isolates of *B. pertussis* which had not been extensively passaged in the laboratory were examined for Bsp22 secretion. Table 3.1 shows, where known, the source, site of isolation, year of isolation and patient age in years of 16 clinical isolates of *B. pertussis* obtained from Prof. Frits R. Mooi, Laboratory for Vaccine Preventable Diseases, RIVM, Netherlands. All 16 strains of B. *pertussis*, in addition to *B. pertussis* Wellcome 28, Tohama I and 18323, were grown to stationary phase. Supernatant protein samples from each strain were prepared following precipitation with 10% TCA, separated in 10% SDS gels and probed with anti-Bsp22 antibodies by Western blotting. Significant Bsp22 secretion was detected in supernatants of BP 0304, BP 0305, BP 0546, BP 0780 and BP 0640, BP 0950, BP 0558, BP 0597, BP 2913, BP 2778 and BP 2777. In contrast, Bsp22 secretion was not detected in the supernatants of two of the clinical isolates, BP 2890 and BP 1868, and the laboratory adapted BP Tohama I and Wellcome 28 (Fig 3.11). Three of the isolates tested, BP 0888, BP 1926 and BP 2740 consistently grew poorly during *in vitro* growth in repeated experiments, and yet a very slight detection of Bsp22 was observed for these strains, in contrast to the significant secretion of Bsp22 observed for BP 18323 (Fig 3.11 C). Although it is likely that these three strains, BP 0888, BP 1926 and BP 2740, do possess T3SS activity *in vivo*, it is not possible to definitively conclude that these clinical isolates of *B. pertussis* secrete significant quantities of Bsp22 *in vitro*.

These results demonstrate that a large proportion (~70%) of the unpassaged clinical isolates of *B. pertussis* investigated in this study secrete significant quantities of Bsp22 *in vitro*, and therefore possess a functional T3SS. These results also suggest that

T3SS activity of *B. pertussis* spp may be related to *in vivo* growth, and that the lack of T3SS activity seen by well-studied strains of *B. pertussis* such as Tohama I and Wellcome 28 may be a result of long-term laboratory passage.

3.3 Discussion

T3SS of gram negative bacteria such as *Bordetella* spp. are complex virulence related secretion systems involved in bacterial subversion of host cells for the purpose of facilitating host colonisation and prolonging bacterial survival. Since the identification of the highly conserved genes encoding the T3SS machinery in the genomes of *B. bronchiseptica* RB50 (Yuk *et al.* 1998) and *B. pertussis* Tohama I (Kerr *et al.* 1999), a range of T3SS-dependent effects have been reported for *B. bronchiseptica* RB50 including *in vitro* secretion of T3SS effector proteins (Yuk *et al.* 2000), host cell cytotoxicity (Yuk *et al.* 2000; Kuwae *et al.* 2003; Stockbauer *et al.* 2003; Panina *et al.* 2005; Kuwae *et al.* 2006), modulation of DC maturation and macrophage activation (Skinner *et al.* 2004; Reissinger *et al.* 2005) and enhanced lung and tracheal colonisation (Yuk *et al.* 2000; Skinner *et al.* 2005; Pilione and Harvill 2006). However, though transcription of the T3SS loci has been observed in *B. pertussis* Tohama I (Hot *et al.* 2003; Mattoo *et al.* 2004), there have been no reports of T3SS protein secretion *in vitro*. In addition, a study conducted by Fauconnier *et al* reported no difference in protein secretion, cellular invasion, cytotoxicity or mouse lung colonization between a T3SS mutant of *B. pertussis* Tohama I and the parental strain (Fauconnier *et al.* 2001). The present study has demonstrated that while the T3SS of laboratory adapted strains of *B. pertussis* such as Tohama I and Wellcome 28 is not functionally active *in vitro*, nor can it be activated by modulating growth conditions, the T3SS of a range of clinical isolates of *B. pertussis* not extensively passaged in culutre is active during *in vitro* growth.

The secretion of several proteins by *B. bronchiseptica* has been shown to be T3SS-dependent, including the two translocators BopB (Kuwae *et al.* 2003) and BopD (Yuk *et al.* 2000; Nogawa *et al.* 2004), the sensor/plug protein BopN (Yuk *et al.* 2000), the cytotoxin BteA/BopC (Panina *et al.* 2005; Kuwae *et al.* 2006) and Bsp22 (Yuk *et al.* 2000). Bsp22 is secreted in high abundance by the T3SS and as such is a useful marker for T3SS activity *in vitro*. The present study employed the use of Bsp22 as a marker of T3SS activity *in vitro*. Anti-Bsp22 polyclonal anti-serum was generated and used to monitor the secretion of Bsp22 by *B. bronchiseptica* RB50 and *B. pertussis* Tohama I. The results shown in this chapter confirm that while *B. bronchiseptica* readily secretes

Bsp22 during the course of *in vitro* growth, *in vitro* T3 secretion could not be detected in culture supernatants of *B. pertussis* Tohama I.

It has been reported that modulation of growth conditions can induce T3 secretion *in vitro* in other human pathogens such as *Yersinia*, *Shigella* and *Salmonella*. This has involved the use of a specific chemical signal, such as the low Ca^{2+} response of *Yersinia* spp. (Michiels *et al.* 1990), the congo red binding of *Shigella* spp. (Bahrani *et al.* 1997), or the alkaline pH and low Mg^{2+} triggers of the *Salmonella* spp. SPI-1 and SPI-2 T3SS respectively (Daefler 1999) (Francis *et al.* 2002). Since *B. pertussis* is generally transmitted directly from host to host and outside of the laboratory does not survive *ex vivo*, it has been hypothesised that the specific signals required for T3SS activation in this species are not present during normal *in vitro* culture conditions. However, in the present study secretion of Bsp22 by *B. pertussis* Tohama I could not be detected following the addition of mammalian proteins, excess carbohydrate, chelating agents or pH regulators to S+S medium. These results demonstrate that the T3SS of *B. pertussis* can not be activated *in vitro* by modulating growth conditions and confirm that, while the T3SS loci may be transcribed, T3SS activity is not a feature of *B. pertussis* Tohama I during *in vitro* growth.

The T3SS of B. *bronchiseptica* RB50 has been implicated in facilitating long-term respiratory tract colonisation of this bacteria (Yuk *et al.* 2000; Skinner *et al.* 2005) (Pilione and Harvill 2006). Infection with *B. pertussis* is not as persistent as that with *B. bronchiseptica*, and the difference in the severity and duration of infection caused by the *Bordetellae* has largely been attributed to differences in virulence factor expression between the species. Since large-scale gene loss is a feature of the recent evolution of *B. pertussis* from *B. bronchiseptica* to facilitate human host restriction (Parkhill *et al.* 2003), it has been suggested that T3SS expression, and therefore host persistence, has been lost in *B. pertussis* in favour of expression of virulence factors, such as TcfA and PT, that increase host transmission (Preston *et al.* 2004). However, this suggestion is inconsistent with the observation of transcription of the *bsc* loci *B. pertussis* Tohama I (Hot *et al.* 2003; Mattoo *et al.* 2004), which points to evolutionary pressure to conserve the T3SS in *B. pertussis*.

In contrast to well studied *B. pertussis* isolates such as BP Tohama I and BP Wellcome 28, this study has demonstrated T3SS activity by two strains of *B. pertussis* available from the ATCC and deposited as clinical isolates by Grace Elderling and Margaret Pittman in the 1950s, BP 12743, BP 9340, as well as BP 18323. BP 12743 was found to secrete Bsp22 in the same manner as *B. bronchiseptica* RB50 over the course of *in vitro* growth. This study has also detected the secretion of Bsp22, and therefore T3SS activation, during the *in vitro* growth of a significant portion (~70%) of clinical isolates of *B. pertussis* derived from infected individuals in the Netherlands. These strains of *B. pertussis* originated in nosocomial infections and none had been extensively characterised or passaged in the laboratory. The one laboratory-adapted strain of *B. pertussis* where T3SS activity was detected, BP 18323, has been studied in detail in the laboratory. It has long been recognised that BP 18323 is more virulent in mice than other *B. pertussis* isolates (Adams 1970), and it has been demonstrated that this strain is genetically more closely related *B. bronchiseptica* than to *B. pertussis* (Musser *et al.* 1986). Consequently, concerns have been raised as to whether this strain should be classified as *B. pertussis* (Preston *et al.* 2004). *B. pertussis* Tohama I, obtained from the ATCC, was first isolated in the 1950s (Kasuga *et al.* 1954) and has been extensively characterised since then. Thus, it is unlikely that original stocks of the bacteria are available, and the *B. pertussis* Tohama I isolates in use by researchers today have been through extensive laboratory passage. Since T3SS activity is regulated and activated *in vivo* by eukaryotic cell contact, it is possible that the lack of T3SS activity observed by *B. pertussis* Tohama I and Wellcome 28 during *in vitro* growth is a feature of long term laboratory passage in the absence of eukaryotic cell contact or *in vivo* infection.

The results presented in this chapter have demonstrated that most clinical isolates of *B. pertussis* express T3SS activity during *in vitro* growth, but that the expression of the T3SS among *B. pertussis* isolates may be influenced by prolonged laboratory cultivation and passage. These results suggest, given the importance of the T3SS during infection with *B. bronchiseptica*, that the T3SS may be an important and previously uncharacterised virulence determinant for *B. pertussis* spp. during infection.

```
Bp TohamaI_bsp22   AGCATGGATATCCAGACTCTCATGGTGTATGTGCAGGGTCGTCGCGCCGAAC
Bp 12743_bsp22     AGCATGGATATCCAGACTCTCATGGTGTATGTGCAGGGTCGTCGCGCCGAAC

                   TCCTCACGGCTCAAATGCAGACCCAGGCCGAAGTGGTGCAGAAGGCCAATG
                   TCCTCACGGCTCAAATGCAGACCCAGGCCGAAGTGGTGCAGAAGGCCAATG

                   AACGCATGGCGCAGCTCAACGAGGTCCTGTCCGCGCTGTCCCGGGCCAAGG
                   AACGCATGGCGCAGCTCAACGAGGTCCTGTCCGCGCTGTCCCGGGCCAAGG

                   CCGAGTTTCCGCCCAATCCGAAGCCGGGCGACACCATCCCGGGCTGGGACA
                   CCGAGTTTCCGCCCAATCCGAAGCCGGGCGACACCATCCCGGGCTGGGACA

                   GCCAGAAGATCAGCCGGATCGAGGTTCCTCTCAATGATGCGCTGCGTGCCG
                   GCCAGAAGATCAGCCGGATCGAGGTTCCTCTCAATGATGCGCTGCGTGCCG

                   CCGGCCTGACGGGCATGTTCGAAGCGCGCGATGGCCGGGTGACCGGCCCCG
                   CCGGCCTGACGGGCATGTTCGAAGCGCGCGATGGCCGGGTGACCGGCCCCG

                   ACGGC
                   ACGGC
```

FIGURE 3.1 Nucleotide sequence alignment of base pairs 67 - 378 of *bsp22* from *B. pertussis* Tohama I compared with the same region of *bsp22* from *B. pertussis* 12743. PCR products were amplified from chromosomal DNA from *B. pertussis* Tohama I and *B. pertussis* 12743 respectively. PCR product sequence analysis was performed by MWG-biotech (Germany).

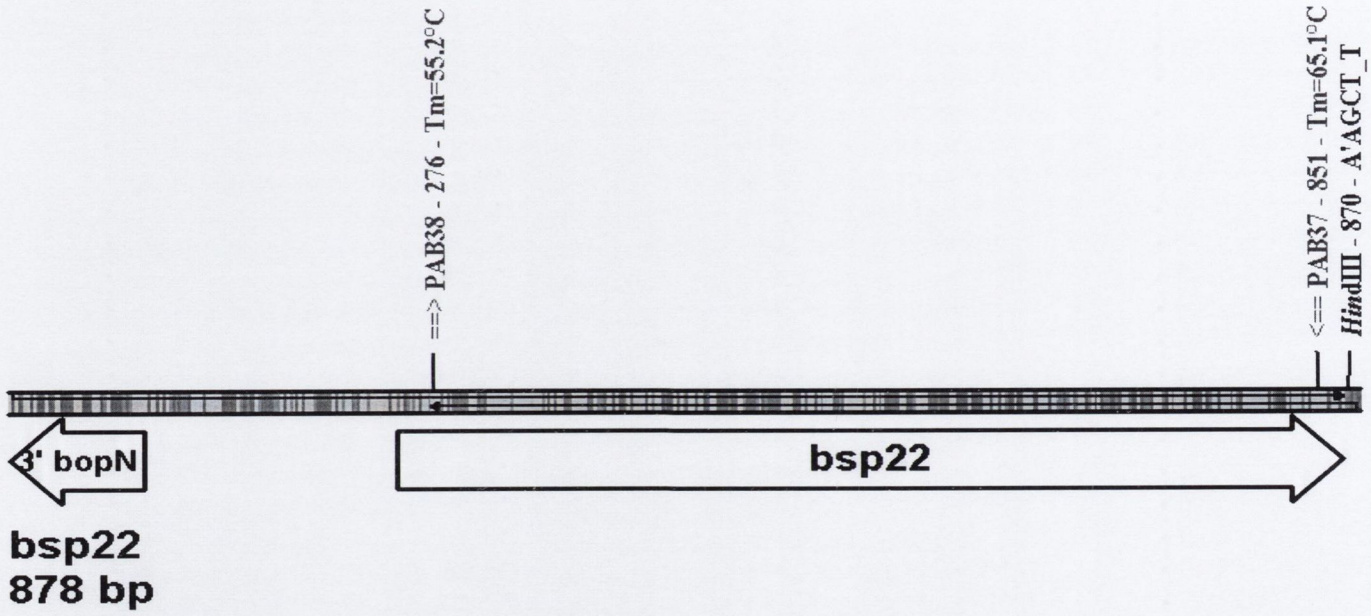


FIGURE 3.2 Bsp22 - Schematic of part of the T3SS locus, showing *bsp22* and part of 5' flanking gene *bopN*. The primer binding sites for PAB38 and PAB37 and the *Hin*dIII restriction site used for cloning are also shown. The other restriction site, *Bam*HI, was engineered into the 5' region of PAB38, and so does not appear on this schematic.

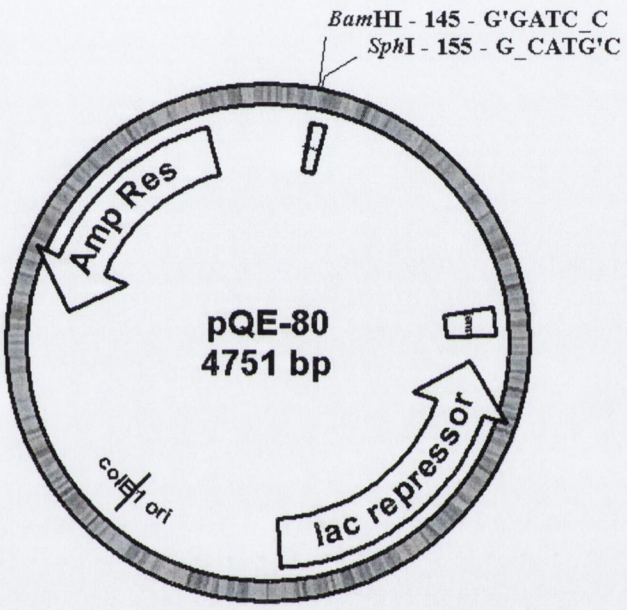


FIGURE 3.3 – Plasmid pQE-80. Cloning vector containing the *Bam*HI and *Hin*dIII restriction sites for insertion of the 575 bp *bsp22* PCR fragment. Plasmid also contains an Ampicillin resistance cassette, T5 promoter and Lac repressor for protein expression, and the colE1 origin of replication.

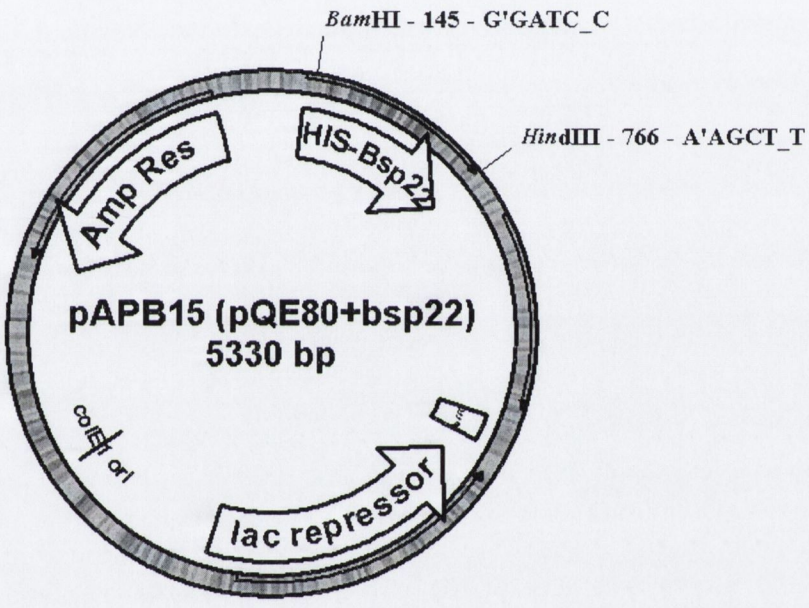


FIGURE 3.4 – Plasmid pAPB15. pQE-80 expression vector containing the 621 bp His-*bsp22*, ampicillin resistance cassette, T5 promoter and Lac repressor for protein expression, and the colE1 origin of replication. The *Bam*HI and *Hin*dIII restriction sites used for cloning are also shown.

FIGURE 3.5 Purification of rHis-Bsp22 and its use to raise anti-Bsp22 antibodies. (A) A coomassie stained SDS-PAGE gel of protein fractions from various stages of chromatography; lane 1, *E. coli* bacterial lysate prior to loading on a Nickel-exchange chromatography column; lanes 2-4, samples of the flow-through collected from the first fraction; lanes 5-9, eluted rHis-Bsp22 protein in the later fractions.

(B) Recognition of rHis-Bsp22 by anti-His antibody; Lanes 1 and 2, two separate samples of rHis-Bsp22 purified from *E. coli* and eluted from a Nickel exchange chromatography column; Lane 3, molecular weight markers. The 23 k Da rHis-Bsp22 is between the 20 kDa and 25 kDa markers.

(C) Recognition of rHis-bsp22 by rabbit anti-Bsp22 antiserum. 0.5, 0.05 and 0.005 μg of purified rHis-Bsp22 were loaded in lanes 1, 2, and 3 respectively, and probed by western blotting with anti-Bsp22 antiserum.

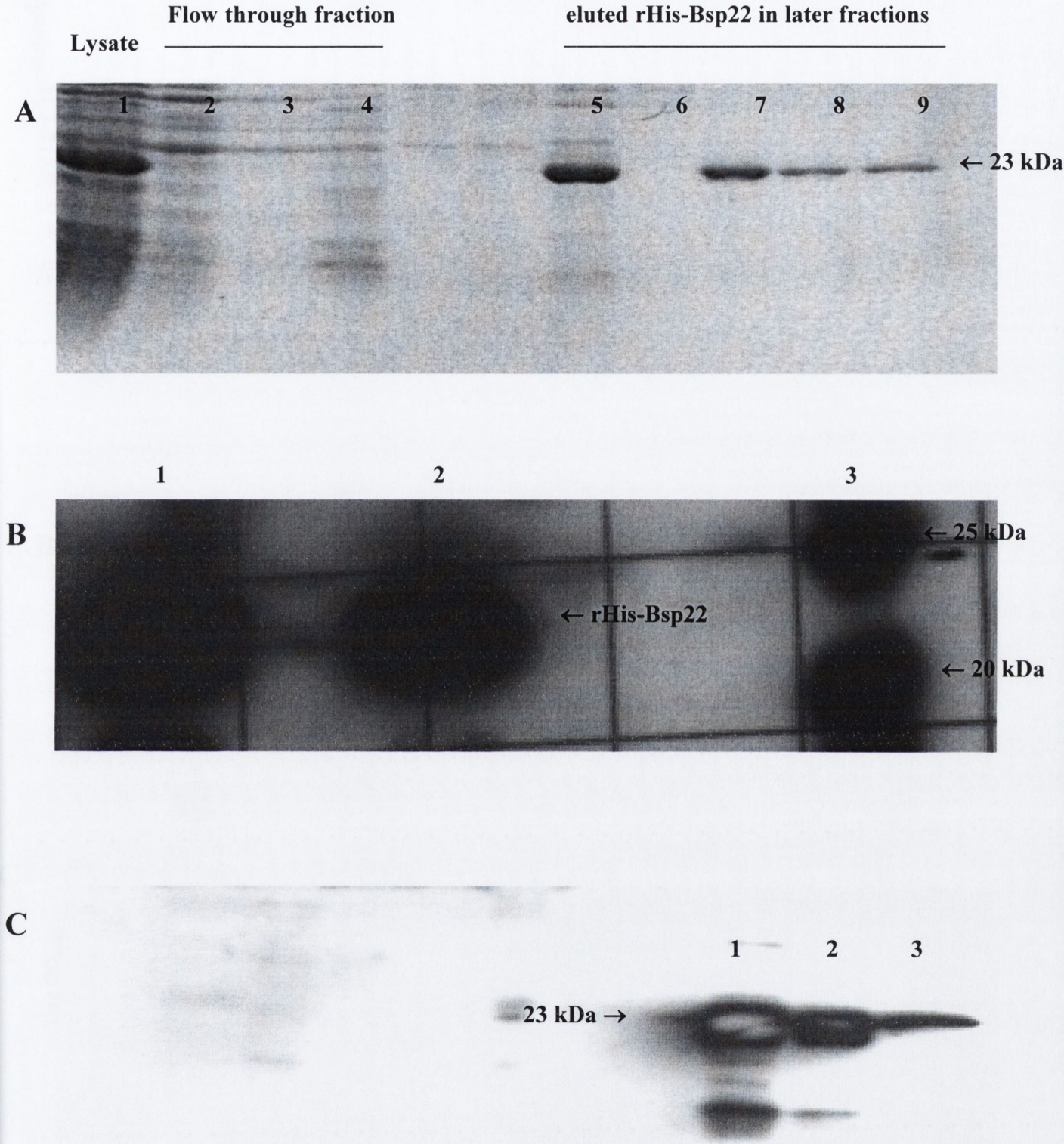
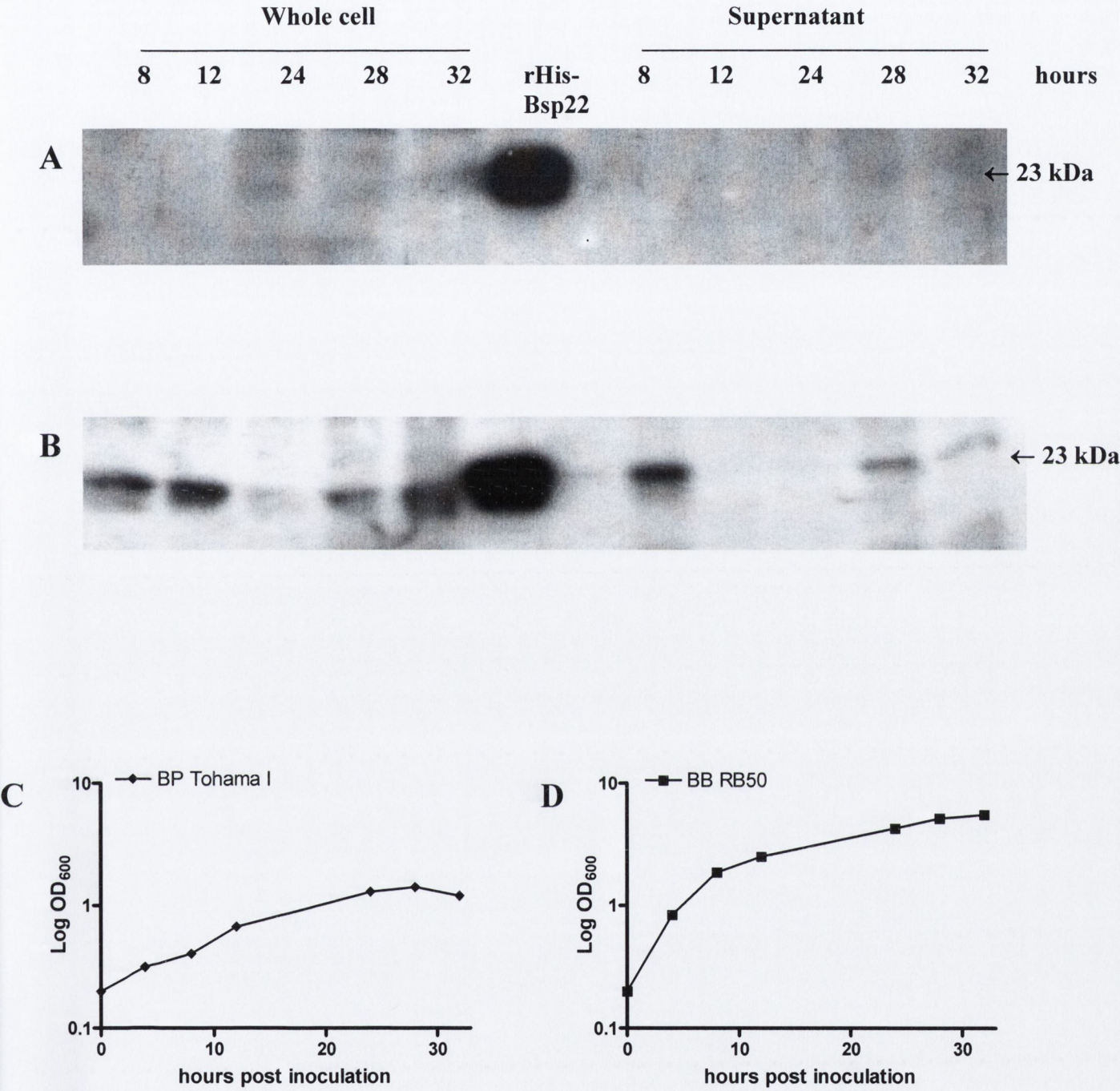

Flow through fraction
Lysate
eluted rHis-Bsp22 in later fractions
A
1
2
3
4
5
6
7
8
9
← 23 kDa
1
2
3
B
← 25 kDa
← rHis-Bsp22
← 20 kDa
C
1
2
3
23 kDa →

FIGURE 3.6 Bsp22 is produced and secreted during *in vitro* growth of *B. bronchiseptica* RB50 but not *B. pertussis* Tohama I. *B. pertussis* Tohama I (A and C) and *B. bronchiseptica* RB50 (B and D) were inoculated at a starting OD_{600} of 0.2 from actively growing precultures. Whole cell (lanes 1-5) and supernatant (lanes 7-11) protein samples were prepared following precipitation with 10% TCA after 8, 12, 24, 28 and 32 hours of growth. Protein samples from *B. pertussis* Tohama I (A) and *B. bronchiseptica* RB50 (B) were probed with anti-Bsp22 antibodies by western blot. The course of *in vitro* growth was monitored by OD_{600} measurements (C and D).


Whole cell
Supernatant
8
12
24
28
32
rHis-Bsp22
8
12
24
28
32
hours
A
← 23 kDa
B
← 23 kDa
C
BP Tohama I
Log OD600
10
1
0.1
0
10
20
30
hours post inoculation
D
BB RB50
Log OD600
10
1
0.1
0
10
20
30
hours post inoculation

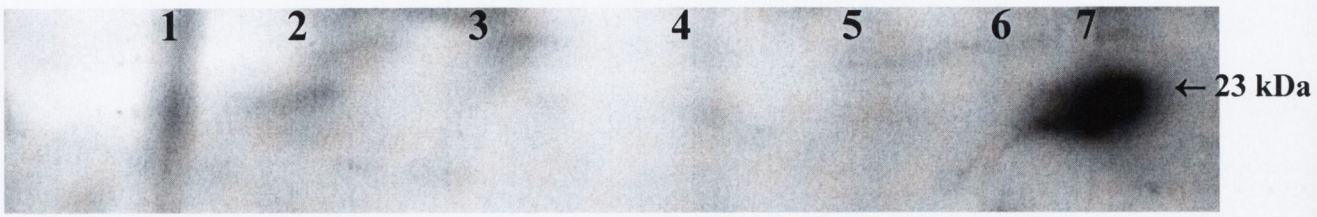


FIGURE 3.7 The T3SS of *B. pertussis* Tohama I cannot be activated *in vitro* by modulating growth conditions. Mid-log cultures (OD_{600} ~0.7) of *B. pertussis* Tohama I were grown for 4 hours in S+S supplemented with 5% FCS as a source of mammalian proteins (lane 1), 1% glycerol as a source of excess carbohydrate (lane 2), 1 mM EDTA as a chelating agent (lane 3), 1 μg/ml BSA as a source of excess protein to mop up protease activity (lane 4), 100 mM Na_2PO_4 as an additional buffering agent to regulate pH (lane 5) or unmodified S+S (lane 6). Supernatant protein samples were prepared following precipitation with 10% TCA, separated by 10% SDS gel and probed with anti-Bsp22 antibodies. The positive control, rHis-Bsp22 (2 μg), was loaded in lane 7.

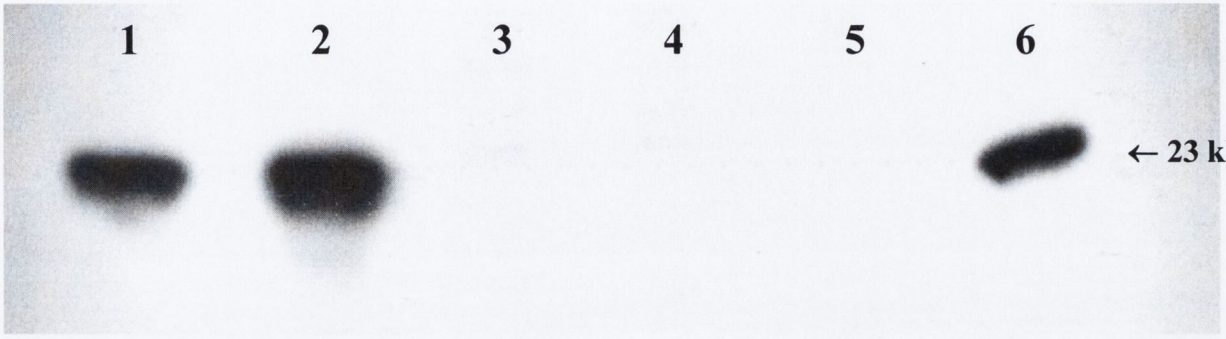


FIGURE 3.8 *In Vitro* T3 secretion by *B. pertussis* clinical isolates 9340 and 12743, but not 12742, or lab adapted strains Tohama I or Wellcome 28. Protein samples from supernatants of stationary phase cultures of *B. pertussis* 9340 (lane 1), 12743 (lane 2), 12742 (lane 3), Tohama I (lane 4) and Wellcome 28 (lane 5) prepared following precipitation with 10% TCA, in addition to 2 μg rHis-Bsp22, were separated by 10% SDS gel and probed with anti-Bsp22 antibodies by Western blotting.

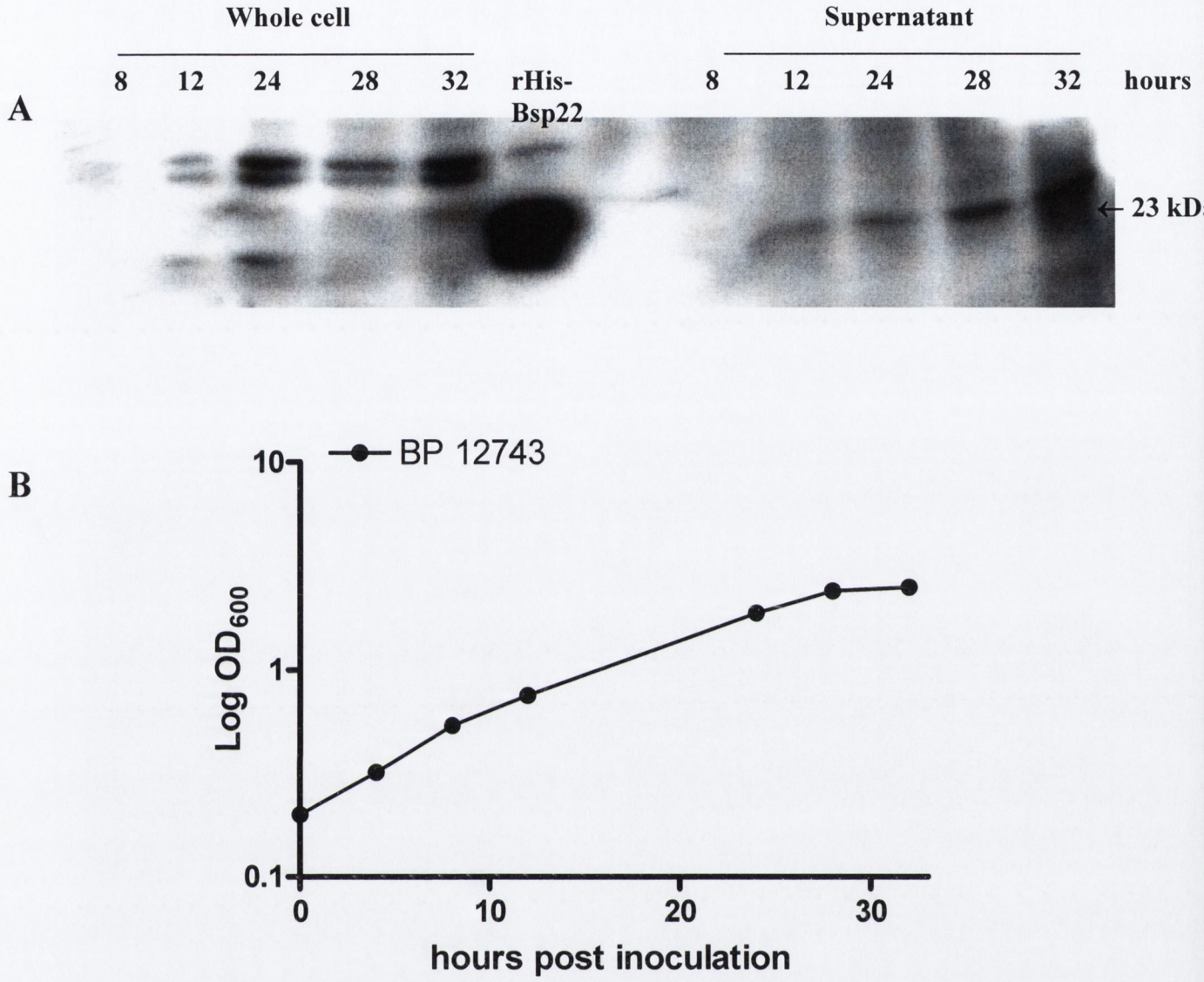


FIGURE 3.9 Bsp22 is produced and secreted by *B. pertussis* 12743 during *in vitro* growth. *B. pertussis* 12743 was inoculated at a starting OD_{600} of 0.2 from an actively growing preculture. The course of *in vitro* growth was monitored by OD_{600} determination (B). Protein samples from whole cell and supernatant were prepared following precipitation with 10% TCA after 8, 12, 24, 28 and 32 hours of growth. Protein samples from *B. pertussis* 12743 and 2 μg rHis-Bsp22 (lane 6) were separated by 10% SDS gel and probed with anti-Bsp22 antibodies by Western blotting.

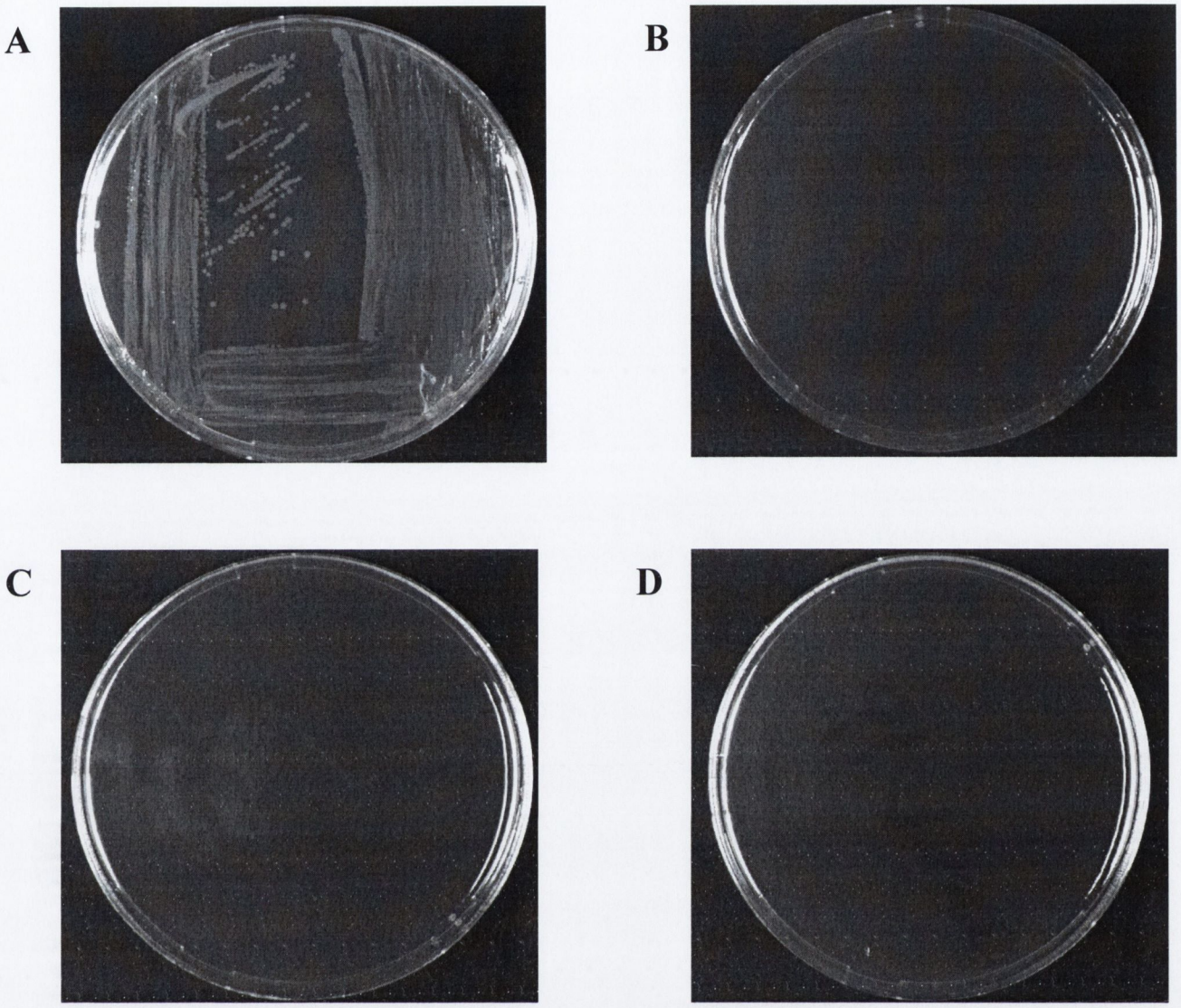


FIGURE 3.10 Growth of *B. bronchiseptica* RB50, but not *B. pertussis* 12743, 9340 or Tohama I, on Macconkey agar.

Macconkey agar plates streaked with *B. bronchiseptica* RB50 (A), *B. pertussis* Tohama I (B), *B. pertussis* 12743 (C) or *B. pertussis* 9340 (D). Bacteria were plated from glycerol stocks onto BG agar and after 3 days growth were subcultured onto Macconkey agar and incubated at 37°C for a further 3 days.

