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**Evolution of the OmpR regulon in *Salmonella enterica* serovar
Typhimurium and *Escherichia coli* in response to low pH**

by

Heather J. Quinn

A dissertation presented for the degree of Doctor of Philosophy, in the Faculty of
Science, University of Dublin, Trinity College

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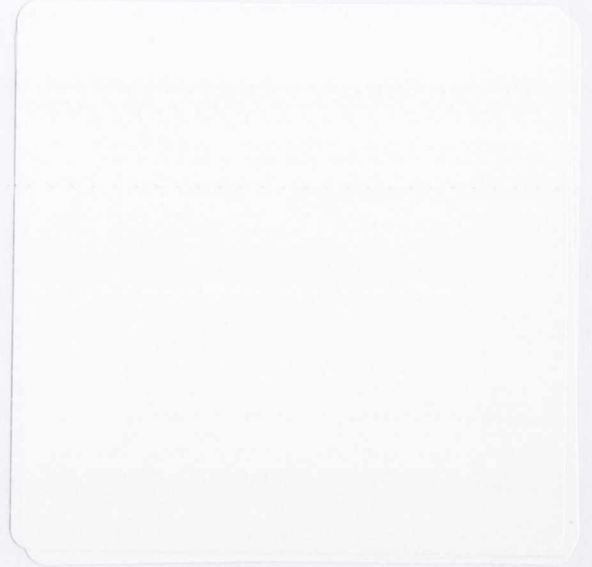


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Summary

Two-component systems (TCS) enable bacteria to sense, respond and adapt to a wide range of environmental stimuli. The OmpR/EnvZ TCS is composed of the inner membrane sensor kinase EnvZ and the response regulator OmpR. In *Escherichia coli* (*E. coli*) EnvZ is stimulated by changes in osmotic pressure; EnvZ phosphorylates the transcription factor OmpR, which differentially expresses the outer membrane porins *ompF* and *ompC*. Comparatively, the orthologous OmpR/EnvZ TCS in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) does not respond to osmolarity. Instead, studies have shown the *ompR* gene is activated by low pH and is essential for the expression of pathogenicity genes. These findings raised the question does *ompR* in *E. coli* respond to low pH?

In this study, comparative analysis of *ompR* expression in both *S. Typhimurium* and *E. coli* was performed. Results revealed *ompR* gene expression and OmpR protein levels were upregulated in *S. Typhimurium* in acidic pH. In *E. coli*, *ompR* expression and OmpR protein levels were insensitive to these changes; thus, *ompR* gene is differentially expressed in these closely-related species. Bioinformatic analysis revealed differences between the *ompR/envZ* operon in both organisms. Genetic rewiring experiments identified the *ompR* regulatory region was responsible for differential expression of *ompR* in each organism.

An investigation was carried out to identify what factor(s) stimulate *ompR* expression at low pH in *S. Typhimurium*. Previous studies have shown the global regulator H-NS represses *ompR* expression. Furthermore, *ompR* is positively autoregulated; OmpR binds to four sites in the *ompR* promoter. This study has identified the OmpR-II site is critical for *ompR* expression and H-NS antagonism at low pH. Previous work has shown the *ompR* gene in *E. coli* is regulated by the integration host factor (IHF) and the c-AMP receptor protein (CRP). This study investigated if these proteins regulate expression of *ompR* in *S. Typhimurium*. Results revealed the IHF protein plays a role in fine-tuning *ompR* expression. CRP acts as a dual regulator of *ompR* mediating both positive and negative effect on expression at pH 7 and pH 4.5 respectively.

This study mapped the genome-wide distribution of OmpR in both *S. Typhimurium* and *E. coli* and investigated the influence of low pH on OmpR binding in both organisms. OmpR binding was mapped using chromatin immunoprecipitation and microarray analysis (ChIP-on-chip). This study reports the genomic targets of OmpR at pH 7 and pH 4.5 in both organisms. In *E. coli*, the number of OmpR bound targets decreased at low pH; in contrast, the number of OmpR targets increased in *S. Typhimurium*. There was limited overlap between the OmpR regulons in both species and many of the genes targeted in *S. Typhimurium* have no orthologues present in *E. coli*, indicating the OmpR regulon has evolved since the divergence of *E. coli* and *S. Typhimurium*. Genes targeted by OmpR in *S. Typhimurium* included those involved in virulence present in the *Salmonella* pathogenicity islands. OmpR was bound at novel gene targets and increased OmpR enrichment at these targets highlights the importance of pH as an environmental signal for OmpR binding.

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For my parents, Philip and Judy Quinn

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List of Abbreviations

ATP	adenosine triphosphate
ADP	adenosine diphosphate
ASP	acid shock protein
ATR	acid tolerance response
BLAST	basic local alignment search tool
Bp	base pair
BSA	bovine albumin serum
CA	catalytic and ATP binding domain
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
DBD	DNA binding domain
DHp	dimerisation and histidine phosphotransfer
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GTP	guanosine triphosphate
HK	histidine kinase
HRP	horse radish peroxidase
kDa	kilodalton
LPS	lipopolysaccharide
M cells	microfold cells
mRNA	messenger RNA
NCBI	national center for biotechnology information
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear
qPCR	quantitative PCR

RACE	rapid amplification of cDNA ends
RD	receiver domain
RR	response regulator
RNA	ribonucleic acid
RNAP	RNA polymerase
Rpm	revolutions per minute
SCV	<i>Salmonella</i> -containing vacuole
SDS	sodium dodecyl sulphate
SPI-1	<i>Salmonella</i> pathogenicity island 1
SPI-2	<i>Salmonella</i> pathogenicity island 2
T1SS	type one secretion system
T3SS	type three secretion system
TCS	two-component system
TSS	transcriptional start site
UV	ultraviolet
V	volts
WT	wild type

Chapter 1 General introduction

1.1 The genus *Salmonella*

Salmonella is a Gram-negative rod-shaped bacterium belonging to the family *Enterobacteriaceae* in the Gamma-proteobacteria subdivision (Ellermeier and Schlauch, 2006). Two species are recognised: *Salmonella enterica* and *Salmonella bongori* (Reeves *et al.*, 1989). The species *S. enterica* is further divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* (Ellermeier and Schlauch, 2006). Subspecies V is now recognised as the separate species *S. bongori*. *Salmonella* subspecies are further divided into serovars based on serological classification of the surface structures, *O*-polysaccharide (*O* antigen) and flagellin protein (*H* antigen). Currently over 2,500 serotypes have been described (Popoff *et al.*, 2004).

1.2 Habitat, disease and treatment

The *Salmonella* species are facultative intracellular pathogens, primarily associated with the intestinal tracts of animal hosts. Serovars that are associated with disease in warm-blooded animals typically belong to *Salmonella enterica* subsp. *enterica* (Uzzau *et al.*, 2000). *Salmonella bongori* are commensals of cold-blooded animals and rarely infect humans (Giammanco *et al.*, 2002). *Salmonella* serovars that primarily infect one host but can cause disease in others are called ‘host-adapted’, those that are associated with a single host are called ‘host-restricted’, and those that have a broad host range are referred to as ‘generalists’ (Uzzau *et al.*, 2000; Sabbagh *et al.*, 2010) (see Table 1.1).

Infection with *Salmonella*, called salmonellosis, can manifest as typhoid fever or gastroenteritis. Typhoid fever is a severe systemic infection caused by *Salmonella enterica* serovar Typhi; serovar Paratyphi causes a clinically indistinguishable condition called paratyphoid (Maskey *et al.*, 2006). *S. Typhi* is restricted to humans; consequently a suitable animal model for infection studies is lacking. *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S. Typhimurium*) is a non-typhoidal strain in humans; however, it causes a systemic and typhoid-like disease in mice. For this reason, *S. Typhimurium* has been the serovar of choice for genetic studies aiming to understand typhoid fever pathogenesis (Haraga *et al.*, 2008). Each year an estimated 22 million cases of typhoid fever occur world-wide with an approximate 10% mortality rate if left untreated (Crump *et al.*, 2004). Infection with *Salmonella* can also manifest as

Table 1.1. Host range of *Salmonella enterica* subsp. *enterica* serovars

Classification	Serovar	Natural host	Rare host
Host restricted	Typhi	Humans	None
	Paratyphi A, C	Humans	None
	Sendai	Humans	None
	Abortusovis	Ovines	None
	Typhisuis	Poultry	None
	Abortusqui	Equines	None
Host adapted	Choleraesuis	Swine	Humans
	Dublin	Bovines	Humans and ovines
Generalist	Typhimurium	Humans, poultry, swine, bovines and rodents	None
	Enteritidis	Humans, poultry and rodents	Swine and bovines

(adapted from Uzzau *et al.*, 2000; Ellermeier and Schlauch, 2006)

gastroenteritis; a self-limiting infection primarily caused by non-typhoidal strains *S. Typhimurium* and *Salmonella enterica* serovar Enteritidis. Annually, there is an estimated 1.3 billion cases world-wide of non-typhoidal salmonellosis of which 3 million are fatal (Pang *et al.*, 1995).

Antibiotics are the standard treatment for cases of typhoid, paratyphoid and gastroenteritis. Treatment with chloramphenicol began in 1948, however by 1972 chloramphenicol resistance had been reported (Woodward *et al.*, 1948; Mirza *et al.*, 1996). Treatment with fluoroquinolones such as ciprofloxacin then came into use, however, by 1993 fluoroquinolone resistant strains had also emerged (Parry and Threlfall, 2008). With multi-drug resistance on the rise, efforts have focussed on prevention by vaccinology. Currently two vaccines against typhoid have been licensed, the live-attenuated vaccine Ty21a and the Vi antigen subunit vaccine (Marathe *et al.*, 2012). The Ty21a vaccine is a chemically mutagenised form of the *Salmonella* Typhi strain Ty2 (Germanier and Furer, 1983). This vaccine has drawbacks as it requires a multiple dosing regime, it has a strict cold chain requirement for distribution and is not effective in children younger than 2 years old or immune-compromised individuals (Guzman *et al.*, 2006). The Vi antigen vaccine is composed of purified capsular polysaccharide Vi antigen. It is administered in a single dose by an intramuscular injection (Hindle *et al.*, 2002). This vaccine is safe, inexpensive and relatively effective. However, this vaccine also has its drawbacks; it is ineffective in children under 2 years old and generates antibodies against the Vi antigen only. Improved protection requires vaccines that generate antibody responses against additional antigens such as lipopolysaccharide (LPS) and flagellin (reviewed in Bishop *et al.*, 2011).

New vaccines that are cost-effective, immunogenic in children and offer cross-protection against Paratyphi strains are required (Bishop *et al.*, 2011). Current candidates include live-attenuated *S. Typhi*, these strains carry multiple mutations such as mutations in the *aro* genes involved in synthesis of aromatic compounds (Bishop *et al.*, 2011). Candidate strains with additional mutations in virulence regulators such as *ompR*, *phoP* (Dorman *et al.*, 1989; Hohmann *et al.*, 1996) or the virulence gene *ssaV* (Hindle *et al.*, 2002) have been constructed and some are currently in clinical trials. However, there is still a need for a licenced vaccine against typhoid fever that is

effective in children under 2 years old which provides humoral and cellular immunity (Marathe *et al.*, 2012).

1.3 The biology of infection

1.3.1 Overview

Typically, infection with *Salmonella* occurs due to ingestion of contaminated food or water. Inside the human host *Salmonella* is subjected to multiple environmental changes such as temperature, fluctuations in pH and oxygen levels (Rychlik and Barrow, 2005). In the small intestine *Salmonella* must survive exposure to bile, cationic antimicrobial peptides and secretory IgA to cross the upper mucosal layer and reach the underlying epithelium (Haraga *et al.*, 2008). Here, *Salmonella* can adhere to the apical cell surface using fimbrial and non-fimbrial adhesins (Baumler *et al.*, 1996b; Gerlach *et al.*, 2007b) and enter host cells by bacterial-mediated invasion or by phagocytosis. *Salmonella* preferentially target microfold (M) cells of the follicle-associated epithelium in the Peyer's patches (see Fig. 1.1) (Sansone, 2004). M cells are specialised epithelial cells which sample intestinal antigens by pinocytosis and deliver them to lymphoid cells in the underlying Peyer's patches (Haraga *et al.*, 2008).

Invasion involves denuding of the microvilli and ruffling of the cell surface (Takeuchi, 1967); this is mediated by a type 3 secretion system (T3SS) which drives bacterial-mediated endocytosis (Francis *et al.*, 1992; Finlay and Brummel, 2000). This T3SS is encoded within *Salmonella* pathogenicity island 1 (SPI-1) and secretes effector proteins into host cells that interfere with host cell signalling and drive internalisation of the bacterium. Dendritic cells may also assist *Salmonella* invasion; these cells can internalise bacteria from the lamina propria by sending pseudopods between adjacent epithelial cells to open tight junctions and directly sample bacteria (Jepson *et al.*, 1995).

After uptake through M cells, non-typhoidal *Salmonella* can interact and enter epithelial cells through the basolateral surfaces. Here, secretion of effector proteins into the host

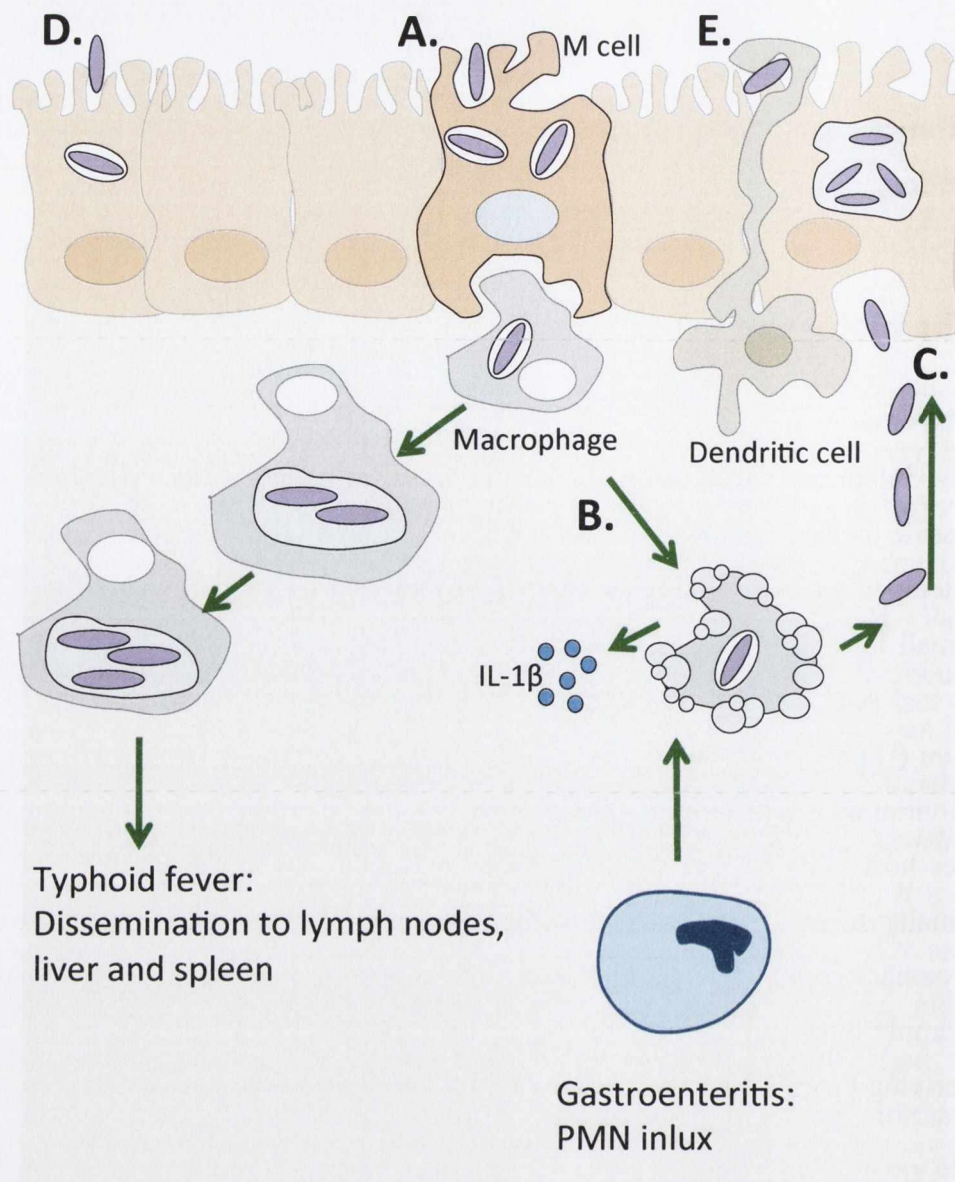


Fig. 1.1. Strategies employed by *Salmonella* to cross the intestinal epithelium barrier. **A.** *Salmonella* (purple ovals) cross M (microfold) cells of epithelium in the Peyer's patches. *Salmonella* are then phagocytosed by submucosal macrophage (grey) where it can survive and replicate inside a *Salmonella*-containing vacuole within host macrophage. **B.** *Salmonella* can also induce macrophage apoptosis; the SPI-1 encoded bacterial effector SipB is secreted into host macrophage and binds caspase-1 which converts interleukin (IL)-1 β to its active form, this adds to inflammation and PMN influx (blue). **C.** *Salmonella* is also capable of entering epithelial cells, adjacent to infected M cells, by their basolateral surfaces. **D.** Epithelial cells can be invaded directly; using the SPI-1 encoded T3SS which induces bacterial-mediated endocytosis. **E.** *Salmonella* may also be taken up directly by dendritic cells that open tight junctions between epithelial cells to directly sample bacteria in the lumen (adapted from Sansonetti, 2004; Haraga *et al.*, 2008).

cytosol causes release of cytokines which induces an early inflammatory response and attracts polymorphonuclear leukocytes (PMNs) into the intestinal lumen (Fig. 1.1) (McCormick *et al.*, 1995). Stimulation of PMN transmigration initiates an inflammatory response, eventually leading to diarrhoea and the symptoms of gastroenteritis (Galan, 2001).

Once across the epithelium barrier, typhoidal strains of *Salmonella* are phagocytosed by submucosal macrophages (Alpuche-Aranda *et al.*, 1994). Following uptake, *Salmonella* reside in a large vacuole; this can be remodelled using a second T3SS to create a distinct intracellular compartment known as the *Salmonella*-containing vacuole (SCV). Expression of effectors, via the T3SS encoded in SPI-2, support survival and replication of *Salmonella*, thereby evading the transition to a lysosome (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Holden, 2002). Subsequently, the bacteria are disseminated throughout the reticuloendothelial system causing systemic infection (Haraga *et al.*, 2008).

Studies *in vitro* have shown *Salmonella* can also invade macrophages using SPI-1 T3SS, instead of phagocytosis (Chen *et al.*, 1996; Monack *et al.*, 1996). Secretion of the effector protein SipB into macrophages induces apoptosis by activating the caspase-1, this protease converts interleukin (IL)-1 β to its mature active form, leading to cell death and induction of a pro-inflammatory response (Hersh *et al.*, 1999).

1.4 The genetics of infection

1.4.1 Type 3 secretion systems (T3SSs)

T3SSs are complex, highly specialised secretion systems that are evolutionarily related to bacterial flagella (Paul *et al.*, 2008). These needle-like structures span both the inner and outer membrane of the bacterial envelope and establish contact with host cells. Upon contact, bacterial effector proteins are translocated into host tissues where they can interfere with host cell signalling and promote bacterial survival and colonisation (reviewed by Kosarewicz *et al.*, 2012). T3SS systems are widespread amongst Gram-negative pathogenic bacteria and plant pathogens (Kosarewicz *et al.*, 2012). *S. enterica* contain two characterised T3SSs, which are required at different stages during infection (Haraga *et al.*, 2008). These are encoded in the pathogenicity islands SPI-1 and SPI-2;

pathogenicity islands are regions of DNA between 10-200 kb in length and are present in pathogenic bacteria but absent from non-pathogenic bacteria (Hentschel and Hacker, 2001). They are acquired by horizontal gene transfer; this is supported by evidence showing the G+C content of these islands differ from the host chromosome, they are flanked by short direct repeats and are frequently associated with tRNA genes which act as targets for integration events (Hentschel and Hacker, 2001).

1.4.2 SPI-1

SPI-1 is 40 kb in length and is located at 63 minutes on the *S. Typhimurium* chromosome. SPI-1 is important during the invasion of host epithelium cells; Galan and Curtiss (1989) have shown a SPI-1 mutant (originally described as an *inv* mutant) was unable to invade M cells. The same mutant was attenuated for virulence when orally ingested in a mouse model, but was unaffected when delivered intraperitoneally, thus highlighting the importance of SPI-1 for cell mediated invasion (Galan and Curtiss, 1989).

The SPI-1 locus encodes the genes necessary to create a functional T3SS as well as several effectors and regulators chaperones (Haraga *et al.*, 2008). The structural genes are encoded by the *prg/org* and *inv/spa* operons while the genes coding for proteins of the translocon and the effectors are located in the *sic/sip* operon (reviewed by Ellermeier and Slauch, 2007). The structure of the SPI-1 T3SS is composed of several proteins; the filamentous needle is made of PrgI protein and extends ~50 nm from the bacterial cell surface (Marlovits *et al.*, 2004). The membrane bound base is made of four distinct rings, the outer membrane rings are composed of InvG protein while the inner membrane rings are composed of PrgH and PrgK (Marlovits *et al.*, 2004). The proteins SipD, SipB and SipC are transported by the T3SS to form the needle tip and translocon which is embedded into the eukaryotic host cell membrane (Fig. 1.2) (Mueller *et al.*, 2008). The process of recognising effector proteins destined for secretion is not fully understood, however some substrates require a cognate chaperone, which deliver the effectors to the T3SS (Kosarewicz *et al.*, 2012). In addition, a recent study has shown the SpaO/OrgA/OrgB proteins form a complex believed to act as a binding platform for chaperone-effector complexes (Wagner *et al.*, 2010). InvC is an ATPase which dissociates effectors from cognate chaperones and partially unfolds

effectors prior to translocation into host tissues (Fig. 1.2) (Akeda and Galan, 2005). Once inside host cells the effector proteins SopE, SopE2 and SopB activate host Rho GTPases: Cdc42, Rac1 and RhoG. This induces host actin cytoskeletal rearrangements, membrane ruffling and bacterial uptake (Hardt *et al.*, 1998a; Bakshi *et al.*, 2000; Stender *et al.*, 2000; Patel and Galan, 2006). This promotes the production of pro-inflammatory cytokines and stimulates PMN transmigration leading to the symptoms of gastroenteritis (Haraga *et al.*, 2008).

SPI-1 encodes regulators that control the expression of genes encoded both inside and outside of the SPI-1 locus. HilA is a member of the OmpR/ToxR protein family and is the major activator of genes within SPI-1. Deletion of *hilA* results in a phenotype similar to deleting the entire SPI-1 locus (Ellermeier *et al.*, 2005). HilA directly activates the expression of genes in the *prg/org* and *inv/spa* operons and transcriptional read through indirectly activates the *sic/sip* operon (Ellermeier and Slauch, 2007). InvF is an AraC-like protein; InvF acts alongside the chaperone SicA to activate the expression of effectors within the *sic/sip* operon and located elsewhere on the chromosome (Darwin and Miller, 1999; Darwin and Miller, 2001). HilC and HilD are also AraC-like proteins; these proteins regulate the expression of their own genes and each other. Importantly, both proteins activate the expression of *hilA* (Schechter and Lee, 2001; Ellermeier *et al.*, 2005) and HilA negatively autoregulates its own expression by an unknown mechanism (De Keersmaecker *et al.*, 2005).

SPI-1 genes are subject to complex control by multiple regulators encoded outside of SPI-1, some of which are mentioned here. RtsA acts positively alongside HilC and HilD to activate HilA expression. The *hilE* gene encoding Hile is unique to *Salmonella* and is located on part on the chromosome that resembles a pathogenicity island; Hile interacts with HilD to negatively affect *hilA* expression (Baxter *et al.*, 2003). The global regulator H-NS (section 1.7.4) represses *hilA* expression (Schechter *et al.*, 2003). The transcriptional regulators OmpR (section 1.6.5) and Fis (section 1.7.5) also regulate SPI-1 expression. Specifically, OmpR activates *hilC* and represses *hilD* expression (Cameron and Dorman, 2012). Fis has been shown to have a positive affect on *hilA*, *invF*, *hilC* and *hilD* expression (Wilson *et al.*, 2001; Cameron and Dorman, 2012).