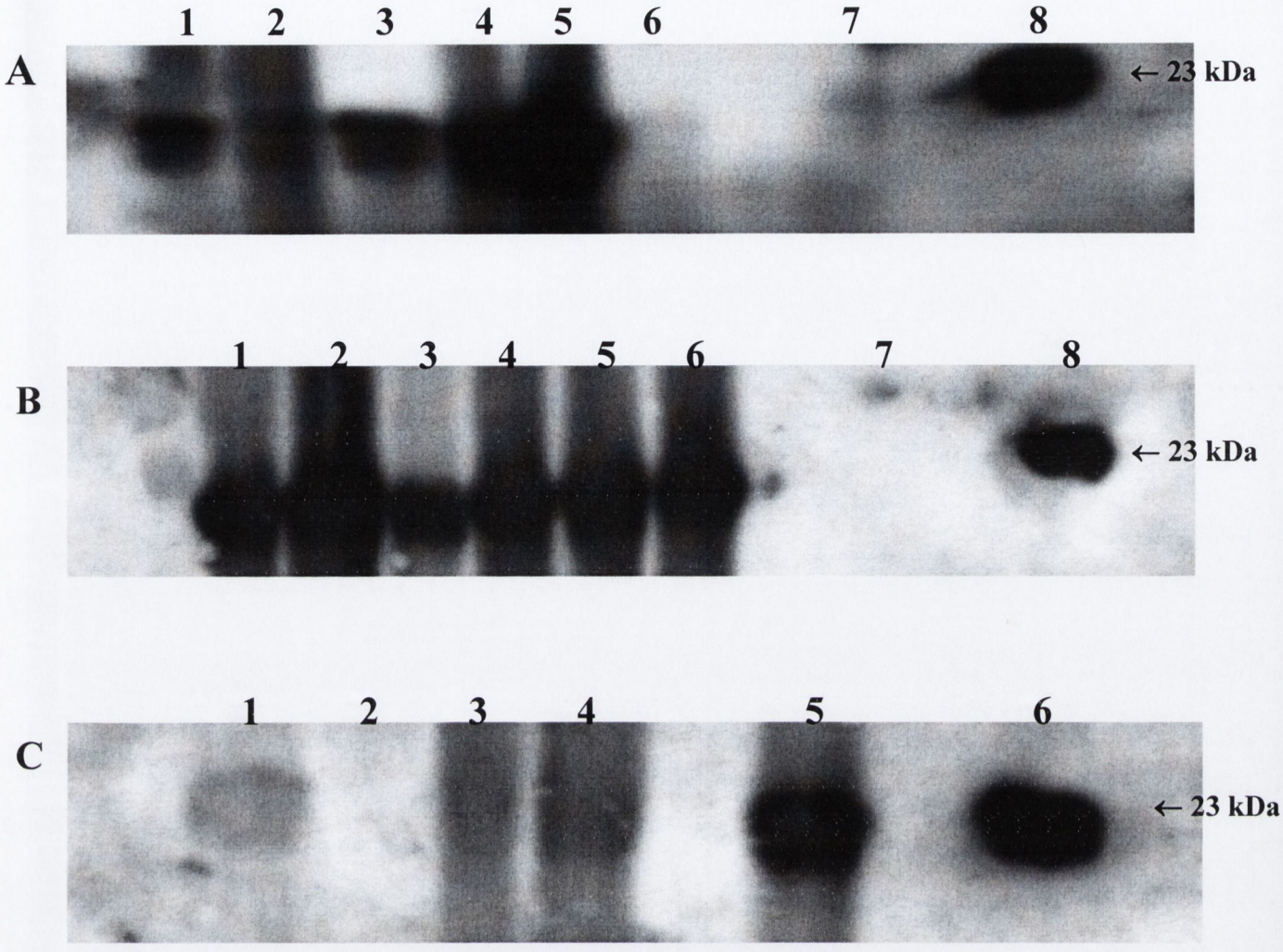


FIGURE 3.11 *In vitro* Bsp22 secretion by recent clinical isolates of *B. pertussis*. Supernatant protein samples were prepared from stationary phase cultures from 19 different strains of *B. pertussis*, separated by 10% SDS-PAGE and probed with anti-Bsp22 antibodies. **(A)** BP 0304 (lane 1), BP 0335 (lane 2), BP 0546 (lane 3), BP 0780 (lane 4), BP 0640 (lane 5), BP 2890 (lane 6), BP Wellcome 28 (lane 7) and 2 µg rHis-Bsp22 (lane 8). **(B)** BP 0950 (Lane 1), BP 0558 (lane 2), BP 0597 (lane 3), BP 2913 (lane 4), BP 2778 (lane 5), BP 2777 (lane 6), BP Tohama I (lane 7) and 2 µg rHis-Bsp22 (lane 8). **(C)** BP 0888 (lane 1), BP 1868 (lane 2), BP 1926 (lane 3), BP 2740 (lane 4), BP 18323 (lane 5) and 2 µg rHis-Bsp22 (lane 6).

Table 3.1 Bsp22 secretion by clinical isolates of *B. pertussis*

Strain	Source	Site of Isolation	Year of Isolation	Patient Age in Years	Bsp22 Secretion
0558	Human	?	1949	?	+
0304	Human	?	1950	?	+
0335	Human	?	1951	?	+
0888	Human	?	1991	0	+/-
0546	Human	Nasopharynx	1993	0	+
0597	Human	Sputum	1995	49	+
0640	Human	Throat	1996	0	+
0780	Human	Sputum	1996	2	+
0950	Human	Nasopharynx	1996	2	+
1868	Human	Nose	2000	2	-
1926	Human	?	2000	0	+/-
2740	Human	?	2004	2	+/-
2890	Human	Nose	2005	2	-
2913	Human	Nasopharynx	2005	?	+
2777	Human	Nose	2005	2	+
2778	Human	Nose	2005	13	+

The source, site of isolation, year of isolation, patient age in years and *in vitro* T3SS activity of 16 recent clinical isolates of *B. pertussis* obtained from the Netherlands. Supernatant samples from stationary phase cultures of each of the strains were precipitated with 10% TCA, separated by 10% SDS-PAGE and probed with anti-Bsp22 antibodies by Western blotting as described in Fig. 3.11.

+ , significant Bsp22 secretion, +/- slight Bsp22 secretion, -, no Bsp22 secretion (See Fig 3.11)

Chapter 4

The secreted effector proteins of the *Bordetella* T3SS

4.1 Introduction

T3SS function to deliver effector proteins directly to the eukaryotic cytosol, by means of an injectisome, which is assembled on the cell surface under permissive (*i.e* virulent) growth conditions. It is clear from the work of several groups to date that the T3SS of *B. bronchiseptica* is of critical importance to the bacteria in terms of establishing persistent respiratory tract colonization (Yuk *et al.* 2000) (Skinner *et al.* 2005) (Pilione and Harvill 2006). A number of T3-mediated effects on target cells *in vitro* have been reported, ranging from pore formation and cytotoxicity (Yuk *et al.* 1998) (Stockbauer *et al.* 2003); (Kuwae *et al.* 2003); (Nogawa *et al.* 2004); (Panina *et al.* 2005) to disruption of MAPK signalling pathways (Skinner *et al.* 2004); (Reissinger *et al.* 2005) and NF-kB aggregation (Yuk *et al.* 2000).

The specific effectors mediating this range of effects are not clear. Of the 5 proteins identified as substrates for the *Bordetella bronchiseptica* T3SS to date, 3 bear strong homology to well conserved groups of T3SS proteins, which have well defined roles in secretion regulation- BopN (Forsberg *et al.* 1991), and translocation pore formation- BopB and BopD (Hakansson *et al.* 1996); (Neyt and Cornelis 1999) (Tardy *et al.* 1999). A role for these proteins in translocation pore formation by *B. bronchiseptica* has already been demonstrated (Kuwae *et al.* 2003) (Nogawa *et al.* 2004). Bsp22, the 4th *B. bronchiseptica* T3SS substrate, is generally believed to be part of the translocation apparatus and though exported by the T3SS, is not thought to be translocated into target cells. Thus the only true *Bordetella* T3SS effector identified to date is BteA (Panina *et al.* 2005) or BopC as it has been named by Kuwae and colleagues (Kuwae *et al.* 2006). So far the only T3-mediated effect that has been demonstrated for this protein is the *in vitro* cytotoxicity of epithelial cells. Thus, since many of the most extensively studied T3SS, such as those of *Yersinia* and *Salmonella* spp., export multiple effector proteins, and since the full range of T3-mediated effects demonstrated for *B. bronchiseptica* cannot be solely attributed to BteA/BopC, it is conceivable that the *Bordetella* T3SS may possess multiple, as yet unidentified, effector proteins.

The energy requirement for the export of T3SS substrates is believed to be met by a cytoplasmic ATPase, first described by Woestyn *et al* in *Yersinia spp* (Woestyn *et al.* 1994). This protein, designated YscN, was found to have 29% identity to the α-subunit and 25% identity to the β-subunit of the bacterial F_0F_1 proton-translocating ATPase. The highly conserved regions of these subunits, termed Walker boxes A and B (Walker *et al.* 1982), are believed to bind and hydrolyse ATP respectively (Senior *et al.* 1993) (Story and

Steitz 1992), and *in vitro* ATP hydrolysis has been demonstrated for a homolog of YscN, the InvC protein of *Salmonella* spp. (Eichelberg *et al.* 1994). The YscN ATPase is one of the most conserved of all T3SS related proteins, with significant homologs in *Pseudomonas, Bordetella, Xanthomonas, Ralstonia, Mesorhizobium, Bradyrhizobium, Chlamydiaphila, Salmonella, Escherichia, Burkholderia, Erwinia, Pantoea,* and *Shigella* (Gophna *et al.* 2003). The high level of conservation of this protein is probably due to constraints on its ATP and Mg^{2+} binding sites. Mutation of the gene encoding YscN or its homologues has been shown to prevent TTSS effector secretion and significantly impair pathogenesis in *Yersinia* spp. (Woestyn *et al.* 1994), *Salmonella* spp. (Eichelberg *et al.* 1994; McKinney *et al.* 2004), *Shigella* spp. (Tamano *et al.* 2000); (Jouihri *et al.* 2003) and *Bordetella bronchiseptica* (Yuk *et al.* 2000), demonstrating the critical importance of YscN for the type three secretion processs. The *Bordetella* homolog, BscN, shows 64% identity to YscN at the amino acid level (Fig 4.1).

In most cases the identification of T3SS effector proteins preceded the identification of the T3SS machinery itself. For the most part, effector identification has relied on random transposon mutagenesis, coupled with growth conditions known to be involved in effector release, such as the $[Ca^{2+}]$ and temperature dependency of *Yersinia* spp. virulence (Cornelis *et al.* 1998) (Cornelis *et al.* 1989), or the epithelial cell invasion of *Salmonella* and *Shigella* spp. (Groisman and Ochman 1993). However, it does not appear that the *Bordetella* T3SS responds to any particular set of growth conditions resulting in elevated levels of effector release *in vitro*, such as the low $[Ca^{2+}]$ of *Yersinia* spp. (Heesemann *et al.* 1984), low O_2 tension of *Salmonella* spp. (Francis *et al.* 1992) or the congo red dye binding of *Shigella* spp. (Bahrani *et al.* 1997). Today, researchers have at their fingertips the entire annotated genome sequences of more than 150 bacterial species (bacterial genome repository at NCBI http://www.ebi.ac.uk/genomes/bacteria.html), which permits by PCR the quick and reliable cloning of genes of interest for further manipulation. The complete genomes of *B. pertussis* strain Tohama I and *B. bronchiseptica* strain RB50 have been sequenced and are available for download from the Sanger pathogen sequencing unit (www.sanger.ac.uk/projects/b_pertussis/bronchiseptica), Cambridge, UK.

Allelic exchange (Fig 2.1) is a process that relies on 2 separate recombination events to replace a chromosomal wild type allele with an extrachromosomally-located mutant allele lacking an internal portion of the wild type gene (deletion mutant) or harbouring an antibiotic resistance cassette (insertion mutant) (Stibitz 1998). A detailed description of the process is given in section 2.8.10. Two widely used counterselectable markers are the *rpsL* gene of *E. coli*, which confers streptomycin (Sm) sensitivity upon

strains otherwise resistant to Sm, and the *sacB* gene of *Bacillus subtilis*, which renders strains sensitive to sucrose in the growth medium (Stibitz 1998). Of these, the *rpsL* Sm sensitivity system has been used the most extensively for gene replacement in *Bordetella* (Black and Falkow 1987; Roberts *et al.* 1991; Zealey *et al.* 1992; Carbonetti *et al.* 1993; Carbonetti *et al.* 1994; Brickman and Armstrong 1996; Allen *et al.* 1998; Kang and Armstrong 1998; Pradel *et al.* 1998; Pradel *et al.* 2000; Pradel and Locht 2001; Vanderpool and Armstrong 2001).

This study set out to inactivate the T3SS of *B. pertussis* Tohama I, *B. pertussis* 12743 and *B. bronchiseptica* RB50, by mutating *bscN,* for the purpose of identifying the complete pool of effector proteins secreted by these bacteria during *in vitro* growth. The findings demonstrate that mutation of the *bscN* gene completely abolishes T3 secretion *in vitro* and has no adverse effects on bacterial growth or virulence factor expression. Further, the results show that *in vitro* secretion of the *Boredetella* T3SS effector, BteA/BopC, may be specific to *B. bronchiseptica.*

4.2 Results

4.2.1 Cloning of the *B. pertussis* Tohama I *bscN* gene.

In order to inactivate the *bscN* gene on the *Bordetella* chromosome, a mutant copy of the allele was first cloned into plasmid pSS1129, to facilitate allelic exchange. Plasmid pSS1129 (Fig 4.6) is a suicide vector, incapable of replicating in *Bordetella*, that contains both a selectable gentamicin (Gm), and a counter-selectable streptomycin (Sm) antibiotic resistance marker to facilitate allelic exchange. Initial attempts to amplify the entire *bscN* ORF from *B. pertussis* chromosomal DNA (strain Tohama I), for removal of an internal portion of the gene by endonuclease digestion, proved unsuccessful. Instead the gene was cloned in two separate parts. The 5' end of the allele was amplified with oligonucleotides PAB20 5'- GCCCTGCGGATCCCGCG - 3' and NF5 5'- TACTGACGCATGCCCCTATCC - 3'. These primers anneal to bp 65-81 of *bscL* (5' flanking gene to *bscN*) and bp 1-12 of *bscN* respectively. The resulting product, designated *bscN1*, was digested with *Bam*HI and *Sph*I (recognition sequence underlined in primer sequence), and this 589 bp fragment (Fig 4.2) was inserted into the corresponding sites of the cloning vector pQE-80 (Qiagen, UK; Fig 4.3), to create plasmid pNF2 (Fig 4.4).

The 3' end of the allele was amplified using oligonucleotides NF4 5'- GCTGGGCATGCTGGTCAAGGGC - 3' and PAB21 5'- GCCGGCTCGCGATGCATCG - 3', which anneal to bp 560-582 of *bscN* and bp 470-489 of *bscO* (3' flanking gene to *bscN*), respectively. The resulting product was digested with *Sph*I and *Nsi*I (recognition sequence underlined in primer sequence), and this 1262 bp fragment (Fig 4.2) was inserted into the corresponding *Sph*I and *Pst*I sites on plasmid pNF2, to create plasmid pNF3 (Fig 4.5). This plasmid was then digested with *Bam*HI and *Nhe*I to remove the 1974 bp mutated *bscN* allele, and this fragment was then inserted into the corresponding sites of the suicide vector pSS1129 (Fig 4.6), to create plasmid pNF4 (Fig 4.7 A). Plasmid pSS1129 is a suicide vector incapable of replicating in *Bordetella* spp., which contains a gentamicin (Gm) resistance cassette for first recombinant selection and the *rpsL* gene, which encodes streptomycin sensitivity, for counterselection in *Bordetella* spp.

4.2.2 Allelic exchange of the *bscN* mutant allele

In order to utilise the counter-selectable marker encoded on plasmid pNF4 for allelic exchange of the *bscN* mutant allele *B. pertussis* Tohama I was rendered streptomycin resistant by culturing the bacteria first in the presence of sub-lethal concentrations of streptomycin (20 μg/ml). Upon subculturing, the concentration of streptomycin (Sm) was then gradually increased by two-fold increments to a final concentration of 200 μg/ml and this strain was then designated *B. pertussis* Tohama I Sm^r. This was not necessary for the naturally streptomycin resistant *B. bronchiseptica* RB50 strain. A successful electroporation protocol was developed for the introduction of pNF4 into electrocompetent *Bordetella* cells. However, while this approach was successful for the introduction of replicating plasmids into *Bordetella*, it was very inefficient for inducing first recombination of suicide plasmids. Instead allelic exchange was performed by mating *Bordetella* with *E. coli* SM10 λ pir, harbouring plasmid pNF4. First recombinant *Bordetella* were selected on BG agar plates containing Gm and second recombinants were selected by growth on sucrose. Second recombinant bacteria were analysed by PCR using oligonucleotides PAB20 5'- GCCCTGCGGATCCCGCG – 3' which binds bp 65 – 81 of *bscL* (5' flanking gene to *bscN*) and BscN_test_rev 5' – ACCGCGGCGCCCTTGACCA – 3' which binds bp 550 – 569 of *bscN* (Fig 4.2). Repeated analysis of second recombinants showed consistent regeneration of the 1128 bp wild type allele, in place of the 582 bp mutant *bscN* allele (Fig 4.7 B and C).

4.2.3 Re-sizing of the *bscN* mutant allele

Due to the relative sizes of the recombination sites on the mutant *bscN* allele, it was possible that both the first and second recombinations were occurring through the larger 1.3 kb 3' *bscN2* (Fig 4.2; Fig 2.1) portion of the allele, such that the second recombination would merely reverse the first, consistently regenerating the wild-type allele. In light of this a new *bscN* mutant allele was constructed, in the hope that similar sized *bscN1* and *bscN2* might have a more balanced outcome in terms of recombination following allelic exchange. Plasmid pNF3 (Fig 4.5) was digested with *Age*I and *Sph*I to remove a smaller, 423 bp, 3' *bscN2* portion of the allele (Fig 4.2). This was then inserted into the corresponding *Sph*I and *Xma*I sites on plasmid pNF2 (Fig. 4.4) to create plasmid pNF7 (Fig 4.8). Digestion of plasmid pNF7 with *Bam*HI and *Nhe*I yielded a new 1151 bp insert (containing the new 1013 bp *bscN* mutant allele and 138 bp of vector sequence), which

was then inserted into the corresponding sites on plasmid pEGBR (Fig 4.9) to create plasmid pNF11 (Fig 4.10 A). Plasmid pEGBR is, like pSS1129, a suicide vector incapable of replicating in *Bordetella* spp., which contains a kanamycin (Kn) resistance cassette for first recombinant selection and the *sacBR* cassette, which encodes sucrose sensitivity, for counterselection in *Bordetella* spp. In addition to the possibility that the unequal sizes of the *bscN1* and *bscN2* alleles were hindering successful allelic exchange, it was also possible that the colonies screened in section 4.2.2 had arisen due to spontaneous Gm resistant mutants being selected as first recombinants. Therefore, plasmid pEGBR was chosen for subsequent allelic exchange in place of plasmid pSS1129.

Plasmid pNF11 was then introduced into heat shock competent *E. coli* SM10 λ pir, and these *E. coli* harbouring pNF11 were mated with *B. pertussis* Tohama I Sm^r and *B. bronchiseptica* RB50 in the same manner as described for pNF5 (see Ch.2). In this case, however, bacteria were selected after mating with Kn and 2X cephalexin (Cx) to kill *E. coli*. First recombinant bacteria were confirmed by streaking colonies separately onto Kn and sucrose containing plates and selecting Kn^r Suc^s colonies. A very large proportion of Kn^r Suc^r colonies were also obtained, and discarded, as these bacteria were likely either spontaneous resistance mutants of Kn or Kn^r colonies that spontaneously became Suc^r, as the Suc^s phenotype reverts easily. Kn^r Suc^s first recombinants were then grown to induce a second recombination event and all Kn^s Suc^r colonies screened by PCR once again showed consistent regeneration of the 1128 bp wild type allele (Fig 4.10 B and C).

4.2.4 Re-design of the *bscN* mutant allele to incorporate Gm resistance

The extremely low level of Kn^r Suc^s colonies obtained after repeated mating attempts using plasmid pNF11 raised the possibility that the *sacB* cassette conferring sucrose sensitivity present on pNF11 was not functioning optimally in *Bordetella.* Therefore we decided to introduce the Gm resistance cassette from plasmid pNF4 into the centre of our *bscN* mutant allele, in place of the original deleted coding sequence. This would remove the need for sucrose counter-selection from our allelic exchange procedure. Since all wild type second recombinants could be efficiently killed off at the end of the procedure by selecting on Gm, only colonies that have successfully generated the mutant allele after second recombination should remain.

To facilitate this, the entire 770 bp Gm resistance cassette was amplified from plasmid pNF4 using oligonucleotides Gmr_for_2 5' – ATAGCATGCTGACGCACACCG – 3' and Gmr_rev 5' – GCATGCTTAGGTGGCGGTAC – 3' (Fig 4.7 A). It was necessary to

include the upstream untranslated region of the Gm^r gene located on plasmid pNF5, as this sequence would likely contain the promoter and ribosomal binding sequence for the Gm^r cassette. Therefore we designed oligonucleotide Gmr_for_2 5' – ATAGCATGCTGACGCACACCG – 3', which primes approximately 232 bp upstream of the start codon of the Gmr gene. A *Sph*I restriction site (underlined) was placed at the beginning of each primer to facilitate insertion of the 770 bp PCR product into the *Sph*I site in the centre of the *bscN* mutant allele (see Fig 4.8). It was not possible to clone the Gm^r gene directly into plasmid pNF11 due to the presence of additional *Sph*I restriction sites located on plasmid pNF11 (Fig 4.10 A). Therefore we inserted the 770 bp Gm^r gene into the centre of the *bscN* mutant allele in plasmid pNF7 using the unique *Sph*I site on this vector (Fig 4.8)

Due to the ease of ligation into commercial "T" vectors and the common cloning observation that transferring DNA from one vector to another is generally more efficient than transferring a digested PCR product to a vector, we decided to insert the 770 bp Gm^r PCR product into the commercial "T" vector pcDNA3.1/His-TOPO (Fig 4.11; Qiagen, UK). PCR products amplified by *taq* polymerase can be ligated into these vectors with very high efficiency (Fig 4.12). This created plasmid Gmr-TOPO (Fig 4.13). The 770 bp Gm^r cassette was then removed from this vector using the S*ph*I restriction sites located on either end and inserted into the unique *Sph*I site on plasmid pNF7 to create plasmid pNF12 (Fig 4.14) containing the new 1795 bp *bscN*::Gm^r mutant allele. This new mutant allele was then removed from plasmid pNF12 using the flanking *Bam*HI and *Nhe*I restriction sites and this 1933 bp fragment (containing the 1795 bp *bscN*::Gm^r mutant allele and 138 bp of vector DNA) was inserted into the corresponding sites on plasmid pEGBR (Fig 4.9) to create the suicide vector pNF13 (Fig 4.15 A).

4.2.5 Successful allelic exchange of the *bscN*::Gm^r allele.

Plasmid pNF13 was introduced into $CaCl_2$ competent *E. coli* SM10λpir, and *E. coli* harbouring pNF13 were mated with *B. pertussis* Tohama I Sm^r or *B. bronchiseptica* RB50 as described for pNF11. After mating, Kn^r Gm^r colonies (first recombinants) were obtained, but, as was the case for pNF11, at a very low efficiency. It is possible that the mutation of the *bscN* gene may have had a deleterious effect on cells expressing the T3SS, resulting in lethality. It has been reported that switching off transcription of the *bsc* loci by rendering the bacteria avirulent in bvg negative Stainer + Scholte (S+S) medium can overcome this difficulty (Mattoo *et al.* 2004). Therefore, first recombinants were grown to

induce second recombination as before, but this time they were grown in bvg negative S+S medium, and subsequently selected for with Gm. All colonies tested by PCR with oligonucleotides PAB20 5'- GCCCTGCGGATCCCGCG – 3' and BscN_test_rev 5' – ACCGCGGCGCCCTTGACCA – 3' (Fig 4.2), consistently re-generated the 1128 bp wild-type allele (Fig 4.15 B and C). The presence of colonies on plates containing Gm at the end of the allelic exchange procedure suggested that these colonies were either first recombinants or spontaneous Gm resistant mutants. Therefore, rather than attempting further allelic exchange, we decided to investigate the genotype of our first recombinant bacteria.

Oligonucleotides recombtest_P1 5' – ACGAGATAGCGCTGCGTTGC – 3', recombtest_P2 5' – CTGGGTTCGTGCCTTCATCC – 3', recombtest_P3 5' - GCA TACGGGAAGAAGTGATGC -3' and recombtest_P4 5' - CCACAACGATACGGG CTTCG - 3' were designed to prime various regions of the *Bordetella* chromosome outside of the mutant *bscN* allele and to prime internal portions of the Gmr cassette (Fig 4.16). Three PCR reactions were set up using primers P1 + P4 (reaction A), P1 + P2 (reaction B) and P3 + P4 (reaction C). Thus as outlined in Fig 4.16, a 2896 bp PCR product from reaction A would indicate a wild type genotype, a 765bp PCR product from reaction B would indicate first recombination having occurred through the *bscN1* portion of the allele, while a 1618 bp PCR product from reaction C would indicate first recombination having occurred through the *bscN2* portion of the allele (Fig 4.16). A PCR product from both reactions B and C would indicate a second recombinant, while a PCR product from reaction A in addition to reaction B or C would indicate a mixed population of first recombinant bacteria and spontaneous mutants. Colonies of *B. pertussis* Tohama I Sm^r harbouring pNF13 (*i.e* first recombinants hereafter referred to as Bp: pNF13) and wild type *B. pertussis* Tohama I Sm^r were harvested and tested by PCR with all three sets of primers. *B. pertussis* Tohama I Sm^r proved positive only for reaction A indicating, as expected, a wild type genotype, while Bp::pNF13 proved positive for both reactions A and C (Fig 4.17), indicating a mixed population of spontaneous Kn^r and Gm^r wild-type bacteria and first recombinant bacteria through the *bscN2* portion of the mutant allele only (Fig 4.18).

In light of this inducement of spontaneous antibiotic resistant bacteria, the allelic exchange protocol was revised. By using dilutions of mated bacteria to select for first recombinants, and thus lowering the amount of bacteria used for selection, it was possible to reduce the likelihood of inducing spontaneously resistant bacteria. Dilutions of matings between *E. coli* SM10λpir harbouring pNF13 and *B. bronchiseptica* RB50, *B. pertussis* Tohama I Sm^r and the T3SS active *B. pertussis*12743 Sm^r were used to select Gm resistant

colonies. These colonies were then subsequently screened for Kn sensitivity by streaking simultaneously onto agar plates supplemented with either Gm or Kn. All Kn^s Gm^r colonies of *B. pertussis* Tohama I Sm^r and *B. bronchiseptica* RB50 (Fig 4.19) and *B. pertussis* 12743 Sm^r (Fig 4.20) tested by PCR with oligonucleotides PAB20 5'-GCCCTGCGGATCCCGCG – 3' and BscN_test_rev 5' – ACCGCGGCGCCCTTGACCA – 3' (Fig 4.2) were found to have successfully generated the 1364 bp mutant *bscN*::Gm^r allele. These bacteria were designated *B. pertussis* Tohama I bscN, *B. bronchiseptica* RB50 bscN and *B. pertussis* 12743 bscN respectively.

4.2.6 Mutation of *bscN* abolishes *in vitro* T3 secretion of Bsp22 in *B. bronchiseptica* RB50 and *B. pertussis* 12743 but not *B. pertussis* Tohama I.

Mutation of *bscN* in *B. bronchiseptica*, or its homologs such as *yscN* in *Yersinia* or *invC* in *Salmonella*, has previously been shown to result in the inhibition of T3SS activity both *in vitro* and *in vivo* (Eichelberg *et al.* 1994; Woestyn *et al.* 1994; Yuk *et al.* 1998; Yuk *et al.* 2000). However, there are no previous reports of the construction of *bscN* mutant bacteria in *B. pertussis*. Bsp22 has been shown to be secreted by *B. bronchiseptica* (Yuk *et al.* 2000) and the present study has shown it is secreted by *B. pertussis* 12743 (Chapter 3). Therefore, the effect of *bscN* mutation on the *in vitro* secretion of the *Bordetella* T3SS effector protein Bsp22 was examined. Cultures of *B. bronchiseptica* RB50, *B. bronchiseptica* RB50 bscN, *B. pertussis* 12743, *B. pertussis* 12743 bscN, *B. pertussis* Tohama I and *B. pertussis* Tohama I bscN were grown to stationary phase in S+S liquid broth and supernatants were analysed for the presence of Bsp22 by western blotting with anti-Bsp22 antibodies. As can be seen from Fig 4.21, significant quantities of secreted Bsp22 protein were detected in the supernatants of wild-type *B. bronchiseptica* RB50 (Fig 4.21 A) and *B. pertussis* 12743 (Fig 4.21 B). In contrast, Bsp22 was undetectable in the supernatants of T3SS deficient *B. bronchiseptica* RB50 bscN (Fig 4.21 A) and *B. pertussis* 12743 bscN (Fig 4.21 B). In agreement with previous results (Chapter 3), no secreted Bsp22 was detected in the supernatants of either wild-type *B. pertussis* Tohama I or T3SS deficient *B. pertussis* Tohama I bscN (Fig 4.21).

These results demonstrate that mutation of the *bscN* gene abolishes T3SS activity in *B. bronchiseptica* RB50 and *B. pertussis* 12743. The results also demonstrate that BscN is an essential component of the *B. pertussis* T3SS, as it is in *B. bronchiseptica*.

4.2.7 Mutation of *bscN* does not significantly affect bacterial growth rate or secretion of the virulence factors, FHA and PT

It has been previously reported that mutation of the *bscN* gene has no effects on the growth of *B. bronchiseptica* RB50 *in vitro* (Yuk *et al.* 1998). However, there are no previous reports on the effect of mutation of the *bscN* gene on the *in vitro* growth characteristics of *B. pertussis* 12743 or *B. pertussis* Tohama I. Therefore, the effect of mutation of the *bscN* gene on the *in vitro* growth of these 2 strains of *B. pertussis* in comparison to *B. bronchiseptica* RB50 was investigated.

Wild-type and T3SS deficient *B. bronchiseptica* RB50, *B. pertussis* 12743 and *B. pertussis* Tohama I were plated on BG agar and used to inoculate precultures. Actively growing precultures were then used to inoculate 100 ml cultures at a starting OD_{600} of 0.2, and the growth of all 6 strains was monitored to stationary phase by measuring the OD_{600} of the cultures after 4, 8, 12, 24, 28, 32, and 40 hours of growth. As can be seen from Fig 4.22, the growth curves obtained for wild-type and T3SS deficient *B. bronchiseptica* RB50 (A), *B. pertussis* 12743 (B) and *B. pertussis* Tohama I (C), show no differences in growth rate between wild-type and *bscN* mutant bacteria (Fig 4.22).

It has also previously been reported that mutation of the *bscN* gene in *B. bronchiseptica* has no effects on the secretion of the *Bordetella* virulence factor, FHA (Yuk *et al.* 1998). However, there are no previous reports detailing the effect of mutation of the *bscN* gene on the secretion of FHA in *B. pertussis* 12743 or *B. pertussis* Tohama I. Likewise, since *B. bronchiseptica* does not produce PT, there are no reports to date of the effect of mutation of the *bscN* gene on PT secretion by *Bordetella* spp. Therefore the secretion of FHA and PT, in addition to Bsp22, by wild-type and T3SS deficient *B. bronchiseptica* RB50, *B. pertussis* 12743 and *B. pertussis* Tohama I was analysed during *in vitro* growth. Bacteria were grown to stationary phase and total proteins from supernatants were precipitated with 10% TCA and subjected to western blot analysis with anti-Bsp22, anti-FHA and anti-PT antibodies (Fig 4.23). Consistent with earlier observations, we detected significant levels of Bsp22 in supernatants from wild-type BB RB50 and BP 12743 but not from BP tohama I (Fig 4.23 A). In addition, similar amounts of FHA was

detected in supernatants from wild-type and T3SS deficient BB RB50, BP 12743 and BP Tohama I (Fig 4.23 B). As expected, PT was not detected in supernantants from either wild-type or T3SS deficient BB RB50 (Fig 4.23 C), while similar levels of PT were observed in the supernatants from wild-type and T3SS deficient BP 12743 and BP Tohama I (Fig 4.23 C).

These results demonstrate that inactivation of the *Bordetella* T3SS, through mutation of the *bscN* gene, has no adverse effects on bacterial growth, or the secretion of the virulence factors, FHA or PT.

4.2.8 Secretion profile of wild-type and T3SS deficient *Bordetella* spp.

In order to further examine the secretion profile of wild-type and T3SS deficient *Bordetella* spp., to facilitate the identification of previously unidentified *Bordetella* T3SS effector proteins, the secreted proteins of *Bordetella* spp. were identified by mass spectroscopy. Total secreted proteins in supernatants from stationary phase cultures of *B. bronchiseptica* RB50, *B. bronchiseptica* RB50 bscN, *B. pertussis* 12743, *B. pertussis* 12743 bscN, *B. pertussis* Tohama I, *B. pertussis* Tohama I bscN and *B. pertussis* 9340 were precipitiated with 30% ammonium chloride, and subjected to MALDI-TOF analysis. The identified peptide fragments were then searched against the complete *B. bronchiseptica* RB50 and *B. pertussis* Tohama I genomes available from the Sanger centre, UK (www.sanger.ac.uk/projects/b_pertussis/bronchiseptica). Their corresponding ORFs that were identified as known virulence factors are summarised in table 4.1. Other proteins not implicated in virulence were also identified and are not discussed here. Consistent with the detection of FHA and PT by western blotting, these proteins were detected in the supernatants of BP 12743 and BP 12743 bscN cultures, as evidenced by their corresponding ORFs *fhaB* and *ptxA, B, D*, and *E* (PT sub-units 1, 2, 4 and 5 respectively) (Table 4.1). Several other *Bordetella* virulence factors, including ACT (*cyaA*), serum resistance protein (*brkA*), tracheal colonisation factor (*tcfA*) and PRN (*prn*) were also detected in these supernatants (Table 4.1). As expected, FHA, ACT and PRN, but not TcfA, BrkA and PT, were detected in supernatants from BB RB50 and BB RB50 bscN. PT and TcfA are *B. pertussis*-specific virulence factors, and while BrkA expression has been demonstrated for some strains of *B. bronchiseptica*, it has been reported that strain RB50 lacks expression of this protein (Rambow *et al.* 1998). In contrast to results obtained by western blotting, PT was not detected by MALDI-TOF analysis in supernatants from wild-

type or BscN mutant BP Tohama I, or from BP 9340 (Table 4.1). However, these proteins samples were analysed at a 1/10 dilution of the neat sample. Though PT was detected following analysis of the neat BP 12743 protein sample, it was not detected when this sample was diluted 1/10 prior to analysis, in contrast to other proteins such as FHA, ACT and Bsp22 (Table 4.1). This suggests that the low level of PT present in the supernatants of *B. pertussis* cultures is undetectable at the 1/10 dilution, and this may explain the lack of PT detection in the supernatants of BP Tohama I and BP 9340 (Table 4.1).

The MALDI-TOF analysis allowed us to identify T3SS substrates of *B. bronchiseptica* and *B. pertussis*. In agreement with western blot analysis, Bsp22 was observed in supernatant from wild-type BP 12743, BB RB50 and BP 9340, but not from BP 12743 bscN, BB RB50 bscN or either BP Tohama I or BP Tohama I bscN (Table 4.1). This confirms the expression and activity of the T3SS in *B. pertussis*. In addition, the BopD T3SS translocator protein was also detected in the supernatant from wild-type BP 12743 and BB RB50, and the BopN sensor/plug protein was also detected only in the supernatant from BP 12743 (Table 4.1). Neither BopN or BopD were detected from BP 9340. In contrast to *B. bronchiseptica*, the *Bordetella* T3SS effector protein, BteA/BopC, was absent from the supernatants of BP 12743 and BP 9340, which suggests that although these strains of *B. pertussis* possess T3SS activity *in vitro*, they do not secrete this effector protein. Consistent with previous observations, no known T3SS secreted proteins were detected in the supernatants from wild-type or BscN mutant BP Tohama I.

Since BP 12743 secretes Bsp22, BopN and BopD, whereas BP Tohama I does not, these results provide further evidence that BP 12743 posseses T3SS activity *in vitro*. The results also confirm that mutation of the *bscN* gene has no adverse effects on secretion of other virulence factors. These results, however, suggest that the T3SS of *B. pertussis* spp. does not secrete BteA/BopC. These results also show that *B. pertussis* secretes BopD and BopN in a T3SS dependent manner, showing for the first time that these proteins are substrates of the *B. pertussis* T3SS.

4.3 Discussion

The aims of this study were to identify the T3SS effectors secreted by *Bordetella* spp. and to investigate the effects of individual effector molecules, and the system as a whole, on host immune cell function. An essential part of this study therefore, was the irreversible inactivation of the *Bordetella* T3SS. Mutation of the gene encoding a critical component of the T3SS injectisome, which abolishes T3SS effector output, is a common strategy used to identify T3SS effector proteins. For example, mutation of *invG* in *Salmonella*, a homolog of the *yscC* secretin of *Yersinia spp*, (Burghout *et al.* 2004) was used to identify the effectors SipB and SipC, (homologs of IpaB and IpaC from *Shigella* spp. respectively) (Kaniga *et al.* 1995) and the SipA and D effectors (Kaniga *et al.* 1995), as well as SptP, which bears homology to regions of both YopE and YopH of *Yersinia* spp. (Kaniga *et al.* 1996). Mutation of the *Salmonella* translocator SipB, homologous to *Yersinia* YopB, allowed the identification of the effectors SopA-E (Lee and Galan 2003), while mutation of its *Bordetella* homolog, BopB was used to identify BteA/BopC (Panina *et al.* 2005) (Kuwae *et al.* 2006). A functional BscN protein has been shown to be essential for the function of the T3SS in numerous bacteria, including *B. bronchiseptica* (Yuk *et al.* 2000 {Kuwae, 2003 #112), and the generation of *bscN* mutants allowed the detection of Bsp22, BopB, BopD and BopN as T3SS effectors in *B. bronchiseptica* by Yuk *et al* (Yuk *et al.* 2000).

The present study has successfully generated *bscN* mutant strains of *B. bronchiseptica* RB50, *B. pertussis* 12743 and *B. pertussis* Tohama I through allelic exchange of a mutant *bscN* allele, and BP 12743 bscN and BB RB50 bscN bacteria have been shown to be deficient in Bsp22 secretion relative to their wild-type counterparts. *B. pertussis* Tohama I, as shown in Chapter 3, does not secrete Bsp22 *in vitro*. In addition, this study has demonstrated that mutation of the *bscN* gene has no adverse effect on the secretion of other virulence factors or on bacterial growth, and, significantly, that secretion of the only true *Bordetella* T3SS effector protein identified to date, BteA/BopC (Panina *et al.* 2005) (Kuwae *et al.* 2006), is restricted to *B. bronchiseptica.* The present study also demonstrates that BopD and BopN are substrates of the *B. pertussis* T3SS.

Initial attempts at allelic exchange were unsuccessful, primarily due to non-optimal mutant allele design. The large size of the 1262 bp 3' portion of the mutant allele appeared to ensure efficient recombination and therefore insertion of the linearised pNF4 vector into the *Bordetella* chromosome, confirmed by the large number of Gm^r colonies obtained. However, allelic exchange relies on a second recombination event occurring between the wild type chromosome and the opposite portion of the mutant allele to where first

recombination occurred (in this case the smaller 5' *bscN1* portion). If second recombination occurs at the same site as first recombination, it merely serves to reverse the first recombination, thus regenerating the wild type allele (see Fig 2.1). It is likely that both recombination events were consistently occurring through the larger 3' *bscN2* portion of the allele, due to its larger size.

Consequently, a new *bscN* mutant allele was constructed from 589 bp from the 5' *bscN1* portion, and 423 bp from the 3' *bscN2* portion of the allele. It was expected that the new similar sizes of the *bscN1* and *bscN2* alleles would likely promote recombination equally through each portion of the allele during allelic exchange. This new *bscN* mutant allele was inserted into plasmid pEGBR, which carries the *sacB* gene. The product of the *sacB* gene converts sucrose to products toxic to gram negative bacteria and thus can be used as a counter-selectable marker in the presence of sucrose. Though the similar sizes of the *bscN* portions were expected to have an effect on the outcome of allelic exchange in favour of the new *bscN* mutant allele, after mating all colonies consistently demonstrated regeneration of the wild-type chromosomal allele. Moreover, though we could easily obtain Kn^r colonies after mating (suggesting correct first recombination), the number of suc^s colonies obtained in all cases was very low. The sucrose sensitivity counter-selection system has not been used extensively in *Bordetella* spp. However, this apparent difficulty in rendering *Bordetella* sucrose sensitive has previously been reported by Pradel *et al*, who, after obtaining only sucrose resistant colonies after allelic exchange, concluded that, in contrast to the case for *E. coli*, the *sacB* cassette may not be expressed optimally from its own promoter in *Bordetella* spp. (Pradel *et al.* 1998). We concluded that this may also be the case in our allelic exchange experiments. Therefore, in the absence of counter-selection, an alternative method was required to select for the incorporation of the mutant allele into the *Bordetella* chromosome at the end of the allelic exchange procedure.

Since double recombination events in most bacteria are rare, in the absence of a readily detectable marker in the mutant allele (such as an antibiotic resistance cassette), it is usually necessary to initially select first recombinant bacteria (in this case Kn^r bacteria). This facilitates the removal of wild-type colonies, and therefore greatly increases the likelihood of selecting for a correct second recombination event. The incorporation of a Gm^r cassette into the *bscN* mutant allele should have resulted in the efficient killing of all wild-type colonies at the end of the allelic exchange process and allowed selection of double recombinants immediately after mating. However, as explained in section 4.2.5, the majority of Gm^r colonies obtained at the end of the allelic exchange procedure still

possessed the Kn^r genotype, indicating they were not double recombinants, but first recombinants. When screened by PCR all of these colonies revealed the wild-type allele.

This suggested a mixed population of both wild-type and first recombinant bacteria, and consequently, a PCR system was designed in order to determine the exact genotype of these bacteria after mating. The PCR analysis indicated a mixed population of spontaneous Kn^r and Gm^r bacteria bearing the wild-type *bscN* allele, and bacteria having undergone first recombination through the *bscN*2 portion of the mutant allele. It appeared that the high density of bacterial plating during allelic exchange was inducing spontaneous resistant mutants. Thus, after mating, the vast majority of colonies returned were spontaneous Gm^r variants of the wild-type with relatively few first recombinants and even fewer double recombinants. The likelihood of selecting a correct exconjugant in these conditions was extremely low. By plating dilutions of bacteria after mating, the number of spontaneous Gm^r mutants obtained was dramatically reduced, with the majority of Gm^r colonies obtained having incorporated the mutant allele, as evidenced by Kn^s and PCR screening.

Consistent with previous reports on the effect of *bscN* mutation on *in vitro* secretion of T3SS effectors by *B. bronchiseptica* (Yuk *et al.* 1998; Yuk *et al.* 2000; Kuwae *et al.* 2003; Kuwae *et al.* 2006), the present study showed that mutation of *bscN* abolished T3SS activity *in vitro*; Bsp22, BteA/BopC and BopD were absent from the culture supernatants of T3SS deficient BB RB50 bscN bacteria. Similar to reports by Yuk *et al* (Yuk *et al.* 1998), this study confirmed that mutation of *bscN* in *B. bronchiseptica* does not significantly effect bacterial growth or the secretion of other virulence factors such as FHA or ACT. In addition, the results indicate that the production of PRN is also unaffected.

This study has shown that, similar to *B. bronchiseptica*, mutation of *bscN* in *B. pertussis* 12743 and *B. pertussis* Tohama I does not significantly effect growth rate or the secretion of other virulence factors such as FHA, PT, ACT, TcfA, BrkA and PRN. Furthermore, mutation of *bscN* in *B. pertussis* 12743 abolished T3SS activity *in vitro*; Bsp22, BopN and BopD were absent from the supernatant of BP 12743 bscN bacteria. Interestingly, the *Bordetella bronchiseptica* T3SS effector, BteA/BopC was not secreted by two of the strains of *B. pertussis* shown in Chapter 3 to possess T3SS activity, BP 12743 and BP 9340. This suggests that BteA/BopC may be a *B. bronchiseptica*-specific T3SS effector, although the gene encoding BteA/BopC, *BP0500*, like the rest of the T3SS loci, is present and intact in the genome of *B. pertussis* Tohama I. It is unknown if this gene is present or intact in the genomes of BP 12743 or BP 9340 as, to our knowledge, no sequence data exists on these species. These results also confirm the lack of T3SS

expression by *B. pertussis* Tohama I, as no T3SS substrates were detected in the supernatants of both wild-type and T3SS deficient *B. pertussis* Tohama I. Interestingly, in contrast to BP 12743, no PT was detected by MALDI-TOF analysis of diluted supernatants of BP 12743, BP Tohama I or BP 9340, suggesting a lower level of PT secretion by *B. pertussis* spp. when compared to other proteins such as FHA or Bsp22.

In conclusion this study has demonstrated that, in contrast to *B. pertussis* Tohama I, the T3SS of low passage *B. pertussis* isolates 12743 and 9340, is functionally active *in vitro*, and for the first time, Bsp22, BopN and BopD have been identified as substrates of the *B. pertussis* T3SS. However, despite the presence of a functional T3SS these strains do not secrete the *Bordetella* effector, Bte/BopC, the expression of which may be specific to *B. bronchiseptica.*

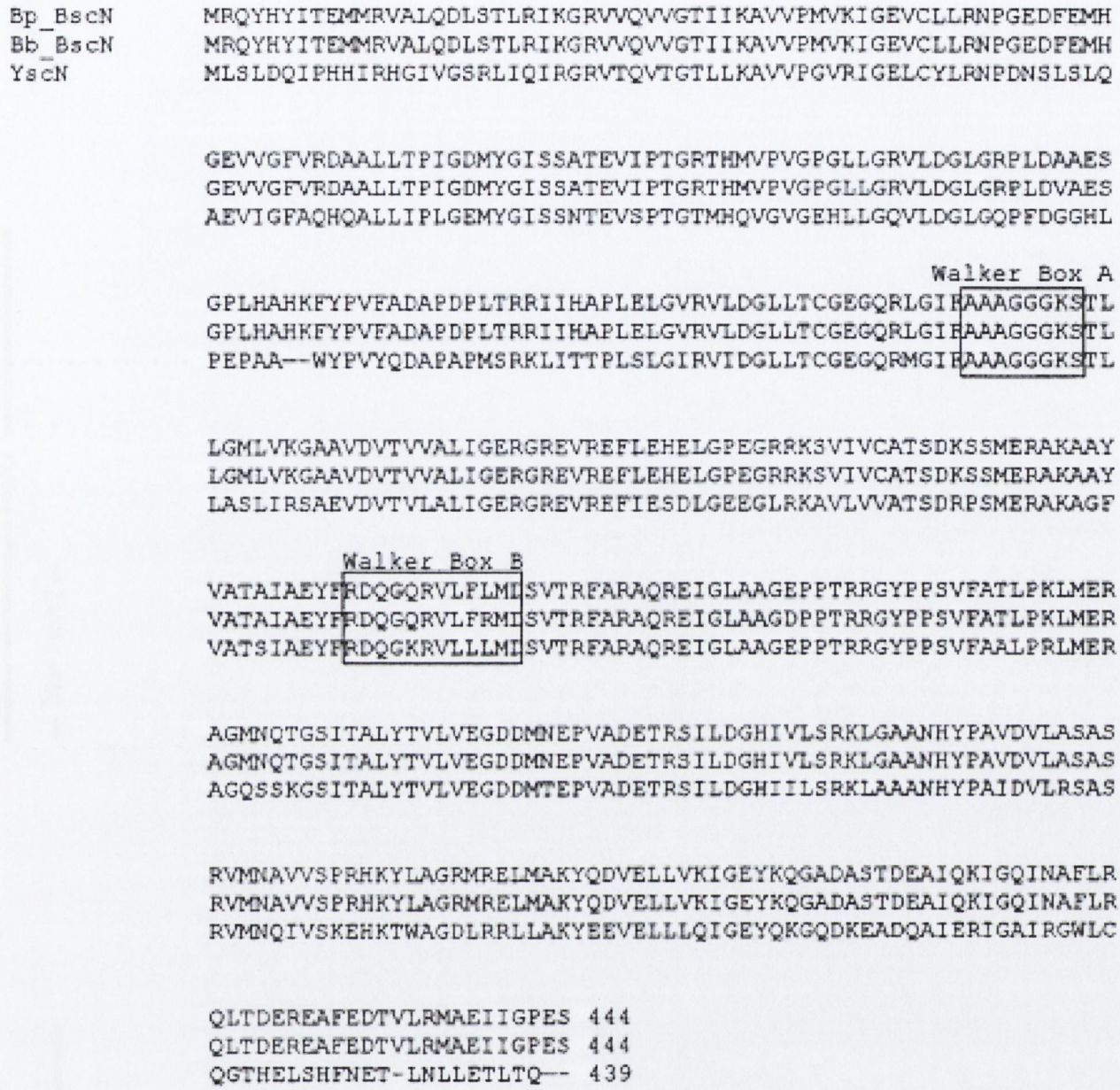


FIGURE 4.1 Protein sequence of *B. pertussis* Tohama I BscN compared with *B. bronchiseptica* RB50 bscN and *Yersinia* YscN. The two conserved Walker boxes are outlined. Figure adapted from Kerr *et al* (1999).

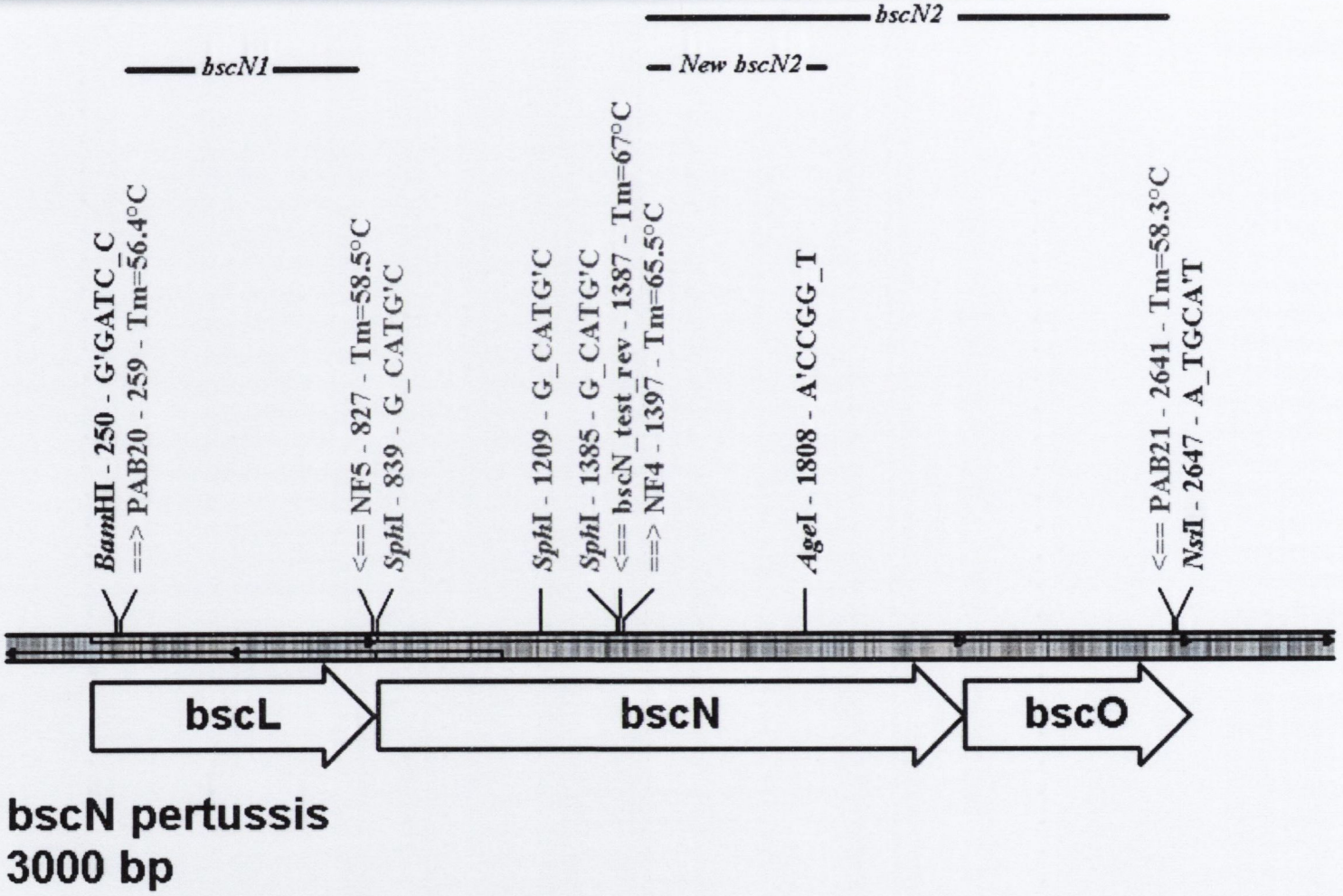


FIGURE 4.2 – The *bscN* gene. Schematic of part of T3SS operon containing ***bscN,*** *bscL* and *bscO*. The primer binding sites for *bscN1, bscN2* and redesigned *new bscN2* portions of the allele are indicated. (Table 2.1). Also shown are the *Sph*I restriction sites originally intended to be used to remove an internal portion of the allele, and the S*ph*I and *Age*I restriction sites used to generate the *new bscN2* allele and the *Bam*HI and *Nsi*I restriction sites used to clone into pSS1129.

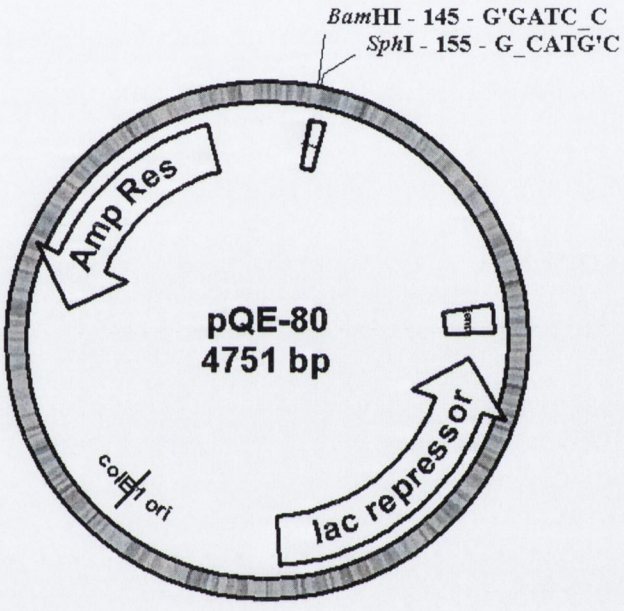


FIGURE 4.3 – Plasmid pQE-80. Cloning vector containing the *Bam*HI and S*ph*I restriction sites for insertion of the 589 bp *bscN1* PCR fragment. The plasmid also contains an Ampicillin (Amp) resistance cassette, T5 promoter and Lac repressor for protein expression, and the colE1 origin of replication.

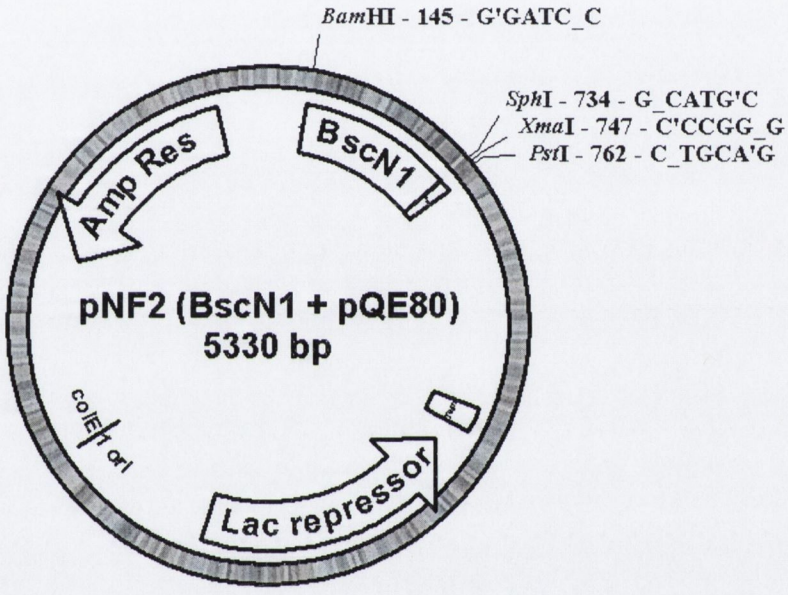


FIGURE 4.4 – Plasmid pNF2. Cloning vector containing the 589 bp *bscN1* fragment, and corresponding *Sph*I and *Pst*I restriction sites for insertion of the original 1262 bp *bscN2* fragment, and sites *Sph*I and *Xma*I for insertion of the re-designed shorter 423 bp *bscN2* fragment. Also shown is an ampicillin resistance cassette, T5 promoter and Lac repressor for protein expression and the colE1 origin of replication.

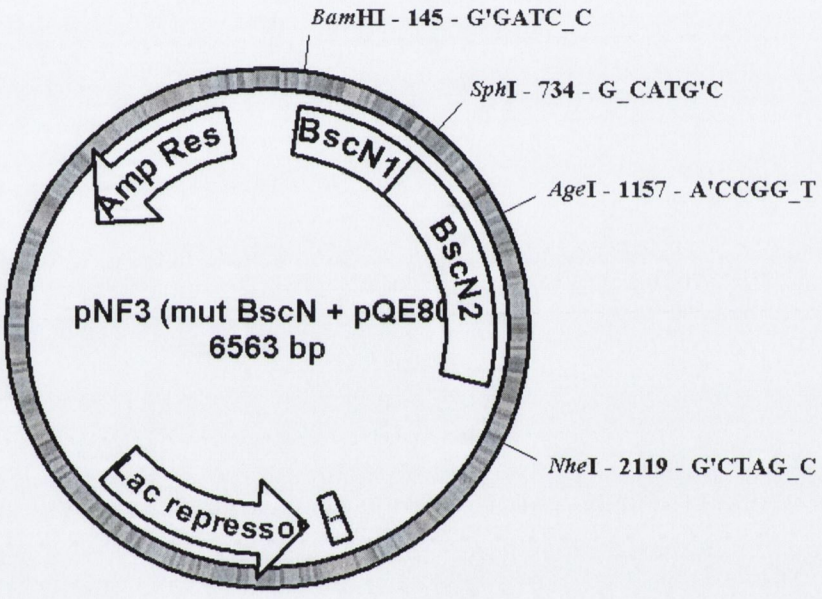


FIGURE 4.5 – Plasmid pNF3. Cloning vector containing the original 1974 bp *bscN* mutant allele, and the *Sph*I and *Age*I restriction sites used to remove the re-designed 423 bp *bscN2* portion of the allele. Also shown is an ampicillin resistance cassette, T5 promoter and Lac repressor for protein expression and the colE1 origin of replication.

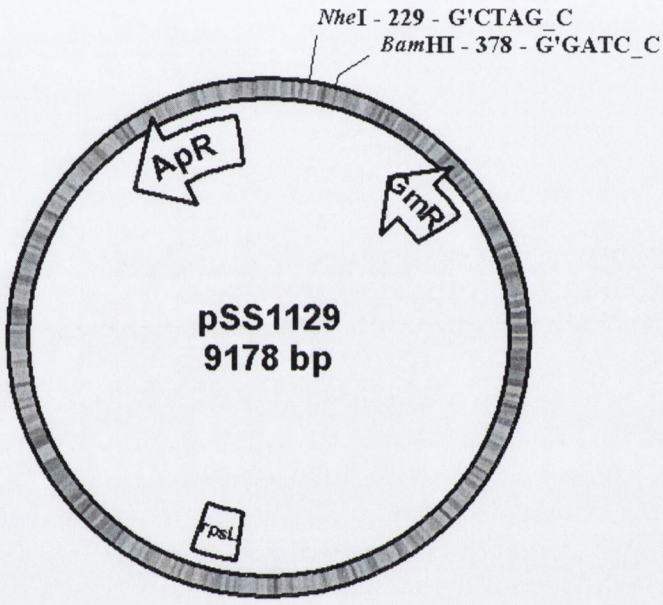


FIGURE 4.6 – Plasmid pSS1129. The suicide vector used for introduction of the 1974 bp *bscN* mutant allele into *Bordetella*, containing the *Bam*HI and *Nhe*I restriction sites for insertion of the mutant allele into pSS1129, gentamicin resistance cassette for selection of first recombinants, the *rps*L gene for selection of second recombinants and an ampicillin resistance cassette.

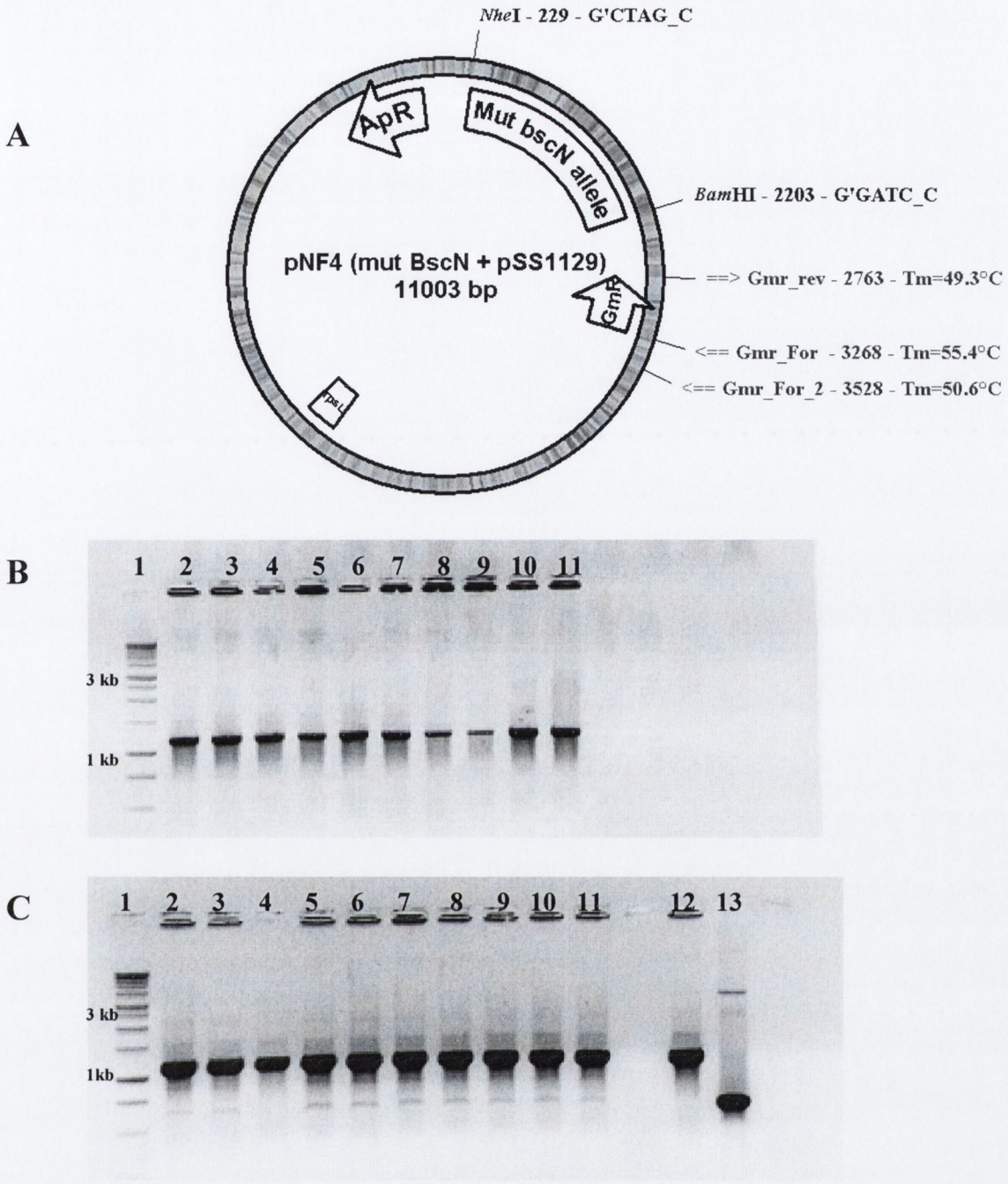


FIGURE 4.7 (A) Plasmid pNF4. Suicide Vector containing the original 1974 bp *bscN* mutant allele, used for introduction of the allele into *Bordetella*. Also shown is an ampicillin resistance cassette, gentamicin resistance cassette for selection of first recombinants and the *rpsL* gene for selection of second recombinants in *Bordetella*. **(B) Agarose gel electrophoresis of PCR analysis of putative second recombinants from *B. bronchiseptica* RB50 and (C) *B. pertussis* Tohama I.** The absence of the 582 bp *bscN* mutant allele is noted, while the 1128 bp *bscN* wild type allele is present in all clones screened. Lane 1 shows 1 kb DNA ladder (Promega, USA), while lanes 12 and 13 (C) show wild type and mutant allele controls respectively. All other lanes show individual colonies. The results are representative from 10 colonies from each species. 50 colonies of each species were tested in total.

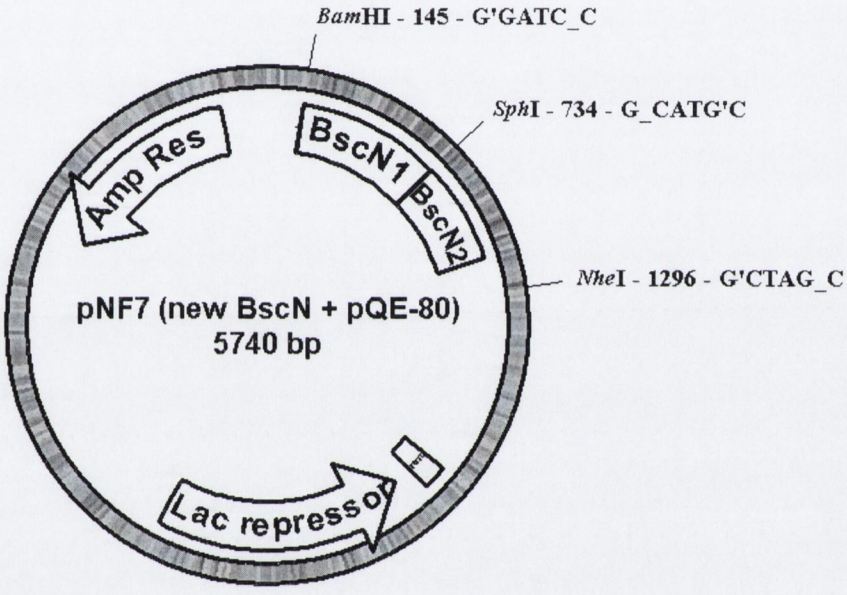


FIGURE 4.8 – Plasmid pNF7. pQE-80 cloning vector containing the re-designed 1013 bp *bscN* mutant allele, inserted at sites *Sph*I and *Xma*I. The *Xma*I restriction site was not regenerated. The *Bam*HI and *Nhe*I sites used for removal of the allele are shown. Also shown is an ampicillin resistance cassette and the T5 promoter and Lac repressor for protein expression.

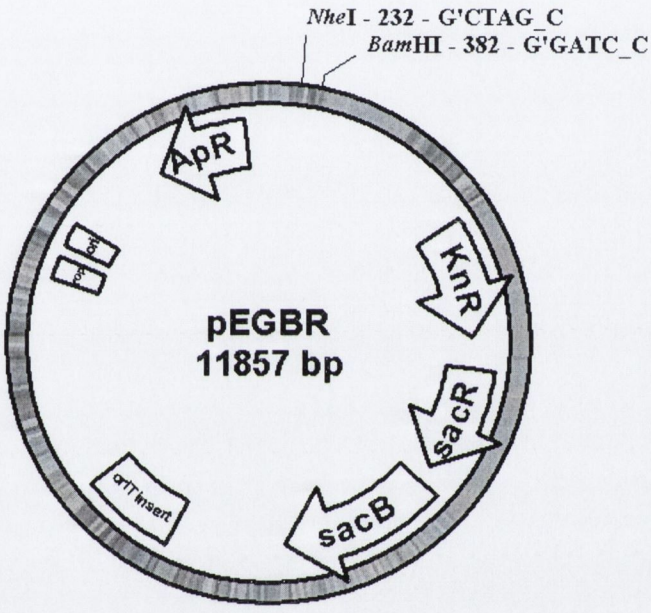
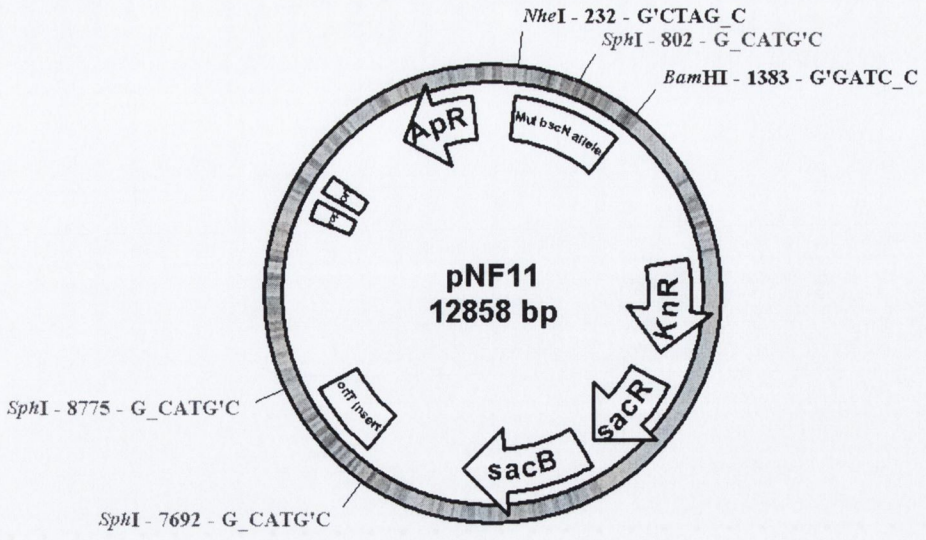
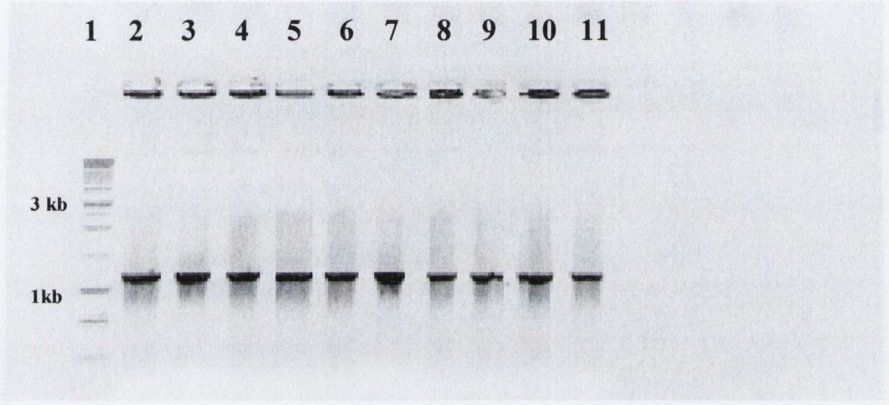


FIGURE 4.9 – Plasmid pEGBR- A sucrose sensitive suicide vector used for allelic exchange. The *BamH*I and *Nhe*I cloning sites used for insertion of the 1151 bp *bscN* insert are shown. Also shown is an ampicillin resistance cassette, kanamycin resistance cassette for selection of first recombinants and the *sacBR* gene used for selection of second recombinants in *Bordetella*.

A



B



C

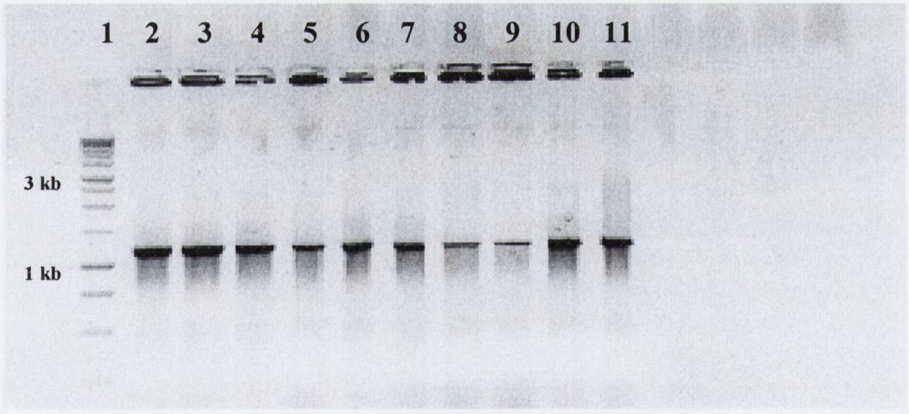


FIGURE 4.10 – (A) Plasmid pNF11. A suicide vector containing the re-designed 1013 bp *bscN* mutant allele used for allelic exchange. Also shown are an ampicillin resistance cassette, kanamycin resistance cassette for selection of first recombinants, SacBR cassette for selection of second recombinants in *Bordetella* and the oriT origin of replication. **(B) Agarose gel electrophoresis of PCR analysis of putative second recombinants of *B. bronchiseptica* RB50 and *B. pertussis Tohama* I (C) generated via allelic exchange using vector pNF11.** The absence of the 589 bp *bscN* mutant allele is noted, while the 1128 bp *bscN* wild type allele is present in all clones screened. Lane 1 shows 1 kb DNA ladder (Promega, USA) while all other lanes show individual colonies. The results are representative from 10 colonies from each species. 50 colonies of each species were tested in total.

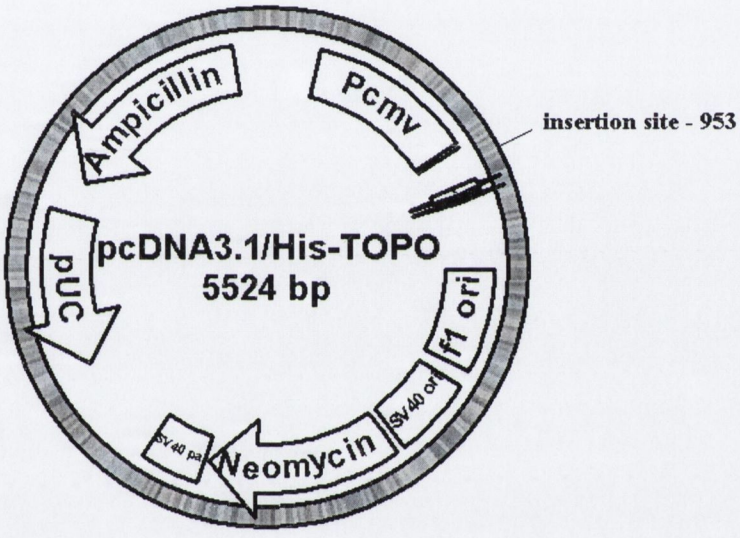


FIGURE 4.11 – Plasmid pcDNA3.1/His-TOPO. A commercial mammalian expression "T" vector. The vector is supplied linearized between base pairs 953 and 954. This is the TOPO insertion site used for efficient ligation of *Taq*-polymerase amplified PCR products. The vector also contains the human cytomegalovirus promoter, the SV40 promoter, f1 and pUC derived origins of replication and both neomycin and ampicillin resistance cassettes.

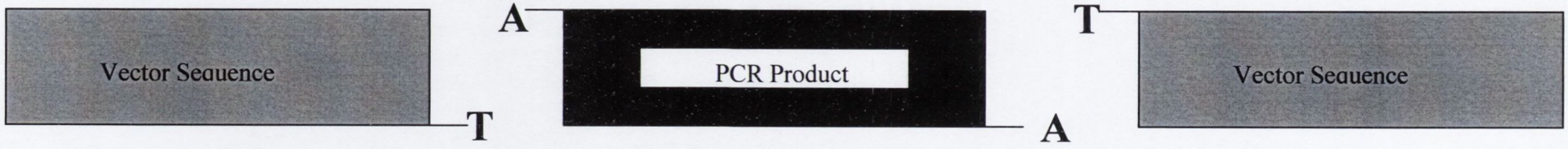


FIGURE 4.12 – TOPO cloning schematic. *Taq* DNA polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized pcDNA3.1/His-TOPO vector contains single, overhanging 3' deoxythymidine (T) residues, which allows PCR products to efficiently ligate with the vector.

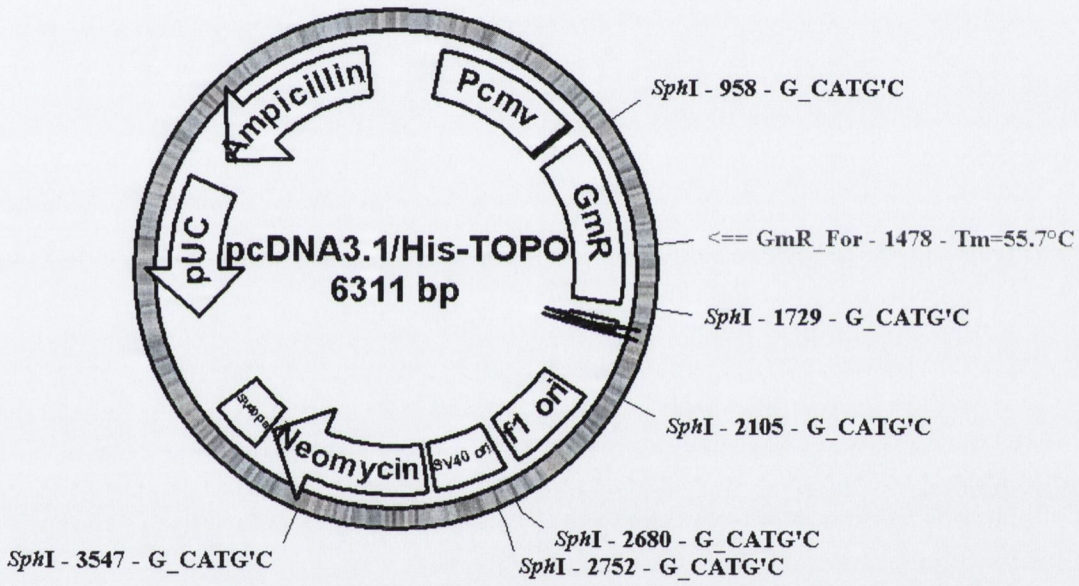


FIGURE 4.13 – Plasmid pcDNA3.1/HisTOPO containing GmR. A derivative of plasmid pcDNA3.1/His-TOPO. The S*ph*I sites at positions 958 bp and 1729 bp used to remove the Gmr cassette for insertion into pNF7 are shown. The start codon for the Gmr cassette is represented by the priming site for oligonucleotide Gmr_For at position 1478 bp. The upstream UTR of the Gmr cassette containing its promoter and RBS is thus indicated between positions 1478 – 1729 bp. The vector also contains the human cytomegalovirus promoter, the SV40 promoter, f1 and pUC derived origins of replication and both neomycin and ampicillin resistance cassettes.

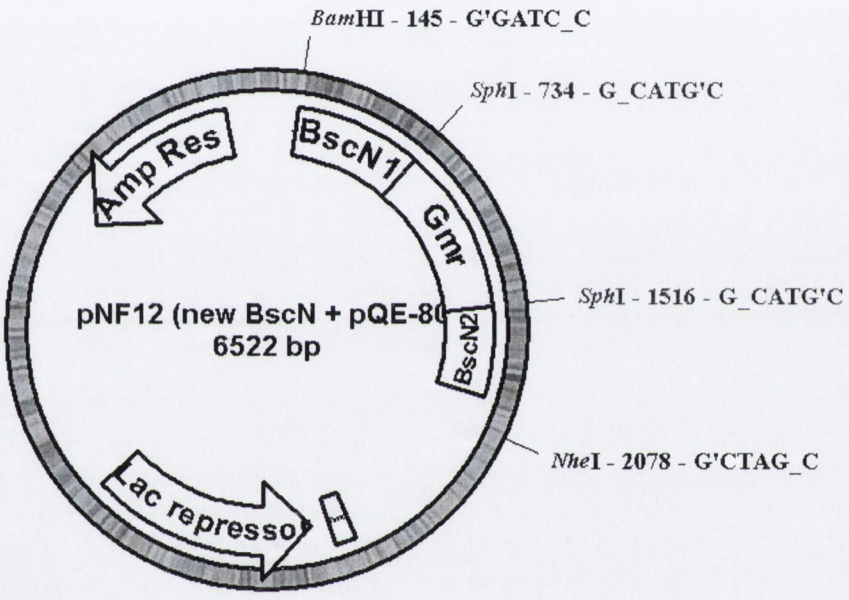


FIGURE 4.14 – Plasmid pNF12. Cloning vector containing the re-designed 1795 bp *bscN* mutant allele with a Gm-resistance cassette inserted in place of the deleted portion of the original wild type allele. The Gm-cassette was amplified from plasmid pNF4. The S*ph*I restriction sites used to insert the Gm-cassette, and the *Bam*HI and *Nhe*I sites used to remove the entire 1933 bp *BscN*::Gmr insert are indicated. Also shown is an ampicillin resistance cassette and the T5 promoter and Lac repressor for protein expression.

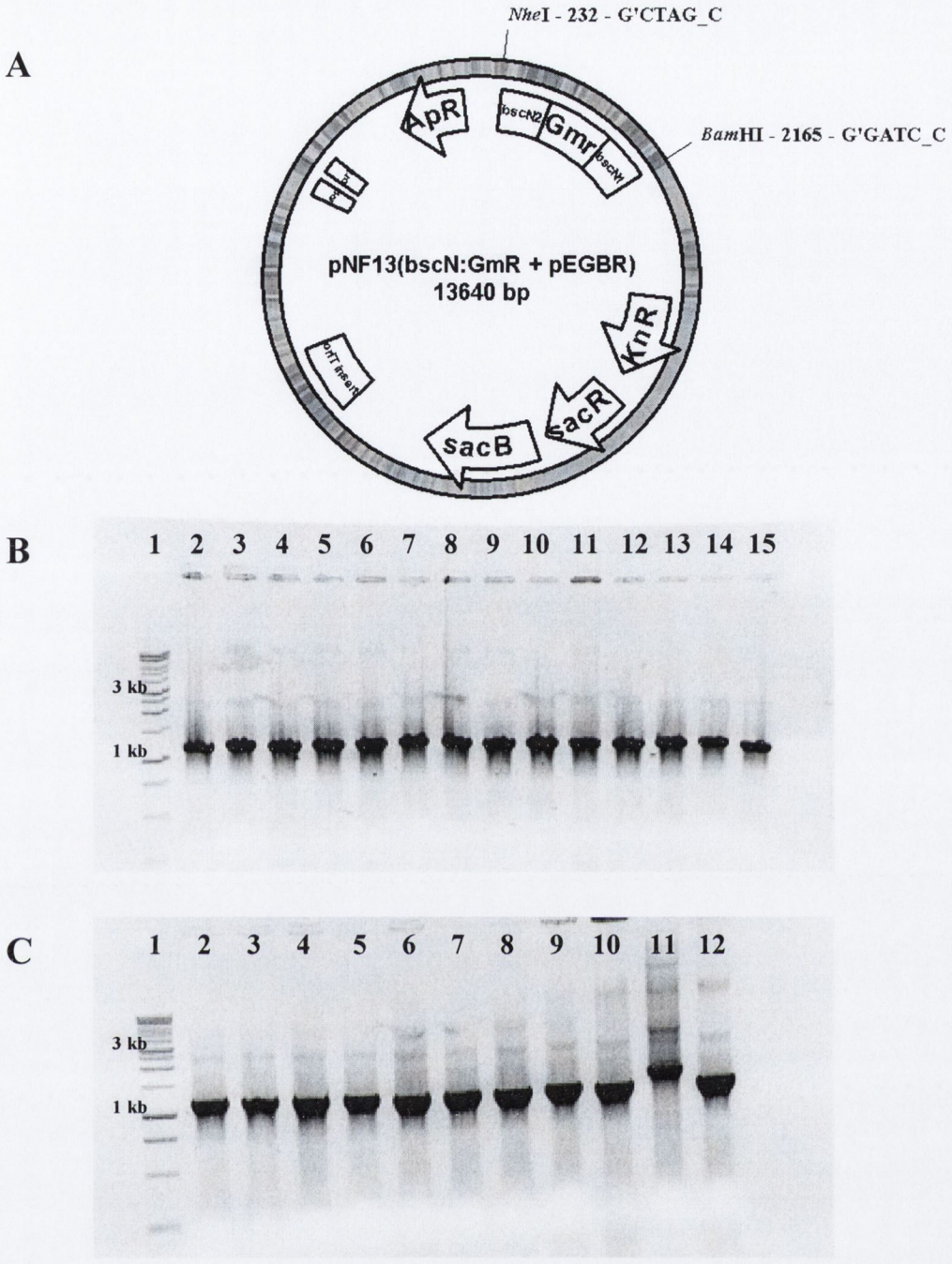


FIGURE 4.15 – (A) Plasmid pNF13. pEGBR suicide vector containing the re-designed 1795 bp *bscN* mutant allele with Gm-resistance cassette inserted in place of the deleted portion of the original wild type allele. The *Bam*HI and *Nhe*I cloning sites used for insertion of the 1933 bp *bscN*::Gmr insert are shown. Also shown is an ampicillin resistance cassette, kanamycin resistance cassette for selection of first recombinants and the *sacBR* gene for the selection of second recombinants in *Bordetella.* **(B)Agarose gel electrophoresis of PCR analysis of putative second recombinants of *B. bronchiseptica* RB50 and (C) *B. pertussis* Tohama I**, generated by allelic exchange using vector pNF13. The absence of the 1364 bp *bscN* mutant allele is noted, while the 1128 bp *bscN* wild type allele is present in all clones screened. Lane 1 shows 1 kb DNA ladder (Promega, USA), while lanes 11 and 12 (C) show mutant allele and wild type controls respectively. All other lanes show individual colonies. The results shown are representative from 14 (*B. bronchiseptica* B) and 9 (*B. pertussis* C) colonies. 50 colonies of each species were tested in total.