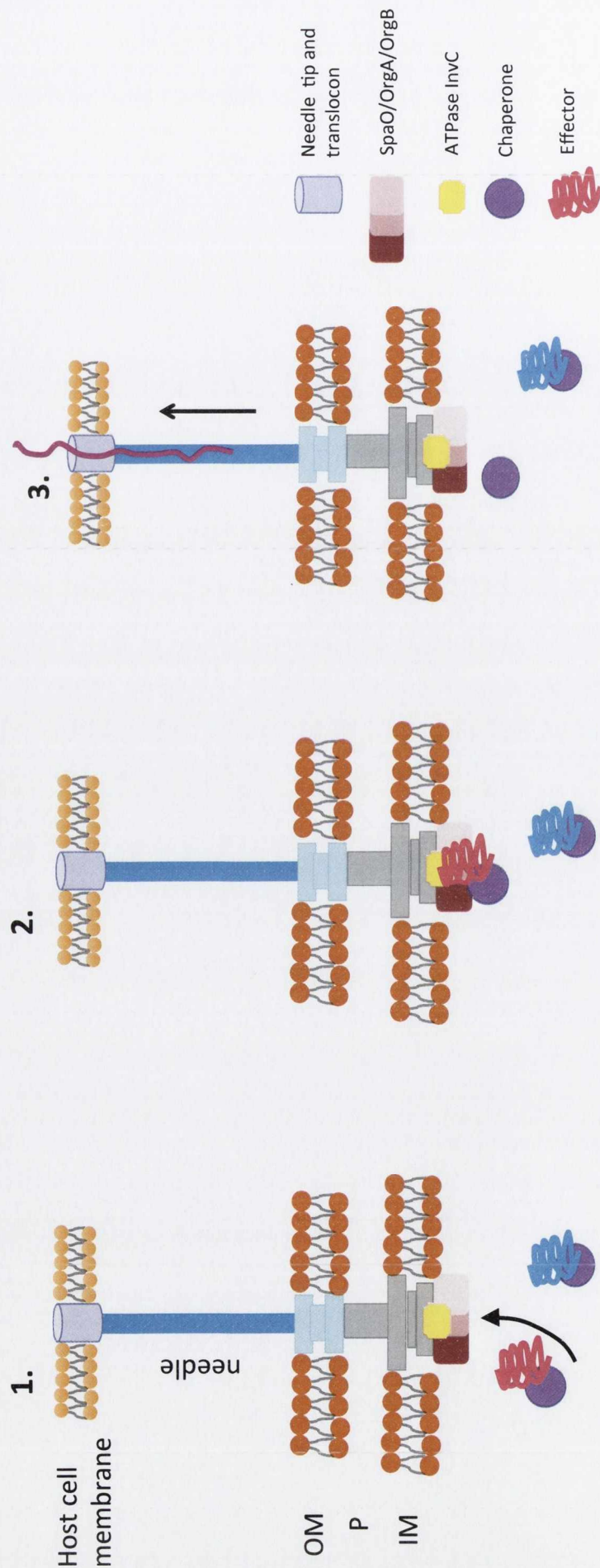


Fig. 1.2. Secretion of bacterial effectors by the SPI-1 type 3 secretion system (T3SS) into host cells. 1. The effector proteins are delivered to secretion apparatus by their cognate chaperones and assisted by the SpaO/OrgA/OrgB sorting platform. 2. The effectors are recognised by the ATPase (InvC), ATP hydrolysis drives chaperone release and partially unfolds the effector. 3. The unfolded effector is then translocated through the base and needle filament and secreted into the host cytoplasm. OM, outer membrane; P, periplasm and IM, inner membrane. Legend is displayed on right (adapted from Kosarewicz *et al.*, 2012).

1.4.3 SPI-2

Salmonella can proliferate inside epithelial cells and non-activated macrophages, but primarily replicate in the latter. After internalisation *Salmonella* resides within a membrane-bound compartment called the SCV (*Salmonella*-containing vacuole) (Unsworth and Holden, 2000). The SCV accommodates bacterial replication and survival, enabling *Salmonella* to establish systemic disease (Unsworth and Holden, 2000). Inside the SCV, *Salmonella* actively manipulate the host cell to maintain the vacuolar compartment and avoid host anti-microbial activities (Haraga *et al.*, 2008). This compartment acidifies between pH 4 and pH 5 within 1 hour after formation (Rathman *et al.*, 1996) and contains magnesium and calcium in the millimolar range. Furthermore, this compartment contains antimicrobial peptides, reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) which are capable of damaging bacterial macromolecules (Alpuche Aranda *et al.*, 1992; Haraga *et al.*, 2008).

Adaptation to an intracellular life and the establishment of systemic disease requires the SPI-2 encoded T3SS; SPI-2 mutants do not persist after infection (Linehan *et al.*, 2005; Jones *et al.*, 2007). SPI-2 is a 40 kb island located at 31 minutes on the *S. Typhimurium* chromosome next to the tRNA gene *valV* (Shea *et al.*, 1996). It is composed of two structural elements 23.5 kb and 14.5 kb in size; the 14.5 kb element is present in *S. enterica* and the phylogenetically older species *S. bongori* and contains genes encoding a tetrathionate reductase (Hensel *et al.*, 1999). Mutations in these genes does not attenuate virulence in mice (Hensel *et al.*, 1999). The 23.5 kb element contains genes essential for virulence and systemic infection but is not present in *S. bongori* serotypes (Hensel *et al.*, 1999). This element encodes structural, regulatory, effectors and chaperone elements for a T3SS (Fig.1.3). As with SPI-1, there are several SPI-2 secreted effectors located outside the SPI-2 locus (Heffron *et al.*, 2011).

The 23.5 kb region encodes a two-component system (SsrA/B). An unidentified stimulus prompts the sensor kinase, SsrA, to phosphorylate its cognate response regulator SsrB which activates expression of SPI-2 (Fass and Groisman, 2009). *ssrA* and *ssrB* are secretion system regulators (*ssr*) and are divergently transcribed from the remaining genes within SPI-2. These genes were originally named based on their

SPI-2

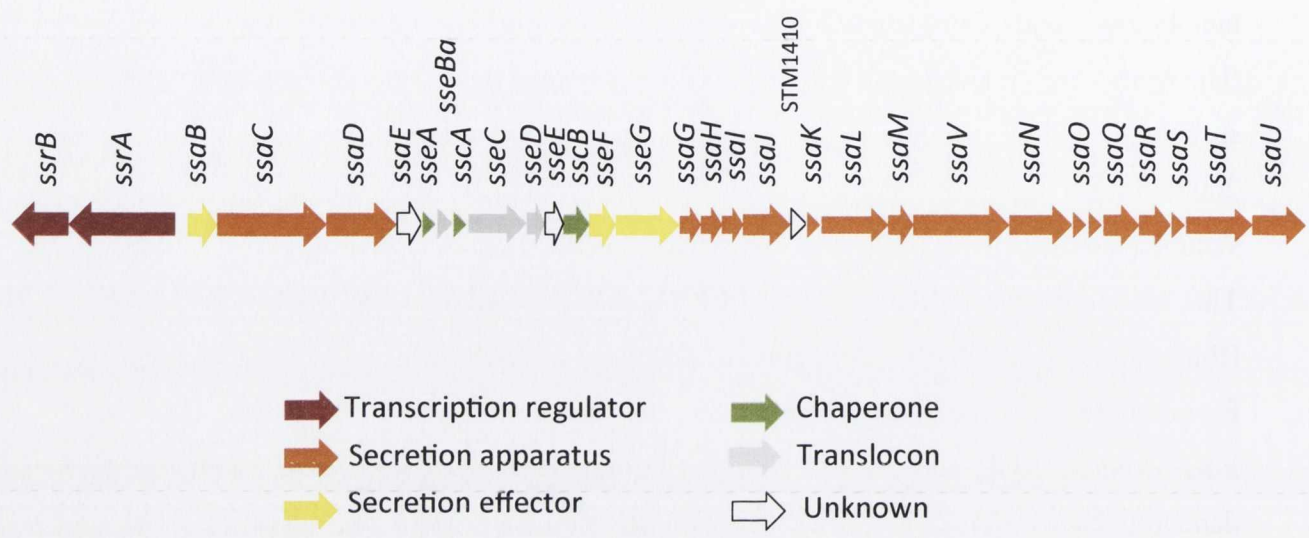


Fig. 1.3. Structural organisation of the 23.5 kb region of SPI-2. Genes are coloured based on functional class according to the key (adapted from Tomljenovic-Berube *et al.*, 2010).

function within SPI-2: the secretion system apparatus (*ssa*) genes, secretion system effectors (*sse*) and secretion system chaperones (*ssc*). In retrospect some genes have been incorrectly named (Hansen-Wester and Hensel, 2001).

SsaL, SsaM and SpiC proteins are required for secretion of the proteins that form the translocon tip of the needle complex (Yu *et al.*, 2004; Yu *et al.*, 2010). The translocon is composed of the proteins SseB, SseC, SseD. SsaV is an essential component of the secretion system located in the inner membrane. A recent study has shown acidic pH is required for secretion of proteins belonging to the translocon; a shift to higher pH then stimulates the secretion of effectors (Yu *et al.*, 2010). This study proposed the needle filament of the secretion system may act as a pH sensor that co-ordinates the timely expression of effectors into the host cell, however this remains to be tested (Yu *et al.*, 2010).

Secretion of effectors into host cell cytoplasm interferes with host cell processes such as intracellular transport and both actin cytoskeletal and microtubular arrangements (Heffron *et al.*, 2011). The SifA effector was the first SPI-2 effector identified. SifA plays an important role in the formation of *Salmonella*-induced-filaments (Sifs) (Stein *et al.*, 1996). These filamentous structures spread from the SCV throughout the host cell. Their formation requires host microtubules to facilitate growth of the SCV to accommodate bacterial replication inside the vacuole (Haraga *et al.*, 2008). SifA was found to bind the host cell protein called SKIP (SifA and kinesin interacting protein) that attaches the SCV to microtubules (Boucrot *et al.*, 2005). The effectors SseF and SseG have been shown to interact and direct the SCV to the Golgi apparatus enabling *Salmonella* to acquire nutrients for growth and evade host-defence mechanisms (Abrahams *et al.*, 2006; Ramsden *et al.*, 2007; Haraga *et al.*, 2008).

Regulation of SPI-2 expression is dependent on the SsrA/B two-component system; SsrB binds and regulates expression of all SPI-2 operons (Deiwick *et al.*, 1999; Walthers *et al.*, 2007; Tomljenovic-Berube *et al.*, 2010). In addition to the SPI-encoded regulators several regulators located outside SPI-2 have been shown to control SPI-2 expression. The OmpR/EnvZ two-component system has been shown to regulate expression of *ssrA/B* expression in conditions of low pH, DNA relaxation and in host macrophages (Lee *et al.*, 2000; Cameron and Dorman, 2012). H-NS and YdgT have

been shown to negatively affect the expression of SPI-2; YdgT and H-NS share similar N-terminal oligomerisation domains and can form heterodimers, which are thought to repress SPI-2 expression (Coombes *et al.*, 2005b; Walthers *et al.*, 2007). The SsrB protein relieves H-NS-mediated repression at SPI-2 genes; single molecule experiments have demonstrated the SsrB protein displaces H-NS that is polymerised along DNA (Walthers *et al.*, 2011). The ancestral regulator SlyA positively affects SPI-2 expression and is required for survival inside macrophages (Linehan *et al.*, 2005). It is thought SlyA antagonises H-NS mediated repression at SPI-2 in a similar manner observed at the promoters of horizontally acquired genes *ugtL* and *pagC* located outside SPI-2 (Perez *et al.*, 2008; Fass and Groisman, 2009). Studies have also demonstrated the Fis protein positively affects SPI-2 expression (Kelly *et al.*, 2004; Cameron and Dorman, 2012).

1.4.4 SPI-3

SPI-3 is a 17 kb insertion at the *selC* tRNA locus located at 82 minutes on the *S. Typhimurium* chromosome (Blanc-Potard *et al.*, 1999). SPI-3 has a mosaic structure, harbouring multiple regions with unrelated roles in pathogenicity. The best characterised operon in SPI-3 is the *mgtCBR* operon. This encodes the virulence protein MgtC that is required for intramacrophage survival, virulence in mice and growth in low Mg^{2+} concentrations (Blanc-Potard and Groisman, 1997). MgtB encodes an Mg^{2+} transporter and MgtR encodes a regulatory peptide; neither of these proteins are required for virulence in mice or macrophage survival (Blanc-Potard and Groisman, 1997; Alix and Blanc-Potard, 2008). Expression of these genes is induced in conditions of low Mg^{2+} (Garcia Vescovi *et al.*, 1996), low pH (Prost *et al.*, 2007) and in the presence of antimicrobial peptides (Bader *et al.*, 2005). Transcription requires the PhoP/Q two-component system (Soncini *et al.*, 1996). Recent studies have shown this operon encodes a long leader sequence that encodes two short open-reading frames (ORFs) *mgtM* and *mgtP*. These ORFs regulate expression by forming alternative stem-loop structures in response to high intracellular ATP and low levels of proline, respectively (Lee and Groisman, 2012a; Lee and Groisman, 2012b). Other SPI-3 encoded proteins include the putative autotransporter MisL that shares similarity to VirG in *Shigella flexneri* and MarT that shares similarity with the ToxR protein in *Vibrio cholerae* (Blanc-Potard *et al.*, 1999). A study has shown MisL binds to

fibronectin and may play a role during intestinal colonisation of mice (Dorsey *et al.*, 2005).

1.4.5 SPI-4

SPI-4 is a 27 kb region located at 92 minutes on the *S. Typhimurium* chromosome; this region harbours the *siiABCDEF* operon, which encodes a type 1 secretion system (T1SS) (Wong *et al.*, 1998; Gerlach *et al.*, 2007b). The proteins SiiC, SiiD and SiiF make up the T1SS and function as the outer membrane secretin, periplasmic adaptor protein and cytoplasmic ATPase respectively (Gerlach *et al.*, 2007b; Gerlach *et al.*, 2008). The SiiE protein encodes a large (595 kDa) non-fimbrial adhesin that is secreted by this T1SS (Gerlach *et al.*, 2007b). The two remaining genes *siiAB* encode inner membrane proteins, neither of which are secreted or required for SiiE secretion (Gerlach *et al.*, 2007b). The non-fimbrial adhesion SiiE was identified as a factor required for colonisation of cattle and intestinal infection in mice (Morgan *et al.*, 2004; Lawley *et al.*, 2006). SiiE adheres to the apical poles of polarised epithelial cells and establishes close contact with host cells prior to invasion (Wagner *et al.*, 2011).

1.4.6 SPI-5

SPI-5 is a 7 kb insertion at *serT* tRNA gene and is located at 25 minutes on the *S. Typhimurium* chromosome (reviewed by Marcus *et al.*, 2000). This region encodes the SPI-1 effectors SopD and SopB and the SPI-2 secreted effector PipB (reviewed by Marcus *et al.*, 2000). SopD and SopB induce inflammation in the ileal mucosa of calves (Jones *et al.*, 1998). SopB activates Cdc42, RhoG, AktA and chloride secretion through its inositol phosphatase activity and opens tight junctions (Zhou *et al.*, 2001; Boyle *et al.*, 2006; Patel and Galan, 2006). PipB is related to the effector protein PipB2, both proteins are targeted to detergent resistant lipid rafts on the SCV and SIF (Knodler *et al.*, 2003; Knodler and Steele-Mortimer, 2005; Steele-Mortimer, 2008) however PipB is not required for virulence in an acute infection model but PipB2 is required.

To date 17 *Salmonella* pathogenicity islands have been described between *S. Typhimurium* and *S. Typhi* and these are reviewed in by Sabbagh *et al.* (2010). Importantly, not all pathogenicity islands are conserved between both serovars; SPI-7 is the largest SPI identified and is present in *S. Typhi* and absent in *S. Typhimurium*

(Sabbagh *et al.*, 2010). This island is 134 kb in size and contains the genes necessary to synthesise the Vi capsule. The Vi capsule inhibits activation of the complement system and promotes resistance to killing by serum and phagocytosis (Hashimoto *et al.*, 1993; Sabbagh *et al.*, 2010) and is the component to the Vi antigen vaccine. In addition this island harbours the SPI-1 effector SopE in *S. Typhi*; this effector is also present in *S. Typhimurium* strains SL1344 and 14028 where it is located on a prophage (Hardt *et al.*, 1998b; Miold *et al.*, 1999; Pelludat *et al.*, 2003).

1.4.7 Plasmids of *S. enterica*

S. Typhimurium strains carry a large (90 kb) virulence plasmid (pSLT). This plasmid harbours virulence genes such as the *spv* operon that is required for systemic infection. *S. Typhimurium* cured of this plasmid is attenuated for virulence inside mice (Jones *et al.*, 1982) and this phenotype can be complemented with the *spv* operon encoding the SpvB toxin (Jones *et al.*, 1982; Gulig *et al.*, 1992). The pSLT plasmid also harbours the *pef* fimbrial operon. Mutations in *pef* genes reportedly decrease adhesion to murine small intestine (Baumler *et al.*, 1996a). The pSLT plasmid is absent in *S. Typhi* strains, where the IncHI plasmids involved in multi-drug resistance are found (Sabbagh *et al.*, 2010).

1.4.8 Phages of *S. enterica*

Most *Salmonella* strains contain prophages or remnant phages, in *S. enterica* some of these phages carry moron-encoded virulence factors. Different strains of *S. Typhimurium* harbour different phages, for example, *S. Typhimurium* strain LT-2 harbours four prophages: Gifsy-1, Gifsy-2, Fels-1 and Fels-2 (McClelland *et al.*, 2001). *S. Typhimurium* strain SL1344 harbours Gifsy-1 and -2 but does not harbour Fels-1 and -2. SL1344 harbours additional prophages, ST64B and SopE Φ , which are absent from strain LT-2 (Figueroa-Bossi *et al.*, 2001). Several of these prophages encode T3SS effector proteins, for example Gifsy-2 encodes the SPI-2 secreted effector SseI. The prophage SopE Φ encodes the SPI-1 secreted effector protein SopE, which is an activator of host GTPases (as discussed in section 1.4.2) and contributes to host cell invasion. This is supported by a study that found lysogenic conversion of *S. Typhimurium* strain 14028 with SopE Φ enhances the capacity of strain 14028 to invade cultured cells (Zhang *et al.*, 2002).

1.5 The genus *Escherichia*

1.5.1 *Escherichia coli*

Escherichia coli (referred to *E. coli* hereafter) is a Gram-negative, facultative anaerobe that is a member of the *Enterobacteriaceae* family. *E. coli* is the representative species of the genus *Escherichia* that includes the genetically similar *E. fergusonii* species and the less similar *E. albertii* (Walk *et al.*, 2009). *Escherichia* and *Shigella* have historically been considered two separate genera, however comparison of *E. coli* and *Shigella* using DNA hybridisation analysis reveals a high degree of similarity between these organisms (Brenner *et al.*, 1972). For this reason, many bacteriologists believe *Shigella* should be grouped within the genus *Escherichia* (Chaudhuri and Henderson, 2012).

E. coli is a generalist that inhabits various hosts including mammals, birds, reptiles and fish (Escobar-Paramo *et al.*, 2006). In addition, *E. coli* is broadly distributed in the environment where it is found in water, soil and sediment (reviewed by Tenaillon *et al.*, 2010). In the mammalian host, *E. coli* is a member of gut microbiota where it lives as commensal primarily in the mucous layer of the colon. *E. coli* is considered an important human commensal that reduces colonisation by pathogens through the production of bacteriocins and other mechanisms (reviewed by Tenaillon *et al.*, 2010). However, *E. coli* also exists as a pathogen; several highly adapted strains that have acquired virulence traits are capable of causing a broad spectrum of disease. Infection with pathogenic *E. coli* can manifest as diarrhoeal, urinary tract infections and sepsis/meningitis (reviewed by Kaper *et al.*, 2004). *E. coli* pathotypes associated with intestinal infection include: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Pathotypes associated with extraintestinal infection include uropathogenic *E. coli* (UPEC) and meningitis-associated *E. coli* (MNEC). The biology and genetics of *E. coli* infection are beyond the scope of this study but a comprehensive review is available elsewhere (Kaper *et al.*, 2004).

E. coli is a member of the closest known genus to *Salmonella* (McClelland *et al.*, 2001), these organisms are thought to have diverged from a common ancestor 100-150 million

years ago (Doolittle *et al.*, 1996). *E. coli* is one of the best-characterised model organisms, with many important advancements in molecular biology and bacterial physiology conducted in derivatives of the reference strain *E. coli* K-12.

1.6 Two-component systems

1.6.1 Overview

Two-component systems (TCS) are the primary means by which bacteria sense and adapt to changes in their environment. These systems are widespread in bacteria and are also found in archaea, yeast and plants (reviewed by Casino *et al.*, 2010; Capra and Laub, 2012).

The prototypical TCS consists of a membrane-bound histidine kinase sensor and its cognate response regulator (Fig. 1.4, A). The sensor kinase (SK) responds to a stimulus by catalysing autophosphorylation on a conserved histidine residue (reviewed by Casino *et al.*, 2010; Capra and Laub, 2012). The phosphoryl group is then transferred to a conserved aspartate residue on the cognate response regulator (RR). Phosphorylation of the response regulator typically induces a conformational change that activates an output response through protein-protein interactions (e.g. chemotaxis) or protein-DNA interactions that results in a change in gene expression (Mascher *et al.*, 2006). The functional state of the SK and the RR depends on three phosphotransfer reactions: (1) autophosphorylation at a conserved histidine residue in the transmitter domain of the SK (2) phosphotransfer to an aspartate residue in the receiver domain of the RR (3) dephosphorylation of the RR which resets the system. Dephosphorylation can be mediated by the SK, by an additional non-cognate phosphatase or this may be an intrinsic property of the RR (Mascher *et al.*, 2006).

Sensor kinases possess multiple enzymatic activities. In response to a specific stimulus, the sensor kinase binds ATP during a process called autophosphorylation. Autophosphorylation can occur either in *trans* or in *cis*. Autophosphorylation in *trans* is the predominant mode of autophosphorylation and has been documented for EnvZ and CheA (Swanson *et al.*, 1993; Wolfe and Stewart, 1993; Cai and Inouye, 2003; Krell *et al.*, 2010). Autophosphorylation in *trans* means the CA domain of one subunit in a

homodimer phosphorylates the DHp domain of another. Autophosphorylation in *cis* means the CA domain from one subunit phosphorylates the DHp domain of the same subunit (Yang and Inouye, 1991). This has been documented in *Thermotoga maritime* HK853, *Staphylococcus aureus* PhoR protein and *E. coli* ArcB (Casino *et al.*, 2009; Krell *et al.*, 2010; Pena-Sandoval and Georgellis, 2010). Sensor kinases can also catalyse phosphotransfer to their cognate response regulator and dephosphorylation of their response regulator. In doing so, the sensor kinase serves to modulate the output of the response to a specific signal.

Many SKs are located in the cell membrane but some exist as cytosolic proteins. Membrane located SKs possess transmembrane (TM) domains involved in signal perception. The prototypical sensor kinase consists of a periplasmic sensor domain and a cytosolic autokinase domain connected by a linker called the HAMP domain. Typically, SKs function as dimers (Krell *et al.*, 2010) however, deviations from the prototypical structure are quite common. ~17% of SKs are cytosolic proteins (e.g. NtrB) that lack a TM domain. 83% of SKs harbour TM regions with 1-10 TM domains frequently observed and SKs with 20 TM domains have been reported (Krell *et al.*, 2010).

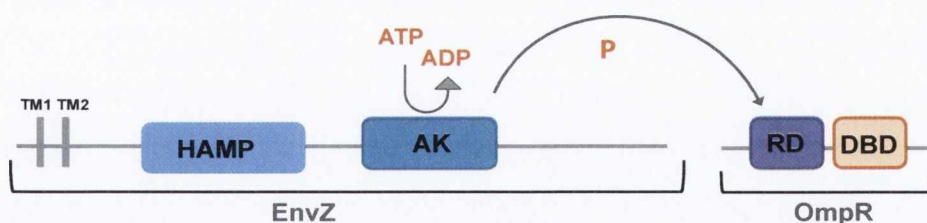
Typically, sensor kinases harbour a transmitter region made of two conserved cytosolic domains; the dimerisation and histidine phosphotransfer (DHp) domain, which harbours the conserved histidine residue involved in autophosphorylation and phosphotransfer. The second domain is the catalytic and ATP-binding domain (CA) (Fig. 1.4, A) (Capra and Laub, 2012). Collectively, these regions make up a region called the autokinase domain. Usually, the DHp domain is made of two helical hairpin structures that form a four-helical bundle; each protomer contains a two helical structure. This region is involved in the transfer of high-energy phosphoryl groups to the conserved histidine residue. Upon interaction with the cognate response regulator the high-energy phosphoryl group is transferred to the conserved aspartate residue of the response regulator (Capra and Laub, 2012). This process is initiated in response to sensing of a specific stimulus.