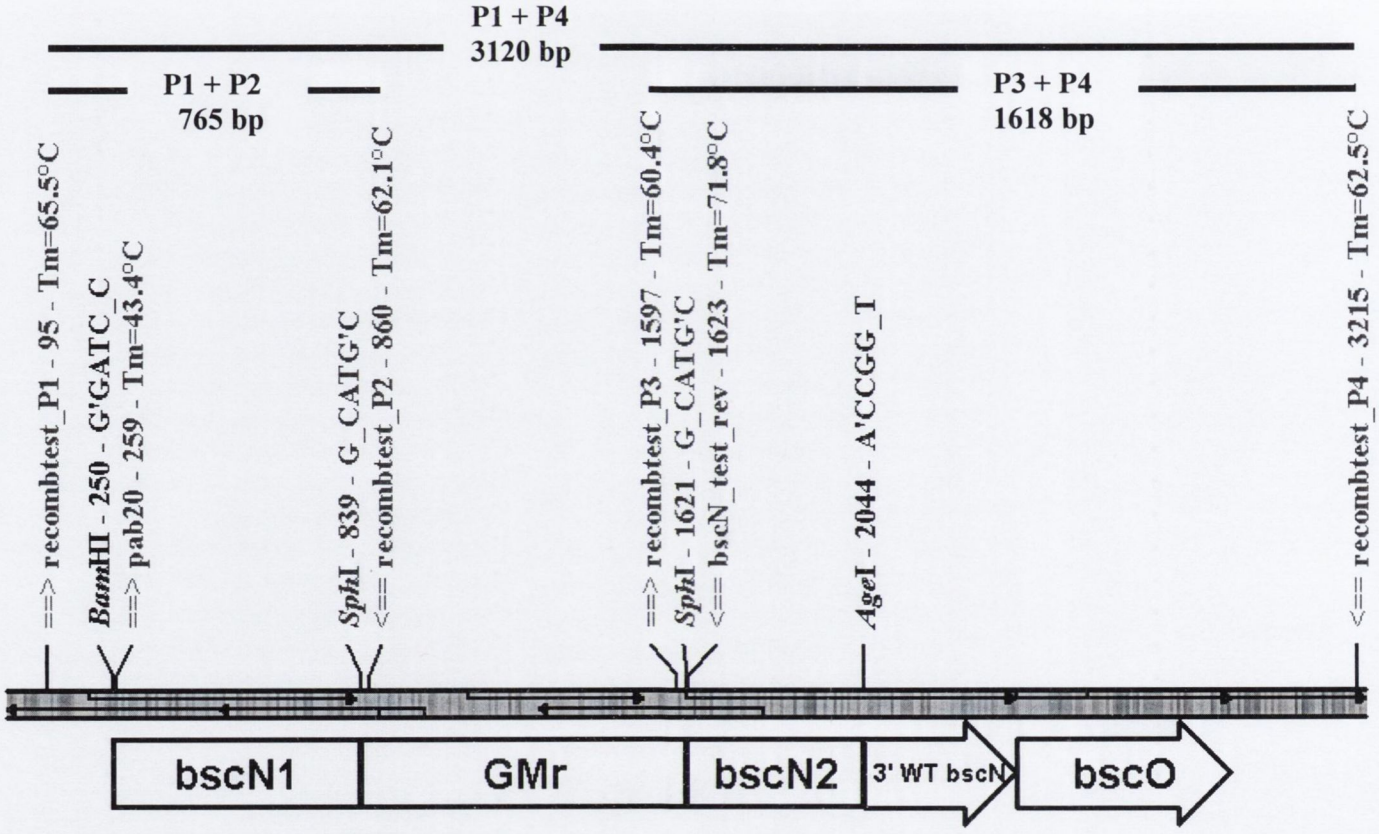


Figure 4.16 – Recombination test schematic of the *bscN* locus of the *Bordetella* chromosome following successful allelic exchange of the 1795 bp *bscN* mutant allele. The binding sites and the PCR product sizes resulting from the various combinations of the recombtest primers (P1-P4) are shown above.

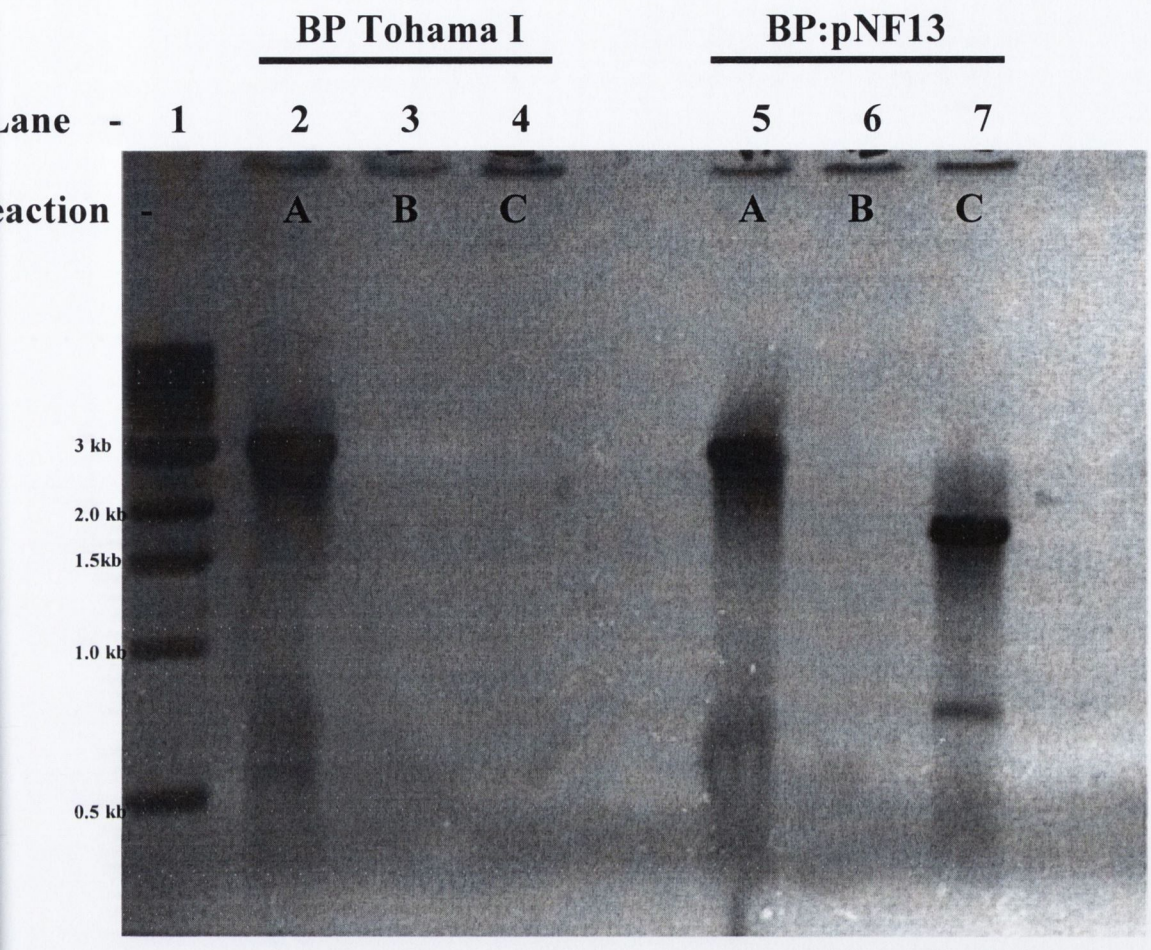


FIGURE 4.17 – PCR recombination test of BP::pNF13. Agarose gel electrophoresis of PCR products of BP Tohama I (lanes 2-4) and Bp: pNF13 (lanes 5-7) generated using recombtest primers P1-P4. Reactions are labelled A (P1 + P4), B (P1 + P2) and C (P3 + P4). BP Tohama I is positive only for reaction A indicating a wild type genotype (lane 2), while Bp::pNF13 is positive for both reaction A and C (lanes 5 and 7), indicating a mixed population of spontaneous Kn^r and Gm^r wild-type bacteria, and first recombinant bacteria. Lane 1 shows 1 kb DNA ladder (New England Biolabs, USA).

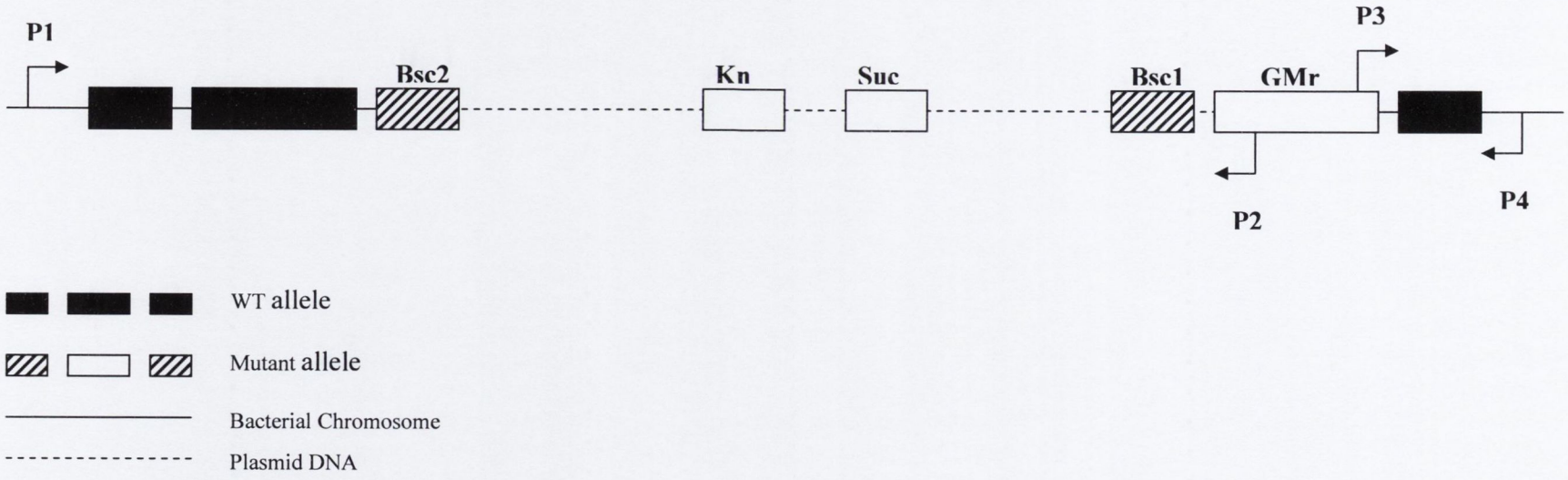
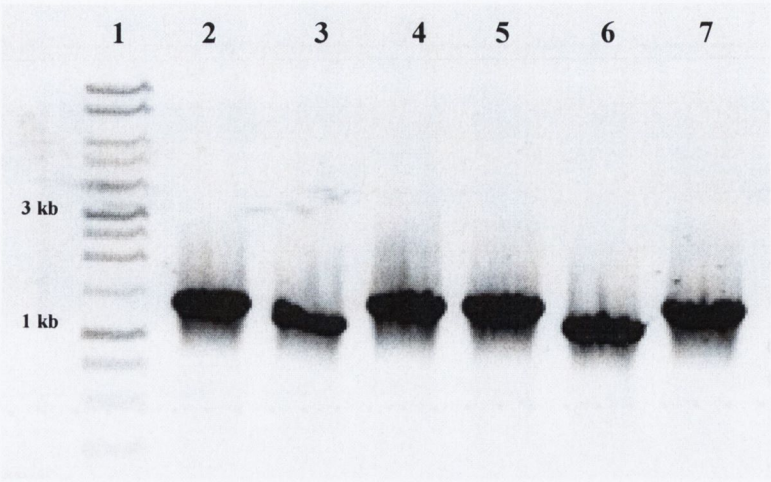


FIGURE 4.18 – First recombination through *bscN2* – Schematic of the chromosome of *B. pertussis* harbouring the linearised plasmid DNA of plasmid pNF13 (Bp::pNF13), inserted into the chromosome through first recombination between the *bscN2* portion of the mutant allele on pNF13 and the corresponding bases of the wild type *bscN* allele on the bacterial chromosome. The inserted plasmid DNA is shown (dashed line), as are the binding sites of the recombtest primers (P1 – P4)



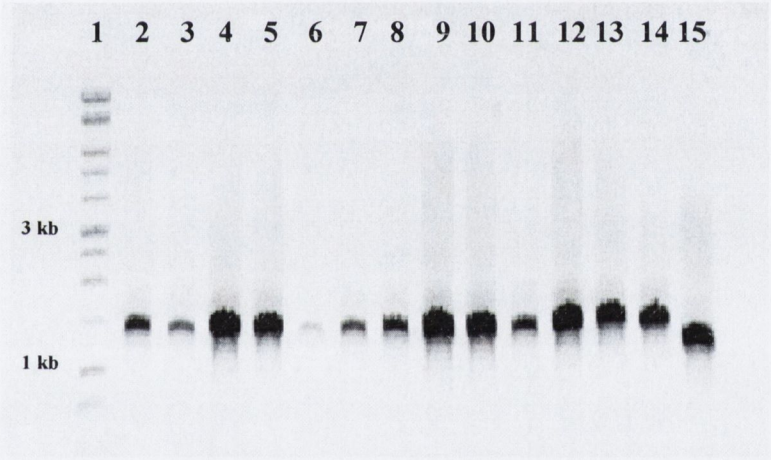


FIGURE 4.19 – Agarose gel electrophoresis of PCR analysis of putative second recombinants of *B. bronchiseptica* RB50 (A) and *B. pertussis* Tohama I (B), generated by allelic exchange using vector pNF13. Lane 1 shows 1 kb DNA ladder (Promega, USA). Lanes 2-5 (A) show the presence of the 1364 bp mutant allele in each colony tested while lanes 6 and 7 show wild type and mutant allele controls respectively.
(B) Lanes 2- 13 show the presence of the 1364 bp mutant allele in all colonies tested. Lanes 14 and 15 show mutant allele and wild type controls respectively.

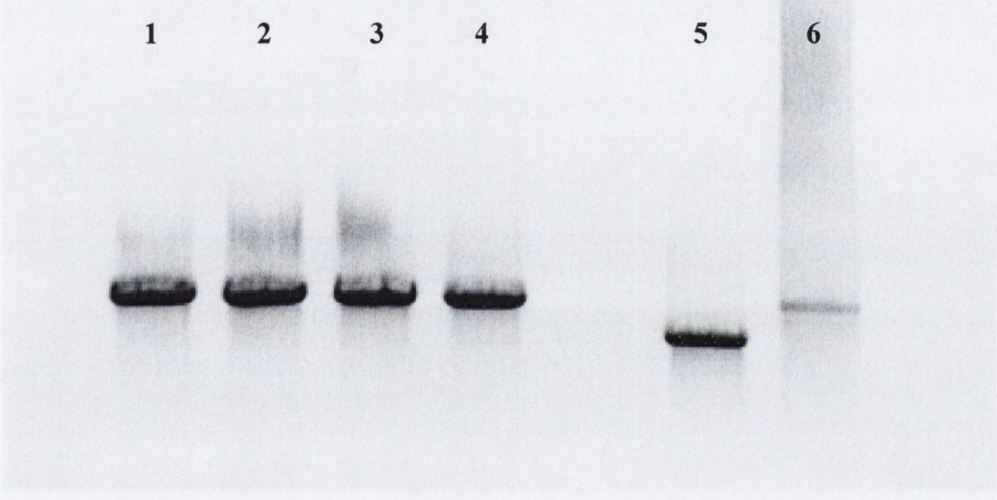


FIGURE 4.20 – Agarose gel electrophoresis of PCR analysis second recombinants of *B. pertussis* 12743 generated by allelic exchange using vector pNF13. Lanes 1-4 show the presence of the 1364 bp mutant allele in each of the four colonies tested. Lanes 5 and 6 show wild type and mutant allele controls respectively.

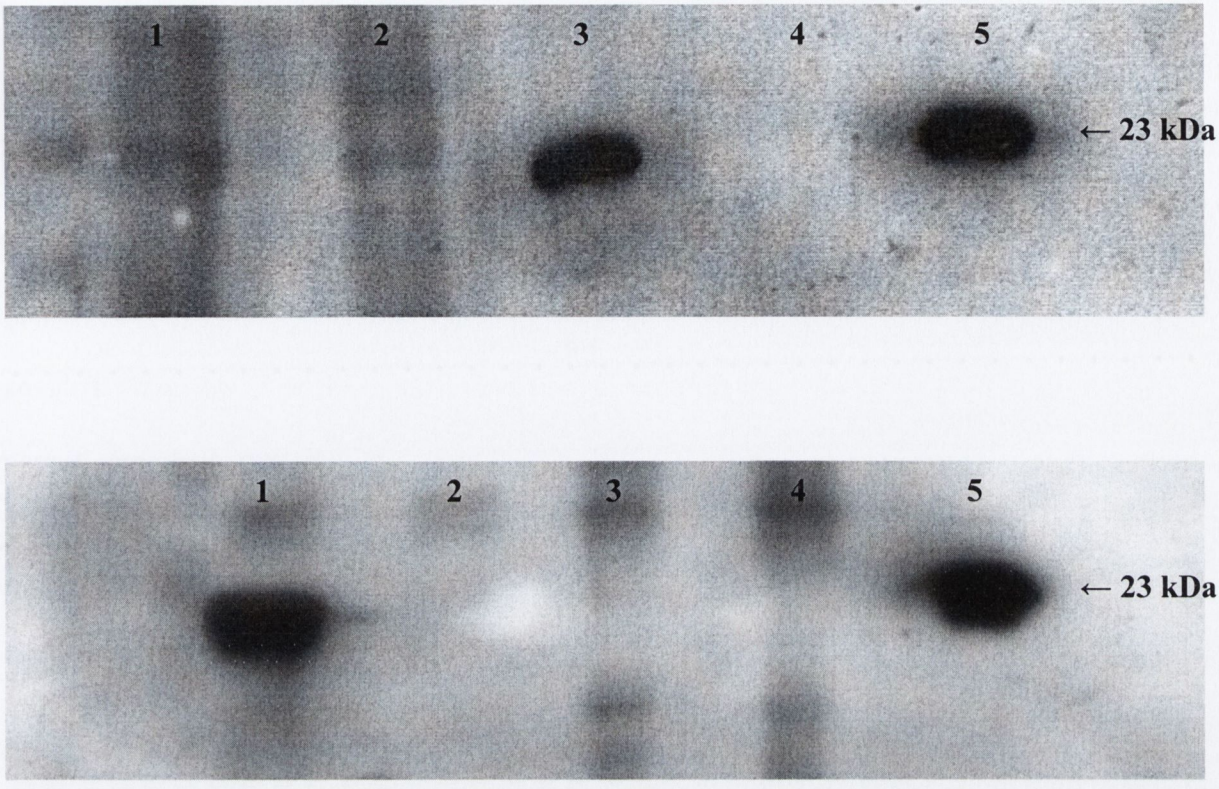
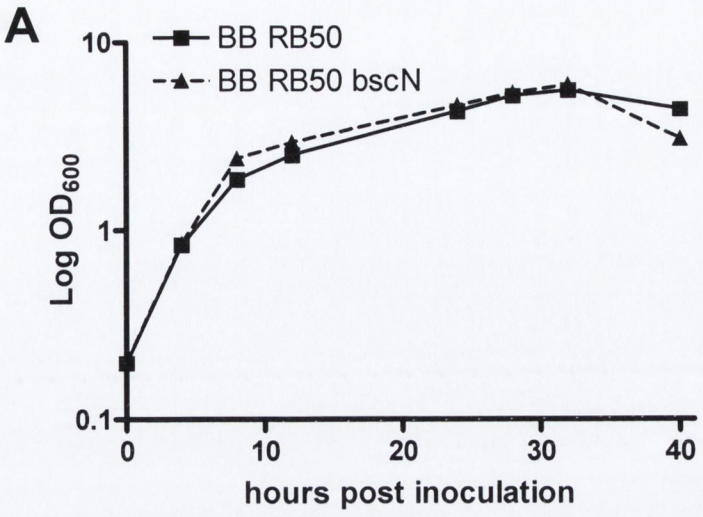
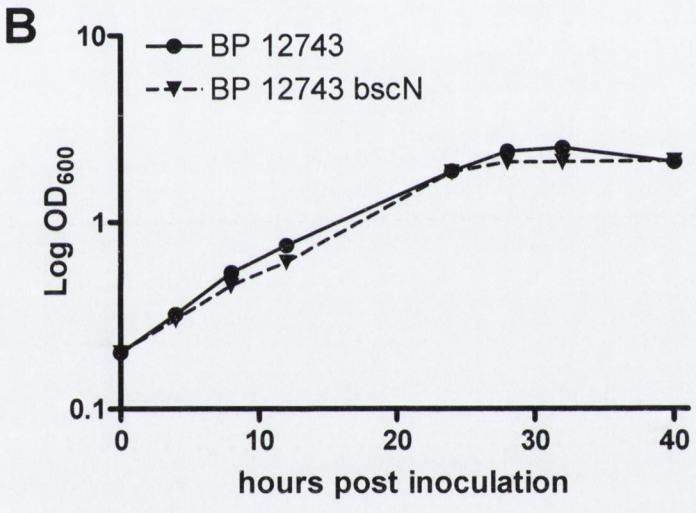
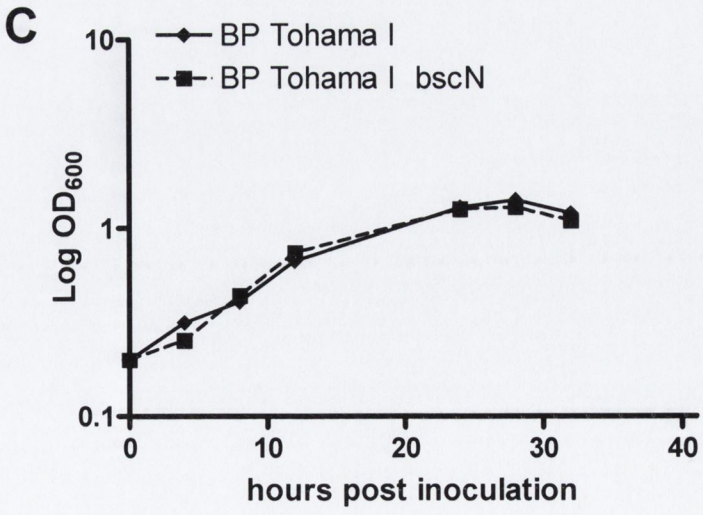


FIGURE 4.21 Mutation of *bscN* in *Bordetella* spp. abolishes Bsp22 production *in vitro*. (A) Western blot analysis of supernatants from stationary phase cultures of BP Tohama I (lane 1), BP Tohama I bscN (lane 2), BB RB50 (lane 3), BB RB50 bscN (lane 4) or 2 μg rHis-Bsp22 (lane 5). (B) Western blot analysis of supernatants from stationary phase cultures of BP 12743 (lane 1), BP 12743 bscN (lane 2), BP Tohama I (lane 3), BP Tohama I bscN (lane 4) or 2 μg rHis-Bsp22 (lane 5). Protein samples were prepared from an equal volume (2 ml) of bacterial culture supernantant following precipitation with 10% TCA, and probed with anti-Bsp22 antibodies.

FIGURE 4.22 Mutation of *bscN* does not significantly affect the rate of bacterial growth. 100 ml cultures of *B. bronchiseptica* RB50 or *B. bronchiseptica* RB50 bscN (A), *B. pertussis* 12743 or *B. pertussis* 12743 bscN (B) and *B. pertussis* Tohama I or *B. pertussis* Tohama I bscN (C) were inoculated at a starting OD_{600} of 0.2 from actively growing precultures as described in material and methods. Cultures were grown to stationary phase and OD_{600} measurements were taken after 4, 8, 12, 24, 28, 32 and 40 hours.


A
BB RB50
BB RB50 bscN
Log OD600
10
1
0.1
0
10
20
30
40
hours post inoculation


B
BP 12743
BP 12743 bscN
Log OD600
10
1
0.1
0
10
20
30
40
hours post inoculation


C
BP Tohama I
BP Tohama I bscN
Log OD600
10
1
0.1
0
10
20
30
40
hours post inoculation

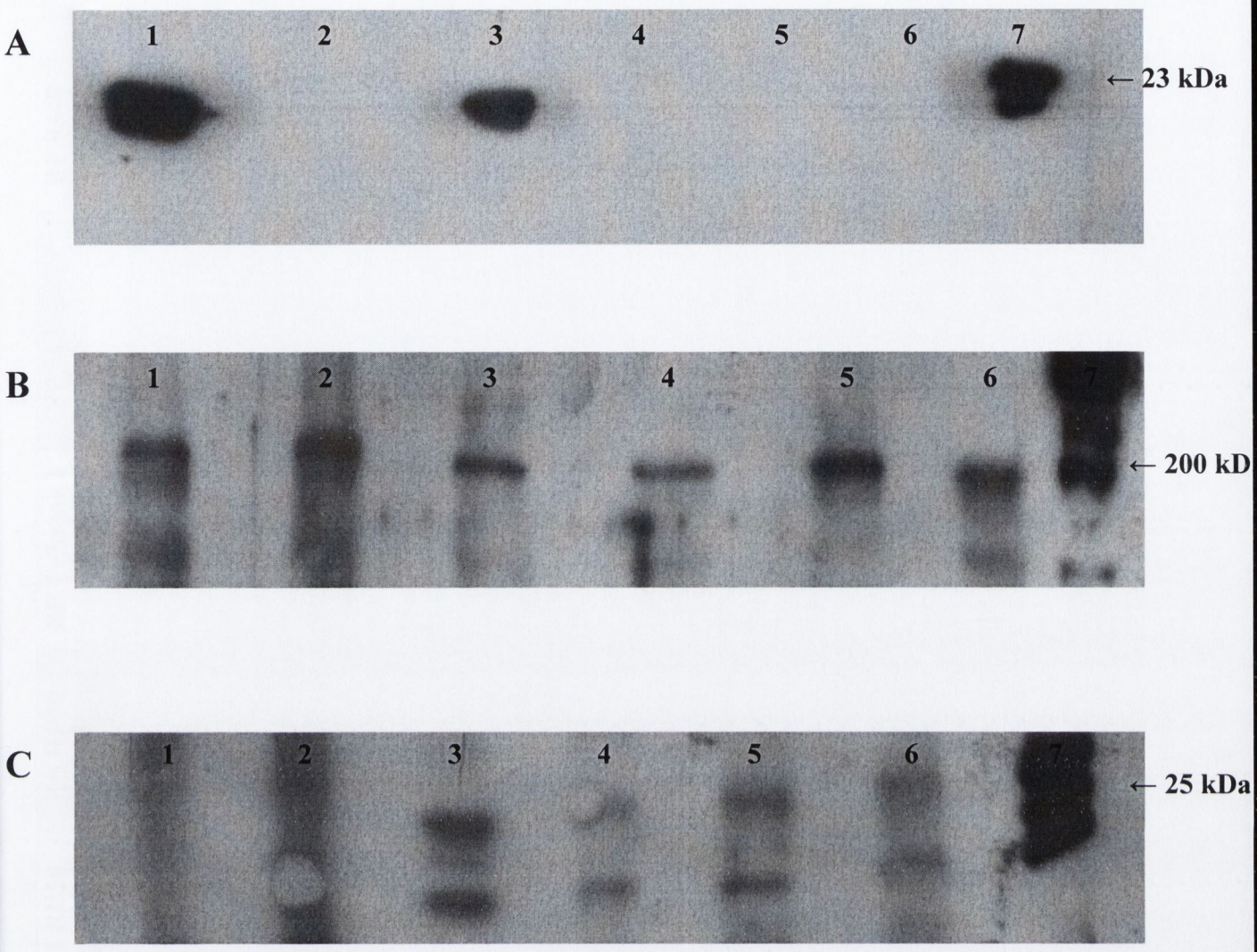


FIGURE 4.23 Mutation of bscN does not affect secretion of the *Bordetella* virulence factors FHA and PT. Western blot analysis of supernatants from 24 hour cultures of BB RB50 (lane 1), BB RB50 bscN (lane 2), BP 12743 (lane 3), BP 12743 bscN (lane 4), BP Tohama I (lane 5) or BP Tohama I bscN (lane 6). Protein samples were prepared from an equal volume of bacterial culture supernatant (2 ml) following precipitation with 10% trichloroacetic acid TCA, and subjected to western blotting with anti-Bsp22 (A), anti-FHA (B) and anti-PT (C) antibodies. Lane 7; rHis-Bsp22 (~2 μg) (A), purified FHA (~5 μg) (B) and purified PT (~2 μg)(C)

ORF	BP 12743 (Neat)	BP 12743 bscN (Neat)	BP 12743 (1/10)	BB RB50 (1/10)	BB RB50 bscN (1/10)	BP Tohama I (1/10)	BP Tohama I bscN (1/10)	BP 9340 (1/10)
fhaB	+	+	+	+	+	+	+	+
cyaA	+	+	+	+	+	+	+	+
brkA	+	+	+	-	-	+	+	-
tcfA	+	+	+	-	-	+	+	+
ptxA	+	+	-	-	-	-	-	-
ptxB	+	+	-	-	-	-	-	-
ptxD	+	+	-	-	-	-	-	-
ptxE	+	+	-	-	-	-	-	-
Prn	+	+	+	+	+	+	+	+
bopD	+	-	+	+	-	-	-	-
bsp22	+	-	+	+	-	-	-	+
bopN	+	-	-	-	-	-	-	-
bteA/bopC	-	-	-	+	-	-	-	-

Virulence factors of *Bordetella* spp. identified in culture supernatants by MALDI-TOF analysis. Total proteins were precipitated from an equal volume of bacterial culture supernatant (10 ml) from each strain with 30% ammonium chloride. MALDI-TOF analysis of protein pellets was carried out in The Laboratory of Mass Spectroscopy, Polish academy of Sciences, Warsaw and identified peptides were searched against the complete *B. bronchiseptica* RB50 and *B. pertussis* Tohama I genome sequences available from the Sanger centre, UK (www.sanger.ac.uk/projects/b_pertussis/bronchiseptica). BP 12743 and BP 12743 bscN protein samples were analysed neat as indicated. The BP 12743 protein sample was also analysed at a 1/10 dilution of the neat sample. Due to excessively high protein concentration, all other protein samples were analysed at a 1/10 dilution.

Chapter 5

The role of the T3SS of *Bordetella* spp. in uptake, cytotoxicity and cytokine production by innate immune cells *in vitro*

5.1 INTRODUCTION

T3SS of animal pathogens represent a sophisticated weapon used by invading bacteria to circumvent target cell function, in order to favour survival of the bacteria in the host. Adherence of the pathogen to the target cell is necessary in order to facilitate effector delivery, and eukaryotic cell contact is thought to be the main trigger of T3 secretion *in vivo*. This cell contact dependent T3SS activation can also be triggered *in vitro*, following co-incubation of pathogens and mammalian cells. Thus, the interaction of pathogens such as *Bordetella* spp. with cultured mammalian cells *in vitro* represents a simple yet effective way to gain insight into the cellular impact of *Bordetella* T3 secretion and therefore possible T3SS function during infection *in vivo*.

Cell types encountered by bacteria that infect the host through mucosal surfaces such as *Bordetella, Yersinia* and *Salmonella* spp. include epithelial cells, and tissue resident and infiltrating cells of the immune system, such as macrophage and DC. The latter cells represent an interesting target for pathogens as they are the bridge between innate and adaptive immunity and therefore have crucial roles in pathogen clearance. For example, during *Bordetella* infection, DC function to counteract the infection, being rapidly recruited to the upper respiratory tract, before being delivered to the lymph nodes draining the respiratory mucosa, where they direct the subsequent adaptive immune response (Gueirard *et al.* 2003). Macrophages and neutrophils play a critical role in the clearance of intracellular pathogens. Furthermore Th1 cells, which help to recruit and activate phagocytic cells have been shown to play a critical role in protection against *B. pertussis* infection in mice (Mills *et al.* 1993; Redhead *et al.* 1993). Thus the interactions of *Bordetella* and other T3SS-bearing pathogens with innate immune cells afford the bacteria an opportunity to subvert the host immune response and promote pathogen survival.

The most extensive studies performed on the effect of T3SS *in vitro* have been conducted with *Yersinia* spp. These primarily extracellular pathogens secrete a range of effectors, which act in concert to prevent bacterial uptake (Cornelis *et al.* 1998; Cornelis 2002). The mechanism of inhibition of phagocytosis by the T3SS involves host cell cytoskeleton paralysis and is extremely important for *Yersinia* pathogenesis, since it allows the bacteria to avoid phagocyte killing (Hueck 1998). In contrast, the primarily

intracellular pathogens *Salmonella* and *Shigella*, utilise a T3SS to induce uptake into epithelial cells, also through the disruption of the target cell cytoskeleton (Galan and Curtiss 1989) (Sansonetti *et al.* 1982; Menard *et al.* 1993; Hueck 1998). This T3SS-mediated invasion of epithelial cells allows these pathogens to penetrate the intestinal mucosa and is a key step in their pathogenesis. Thus the host cell cytoskeleton is a common target of T3SS from multiple pathogens who manipulate its dynamics for their own specific needs.

A common outcome of the interaction of T3SS expressing bacteria with host cells is host cell death, through the T3SS-mediated induction of both necrosis and apoptosis. T3SS-mediated apoptosis of resident macrophage in the lymphoid tissue underlying the intestinal epithelium during *Shigella* (Zychlinsky *et al.* 1996), and *Salmonella* (Guiney 2005) infection results in a significant influx of PMN, through a mechanism involving caspase 1 activation and IL-1β release. This destabilises the integrity of the intestinal barrier, allowing these pathogens to further penetrate the intestinal submucosa (Perdomo *et al.* 1994; Guiney 2005). T3SS-mediated macrophage apoptosis is also displayed by *Yersinia* spp. (Mills *et al.* 1997), in addition to having a cytotoxic effect on epithelial cells, through a mechanism involving disruption of actin microfilament structures (Rosqvist *et al.* 1991). Interestingly, *B. bronchiseptica*, another primarily extracellular pathogen, has been reported to display both T3SS-dependent cytotoxicity of epithelial cells (Yuk *et al.* 1998), and T3SS-dependent induction of apoptosis in J774 macrophage (Yuk *et al.* 2000). However, a more recent study reported T3SS-dependent killing of peritoneal macrophage from caspase 1 knockout mice, suggesting that the *B. bronchiseptica* T3SS induces necrotic, rather than apoptotic cell death (Stockbauer *et al.* 2003).

As innate immune cells have the capacity to direct subsequent antigen-specific adaptive immune responses after encountering pathogens, the suppression of intracellular signalling that leads to cytokine release and DC maturation can be of significant benefit to the pathogen. T3SS mediated modulation of signalling in macrophage and DC can result in the upregulation of anti-inflammatory cytokines, such as the *Yersinia* T3SS-mediated enhancement of IL-10 release (Nedialkov *et al.* 1997; Sing *et al.* 2002), and inhibition of the pro-inflammatory cytokines IFNγ and TNFα (Nakajima and Brubaker

1993). This has been attributed to the ability of *Yersinia* to inhibit MAP kinase signalling pathways in macrophage (Ruckdeschel *et al.* 1997). Recently, the T3SS of internalised *Salmonella* spp. has been shown to inhibit the presentation of antigenic peptides by MHC class II molecules on DC, thereby preventing the generation of *Salmonella*-specific adaptive immune responses (Cheminay *et al.* 2005). A similar strategy has also recently been demonstrated for the T3SS of *B. bronchiseptica*. Reissinger *et a*l have demonstrated T3SS dependent downregulation of ERK and p38 activation in macrophage, resulting in attenuated production of IL-6 and IL-10 (Reissinger *et al.* 2005). Skinner *et al* have shown the T3SS-dependent activation of the ERK MAPK pathway in DC, resulting in increased expression of MHC class II, CD80 and CD86, and an altered DC maturation status (Skinner *et al.* 2004).

While the cellular impact of the T3SS of *B. bronchiseptica* has been investigated, there are no reports to date detailing the role of the T3SS of *B. pertussis* on infection of cells *in vitro*. Fauconnier *et al* reported no differences in cytotoxicity of J774 macrophage cells following incubation with wild-type and T3SS deficient *B. pertussis* Tohama I (Fauconnier *et al.* 2001). However, as this thesis has already demonstrated, this strain of *B. pertussis* does not possess T3SS activity *in vitro.* The present study set out to examine the role of the T3SS of *B. pertussis* 12743, in comparison with the role of the T3SS of *B. bronchiseptica* RB50, in the induction of cytotoxicity in epithelial, macrophage and DC, and its effect on cytokine production following infection of DC. In addition, the role of the *Bordetella* T3SS in modulating bacterial uptake by macrophage was also investigated.

5.2 RESULTS

5.2.1 The T3SS of *B. bronchiseptica* RB50 but not *B. pertussis* 12743 or *B. pertussis* Tohama I mediates cytotoxicity against cultured macrophage, epithelial and dendritic cells.

Previous reports have demonstrated that the T3SS of *B. bronchiseptica* can mediate cytotoxicity in a wide range of cultured cells (Yuk *et al.* 1998; Yuk *et al.* 2000; Kuwae *et al.* 2003; Stockbauer *et al.* 2003; Nogawa *et al.* 2004; Skinner *et al.* 2004) and that this cytotoxicity is dependent on the expression of the *Bordetella* T3SS effector, BteA (Panina *et al.* 2005), also named BopC (Kuwae *et al.* 2006). The present study examined the role of the T3SS of BP 12743 and BP Tohama I, in comparison to that of the T3SS of BB RB50 on cytotoxicity of macrophage, DC and epithelial cells. Monolayers of murine J774 macrophage, murine BMDC and human HEK 293T epithelial cells were infected with wild-type and T3SS deficient *Bordetella* spp. for a period of 2 and 4 hours at an MOI of 20, 100 and 500. As can be seen from Fig. 5.1 and 5.2, wild type BB RB50 was cytotoxic to all 3 cell types, as measured by LDH release. Furthermore, the level of cytotoxicity induced increased with increasing MOI and duration of infection. In contrast, no LDH release was detected from monolayers infected with T3SS deficient BB RB50 bscN, even at the highest MOI of 500 for all 3 cell types (Fig 5.1 and 5.2).

In contrast to *B. bronchiseptica*, no significant cytotoxicity was detected in any cell type infected with wild-type or T3SS deficient BP 12743 or BP Tohama I, even at the highest MOI of 500. These results demonstrate that the T3SS of *B. bronchiseptica*, but not *B. pertussis* 12743 or *B. pertussis* Tohama I, mediates significant cytotoxicity to cultured macrophage, dendritic and epithelial cells.

5.2.2 The T3SS of *B. bronchiseptica* RB50, but not *B. pertussis* 12743, inhibits phagocytic uptake of the bacteria by murine macrophage.

The T3SS of *Yersinia* spp. plays a crucial role in preventing bacterial uptake during infection (Cornelis 2002), while the T3SS of *Salmonella* spp. mediates bacterial uptake to facilitate tissue invasion and intracellular replication (Galan and Curtiss 1989) (Galan and Curtiss 1991) (Abrahams and Hensel 2006). The modulation of host cell cytoskeletal dynamics is a common theme amongst T3SS of different bacterial species. To our knowledge there are no previous reports that have examined the role of the T3SS in phagocytic uptake of *Bordetella* spp. In order to investigate the role of the *Bordetella* T3SS in mediating or preventing bacterial uptake, murine J774 macrophage were infected with wild-type and T3SS deficient BB RB50 and BP 12743 at an MOI of 100 for 3 hrs. Following 2 hours of bacterial contact, cells were washed and centrifuged to remove non-surface associated bacteria and treated for 1 hour with medium only or kanamycin to kill surface-associated bacteria. CFU counts were determined by plating on BG agar.

Significantly higher CFU of T3SS deficient BB RB50 bscN were detected in kanamycin treated J774 in comparison to wild-type BB RB50, indicative of a higher number of internalised viable bacteria (Fig 5.3 A). In contrast, the number of bacteria recovered following treatment with medium were similar following infection with wild-type and T3SS deficient *B. bronchiseptica*, indicative of similar numbers of wild-type and T3SS deficient bacteria bound at the cell surface (Fig 5.3 A). The higher CFU of T3SS deficient BB RB50 bscN detected in Kn treated J774 could be due to a higher number of viable J774 cells at the end of the assay, as a result of a lack of T3SS-mediated cell death. Having previously demonstrated a significant level of T3SS mediated cell death at an MOI of 100 (~ 40% following 4 hours of wild-type bacterial contact and ~ 20% following 2 hours of wild-type bacterial contact, for murine J774 cells; Fig 5.3 A), we corrected the CFU to allow for an estimated level of cell death of 30% following 3 hours of bacterial contact. Figure 5.3 B shows the corrected numbers of viable bacteria recovered from the assay, when T3SS mediated cell death as outlined above is taken into account. The data shows the same trend to uncorrected CFU shown in Fig 5.3 A.

In contrast to *B. bronchiseptica*, the numbers of viable wild-type and T3SS deficient *B. pertussis* 12743 recovered from kanamycin treated J774 were very similar,

indicative of a similar number of internalised *B. pertussis* (Fig 5.4). However, in contrast to *B. bronchiseptica*, the number of viable colonies recovered following treatment with medium alone was significantly lower from the T3SS deficient BP 12743 when compared to wild-type bacteria, indicating a lower number of cell-associated bacteria following infection (Fig 5.4).

These results demonstrate that the T3SS of *B. bronchiseptica* RB50 reduces bacterial uptake by murine J774 macrophage following *in vitro* infection, whereas the T3SS of *B. pertussis* 12743 may promote adherence of the bacteria to macrophage *in vitro*.

5.2.3 Differential cytokine expression by BMDC following infection with wild-type and T3SS deficient *B. bronchiseptica* RB50.

Recent reports have demonstrated that *B. bronchiseptica* RB50 can modulate MAP kinase signalling pathways in macrophage and DC (Skinner *et al.* 2004; Reissinger *et al.* 2005). These pathways control the expression of a large number of inflammatory mediators, including inflammatory cytokines (Kyriakis and Avruch 2001). The present study examined the modulatory effect of the T3SS of *B. bronchiseptica* RB50 on cytokine production following infection of murine BMDC. BMDC cell suspensions were infected with wild-type and T3SS deficient *B. bronchiseptica* spp. at a range of MOI ranging from 0.1 to 1000, and the production of the pro-inflammatory cytokines, IL-12p70, IL-12p40, IL-23, TNFα and IL-1β, and the anti-inflammatory cytokine, IL-10, were measured 6 hours later (Fig 5.5).

In general, the concentrations of cytokines produced by BMDC in response to infection with T3SS deficient *B. bronchiseptica* were significantly higher when compared with those produced in response to wild-type *B. bronchiseptica* 6 hours post infection (Fig 5.5). This includes the anti-inflammatory cytokine IL-10, as well as the pro-inflammatory cytokines IL-12p40, IL-23 and TNFα (Fig 5.5). The exception was IL-1β, where significantly lower levels of IL-1β were detected in response to T3SS mutant BB RB50 bscN 6 hours post infection at the higher MOI's. Interestingly, this trend was reversed with reducing MOI, with significantly higher IL-1β production in response to

T3SS mutant BB RB50 bscN infection at an MOI of 1 (Fig 5.5). IL-12p70 was not detected 6 hours post infection.

These results suggest that the T3SS of *B. bronchiseptica* may have the ability to modulate cytokine production during infection of BMDC, though the lower level of cytokines produced following wild-type BB RB50 infection could possibly be attributed to the significant cell death induced in these cells.

5.2.4 Cytokine production by DC following infection with wild-type and T3SS deficient *B. pertussis* 12743 and Tohama I.

We next examined the ability of the T3SS of *B. pertussis* 12743 to modulate cytokine production by BMDC in comparison to *B. bronchiseptica* RB50 and *B. pertussis* Tohama I. BMDC cell suspensions were infected with wild-type and T3SS defcient *B. pertussis* 12743 and Tohama I at a range of MOI ranging from 0.1 to 1000, and supernatents were assayed for pro and anti-inflammatory cytokine production. The data shown in Fig. 5.5 reveal that the concentrations of the pro-inflammatory cytokines, IL-12p70, IL-12p40, TNFα and IL-1β, and the anti-inflammatory cytokine, IL-10, produced by BMDC are similar in response to infection with wild-type and T3SS deficient *B. pertussis* 12743 (Fig 5.6). However, higher concentrations of IL-23 were produced by BMDC 6 hours post infection with T3SS deficient *B. pertussis* 12743 when compared to wild-type bacteria at high MOI (Fig 5.6).

Similarly, the data shown in Fig. 5.7 demonstrate that the concentrations of the pro-inflammatory cytokines IL-12p70, IL-12p40, TNFα and IL-1β produced by BMDC are similar in response to infection with wild-type and T3SS deficient *B. pertussis* Tohama I. In contrast to *B. pertussis* 12743, significantly higher concentrations of the anti-inflammatory cytokine, IL-10, were detected with T3SS deficient BP Tohama I when compared to wild-type bacteria (Fig 5.7).

These results suggest that the T3SS of *B. pertussis* 12743 may inhibit IL-23 production by BMDC following infection. In addition, the data also suggest that the T3SS of *B. pertussis* Tohama I may inhibit IL-10 production by DC following infection.

5.2.5 Enhanced pro-inflammatory cytokine production by DC in response to live or heat-killed T3SS deficient *B. bronchiseptica* compared with wild-type bacteria.

The results shown in section 5.2.3 showed that T3SS deficient *B. bronchiseptica* induced higher cytokine production in BMDC than wild-type bacteria. Here the requirement for viable bacteria (and therefore a functional T3SS) was assessed by analysing cytokine production by BMDC either infected with live wild-type and T3SS deficient *B. bronchiseptica* spp. at an MOI of 100 for 6 hours, or treated with heat-killed wild-type and T3SS deficient *B. bronchiseptica.* Consistent with the data in Fig 5.5 significantly higher IL-12p40, IL-12p70, IL-23 and TNFα was detected in supernatants from BMDC infected with T3SS deficient BB RB50 bscN when compared to those from BMDC infected with wild-type BB RB50 (Fig 5.8). In contrast, the concentrations of these cytokines induced by heat killed wild-type and T3SS deficient BB RB50 were similar (Fig 5.8), suggesting the higher concentrations of cytokines detected following infection with T3SS deficient BB RB50 bscN is due to the action of live bacteria. The concentrations of IL-1β produced by DC were similar following infection with wild-type and T3SS bacteria, but were much lower with the equivalent heat killed bacteria, suggesting the production of high concentrations of IL-1β requires live bacteria (Fig 5.8). Although significantly higher concentrations of the anti-inflammatory cytokine, IL-10, were detected in the supernatants from BMDC infected with T3SS deficient BB RB50 bscN, we detected significantly lower IL-10 from DC incubated with heat-killed T3SS deficient BB RB50 bscN when compared with killed wild-type bacteria (Fig 5.8).

These results demonstrate that the T3SS of *B. bronchiseptica* functions to inhibit pro-inflammatory cytokine production from BMDC following *in vitro* infection. The results also demonstrate that heat treatment of the bacteria enhances pro-inflammatory cytokine production to concentrations greater than that observed following infection with live T3SS deficient *B. bronchiseptica*, suggesting that other heat-labile components of the bacteria contribute to suppression of pro-inflammatory cytokine production.

5.2.6 Pro-inflammatory cytokine production by DC in response to live or heat-killed T3SS deficient *B. pertussis* 12743 compared with wild-type bacteria.

To further investigate the role of the T3SS of *B. pertussis* 12743 in the modulation of pro-inflammatory cytokine production, DC were exposed to either live or heat-killed T3SS deficient and wild-type *B. pertussis* 12743. Significantly higher concentrations of IL-12p40, IL-12p70 and IL-23, and significantly lower IL-10, were detected in supernatants from BMDC treated with heat-killed T3SS deficient BP 12743 bscN when compared with the wild-type bacteria (Fig. 5.9). Consistent with data shown in Fig 5.6, with the exception of IL-12p40, the concentrations of cytokines secreted by BMDC were not significantly different following infection with T3SS deficient BP 12743 bscN and wild-type bacteria.

These results demonstrate that the T3SS of *B. pertussis* 12743 does not have a role in the inhibition of cytokine production following *in vitro* infection of DC.

Discussion

T3SS function by delivering effector proteins directly to the target cell cytoplasm following binding at the host cell surface; therefore adherence to the host cell is necessary for this process. Several recent studies on *in vitro* infection of murine cells with *B. bronchiseptica* have suggested a role for the T3SS of this species in inducing host cell death and modulating intracellular signalling pathways involved in immune cell activation (Kuwae *et al.* 2006) (Panina *et al.* 2005) (Reissinger *et al.* 2005) (Skinner *et al.* 2004). However, to date there are no reports concerning the role of the *B. bronchiseptica* T3SS in altering bacterial phagocytic uptake by macrophage, a common T3SS function in other species such as *Yersinia*, *Salmonella* and *Shigella* (Hueck 1998) (Cornelis *et al.* 1998). In addition, consistent with a proposed lack of T3SS function in the pathogenesis of *B. pertussis*, there are no reports on the role of the T3SS on infection with *B. pertussis in vitro*. This study has demonstrated a role for the T3SS of *B. bronchiseptica* in the induction of host cell death, reduction of pro-inflammatory cytokine production from BMDC, and in the prevention of phagocytic uptake of the bacteria by murine macrophage *in vitro*. Furthermore, this study showed that, in contrast to *B. bronchiseptica*, the T3SS of *B. pertussis* 12743 is unable to induce host cell death, reduce pro-inflammatory signalling from BMDC or prevent phagocytic uptake by murine macrophage, but may have a role in promoting adherence to macrophage *in vitro*.

Consistent with previous observations, (Kuwae *et al.* 2006) (Panina *et al.* 2005) (Kuwae *et al.* 2003) (Stockbauer *et al.* 2003) (Yuk *et al.* 2000) (Yuk *et al.* 1998) this study found that the T3SS of *B. bronchiseptica* RB50 could induce cell death in multiple cell types. Wild-type BB RB50 to induced significant cytotoxicity in murine BMDC, murine J774 macrophage and human HEK 293T epithelial cells, and this cytotoxicity was T3SS dependent, since no cell death was observed with T3SS deficient BB RB50 bscN, even at the highest MOI of 500. In contrast to *B. bronchiseptica*, this study demonstrated that *B. pertussis* 12743 or Tohama I did not induce T3SS-dependent host cell cytotoxicity during *in vitro* infection of macrophage, DC or epithelial cells. In fact, there is no cytotoxicity associated with *B. pertussis* infection (Harvill *et al.* 1999) Recently, the first *Bordetella* T3SS effector protein was identified independently by two groups, and named BteA or BopC (Panina *et al.* 2005) (Kuwae *et al.* 2006). This protein was reported to

induce necrotic cell death associated with the T3SS of *B. bronchiseptica* in multiple cell types (Stockbauer *et al.* 2003). However, BP 12743 and Tohama I did not secrete this protein *in vitro* and this may explain the lack of cytotoxicity seen with BP 12743 and Tohama I, and further suggests that the cytotoxicity associated with the T3SS of *B. bronchiseptica* is BteA/BopC-mediated.

Another reported *in vitro* effect of the *B. bronchiseptica* T3SS is the altered maturation or activation status of macrophage and DC, and this effect has been attributed to the ability of the T3SS of *B. bronchiseptica* to interfere with MAPK signalling pathways (Skinner *et al.* 2004; Reissinger *et al.* 2005). In addition to co-stimulatory molecule expression, these signalling pathways control the expression of a large number of inflammatory mediators (Kyriakis and Avruch 2001). The T3SS of *Yersinia* has been shown to inhibit TNFα and IFNγ release from macrophage (Nakajima and Brubaker 1993; Boland and Cornelis 1998). The present study demonstrated significantly higher production of the pro-inflammatory cytokines IL-12p40, IL-12p70, IL-23 and TNFα from murine BMDC in response to infection with T3SS deficient BB RB50 bscN, when compared with wild-type BB RB50. This effect was dependent on live bacteria, as no differences in cytokine production were observed following exposure to heat-killed wild-type and T3SS deficient BB RB50. The T3SS deficient BB RB50 bscN also promoted the production of higher concentrations of the anti-inflammatory cytokine, IL-10, from BMDC than the wild-type BB RB50.

Consistent with the absence of host cell cytotoxicity, cytokine production was not significantly different following infection of BMDC with wild-type *B. pertussis* 12743 and *B. pertussis* Tohama I in comparison with T3SS deficient bacteria. This suggests that the lower concentrations of inflammatory cytokines observed with BB RB50 6 hours post-infection of BMDC, though T3SS-mediated, are likely a result of BteA/BopC-mediated cell death rather than a specific targeting of cytokine production by the T3SS. It is unclear why this effect is not seen with IL-1β. However, this cytokine has no classical secretion signal sequence, and therefore is secreted in a different manner to other pro-inflammatory cytokines, through a mechanism involving accumulation of its precursor, pro-IL-1β, in intracellular vesicles prior to cleavage and subsequent export (Rubartelli *et al.* 1990). Thus the elevated level of IL-1β, when compared to the levels of the other

cytokines, detected following infection of BMDC with live wild-type RB50 may represent the release of an intracellular store of the cytokine following *B. bronchiseptica* induced necrotic cell death.

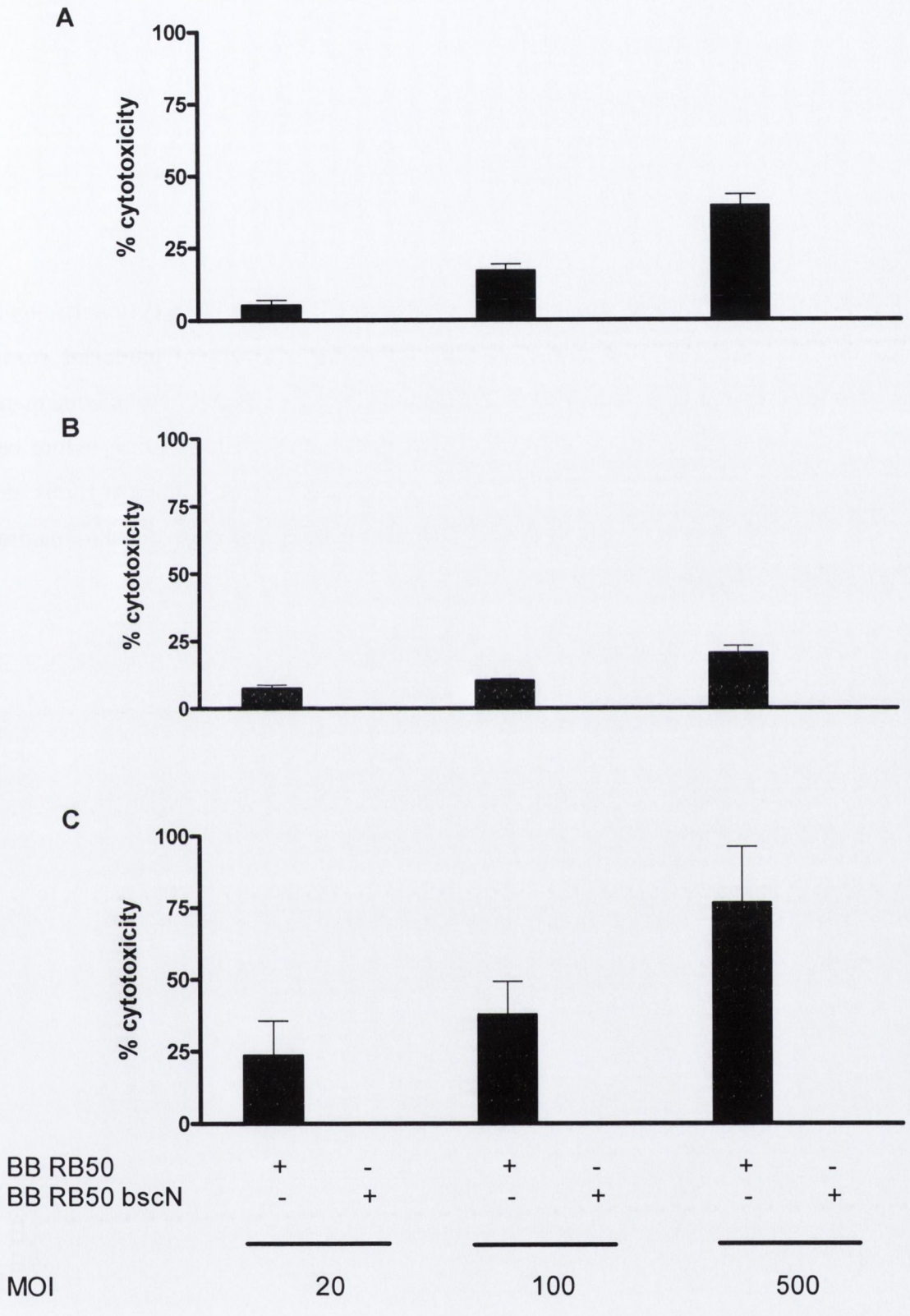
The modulation of host cytoskeleton dynamics is a common theme amongst T3SS expressing bacteria. For example, *Yersinia* spp. strongly prevent their uptake into macrophage (Rosqvist *et al.* 1988; Rosqvist *et al.* 1990; Bliska and Black 1995) and PMN(Visser *et al.* 1995; Andersson *et al.* 1999) in a T3SS-dependent manner. In contrast, both *Salmonella* and *Shigella* spp. utilise a T3SS to promote epithelial cell invasion (Galan and Curtiss 1989) (Watarai *et al.* 1995). In addition, once internalised, both *Salmonella* (Kuhle and Hensel 2004) and *Yersinia* (Cornelis *et al.* 1998) are capable of inhibiting respiratory burst in macrophage and neutrophils in a T3SS-dependent manner. The present study has demonstrated that the T3SS of *B. bronchiseptica* prevent phagocytic uptake of the bacteria by murine macrophage. Significantly higher numbers of viable intracellular bacteria were recovered following infection of murine J774 macrophage with T3SS deficient BB RB50 bscN when compared with wild-type bacteria. Furthermore, the difference in the numbers of CFU recovered was far greater than could be accounted for by T3SS-mediated cell death. To our knowledge this is the first report to identify a role for the T3SS of *B. bronchiseptica* in the suppression of macrophage phagocytosis. A previous study reported the *B. bronchiseptica* T3SS-dependent tyrosine dephosphorylation of host cell proteins (Yuk *et al.* 1998), in a manner similar to that of the *Yersinia* YopH effector (Andersson *et al.* 1996) (Black and Bliska 1997) (Persson *et al.* 1997). Interestingly, the expression of YopH has been shown to be necessary for the inhibition of bacterial uptake by J774 cells (Andersson *et al.* 1996).

In contrast to *B. bronchiseptica*, similar numbers of viable wild-type and T3SS deficient *B. pertussis* 12743 were recovered from infected macrophages following removal of all extracellular bacteria. This indicates a similar number of internalised bacteria and suggests that, unlike the T3SS of *B. bronchiseptica*, the T3SS of *B. pertussis* 12743 does not prevent phagocytic uptake by macrophage. However, a significantly lower number of viable T3SS deficient BP 12743 bscN bacteria, when compared with wild-type, were recovered in the absence of antibiotic treatment to remove extracellular bacteria. This indicates that fewer BP 12743 bscN bacteria bound to the macrophage cell

surface, and suggests that the T3SS of *B. pertussis* 12743 may mediate adherence of *B. pertussis* to macrophage. The overall lower number of CFU recovered following infection with wild-type *B. pertussis* 12743, when compared with wild-type B. *bronchiseptica*, is consistent with previous reports that *B. bronchiseptica,* but not *B. pertussis*, can survive and persist within macrophage over time (Forde *et al.* 1998). Intracellular survival has been attributed, in part, to the *B. bronchiseptica*-specific expression of acid phosphatase and urease, enzymes involved in inducing tolerance to the acidic pH of phagolysosomes (Schneider *et al.* 2000). Thus, survival and persistence within macrophage may be a specific strategy employed by *B. bronchiseptica* in order to persist in its host.

In conclusion, this study has demonstrated that similar to the T3SS of *Yersinia* spp., the T3SS of *B. bronchiseptica* RB50 causes host cell death, a reduction in pro-inflammatory cytokine production and a reduction in phagocytic uptake following interaction with DC and macrophage. Though these T3SS-specific effects were not observed with *B. pertussis* 12743, the results demonstrate that the T3SS of *B. pertussis* may promote adherence, thus contributing to persistence *in vivo* following infection.

FIGURE 5.1 The T3SS of *B. bronchiseptica* RB50 mediates *in vitro* cytoxicity against cultured macrophage, DC and epithelial cells following 2 hours of bacterial contact. Murine J774 macrophage (A), human HEK 293T epithelial cells (B) and murine bone marrow derived DC (C) were cultured overnight in 24-well plates at 2 x 10^5 cells/ml, before being infected with wild-type or T3SS deficient *B. bronchiseptica* RB50 for 2 hours at MOIs of 20, 100 or 500. Cytotoxicity was measured by an LDH release assay and normalized to maximum lysis induced by 1% Triton-X-100.


A
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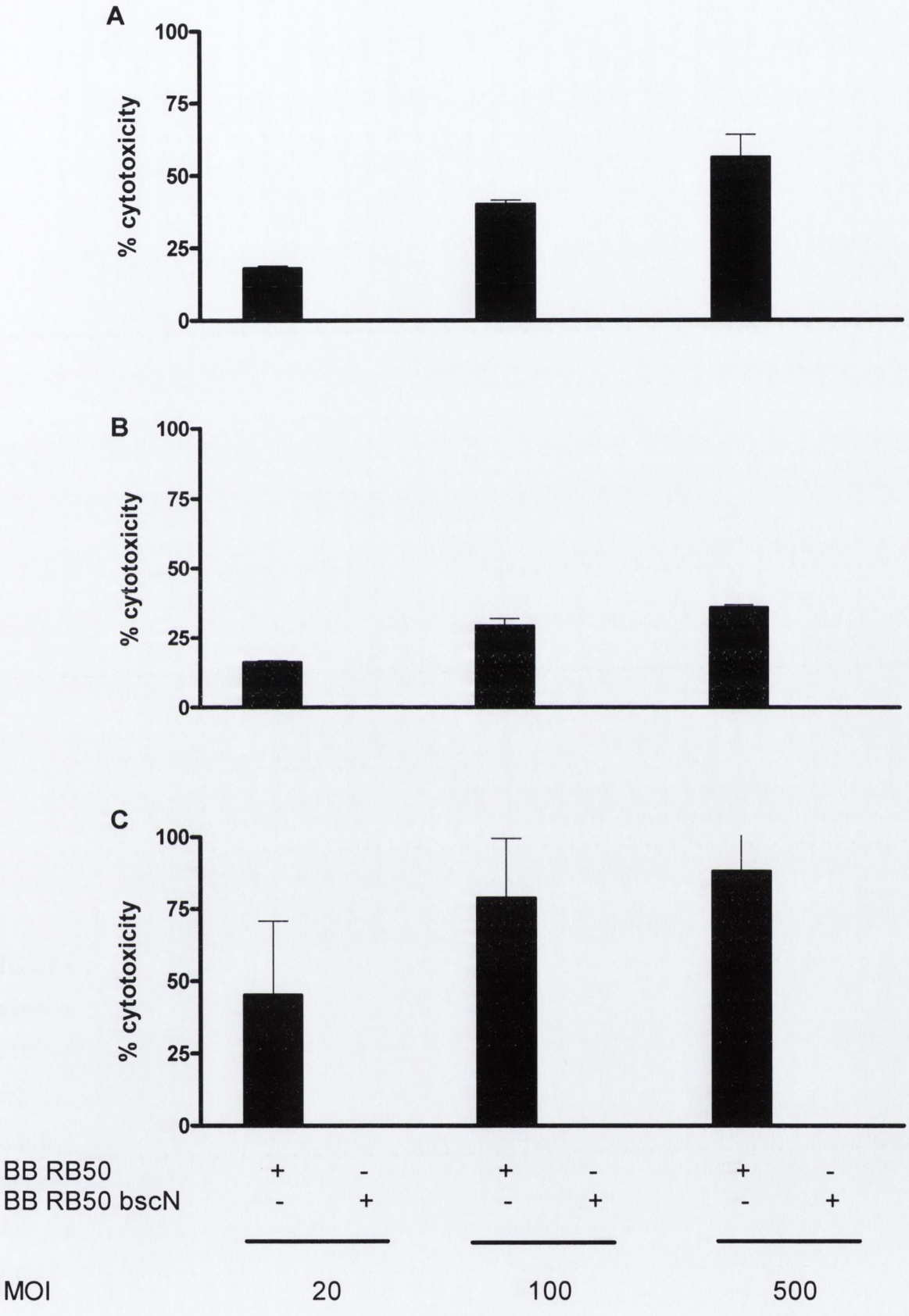
MOI
20
100
500

FIGURE 5.2 The T3SS of *B. bronchiseptica* RB50 mediates *in vitro* cytoxicity against cultured macrophage, DC and epithelial cells following 4 hours of bacterial contact. Murine J774 macrophage (A), human HEK 293T epithelial cells (B) and murine bone marrow derived DC (C) were cultured overnight in 24-well plates at 2 x 10^5 cells/ml, before being infected with wild-type or T3SS deficient *B. bronchiseptica* RB50 for 4 hours at MOIs of 20, 100 or 500. Cytotoxicity was measured by an LDH release assay and normalized to maximum lysis induced by 1% Triton-X-100.


A
100
75
50
25
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% cytotoxicity
B
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75
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C
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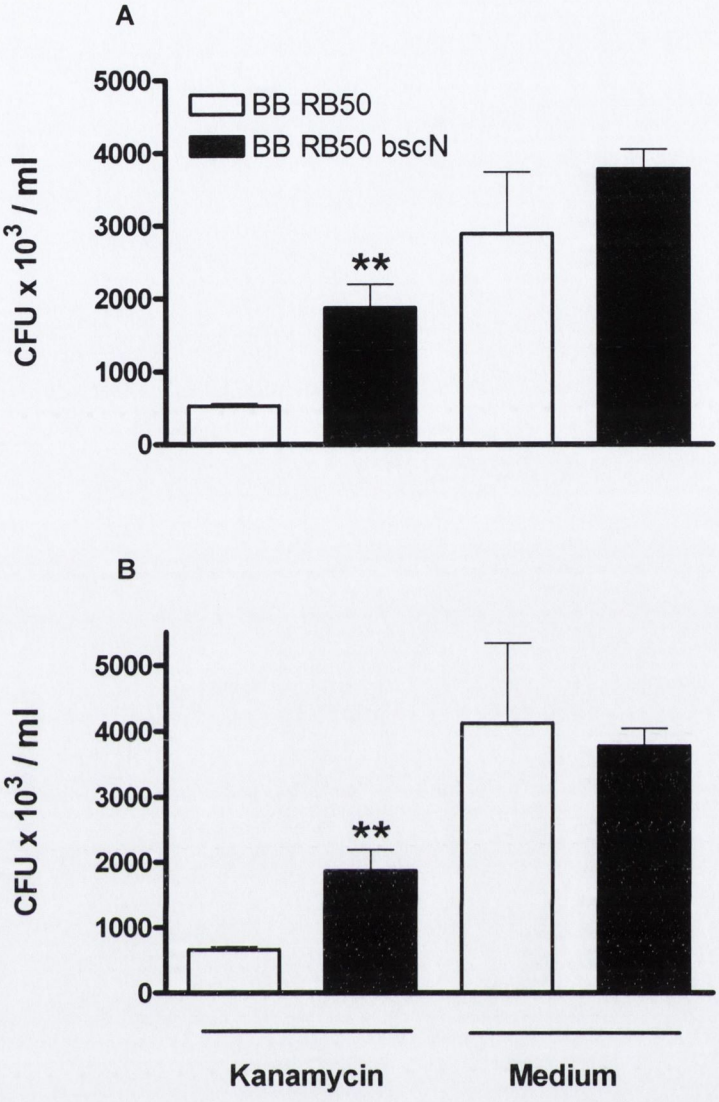


FIGURE 5.3 The T3SS of *B. bronchiseptica* RB50 reduces *in vitro* phagocytic uptake of bacteria by murine J774 macrophage. (A) J774 macrophage were incubated overnight at 1 X 10^6 cells/ml before being infected with *B. bronchiseptica* RB50 or *B. bronchiseptica* RB50 bscN at an MOI of 100. 2 hours post infection, cells were treated with 100 μg/ml kanamycin (to kill extracellular bacteria) or medium only for 1 hour, and centrifuged prior to being lysed and plated in triplicate on BG agar plates. (B) cells treated as in (A) but values corrected to allow for estimated cell death of 30 % following 2 hrs of bacterial contact. **P<0.01 mutant vs wild-type *B. bronchiseptica* RB50.

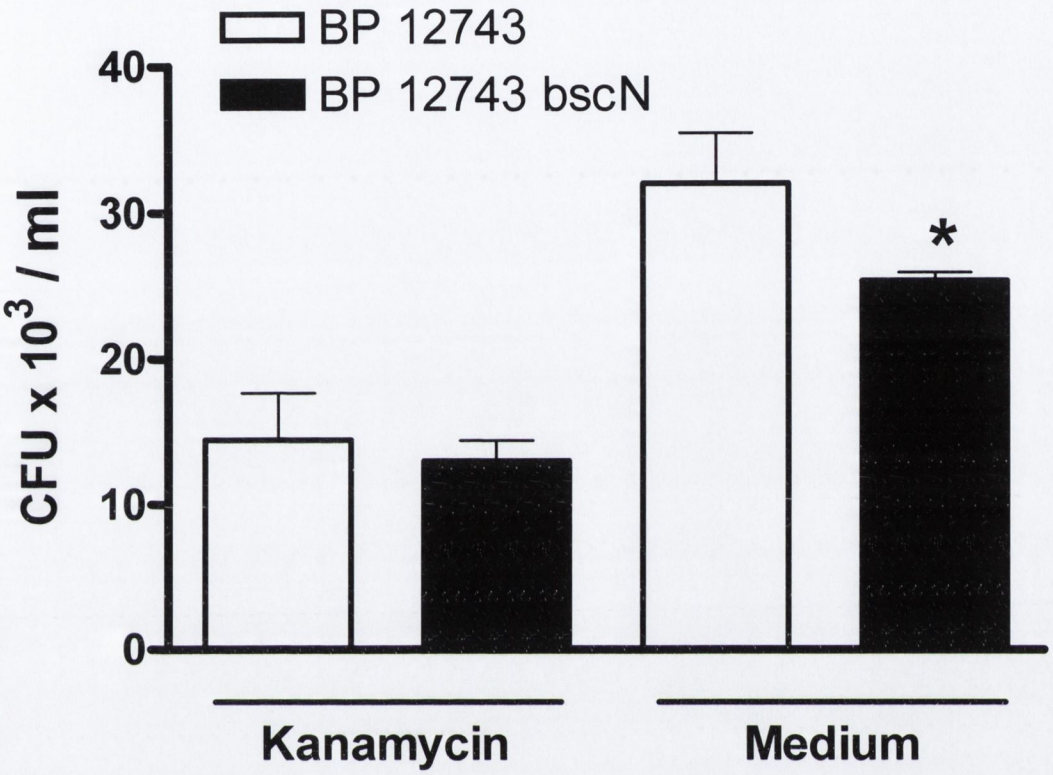


FIGURE 5.4 The T3SS of *B. pertussis* 12743 does not affect *in vitro* phagocytic uptake of bacteria by murine J774 macrophage. J774 macrophage were plated overnight at 1 X 10^6 cells/ml before being infected with *B. pertussis* 12743 or *B. pertussis* 12743 bscN at an MOI of 100. 2 hours post infection, cells were treated with 100 μg/ml kanamycin (to kill extracellular bacteria) or medium only for 1 hour, and centrifuged prior to being lysed and plated in triplicate on BG agar plates. *P<0.05 mutant vs wild-type *B. pertussis* 12743

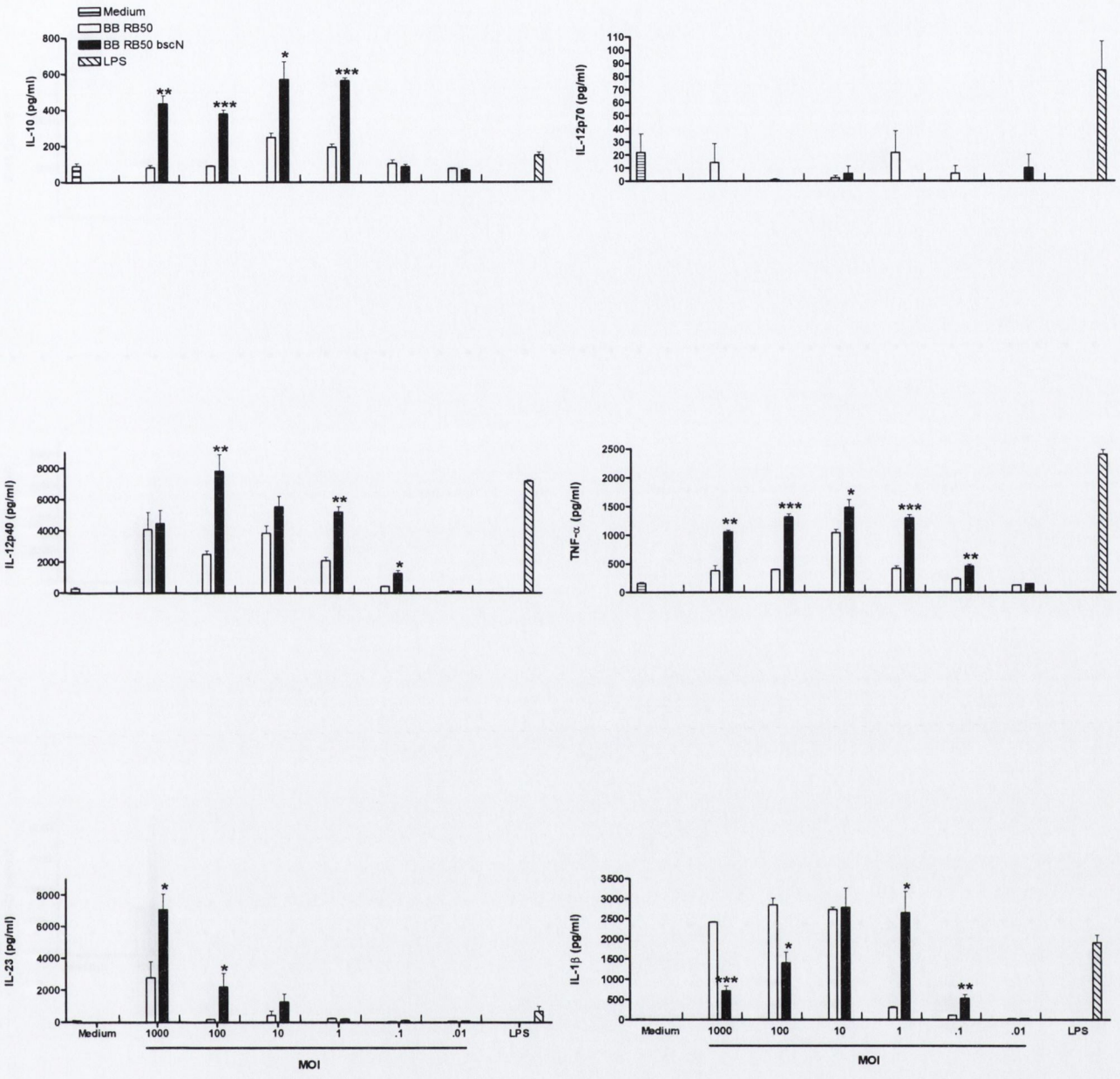


FIGURE 5.5 Differential cytokine production by murine BMDC following infection with *B. bronchiseptica* RB50 or *B. bronchiseptica* RB50 bscN. BMDC were cultured overnight at 1 x 10^6 cells/ml before being challenged with *B. bronchiseptica* RB50 or *B. bronchiseptica* RB50 bscN in the range of MOIs 0.1-1000. DC incubated with medium only or LPS (10 ng/ml) served as negative and positive controls respectively. Supernatants were removed 6 hours post infection and IL-10, IL-12p70, IL-12p40, TNFα, IL-23 and IL-1β were determined by ELISA. *P<0.05, **P<0.01 and ***P<0.001 T3SS mutant vs wild-type bacteria.