Within the sensory domain of sensor kinases the most frequently encountered domain is the PAS (PER-ARNT-SIM) domain. PAS domains are named after repeat sequences

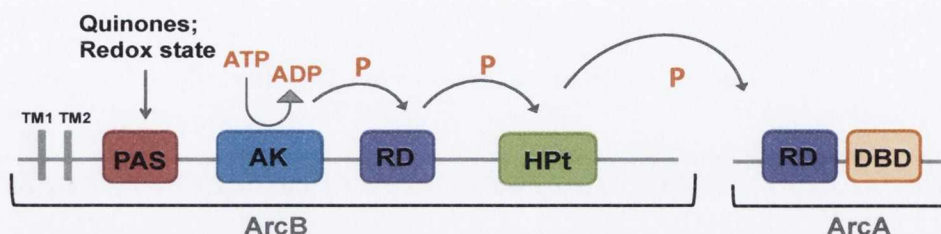
first identified in *Drosophila*; the protein period clock protein (PER), single-minded protein (SIM) and the vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT) (Taylor and Zhulin, 1999). These domains are identified based on their structure that contains an α/β -fold, to date no conserved amino acid sequence has been described (Taylor and Zhulin, 1999). PAS domains are important for sensing stimuli; these domains can function through protein-protein interactions or bind cofactors to regulate protein-protein interactions in response to stimuli. Histidine kinases can also harbour additional domains between their transmembrane and DHp regions e.g. the HAMP domain. This region is important for signal transduction from periplasmic sensory domains to the transmitter region (Parkinson, 2010). HAMP domains (histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis, proteins and some phosphatases) consist of two helices function as an important linker domain for signal transduction from periplasmic input to the cytoplasmic kinase domain. These domains are found in the methyl-accepting chemotaxis proteins (MCP) Tar and Tsr and other response regulators such as EnvZ and NarX. HAMP domains are thought to regulate the phosphorylation or methylation of homodimeric receptors by transmitting the conformational changes in periplasmic ligand-binding domains to cytoplasmic signalling kinase and methyl-acceptor domains (Parkinson, 2010). The chemoreceptors Tar and Tsr mediate attractant responses to amino acids aspartate and serine, respectively. These chemoeffectors bind to the periplasmic sensing domain and induce conformational changes that are transduced to the HAMP domain. The HAMP domain is connected to the output methylation helices which in turn promote binding with histidine kinase CheA (Parkinson, 2010).

Response regulators contain a conserved receiver domain that catalyses the transfer of the phosphoryl group from the sensor kinase. Phosphorylation induces a conformational change on one face of the receiver domain which affects the output of the system (Gao *et al.*, 2007). In most cases, phosphotransfer leads to homodimerisation of the receiver domain and enhances activity of the DNA binding domain. The majority of response regulators contain a DNA binding domain (DBD) enabling the RR to bind DNA and regulate gene expression in response to an input signal (Stock *et al.*, 2000). Other response regulators act as enzymes, such as the CheB methyl-esterase involved in chemotaxis (Simms *et al.*, 1985). In some cases the response regulator is composed of a

A. EnvZ/OmpR



B. ArcB/ArcA



C. KinA/Spo0F/Spo0B/Spo0A

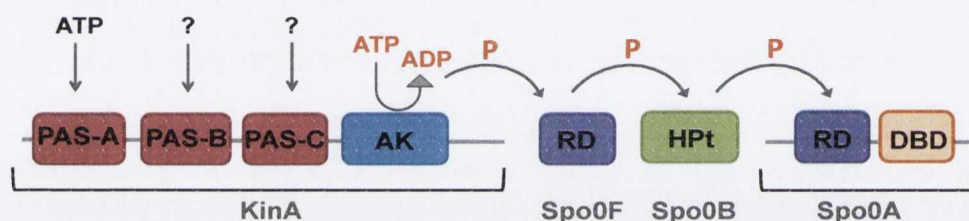


Fig.1.4. Overview of two-component signal transduction. **A.** A prototypical two-component system (TCS). The sensor kinase (SK) EnvZ senses a stimulus. Using ATP the SK autophosphorylates at a conserved histidine residue in the AK (autokinase) domain. This phosphoryl group is then transferred to the RD (receiver domain) of the cognate response regulator (RR) OmpR. This induces conformational changes in output domain (e.g. DNA-binding domain, DBD). The response regulator is responsible for eliciting cellular changes, usually by affecting gene expression. **B+C.** The phosphorelay system of the tripartite kinase. **B.** The ArcB/A TCS. The SK, ArcB, autophosphorylates in the AK domain. This phosphoryl group is transferred first to an intramolecular RR domain followed by transfer to an intramolecular HPt domain. Finally the phosphoryl group is transferred to the RR ArcA. **C.** The KinA/Spo0F/Spo0B/Spo0A TCS. The SK, KinA, autophosphorylates in the AK domain and transfers the phosphoryl group to the intermediate RR Spo0F. The phosphoryl group is subsequently transferred to the HPt Spo0B and finally to the RD domain of the RR Spo0A (Adapted from Krell *et al.*, 2010).

just a receiver domain. For example, the chemotaxis protein CheY associates with the effector protein FlhM of the flagellar motor (Welch *et al.*, 1993). The prevalence of TCS in bacteria is variable; in *E. coli* there are 30 histidine kinases and 32 response regulators. *Mycoplasma genitalium* possess no TCS, however *Myxococcus xanthus* possesses 136 histidine kinases and 127 response regulators (reviewed by Stock *et al.*, 2000; Capra and Laub, 2012). The large number of TCSs in some bacteria raises the question of specificity i.e. does cross-talk with non-cognate response regulators occur? Yamamoto *et al.* (2005) observed cross-talk between response regulators that were phosphorylated by non-cognate sensor kinases but this occurrence was rare. In addition, sensor kinases promoting dephosphorylation by non-cognate response regulator was also observed (Yamamoto *et al.*, 2005). Another study quantified cross-talk using the sensor kinase EnvZ. Using a phosphotransfer profiling technique Skerker *et al.* (2005) found EnvZ phosphorylated 16 non-cognate response regulators after 60 min of transphosphorylation. In contrast, after 10 s of transphosphorylation, EnvZ phosphorylated its cognate response regulator OmpR (Skerker *et al.*, 2005). These results highlight the specificity and insulation amongst TCSs, indicating that cross-talk occurs rarely.

Hybrid kinases constitute a large number of the histidine kinases. These resemble the classic sensor kinase, however they harbour response-regulator-type receiver domains fused to their C-terminus (Fig. 1.4, B) (Cock and Whitworth, 2007). The hybrid kinases (also called tripartite kinases) participate in sequential series of reactions called phosphorelay. The reaction begins with phosphorylation of the conserved histidine residue in the transmitter domain within the SK. This phosphoryl group is either transferred to a cognate response regulator or to the receiver domain fused to the sensor kinase itself. The chemistry of the transfer is conserved and consists of His >Asp >His >Asp sequence but the execution of this is variable (Krell *et al.*, 2010).

The ArcB/A TCS modulates the expression of genes in response to oxygen levels and redox states during growth (Fig. 1.4, B) (Malpica *et al.*, 2004). It is composed of the response regulator ArcA and the sensor kinase ArcB (Iuchi *et al.*, 1990). ArcA contains an N-terminal response regulator domain and a C-terminal DNA binding domain. ArcB is a tripartite sensor kinases that possesses two transmembrane domains and a complex cytoplasmic domain composed of three cytoplasmic domains; a catalytic domain, a

receiver domain and a C-terminal histidine phosphotransfer domain (HPt) (Krell *et al.*, 2010). Anoxic conditions stimulate phosphorelay from His >Asp >His within ArcB and a final transfer to the conserved Asp residue in ArcA (Iuchi *et al.*, 1990; Ishige *et al.*, 1994). Quinones are thought to relate the redox state of the cell to the cytoplasmic domain of ArcB. Oxidised quinones (under oxic conditions) inhibit autophorylation of ArcB by oxidising two Cys thiol residues (Cys180 and Cys241 in the PAS domain) that creates a disulphide bond that reduces ArcB kinase activity thus dampening the systems output (Mascher *et al.*, 2006; Bekker *et al.*, 2010).

A more complex phosphorelay system present in *B. subtilis* is the KinA/Spo0F/Spo0B/Spo0A system (Fig. 1.4, C) (Burbulys *et al.*, 1991; Hoch, 1993). This phosphorelay is activated when *B. subtilis* regulates sporulation and is activated during nutrient starvation. This system is composed of four proteins; Spo0A is a regulator of gene expression in the sporulating predivisional cell. Phosphorylation levels of Spo0A are controlled by Spo0F and Spo0B (Krell *et al.*, 2010). Spo0F is a single domain response regulator that lacks a DNA binding domain. Spo0B is a phosphotransferase that facilitates transfer of phosphoryl groups from Spo0F to the regulator Spo0A (Krell *et al.*, 2010). The KinA sensor kinases, in addition with two others (KinB and KinC) all feed information into this phosphorelay system. In addition the phosphatases RapA and Spo0E regulate this phosphorylation levels of Spo0A (Perego and Hoch, 2002).

This study focused on the prototypical OmpR/EnvZ TCS of both *S. Typhimurium* and *E. coli* (Fig. 1.4, A). The structure, function and regulation of this system are discussed in the following sections.

1.6.2 OmpR/EnvZ

The OmpR/EnvZ TCS is a paradigm of two-component systems; this system has been intensively studied in *E. coli* and for this reason the following information is based on studies performed in *E. coli*, unless otherwise stated.

The OmpR/EnvZ TCS is composed of the histidine kinase EnvZ and the response regulator OmpR. EnvZ is located in the inner membrane of the bacterial envelope

(Hidaka *et al.*, 1997). Upon sensing a change in osmolarity (section 1.6.3), EnvZ undergoes intramolecular autophosphorylation at the highly conserved histidine residue present at position 243 (Forst *et al.*, 1989; Yang and Inouye, 1991). This phosphoryl group is subsequently transferred to OmpR (OmpR-P), specifically at a conserved aspartate residue at position 55 (Delgado *et al.*, 1993; Tomomori *et al.*, 1999). OmpR consists of a N-terminal receiver domain connected to a C-terminal DNA binding domain by a flexible linker. Phosphorylation of OmpR at the aspartate residue enhances its affinity for DNA enabling OmpR to regulate gene expression (Kato *et al.*, 1989; Kenney *et al.*, 1995).

OmpR/EnvZ is best known for its role in regulating the expression of outer membrane porin genes *ompF* and *ompC* (Alphen and Lugtenberg, 1977; Sarma and Reeves, 1977; Slauch *et al.*, 1988). The *ompB* locus (described in section 1.6.5) encodes the *ompR* *envZ* genes. This locus plays an important regulatory role for porin expression (Hall and Silhavy, 1981b; Hall and Silhavy, 1981c). OmpF and OmpC are major porins of the cell that form stable homotrimers in the outer membrane (De la Cruz and Calva, 2010). Both porins produce aqueous channels in the outer membrane enabling the passive diffusion of small hydrophilic solutes. Both proteins share extensive amino acid homology (Mizuno *et al.*, 1983). OmpF appears to have a larger channel than OmpC, which contributes to a faster diffusion rate (Nikaido and Rosenberg, 1983). The total amount of both OmpF and OmpC is kept constant in the cell. However, the relative levels of these proteins fluctuate in response to osmolarity; both proteins are reciprocally expressed at high and low levels of osmolarity. At low osmolarity OmpF is predominantly expressed, whereas at high osmolarity OmpC is predominantly expressed (Alphen and Lugtenberg, 1977). The reciprocal expression of these porins is regulated at the level of transcription by the OmpR/EnvZ TCS and is discussed in detail in section 1.6.6. The transcriptional activity of OmpR is mediated by the control of the cellular levels of OmpR through the kinase and potentially the phosphatase activity of EnvZ (Mattison and Kenney, 2002; Yoshida *et al.*, 2002). The enzymatic activities of EnvZ regulate the ratio of OmpF and OmpC. It is thought EnvZ preferentially adopts different enzymatic states as osmolarity changes. Low levels of OmpR-P are present at low osmolarity, this is presumably due to an increase in EnvZ phosphatase activity or a decrease in EnvZ kinase activity (Pratt and Silhavy, 1995).

Porin regulation in *Salmonella* Typhi is different to *E. coli*. OmpF is osmoregulated in the same manner as described for *E. coli*. However, OmpC expression is insensitive to osmolarity; OmpC levels were constant at both high and low levels of osmolarity (Martinez-Flores *et al.*, 1999). This insensitivity to osmolarity was also observed at the level of transcription; *lacZ* fusions to the *ompC* gene showed no difference in *ompC* expression at high or low osmolarity (Martinez-Flores *et al.*, 1999). Complementation experiments between *E. coli* and *S. Typhi* showed *S. Typhi ompC-lacZ* and *E. coli ompC-lacZ* fusions were expressed at constant levels (i.e. insensitive to changes in osmolarity) in a *S. Typhi* background. Interestingly, both fusions were osmoregulated when the *E. coli ompB* locus was moved into $\Delta ompB$ *S. Typhi* (Martinez-Flores *et al.*, 1999). These results indicate the *E. coli ompB* regulatory component is required for osmoregulation of *ompC*. However, both fusions were osmoregulated in an $\Delta ompB$ *E. coli* mutant harbouring the *ompB* locus from *S. Typhi*. Therefore, the insensitivity of *S. Typhi ompC* gene expression to osmolarity is due to *ompB* regulatory locus and other *trans*-acting factors present in *S. Typhi* (Martinez-Flores *et al.*, 1999). Furthermore, this study highlights the differences in the OmpR/EnvZ TCS in *Salmonella* compared to *E. coli*.

1.6.3 EnvZ sensor kinase

The EnvZ protein is composed of two monomers that can form a homodimeric protein. Each monomer is composed of 450 amino acids organised as two transmembrane domains located within the N-terminus (Fig. 1.5 A) (Comeau *et al.*, 1985). The C-terminus of EnvZ (EnvZc) is composed of a cytoplasmic domain that extends from amino acid residues 180-450. EnvZc consists of a linker region (residues 180-222), a DHp domain (residues 223-289) and a CA domain (residues 290-450) (Cai and Inouye, 2002). The DHp domain contains the site of autophosphorylation (His²⁴³) and the CA domain contains the ATP-binding site and the highly conserved G1, G2, F and N boxes (Tanaka *et al.*, 1998). G1 and G2 are located in the catalytic core and are required for kinase activity (Tanaka *et al.*, 1998). Nuclear magnetic resonance (NMR) spectroscopy has been used to solve the structures of the DHp and CA domains of EnvZ (Tanaka *et al.*, 1998). In its dimeric form the DHp domain forms a four helix bundle (Tomomori *et al.*, 1999), the CA domain forms an α/β sandwich fold; one layer consists of a five-stranded β -sheet and the second layer consists three α -helices (Tanaka *et al.*, 1998).

The EnvZ protein possesses several enzymatic activities. EnvZ autophosphorylates *in trans*; the CA domain from one subunit of an EnvZ homodimer phosphorylates the DHP domain of the other subunit (Yang and Inouye, 1991). EnvZ also possesses phosphotransfer activity and phosphatase activity towards the response regulator OmpR (Yang and Inouye, 1991). One study found the DHP domain was responsible for phosphatase activity and this activity is enhanced when the DHP domain is covalently linked to the CA domain (Zhu *et al.*, 2000). This same study found His²⁴³ was required for EnvZ phosphatase activity (Zhu *et al.*, 2000). However, the experimental set up used high EnvZ to OmpR ratio (2:1), which does not reflect the ratio *in vivo* (1:35-40) (Cai and Inouye, 2002; Kenney, 2010). An additional study hypothesised EnvZ should have a higher affinity for OmpR-P for the phosphatase activity to have a significant affect *in vivo* (Mattison and Kenney, 2002). It was speculated that EnvZ would preferentially bind OmpR-P prompting dephosphorylation of OmpR; accordingly, EnvZ would release OmpR and bind to another OmpR-P molecule. Studies have found EnvZ has a higher affinity for OmpR and concluded EnvZ phosphatase activity does not play a significant role *in vivo* (Mattison and Kenney, 2002; King and Kenney, 2007; Kenney, 2010). However, another group have found EnvZc interacts with OmpR and OmpR-P with similar affinities and conclude the phosphatase activity of EnvZ modulates OmpR levels in response to osmotic signalling (Yoshida *et al.*, 2002). It is evident that the EnvZ protein possesses phosphatase activity, however the physiological relevance of the activity remains to be elucidated.

When the EnvZ protein is stimulated by an appropriate signal it then relays this information to OmpR. Despite the intensive level of study, there are a limited number of studies demonstrating the mechanism of osmosensing by EnvZ (Tabatabai and Forst, 1995; Wang *et al.*, 2012). The stimulus is known for many well-characterised TCS. For example, the chemotaxis systems binds serine and aspartate via the chemoreceptors Tsr and Tar, respectively (Hazelbauer, 2012). Additional molecules such as maltose, galactose, ribose and dipeptides interact with the chemoreceptors Tar, Tap and Trg through interactions with periplasmic binding proteins (Hazelbauer, 2012). It is known that the sensor kinase EnvZ senses changes in osmolarity; the osmolarity of a solution is varied by changing the concentrations ionic compounds such as sodium chloride (NaCl) or non-ionic compounds such as sucrose in a solution. EnvZ is stimulated by changes in either sucrose or NaCl concentrations (Cai and Inouye, 2002; Wang *et al.*, 2012). In

addition procaine, which is an anesthetic and membrane perturbant, stimulates porin expression in an EnvZ dependent manner (Granett and Villarejo, 1982; Rampersaud and Inouye, 1991). Rampersaud and Inouye (1991) have speculated that both osmolarity and local anesthetics may share a common pathway leading to porin regulation by a mechanism that perturbs normal membrane function (Rampersaud and Inouye, 1991). These data raise the question what stimulating factor is EnvZ sensing? and how is EnvZ sensing this stimulus? The former remains unanswered, however, recent studies have offered insights into the sensing mechanism of EnvZ.

Initially, the periplasmic domain of EnvZ was hypothesised as the signal-sensing domain this has since been challenged (Reviewed by Krell *et al.*, 2010). The homologous EnvZ protein in *Xenorhabdus nematophilus* (EnvZ^{X.n}) is composed of 342 amino acids (108 amino acids shorter than EnvZ in *E. coli*) (Tabatabai and Forst, 1995). Importantly, EnvZ^{X.n} does not possess a periplasmic domain. The cytoplasmic domain of EnvZ^{X.n} shares 57% amino acid identity with cytoplasmic domain of EnvZ in *E. coli*. Tabatabai and Forst (1995) demonstrated that EnvZ^{X.n} can complement an *envZ* mutant in *E. coli*, such that EnvZ^{X.n} phosphorylated OmpR in *E. coli* and osmo-regulated expression of both OmpF and OmpC (Tabatabai and Forst, 1995). These results provide evidence that the periplasmic domain of EnvZ in *E. coli* is not required for signal sensing. An additional study showed replacement of the EnvZ periplasmic region with periplasmic region of the sensor kinase PhoR from *Bacillus subtilis* did not significantly affect osmoregulation of OmpF and OmpC (Leonardo and Forst, 1996). Cumulatively, these results indicate the cytoplasmic domain of EnvZ is required for signal sensing.

A more recent study provides structural evidence that EnvZc responds to changes in osmolarity (Wang *et al.*, 2012). Using amide hydrogen deuterium exchange mass spectrometry Wang *et al.* demonstrated the N-terminally located four-helix subdomain and dimer interface was responsible for sensing changes in osmolarity (Fig. 1.5, A). Furthermore, EnvZc did not require membrane localisation for this activity (Wang *et al.*, 2012). High osmolarity altered the conformation of the four-helix bundle, which includes the helix containing the His²⁴³ residue. This study also demonstrated the importance of the His²⁴³ residue in osmosensing using a His²⁴³ mutant (H243A). The His²⁴³ mutant was insensitive to changes in osmolarity due to its inability to autophosphorylate (Wang *et al.*, 2012). At high osmolarity, there was reduced amide

hydrogen exchange rates. This resulted in increased stabilisation of intrahelical H-bonding and increased folding of the four-helix bundle which catalyses autophosphorylation of EnvZ and phosphotransfer to OmpR (Wang *et al.*, 2012).

Upon detecting a change in osmolarity EnvZ must communicate this information to OmpR and EnvZ does this at both high and low osmolarity. Early studies found *envZ* null mutants resulted in an OmpF⁺/OmpC⁻ phenotype (Garrett *et al.*, 1983), as this phenotype does not correspond with phenotypes at high or low osmolarity it is assumed that EnvZ is active under both conditions (Pratt and Silhavy, 1995).

1.6.4 OmpR response regulator

Response regulators are grouped into three subfamilies based on the similarities of their effector domains; these are the OmpR/PhoB, NtrC/DctD and NarL/FixJ families (Stock *et al.*, 2000). OmpR is a 27.3 kDa transcription factor and a member of the largest subfamily of response regulators (OmpR/PhoB). OmpR is composed of an N-terminal receiver domain (RD) that harbours the Asp⁵⁵ residue which is phosphorylated by EnvZ, and a C-terminal DNA binding domain (DBD) joined by a flexible linker (Fig. 1.5, B) (Kato *et al.*, 1989; Kenney *et al.*, 1995). This flexible linker is 15 amino acids long and is sensitive to proteolysis. Mutations that shorten the length of this linker are defective in activation at *ompF* and *ompC* expression, highlighting the importance of the linker length (Kenney *et al.*, 1995; Kenney, 2002). Furthermore, the structure of the linker is altered by both phosphorylation and DNA binding (Kenney *et al.*, 1995; Ames *et al.*, 1999). This communication is bi-directional; phosphorylation of the N-terminal receiver domain enhances the affinity of the C-terminus for DNA and DNA binding stimulates phosphorylation (Aiba *et al.*, 1989; Harlocker *et al.*, 1995; Huang and Igo, 1996; Head *et al.*, 1998; Ames *et al.*, 1999).

The crystal structure of the C-terminus of OmpR (OmpRc; amino acid residues 130-239) revealed a winged helix-turn-helix structure (wHTH) that is conserved amongst members of the OmpR/PhoB subfamily (Kondo *et al.*, 1997; Martinez-Hackert and Stock, 1997; Stock *et al.*, 2000). OmpRc forms an N-terminal four-stranded β sheet, a central three- α helical bundle and a C-terminal β hairpin. NMR studies show the α -helix 3, which is part of the HTH (α 2- α -loop- α 3) makes contact with DNA (Rhee *et al.*,

2008). Specifically, OmpR contacts T and G bases via Val²⁰³ and Arg²⁰⁷ and contacts the phosphate backbone via Arg²⁰⁹ which are located in α -helix 3 of the HTH (Rhee *et al.*, 2008). Binding of a single OmpR monomer to DNA in this manner stimulates binding of a second monomer. Structural analysis of other members of the OmpR/PhoB families suggest dimerisation between OmpR monomers occurs in the α 4- β 5- α 5 structure in the N-terminus (Robinson *et al.*, 2003; Toro-Roman *et al.*, 2005). This region is highly conserved amongst members of the OmpR/PhoB family (Robinson *et al.*, 2003).