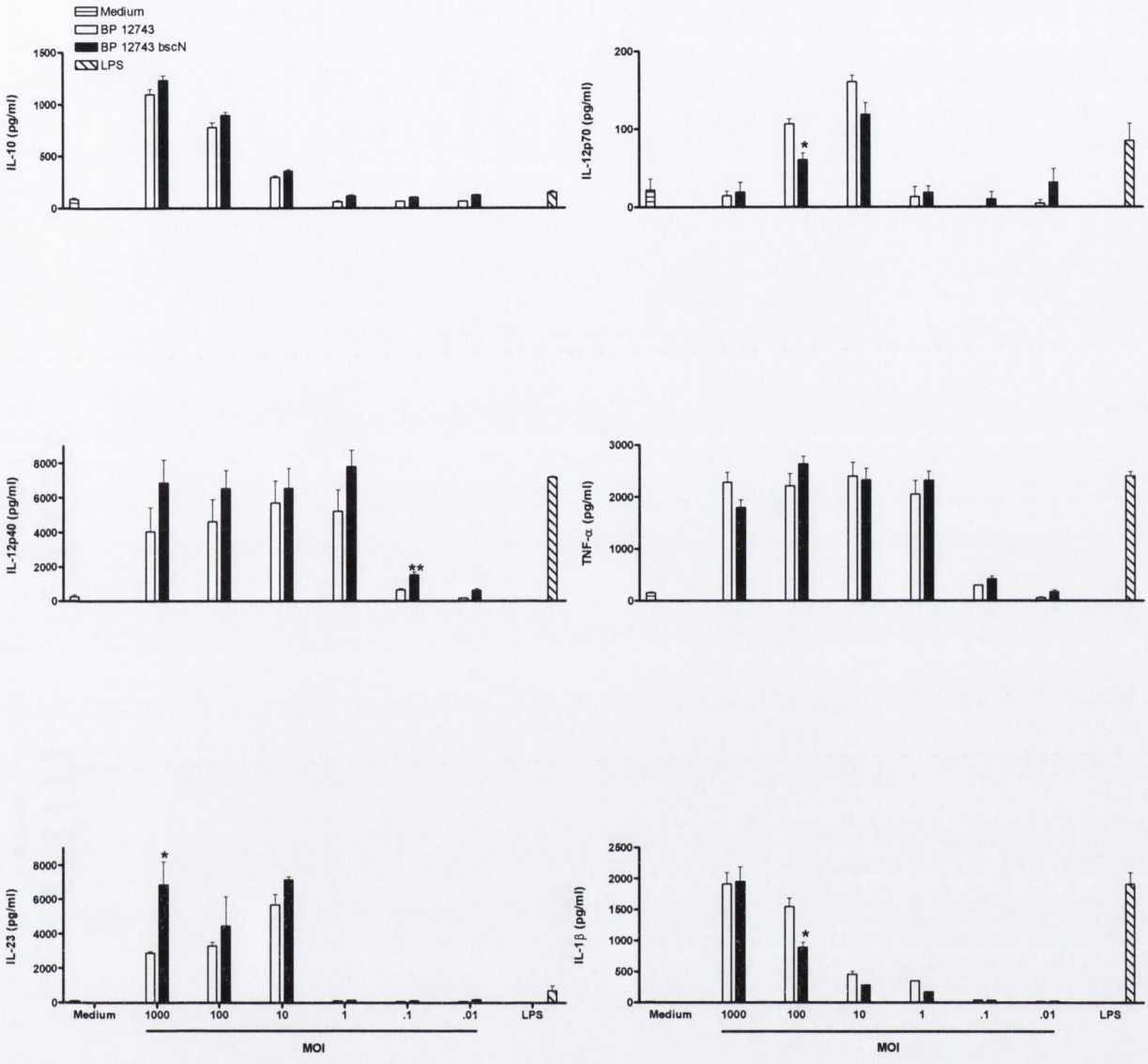


FIGURE 5.6 Similar cytokine production by DC following infection with *B. pertussis* 12743 and *B. pertussis* 12743 bscN. BMDC were cultured overnight at 1 x 10^6 cells/ml before being challenged with *B. pertussis* 12743 or *B. pertussis* 12743 bscN in the range of MOIs 0.1-1000. DC incubated with medium only or LPS (10 ng/ml) served as negative and positive controls respectively. Supernatants were removed 6 hours post infection and IL-10, IL-12p70, IL-12p40, TNFα, IL-23 and IL-1β concentrations were determined by ELISA. *P<0.05 and **P<0.01 T3SS deficient vs wild-type bacteria

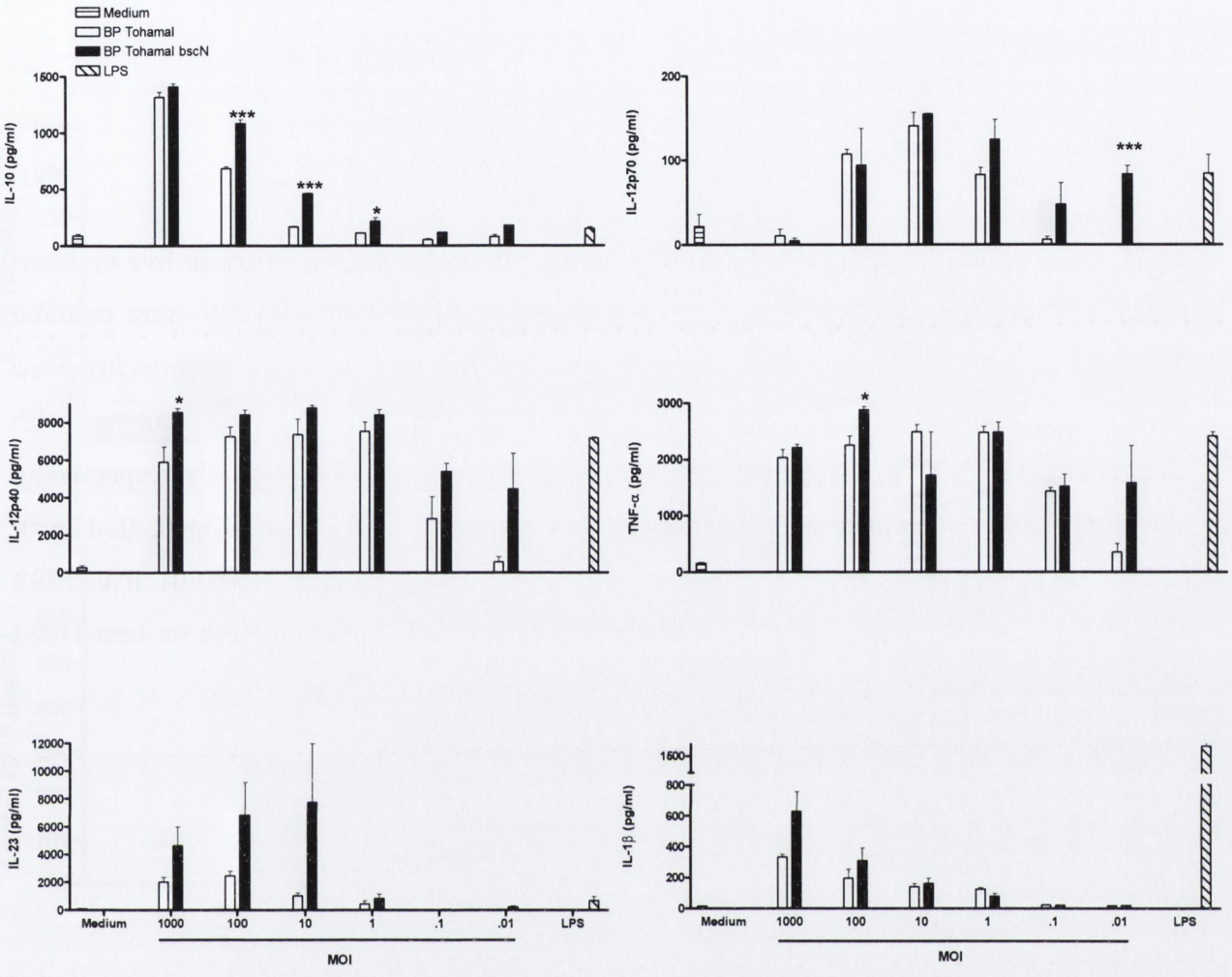
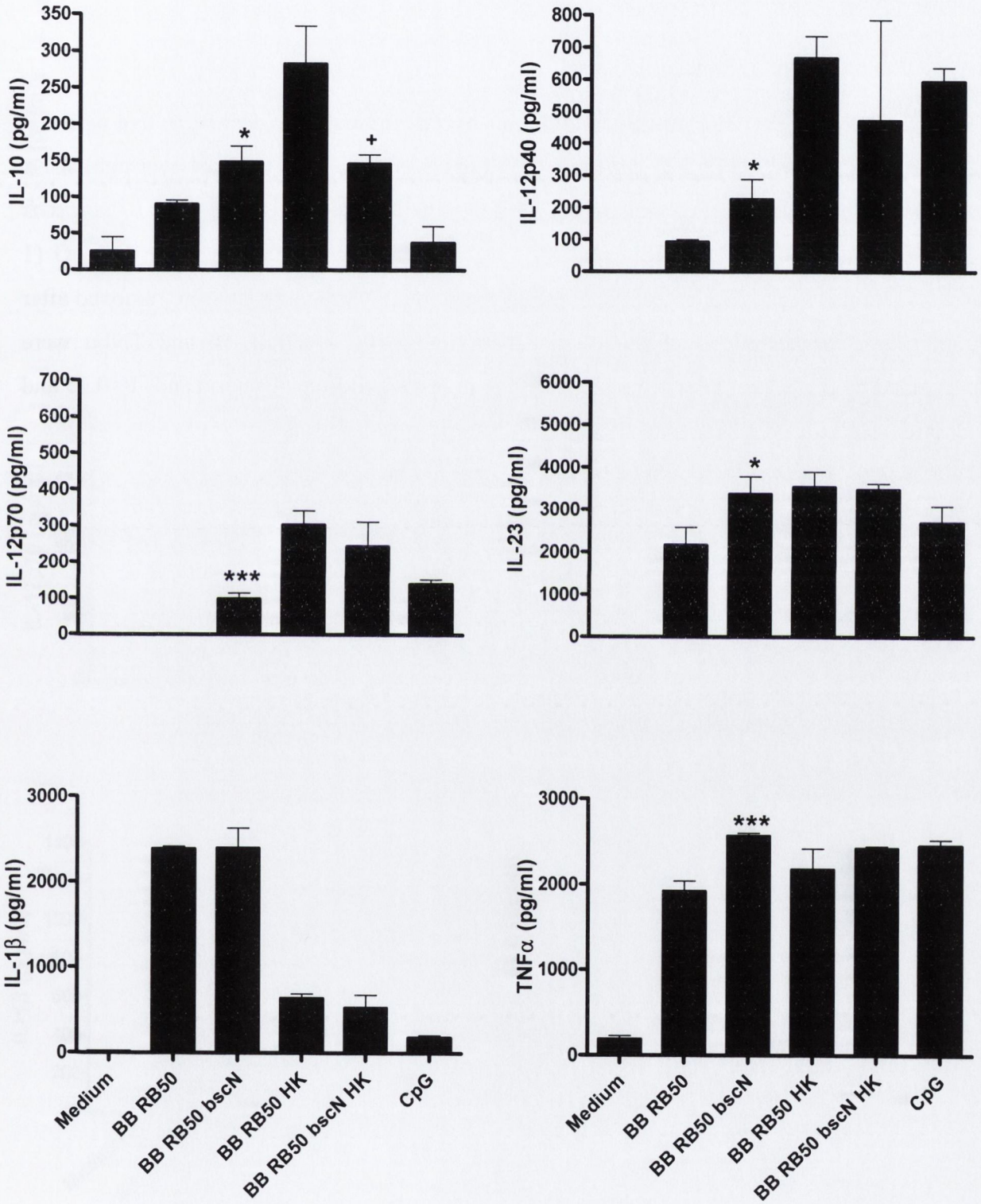


FIGURE 5.7 Similar cytokine production by DC following infection with *B. pertussis* Tohama I and *B. pertussis* Tohama I bscN. BMDC were cultured overnight at 1 x 10^6 cells/ml before being challenged with *B. pertussis* Tohama I or *B. pertussis* Tohama I bscN in the range of MOIs 0.1-1000. DC incubated with medium alone or LPS (10 ng/ml) served as negative and positive controls respectively. Supernatants were removed 6 hours post infection and IL-10, IL-12p70, IL-12p40, TNFα, IL-23 and IL-1β concentrations were determined by ELISA. *P<0.05 and ***P<0.001 T3SS deficient vs wild-type bacteria.

FIGURE 5.8 Differential cytokine production by DC following exposure to live or heat-killed *B. bronchiseptica* RB50 and *B. bronchiseptica* RB50 bscN. BMDC were cultured overnight at 1 x 10^6 cells/ml before being exposed to live or heat-killed *B. bronchiseptica* RB50 or *B. bronchiseptica* RB50 bscN at a bacteria:DC ratio of 100:1. DC incubated with medium alone or CpG (1 μg/ml) served as negative and positive controls respectively. Supernatants were removed after 6 hours and concentrations of IL-10, IL-12p40, IL-12p70, IL-23, IL-1β and TNFα were determined by ELISA. *P<0.05 and ***P<0.001 live T3SS deficient vs live wild-type bacteria, and $^{+}$P<0.05 heat-killed T3SS deficient vs heat-killed wild-type bacteria.


IL-10 (pg/ml)
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IL-1β (pg/ml)
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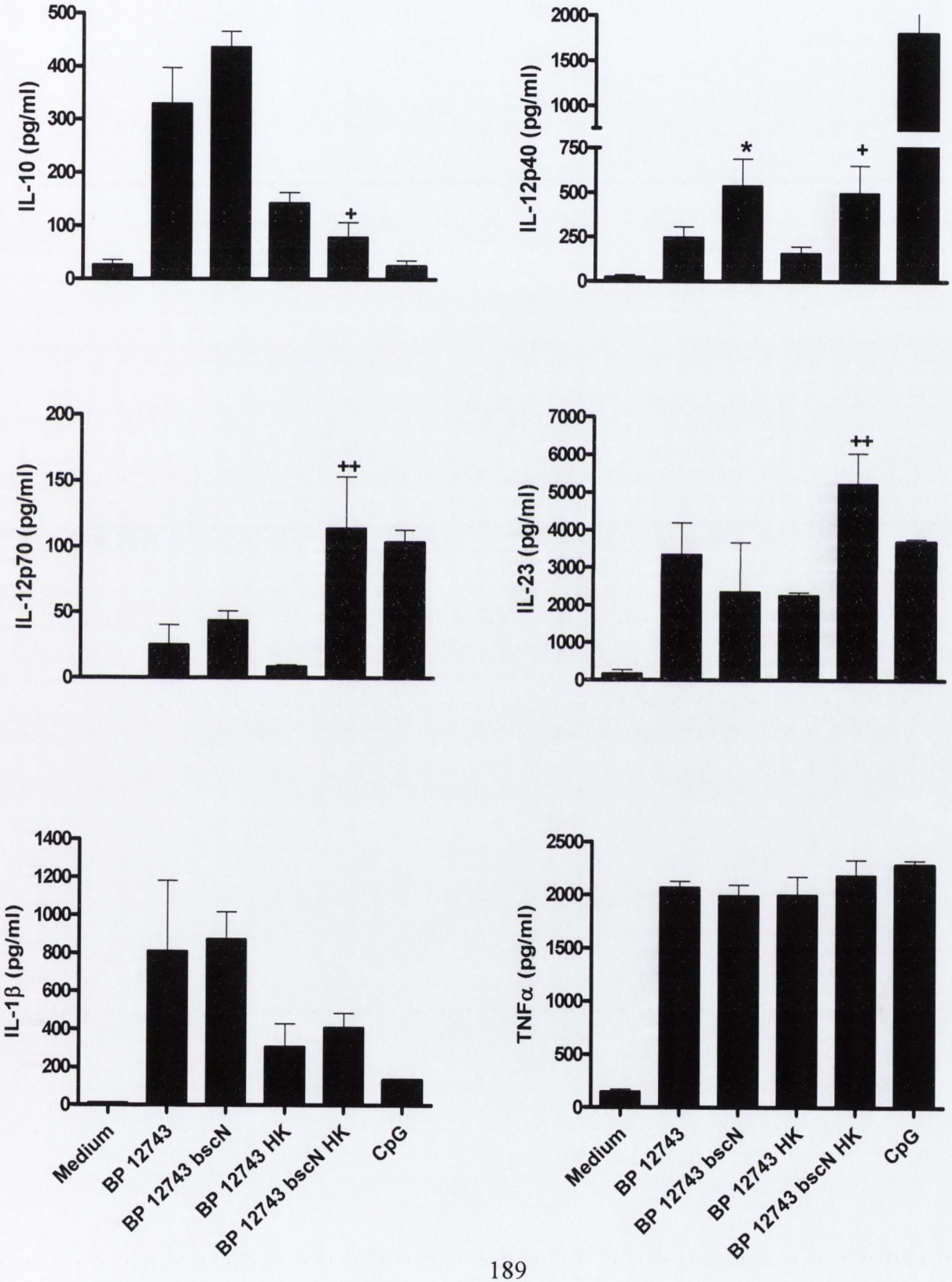
2000
1000
0
TNFα (pg/ml)
3000
2000
1000
0
***
Medium
BB RB50
BB RB50 bscN
BB RB50 HK
BB RB50 bscN HK
CpG

FIGURE 5.9 Differential cytokine production by DC following exposure to live or heat-killed *B. pertussis* 12743 and *B. pertussis* 12743 bscN. BMDC were cultured overnight at 1 x 10^6 cells/ml before being exposed to live or heat-killed *B. pertussis* 12743 or *B. pertussis* 12743 bscN at a bacteria:DC ratio of 100:1. DC incubated with medium alone or CpG (1 μg/ml) served as negative and positive controls respectively. Supernatents were removed after 6 hours and concentrations of IL-10, IL-12p40, IL-12p70, IL-23, IL-1β and TNFα were determined by ELISA. *$P<0.05$ live T3SS deficient vs live wild-type bacteria and $^{+}P<0.05$ and $^{++}P<0.01$ heat-killed T3SS deficient vs heat-killed wild-type bacteria.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++
IL-23 (pg/ml)
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6000
5000
4000
3000
2000
1000
0
++
IL-1β (pg/ml)
1400
1200
1000
800
600
400
200
0
TNFα (pg/ml)
2500
2000
1500
1000
500
0
Medium
BP 12743
BP 12743 bscN
BP 12743 HK
BP 12743 bscN HK
CpG

Chapter 6

The role of the T3SS of *Bordetella* spp. during *in vivo* infection

6.1 INTRODUCTION

All animal pathogens are constantly subject to the threat of eradication by the host immune systems. Consequently, bacterial pathogens such as *Bordetella* spp. have evolved complex mechanisms to overcome or subvert host immunity in order to ensure survival. The ability of *B. bronchiseptica* to often cause life-long infection in animal hosts and the endemic nature of *B. pertussis* infection in largely vaccinated human populations is consistent with the presence of a complex array of virulence factors which help the bacteria to suppress host immune responses (Discussed in section 1.3).

The recognition of pathogens at mucosal sites or tissues such as the lung leads to innate immune activation and inflammation, which helps to contain the infection until the clearing adaptive immune response can be mounted, which effectively clears the infection from the host. This level of inflammation must be tightly controlled, as strong pro-inflammatory response can result in damage to host tissues, while strong anti-inflammatory response may suppress protective effector response and result in excessive bacterial growth and lethal dissemination. Thus the host must achieve the correct balance between pro and anti-inflammatory signals required to bring about eradication of the pathogen, with minimal collateral damage to host tissues.

Rapid and potent inflammation at critically important mucosal sites like the lung, serves to protect the host immediately following infection or acute injury. In addition, an efficient innate inflammatory response is necessary, not only to prevent pathogen dissemination but also for the recruitment and subsequent priming of T cells and B cells that mediate humoral and cell-mediated adaptive immune responses, which ultimately clear the infection. The disruption of immune-related signalling and inflammation is therefore a common strategy evolved by pathogens to subvert immune responses. A good example of this is the action of the *Yersinia* T3SS effector YopJ/YopP, which blocks NF-κB activation, resulting in inhibition of pro-inflammatory cytokine or chemokine production and adhesion molecule expression, and eventual macrophage apoptosis (Brubaker 2003).

Recently, it has been shown that the T3SS of *B. bronchiseptica* can modulate the balance between pro and anti-inflammatory immune responses to favour bacterial survival within the host. Specifically, the T3SS has been implicated in synergising with ACT to induce production of the anti-inflammatory IL-10, early in infection, and this has been correlated with an inhibition of IFNγ production and bacterial persistence. (Skinner *et al.* 2004) (Skinner *et al.* 2005) (Pilione and Harvill 2006). IL-10 can inhibit activation and inflammatory cytokine production by Th1 cells (Fiorentino *et al.* 1991) (Moore *et al.*

1990), and has a critical role in controlling inflammatory pathology, including during respiratory infection in the lung (Higgins *et al.* 2003). Therefore the T3SS and ACT-mediated induction of IL-10- secreting T-cells by *B. bronchiseptica* represents a specific strategy employed by the bacterium in order to tip the balance in favour of inflammatory signals, thus delaying its clearance from the host.

The selective upregulation of IL-10 by the T3SS is not unique to *B. bronchiseptica.* The oldest known T3SS effector, LcrV of *Yersinia* spp., has been associated with the suppression of IFNγ and TNFα production (Nakajima and Brubaker 1993), through a mechanism involving the specific upregulation of IL-10 (Nedialkov *et al.* 1997; Sing *et al.* 2002). Similarly, it has recently been shown that the SPI-2 T3SS of *Salmonella* spp. encodes an effector directly involved in the upregulation of IL-10 production from macrophage, through a mechanism involving protein kinase A upregulation and CREB phosphorylation (Uchiya *et al.* 2004).

As discussed in section 1.2.2.3, during *B. pertussis* infection regulatory T-cells are induced in order to subvert host inflammation. However, the mechanisms of Treg induction have been attributed to virulence factors other than the T3SS, such as FHA (McGuirk *et al.* 2002), LPS (Higgins *et al.* 2003) and ACT (Ross *et al.* 2004) (Boyd *et al.* 2005). Clearance of *B. pertussis* following natural infection in children and mice correlates with the induction of a strong Th1 responses (Ryan *et al.* 1997; Hafler and Pohl-Koppe 1998), mirrored by the presence of IFNγ in the BAL fluid of mice recovering from *B. pertussis* infection (Torre *et al.* 1993). Thus suppression of the protective Th1 response through the induction of Treg cells is one explanation for the persistence of *B. pertussis* for weeks in the infected host, despite the fact that it induces a significant local inflammatory response.

A previous investigation has failed to define a role for the T3SS of *B. pertussis* Tohama I during *in vivo* infection of mice (Fauconnier *et al.* 2001). However, there are conflicting reports in the literature as to whether the T3SS loci are transcribed in this strain of *B. pertussis* (Yuk *et al.* 1998; Fauconnier *et al.* 2001; Hot *et al.* 2003). To date no *in vivo* studies have been performed with *B. pertussis* strains expressing a functional T3SS *in vitro*. Having already demonstrated a functional T3SS in recent clinical isolates of *B. pertussis* such as BP 12743, in contrast to lab-adapted strains such as BP Tohama I and BP Wellcome 28, this study set out to examine the role of the T3SS of *B. pertussis* 12743, in comparision to the T3SS *B. bronchiseptica* RB50 following aerosol infection of BALB/c mice.

6.2 RESULTS

6.2.1 The T3SS of *B. pertussis* 12743 enhances bacterial persistence in the lungs of mice following aerosol challenge.

In order to examine the role of a functional T3SS on the course of *Bordetella* infection, groups of 24 BALB/c mice were challenged by exposure to an aerosol of BB RB50, BB RB50 bscN, BP 12743 and BP 12743 bscN. The course of infection was monitored by performing CFU counts on lung homogenates of 4 mice from each group 3 hours, 3, 7, 14, 21, and 28 days after challenge. Neither of the T3SS deficient strains colonised the lungs as effectively as their wild type counterparts (Figures 6.1 and 6.2). After an initial rise in bacterial numbers, peaking at day 7, wild-type BP 12743 began to be steadily cleared from the lungs between days 7 and 21, with complete clearance of the bacteria occurring 28 days post infection (Fig 6.1). In contrast, its T3SS-deficient mutant, BP 12743 bscN, colonised the lungs to a significantly lower degree at each timepoint (Fig 6.1) and began to be cleared from the lung earlier in infection. A steady decline in bacterial numbers of BP 12743 bscN, beginning at day 3, resulted in complete clearance from the lung 1 week earlier than the wild-type bacteria, at 21 days post infection (Fig 6.1).

Similar to wild-type *B. pertussis*, we detected an initial rise in the numbers of *B. bronchiseptica* in the lungs, peaking at 7 days post infection. Both strains of bacteria began to be cleared between day 7 and 14, with bacterial numbers dropping sharply for both strains of *B. bronchiseptica*. However, in contrast to the T3SS-deficient mutant, BB RB50 persisted for 14 to 28 days, with CFU counts dropping only slightly after this period. The numbers of viable BB RB50 bscN continued to decline, with the bacteria completely cleared from the lungs 28 days post infection (Fig 6.2).

These results suggest that the *Bordetella* T3SS is crucial for both the initial colonization, and persistence of BB RB50 and BP 12743 in the lungs following aerosol challenge of BALB/c mice.

6.2.2 The T3SS of *B. bronchiseptica* RB50 but not *B. pertussis* 12743 enhances bacterial colonization of the trachea following aerosol challenge.

In addition to colonising the lungs of susceptible hosts, *Bordetella* spp. efficiently colonise the entire respiratory epithelium. In order to assess the possible role of the *Bordetella* T3SS in the colonization of the upper respiratory tract, the course of infection in the trachea was also monitored at regular intervals post aerosol challenge. The course of infection in the trachea for BB RB50 was quite similar to that in the lung, although the overall level of persistence in the trachea was slightly higher on day 28 (Trachea log 4.2 vs. Lung log 3; Fig 6.2). However, in contrast to the lung, BB RB50 bscN was not completely cleared from the trachea 28 days post challenge (Fig 6.2). In contrast, out of 3 aerosols performed, we only recovered viable colonies of wild-type BP 12743 from the tracheas of infected mice during one of these aerosols, and only at 3 hrs post-infection. It was not possible to detect any CFU at any later timepoints. In contrast, we did not detect any viable colonies of T3SS deficient BP 12743 bscN in the trachea at any timepoint in all 3 aerosols.

These results suggest that the T3SS enhances colonization, but not persistence of *B. bronchiseptica* in the tracheas of infected mice following aerosol challenge. The role, if any, of the T3SS of *B. pertussis* 12743 in the tracheal colonisation is unclear from this experiment, since it would appear that BP 12743 is unable to colonize the tracheas of infected mice to any great extent following aerosol challenge.

6.2.3 Differential inflammatory cytokine and chemokine responses in the lungs of mice infected with wild type and T3SS-deficient *B. pertussis* 12743.

Since T3SS of other bacteria have been shown to function by subverting various functions of target cells following contact with the bacterium, and since *B.pertussis* is known to interact with macrophage and DC during infection, we examined the local inflammatory response in the lungs of mice infected with BP 12743 and BP 12743 bscN at 3 hours, 3, 7 and 14 days following aerosol challenge.

In contrast with the lower level of bacteria in the lungs of T3SS-deficient *B. pertussis* infected mice throughout the course of infection, we detected significantly higher concentrations of the pro-inflammatory cytokines IL-6 ($P<0.05$), TNFα and IL-1β

(P<0.01), and pro-inflammatory chemokines MIP1α (P<0.01) and MIP2 (P<0.05) in the lungs 3 hours post challenge (Fig 6.3). Furthermore, the concentrations of IL-1β and MIP1α remained significantly higher in the lungs of mice infected with the T3SS deficient mutant up to 14 days post challenge (Fig 6.3). In addition the concentration of MIP2 remained significantly higher in the lungs of mice infected with the T3SS-deficient mutant up to 7 days post challenge (Fig 6.3). We also detected significantly higher concentrations of IL-6 and IL-12p40 in the lungs of mice 7 days post challenge with the T3SS mutant bacteria (Fig 6.3). In contrast, the concentrations of IL-10 were consistently lower, though not significantly, except at day 14 post challenge, in mice infected with BP 12743 bscN when compared with wild-type bacteria.

These results suggest a role for the T3SS of B. pertussis 12743 in the down-regulation of innate pro-inflammatory cytokine and chemokine responses in the lung early in infection.

6.2.4 Differential inflammatory cytokine and chemokine responses in the lungs of mice infected with wild type and T3SS-deficient *B. bronchiseptica* RB50.

We next examined the role of the T3SS of *B. bronchiseptica* RB50 on the induction of pro-inflammatory responses in the lung during infection. In contrast to *B. pertussis*, there were lower concentrations of pro-inflammatory cytokines and chemokines in the lungs of T3SS deficient BB RB50 bscN infected mice when compared with wild-type infected, and this correlated with the lower number of bacteria in the lungs (Fig 6.1). We detected significantly lower concentrations of MIP1α, MIP2 (P<0.001), IL-6 and IL-1β (P<0.05) at 3 hours post challenge, and significantly lower concentrations of Mip1α, TNFα, IL-6 (P<0.05), Mip2 (P<0.001) and IL-1β (P<0.01) 7 days post challenge in the lungs of mice infected with BB RB50 bscN (Fig 6.4). No significant differences were detected at day 14 with the exception of IL-1β (P<0.01 vs. BB RB50 bscN). Although there were no significant differences early in infection, the concentration of IL-10 was significantly higher at 14 days post challenge in the lungs of mice infected with T3SS deficient bacteria when compared with wild-type bacteria (Fig 6.4)

These data suggest that the T3SS of BB RB50, in contrast to that of *B. pertussis* 12743, may not have a role in downregulating pro-inflammatory cytokine and chemokine responses in the lung during infection.

6.2.5 Enhanced $CD25^+$ $FoxP3^+$ expressing T cells in the lungs of mice following infection with wild-type or T3SS deficient *B. pertussis* 12743 and *B. bronchiseptica* RB50 bacteria.

B. pertussis infection of mice following aerosol challenge is known to be associated with the induction of Treg cells in the lungs (McGuirk *et al.* 2002). Moreover, the T3SS of *B. bronchiseptica* has been implicated in the induction of IL-10 secreting T cells following intra-nasal challenge (Pilione and Harvill 2006). Since FoxP3 expression is a reliable marker for natural Treg cells (Khattri *et al.* 2003), the expression of Foxp3 on $CD4^+$ and $CD4^+$ $CD25^+$ T cells in the lungs was examined following aerosol challenge with wild-type and T3SS deficient *B. pertussis* 12743 and *B. bronchiseptica* RB50, using the protocol described in Figure 6.5.

Infection of mice with *B. pertussis* or *B. bronchisptica* was associated with a transient increase in the percentage of $CD4^+$ T cells expressing FoxP3 at day 3, which declined to preinfection levels by day 7, and increased again later in infection peaking at day 21 (Fig 6.6; Fig6.7). A similar percentage of $CD4^+$ $FoxP3^+$ T cells was detected in the lungs of mice infected with wild-type and T3SS deficient *B. bronchiseptica* and *B. pertussis* 12743 at each timepoint after aerosol challenge (Fig 6.7). An examination of CD25 expression on $CD4^+$ T cells in the lung revealed a dramatic increase 7 days post infection with *B. pertussis* or *B. bronchiseptica*, with 75-90 % of the CD4+ cells expressing CD25, and this had returned to baseline levels by day 14 (Fig 6.6; Fig 6.7). When these cells were also analysed for FoxP3 expression, a transient increase in the percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells was detected in the lungs of wild-type and T3SS deficient *B. bronchiseptica* RB50 and *B. pertussis* 12743 7 days post challenge (Fig 6.6; Fig 6.7) Interestingly, the study revealed the presence of $CD4^+$ $FoxP3^+$ $CD25^-$ T cells in the lung in naïve mice (approximately 9% of $CD4^+$ cells), which were enhanced to 16% 3 hours, and 27% 3 days post challenge (Fig 6.6; compare A and C). and only minor differences were observed between wild-type and T3SS deficient bacteria (Fig 6.6; Fig 6.7).

These results demonstrate that $FoxP3^+$ regulatory T-cells are recruited to the lung early in *Bordetella* infection, and that this recruitment is independent of T3SS function.

6.2.6 Different antigen specific IL-10 and IFNγ spleen cell responses in mice infected with wild-type and T3SS deficient *B. pertussis* 12743.

The balance between the production of the anti-inflammatory cytokine, IL-10, and the pro-inflammatory cytokine, IFNγ, is of critical importance to both the pathogen and the host, in terms of mediating complete bacterial clearance, without causing excessive pathology to the host. It has previously been reported that the T3SS of *B. bronchiseptica* may contribute to the induction of higher concentrations of antigen-specific IL-10 from spleen cells, with a corresponding reduction in IFNγ production, thus facilitating bacterial persistence in the lung (Skinner *et al.* 2005; Pilione and Harvill 2006). Having already established a critical role for the T3SS of *B. pertussis* 12743 in the lung (Fig 6.1), we next set out to examine the role of T3SS in the production of antigen specific IL-10 and IFNγ during the course of aerosol infection.

Spleens were removed from mice 7, 14, 21 and 28 days post challenge and spleen cells were stimulated with sonicated *B. pertussis* 12743 antigen, or purified heat-inactivated FHA and PT antigens. As can be seen from figure 6.8 the IL-10 response detected from spleen cells taken from mice at day 14 (Fig 6.8 B) and day 28 (Fig 6.8 D) infected with wild-type and T3SS-deficient BP 12743 was very similar, and not significantly higher than the response detected from spleen cells taken from uninfected animals (Fig 6.8 A). Interestingly, we detected significantly higher levels of IL-10 production by spleen cells taken from mice infected with T3SS deficient BP 12743 21 days post infection, following *in vitro* stimulation with sonicated BP 12743 antigen (Fig 6.8 C).

In contrast, we detected significantly higher levels of antigen-specific IFNγ production by spleen cells taken from mice infected with wild-type or T3SS-deficient BP 12743 in response to sonicated BP 12743 antigen, when compared to spleen cells from uninfected mice (Fig 6.9 A). Further, the spleen cell IFNγ production induced by *B. pertussis* sonicate, FHA or PT was significantly higher by spleen cells from mice removed 14, 21 or 28 days after infection with T3SS mutant B. pertussis when compared with wild-type bacteria (Fig 6.9). Most significantly, we detected dramatically higher levels of pro-inflammatory IFNγ from spleen cells from T3SS-deficient BP 12743 infected mice 21 days post infection when stimulated with PT antigen and compared to those from wild-type BP 12743 infected animals (Fig 6.9 C).

These results suggest that the T3SS of BP 12743 contributes to the down-regulation of antigen-specific IFNγ production responses over the course of infection following aerosol challenge.

6.2.7 Elevated antigen specific IL-17 spleen cell responses in mice infected with T3SS-deficient *B. pertussis* 12743.

IL-17 producing T-cells are thought to enhance a potent inflammatory response by activating endothelial cells and monocytes at mucosal tissues, such as the lung, to produce key inflammatory mediators such as IL-8, IL-1, IL-6, TNFα and GM-CSF (McKenzie *et al.* 2006). Having previously observed dramatically increased IL-1β production in the lungs of T3SS deficient BP 12743 infected mice when compared to wild-type mice following aerosol challenge (Fig 6.3), we next examined the level of antigen-specific IL-17 production from *B. pertussis* antigen stimulated spleen cells during BP 12743 infection.

As shown in Fig 6.10, we observed significantly elevated levels of IL-17 production from antigen-stimulated spleen cells from mice infected with T3SS deficient BP 12743 when compared with spleen cells from mice infected with wild-type BP 12743 or those from uninfected animals (Fig 6.10 A) at 14 (Fig 6.10 B), 21 (Fig 6.10 C) and 28 (Fig 6.10 D) 28 days post aerosol challenge. Similar to the IFNγ response (Fig 6.9 C), the most dramatic increase in IL-17 production was observed with spleen cells stimulated with heat inactivated PT antigen at 21 days (Fig 6.10 C) post challenge.

These results demonstrate that the T3SS of *B. pertussis* 12743 functions to reduce the activation of pro-inflammatory IL-17 producing T-cells following aerosol challenge.

6.2.8 Similar antigen specific IL-10 and IFNγ spleen cell responses in mice infected with wild-type versus T3SS deficient *B. bronchiseptica* RB50.

It has previously been reported that the T3SS of *B. bronchiseptica* may contribute to the induction of higher concentrations of antigen specific IL-10 from spleen cells, with a corresponding reduction in IFNγ production, thus facilitating bacterial persistence in the lung (Skinner *et al.* 2005; Pilione and Harvill 2006). Having already established a critical role for the T3SS of *B. bronchiseptica* RB50 in mediating host persistence (Fig 6.2), we next examined the role of the T3SS in the production of antigen-specific IL-10 and IFNγ following aerosol challenge.

Spleen cells from infected mice were stimulated with a range of concentrations of heat killed wild-type BB RB50 or formalin treated sonicated *B. pertussis* Wellcome 28 antigen. As can be seen from figure 6.11, we detected no significant differences in production of the anti-inflammatory cytokine, IL-10, from spleen cells from mice 14 days

post infection with wild-type or T3SS deficient BB RB50 (Fig 6.11 B). Interestingly, and similar to what was observed for *B. pertussis* 12743 21 days post infection (Fig 6.8 C), we detected significantly higher concentrations of IL-10 production by spleen cells from mice infected with T3SS deficient BB RB50 in response to sonicated *B. pertussis* antigen 21 days following aerosol challenge (Fig 6.11 C). However, we detected significantly lower concentrations of IL-10 28 days post infection by ACT-stimulated spleen cells with wild-type BB RB50 when compared with T3SS deficient bacteria (Fig 6.11 D).

In addition, we observed significantly higher levels of IFNγ production by spleen cells from mice infected with T3SS deficient BB RB50 21 days post infection in response to sonicated *B. pertussis* Wellcome 28 antigen (Fig 6.12 C). In contrast to *B. pertussis*, no other significant differences in concentrations of IFNγ were observed at day 14 (Fig 6.12 B) or day 28 (Fig 6.12 D) between responses from mice infected with wild-type and T3SS deficient BB RB50.

These results suggest that the T3SS of *B. bronchiseptica* RB50 may be capable of increasing production of the anti-inflammatory cytokine IL-10 in response to purified ACT antigen only, and this increased IL-10 production may affect IFNγ production following aerosol challenge.

6.2.9 Elevated serum antibody responses in mice infected with T3SS deficient versus wild-type *Bordetella* spp.

Antibodies play an important role in clearance of extracellular bacteria from tissues. Significantly higher levels of total serum IgG have been reported in C57/BL6 mice following intra-nasal challenge with T3SS deficient *B. bronchiseptica* when compared to responses in mice infected with the wild-type bacteria. Here, *B. pertussis*-specific IgG antibody responses were quantified in serum from mice 7, 14, 21 and 28 days following aerosol infection with wild-type and T3SS deficient bacteria.

B. pertussis-specific serum antibodies were detected in infected mice 14-28 days after challenge. 28 days post infection significantly higher *B. pertussis*-specific serum IgG was detected in mice infected with BP 12743 bscN when compared to wild-type bacteria (Fig 6.13 A). In addition, and consistent with the higher local innate inflammatory anf Th1 responses in the mice infected with BP 12743 bscN, we also detected significantly higher titres of serum IgG2a in these mice (Fig. 6.13 B), where no differences in serum IgG1 were detected (Fig 6.13 C). We also observed higher *B. bronchiseptica*-specific IgG titres in mice infected with T3SS-deficient BB RB50 bscN when compared with wild-type BB

RB50 and this was statistically significant 21 days post infection (Fig 6.14). These results suggest that the *Bordetella* T3SS may have a role in reducing the humoral immune response, possibly as a consequence of suppressing inflammatory Th1 cells, following respiratory challenge.

6.3 Discussion

T3SS of animal pathogens are complex multi-protein organelles that function to subvert host cellular functions during infection. The identification of these systems in the genomes of *Bordetella* spp. has subsequently led to the characterisation of the effects of the T3SS during *B. bronchiseptica* infection by several groups (Yuk *et al.* 2000; Skinner *et al.* 2005; Pilione and Harvill 2006). However, to date there have been no previous reports detailing an active role for the T3SS in the pathogenesis of *B. pertussis.* This study has demonstrated an immunomodulatory role for the T3SS of a clinical *B. pertussis* isolate, BP 12743, during *in vivo* infection of BALB/c mice. Furthermore, the study shows that the T3SS of *B. pertussis* 12743 functions in a distinct manner from that of *B. bronchiseptica.* Although the T3SS of *B. pertussis* did not mediate persistence, it appears to suppress the early innate pro-inflammatory responses in the lung, as well as Th1/Th17 and IgG antibody responses, to facilitate a more efficient initial lung colonisation of the host.

Murine respiratory infection with *B. pertussis* by aerosol challenge resembles natural infection in many ways, including infection route, age dependency and severity of disease, duration of clinical symptoms (Sato *et al.* 1980; Mills 2001). Consistent with our observations of a non-functional T3SS phenotype in *B. pertussis* Tohama I, a previous study reported no differences in bacterial colonisation of wild-type Tohama I and a T3SS deficient mutant in the mouse lung following intranasal inoculation (Fauconnier *et al.* 2001). In contrast, the present study has demonstrated a significant role for the T3SS of a clinical *B. pertussis* isolate, BP 12743, in establishing efficient bacterial colonisation following aerosol challenge. T3SS deficient BP 12743 did not colonise the lung as efficiently as the wild-type bacteria, particularly in the earlier stages of infection.

Consistent with recent reports (Skinner *et al.* 2005; Pilione and Harvill 2006), this study has also demonstrated a critical role for the T3SS of *B. bronchiseptica* in mediating bacterial persistence in the lung following respiratory challenge. A functional T3SS has also been shown to be necessary for persistence within the trachea of rats (Yuk *et al.* 1998) and C57BL/6 mice (Yuk *et al.* 2000). Interestingly while the T3SS of *B. bronchiseptica* enhanced tracheal colonisation of BALB/c mice, significant numbers of *bscN* mutant RB50 were recovered from the tracheas of these mice 35 days post respiratory challenge (Yuk *et al.* 2000). Consistent with these reports, this study has also demonstrated significantly lower tracheal colonisation of BALB/c mice with T3SS deficient *B. bronchiseptica* RB50 following aerosol challenge. However, in contrast to the results of Harvill *et al* (Harvill *et al.* 1999), who detected significant tracheal colonisation in BALB/c mice following intra-nasal respiratory challenge with *B. pertussis* Tohama I, we failed to detect colonisation of

the trachea following aerosol cahllenge with either wild-type or T3SS deficient *B. pertussis* 12743. It is possible that these differences may be due to differences between the bacterial strains or the infection route used in the two studies.

Studies conducted using intra-nasal inoculation of C57BL/6 mice with *B. bronchiseptica* RB50 have suggested a role for the T3SS in the downregulation of Th1 responses in favour of more Treg cell responses, leading to bacterial persistence (Pilione and Harvill 2006) (Skinner *et al.* 2005). These studies suggested that the T3SS modulates DC function, resulting in the early induction of IL-10 secreting T-cells following infection. The IL-10 functions to suppress induction of IFNγ secreting Th1 cells, which have been associated with bacterial clearance (Pilione and Harvill 2006). In contrast to these published studies, our data show little evidence for a role for the T3SS of *B. bronchiseptica* RB50 in the early induction of IL-10 secreting T-cells or their subsequent inhibitory effect on IFNγ production following aerosol challenge. However, we did detect significantly higher IL-10 production from spleen cells taken from mice infected with wild-type BB RB50 28 days post infection in response to low-endotoxin heat-inactivated ACT antigen only, which suggests the T3SS of BB RB50 may be involved in the induction of IL-10 late in infection. However, studies have shown that the genetic background of mice is important in the suceptibilty to bacterial infections such as *Yersinia* (Hancock *et al.* 1986) and *Listeria* (Gervais *et al.* 1984). In contrast to the susceptibility of BALB/c mice, C57BL/6 mice appear to be more resistant to infection by these pathogens. A subsequent study in *Listeria* infected mice reported that C57BL/6 mice developed strong IL-12-induced Th1 responses whereas BALB/c mice generated a more polarized IL-4 driven Th2 response (Liu *et al.* 2000). Therefore, it is possible that the effects of the *B. bronchiseptica* T3SS in suppressing IFNγ production are more subtle or not as significant in BALB/c mice.

In addition, we observed the transient recruitment of activated ($CD25^+$) T-cells expressing the regulatory T-cell marker FoxP3 to the lung early in *Bordetella* infection, at 7 days post aerosol challenge. However, the levels and patterns of Treg cell infiltration were almost identical between mice infected with wild-type and T3SS-deficient *Bordetella*, suggesting that the T3SS of *Bordetella* spp. does not have a role in the induction of Treg cells following aerosol challenge.

Similar to *B. bronchiseptica*, we observed no evidence for T3SS-mediated induction of IL-10 secreting T-cells early in infection of BALB/c mice with *B. pertussis* 12743. However, the higher antigen specific IFNγ production observed from spleen cells from BALB/c mice infected with BP 12743 bscN in contrast to the wild-type bacteria

demonstrated that the T3SS of *B. pertussis* may be involved in the inhibition of induction or activation of antigen-specific IFNγ production by spleen cells. IFNγ, as the signature cytokine of Th1 cells, is detectable in the BAL fluid of mice following natural infection with *B. pertussis* (Torre *et al.* 1993), and resolution of infection is dependent on IFNγ and correlates with the induction of Th1 responses (Mills *et al.* 1999). Thus, the weaker Th1 response elicited by mice infected with wild-type *B. pertussis* 12743 in this study, as evidenced by the lower level induction of IFNγ, correlates with the increased bacterial burden in the lungs of these mice.

It has previously been reported that LPS from *B. pertussis* stimulates pro-inflammatory cytokine and chemokine production in the lungs of mice following aerosol challenge (Higgins *et al.* 2003). However, in contrast to the higher bacterial burden seen in the lungs of mice infected with wild-type *B. pertussis* 12743, we detected significantly lower levels of proinflammatory cytokines IL-1β, TNFα, IL-6 and chemokines MIP1α and MIP2 in the lungs of these mice, when compared with those from mice infected with *B. pertussis* 12743 bscN. These inflammatory mediators have important roles in the recruitment and activation of PMNs and monocytes during infection, and are known to be released by epithelial cells following bacterial invasion (Jung *et al.* 1995; McGuirk and Mills 2000). TNFα is chemotactic for monocytes, upregulates adhesion molecule expression and activates killing activities of phagocytes (Vassalli 1992), while MIP1α is a potent chemoattractant and activator of neutrophils (Jung *et al.* 1995). T3SS-mediated inhibition of both TNFα and the human homologue of MIP1α, IL-8, has been demonstrated for *Yersinia* spp. (Schulte *et al.* 1996; Boland and Cornelis 1998; Palmer *et al.* 1998), presumably as a result of the inactivation of NF-κB nuclear transloction by YopP/J. The T3SS-mediated inactivation of NF-κB in epithelial cells has also been reported for *B. bronchiseptica* (Yuk *et al.* 2000), and a homologous T3SS effector in *Shigella* and *Salmonella*, IpaH9.8 (Okuda *et al.* 2005) and SspH1 (Haraga and Miller 2003) respectively. Interestingly, studies in a lung infection model have recently shown that IpaH9.8 is directly responsible for the inhibition of PMN recruitment following *Shigella* infection, through the inhibition of MIP2, IL-1β, RANTES and GM-CSF.

It recently has been demonstrated that Th17 cells play an important role in vaccine induced immunity to *B. pertussis* (Higgins *et al.* J immunol. In press). IL-17 production by Th17 cells mediates neutrophil recruitment to the site of infection, suggesting a role in containing infection, until a subsequent Th1 response can be generated by the host, which takes several days to develop (McKenzie *et al.* 2006). This more potent and rapid IL-17-

driven inflammatory response could be required in tissues of vital organs, such as the lung, where significant or sudden pathogen invasion can be life threatening. Recently IL-1β, together with IL-23 has been shown to be involved in the induction of IL-17 producing T-cells, and interestingly TNFα was also shown to synergise with IL-23 to promote IL-17 production (Sutton *et al.* 2006). The present study shows dramatically higher IL-1β 3-14 days post infection with T3SS deficient *B. pertussis* 12743 bscN. In addition, these results also demonstrate a corresponding increase in antigen specific IL-17 production by T-cells from the mice infected with T3SS deficient *B. pertussis* 12743 bscN. To our knowledge, this is the first report of T3SS mediated IL-1β or IL-17 inhibition in *Bordetella*, and in conjunction with inhibition of MIP1α, MIP2 and early TNFα, of a possible role for the *Bordetella* T3SS in the inhibition of early local inflammation and PMN recruitment to the lung following aerosol challenge.

In contrast to *B. pertussis*, we did not observe a similar role in the inhibition of pro inflammatory signals in the lung for the T3SS of *B. bronchiseptica*. Levels of pro inflammatory cytokines TNFα, IL-6 and IL-1β, and chemokines MIP1α and MIP2 detected between wild-type and T3SS deficient bacteria were consistent with the higher concentration of bacteria in the lungs of mice infected with wild-type *B. bronchiseptica* RB50. These observations are in agreement with similar reports demonstrating differences in the ability of *B. pertussis* and *B. bronchiseptica* to resist neutrophil recruitment to the lung, and subsequent neutrophil mediated phagocytosis of opsonised bacteria, in the early stages of infection (Kirimanjeswara *et al.* 2005). A notable difference between the two bacterial species is the *B. pertussis* specific expression of PT. Interestingly, PT has been shown to be responsible for blocking migration of neutrophils to the lung during the first week of *B. pertussis* infection (Kirimanjeswara *et al.* 2005). However, the above study was performed using *B. pertussis* Tohama I, a strain of *B. pertussis* we have shown not to possess T3SS activity, yet this proposed function for PT is consistent with the observations in the present study on the role of the T3SS of *B. pertussis* 12743. This apparent similarity or redundancy in the role of the *B. pertussis* T3SS to that of PT may explain why lab-adapted strains, such as *B. pertussis* Tohama I and *B. pertussis* W28, have been able to lose T3SS expression, without a significant loss of virulence or host colonisation.

Interestingly, the most striking T3SS-mediated inhibition of both IFNγ and IL-17 in the present study was observed in response to purified heat-inactivated PT antigen. Therefore, it is possible that the T3SS-mediated downregulation of pro-inflammatory responses in the lung seen with *B. pertussis* 12743 following aerosol challenge is

dependent on expression of PT, and may be a synergistic effect. It has already been demonstrated that high level PT expression during infection confers a significant advantage to *B. pertussis*, due to its effect in preventing neutrophil infiltration to the lung (Kirimanjeswara *et al.* 2005). However, of significant disadvantage to the bacterium would be the enhancement of IL-12 (He *et al.* 2000) and possible IL-23 induction, proposed to result from the cAMP elevating activity of PT (discussed in section 1.2.2.1). Therefore, since IL-12 promotes IFNγ and IL-23 triggers rapid IL-17 production from tissue-resident T-cells in response to LPS (McKenzie *et al.* 2006), and IL-1β is crucial for this process (Sutton *et al.* 2006), the T3SS-mediated inhibition of IL-1β in the lung early in infection may be a mechanism that allows *B. pertussis* to overcome the potent and rapid pro-inflammatory Th17 response that may result from the cAMP elevating activities of PT.

In summary, this chapter has demonstrated a novel role for the T3SS of *B. pertussis* 12743 during *in vivo* infection of BALB/c mice. We show that the *B. pertussis* T3SS, in contrast to that of *B. bronchiseptica*, does not mediate bacterial persistence in the lung, but enhances the bacterial burden early in infection by suppressing early inflammatory responses associated with PMN influx to the lung.

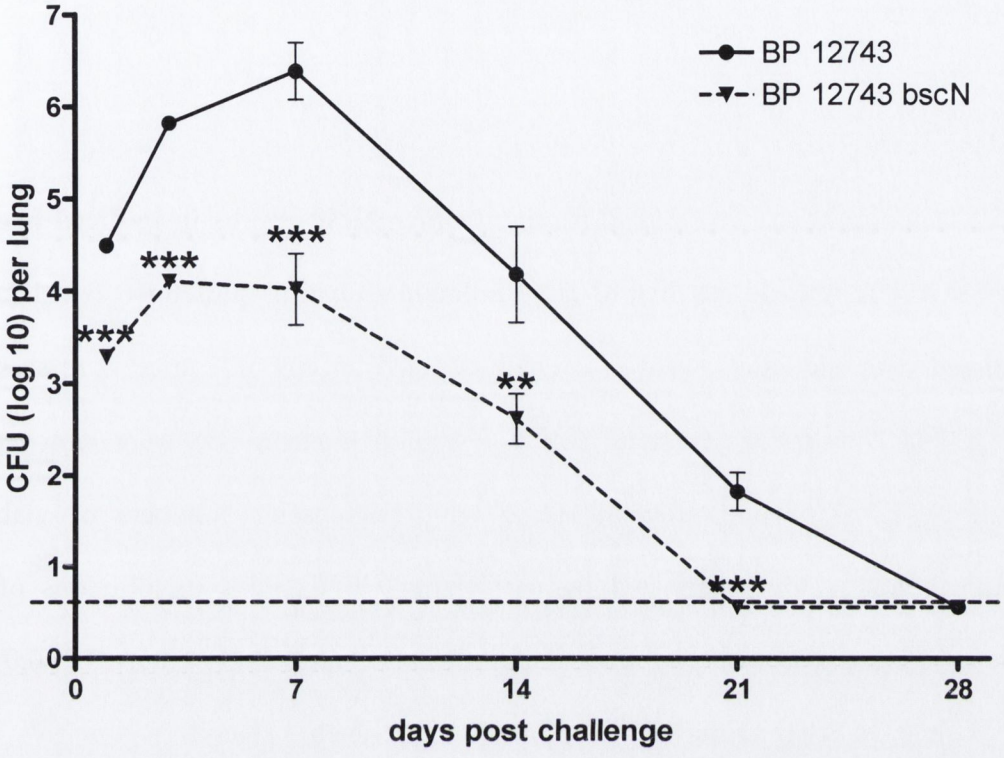
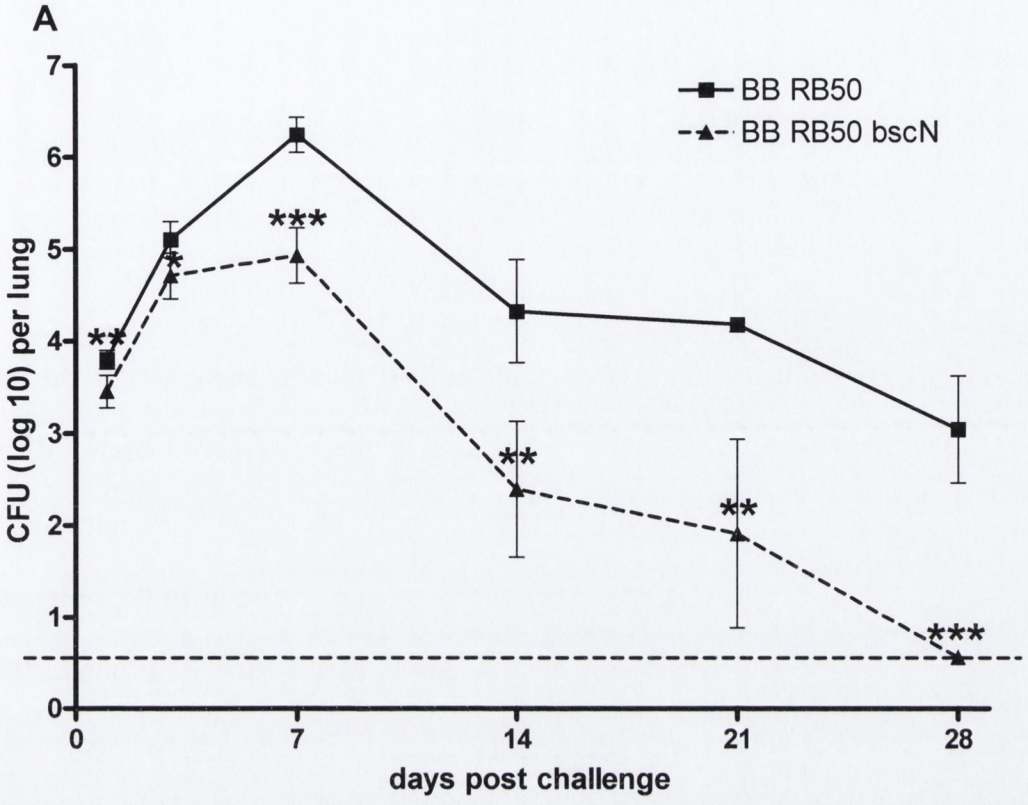


FIGURE 6.1 Deletion of the T3SS of *B. pertussis* 12743 results in reduced bacterial load in the lungs of mice following aerosol challenge. BALB/c mice were challenged with BP 12743 and BP 12743 bscN, and groups of 4 mice were sacrificed 3 hours, 3, 7, 14, 21 and 28 days later. Lungs were removed and homogenised in physiological saline with 1% casein. Numbers of viable *B. pertussis* colonies were estimated by performing CFU counts on dilutions of lung homogenates. The dashed line represents the limit of detection. **P<0.01 and ***P<0.001 wild-type *B. pertussis* 12743 vs T3SS-deficient bacteria.

FIGURE 6.2 Deletion of the T3SS of *B. bronchiseptica* RB50 results in reduced bacterial colonisation in the lungs and trachea of mice following aerosol challenge. BALB/c mice were challenged with BB RB50 and BB RB50 bscN, and groups of 4 mice were sacrificed 3hours, 3, 7, 14, 21 and 28 days later. Lungs (A) and tracheas (B) were removed and homogenised in 1 ml of physiological saline with 1% casein. Numbers of viable *B. bronchiseptica* colonies were estimated by performing CFU counts on dilutions of lung homogenates. The dashed line represents the limit of detection. *P<0.05, **P<0.01 and ***P<0.001 wild-type *B. bronchiseptica* RB50 vs T3SS-deficient bacteria.


A
BB RB50
BB RB50 bscN
CFU (log 10) per lung
days post challenge
0
1
2
3
4
5
6
7
0
7
14
21
28
*
**
***
**
**
***

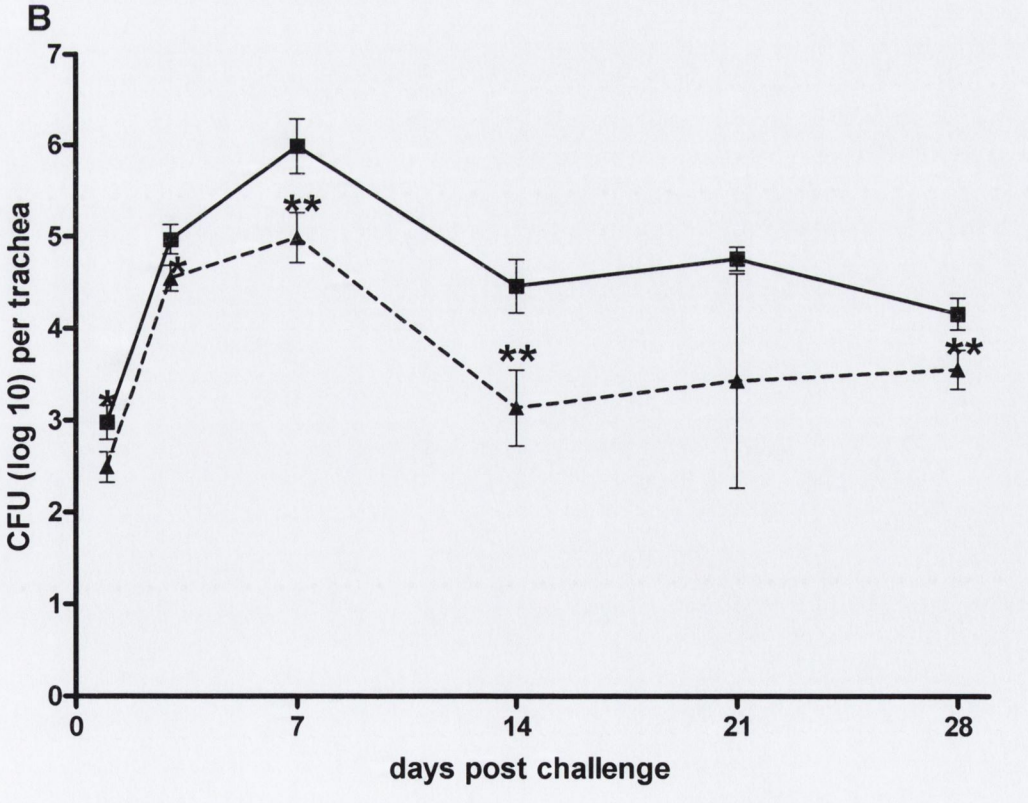

B
CFU (log 10) per trachea
days post challenge
0
1
2
3
4
5
6
7
0
7
14
21
28
*
**
**
**

FIGURE 6.3 Differential inflammatory cytokine and chemokine responses in the lungs of BALB/c mice infected with *B. pertussis* 12743 and *B. pertussis* 12743 bscN. Mice were challenged as described in Figure 6.1. Cytokine and chemokine concentrations were determined by ELISA of lung homogenates from mice 3 hours, 3, 7 and 14 days after aerosol challenge. Results are mean (+/- SD) for 4 mice per group at each time point. *P<0.05, **P<0.01 and ***P<0.001 wild-type *B. pertussis* 12743 vs T3SS-deficient bacteria.

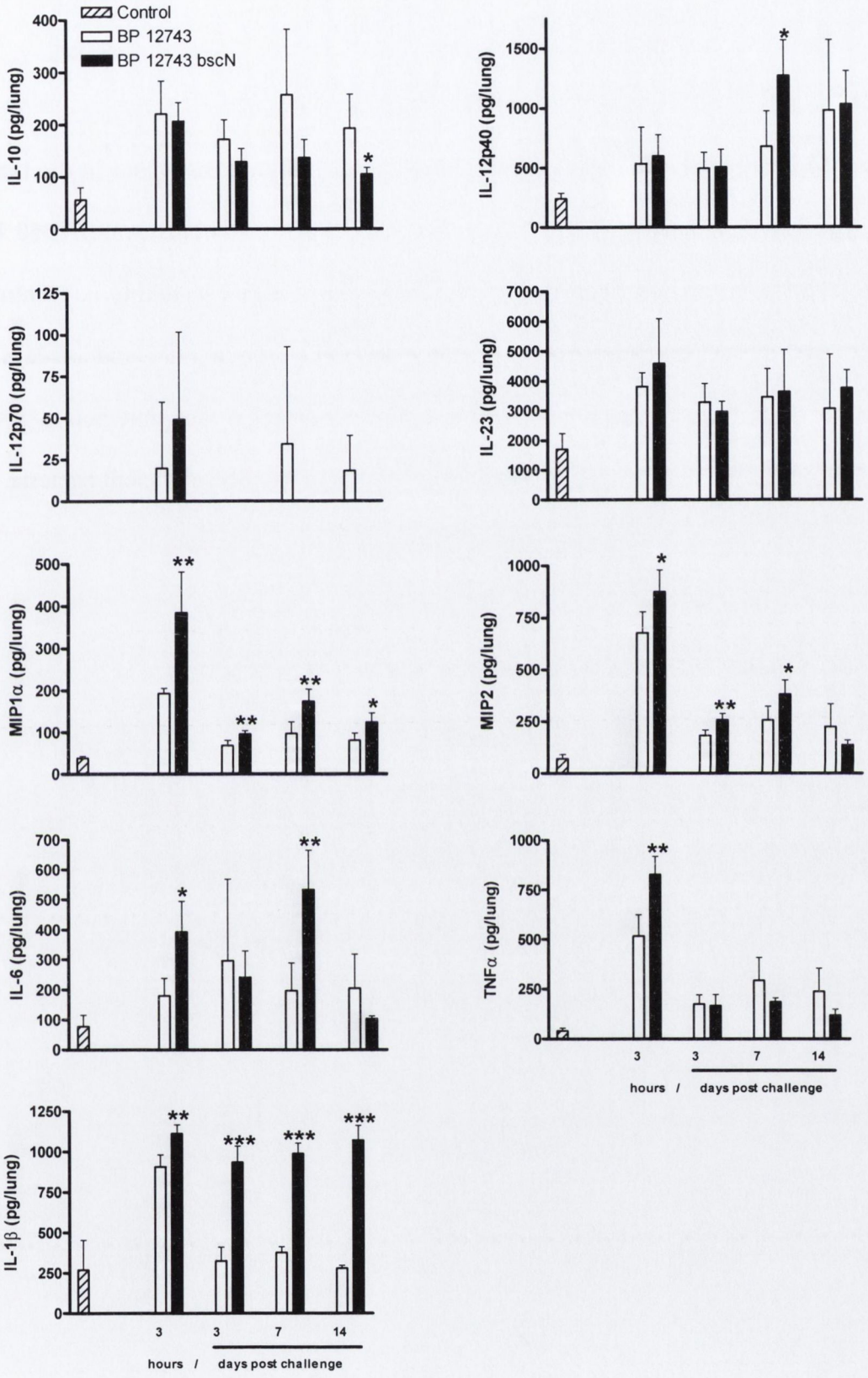
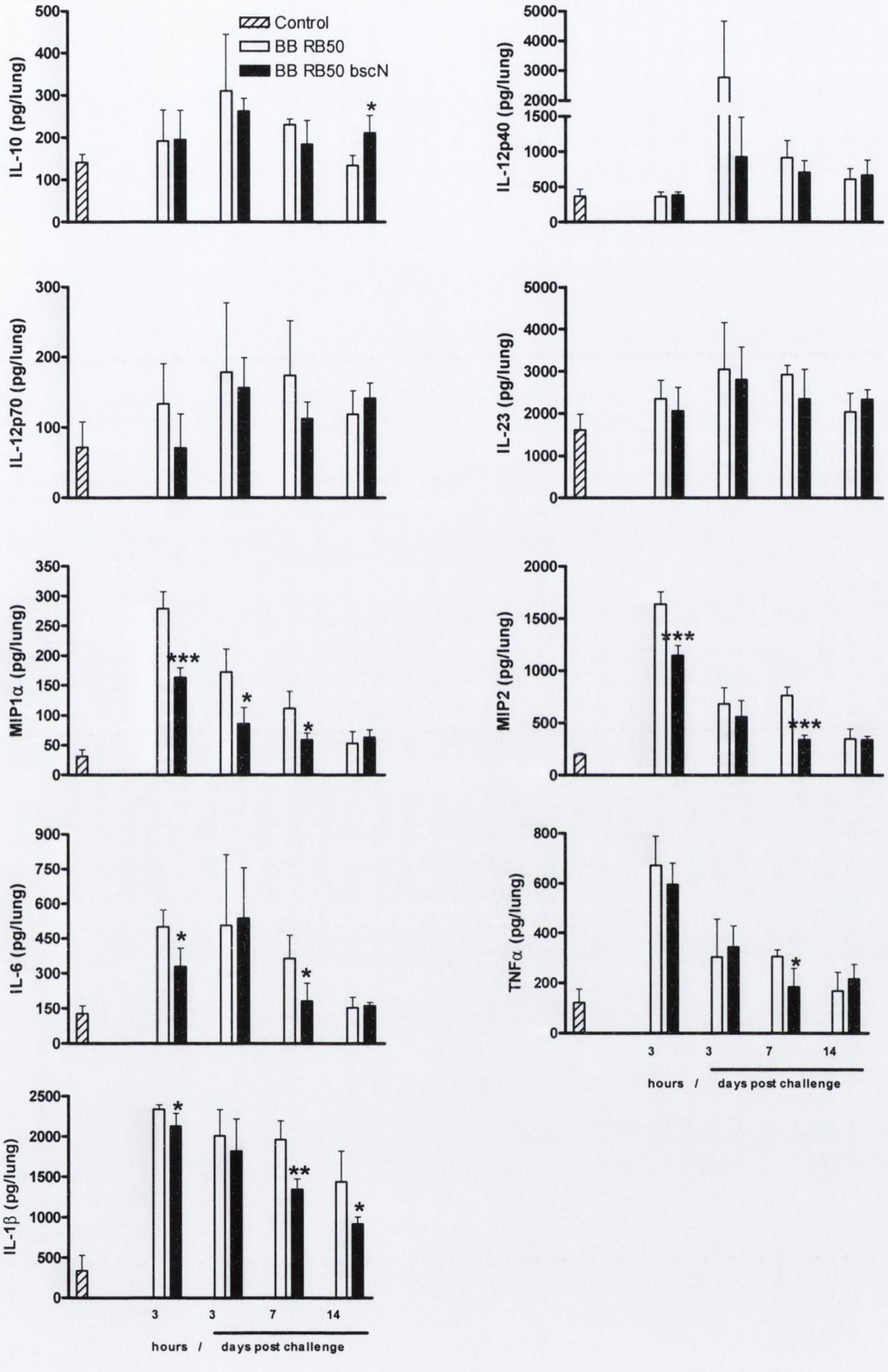

Control
BP 12743
BP 12743 bscN
IL-10 (pg/lung)
IL-12p40 (pg/lung)
IL-12p70 (pg/lung)
IL-23 (pg/lung)
MIP1α (pg/lung)
MIP2 (pg/lung)
IL-6 (pg/lung)
TNFα (pg/lung)
IL-1β (pg/lung)
3
3
7
14
hours / days post challenge

FIGURE 6.4 Differential inflammatory cytokine and chemokine responses in the lungs of BALB/c mice infecteted with *B. bronchiseptica* RB50 and *B.bronchiseptica* RB50 bscN. Mice were challenged as described in Figure 6.2. Cytokine and chemokine concentrations were determined by ELISA of lung homogenates from mice 3 hours, 3, 7 and 14 days after aerosol challenge. Results are mean (+/- SD) for 4 mice per group at each time point. *P<0.05, **P<0.01 and ***P<0.001 wild-type *B. bronchiseptica* RB50 vs T3SS-deficient bacteria.


Control
BB RB50
BB RB50 bscN
IL-10 (pg/lung)
IL-12p40 (pg/lung)
IL-12p70 (pg/lung)
IL-23 (pg/lung)
MIP1α (pg/lung)
MIP2 (pg/lung)
IL-6 (pg/lung)
TNFα (pg/lung)
IL-1β (pg/lung)
3
3
7
14
hours / days post challenge

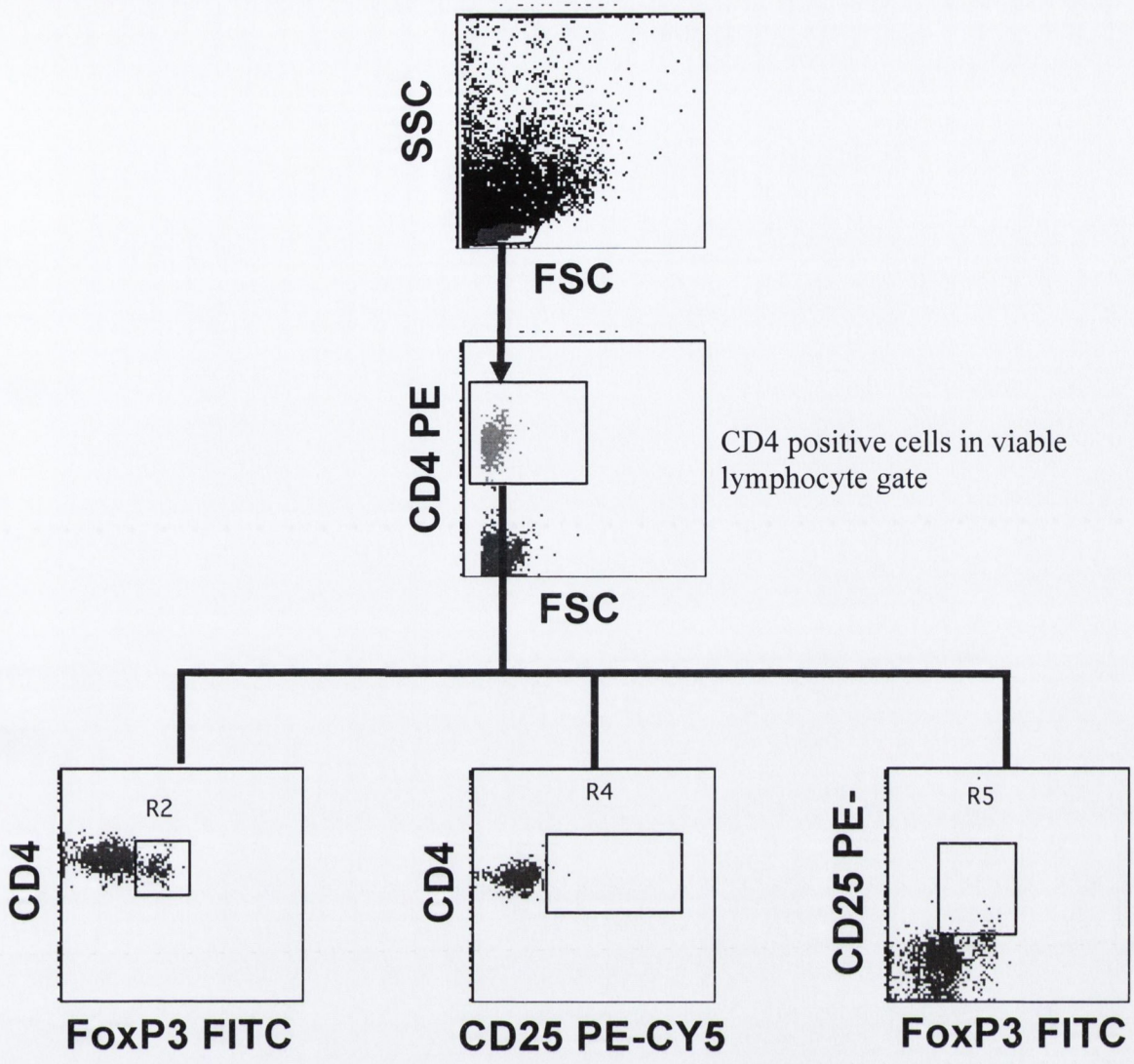


FIGURE 6.5 Schematic of flow cytometry gating events performed on lung cells from mice infected with wild-type or T3SS deficient *B. bronchiseptica* RB50 or *B. pertussis* 12743 following aerosol challenge. Viable lymphocytes were identified on the basis of size (forward scatter- FSC) and granularity (side scatter- SSC), followed by gating on the total $CD4^+$ T-cell population within this cluster. Next, expression of both CD25 and FoxP3 on the total $CD4^+$ T cell population was determined. Finally, CD25 expression was determined on the total $CD4^+$ $FoxP3^+$ T cell population.

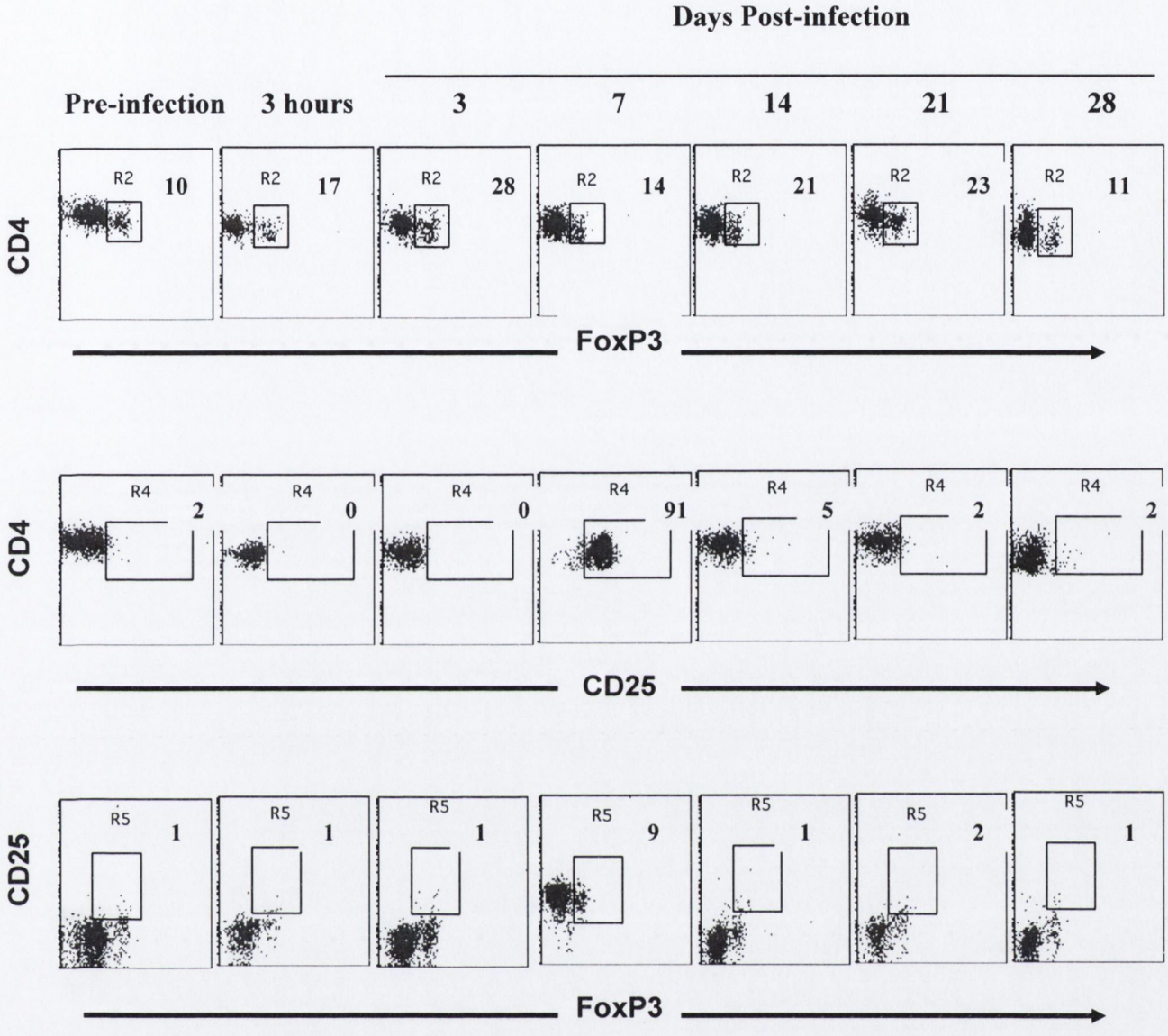


FIGURE 6.6 Regulatory T cell populations in the lung during infection with *Bordetella* spp. BALB/c mice were infected with wild-type and T3SS deficient *B bronchiseptica* RB50 or *B. pertussis* 12743 by aerosol challenge as described in figure 6.1. Flow cytometry was performed on lung cells for the detection of $CD4^+$ T-cells in combination with antibodies specific for FoxP3, CD25 or both. Results are generated from pooled lung cells from 4 mice at the indicated timepoints post infection. The results above are from *B. pertussis* 12743 infected mice and are representative for all 4 strains of bacteria

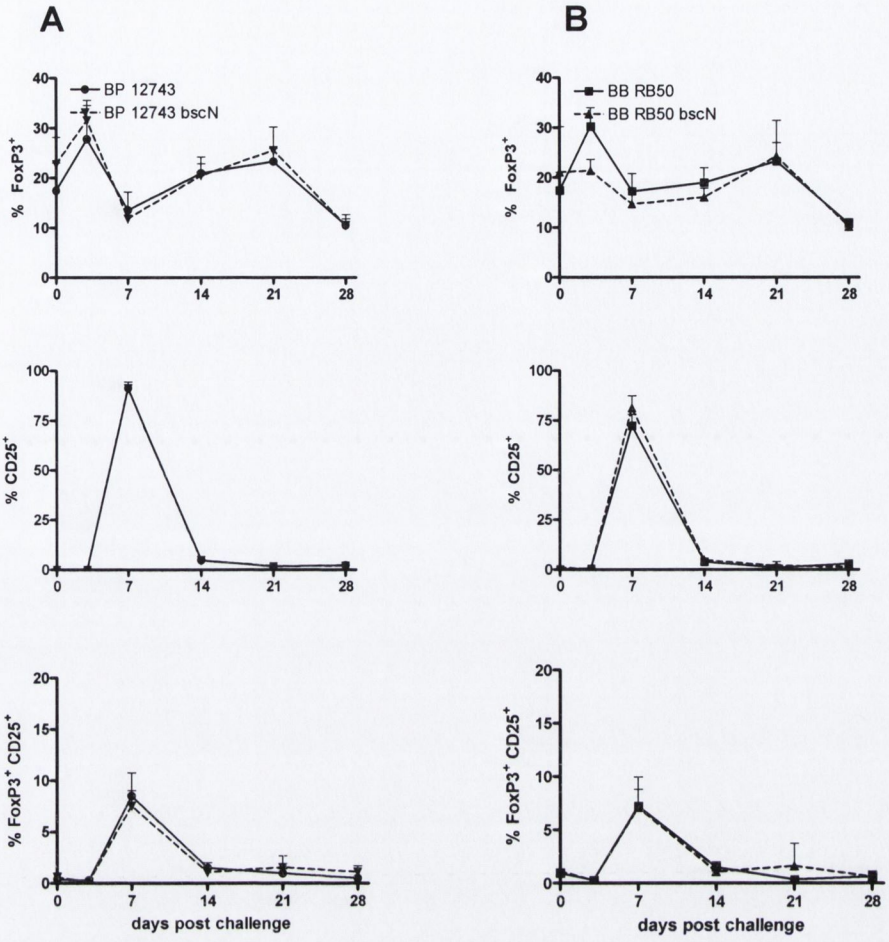


FIGURE 6.7 Enhanced $CD4^+$ $FoxP3^+$ expressing T cells in the lungs of mice following infection with wild-type or T3SS deficient *B. pertussis* 12743 or *B. bronchiseptica* RB50 bacteria. BALB/c mice were infected with wild-type and T3SS deficient *B. pertussis* 12743 (A) and *B. bronchiseptica* RB50 (B) as described in figures 6.1 and 6.2 respectively. The left lung lobes were removed 3 h, 3, 7, 14, 21 and 28 days post challenge and digested with collagenase D to obtain cell suspensions which were then labelled with fluorescent antibodies specific for CD4, CD25 and FoxP3. Results represent the % of $CD4^+$ cells expressing FoxP3, CD25 or FoxP3 and CD25 (+/- SD) for four mice per group at each timepoint

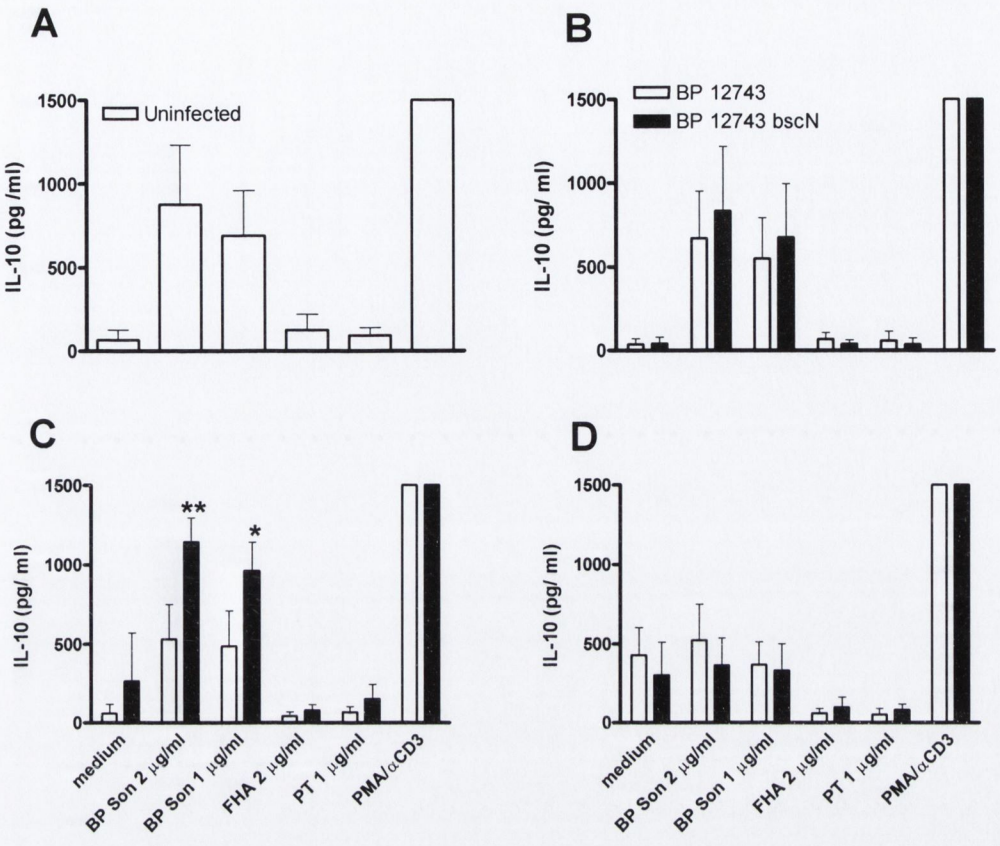


FIGURE 6.8 Different antigen specific IL-10 spleen cell responses in mice infected with T3SS-deficient and wild-type *B. pertussis* 12743. BALB/c mice were challenged as described in figure 6.1. Spleens were removed from naïve (A) or infected mice 14 (B), 21 (C) and 28 (D) days after aerosol challenge. Splenocytes were stimulated with sonicated *B. pertussis* 12743 (BP Son), FHA and PT, as well as PMA/αCD3 or medium only as positive and negative controls respectively. Supernatents were removed after 3 days and IL-10 concnetrations were determined by ELISA. Results are mean (+/- SD) for 4 mice per group. *P<0.5 and **P<0.01 wild-type *B. pertussis* 12743 vs T3SS-deficient bacteria.

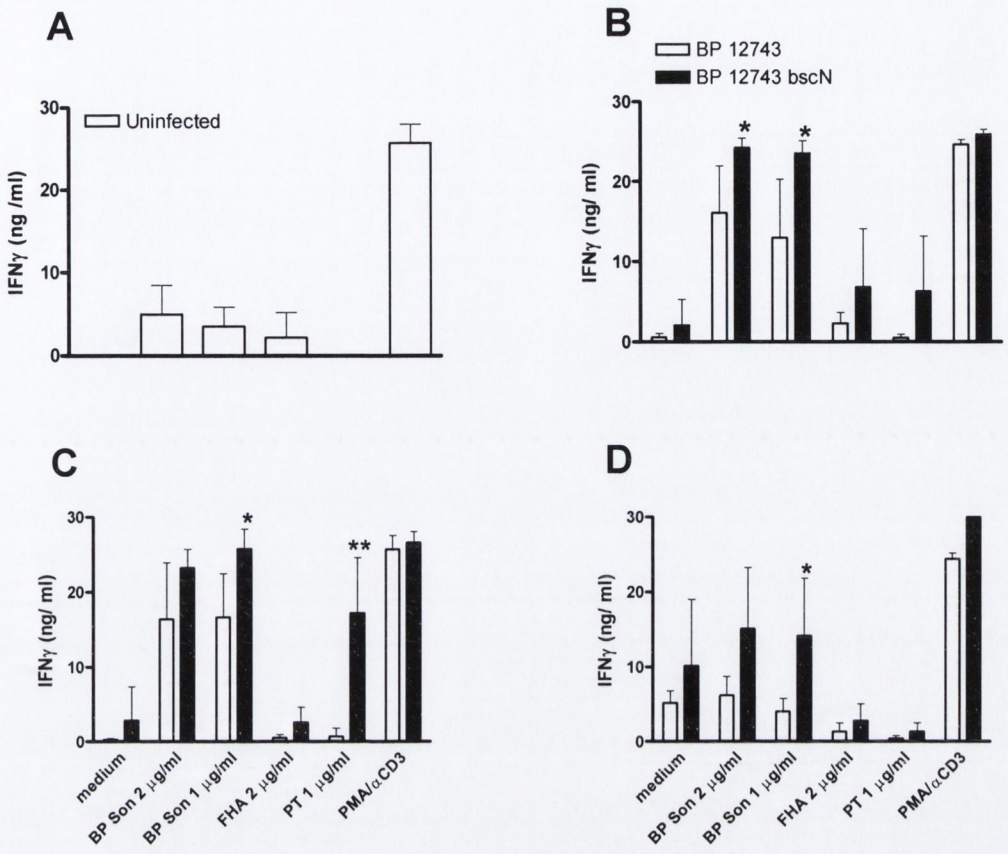


FIGURE 6.9 Different antigen specific IFNγ spleen cell responses in mice infected with T3SS-deficient and wild-type *B. pertussis* 12743. BALB/c mice were challenged as described in figure 6.1. Spleens were removed from naïve (A) or infected mice 14 (B), 21 (C) and 28 (D) days after aerosol challenge. Splenocytes were stimulated with sonicated *B. pertussis* 12743 (BP Son), FHA and PT, as well as PMA/αCD3 or medium only as positive and negative controls respectively. Supernatents were removed after 3 days and IFNγ concentrations determined by ELISA. Results are mean (+/- SD) for 4 mice per group. *P<0.5 and **P<0.01 wild-type *B. pertussis* 12743 vs T3SS-deficient bacteria.