1.6.5 The *ompR/envZ (ompB)* operon

The *ompB* locus was identified in a study investigating genes that controlled the expression of OmpF and OmpC (Sarma and Reeves, 1977). Subsequent studies found the *ompB* locus contained the *ompR* and *envZ* genes organised in an operon and expression of both genes is dependent on the promoter upstream of *ompR* (Hall and Silhavy, 1981c; Comeau *et al.*, 1985). Experiments performed in *S. Typhimurium*, using translational *lacZ* fusions to both *ompR* and *envZ*, showed that *ompR* is translated 10 times more efficiently than *envZ* (Liljestrom *et al.*, 1988). This is a result of the four base pair overlap between the *ompR* stop codon and the *envZ* start codon and the lack of a good ribosome-binding site in the *envZ* message. It is thought that translation of *envZ* depends on reinitiation of the ribosome on the polycistronic mRNA. Therefore translation of *envZ* may depend on the termination of *ompR* translation and initiation of *envZ* translation. This process may be inefficient and therefore account for the lower levels of *envZ* translation compared to *ompR*, thus both *ompR* and *envZ* mRNA may be regulated differently at the level of translation (Comeau *et al.*, 1985; Liljestrom *et al.*, 1988). A subsequent study calculated the intracellular levels of OmpR and EnvZ by quantitative analysis of western blot. OmpR and EnvZ levels were estimated to be 3,500 molecules and 100 molecules per cell, respectively (Cai and Inouye, 2002). Although the total levels of OmpR and EnvZ were elevated at high osmolarity compared to low osmolarity, the ratio of OmpR to EnvZ remained constant in both media tested (i.e. 30-35 OmpR monomers for every EnvZ monomer) (Cai and Inouye, 2002). This same study estimated the cellular concentrations of OmpR and EnvZ to be 6 μ M and 0.18 μ M, respectively. Furthermore, the K_d for the interaction of both proteins was calculated

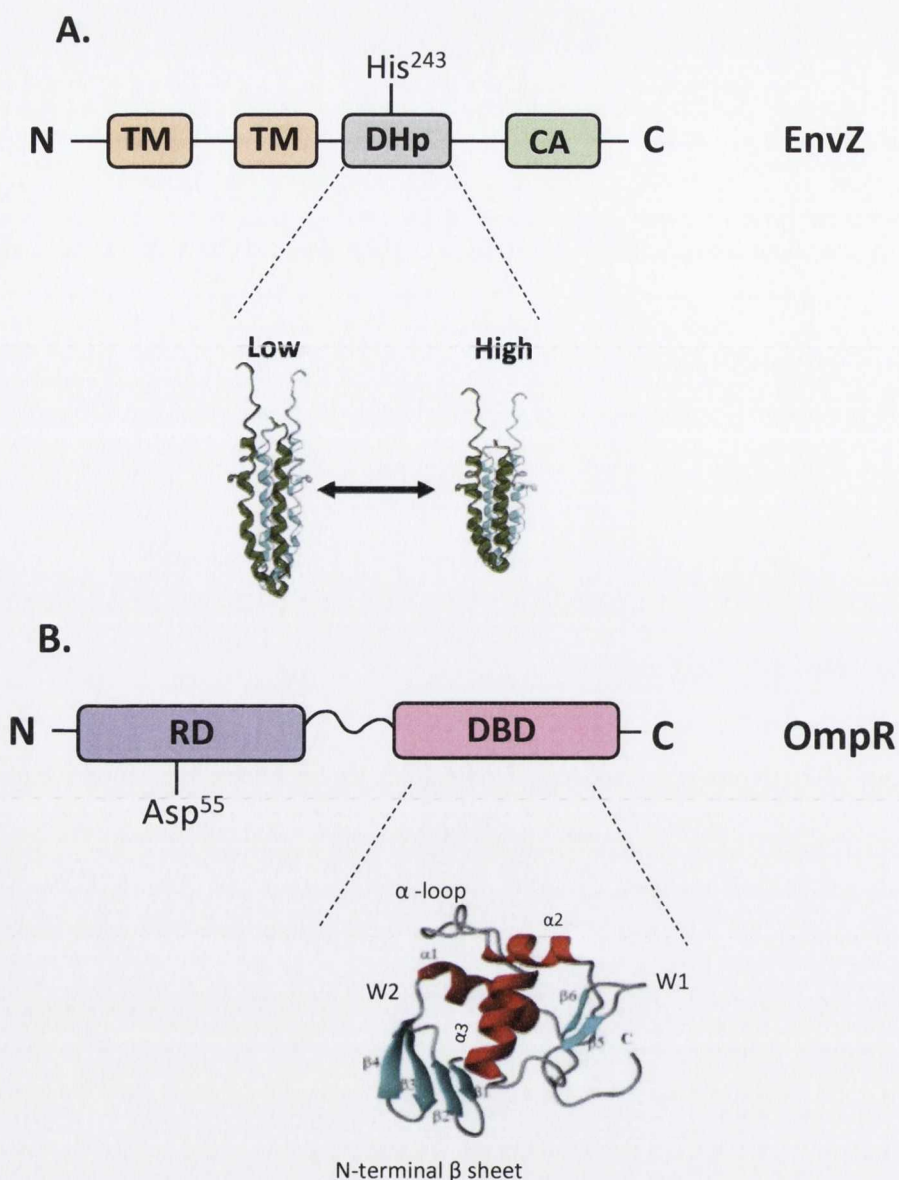


Fig. 1.5. Domain organisation of EnvZ and OmpR. **A.** EnvZ is composed of two transmembrane domains (TM) located in the inner membrane. This is followed by a cytoplasmic domain composed of a linker, DHp (dimerisation and histidine phosphotransfer) domain and a CA (catalytic and ATP-binding) domain. Below; a ribbon diagram of the DHp dimer (Wang *et al.*, 2012) responsible for osmosensing in *E. coli*. At low osmolarity (left) a stretched helical bundle with weak intrahelical hydrogen (H) bonding predominates. At high osmolarity, a more folded conformation is favoured, with more stable intrahelical H-bonds resulting in increased autophosphorylation and phosphotransfer from the His²⁴³ residue to OmpR **B.** OmpR is composed of a receiver domain (RD) containing the Asp⁵⁵ residue for phosphorylation. This is connected to the DNA binding domain (DBD) by a flexible linker. Below, a ribbon diagram illustrating the secondary structure of the DBD domain, see text for details (Rhee *et al.*, 2008).

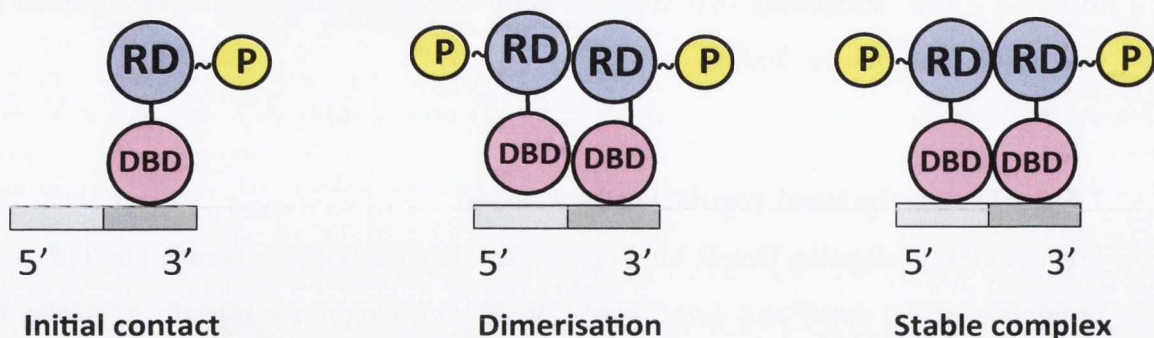
to be $\sim 1.2 \mu\text{M}$, suggesting that approximately 85% of cellular EnvZ is occupied by OmpR (Cai and Inouye, 2002).

1.6.6 Transcriptional regulation by OmpR

Most work investigating OmpR binding to DNA has focussed on OmpR binding to the promoters of the *ompF* and *ompC* genes. In *E. coli*, OmpR reciprocally regulates the expression of *ompF* and *ompC* in response to changes in osmolarity (Forst *et al.*, 1990; Cai and Inouye, 2002). At low osmolarity, low levels of phosphorylated OmpR (OmpR-P) activate expression of *ompF*. At high levels of osmolarity there are increased levels of OmpR-P which represses *ompF* expression with the concomitant activation of *ompC* (Fig. 1.6, B). This reciprocal regulation is due to the hierarchical occupancy of the OmpR binding sites located upstream of these genes (Rampersaud *et al.*, 1994; Bergstrom *et al.*, 1998). OmpR binds to four sites in the *ompF* promoter (F1, F2, F3, F4) and to three sites in the *ompC* promoter (C1, C2, C3). At low osmolarity, OmpR-P occupies the F1-F2/F1-F2-F3 sites and activates *ompF* expression (Fig. 1.6, B). Under these same conditions OmpR-P occupies only the C1 site, which is insufficient to activate expression. Increased levels of OmpR-P, at high osmolarity, leads to occupation of the F4 site at *ompF*, which is thought to repress *ompF* expression by DNA loop formation facilitated by integration host factor (IHF) protein (Huang *et al.*, 1994). Concurrently, OmpR-P occupies the remaining C2 and C3 sites and activates *ompC* expression (Fig. 1.6, B) (Rampersaud *et al.*, 1994; Bergstrom *et al.*, 1998).

Reciprocal regulation of *ompF* and *ompC* is mediated by the co-operative and step-wise manner in which OmpR binds to DNA (Yoshida *et al.*, 2006). OmpR binds to a 20 bp binding site, which typically contain a $G_1X_2X_3A_4C_5A_6$ motif (Harlocker *et al.*, 1995; Yoshida *et al.*, 2006). Each site is composed of two tandem 10-bp half sites that accommodates a single OmpR-P molecule. OmpR-P has a high affinity for the 3' half site and weaker affinity for the 5' half site (Yoshida *et al.*, 2006). The current model for OmpR binding to DNA is as follows; a single OmpR-P molecule occupies the 3' half site (Fig. 1.6, A), initial binding of the single OmpR-P molecule promotes binding of a second OmpR-P molecule to the weaker 5' half site. In the presence of DNA two OmpR-P molecules dimerise via interactions between the N-terminal domains of each monomer (Fig. 1.6, A) (Yoshida *et al.*, 2006).

A.



B.

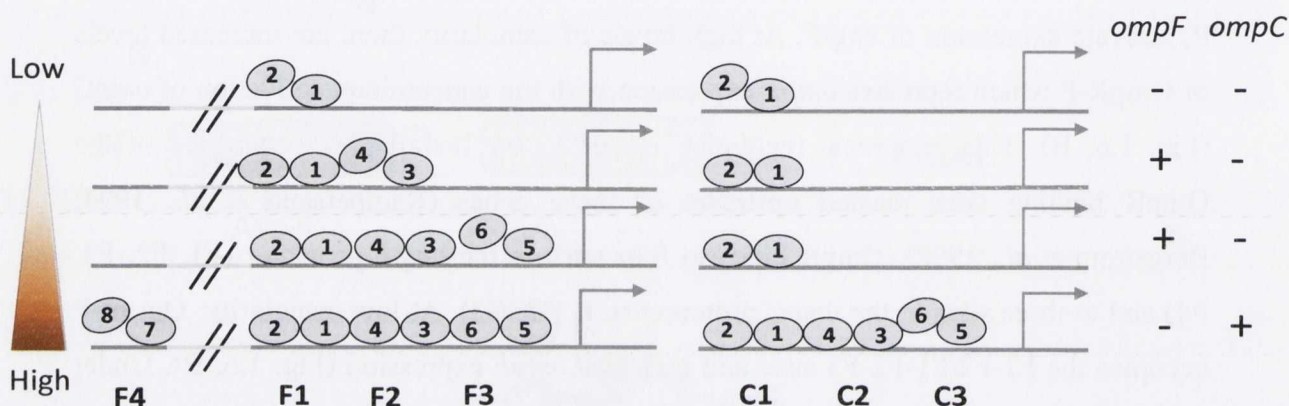


Fig. 1.6. DNA binding and transcriptional regulation by OmpR. **A.** A single phosphorylated OmpR molecule (OmpR-P) binds to the 3' end of the binding site with high affinity. This recruits a second OmpR-P molecule to the 5' end of the binding site; both molecules form a dimer and a stable DNA binding complex. **B.** OmpR-P single molecules (grey ovals) bind fill the binding sites at the *ompF* promoter (F1-F4) and the *ompC* promoter (C1-C3) as described in A. The order in which the sites are filled is based on the affinity OmpR has for each site. At low osmolarity the high affinity sites in both promoters are filled. As osmolarity increases, OmpR fills the remaining sites in a hierarchical manner. The effect this has on transcription is shown on the right; - low/no expression, + high expression. Bent arrow represents the transcription start site, the F4 site is located upstream of the remaining sites, this is denoted by a break in the line (adapted from Yoshida *et al.*, 2006).

The mechanism of transcription activation by OmpR is not well understood. Genetic studies indicate OmpR-P activates transcription by making direct contact with RNA polymerase. Using the transcriptionally defective *envZ* mutant, *envZ473*, one study mapped several suppressor mutations in the *rpoA* gene which codes for the α subunit of RNA polymerase (RNAP) (Garrett and Silhavy, 1987). Another study found suppression of the *envZ11* mutation by a mutation in *ompR* could be appeased by an additional mutation in the α subunit of RNAP (Matsuyama and Mizushima, 1987). An additional study characterised several mutations in *rpoA* that affect expression of the porin genes *ompF* and *ompC* (Slauch *et al.*, 1991). Furthermore, *in vitro* transcription assays with a truncated α -CTD subunit of RNAP failed to activate transcription at *ompC* (Igarashi *et al.*, 1991). These results further indicate OmpR-P contacts the α -CTD of RNAP to activate transcription, however, the region(s) of OmpR that make direct contact with RNAP are not known.

It is worth mentioning that although OmpR is an essential regulator of *ompF* and *ompC* transcription, additional regulators, listed in Table 1.2, affect expression of these genes. The expression of *ompF* and *ompC* is also regulated post-transcriptionally by small untranslated regulatory RNAs (sRNAs); the best described are MicF and MicC. In bacteria a large proportion of sRNAs act as post-transcriptional regulators by base-pairing with target mRNAs. Frequently, sRNAs target the 5' untranslated region (UTR) or the early coding sequence of mRNAs and positively but more often negatively affects their translation (reviewed by Guillier *et al.*, 2006). MicF was the first identified sRNA in 1984 (Mizuno *et al.*, 1984). MicF is 93 nucleotides (nt) in length and is divergently transcribed from the *ompC* gene; both genes are expressed by high levels of phosphorylated OmpR. MicF acts by forming a 20-bp imperfect RNA duplex with the translation-initiation site of *ompF* mRNA and negatively affects *ompF* translation (Schmidt *et al.*, 1995). The 109 nucleotide MicC sRNA represses *ompC* expression by base-pairing with the *ompC* mRNA and preventing translation complex forming. Studies have shown MicC levels increase in an *ompR* mutant indicating OmpR acts as a negative regulator (Chen *et al.*, 2004; Pfeiffer *et al.*, 2009).

1.6.7 Acetyl phosphate

Previous studies have shown that *envZ* mutants are still capable of expressing OmpF

Table 1.2. Regulators of *ompF* and *ompC*

Regulator	OmpF	OmpC	References
IHF	+/-	-	(Huang <i>et al.</i> , 1990; Ramani <i>et al.</i> , 1992)
LRP	+	-	(Ferrario <i>et al.</i> , 1995)
H-NS	NK	-	(Suzuki <i>et al.</i> , 1996)
CpxR	-	+	(Batchelor <i>et al.</i> , 2005)
CRP	+	NK	(Huang <i>et al.</i> , 1992)
StpA	-	NK	(Deighan <i>et al.</i> , 2000)
sRNAs	OmpF	OmpC	References
MicC	NK	-	(Chen <i>et al.</i> , 2004)
MicF	-	NK	(Schmidt <i>et al.</i> , 1995)
RseX	NK	-	(Douchin <i>et al.</i> , 2006)
RybB	-	-	(Papenfort <i>et al.</i> , 2006)
IpeX	-	-	(Castillo-Keller <i>et al.</i> , 2006)

+, positive regulator. -, negative regulator. NK; not known.

(Villarejo and Case, 1984; Mizuno and Mizushima, 1987), suggesting that OmpR can be phosphorylated by an alternative phosphodonor in the absence of EnvZ. Like other response regulators such as CheY and PhoB, the OmpR protein is also phosphorylated by the small phosphor-donor acetyl phosphate (Lukat *et al.*, 1992; McCleary and Stock, 1994). Acetyl phosphate synthesises from acetyl-CoA and P_i (inorganic phosphate) is catalysed by phosphotransacetylase encoded by the *pta* gene (McCleary *et al.*, 1993). Synthesis of acetyl phosphate from acetic acid and ATP requires the enzyme acetate kinase, which is encoded by the *ackA* gene. As such, *pta ackA* double mutants do not produce acetyl phosphate (McCleary *et al.*, 1993).

A systematic study performed by Matsubara and Mizuno (1999) using Medium A with or without sucrose (15%). This work showed expression of OmpF and OmpC in an *pta ackA* double mutant was mildly lower than wild-type. However, OmpF and OmpC expression was barely detectable when the *pta ackA* mutant was coupled with an *envZ* mutant (Matsubara and Mizuno, 1999). Complementation of *ack pta envZ* mutant with multicopy plasmid expressing the *ackA pta* genes restored osmoregulation of OmpF and OmpC (Matsubara and Mizuno, 1999). Thus, supporting the view that, in the absence of *envZ*, OmpR is phosphorylated *in vivo* by acetyl phosphate (Matsubara and Mizuno, 1999).

1.6.8 Acidic pH and the OmpR/EnvZ system

Orally ingested enteric bacteria such as *S. Typhimurium* and *E. coli* must survive the acidic pH of the stomach inside the host. In addition, *S. Typhimurium* must also survive acidification within the SCV in order to elicit infection (Haraga *et al.*, 2008). employ a number of mechanisms to survive with acidic pH. Some of these are general resistant mechanism, some are enzyme-based systems that pump protons out of the cell to maintain pH homeostasis, while others are classified based on the conditions in which they are induced.

These systems are required to maintain pH homeostasis and enable bacteria to tolerate and/or grow at external pH values outside the preferred cytoplasmic range. Regulation of cytoplasmic pH is important for cell physiology. For example, cellular proteins have a range of pH values within which they maintain function and protonation as a result of

changes in cytoplasmic pH can damage the function of both proteins and nucleic acids. Furthermore, elevated levels of intracellular protons can affect enzyme activity and processes such as DNA transcription and protein synthesis (Madshus, 1988; Baker-Austin and Dopson, 2007).

Regulation of cytoplasmic pH requires control of the cells permeability to protons (H^+) (Booth, 1985). Neutralophiles, like *Salmonella* and *E. coli*, preferentially grow at pH values between ~5.5-9.0 and maintain their cytoplasmic pH (pH_i) between ~7.5-7.7. Acidophiles maintain pH_i ~6.5-7.0 and alkalophiles have pH_i values between ~8.4-9.0 (Booth, 1985). The pH gradient ($\Delta pH = pH_i - pH_{out}$) contributes to the total proton motive force (PMF) in the cell and this drives cellular bioenergetic work (Foster, 2004). PMF is a transmembrane electrochemical gradient across the bacterial cell membrane. PMF is affected by two factors, the difference in proton gradient (ΔpH) and the difference in electrical charge (i.e. the difference in membrane potential; $\Delta \Psi$, this is a measurement of all cellular charges including H^+) (Foster, 2004). PMF energises many active processes which contribute to pH homeostasis including driving ATP synthesis. Bacteria use both active and passive mechanisms to maintain pH homeostasis. Active strategies include the use of transporters that catalyse active proton transport such as proton pumps (e.g. ATPases) and cation-proton antiporters (Krulwich *et al.*, 2011). Cation-proton antiporters use PMF from respiration or ATPases to facilitate proton uptake in exchange for cytoplasmic cations Na^+ or K^+ (Krulwich *et al.*, 2011). At low pH in *E. coli* the expression of respiratory chain complexes (that extrudes protons from the cell) is increased. In addition expression of ATP synthase (that transports protons into the cell) is decreased (Krulwich *et al.*, 2011).

The passive homeostatic mechanism includes low permeability of the membrane to protons and the buffering capacity of the cytoplasm. As such, the capacity for pH homeostasis is reduced if the impermeability of the membrane to protons is perturbed by ionophores such as dinitrophenol (Khan and Macnab, 1980). Maintaining a cellular membrane which is impermeable to protons ensures PMF is not rapidly dissipated by the movement of protons across the membrane. The ability of cells to recover from changes in pH_i depends on the magnitude of the change; this change is determined by the intracellular buffering capacity. The cells buffering capacity is defined as the capacity of the cytoplasmic contents to resist changes in pH (Krulwich *et al.*, 2011).

The cytoplasm is buffered by small organic molecules such as amino acids, ionisable side chains on proteins and inorganic phosphates that may sequester or release protons as necessary to maintain internal pH.

There are four described acid resistance systems (AR) systems (AR1-4) described in *E. coli*. These systems are induced at low pH and require additional supplementation such as amino acids. AR1 functions in stationary phase cells grown in LB broth at pH 5.5 without glucose. This system is dependent on the stationary phase alternative sigma factor RpoS and the global regulator CRP (Bearson *et al.*, 1997; Castanie-Cornet *et al.*, 1999; Audia *et al.*, 2001; Foster, 2004; Ramos-Morales, 2012). AR2-4 are decarboxylase(s)/antiporter systems that are induced in the presence of specific amino acids and at low pH (see Table 1.3). These systems catalyse the decarboxylation of amino acid substrates, consuming intracellular protons and producing CO₂ in the process. The decarboxylated end-product is removed from the cell by its cognate antiporter and exchanged for more substrate. It is thought that consumption of intracellular protons by these systems helps to maintain pH homeostasis for the cell and prevent damage to cellular components (Foster, 2004). *Salmonella* also possesses decarboxylases that contribute to survival at low pH (see Table 1.3). In the presence of lysine, arginine or ornithine the antiporters CadA, AdiA and SpeF expel cadaverine, agmatine and putrescine respectively (Fig. 1.7) (Viala *et al.*, 2011).

To survive acid challenge, *S. Typhimurium* employs both the log and the stationary phase acid tolerance responses ATRs that are induced in minimal media. These differ from the AR systems in that they require no additional supplementation for their induction. Stimulation of these systems is dependent upon the growth phase in which the cells are challenged with low pH (Audia *et al.*, 2001; Foster, 2004; Ramos-Morales, 2012).

The log phase ATR is induced when exponentially growing cells are exposed to low pH (pH 4.5-5.8). Induction of the log phase ATR protects the cells against subsequent challenges with otherwise lethal pH values (pH 2.5-3). These systems induce the synthesis of over 40 acid-shock proteins ASPs. The genes encoding these ASPs are regulated by both RpoS and the iron regulator Fur (ferric uptake regulator) protein

Table 1.3. The decarboxylases of *E. coli* and *S. Typhimurium*

	AR system	Amino acid	Decarboxylase(s)	End-product	Antiporter
<i>Escherichia coli</i>					
Order of efficiency ↓	AR2	Glutamate	GadA, GadB	GABA	GadC
	AR3	Arginine	AdiA	Agmatine	AdiC
	AR4	Lysine	CadA	Cadaverine	CadB
<i>S. Typhimurium</i>					
Order of efficiency ↓	1	Arginine	AdiA	Agmatine	AdiC
	2	Lysine	CadA	Cadaverine	CadB
	3	Ornithine	SpeF	Putrescine	PotE

GABA; gamma amino butyric acid.

(Foster and Hall, 1992; Lee *et al.*, 1995). Although many of these ASPs remain uncharacterised, it is thought that these proteins may offer protection to the cell against threats posed by acidic pH that in turn affects pH_i (Audia *et al.*, 2001). Changes in pH_i can affect enzyme activity, protein stability, nucleic acid structure and other molecules.

The stationary phase ATR is induced when cells in stationary phase are exposed to low pH (Lee *et al.*, 1994). 15 ASPs are induced in the stationary phase ATR, 4 of which are also induced in the log phase ATR (Lee *et al.*, 1994). This system does not require RpoS and is distinct from the RpoS-dependent general stress response. A study conducted by Bang *et al.* (2000) searching for regulators of the stationary phase ATR identified OmpR as an important regulator. Genetic screens identified two mutants that were capable of inducing a log phase ATR but were defective for inducing a stationary-phase ATR (Bang *et al.*, 2000). Mutations in both mutants mapped within the *ompR* open-reading frame thus identifying a requirement for OmpR during the stationary phase ATR (Bang *et al.*, 2000). Bang *et al.* found that *ompR* gene expression increased 3.7 fold upon exposure to low pH (Bang *et al.*, 2002). Furthermore, *ompR* mutants were 500-fold more pH sensitive than wild type (Bang *et al.*, 2000). Importantly, *ompR* expression in *S. Typhimurium* was not induced by high osmolarity (Bang *et al.*, 2000; Bang *et al.*, 2002). Thus, the OmpR/EnvZ system in *S. Typhimurium* does not respond to osmolarity like its orthologue in *E. coli*. Instead this system responds to acidic pH (Bang *et al.*, 2000; Bang *et al.*, 2002). The effect of acidic pH on *ompR* expression in *E. coli* was not investigated.