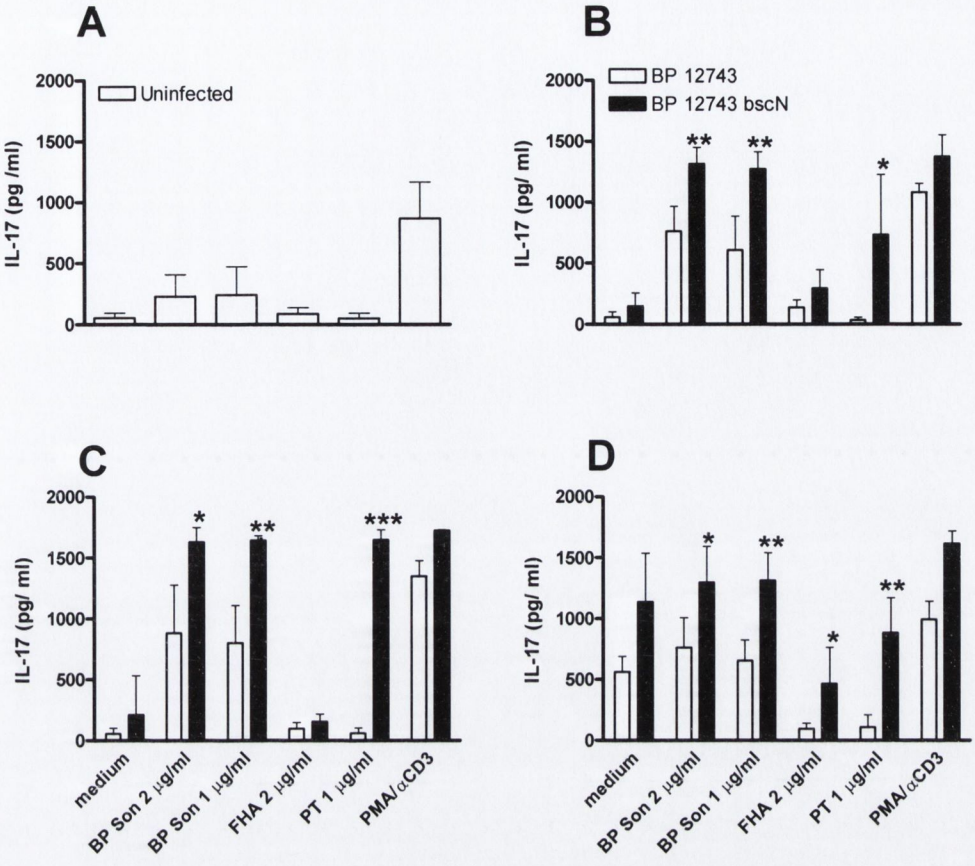


FIGURE 6.10 Different antigen specific IL-17 spleen cell responses in mice infected with T3SS-deficient and wild-type *B. pertussis* 12743. BALB/c mice were challenged as described in figure 6.1. Spleens were removed from naïve (A) or infected mice 14 (B), 21 (C) and 28 (D) days after aerosol challenge. Splenocytes were stimulated with sonicated *B. pertussis* 12743 (BP Son), FHA and PT, as well as PMA/αCD3 or medium only as positive and negative controls respectively. Supernatants were removed after 3 days and IL-17 concentrations were determined by ELISA. Results are mean (+/- SD) for 4 mice per group. *P<0.5, **P<0.01 and ***P<0.001 wild-type *B. pertussis* 12743 vs T3SS-deficient bacteria.

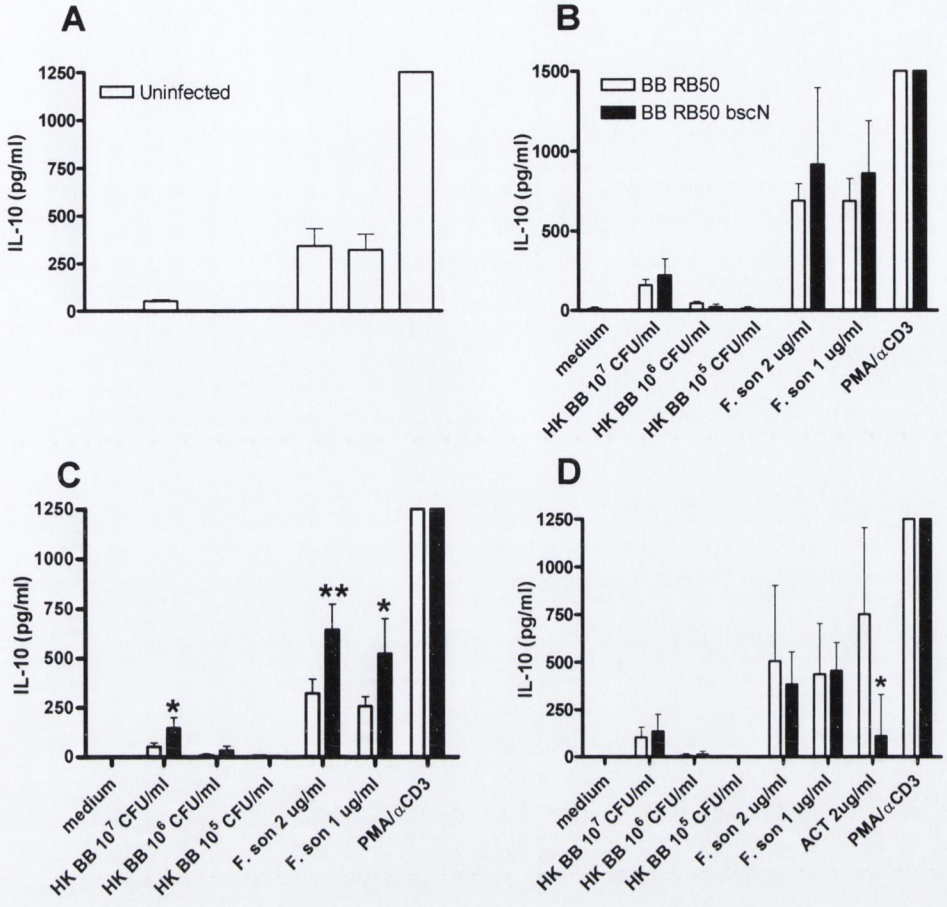


FIGURE 6.11 Different antigen specific IL-10 spleen cell responses in mice infected with T3SS-deficient and wild-type *B. bronchiseptica* RB50. BALB/c mice were challenged as described in figure 6.2. Spleens were removed from naïve (A) or infected mice 14 (B), 21 (C) and 28 (D) days after aerosol challenge. Splenocytes were stimulated with heat-killed *B. bronchiseptica* RB50 (HK BB) and formalin treated sonicated *B. pertussis* W28 antigen (F. son), as well as PMA/αCD3 or medium only as positive and negative controls respectively. Supernatents were removed after 3 days and IL-10 concentrations were determined by ELISA. Results are mean (+/- SD) for 4 mice per group. *P<0.5 and **P<0.01 wild-type *B. bronchiseptica* RB50 vs T3SS-deficient bacteria.

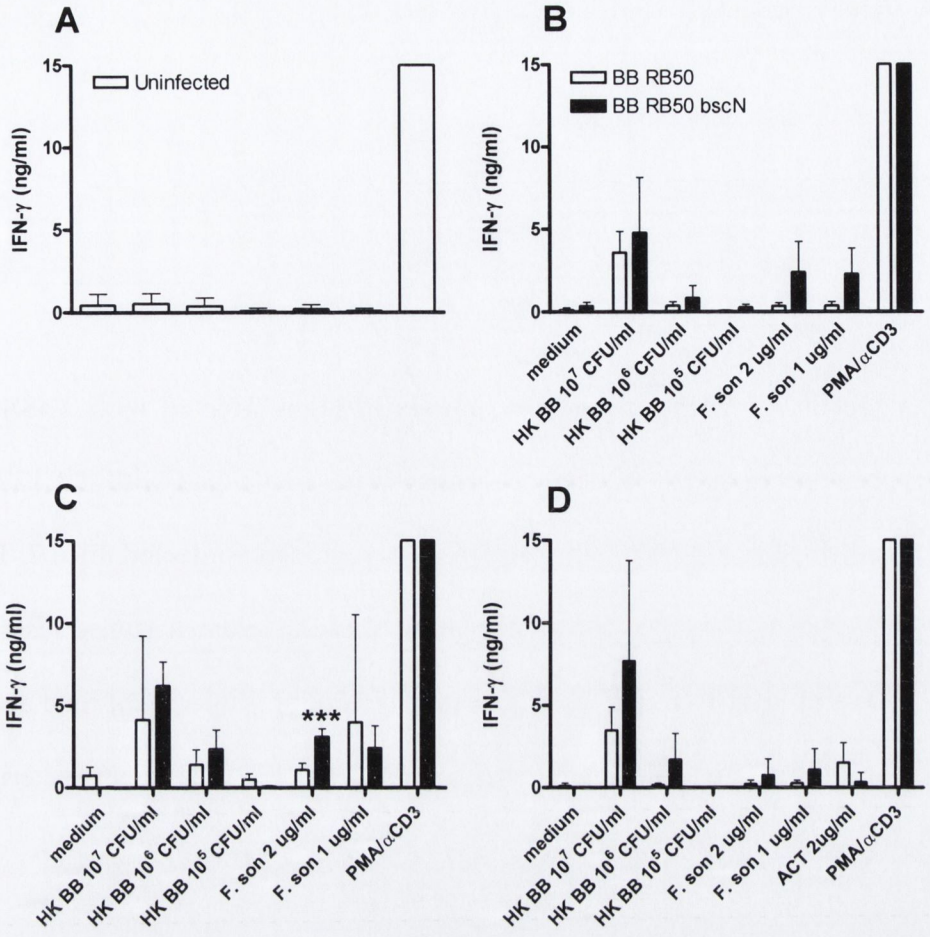
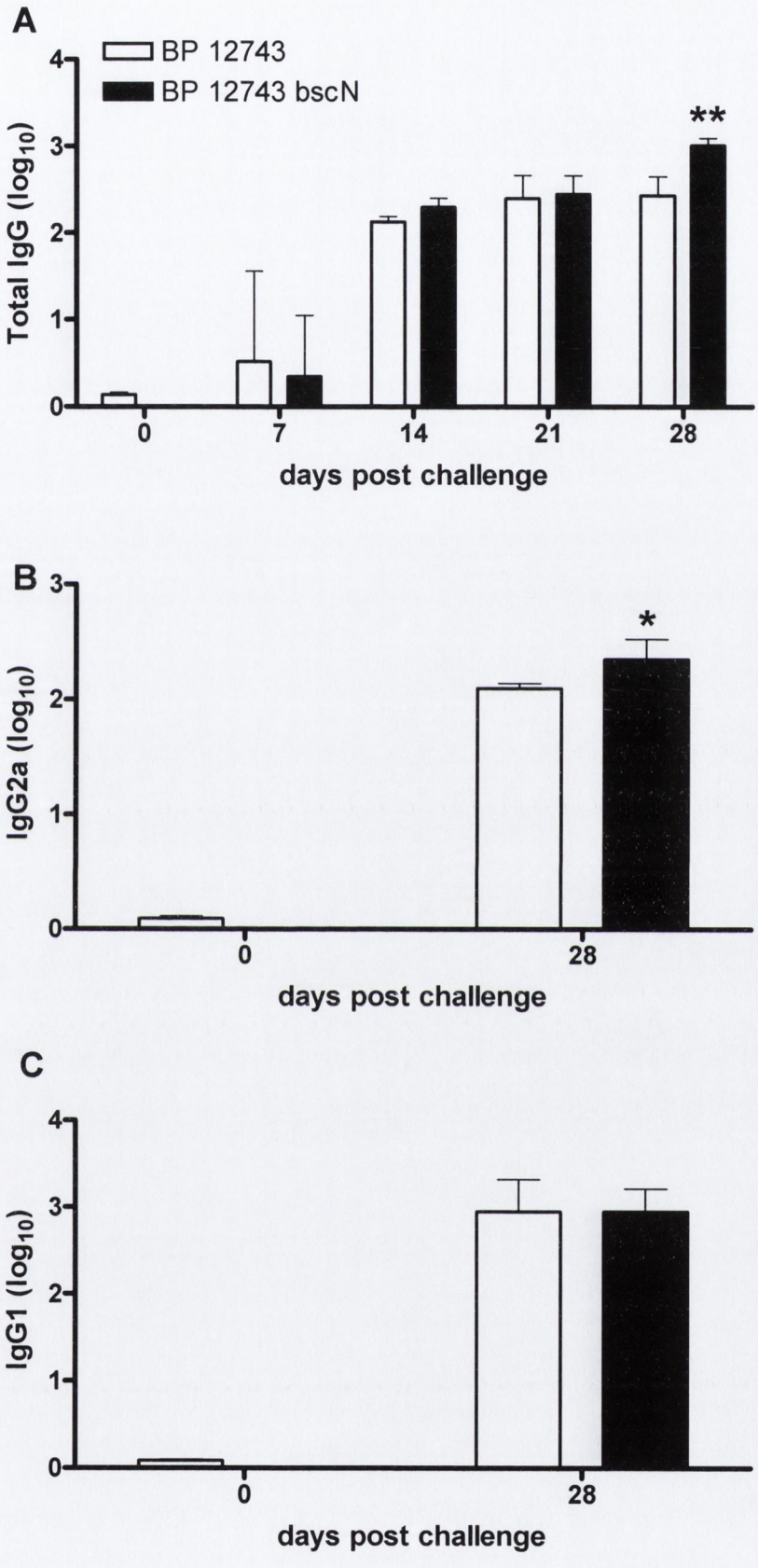


FIGURE 6.12 Different antigen specific IFNγ spleen cell responses in mice infected with T3SS-deficient vs wild-type *B. bronchiseptica* RB50. BALB/c mice were challenged as described in figure 6.2. Spleens were removed from naïve (A) or infected mice 14 (B), 21 (C) and 28 (D) days after aerosol challenge. Splenocytes were stimulated with heat-killed *B. bronchiseptica* RB50 (HK BB) and formalin treated sonicated *B. pertussis* W28 antigen (F. son), as well as PMA/αCD3 or medium only as positive and negative controls respectively. Supernatents were removed after 3 days and IFNγ concentrations were determined by ELISA. Results are mean (+/- SD) for 4 mice per group. *P<0.5 and **P<0.01 wild-type *B. bronchiseptica* RB50 vs T3SS-deficient bacteria.

FIGURE 6.13 Elevated serum antibody responses in mice infected with T3SS-deficient versus wild-type *B. pertussis* 12743. BALB/c mice were challenged with BP 12743 or BP 12743 bscN as described in Figure 6.1. Serum was recovered from infected mice 7, 14, 21 and 28 days post challenge and assayed for total IgG(A). Day 28 serum was also tested for BP-specific IgG2a (B) and IgG1 (C). Results are mean (+/- SD) for 4 mice per group at each tine point. *P<0.05 and **P<0.01 wild-type *B. pertussis* 12743 vs T3SS-deficient bacteria.


A
BP 12743
BP 12743 bscN
Total IgG (log10)
4
3
2
1
0
**
0
7
14
21
28
days post challenge
B
IgG2a (log10)
3
2
1
0
*
0
28
days post challenge
C
IgG1 (log10)
4
3
2
1
0
0
28
days post challenge

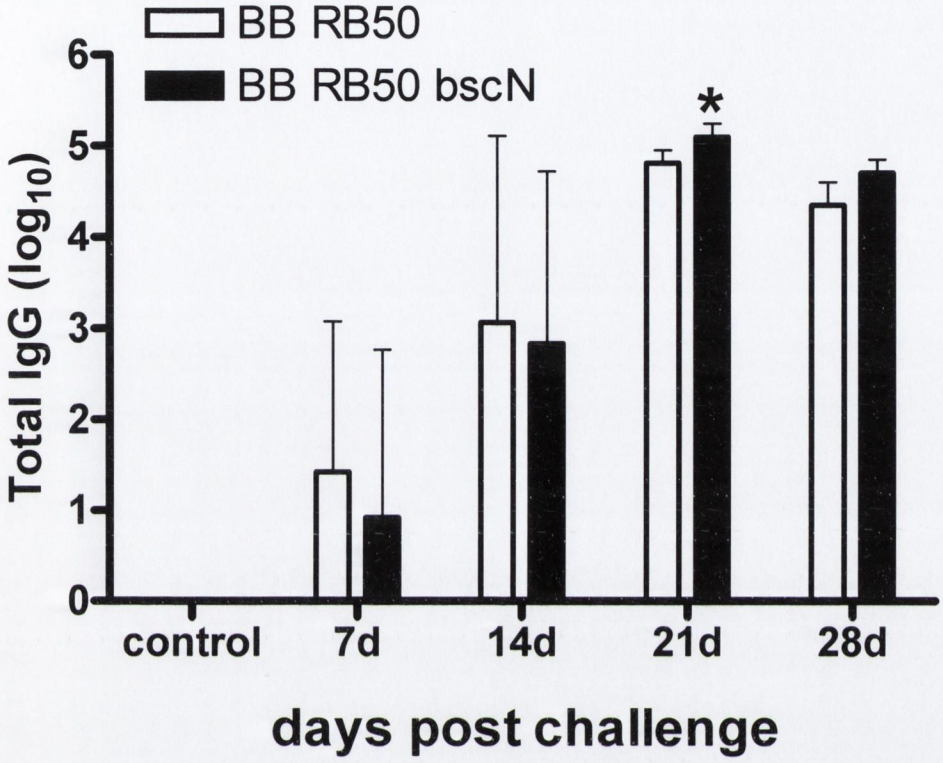


FIGURE 6.14 Serum antibody responses in mice infected with T3SS-deficient and wild-type *B. bronchiseptica* RB50. BALB/c mice were challenged as described in Figure 6.2. Serum recovered from infected mice was assayed for total IgG 7, 14, 21 and 28 days post challenge. Results are mean (+/- SD) for 4 mice per group at each time point. *P<0.5 wild-type *B. bronchiseptica* RB50 vs T3SS-deficient bacteria.

Chapter 7

General Discussion

7. General Discussion

Despite extensive vaccination programs, *B. pertussis* remains a significant cause of childhood disease worldwide. The recent observations that increasing numbers of adults are being diagnosed with *B. pertussis*, and may be transmitting the disease to infants, have called into question the level of protection provided by current *B. pertussis* vaccines, and highlighted the need for a better understanding of the molecular mechanisms of *B. pertussis* pathogenesis during infection. This thesis has investigated the activities of the T3SS of *B. pertussis* during *in vitro* growth and infection of mice has provided significant new information on the pathogenesis of *B. pertussis*.

The study demonstrated for the first time that a *B. pertussis* virulence factor, the T3SS, is functionally active in a large proportion of low passage clinical isolates and identified several substrates of the *B. pertussis* T3SS. The study also showed that the T3SS of *B. pertussis* facilitates more efficient bacterial lung colonisation, by suppressing protective inflammatory responses during infection of BALB/c mice. It appears that the T3SS of *B. pertussis* may facilitate survival in the host by (a) promoting binding of *B. pertussis* to host cells, (b) inhibiting early inflammatory responses in the lung which promote PMN recruitment and innate cell activation (possibly in concert with PT), and (c) inhibiting Th17, Th1 and antibody responses which function to clear the bacteria.

B. pertussis and *B. bronchiseptica* both produce a range of adhesins and toxins, collectively referred to as virulence factors, most of which have well-defined roles in establishing and maintaining *Bordetella* infection. The two closely related pathogens have evolved different strategies for survival in the host. The relatively short-lived and acute severity of *B. pertussis* infection of humans, which favours a high rate of host to host transmission, is in contrast to the asymptomatic and often life-long persistence of *B. bronchiseptica* infection of animals. In recent years a number of studies have described a role for an additional less well characterised virulence factor, the T3SS, in modulating innate immune cell signalling, and therefore subsequent adaptive immune responses, to facilitate long-term bacterial persistence of *B. bronchiseptica* (Yuk *et al.* 2000; Skinner *et al.* 2005; Pilione and Harvill 2006). Thus, the apparent lack of T3SS expression by *B. pertussis*, in contrast to that of the more persistent *B. bronchiseptica*, is therefore in agreement with the suggested survival strategy of these two respiratory pathogens. The

significant finding of the present study is that the T3SS of unpassaged clinical isolates of *B. pertussis* is functionally active and plays a role in promoting more efficient bacterial colonisation and persistence, through immunosuppression following respiratory challenge.

The present study showed significant secretion of Bsp22, a reliable marker for T3SS activity in *Bordetella* spp, by 11 nosocomial isolates of *B. pertussis* (listed in Table 3.1), in addition to three isolates obtained from the ATCC, BP 12743, BP 9340, and BP 18323, two of which had not undergone extensive laboratory passage. The results showed secretion of two additional known *Bordetella* T3SS substrates, BopN and BopD, by BP 12743. This is the first demonstration of T3SS activity in *B. pertussis* spp. The secretion of these T3SS substrates by clinical isolates of *B. pertussis* was in marked contrast to the absence of T3SS substrate secretion by two well studied lab adapted strains of *B. pertussis*, Tohama I and Wellcome 28. It has been reported that while the T3SS loci are conserved and transcribed in *B. pertussis* Tohama I during *in vitro* growth, a post-transcriptional block prevents protein translation (Mattoo *et al.* 2004). *B. pertussis* Tohama I, first isolated in Japan in 1954 (Kasuga *et al.* 1954), is the most well studied and well characterised of *B. pertussis* isolates, which is partly why it was chosen for genomic sequencing (Parkhill *et al.* 2003). The sequencing of the *B. pertussis* Tohama I genome revealed extensive expansion of the IS element, IS481, indicating recent large scale genome rearrangements and thus a high level of genome plasticity.

Due to its extensive study and characterisation, original stocks of *B. pertussis* Tohama I are not available. Thus it is likely that most isolates of *B. pertussis* Tohama I and Wellcome 28 in use by researchers today have been through extensive laboratory passage. It has been reported that long-term laboratory passage has led to significant changes in gene expression and virulence factor production in routinely cultured bacterial species such as *E. coli*, *Pseudomonas* and *Staphylococcus* spp. (Fux *et al.* 2005). A recent study reported that a variant clinical isolate of *P. aeruginosa*, isolated from the lung of a cystic fibrosis patient, showed a pronounced upregulation of the T3SS and the respective effector proteins when compared with its non-variant lab-cultured wild-type (von Gotz *et al.* 2004). This upregulation of the T3SS correlated with increased *in vitro* cytotoxicity and enhanced virulence in murine respiratory tract infection (von Gotz *et al.*

2004). Furthermore, it has been reported that the *Yersinia* YopT T3SS effector protein is not expressed by serotype O3 strains of *Y. pseudotuberculosis* that have been extensively passaged *in vitro* during *Yersinia* research (Viboud and Bliska 2005). In addition, significant changes in gene transcription by clinical isolates of *B. pertussis* were reported after as few as 12 laboratory passages (Brinig *et al.* 2006), indicating that *B. pertussis*, like other bacterial species, can alter gene expression when introduced into a new environment. It is therefore conceivable that the absence of T3SS activity in *B. pertussis* Tohama I and Wellcome 28 is a consequence of long-term laboratory culture in the absence of eukaryotic cell contact or *in vivo* infection, which has resulted in a block in T3SS activity. Though we detected Bsp22 secretion by another extensively passaged *B. pertussis* strain, BP 18323, concerns have been raised as to whether this strain should be classified as *B. pertussis* (Preston *et al.* 2004). It has long been recognised that BP 18323 possesses virulence for mice unequalled by other *B. pertussis* isolates (Adams 1970), and it has been demonstrated that this strain is genetically more closely related to *B. bronchiseptica* isolates than to *B. pertussis* isolates (Musser *et al.* 1986).

The tailoring of virulence factor expression to favour bacterial survival is common amongst the classical *Bordetella* spp. For example the serum resistance protein, BrkA is expressed by some strains of *B. bronchiseptica*, but not by others, such as BB RB50 (Rambow *et al.* 1998). Furthermore, some *B. bronchiseptica* isolates lose expression of ACT (Gueirard *et al.* 1995,) and the LPS O-antigen (Le Blay *et al.* 1997; Gueirard *et al.* 1998) during the transition from an animal to human host. The expression of PT and TcfA, two virulence factors that promote respiratory tract adherence and possibly host transmission, is specific to *B. pertussis* (Arico and Rappuoli 1987; Hausman *et al.* 1996; Gerlach *et al.* 2001). Interestingly, though *B. parapertussis* does not produce PT during growth *in vitro*, modest anti-PT antibody titres have been observed in children infected with *B. parapertussis* (Hausman et al. 1996). This suggests that *Bordetella* spp. fine-tune virulence factor expression in response to their immediate environment.

The present study did not detect the *in vitro* secretion of any additional and previously unknown *Bordetella* T3SS effectors. The exact signals regulating *in vitro* T3SS activation in *Bordetella* spp. are not currently understood, and of the currently

known *Bordetella* T3SS substrates, BopN, BopD, BopB, Bsp22 and BteA/BopC, only BopN, BopD, BopB and Bsp22 were initially identified by their relative abundance in culture supernatants (Yuk *et al.* 2000) (Kuwae *et al.* 2003). BopD and BopB are known to complex together with their T3SS chaperone, BcrH2, in the *Bordetella* cytoplasm prior to secretion (Nogawa *et al.* 2004). T3SS chaperones have been implicated in guiding T3SS effectors to the T3SS injectisome and once secreted, BopB and BopD, have a joint function in translocation pore formation. Though BopB was not detected in culture supernatant from *B. pertussis* 12743 in this study, Bsp22, BopN and BopD were detected and the presence of BopD suggests that BopB is also produced and secreted by this strain. BteA/BopC was initially identified by a bioinformatic approach by Panina *et al* (Panina *et al.* 2005) and with the assistance of MALDI-TOF analysis by Kuwae *et al* (Kuwae *et al.* 2006).

In contrast to *B. bronchiseptica*, BteA/BopC secretion was not detected in *B. pertussis* 12743 in this study. The *B. bronchiseptica* RB50 genome contains in excess of 1000 genes that are not present or expressed in *B. pertussis* Tohama I (Parkhill *et al.* 2003). The majority of these genes are involved in providing *B. bronchiseptica* with a far greater metabolic versatility and resistance to detrimental environmental conditions, and correlate with the more fastidious growth requirements of *B. pertussis*, whose genome is heavily weighted in favour of *in vivo*, rather than *in vitro*, growth (Preston *et al.* 2004). Therefore, it is possible that the environmental conditions during *in vitro* growth of *B. pertussis* may not be optimised for the release of BteA/BopC, BopB or other, as yet, unidentified T3SS effectors. In agreement with several studies reporting that *B. bronchiseptica* (Yuk *et al.* 1998) (Yuk *et al.* 2000) (Stockbauer *et al.* 2003) (Kuwae *et al.* 2003), and specifically BteA/BopC (Kuwae *et al.* 2006) (Panina *et al.* 2005), induces cytotoxicity in a wide variety of cultured cell types, the present study demonstrated *B. bronchiseptica* T3SS-dependent cell death of macrophage, DC and epithelial cells. However, consistent with the absence of BteA/BopC secretion, BP 12743 did not display this cytotoxic effect when incubated with eukaryotic cells *in vitro*. T3SS–mediated cytotoxicity of innate immune cells during *in vivo* infection may result in a reduction of phagocytosis and subsequent bacterial killing, in addition to a reduction of antigen presentation by APC and a reduction of pro-inflammatory cytokine production by DC,

thereby promoting bacterial persistence. In addition to T3SS dependent cytotoxicity, the present study also observed T3SS dependent reduction in pro-inflammatory cytokine production by DC and T3SS dependent bacterial host persistence by *B. bronchiseptica*, in contrast to *B. pertussis*. Taken together, these data suggest that the secretion of BteA/BopC may be specific to *B. bronchiseptica* and may function in promoting bacterial persistence through the cytotoxicity of innate immune cells.

The modulation of host cell cytoskeletal dynamics for the promotion, or prevention, of bacterial uptake is a common effect of T3SS from different species, such as *Yersinia*, *Salmonella* and *Shigella* (Hueck 1998). The present study revealed a novel effect of the T3SS of *B. bronchieptica*, suppression of bacterial uptake by macrophages. One of the simplest ways for a bacterium to avoid the bactericidal activity of a phagocytic cell is to avoid being ingested. This is a strategy utilised by the T3SS of *Yersinia* spp. and is a key factor in its pathogenesis (Cornelis *et al.* 1998). Both *B. pertussis* (Friedman *et al.* 1992) and *B. bronchiseptica* (Forde *et al.* 1998) can survive for up to 3-4 days inside cultured murine macrophage. Intracellular survival by *B. bronchiseptica* has been attributed, in part, to the *B. bronchiseptica*-specific, bvg-independent, expression of acid phosphatase and urease, enzymes involved in inducing tolerance to the acidic pH of phagolysosomes (Schneider *et al.* 2000). Thus, in addition to its reported role in facilitiating bacterial persistence, through the modulation of host adaptive immune responses (Yuk *et al.* 2000; Skinner *et al.* 2005; Pilione and Harvill 2006), the present study has demonstrated that the T3SS of B. *bronchiseptica* may also facilitate bacterial persistence through subversion of phagocytic uptake by macrophage.

Interestingly, in contrast to *B. bronchiseptica*, *B. pertussis* appears to employ a survival strategy involving a more short-term acute infection, resulting in a high level of host to host transmission, possibly facilitated through the expression of *B. pertussis*-specific virulence factors that promote local inflammatory pathology and increased respiratory tract adherence, such as PT and TcfA. In the present study, a significantly lower number of cell surface associated bacteria were detected following exposure of T3SS deficient *B. pertussis* 12743 bscN to murine macrophage when compared with wild-type bacteria. In addition a significantly lower number of bacteria was detected in the lungs of mice when 3 hours after aerosol challenge with T3SS deficient *B. pertussis*

12743 bscN compared with wild-type bacteria. While it cannot be ruled out that the T3SS of *B. pertussis* 12743 may be having an immunosuppresive effect in the lung immediately following aerosol challenge, it is unlikely to have had such significant impact at that early a timepoint. Interestingly, a similar difference in initial lung colonisation was also observed between wild-type and T3SS deficient *B. bronchiseptica*. Taken together, these data suggest that the T3SS of *B. pertussis*, and possibly *B. bronchiseptica*, may function as an additional adherence factor; the presence of multiple T3SS needle-like injectisomes at the cell surface may help to anchor the bacteria to host cells and tissues during infection. The promotion of host cell adherence by the T3SS has also been demonstrated for enteropathogenic *E. coli* (EPEC), which translocates the translocated intimin receptor (Tir) effector protein into intestinal epithelial cells (Kenny *et al.* 1997). The Tir effector protein then inserts into the host cell plasma membrane where it serves as a receptor for intimin, a T3SS associated bacterial adhesin (Rosenshine *et al.* 1996), thus facilitating *E. coli* attachment to the intestinal epithelium.

Consistent with previous reports involving intra-nasal challenge of C57BL/6 mice with *B. bronchiseptica* (Skinner *et al.* 2005; Pilione and Harvill 2006), using an aerosl challenge model the present study found that wild-type *B. bronchiseptica* persisted in the lungs of BALB/c mice, whereas T3SS deficient bacteria were completely cleared after 28 days. In addition, this study has demonstrated a role for the T3SS of *B. pertussis* in promoting colonisation of the lungs of BALB/c mice following aerosol challenge. It has been suggested that the T3SS of *B. bronchiseptica* may promote the early induction of IL-10 secreting T-cells following infection of C57BL/6 mice. These Treg type cells may have an inhibitory effect on innate pro-inflammatory cytokines and IFNγ production by T cells, thereby enhancing bacterial persistence (Skinner *et al.* 2005; Pilione and Harvill 2006). In contrast, there was little evidence of early T3SS dependent recruitment or activaton of IL-10 secreting T-cells following aerosol challenge of BALB/c mice with either *B. bronchiseptica* or *B. pertussis* 12743 in the present study. However, significantly higher IL-10 production was observed in antigen-stimulated spleen cells from mice infected with wild-type *B. bronchiseptica,* compared with T3SS deficient bacteria, later in infection at 28 days post challenge.

A significant finding of this study is that the T3SS of *B. pertussis*, but not *B. bronchiseptica*, is capable of reducing pro-inflammatory cytokine production and antibody and antigen-specific T cell responses following aerosol challenge of BALB/c mice. The deletion of a functional T3SS in *B. pertussis* was associated with enhancement of IL-12p40 production in the lungs and enhanced IFNγ by spleen cells from BALB/c mice following aerosol challenge with BP 12743 bscN. IFNγ, which is secreted by Th1 cells as well as NK cells, is detectable in the BAL fluid of mice following natural infection with *B. pertussis* (Torre *et al.* 1993), and resolution of *B. pertussis* infection is dependent on IFNγ and correlates with the induction of Th1 responses (Mills *et al.* 1999), and recruitment of NK cells into the lung 14-21 days post challenge with *B. pertussis* (Byrne *et al.* 2004). Taken together, these findings suggest that the lower bacterial lung colonisation exhibited by T3SS deficient *B. pertussis* in this study may reflect the higher innate pro-inflammatory and Th1-type response generated in mice infected with the mutant bacteria.

Although higher concentrations of IL-12p40 and IL-12p70 were observed following *in vitro* infection of DC with T3SS deficient *B. bronchiseptica* in comparison with wild-type bacteria, this did not correlate with the production of these cytokines following *in vivo* infection. In most cases, cytokine production in the lungs of BALB/c mice were similar or slightly lower following aerosol challenge with T3SS deficient *B. bronchiseptica*, when compared with wild-type bacteria. Consistent with the lack of effects of the T3SS on IL-10 production, this study did not reveal differences in antigen-specific IFNγ production from spleen cells from mice infected with wild-type and T3SS deficient *B. bronchiseptica*. These findings are at variance with previous reports suggesting that the T3SS of *B. bronchiseptica* may promote IL-10 and inhibit IFNγ production following intra-nasal challenge of C57BL/6 mice (Skinner *et al.* 2005; Pilione and Harvill 2006). The difference may reflect differences in the route of infection. Skinner *et al* and Pilione *et al* used intra-nasal infection of mice, whereas the present study employed aerosol challenge. In addition, genetic differences in the strains of mice used have been reported to significantly influence the outcome of bacterial challenge with pathogens such as *Yersinia* (Hancock *et al.* 1986) and *Listeria* (Gervais *et al.* 1984). In particular, BALB/c mice have been shown to produce a more Th2-type response,

characterised by lower IL-12 and IFNγ production, whereas C57BL/6 mice generated a more Th1 polarized response (Liu *et al.* 2000). It has been reported by Yuk *et* al that T3SS deficient *B. bronchiseptica* were completely cleared from the tracheas of C57BL/6 mice 35 days post respiratory challenge, whereas significant numbers of the same bacteria could still be recovered from the tracheas of BALB/c mice 35 days post respiratory challenge (Yuk *et al.* 2000).

The present study also demonstrated that production of IL-1β, MIP1, MIP2, early TNFα and IL-12p40 was significantly enhanced in the lungs of BALB/c mice following aerosol challenge with T3SS deficient when compared with wild-type bacteria. Furthermore, IL-12p40 and IL-23 production by DC *in vitro* was significantly higher following infection with T3SS deficient *B. pertussis*. This correlated with dramatically higher antigen-specific IL-17 production by spleen cells from BALB/c mice infected with T3SS deficient *B. pertussis* when compared with wild-type bacteria. IL-1β and IL-23 have been shown to synergise for the induction of IL-17 producing T-cells, and interestingly TNFα was also shown to synergise with IL-23 to promote IL-17 production (Sutton *et al.* 2006). IL-17 has been shown to have a role in protective immunity to respiratory infection with *Klebsiella pneumoniae* (Ye *et al.* 2001) and *B. pertussis* by recruiting nuetrophils and activating macrophages (Higgins *et al*; Journal of immunology: in press).

Studies with other pathogens have revealed that the T3SS may inhibit cytokines and chemokines involved in PMN recruitment. The T3SS of *Yersinia* (Schulte *et al.* 1996; Boland and Cornelis 1998; Palmer *et al.* 1998), *Salmonella* (Haraga and Miller 2003) and *Shigella* (Okuda *et al.* 2005) prevents the activation of NF-κB in epithelial cells, which inhibits release of IL-8 (MIP2) and MIP1, chemokines involved in the recruitment of neutrophils and macrophages. The T3SS of *B. bronchiseptica* has also been shown to prevent NF-κB activation in epithelial cells, through a mechanism that interferes with nuclear translocation (Yuk *et al.* 2000). The T3SS of *Shigella* spp was recently shown to be directly responsible for the inhibition of PMN recruitment to the lungs following *Shigella* infection, through the inhibition of MIP2, IL-1β, RANTES and GM-CSF production (Okuda *et al.* 2005). Recently, Th17 cells have been implicated in inflammatory responses that mediate autoimmunity and also function in the rapid

recruitment of neutrophils to the site of infection, where they may help contain the pathogen, until a subsequent clearing IFNγ-producing Th1 response can be generated (McKenzie *et al.* 2006). This more rapid and potent inflammatory response could be required in the tissues of vital organs, such as the lung, and recently it has recently been demonstrated that Th17 cells play an important role in vaccine induced protection against respiratory infection with *B. pertussis* (Higgins *et al.* J immunol. In press). To our knowledge this is the first study to correlate the T3SS-mediated inhibition of IL-1β and TNFα at the site of infection with the inhibition of the subsequent antigen-specific IL-17 response. Therefore, T3SS mediated inhibition of IL-1β, IL-23 and IL-17 production, and consequential inhibition of MIP-1 and MIP-2 could be a mechanism whereby pathogens such as *B. pertussis* and *Shigella* spp., delay early local inflammation and PMN recruitment to the site of infection, facilitating efficient initial bacterial colonisation or tissue invasion.

Interestingly, it has recently been reported that PT plays a role in the prevention of neutrophil recruitment to the lung in the early stages of *B. pertussis* infection (Kirimanjeswara *et al.* 2005), thereby allowing *B. pertussis* to avoid neutrophil mediated clearance of antibody opsonized bacteria. The present study has shown that, like PT, the T3SS of *B. pertussis* may also have a role in inhibiting PMN influx to the lungs of BALB/c mice. Infection of mice with T3SS deficient *B. pertussis* also induced significantly higher *B. pertussis* specific serum IgG and IgG2a responses than the wild-type strain when examined 28 days post aerosol challenge. In contrast, only minor differences in antigen-specific serum IgG titres were observed following infection of BALB/c mice with wild-type and T3SS deficient *B. bronchiseptica* 21 days post aerosol challenge, and the titres were similar 28 days post challenge. In addition, the most striking evidence of T3SS-mediated inhibition of antigen-specific IFNγ and IL-17 production in the present study was observed in response to purified heat-inactivated PT antigen. Therefore, it is possible that the T3SS-mediated suppression of serum antibody responses and of pro-inflammatory responses to *B. pertussis* infection in the lung is dependent on the expression of PT; PT may synergise with the T3SS to suppress protective immune responses. Since *B. bronchiseptica* does not produce PT, this may also partly explain why these effects were not observed with *B. bronchiseptica.*

PT is also known to promote induction of IFNγ through its enhancement of IL-12 production (He *et al.* 2000) and, as an elevator of intracellular cAMP, has been implicated in the enhancement of IL-23, which triggers rapid IL-17 production from tissue-resident T-cells (McKenzie *et al.* 2006). Therefore, since IL-1β is also crucial for promoting IL-17 production (Sutton *et al.* 2006), T3SS-dependent inhibition of IL-1β, TNFα and IL-12p40 in the lung early in infection may be a mechanism that prevents *B. pertussis* from inducing a rapid pro-inflammatory Th17 response, and inducing IFNγ later in infection.

Despite the description of whooping cough as a vaccine-preventable disease and the implementation of large-scale vaccination programmes, *B. pertussis* remains a significant cause of disease in both adults and infants worldwide. The ultimate long-term goal of the prevention of any disease by vaccination should be the elimination of the circulating pathogen within its host reservoir. However, reports have emerged suggesting an increase in the number of cases of pertussis in developed countries, even in the presence of high levels of vaccine coverage (Weber *et al.* 2001; Das 2002; Crowcroft *et al.* 2003; Mitka 2006). This increase in *B. pertussis* infection has largely been attributed to waning vaccine induced immunity, and has called into question the level of protection provided by current pertussis vaccines. It is possible that waning immunity in previously vaccinated adults and adolescents may provide the bacterium with a persistent reservoir from which *B. pertussis* might be transmitted to young infants prior to vaccination, and thus may contribute to a resurgence of whooping cough in infants. This concern about resurgence of whooping cough has prompted recent suggestions that adults and adolescents should receive regular booster doses of pertussis vaccines (Yeh and Mink 2006) (Heininger and Cherry 2006) and that new combinations of vaccine components and adjuvants should be tested for greater efficacy (Mattoo and Cherry 2005).

The identification of new surface located *B. pertussis* proteins is thus an important step in the development of more protective acellular pertussis vaccines. Perhaps the most significant finding of this study is the demonstration that the T3SS of numerous clinical isolates of *B. pertussis* are functionally active and secrete significant quantities of the *Bordetella* T3SS substrate Bsp22. It has been suggested that this protein may be a good candidate for inclusion in future acellular vaccine preparations as it is unique to

Bordetella spp, highly conserved amongst the sequenced strains, and has been shown to be antigenic in rats (Yuk *et al.* 1998), mice (Yuk *et al.* 2000) and rabbits (this study). The use of T3SS proteins as antigens for vaccine development is attractive due to their necessity for virulence, and their surface location, which makes them accessible to circulating antibodies and ideal for opsonization. Recently, immunisation with the YscF injectisome protein of *Yersinia pestis* was shown to provide significant protection against plague infection in mice (Matson *et al.* 2005). Though exported by the T3SS, Bsp22 specifically associates with the T3SS injectisome (Mattoo and Cherry 2005), and has previously been shown to be essential for tracheal colonisation of mice following intra-nasal respiratory challenge with *B. bronchiseptica* (Yuk *et al.* 2000). Thus antibodies targeted against Bsp22 may be useful for opsonization and therefore prevention of *Bordetella* infection.

Chapter 8

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