1.7 Factors that affect *ompR* expression

Despite intensive investigation into the OmpR/EnvZ TCS, there are a few studies investigating regulation of *ompR* in both *S. Typhimurium* and *E. coli*. Furthermore, the evidence that expression of the *ompR/envZ* operon gene in *S. Typhimurium* is activated by low pH highlights the need for more comparative analysis of *ompR* regulation in both species. Studies have shown *ompR* expression in *E. coli* is regulated by the IHF and CRP proteins (Tsui *et al.*, 1991; Huang *et al.*, 1992). While studies in *S. Typhimurium* have revealed *ompR* is regulated by the OmpR, H-NS and Fis proteins (Bang *et al.*, 2002; Cameron and Dorman, 2012). These proteins are all transcription factors but some (IHF, H-NS and Fis) are also categorised as nucleoid-associated

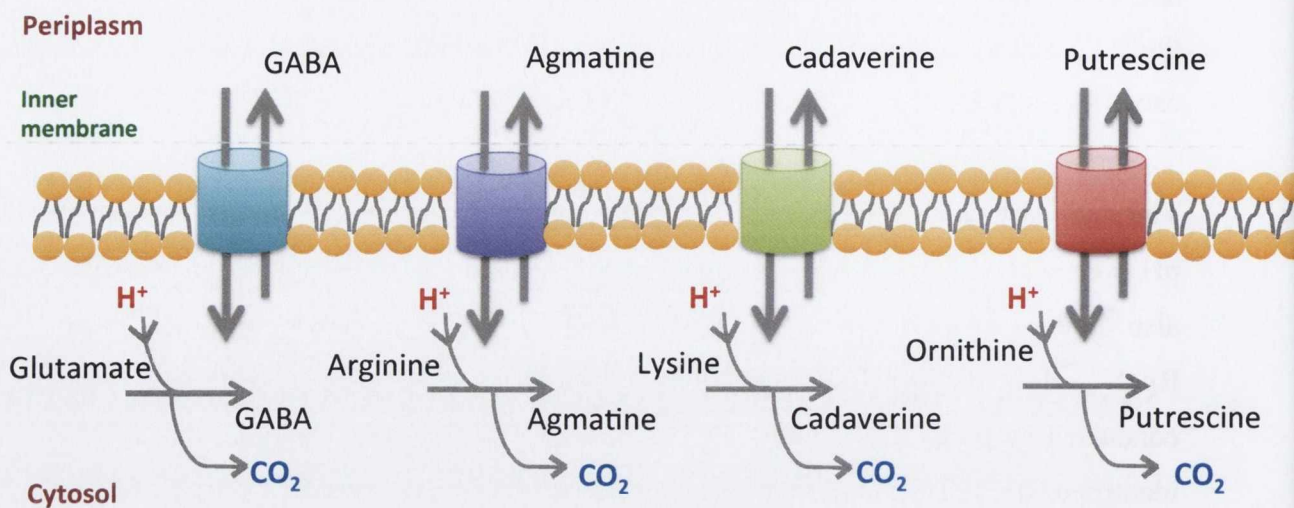


Fig. 1.7. Schematic of the decarboxylases in *E. coli*. Amino acids glutamate, arginine, lysine and ornithine are taken up by the cell by specific antiporters located in the inner membrane. Decarboxylases catalyse the decarboxylation of the amino acids, consuming an intracellular proton (H^+) and producing CO_2 in the process. The decarboxylated product is removed from the cell by the cognate antiporter and exchanged for more substrate. GABA; gamma amino butyric acid.

proteins (NAPs). NAPs are a class of proteins that are highly abundant and share a similar function; most possess DNA binding activity and alter the architecture of the chromosome through DNA bending, wrapping or bridging (Dillon and Dorman, 2010). Before discussing regulation of *ompR* expression in both *E. coli* and *S. Typhimurium* this section will begin with a brief overview of transcription initiation (section 1.7.1) followed by a description of each of these regulators (section 1.7.2-1.7.5). Finally, current knowledge of the effect each of these regulators has on *ompR* expression in *E. coli* and *S. Typhimurium* will be discussed (section 1.7.6).

1.7.1 An overview of transcription and DNA supercoiling

Gene expression begins with the initiation of transcription; this is a multistep process that is catalysed by the RNA polymerase holoenzyme. RNA polymerase contains a catalytically competent core composed of the $\alpha_2\beta\beta'\omega$ subunits (~400 kDa) (Browning and Busby, 2004). Promoter-specific initiation of transcription and holoenzyme formation requires an additional subunit, the σ (sigma factor), which binds to the catalytic core.

The $\beta\beta'$ subunits are the largest subunits and function by binding both DNA and RNA templates during transcription (Korzheva *et al.*, 2000). The α subunits are composed of an amino terminal domain (α -NTD), typically connected to a DNA-binding carboxy terminal domain (α -CTD) by a flexible linker (Gourse *et al.*, 2000). The ω subunit is thought to play a role in folding of the β' subunit.

The σ subunit is composed of four domains (σ 1-4) connected by linkers (Campbell *et al.*, 2002). These domains are involved in promoter recognition, positioning of RNA polymerase and unwinding the DNA duplex (Wosten, 1998). The main housekeeping sigma factor σ^{70} recognises two hexameric sequences in the promoter called the -10 and -35 elements. Specifically, domain 2 of the σ subunit recognises the -10 element and domain 4 recognises the -35 element. Optimally, these elements are separated by a 17 bp 'spacer' and are located 6 bp from the transcription start site, however this can vary (reviewed by Gruber and Gross, 2003; Browning and Busby, 2004). In addition to σ^{70} , bacteria have multiple these alternative sigma factors. These alternative sigma factors can bind to the catalytic core and confer promoter selectivity on the holoenzyme

(reviewed by Gruber and Gross, 2003; Browning and Busby, 2004). Alternative sigma factors recognise distinct promoter sequences and activate expression of genes under specific conditions. For example, the heat shock sigma factor (σ^{32}) is required to activate heat shock promoters; expression of σ^{32} is induced and protein stability is enhanced after a shift from 30 to 42 °C (Straus *et al.*, 1987).

Interaction of the RNAP holoenzyme with promoter DNA initiates subsequent events from the formation of a closed complex structure to formation of a competent open complex. Formation of an open complex occurs when the double-stranded DNA is melted over the transcription start site (+1) and extends approximately 12 base pairs (deHaseth *et al.*, 1998; Tomsic *et al.*, 2001). RNA synthesis begins with the formation of the first phosphodiester bond between the initiating and adjacent nucleoside triphosphates. After the polymerase transcribes approximately 10 base pairs the σ subunit is thought to be released, although studies have shown retention of the σ^{70} subunit after the early phases of transcription (reviewed by Saecker *et al.*, 2011), and elongation with the core polymerase continues to transcribe the RNA message.

Transcription factors can bind at or near the promoter region and have a positive or negative effect on transcription initiation (Browning and Busby, 2004). Regulation can be more complex; transcription factors can act as both activators and repressors and these effects can often be context specific (Browning and Busby, 2004). While some factors regulate the expression of a specific gene (e.g. the repressor LacI at the *lac* operon) (Reznikoff, 1992), other factors bind to multiple promoters and co-ordinate the expression of hundreds of genes, known as a regulon. Transcription factors can bind at promoters and make direct contact with RNAP; this contact can be promote (activator) or prevent (repressor) the initiation of transcription (Browning and Busby, 2004). Alternatively transcription factors that bind in a regulatory region make no contact with the RNAP, instead these proteins may facilitate transcription initiation by modifying DNA topology and facilitating contact between distantly bound proteins and RNAP (e.g. IHF at σ^{54} -dependent promoters) (reviewed by Goosen and van de Putte, 1995). These transcription factors may bend, wrap or bridge the DNA and affect transcription in a positive or negative manner. Transcription factors may also affect the behaviour of each other; binding of one factor may be dependent on the presence of another (i.e. co-operative binding) (Browning and Busby, 2004). Alternatively binding of one factor

may alleviate the repressive effect of another (i.e. anti-repression/de-repression) (Browning and Busby, 2004).

The process of transcription may also be affected by DNA supercoiling. It is known that DNA supercoiling affects the expression of *ompR* in *S. Typhimurium* (Bang *et al.*, 2002; Cameron and Dorman, 2012). DNA supercoiling is the production of an excess or deficit of helical turns in the DNA which results in coiling of the DNA polymer in a 3D space (Sobetzko *et al.*, 2012). Typically, DNA exists as two complementary strands of polynucleotides that are interwound to form a double helix. The conformation of DNA known as B-DNA describes a right-handed helix with a ~ 10.5 bp per turn which is the prevailing conformation of DNA. Locally, DNA can exist in non B-DNA conformations, however the term B-DNA describes the overall conformation of the DNA.

The bacterial chromosome is a closed circular DNA molecule. The ‘closed’ form prevents free rotation of the ends of DNA; any DNA segment where rotation of the ends is not possible is called a topological domain. In eukaryotic cells the chromosomes are linear, however the DNA is constrained tightly and loops around the histone octamer (Higgins *et al.*, 2010). These loops are topological domains and are the topological equivalent to circular DNA. In most organisms DNA exists in a supercoiled form and can exist in a variety of topological conformations. Supercoiled DNA is more compact than linear DNA and therefore aids in packaging the chromatin into the cell. In bacteria, DNA is maintained in a negatively (underwound) supercoiled state (reviewed by Pettijohn, 1996). DNA that is negatively supercoiled contains a deficiency of helical turns, relative to the same molecule that has been nicked on a single strand and is described as ‘relaxed’ (reviewed by Dorman, 2006). DNA that is positively (overwound) supercoiled contains an excess of helical turns. These differences can be quantified in terms of linking number (Lk); this is the number of times that two DNA strands of a covalently closed DNA molecule are intertwined. The linking number can be changed by breaking one or both strands of DNA, rotating the DNA strands relative to one another and re-ligating both ends to seal (Higgins and Vologodskii, 2004). This is the mechanism of action used by enzymes called topoisomerases (described below). The linking number is the sum of the number of twists (Tw) and writhes (Wr) in a DNA molecule;

$$Lk=Tw+Wr \text{ (Travers and Muskhelishvili, 2005)}$$

Tw describes the number of times one DNA strand crosses the other in a duplex and Wr describes the number of times the DNA helix is coiled around itself (Travers and Muskhelishvili, 2005). Superhelical density (σ) is the ratio of linking difference (ΔLk) of a supercoiled molecule relative to that of a relaxed molecule under identical conditions (Higgins *et al.*, 2010). The superhelical density of a molecule can be measured by electrophoresis; the migration of DNA depends on the level of superhelicity (Higgins *et al.*, 2010). The DNA is separated into bands depending on the linking number. Therefore these bands differ by their topology and are referred to as topoisomers. Superhelical density can also be measured by ethidium bromide titration in sucrose gradients. In this method the more supercoiled molecules sediment faster compared to relaxed DNA (Higgins *et al.*, 2010). DNA molecules of different superhelicity can also be visualised by electron microscopy (Higgins *et al.*, 2010).

Maintaining DNA in an underwound state imparts torsional tension to the DNA molecule that can assist in strand separation and favours biological processes such as transcription, replication and recombination (reviewed by Dorman, 1994). DNA supercoiling is regulated by enzymes called topoisomerases. Topoisomerases that break both strands of DNA are called type II enzymes and those which break one strand are called type I enzymes. The best understood topoisomerases are DNA gyrase (TopoII) encoded by *gyrA* and *gyrB* and topoisomerase I (encoded by *topA*) (reviewed by Drlica and Zhao, 1997). DNA gyrase is a type II enzyme that introduces negative supercoils into relaxed DNA by a double stranded break, passage and religation mechanism that requires ATP; it removes positive supercoils in the same manner (Gellert *et al.*, 1976a; Hsieh *et al.*, 1991a; Hsieh *et al.*, 1991b). DNA topoisomerase I (Topo I) is a type I enzyme that relaxes DNA by removing negative supercoils by a single-stranded break in the DNA and uses energy stored in the supercoiled molecule to relax the DNA (Cozzarelli, 1980; DiNardo *et al.*, 1982; Pruss *et al.*, 1982). Topo IV is the second type II enzyme, this enzyme works with Topo I to avoid hyper-supercoiling in the DNA (Menzel and Gellert, 1983). Hyper-supercoiling can result in the formation of R-loops, cruciform or H-DNA. These formations are fundamentally different from B-DNA and can have aberrant affects on cellular processes such as DNA replication and transcription (Higgins and Vologodskii, 2004).

Evidence that DNA supercoiling can affect transcription stems from research monitoring gene expression *in vivo* in the presence of antibiotics that can inhibit the topoisomerases (e.g. novobiocin; inhibits DNA gyrase), or with mutants in the genes encoding the topoisomerases (Gellert *et al.*, 1976b; Pruss *et al.*, 1982; Pruss and Drlica, 1986; Pruss *et al.*, 1986). Furthermore, the expression of the genes encoding the topoisomerases are sensitive to supercoiling. Studies have shown DNA relaxation results in increased expression of *gyrA* and *gyrB* and a decrease in *topA* expression (Menzel and Gellert, 1983; Tse-Dinh, 1985). A genome-wide study using microarrays identified 306 genes that were supercoiling-sensitive in *E. coli* (Peter *et al.*, 2004).

DNA supercoiling levels vary throughout bacterial growth as DNA relaxes with the onset of stationary phase due to metabolic flux in the cell and the reduction of ATP level (Balke and Gralla, 1987; Dorman *et al.*, 1988). As DNA gyrase is an ATPase, this can directly affect the activity of DNA gyrase and in turn alter DNA supercoiling (Hsieh *et al.*, 1991a; Hsieh *et al.*, 1991b). Furthermore, studies have shown changes in environmental stimuli (e.g. temperature, pH, oxygen levels and osmolarity) affect DNA supercoiling levels (Goldstein and Drlica, 1984; Dorman *et al.*, 1988; Higgins *et al.*, 1988; Karem and Foster, 1993). Thus, DNA supercoiling is a global regulator of gene expression; changes in environmental conditions can be relayed throughout the genome to supercoiling sensitive genes by altering DNA supercoiling levels (Dorman, 1991; Dorman, 2006). At the local level, transcription itself can affect DNA supercoiling; movement of RNA polymerase during transcription results in the accumulation of positive supercoils ahead of the transcription apparatus and negative supercoils behind the transcribing polymerase (Liu and Wang, 1987). These transcription-induced changes in local supercoiling can affect the expression of neighbouring genes (Pruss and Drlica, 1989). Transcription factors can also contribute to regulation of gene expression by supercoiling. Both IHF and Fis have demonstrated roles in assisting the transmission of superhelical energy to promoters and activating transcription (Sheridan *et al.*, 1998; Opel *et al.*, 2004), discussed in the following sections.

1.7.2 Integration Host Factor (IHF)

The IHF protein was originally described as a host factor required for the integration of phage λ into the bacterial chromosome (Miller and Nash, 1981). It is now known that

IHF plays a role in DNA replication (Ryan *et al.*, 2002), recombination (Bushman *et al.*, 1985), transposition (Crellin *et al.*, 2004) and has a global effect on gene transcription in both *E. coli* (Arfin *et al.*, 2000) and *S. Typhimurium* (Mangan *et al.*, 2006). The heterodimeric IHF is formed by the α and β subunits encoded by the unlinked genes *ihfA* (*himA*) and *ihfB* (*himD* or *hip*) respectively. Each subunit is ~11 kDa in size and share ~30% amino acid identity (Nash *et al.*, 1987). The IHF heterodimer is the predominant form. However, IHF can also exist as a homodimer which binds DNA with a lower affinity and these homodimers are functional *in vivo* (Goosen and van de Putte, 1995; Mangan *et al.*, 2006). Analysis of intracellular IHF levels show at the onset of stationary phase (Bushman *et al.*, 1985); evidence using immunoblotting suggest IHF is present at approximately 8,500-17,000 dimers per cell in exponential phase and 5 times higher in stationary phase (Bushman *et al.*, 1985; Ditto *et al.*, 1994).

IHF is a site-specific DNA binding protein that binds to the minor groove of DNA and recognises the consensus sequence (W)ATCAANNNTT(R) this is often accompanied by an upstream A/T rich region (Hales *et al.*, 1994). The crystal structure of IHF binding to DNA has been solved (Fig. 1.9, A) (Rice *et al.*, 1996); IHF binds DNA by a mechanism known as indirect readout, where protein-DNA complexes are affected by the sequence-dependent alterations in the conformation of the non-contacted bases in the binding site (Aeling *et al.*, 2006). Hydrophobic residues located on the two extending ribbon 'arms' of IHF intercalate into the minor groove of DNA causing a severe U-turn in the DNA of up to 180° (Fig. 1.9, A) (Rice *et al.*, 1996). IHF remodels DNA at local levels, but it is also considered as an important architect of chromatin topology on a global scale. Specifically, IHF is thought to assist in condensing the chromosome during the onset into stationary phase (Ali *et al.*, 2001; Sarkar *et al.*, 2009).

This IHF-induced architectural change in DNA is thought to facilitate transcriptional activation; for example, IHF has been shown to bind at σ^{54} dependent promoters in different bacterial species (Claverie-Martin and Magasanik, 1991; Macchi *et al.*, 2003). σ^{54} dependent transcription requires an activator to initiate transcription, these activators usually bind 80-150 bp upstream of the promoter (reviewed by Bush and Dixon, 2012). The DNA must bend in order to facilitate contact between the activator and the RNAP σ^{54} holoenzyme this can be facilitated by IHF. Studies have shown IHF sites can be

functionally replaced by intrinsically curved DNA (Molina-López *et al.*, 1994) this demonstrates IHF plays an architectural role in facilitating gene expression (Goosen and van de Putte, 1995). IHF transcriptional activity has also been demonstrated at σ^{70} -dependent promoters such as the *narGHJI* operon where IHF acts as positive regulator (Schroder *et al.*, 1993) and the *ompR* gene in *E. coli* where IHF acts as a negative regulator (Tsui *et al.*, 1991).

IHF also facilitates transcription by a more uncommon mechanism as observed at the *ilvP_G* promoter in *E. coli* (Sheridan *et al.*, 1998). The *ilvP_G* promoter is activated by protein-mediated translocation of superhelical energy. Specifically, Sheridan *et al.* (1988) identified a region in the promoter that was predicted to undergo strand separation when placed under superhelical stress referred to as a SIDD site (stress-induced duplex destabilisation) sites. IHF binds downstream of the SIDD site strand separation in this region, instead IHF binding transmits the superhelical energy in the SIDD site to the next susceptible region, the -10 element in the promoter, leading to enhanced open complex formation (Sheridan *et al.*, 1998).

1.7.3 cAMP receptor protein (CRP)

The cAMP receptor protein CRP, also known as the catabolite activator protein (CAP) was the first transcription factor to be purified and have its structure solved (McKay and Steitz, 1981). CRP is a homodimeric protein that requires an allosteric effector cAMP (cyclic adenosine monophosphate) to bind DNA. cAMP binds in a hydrophobic pocket located in the N-terminal domain, this induces a conformational change and exposes a HTH motif located in the C-terminal domain (Popovych *et al.*, 2009). In *E. coli*, CRP binds to a 22-bp consensus sequence composed of two symmetrical 11bp half sites (consensus half site A₁A₂A₃T₄**G**₅T₆**G**₇**A**₈T₉C₁₀T₁₁; CRP specifically interacts with the nucleotides in bold) (Ebright *et al.*, 1989). CRP binding to DNA introduces an 80-90° kink in the DNA structure (Fig. 1.8, B) (Schultz *et al.*, 1991).

CRP can act as both an activator and a repressor of gene expression but is best known as an activator of genes in response to glucose starvation (Zheng *et al.*, 2004). This is mediated through intracellular levels of cAMP; in the absence of glucose increased activity of adenylate cyclase leads to an increase in cAMP levels. This in turn activates

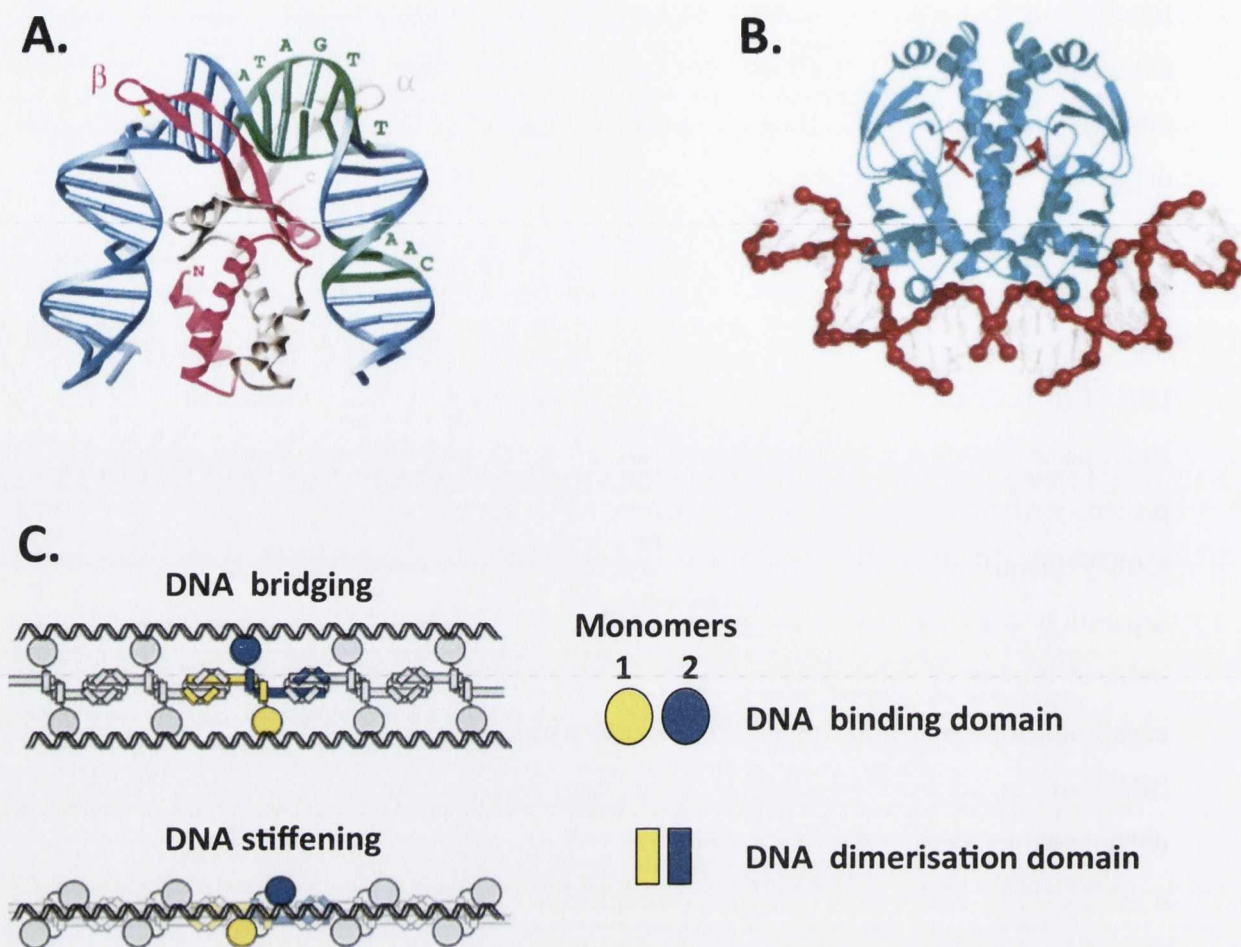


Fig. 1.8. The DNA binding proteins IHF, CRP and H-NS. **A.** Binding of the IHF dimer (pink and grey) to DNA introduces $\sim 180^\circ$ bend into the DNA (blue and green) (Rice *et al.*, 1996). **B.** CRP dimer (blue) with the allosteric effector cAMP (embedded in CRP dimer and highlighted in red) introduce $\sim 80\text{-}90^\circ$ bend into DNA (red) (Lawson *et al.*, 2004). **C.** Diagram illustrating the H-NS (yellow and blue) binding DNA in a bridging and stiffening mode (Ali *et al.*, 2012).

CRP into a DNA-binding functional form which in turn activates genes for transport and metabolism of alternative carbon sources (reviewed by Deutscher, 2008; Gorke and Stulke, 2008). CRP is also involved in other processes such as biofilm formation, competence and iron uptake (Jackson *et al.*, 2002; Zhang *et al.*, 2005; Sinha *et al.*, 2009) and virulence in *S. Typhimurium* (Curtiss *et al.*, 1988).

CRP can recruit RNAP to target promoters where it is capable of making direct contact with RNAP (Busby and Ebright, 1999). Promoters that are activated by CRP are categorised into three groups called class I, class II and class III promoters (Busby and Ebright, 1999). At class I-dependent promoters, the CRP binding site is centred -93, -83, -72 or -62 relative to the transcription start site (Tebbutt *et al.*, 2002). At class II dependent promoters, the CRP-binds near -42 relative to the transcription start. The AR1 (activating region 1; residues 154-164) of CRP makes contact with the α -CTD of RNAP at both class I and II promoters (Benoff *et al.*, 2002). At class II promoters CRP makes additional contact with the α -NTD via the AR2 (residues 19, 21, 96) and AR3 (residues 52-55) sites (Lawson *et al.*, 2004). Class III-dependent promoters contain multiple CRP binding sites or a CRP binding site and additional activators (Beatty *et al.*, 2003; Barnard *et al.*, 2004).

CRP is thought to regulate transcription of ~200 genes (Zheng *et al.*, 2004). Two independent bioinformatic searches for CRP binding sites identified 161 and 220 high affinity CRP sites, however both studies predict different numbers of low affinity sites, 285 and ~10,000 respectively (Robison *et al.*, 1998; Tan *et al.*, 2001). The identification of thousands of low affinity sites by Robinson *et al.* (1998) is supported by a Chromatin immunoprecipitation (ChIP) and microarray (chip) (ChIP-chip) study that identified CRP binds to 68 defined CRP sites in addition to thousands of weak CRP binding sites throughout the chromosome (Grainger *et al.*, 2005). Importantly, binding to these targets was lost when cells were grown in the presence of glucose (recall glucose lowers intracellular cAMP levels and inactivates CRP). Grainger *et al.* (2005) suggest the weaker affinity sites scattered throughout the genome may be the result of CRP forming higher-order structures in the chromosome, thus suggesting CRP may possess NAP-like features.

1.7.4 Histone-like nucleoid structuring protein (H-NS)

H-NS is a small (~15 kDa) DNA binding protein that is present at ~20,000 copies per cell. H-NS is composed of an N-terminal DNA binding domain and a C-terminal oligomerisation domain connected by a flexible linker (Ueguchi *et al.*, 1996; Ali Azam *et al.*, 1999; Rimsky, 2004). In solution H-NS exists as a dimer, however studies have shown H-NS can form higher order oligomers with additional H-NS molecules (Smyth *et al.*, 2000; Arold *et al.*, 2010) and H-NS-like proteins (Deighan *et al.*, 2003; Castang and Dove, 2010).

DNase I footprints showed H-NS protects regions of AT rich DNA (Lucht *et al.*, 1994; Tupper *et al.*, 1994). Subsequent ChIP-on-chip studies performed in *E. coli* and *S. Typhimurium* have shown H-NS binds to AT rich sequences and generally acts as a repressor of transcription (Grainger *et al.*, 2006; Lucchini *et al.*, 2006; Navarre *et al.*, 2006; Oshima *et al.*, 2006). These studies also revealed H-NS selectively silences horizontally acquired DNA in *S. Typhimurium*, which is characterised by its high AT content. Newly acquired DNA has the potential to reduce bacterial fitness. H-NS binds and silences expression of genes on incoming horizontally acquired DNA. In this regard H-NS is regarded as a 'genome sentinel' that silences incoming DNA and 'protects' the fitness level of the bacterium (Dorman, 2007).

Oligomerisation of H-NS is important for mediating repression of gene expression; H-NS proteins that cannot oligomerise are defective for repression (Rimsky *et al.*, 2001). Single molecule experiments using atomic force microscopy and magnetic tweezers have revealed two modes of DNA bindings for H-NS; DNA bridging (Dame *et al.*, 2000) and DNA 'stiffening' (Amit *et al.*, 2003), respectively (see Fig. 1.8, C). The abundance of H-NS coupled with the ability to form DNA bridges supports the hypothesis that H-NS is involved in structuring the chromosome by forming topological barriers called domains (Hardy and Cozzarelli, 2005). Locally, DNA bridging between different sites on the same DNA molecule can act to trap or occlude RNAP from promoters, thereby repressing transcription. DNA 'stiffening' was observed in single molecule experiments with magnetic tweezers; this study found H-NS binding to λ DNA resulted in lengthening of the DNA molecule (Amit *et al.*, 2003). This mechanism involves initial binding of H-NS to DNA followed by a lateral spread along the DNA. A recent study shows H-NS mutants that are unable to repress gene expression are also

unable to form these rigid structures (Lim *et al.*, 2012). An additional study with the SPI-2 activator SsrB, found SsrB could displace H-NS bound in the rigid ‘stiffening’ mode but was unable to displace H-NS in its bridging form (Walthers *et al.*, 2011). These results indicate H-NS polymerisation along DNA is an important repressive mechanism. Although silencing of newly acquired genes by H-NS can initially confer a benefit to host fitness, in order to benefit from these genes in specific environmental conditions efficient displacement of H-NS is required. Comprehensive reviews of the mechanisms used by bacteria to derepress H-NS silenced genes can be found elsewhere (Stoebel *et al.*, 2008; Dorman and Kane, 2009).

1.7.5 Factor for inversion stimulation (Fis)

The Fis protein is a small (~11 kDa) homodimeric DNA binding protein that was originally identified for its role in stimulating site-specific inversion systems catalysed by recombinases of the invertase family (Johnson *et al.*, 1986). Fis binds to a 17 bp AT rich consensus sequence, with preferences for a G and C residue at position 2 and 16 respectively (Cho *et al.*, 2008). DNA binding occurs through the HTH motif in each monomer and introduces a bend between 50 and 90°. DNA bending is an important feature for transcriptional regulation and genome structuring by Fis (Pan *et al.*, 1996; Skoko *et al.*, 2006). Intracellular levels of Fis vary throughout growth; Fis levels are high in early exponential phase (60,000 molecules per cell) and decline rapidly thereafter (Nilsson *et al.*, 1990; Ball *et al.*, 1992; Ali Azam *et al.*, 1999).

Fis can act as a global regulator of transcription and genome organisation. Transcriptomic analysis in *S. Typhimurium* and *E. coli* revealed Fis regulates a number of genes including those involved in metabolism and virulence (Kelly *et al.*, 2004; Bradley *et al.*, 2007). ChIP-chip analysis performed in *E. coli* identified Fis binds throughout the chromosome (Cho *et al.*, 2008). Specifically, 894 sites were identified with 33% of these sites localised within coding regions supporting the view that Fis is involved in genome structure (Cho *et al.*, 2008). Fis binds in the upstream activating sequences (UAS) of stable RNA promoters (rRNA and tRNA) and facilitates recruitment of RNAP to promoters. Fis is also capable of making direct contact with the α -CTD of RNAP (McLeod *et al.*, 2002), and in a similar mechanism described for IHF

(section 1.7.2) the Fis protein has been shown to displace superhelical energy in the SIDD site at the *leuV* promoters (Opel *et al.*, 2004).

Fis also can act as a repressor of transcription and a modulator of DNA supercoiling; Fis is a repressor of *gyrA* and *gyrB* that encode the A and B subunits of the topoisomerase DNA gyrase respectively (Schneider *et al.*, 1999). Furthermore, Fis both positively and negatively affects expression of the *topA* gene, which codes for topoisomerase I and is responsible for removing negative supercoils (Weinstein-Fischer and Altuvia, 2007). Expression of the *fis* gene is induced by negative supercoiling; this reflects the pattern of Fis expression, peaking in early exponential phase when DNA is negatively supercoiled (Schneider *et al.*, 2000). This peak in protein levels places the Fis protein in a good position to regulate expression of stable RNA promoters which are required during exponential growth when the demand for protein synthesis is high (Rochman *et al.*, 2004; Dillon and Dorman, 2010).

1.7.6 *ompR* regulation in *E. coli* and *S. Typhimurium*

Studies performed in *E. coli* have shown IHF and CRP regulate *ompR* expression (Table 1.4). EMSA analysis and DNase I footprinting showed IHF binds to three sites in the *ompR* promoter (IHF-I, IHF-II and IHF-III in Fig. 1.9, A) (Tsui *et al.*, 1991). Analysis of *ompR* transcription *in vitro* revealed IHF repressed expression via the IHF-I site by preventing RNAP binding to the promoter (Tsui *et al.*, 1991). This repressive effect of IHF on *ompR* expression was also demonstrated *in vivo* using *lacZ* transcriptional fusions to measure *ompR* expression in different growth media. An additional performed in *E. coli* study showed CRP binds to a single site in the *ompR* promoter and acts as both an activator and repressor of transcription (Fig. 1.9, A) (Huang *et al.*, 1992). CRP repressed expression from two transcription start sites (TSS-4 and TSS-1) in close proximity to the CRP binding site and activated the expression of two distally located transcription start sites (TSS-2 and TSS-3) (Huang *et al.*, 1992). Measurement of *ompR* expression *in vivo* revealed CRP had an overall positive affect on transcription (Huang *et al.*, 1992).

As described earlier (section 1.6.8) *ompR* expression in *S. Typhimurium* is induced at low pH and by DNA relaxation (Bang *et al.*, 2002). Bang *et al.* have shown *ompR*

expression at pH 4.4 is primarily from TSS-3 (see Fig. 1.9, B). TSS-1 was constitutively active at both pH 8 and pH 4.4 (Bang *et al.*, 2002). Studies have identified OmpR binds to four sites in the *ompR* promoter and positively affects *ompR* expression (Table 1.4 and Fig. 1.9, B) (Bang *et al.*, 2002; Cameron and Dorman, 2012). The H-NS protein represses *ompR* expression. Bang *et al.* found *ompR* expression was de-repressed at pH 7 in a *hns* mutant but no difference in expression was observed at pH 4.5 compared to wild type (Bang *et al.*, 2002). Thus, indicating H-NS represses *ompR* at pH 7 and shifting cells to pH 4.5 relieves H-NS mediated repression (Bang *et al.*, 2002). *ompR* expression increased with novobiocin treatment (relaxes the DNA) and OmpR was required to mediate this positive affect (Bang *et al.*, 2002). Thus, the OmpR protein and DNA relaxation are required to activate *ompR* expression and may alleviate H-NS mediated repression (Bang *et al.*, 2002). A separate study found the Fis protein binds and activates *ompR* expression (Cameron and Dorman, 2012). Detailed analysis of *ompR* expression in response to DNA relaxation has revealed a reciprocal relationship between promoter occupancy by OmpR and Fis. Specifically, Fis occupancy of the *ompR* promoter decreased as DNA became more relaxed with a concomitant increase in OmpR (Cameron and Dorman, 2012). H-NS occupancy was unaffected by DNA relaxation, but it is thought that H-NS oligomers are re-structured to facilitate expression (Cameron and Dorman, 2012). Importantly, the location of OmpR binding sites in the *ompR* promoter of *S. Typhimurium* have been described. In contrast, the location of Fis and H-NS binding is unknown. To date, ChIP analysis has reported both proteins bind to the *ompR* promoter in *S. Typhimurium* but the precise location has not been described (Cameron and Dorman 2012).

1.7.7 Post-transcriptional regulation by sRNAs OmrA and OmrB

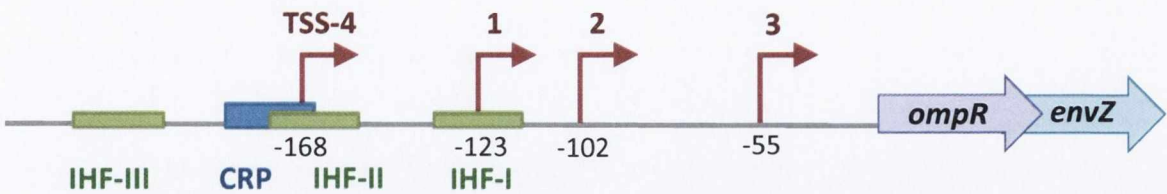
ompR expression is post-transcriptionally regulated by the sRNAs OmrA and OmrB in *S. Typhimurium* and *E. coli* (Vogel and Papenfort, 2006). Both sRNAs are tandemly encoded in the *aas-galR* intergenic region and have a highly similar 5' and 3' regions OmrA (88 nt) is expressed in stationary phase while OmrB (82 nt) is transiently expressed in early stationary phase (Vogel and Papenfort, 2006). Microarray analysis in *E. coli* strains that overexpressed either *omrA* or *omrB* resulted in a 2-fold decrease in *ompR* and *envZ* expression (Guillier and Gottesman, 2006). These results indicated both OmrA and OmrB down-regulate *ompR* and *envZ* expression (Guillier and Gottesman

Table 1.4. Regulators acting at the *ompR* promoter.

Regulator	<i>E. coli</i>	<i>S. Typhimurium</i>	Reference
IHF	-	NT	(Tsui <i>et al.</i> , 1991).
CRP	+/-	NT	(Huang <i>et al.</i> , 1992)
OmpR	NT	+	(Bang <i>et al.</i> , 2002)
Fis	NT	+	(Cameron and Dorman 2012)
H-NS	NT	-	(Bang <i>et al.</i> , 2002)

+, positive regulator, -, negative regulator, NT; not tested to date

A. *E. coli*



B. *S. Typhimurium*

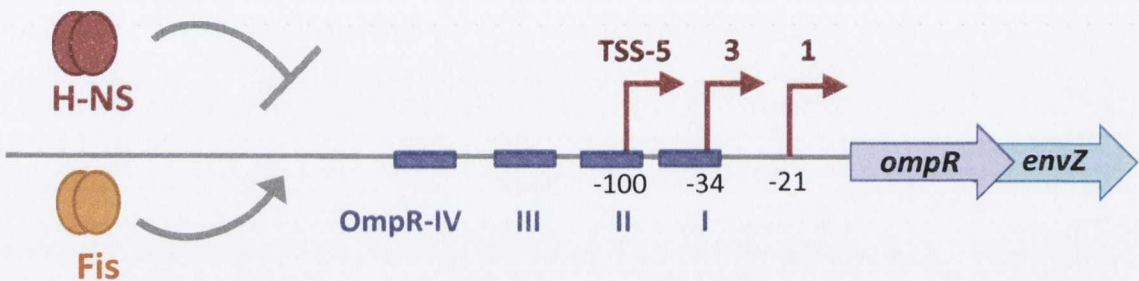


Fig. 1.9. Diagram of regulators acting at the *ompR* promoter. **A.** The *ompR* promoter in *E. coli*. There are four transcription start sites (TSS) reported in the *ompR* promoter of *E. coli* (Mizuno *et al.*, 1982; Wurtzel *et al.*, 1982; Tsui *et al.*, 1991; Huang *et al.*, 1992). These are denoted by a bent arrow and their location upstream of the open reading frame is shown (ATG; +1). The IHF protein binding sites are shown in green (Tsui *et al.*, 1991). The CRP binding site is shown in blue (Huang *et al.*, 1992). **B.** The *ompR* promoter in *S. Typhimurium*. There are five transcription start sites (TSS) reported in the *ompR* promoter of *S. Typhimurium* (Liljestrom *et al.*, 1988; Bang *et al.*, 2002), three are shown. TSS-2 and -4 are mapped to -32 and -36 respectively. Their close proximity to TSS-3 may indicate stuttering of the RNA polymerase; thus, transcripts from TSS-2, -3 and -4 may be the product from a single TSS. OmpR binds to four binding sites (Bang *et al.*, 2002). These are shown in purple. H-NS is a negative regulator of *ompR* expression and Fis is a positive regulator (Bang *et al.*, 2002; Cameron and Dorman 2012.). These proteins bind to the *ompR* promoter, however their location is unknown.

2006). Other OmrA/B targets identified include *cirA*, *fepA* and *fecC* (Fig. 1.10, B) (Guillier and Gottesman, 2006). Bioinformatic analysis revealed the 5' region of these sRNAs target the ribosome binding sequence of *ompR* (Fig. 1.10, A) (Guillier and Gottesman, 2008). Expression of these sRNAs is induced at high osmolarity and is positively affected by OmpR (Guillier and Gottesman, 2006); thus, presenting a negative feedback loop whereby the induction of OmrA/B by OmpR leads to a reduction in *ompR* translation (Fig. 1.10, B) (Guillier and Gottesman, 2008).

1.8 Members of the OmpR regulon

OmpR has a well-characterised role in regulating expression of the porins *ompF* and *ompC* in response to osmolarity (Alphen and Lugtenberg, 1977; Sarma and Reeves, 1977). However, OmpR regulates additional genes with various functions in both *E. coli* and *S. Typhimurium*. Previously identified OmpR targets are listed in Table 1.5. These include regulators of curli fimbriae (*csgD*) that are important for biofilm formation. In *E. coli* OmpR has been shown to activate expression of *csgD* expression (Gerstel *et al.*, 2003; Ogasawara *et al.*, 2010), whereas in *S. Typhimurium* OmpR acts as both an activator and repressor *csgD* (Gerstel *et al.*, 2003). OmpR also regulates the expression of *tppB* that encodes a tripeptide permease in both *E. coli* and *S. Typhimurium*. The *tppB* gene is induced under anaerobic conditions in *S. Typhimurium* and is dependent on OmpR (Jamieson and Higgins, 1984; Jamieson and Higgins, 1986; Gibson *et al.*, 1987). In *E. coli* expression of *tppB* also requires OmpR, however, *tppB* is expressed in aerobic conditions and only modestly induced in in anaerobic conditions (Oshima *et al.*, 2002; Goh *et al.*, 2004). Expression of *flhDC*, the principal regulator of flagellar biosynthesis, is repressed by OmpR in *E. coli* (Shin and Park, 1995). Studies in *S. Typhimurium* demonstrated no role for OmpR in *flhDC* expression (Kutsukake, 1997). Additional genes such as *fadL* involved in uptake of fatty-acids and *bolA* for normal cell morphology are regulated by OmpR in *E. coli* but no role has been defined in *S. Typhimurium* (Higashitani *et al.*, 1993; Yamamoto *et al.*, 2000). Global transcriptomic analysis performed using an $\Delta ompR/envZ$ mutant in *E. coli* grown in LB medium during exponential phase of growth. This study identified 125 genes with altered expression patterns (Oshima *et al.*, 2002). Thus indicating OmpR has a global affect on gene expression, however it is unknown how many of these genes are

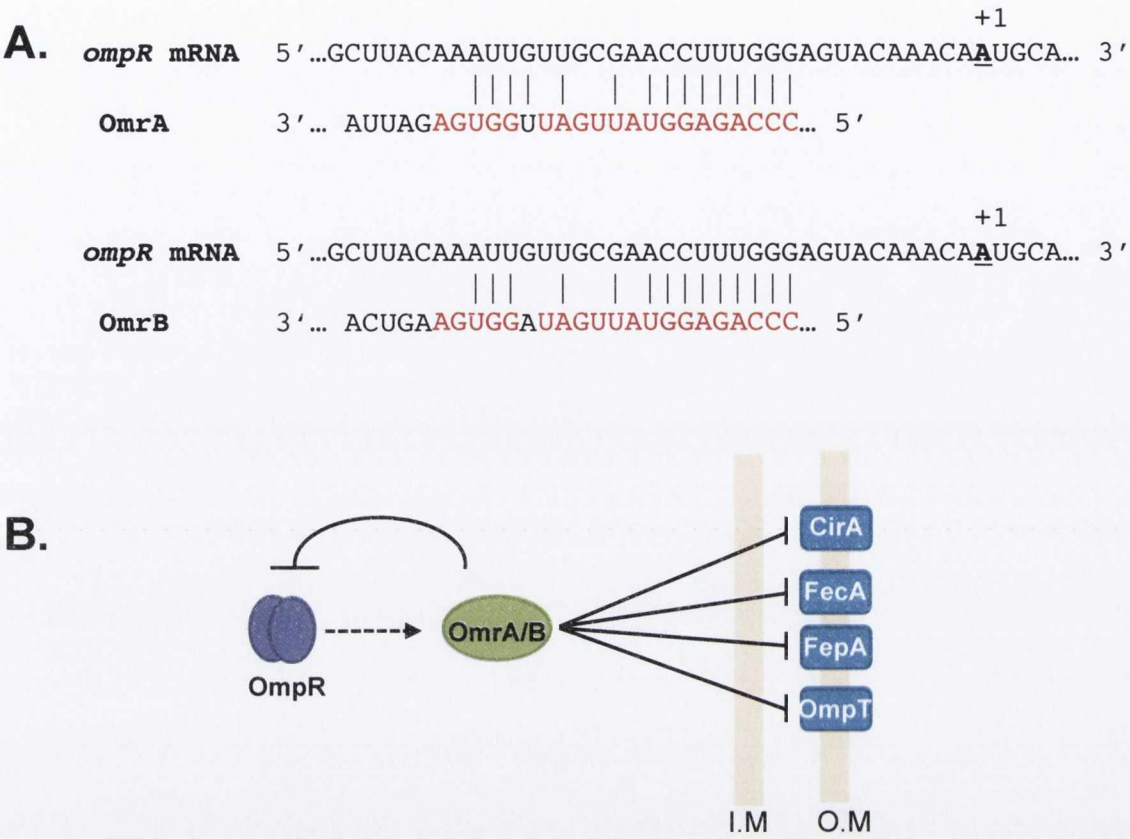


Fig. 1.10. Post-transcriptional regulation of *ompR* by sRNAs OmrA and OmrB. (A) Predicted base-pairing between *ompR* mRNA and OmrA/B. Nucleotides in red are conserved between OmrA and OmrB. The start codon is in bold and underlined (**A**UG; +1) (adapted from Guillier and Gottesman, 2008). (B) Diagram illustrating the negative feedback loop of OmrA/B and OmpR. Additional OmrA/B targets are shown. I.M: inner membrane, OM: outer membrane (adapted from Papenfort and Vogel, 2009).

Table 1.5. Previously identified OmpR targets in *E. coli* and *S. Typhimurium*.

OmpR target	<i>E. coli</i>	<i>S. Typhimurium</i>	Reference
<i>ompF</i>	+	+	(Sarma and Reeves, 1977; Hall and Silhavy, 1981c; Hall and Silhavy, 1981a; Mills <i>et al.</i> , 1998)
<i>ompC</i>	+	+	(Sarma and Reeves, 1977; Hall and Silhavy, 1981c; Hall and Silhavy, 1981a; Mills <i>et al.</i> , 1998)
<i>micF</i>	+	+	(Mizuno <i>et al.</i> , 1984; Delihias and Forst, 2001; Javayel <i>et al.</i> , 2011)
<i>micC</i>	+	+	(Chen <i>et al.</i> , 2004; Pfeiffer <i>et al.</i> , 2009)
<i>fadL</i>	+	NT	(Higashitani <i>et al.</i> , 1993)
<i>flhDC</i>	+	-	(Shin and Park, 1995; Kutsukake, 1997)
<i>tppB</i>	+	+	(Gibson <i>et al.</i> , 1987; Goh <i>et al.</i> , 2004)
<i>csgDEFG</i>	+	+	(Gerstel <i>et al.</i> , 2003; Ogasawara <i>et al.</i> , 2010)
<i>ssrA</i>	NP	+	(Lee <i>et al.</i> , 2000)
<i>hilC</i>	NP	+	(Cameron and Dorman, 2012)
<i>hilD</i>	NP	+	(Cameron and Dorman, 2012)
<i>mcbABCD</i>	+	NT	(Hernandez-Chico <i>et al.</i> , 1982; Connell <i>et al.</i> , 1987)
<i>emrAB</i>	+	N	(Hirakawa <i>et al.</i> , 2003)
<i>bolA</i>	+	NT	(Yamamoto <i>et al.</i> , 2000)
<i>nmpC</i>	+	-	(Dorman <i>et al.</i> , 1989; Coll <i>et al.</i> , 1994)
<i>sra</i>	+	NT	(Izutsu <i>et al.</i> , 2001)
<i>omrA</i>	+	+	(Guillier and Gottesman, 2006; Guillier and Gottesman, 2008)
<i>omrB</i>	+	+	(Guillier and Gottesman, 2006)
<i>ecnB</i>	+	NT	(Yamamoto <i>et al.</i> , 2000)
<i>ompS1</i>	NT	+	(Flores-Valdez <i>et al.</i> , 2003)

+, a regulatory role for OmpR has been reported, NP; genes are not present, NT; not tested.

directly regulated by OmpR. Furthermore, microarray analysis in *E. coli* reveals OmpR affects a large number of genes with various functions in the cell (Oshima *et al.*, 2002). However, the extent of OmpR binding throughout the genomes of both *E. coli* and *S. Typhimurium* is currently unknown. In *S. Typhimurium* OmpR plays a key role in pathogenesis, *ompR* mutants are attenuated for virulence. OmpR is an activator of the *ssrA/B* locus which is responsible for expression of virulence genes encoded within SPI-2 (Lee *et al.*, 2000; Feng *et al.*, 2004). Furthermore, OmpR is an activator and a repressor of SPI-1 regulators *hilC* and *hilD* respectively (Cameron and Dorman, 2012). Based on the functions of the genes described here it is evident OmpR regulates a variety of genes.

1.9 Aims of this study

ompR in *S. Typhimurium* is not osmoreponsive like its *E. coli* counterpart. Instead *ompR* is activated by low pH (Bang *et al.*, 2002). This prompts the question does *ompR* in *E. coli* respond to low pH in a similar manner?

Evidence available shows IHF and CRP are known to target the *ompR* promoter in *E. coli* (Huang *et al.*, 1990; Huang *et al.*, 1992). Furthermore evidence available has shown both OmpR, H-NS and Fis target the *ompR* promoter in *S. Typhimurium* (Bang *et al.*, 2002; Cameron and Dorman, 2012). To date, the role of IHF and CRP at *ompR* in *S. Typhimurium* has not been tested.

Finally, it is evident that OmpR affects the expression of a number of genes in both *E. coli* and *S. Typhimurium*. Most of our current understanding of the OmpR regulon stems from studying the effect of OmpR on single genes, however it is evident from microarray analysis in *E. coli* that OmpR affects a large number of genes. Therefore, the aims of this thesis were to further our current knowledge on the effect of low pH on *ompR* expression and advance our understanding of OmpR function in both *S. Typhimurium* and *E. coli*. With this in mind specific the aims of this thesis were as follows:

1. To conduct comparative analysis of *ompR* expression in both *S. Typhimurium* and *E. coli* at low pH.

2. To investigate the contribution of IHF and CRP on *ompR* expression in *S. Typhimurium* and to identify important regulators required for OmpR expression at low pH.
3. To map genome wide OmpR binding in both *S. Typhimurium* and *E. coli* at low pH.
4. Having identified members of the OmpR regulon for aim 3, to then identify both common and unique OmpR targets in both species.

Chapter 2 Materials and Methods

2.1 Chemicals and enzymes

All chemicals and reagents used in this study were purchased from Ambion, Bio-Rad, Difco, Invitrogen, PALL, Thermo-Scientific, Promega, Qiagen, Roche Molecular Biochemicals, Sigma Chemical Company or Stratagene unless otherwise stated. In addition to the companies listed above, DNA modifying enzymes and restriction enzymes were purchased from New England Biolabs. The molecular biology kits used in this study are described below without detailing the complete protocol provided by the manufacturer.

2.2 Growth media and conditions

2.2.1 Standard methods

Materials for preparing standard growth media were obtained from Difco (BD Diagnostic systems). All media were sterilised by autoclaving at 120°C for 20 min. All quantities listed below are for a 1 litre preparation of medium using deionised water (ddH₂O).

Lennox broth and L-agar plates

LB (Lysogeny broth) media is routinely used for culturing of *Salmonella* and *E. coli* and is a nutrient-rich broth. Throughout this study a low salt variation of LB broth termed Lennox broth (L-broth) was used (Lennox, 1955) and recipes are described in Table 2.1. Bacteria were routinely cultured in 4 ml of L-broth and grown at 37°C and 200 rpm for 18 h unless otherwise stated. L-agar plates were routinely used for culturing of bacterial strains and they were prepared by the addition of 15 g of agar per 1 litre of medium prior to autoclaving. Antibiotics were added as described in Table 2.2.

Table 2.1. Recipe for LB and L-broth

	LB	L-broth
Bacto-tryptone	10 g/l	10 g/l
Yeast extract	5 g/l	5 g/l
NaCl	10 g/l	5 g/l

Aqueous solutions that could be damaged by autoclaving e.g. amino acids, antibiotics, and carbon sources, were filter-sterilised using 0.22 µm SupaTop Syringe Filters (Anachem).

2.2.2 Antibiotics

Antibiotics were made as 1000X stocks and aliquoted into 1 ml volumes, stored at -20°C and used at the final concentration listed below in Table 2.2.

Table 2.2. Antibiotics used in this study

Antibiotic	Solvent	Final concentration
Carbenicillin	H ₂ O	100 µg/ml
Chloramphenicol	Ethanol	20 µg/ml
Kanamycin	H ₂ O	50 µg/ml
Rifampicin*	Methanol	150 µg/ml

*Rifampicin was made fresh each time prior to use and kept in the dark.

2.2.3 Minimal media

E-minimal medium (Vogel and Bonner, 1956) was used as a chemically-defined medium for all studies investigating the effect of pH change. E-minimal medium was routinely made up as a 5X stock as detailed in Table 2.3 and autoclaved. 1X E-minimal medium was supplemented with glucose unless otherwise stated. L-histidine was added to facilitate growth of SL1344, while proline and thiamine were used to facilitate growth of CSH50. 1X stock for growth of bacterial strains was prepared as described in Table 2.4.

Table 2.3. Recipe for 5X E-minimal medium

5X E-minimal	g/l	Final concentration
MgSO ₄ .7H ₂ O	1	4.06 mM
Citric acid.H ₂ O	10	47.59 mM
K ₂ HPO ₄ anhydrous	50	287.06 mM
NaNH ₄ HPO ₄ .4H ₂ O	17.5	83.7 mM

Table 2.4. Recipe for 1 litre of 1X EG-minimal medium

<u>1X EG-minimal</u>	<u>ml</u>	<u>Final concentration</u>
5X E-minimal	200	1X
40% (w/v) Glucose	10	0.4 %
200 mM L-histidine	2.5	0.5 mM
1 mg/ml Thiamine	1	1 µg/ml
100 mg/ml Proline	1	100 µg/ml
ddH ₂ O	78.75	

1X EG minimal medium was then filter sterilised using a Fast PES filter unit (Nalgene, Thermo-scientific) with a 0.22-µm pore size. When necessary, 1X EG minimal medium was acidified from pH 7.2 to pH 4.5 using 3.7 M HCl and filter sterilized using 0.22 µm SupaTop Syringe Filter before use.

2.2.4 Green agar

Green agar plates were used following P22-mediated generalised transduction. Green agar is a differential medium used to distinguish true phage-free bacteria from pseudolysogens. Green agar contains pH indicators that are green at neutral pH and dark blue at low pH. Lysis of cells containing pseudolysogens lowers the pH of the medium changing it dark blue. Thus, phage-free colonies can be seen as pale green colonies. These colonies were routinely picked and re-streaked a second time on green agar to purify phage-free colonies. Green agar was prepared as described in Table 2.5.

Table 2.5. Recipe for 1 litre of Green agar

<u>Green agar</u>	<u>g/l</u>	<u>ml</u>
Tryptone	8	-
Yeast extract	1	-
NaCl	5	-
Agar	15	-
40% (w/v) Glucose *	-	21
2.5% (w/v) Alizarin yellow *	-	25
2% (w/v) Aniline blue *	-	3.3

*The following were added after the melted agar was cooled to 55°C. Antibiotics were added where appropriate.

2.2.5 Culture conditions

Bacterial strains were maintained as stocks in 16% (v/v) glycerol in L-broth and stored at -80°C. Cultures conditions used throughout this study are described below.

2.2.6 Investigating the effect of pH change on gene expression

The conditions described below were used throughout this study unless otherwise stated.

Strains were grown in 6 ml of L-broth in a test tube at 37°C and 200 rpm shaking for 18 h. The culture optical density at 600 nm (OD₆₀₀) was equalised to 0.1 units. Cells were centrifuged at 4,000 rpm for 8 min and the supernatant removed. The pellet was resuspended in 1 ml of 1X EG-minimal (pH 7.2) to begin washing away residual nutrients after growth in L-broth. Cells were collected in a microfuge tube and centrifuged at 13,200 rpm for 1 min and the supernatant removed. This wash step was repeated and the final pellet was resuspended in 1 ml of 1X EG minimal medium (pH 7.2).

The cells were added to a final volume of 40 ml of 1X EG minimal medium pH 7.2 in a 250 ml flask. Cells were grown to OD₆₀₀ ~0.5-0.6. At this point the culture was divided into two 20-ml volumes and centrifuged at 4,000 rpm for 8 min. The supernatant was removed and the two pellets were subjected to different treatments. One was resuspended in 1 ml of pre-warmed 1X EG-minimal medium pH 7.2. The second was resuspended in 1 ml of pre-warmed 1X EG-minimal medium pH 4.5. Each suspension was added to a final volume of 20 ml 1X EG minimal medium of the same pH in a 250 ml flask. Cells were grown at 37°C and 200 rpm shaking for 90 min before harvesting for analysis.

Investigation into the presence of RNA polymerase (RNAP) at the *ompR* promoter using chromatin immunoprecipitation (ChIP) was carried using the culture conditions

described above; however, samples were treated at pH 4.5 for 15 min followed by treatment with rifampicin at 150 µg/ml for 20 min. Rifampicin inhibits bacterial growth by binding the subunit of RNAP upstream of the active site thereby preventing synthesis of RNA more than 2 to 3 nucleotides in length (Campbell *et al.*, 2001). Cells were then harvested for ChIP as described in section 2.15.1.

2.2.7 Monitoring bacterial growth

Bacterial growth was monitored by measurement of the optical density at 600 nm (OD₆₀₀). For routine measurement, 800 µl of the culture to be tested was transferred into a plastic disposable cuvette (Greiner). The OD₆₀₀ measurement was performed on a ThermoSpectronic Genesys 10 UV spectrophotometer. Samples were diluted within the spectrophotometric range of 0.1 to 1 and the values obtained corrected by multiplication by the dilution factor. Sterile medium was used as a blank.

2.2.8 Sugar fermentation tests

Sugar fermentation tests were carried out to confirm phenotypically the construction of SL1344 Δcrp (see section 4.2.8). In brief, as a result of deleting the *crp* gene the cells can no longer produce CRP-cAMP complexes. CRP-cAMP activates genes encoding the enzymes necessary to utilise certain sugars (e.g. lactose, maltose, galactose). In the absence of *crp* the cells can no longer produce these enzymes and as a result cannot ferment certain sugars. However, *crp* mutants should still maintain the ability to ferment sugars such as glucose and sialic acid as activation of the necessary genes for their fermentation is independent of CRP-cAMP.

To test the ability of a strain to ferment a carbon source the following test was performed. Strains were inoculated into peptone broth containing a sugar or sugar alcohol of choice at 0.5% final concentration and bromothymol blue as an indicator. Strains were grown at 37°C and 200 rpm for 18 h. The acid end-product as a result of fermentation lowers the pH of the medium; this is indicated by a colour change from blue to yellow in the medium. If no fermentation occurs the medium remains blue.

2.3 Bacterial strains and plasmids

All bacterial strains used throughout this study were derivatives of *Salmonella enterica* serovar Typhimurium strain SL1344 or *Escherichia coli* K-12 strain CSH50, unless otherwise stated. Strains used are detailed in Table 2.6. All plasmids used in this study are listed in Table 2.7.

2.4 Oligonucleotides

All oligonucleotides used throughout this study were supplied by Integrated DNA Technologies (IDT, Belgium). A list of their DNA sequences can be found in Table 2.8.

2.5 Molecular biology techniques

2.5.1 Plasmid DNA extraction

Plasmid DNA was routinely extracted from overnight cultures using the PureYield Plasmid Miniprep System (Promega) as per the manufacturer's instructions. This procedure is based on a modified alkaline lysis method (Birnboim and Doly, 1979). After harvesting cells by centrifugation the pellet is resuspended in a Tris (hydroxymethyl aminomethane), EDTA (Ethylenediaminetetraacetic acid), glucose buffer in the presence of RNases. The action of EDTA is two-fold; EDTA chelates divalent ions preventing DNases from damaging the plasmid DNA, EDTA also aids in destabilisation of the cell membrane. Bacterial RNA is degraded by RNases upon lysis of the cells while glucose maintains osmotic pressure prior to lysis. The cells are lysed using an SDS (sodium dodecyl sulfate) buffer containing NaOH (sodium hydroxide). SDS solubilises the cell membrane and NaOH denatures plasmid and chromosomal DNA. The alkaline lysate is then neutralized by the addition of KOH (potassium acetate). The high salt concentration also causes the cellular protein to precipitate while the plasmid DNA renatures. Due to the size of chromosomal DNA it takes longer to reanneal and is pelleted with cell debris by centrifugation. Plasmid DNA remains in the supernatant and is added to a silica membrane column where it binds and is washed with alcohol to remove excess salts and EDTA. The plasmid DNA is then eluted from the column in Tris-Cl pH 7 and stored at -20°C.

Table 2.6. *S. Typhimurium* and *E. coli* strains used in this study

Strain	Relevant genotype	Reference/ Source
<i>S. enterica</i> serovar Typhimurium		
SL1344	<i>rpsL</i> , <i>hisG</i>	(Hoiseth and Stocker, 1981)
SL1344 Δ <i>ihfA::cat</i>	Δ <i>ihfA::cat</i> , Cm ^r	This study
SL1344 Δ <i>crp</i>	Δ <i>crp</i>	This study
SL1344 <i>hns::kan</i>	<i>hns::kan</i> , Km ^r	Jay Hinton
SL1344 Δ <i>ompR</i>	Δ <i>ompR</i>	(Cameron and Dorman, 2012)
SL1344 Δ <i>envZ::kan</i>	Δ <i>envZ::kan</i> , Km ^r	(Cameron and Dorman, 2012)
SL1344 Δ <i>PompR::cat</i> pKD46	Δ <i>PompR::cat</i> , Cm ^r , Amp ^r , (Carb ^r)	This study
SL1344 <i>PompR</i> IHF-I ⁻	3 bp substitution in IHF-I	This study
SL1344 <i>PompR</i> IHF-III ⁻	3 bp substitution in IHF-III	This study
SL1344 <i>PompR</i> IHF-I ⁻ IHF-III ⁻	3 bp substitution in both IHF-I and IHF-III	This study
SL1344 <i>PompR</i> CRP-I ⁻	5 bp substitution in CRP-I	This study
SL1344 <i>PompR</i> OmpR-II ⁻	5 bp substitution in OmpR-II	This study
SL1344 <i>PompR</i> OmpR-II ⁻ IHF-I ⁻	5 bp substitution in OmpR-II and a 3 bp change in IHF-I	This study
SL1344 <i>hns::kan</i> OmpR-II ⁻	<i>hns::kan</i> X <i>PompR</i> OmpR-II ⁻	This study
SL1344 <i>ompR::3xFLAG</i>	<i>ompR::3xFLAG</i> epitope fusion	(Cameron and Dorman, 2012)
SL1344 <i>PompR</i> OmpR-II ⁻ <i>ompR::3xFLAG</i>	5 bp change in OmpR-II <i>ompR::3xFLAG</i> epitope fusion	This study
SL1344 pUC18	SL1344 pUC18	This study

Table 2.6. continued.

Strain	Relevant genotype	Reference/ Source
SL1344 <i>crp</i> ::3xFLAG	<i>crp</i> ::3xFLAG	This study
<i>E. coli</i>		
CSH50	F- λ - <i>ara</i> Δ (<i>lac-pro</i>) <i>rpsL thi</i> <i>fimE</i> ::IS1	(Miller, 1972)
CSH50 Δ <i>ompR/envZ</i> :: <i>cat</i>	<i>ompR/envZ</i> , Cm ^r	This study
CSH50 Δ <i>PompR</i> :: <i>cat</i>	<i>PompR</i> , Cm ^r	This study
CSH50 <i>PompR</i> <i>S. enterica</i>	SL1344 <i>PompR</i> in place of native CSH50 <i>PompR</i>	This study
CSH50 <i>ompB</i> <i>S. enterica</i>	SL1344 <i>ompR/envZ</i> in place of native CSH50 <i>ompR/envZ</i>	This study
CSH50 <i>ompR</i> ::3xFLAG	<i>ompR</i> ::3xFLAG epitope fusion	This study
CSH50 pUC18	CSH50 pUC18	This study
XL-1	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> <i>supE44 relA1 lac</i>	Stratagene
DH10B	<i>dam</i> ⁺ <i>dcm</i> ⁺ Δ <i>hsdRMS endA1</i> <i>recA1</i>	Invitrogen

Abbreviations: Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Amp^r, ampicillin resistance; Carb^r carbencilin resistance; X (uppercase) denotes transductional crosses represented as A X B, where A is the donor and B is the recipient.

Table 2.7. Plasmids used in this study

Plasmid	Relevant characteristics	Reference/ Source
pKD46	Ts, Amp ^r , (Carb ^r) encodes λ -Red recombinase genes	(Datsenko and Wanner, 2000)
pKD4	Encodes <i>frt-kan-frt</i> for gene deletions, Km ^r	(Datsenko and Wanner, 2000)
pKD3	Encodes <i>frt-cat-frt</i> for gene deletions, Cm ^r	(Datsenko and Wanner, 2000)
pCP20	Ts, encodes FLP recombinase system, Amp ^r (Carb ^r)	(Cherepanov and Wackernagel, 1995)
pSUB11	3xFLAG epitope, Km ^r	(Uzzau <i>et al.</i> , 2001)
pQE30 <i>crp</i>	<i>S. enterica crp</i> cloned under IPTG inducible promoter, Amp ^r (Carb ^r)	Qiagen
pREP4	Encodes <i>lacI</i> gene, Km ^r	Qiagen
pUC18	Amp ^r (Carb ^r)	(Yanisch-Perron <i>et al.</i> , 1985)
pJET	Amp ^r (Carb ^r)	Thermo Scientific
<i>PompR S. enterica</i> pJET	340 bp <i>ompR</i> promoter region from SL1344 Amp ^r (Carb ^r), Km ^r	This study
<i>PompRIHF-I</i> pJET	3 bp substitution in IHF-I Amp ^r (Carb ^r), Km ^r	This study
<i>PompRIHF-III</i> pJET	3 bp substitution in IHF-III, Amp ^r (Carb ^r), Km ^r	This study
<i>PompRIHF-I</i> IHF-III pJET	3 bp substitution in both IHF-I and IHF-III, Amp ^r (Carb ^r), Km ^r	This study

Table 2.7. continued

Plasmid	Relevant characteristics	Reference/ Source
<i>PompRCRP-I</i> pJET	5 bp substitution in CRP-I, Amp ^r (Carb ^r), Km ^r	This study
<i>PompROmpR-II</i> pJET	5 bp change in OmpR-II, Amp ^r (Carb ^r), Km ^r	This study
<i>PompRIHF-I</i> OmpR-II pJET	3 bp change in IHF-I and a 5 bp change in OmpR-II, Amp ^r (Carb ^r), Km ^r	This study
pET21- <i>ompR</i> (D55E)	<i>ompR</i> (D55E) cloned in frame with C-terminal His-tag.	Cameron and Dorman 2012.

Abbreviations: Ts, Temperature sensitive; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Amp^r, ampicillin resistance; Carb^r, carbenicillin resistance.

Table 2.8. oligonucleotides used in this study

Name	Sequence (5'-3')
<i>ompR_RT_S.e_F</i>	ATCGTCTGCTGACCCGTGAATCTT
<i>ompR_RT_S.e_R</i>	TTACTTTGACTACGCAGGCGACGA
<i>ssrA_RT_F</i>	ACATAAACGGCAAAGGATGGCACG
<i>ssrA_RT_R</i>	TCGGGAAGTTTAACCGTCACCTCA
<i>ihfA_KO_F</i>	TTAAAAGAGCGATTCCAGGCATCATTGAGGGATTGAACC TGTGTAGGCTGGAGCTGCTTC
<i>ihfA_KO_R</i>	TGCCGCAATACACCCTGATGGATGTTATGCCTGGATCTG ACATATGAATATCCTCCTTAG
<i>ihfA_KO_conf_F</i>	CTACCGTCGCCAAATGTGTA
<i>ihfA_KO_conf_R</i>	TGATGGATGTTATGCCTGGA
IHF-I_SDM_F	GGTTGCCGATTAATTGTATATTTGGGCTGCCGTAAATA TGCTTTGTAACAATTTAG
IHF-I_SDM_R	CTAAATTGTTACAAAGCATATTTAACGGCAGCCCAAATA TACAATTAATCGGCAACC
IHF-III_SDM_F	CGCGGGTGATCAGGGGCGTTTTTCGGCTCGTCGATTTACC TTTGCTGTCGATATTGCGC
IHF-III_SDM_R	GCGCAATATCGACAGCAAAGGTAAATCGACGAGCCGAAA ACGCCCCTGATCACCCGCG
Bio_ompR_F	TCACGCCAGAGATAATTGAGC
Bio_ompR_R	CGCATATCGTCATCAACCAC
PompR_S.e_KO_F	CAGACTCGCAGCCCAGGTCACTTTTTTGGTGACCTCCGG GGCGCCTACCTGTGACGGAAG
PompR_S.e_KO_R	TATCGTCATCAACCACCAGAATCTTATAATTCTCTTGCA TTAGGAACCTTCATTTAAATGG
PompR_KO_conf_F	TGACGTACCAGTTTGCTTCG
PompR_S.e_int_F	CAGACTCGCAGCCCAGGTCACTTTTTTGGTGACCTCCGG GGTGTAGGCTGGAGCTGCTTC
PompR_S.e_int_R	GAAGCCCTGCTCGGTCAGATAACGTTCCAGTAGCGCCCG CAGACGCATATCGTCATCAAC

Table 2.8. continued

Name	Sequence (5'-3')
<i>crp</i> _pQE30_F	GTCGATGGATCCGTGCTTGGCAAACCGCAAAC
<i>crp</i> _pQE30_R	GTCGATAAGCTTTTAAACGGGTGCCGTAGACGA
<i>crp</i> _KO_F	TCTGGCTCTGGAGACAGCTTATAACAGAGGATAACCGCG CGTGTAGGCTGGAGCTGCTTC
<i>crp</i> _KO_R	ACAAAAATGGCGCATGATAAAACGCGCCATTCTGACGGA ACATATGAATATCCTCCTTAG
<i>crp</i> _KO_conf_F	TCTGGCTCTGGAGACAGCTT
<i>crp</i> _KO_conf_R	ATAAACGCGCCATTCTGAC
<i>ompR</i> _E.c_RT_F	ATCGCCTGCTGACTCGTGAATCTT
<i>ompR</i> _E.c_RT_R	TTGCTCTGACTACGAAGACGTCGG
<i>ompR::FLAG</i> _E.c_F	GGGTCTGGGCTACGTCTTTGTACCGGACGGCTCTAAAGC AGACTACAAAGACCATGACGG
<i>ompR::FLAG</i> _E.c_R	GGGCAAATGAACTTCGTGGCGAGAAGCGCAATCGCCTCAT CATATGAATATCCTCCTTAG
<i>ompR</i> _KO E.c_F	ACACGCTTACAAATTGTTGCGAACCTTTGGGAGTACAAA CAGTGTAGGCTGGAGCTGCTTC
<i>ompR</i> _KO E.c_R	GGGCAAATGAACTTCGTGGCGAGAAGCGCAATCGCCTCA TCATATGAATATCCTCCTTAG
<i>ompF</i> _E.c_RT_F	AGCGAACTACGGTGAAACCCGTAA
<i>ompF</i> _E.c_RT_R	ACGCAGACCGAAATCGAACTGGTA
<i>ompC</i> _E.c_RT_F	TGGTGAAGGCTTTACTAGTGGCGT
<i>ompC</i> _E.c_RT_R	AGCATCAGTACGTTTGGAGCTGGA
<i>ompR</i> _conf_E.c_F	TAGGCTGAAATTCATACCAGATTT
<i>ompR</i> _conf_E.c_R	GGCTGGCGAACAGCAAGGTGACGATG
CRP-I_SDM_F	ACACGGGGTATAACTTATTCGTCCCTATAGAATAAATAA TGGGG
CRP-I_SDM_R	CCCCATTATTTATTCTATAGGGACGAATAAGTTATACCC CGTGTG

Table 2.8. continued

Name	Sequence (5' -3')
CRP-II_SDM_F	ATTTACCTTTGCTGCGCTTATTGCGGCGCCGGGGTATAA
CRP-II_SDM_R	TTATACCCCGGCGCCGCAATAAGCGCAGCAAAGGTAAAT
OmpR-II_SDM_F	CTGCTGTTAAATTTGCTTTATATGCATTTAGCCTGAAA
OmpR-II_SDM_R	TTTCAGGCTAAATGCATATAAAGCAAATTTAACAGCAG
<i>PompR_KO_E.c_F</i>	GACTTGCGGCCCCAGGTCACCTTTTTTTGTGACCTCCGGGC GGCGCCTACCTGTGACGGAAG
<i>PompR_KO_E.c_R</i>	TGTCGTCATCGACCACCAGAATCTTGTAGTTCTCTTGCA TTAGGAACCTTCATTTAAATGG
<i>PompR_int_E.c_F</i>	GACTTGCGGCCCCAGGTCACCTTTTTTTGTGACCTCCGGGC GGTGTAGGCTGGAGCTGCTTC
<i>PompR_int_E.c_R</i>	GCAGGCGCATGTCGTCATCGACCACCAGAATCTTGTAGT TCTCTTGCAATTGTCTGTACTC
IHF-I_SDM_OmpR-II_F	TTGTATATTTGGGCTGCCGTTAAATTTGCTTTATATG
IHF-I_SDM_OmpR-II_R	CATATAAAGCAAATTTAACGGCAGCCCAAATATACAA
<i>ompB::cat_E.c_F</i>	CACGCTTACAAATTGTTGCGAACCTTTGGGAGTACAAAC ATAGGAACCTTCATTTAAATGG
<i>ompB::cat_E.c_R</i>	ATAGAAAGCAAAACGGGAGGCACCTTCGCCTCCCGTTTA TGCGCCTACCTGTGACGGAAG
<i>ompB-kan_S.e_F</i>	GGCTCGCGTCCAGGGGACGACAAAAGAGGCATAAGAAAG G
<i>ompB-kan_S.e_R</i>	GCGTCATCCGGCGTTGAGAAGAAAGGGAGGGTAATACCT CCATATGAATATCCTCCTTAG
<i>ompR_F</i>	CACGCTTACAAATTGTTGCGAACCTTTGGGAGTACAAAC AATGCAAGAGAATTATAAGAT
<i>PompR_R_ompB_ChIP</i>	TACTCCCAAAGGTTGCAACA
<i>PompR_ChIP_S.e_F</i>	AGCCTGAAATTCATACCAGAA
<i>PompR_ChIP_S.e_R</i>	TTCTCTTGCAATTGTCTGTACT
Bio_PphoP_F	GACTCTGGTCGACGAACTTAAATAA
Bio_PphoP_R	CCTCTACAACCAGTACGCGCATCAT

Table 2.8. continued

Name	Sequence (5'-3')
<i>Kan_R</i>	TATCTTATAGAAAGCAAAACGGGAGGCACCTTCGCCTCC CCATATGAATATCCTCCTTAG
<i>phoP_S.e_RT_F</i>	AGTCGAGGTTCTCAGCTCCG
<i>phoP_S.e_RT_R</i>	CACCTGGGAGGCCAGACCGC
<i>RACE_ompR</i>	GTTTTTGCTAGCTTAGCGACGCTTCGAACCTGGAAG
<i>JVO-0367</i>	ACTGACATGGAGGAGGGA
<i>PphoPpJET_F</i>	ATAGCGGCCGCAATTATATCGGTCGCGCTGT
<i>PphoPpJET_R</i>	GCCGTCTAGAGTGGCGTAATAATGCATTAT
<i>PphoP_SDM_F</i>	AACGCTAGACTGTACTTATTAATATGCCAAGGGAGAAGA G
<i>PphoP_SDM_F</i>	CTCTTCTCCCTTGGCATATTAATAAGTACAGTCTAGCGT T
<i>crp::FLAG_F</i>	CTCCGCGCATGGCAAGACCATCGTCGTCTACGGCACCCGT GACTACAAAGACCATGACGG
<i>crp::FLAG_R</i>	ACAAAAATGGCGCATGATAAAACGCGCCATTCTGACGGAA CATATGAATATCCTCCTTAG
RNA oligonucleotide A4	GACGAGCACGAGGACACUGACAUGGAGGAGGGAGUAGAA A
<i>ompR_F_pJET</i>	CGCTCTTCACGCCAGA
<i>ompR_R_pJET</i>	CGCATATCGTCATCAACC
<i>PstI_pKD4_F</i>	GTCGATCTGCAGGTGTAGGCTGGAGCTGCTTC
<i>XhoI_pKD4_R</i>	GTCGATCTCGAGCATATGAATATCCTCCTTAG
<i>gmk_F_Se</i>	AGCAAATTCGCGAAAAGATG
<i>gmk_R_Se</i>	TGGCAATGACTTCTTCGCTAT
<i>gmk_F_Ec</i>	TATTGTTTCTGCCCCCAGTG
<i>gmk_R_Ec</i>	GTTCGAGGAACGCATCTCTG

The reinstated *envZ* start codon is underlined for *ompR::FLAG_E.c_R*.

2.5.1 Genomic DNA extraction

Chromosomal DNA used to create standard curves for quantitative RT-PCR was purified using a Gentra Puregene kit (Qiagen). Typically, 0.5 ml of an overnight culture was used to purify chromosomal DNA as per the manufacturer's guidelines. Cells were lysed with an anionic detergent in the presence of a DNA stabilising agent that prevents damage to DNA by both cellular and contaminating DNases. Lysis was achieved by incubating samples at 80°C for 5 min. An RNA digesting enzyme was incubated with the cell lysate at 37°C for 60 min to inactivate RNases. Proteins and cellular debris were precipitated using high salt concentrations and removed by centrifugation. Genomic DNA was washed with isopropanol and then with 70% alcohol. The DNA pellet was resuspended in a 1 mM EDTA, 10 mM Tris-Cl pH 7.5 solution and heated to 65°C for 60 min before cooling to room temperature and storing at -20°C.

2.6 RNA extraction

Bacterial RNA is chemically unstable; RNA has a short half-life and is susceptible to degradation by RNase enzymes that are ubiquitous in the environment (Hirashima *et al.*, 1973). Unlike bacterial RNA, RNases are very stable and can remain active even after autoclaving. For this reason preventative measures were taken when isolating and working with RNA. All work surfaces were cleaned with 100% ethanol and latex gloves were changed regularly. In addition, certified nuclease-free filter-pipette-tips and tubes (Sarstedt) were used and RNA was kept on ice where possible.

To isolate RNA from bacterial cells, culture densities were equalised to 10 OD₆₀₀ units (e.g. 10 ml of culture OD₆₀₀ 1.0). Cultures were added to 5% (v/v) phenol (pH 4.5, Sigma): 95% ethanol at 2/5 of the culture volume and incubated on ice for 45 min. The phenol:ethanol solution halts bacterial growth and helps stabilize the RNA and prevent degradation. Cells were pelleted at 4,000 rpm at 4°C for 8 min and the supernatant was removed. Cells were resuspended in the residual volume and transferred to a fresh microfuge tube. Cells were centrifuged at 13,200 rpm for 1 min at 4°C and the supernatant was removed. The pellet was then resuspended 100 µl Tris-EDTA pH 8 buffer containing lysozyme at 50 mg/ml and incubated for 5 min at room temperature.

The following steps were carried out using an SV Total RNA Purification kit (Promega) as per the manufacturer's instructions. Cells were incubated in lysis buffer containing β -mercaptoethanol, guanidine thiocyanate and SDS. β -mercaptoethanol acts as a reducing agent to disrupt di-sulfide bonds in ribonucleases that would otherwise damage the RNA. Guanidine thiocyanate acts to denature proteins and also inhibits RNases. Proteins were precipitated in a high salt RNA dilution buffer after incubation at 70°C for 3 min. Cellular debris and precipitated proteins were cleared by centrifugation at 13,200 rpm for 10 min. RNA was precipitated from the supernatant using 200 μ l of 95% ethanol in a RNase-free microfuge tube (Sarstedt). The supernatant was then transferred to a silica based spin column and centrifuged. RNA bound to the silica membrane was DNase-treated on the spin column with DNase enzyme at 37°C for 30 min. DNase treatment was stopped using a stop solution containing EDTA that chelates divalent cations. The RNA was then washed twice in an ethanol-containing wash buffer and eluted in 50 μ l nuclease-free H₂O.

The RNA was then DNase treated a second time using the Turbo DNA-free™ kit (Ambion). RNA samples were incubated with DNase I at 37°C for 30 min to digest contaminating DNA. RNA concentration was then read on a spectrophotometer (see section 2.6.7). Typically, 5 μ l of sample was loaded in a 1% Tris- borate-EDTA (TBE) agarose gel for analysis of the ribosomal RNAs 16S and 23S.

2.6.1 cDNA synthesis

As RNA cannot serve as a template for PCR it must be reverse transcribed into complementary DNA (cDNA) before being quantified by PCR (Bustin, 2000). This can be performed in one-step or two-step reactions. The one-step reaction uses both RNA and DNA-dependent polymerases with gene-specific primers in a single tube. This combines both first-strand cDNA synthesis and the PCR reaction in a single tube. The two-step reaction uses reverse transcriptase to generate cDNA pools of total RNA using random primers (Bustin, 2000). Following cDNA synthesis PCR is carried out in a second separate reaction using gene-specific primers. Two-step RT-PCR was used throughout this study as it has the added advantage of quantifying multiple gene targets from a single RNA preparation.

Reverse transcription was carried out using the GoScript™ Reverse Transcription system (Promega) as per the manufacturer's guidelines. This system uses Moloney Murine leukaemia virus reverse transcriptase (MMLV-RT). All RNA samples were normalised for 1 µg of RNA and added to 0.5 µg of random primers in a 5 µl reaction volume. Samples were incubated at 70°C for 5 min to denature the primers and template mix, samples were then chilled on ice for 5 min. The following was added to the 5 µl RNA:primer mix (final concentrations are specified): 1X reaction buffer, 1.25 mM MgCl₂, 0.5 mM PCR nucleotides, 1 µl reverse transcriptase and 20 U of recombinant RNasin (ribonuclease inhibitor) in a 20-µl final volume. Samples were collected by brief centrifugation. The following cycle was set up on a PTC-200 Peltier Thermal Cycler (MJ Research):

1. 25°C, 5 min (Primer annealing)
2. 42°C, 60 min (First-strand cDNA synthesis)
3. 70°C, 15 min
4. 4°C, Hold

100 µl was added to each sample and samples were stored at -20°C.

2.6.2 Polymerase chain reaction (PCR)

PCR is dependent on primers that are 5'-3' oligonucleotides, typically 20 nucleotides in length (Saiki *et al.*, 1988). These primers are designed to be complementary and flanking to the region of interest. DNA templates are first heated to break hydrogen bonds and denature the template. Amplification is then carried out by heat-stable polymerases that read the primed DNA template in the 3'-5' direction and synthesises a new strand of DNA in the 5'-3' direction using dideoxy nucleoside triphosphates (dNTPs) (Saiki *et al.*, 1988). Thermal cycles are repeated 25-30 times; during each cycle the DNA is denatured, primers anneal to complementary sequence and DNA synthesis is carried out. Assuming the reaction is 100% efficient the procedure is exponential, doubling the template with each cycle until the reaction becomes limiting and DNA polymerase activity decreases.

Throughout this study two DNA polymerases were used; Phusion™ High-Fidelity DNA polymerase (Finnzymes) and *Thermus aquaticus* (*Taq*) DNA polymerase (New England

Biolabs). Phusion is a *Pyrococcus*-like enzyme with 5'-3' DNA polymerase activity and 3'-5' exonuclease activity. *Taq* also possesses 5'-3' DNA polymerase activity and 3'-5' exonuclease activity, however Phusion has an error rate 50 times lower than *Taq*. Due to the high-fidelity of Phusion DNA polymerase and possibility of non-specific binding and amplification of DNA template *Taq* was preferentially used for PCR amplification of cDNA used for transcription start site mapping (see section 2.9).

PCR reactions were carried out as per the manufacturer's recommendations. Typically, Phusion-based PCR reactions were set up as follows (Final concentrations are specified): 1X HF buffer, 0.2 mM dNTPs, 0.5 μ M of each oligonucleotide, 1 U of Phusion and 5-50 ng of template and the final volume was adjusted to 50 μ l with ddH₂O. The sample was placed in a 500 μ l thin-walled PCR tube (Sarstedt). Reactions were kept on ice where possible. Reactions were thoroughly mixed, collected by brief centrifugation and placed into a PTC-200 Peltier Thermal Cycler (MJ Research).

PCR reactions carried out with *Taq* were set up as above except using 1X *Taq* buffer and 1 U of *Taq* polymerase.

Cycles were routinely set up as follows when using Phusion:

1. 98°C, 30 s (Initial denaturation)
2. 98°C 10 s (Denaturation)
3. Primer annealing temperature^a, 30 s
4. 72°C, appropriate extension time^b
5. Repeat steps 2-4, 30 cycles
6. 72°C, 10 min (Final extension)
7. 4°C, Hold.

Reaction products were stored at -20°C.

^a Annealing temperature of primers was calculated using the T_m calculator web tool provided by Finnzymes (http://www.finnzymes.fi/tm_determination.html), annealing temperatures for primer pairs were typically 5°C below the highest melting temperature calculated

^b15-30 s per 1 kb.

Cycles were routinely set up as follows when using *Taq* DNA polymerase:

1. 95°C, 10 min
2. 95°C, 40 s
3. Primer annealing temperature
4. 72°C, appropriate extension time ^c
5. Repeat steps 2-4 30 cycles
6. 72°C, 10 min
7. 4°C, Hold.

^c 1 min per kb.

2.6.3 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to quantify gene expression. Amplification of cDNA was monitored by fluorescent signal using an enhanced SYBR® Green dye. SYBR Green dye binds to the minor groove of double-stranded DNA and the increase in signal is proportional to the amount of double-stranded DNA. At the beginning of PCR, the fluorescence emission for each cycle does not change significantly. At this point the baseline fluorescence is calculated; this phase is often referred to as 'ground' or 'background' phase (Bustin, 2000; Pfaffl, 2001). This is followed by the 'exponential' phase, in ideal conditions the PCR product doubles after each cycle. The reaction then reaches a 'plateau' phase where reaction components become limiting. qPCR measurements are acquired during the log-linear exponential phase of the reaction when amplification is proceeding most efficiently (Bustin, 2000; Pfaffl, 2001).

This study used the GoTaq PCR Master Mix (Promega) for qPCR. This system contains BRYT Green® dye which is an enhanced SYBR® Green dye. Each reaction was 20 µl in total; 10 µl of a 2X master mix containing BRYT® Green dye, carboxy-X-rhodamine (CXR) reference dye, GoTaq® Hot Start Polymerase, MgCl₂ and dNTPs. Primers were then added; concentrations were typically between 0.3-0.6 µM, the volume was adjusted to 12 µl with nuclease-free H₂O. Finally 8 µl of template cDNA (see section 2.6.1) was added. Reactions were set up in a 96-well plate (Rainin).

Typically a no template control was set up for each primer set used; a well was set up containing 12 µl of master mix described above and 8 µl of nuclease-free H₂O. Any amplification that occurred in this well is indicative of template contamination. In addition a DNA contamination control was also set up using 12 µl of master mix and 8 µl of DNase-treated RNA, which was used for cDNA synthesis. Amplification in this well is indicative of DNA contamination.

Samples were collected in the 96-well plate after centrifugation at 500 rpm for 1 min. The qPCR reactions and fluorescence measurements were performed using the StepOnePlus™ Real-time PCR system (Applied Biosystems). Cycling conditions were as follows:

1. 95°C, 2 min (Hot-start activation of GoTaq polymerase)
2. 95°C, 15 s (Denaturation of template)
3. 60°C, 60 s (Annealing and extension)

Steps 2 – 3 repeated 40 times.

Quantification of gene expression was calculated using the standard curve method. The StepOne software measures amplification of the target in samples and in a standard dilution series using a 10-fold dilution series of genomic DNA. Data from the standard dilution series are used to generate the standard curve and the software interpolates the absolute quantity of target in the samples. The gene *gmk* (guanylate monophosphate kinase) was used as a reference gene (Muller *et al.*, 2009). The amount of *gmk* in each cDNA sample was quantified using the standard curve method. The relative quantity of each experimental gene was then divided by the relative quantity of *gmk* and these values were used to express arbitrary levels of gene expression.

2.6.4 Agarose gel electrophoresis

DNA samples were routinely analysed after PCR or restriction enzyme digestion by agarose gel electrophoresis. Agarose powder was dissolved in TAE buffer (40 mM Tris, 1 mM EDTA, 20 mM glacial acetic acid) at the appropriate percentage. This was heated until in solution and then cooled to 55°C. Ethidium bromide was then added at a final concentration of 1 µg/ml. Ethidium bromide intercalates into the DNA and fluoresces in UV light allowing the DNA to be visualised on a UV imager. Samples were mixed with

loading dye (Promega) at 1X final concentration before loading in the gel. 1 kb or 100 bp ladders (Promega) were analysed alongside the DNA for a size reference. Agarose gels underwent electrophoresis in 1X TAE buffer at 80-100 V.

2.6.5 Chloroquine gel electrophoresis

The high-copy-number plasmid pUC18 was used to measure the level of DNA supercoiling in this study (Hsieh *et al.*, 1991a). Plasmids were isolated from cultures as described in section 2.5.1. Electrophoresis was conducted in 27-cm-long 1% agarose gels containing 2.5 µg/ml chloroquine with 2X Tris Borate EDTA (TBE) used as gel and running buffer. At this concentration of chloroquine more supercoiled plasmids migrate faster in the gel compared to relaxed molecules. Chloroquine intercalates with DNA and relaxes negative supercoils; importantly the final degree of relaxation reached by the DNA depends on the initial supercoiling state (Dougherty, 1983; Esposito and Sinden, 1987).

Approximately 1 µg of plasmid DNA was loaded with 4 µl of loading buffer (80% glycerol, 0.5 mg/ml bromophenol blue) in a final volume of 20 µl. Electrophoresis was carried out at 100 V for 16 h. Chloroquine was removed by washing gels in ddH₂O for 3 h with gentle rocking; ddH₂O was replaced every 30 min. Gels were subsequently stained with ethidium bromide at 1 µg/ml for 1 h. Gels were then destained by washing in ddH₂O for 20 min and visualised with UV light.

2.6.6 Purification of PCR products

Linear DNA from PCR amplification was purified after electrophoresis for use in downstream applications such as cloning into plasmids or for use as bait DNA in electrophoretic mobility shift assays. Long-wave (365 nm) UV light was used to illuminate DNA in agarose gels containing ethidium bromide at 1 µg/ml. Long-wave UV light minimizes the damage to DNA compared to short-wave by reducing the time the DNA is exposed to UV light.

The DNA fragment of interest was excised from the gel using a scalpel and placed in a microfuge tube. The following steps were carried out using the HiYield gel/PCR

fragments extraction kit (RBC). This kit is based on the protocol described elsewhere (Vogelstein and Gillespie, 1979). Gel pieces containing DNA is dissolved in a chaotropic salt-containing buffer and heated to 55°C. Once the gel has dissolved it is added to a silica membrane column. The column is centrifuged at 13,200 rpm for 1 min. The DNA binds to the silica membrane and is washed with an ethanol wash buffer. DNA is eluted in Tris-Cl pH 7 at stored at -20°C.

2.6.7 Quantification of nucleic acids by spectroscopy

Nucleic acids were quantified by spectroscopy using a NanoDrop® ND-1000 spectrophotometer. This was carried out by measuring absorbance at 260 nm (A_{260}) and 280 nm (A_{280}). Nucleic acids absorb light at 260 nm while contaminants such as proteins absorb light as 280 nm. The ratio value OD_{260}/OD_{280} was calculated; a ratio of ~1.8 and 2.0 is considered 'pure' DNA and RNA respectively.

2.7 Manipulation and mutagenesis of DNA

2.7.1 Cloning into pJET1.2

The CloneJet™ PCR cloning kit (Thermo-Scientific) was used to clone DNA sequences prior to site-directed mutagenesis (see section 2.7.2) and PCR products for transcription start-site mapping (see section 2.9). This kit provides linearised pJET cloning vector with 5' phosphoryl groups. Blunt-end PCR products are then ligated to the pJET vector at a 3:1 molar ratio respectively. The ligation mix contains 5 U of T4 ligase and 1X reaction buffer (Thermo-Scientific). PCR products generated by *Taq* DNA polymerase have a 3'-dA overhang, which were blunted using a DNA blunting enzyme prior to ligation.

The pJET vector contains a lethal restriction enzyme gene that is disrupted as a result of positive DNA ligations. Recircularised pJET vector expresses the lethal gene when transformed into cells and as a result is not propagated. The pJET vector expresses the *bla* gene, which encodes β -lactamase that confers resistance to ampicillin and was used as a selectable marker.

2.7.2 Site-directed mutagenesis

Site-directed mutagenesis (SDM) was carried out using the QuikChange II SDM kit (Stratagene) according to the manufacturer's instructions. SDM was used to introduce base pair substitutions into the *ompR* promoter cloned into the pJET vector (*PompRpJET*). Primers were designed complementary to opposite strands of the DNA to be mutated. These primers were overlapping and contained the desired non-homologous sequence to be introduced into the vector in addition to 15-20 bp of homologous sequence to the flanking regions. The QuickChange SDM kit utilises the *PfuTurbo* DNA polymerase to replicate both strands of plasmid DNA creating mutated vectors with staggered nicks. After amplification the sample is treated with *DpnI* endonuclease. This enzyme digests the original parental DNA that was extracted from *Escherichia coli* XL-I (*dam*⁺) strain and is therefore methylated. Digestion with *DpnI* increases the likelihood that transformants will contain PCR generated plasmid containing the desired mutations.

Reactions were set up in thin-walled PCR tubes (Sarstedt) and contained 5-50 ng of plasmid DNA, 125 ng each of primer, 1X reaction buffer, 1 U of *PfuTurbo* and 1 µl of dNTP mix. Tubes were placed in a PTC-200 Peltier Thermal Cycler (MJ Research) and with the following cycle:

1. 95°C, 30 s
2. 95°C, 30 s
3. 55°C, 1 min
4. 68°C, 3 min 30 s
5. Repeat steps 2-4, 15 times

10 U of *DpnI* was then added to the reaction mix and incubated 37°C for 1 h. This reaction mix was then directly transformed into rubidium chloride competent cells (see section 2.10.3).

2.7.3 Lamda Red recombination

Chromosomal DNA mutations and epitope tag fusions were constructed using the Lamda (λ) Red recombination system (Datsenko and Wanner, 2000; Uzzau *et al.*,

2001). This procedure uses the vector pKD46 to integrate PCR products onto the chromosome using short homologous flanking regions. The vector pKD46 encodes the phage λ Red locus. This locus harbours the genes *bet*, *exo* and *gam* (β , *exo*, γ). Exo is a 5'-3' exonuclease that degrades the 5' ends of linear DNA. Bet binds to single-stranded DNA and promotes binding to complementary DNA, while the Gam protein inhibits host exonuclease activity (Murphy, 1998). In pKD46 this locus is under the control of the arabinose-inducible promoter P_{araB} , this prevents unwanted recombination events occurring until arabinose is added to the culture. The pKD46 vector encodes the *bla* gene for ampicillin resistance and has a temperature sensitive replicon allowing recombinants to be cured of the plasmid by an upshift in temperature from 30 to 37°C. Primers, typically 60 bp in length, were designed for the generation PCR products to be integrated into the chromosome. The 5' end of the primers carried 40 bp of sequence homology for the region of integration followed by 20 bp of homology for the genes encoding selectable resistance markers. These primers were used to amplify resistance markers *cat* (chloramphenicol resistance) or *kan* (kanamycin resistance) from vectors pKD3 or pKD4 respectively. PCR products were gel extracted and used directly for transformation into strains harbouring the vector pKD46.

Strains harbouring pKD46 were grown overnight in 4 ml of L-broth containing carbenicillin at 30°C and 200 rpm. The overnight culture was diluted 1 in 100 into fresh L-broth containing carbenicillin in 250 ml flask. Cells were grown at 30°C and 200 rpm to OD₆₀₀ ~0.5 at which point L-arabinose was added to the culture at a final concentration 0.2%. Cells were grown for another hour, allowing induction and expression of the phage λ Red locus. Cells were then made electrocompetent as explained in section 2.10.1 and transformed with 100-200 ng DNA. Cells were recovered with 1 ml of prewarmed of L-broth at 37°C and 200 rpm for 1 hr. Recombinants were selected for on L-agar plates using kanamycin or chloramphenicol where appropriate and grown overnight at 37°C. Transformants were picked and restreaked on L-agar containing appropriate antibiotics. Mutants were screened for loss of pKD46 by checking for growth on L-agar containing carbenicillin. Mutations were transduced into a fresh genetic background as described section 2.11

2.7.4 FLP recombinase by pCP20

Antibiotic resistance cassettes amplified from pKD3 and pKD4 are flanked by short repeats called FRT sites. These sites are the targets of the FLP enzyme of *Saccharomyces cerevisiae*. The FLP enzyme promotes site-specific recombination between the FRT sites and removes enclosed sequences leaving behind a “scar” sequence containing one of the FRT sites (Cherepanov and Wackernagel, 1995). Throughout this study the vector pCP20 was used to remove antibiotic resistance markers. The plasmid pCP20 is ampicillin resistant, temperature-sensitive and encodes the FLP enzyme that is thermally induced at 30°C (Datsenko and Wanner, 2000).

Briefly, cells harbouring an FRT-flanked resistance marker were grown overnight at 37°C and 200 rpm. Cells were diluted 1 in 100 in L-broth in a 250 ml flask and grown to OD₆₀₀ ~0.5. Cells were made electrocompetent as described in section 2.10.1. Cells were then transformed with 50-200 ng of pCP20 (see section 2.10.2) and recovered for 1 h with 1 ml of L-broth at 30°C. Cells were then plated on L-agar containing carbenicillin and grown for overnight at 30°C. Transformants were then re-streaked onto L-agar containing no antibiotics at 42°C for 3 h, followed by a temperature shift to 37°C overnight to cure cells of the plasmid pCP20. Routinely, several colonies were picked and re-streaked onto L-agar containing no antibiotics and grown again at 37°C overnight. Colonies were then screened for growth on carbenicillin and kanamycin or chloramphenicol. Sensitivity to carbenicillin indicated cells were cured of pCP20. Sensitivity to kanamycin or chloramphenicol indicated the resistance marker had been removed from the chromosome.

2.7.5 Epitope tagging

Epitope tagging can be used to fuse antigens, to which there are commercially available antibodies, to a protein of interest (Uzzau *et al.*, 2001). A 3xFLAG sequence was amplified from the vector pSUB11; using the λ -Red recombination system (described in section 2.7.3) the 3x FLAG sequence was fused to the carboxy terminus of OmpR and the amino terminus of CRP. OmpR and CRP protein levels were then detected by western blot (see section 2.13).

2.8 Electrophoretic mobility shift assay

The gel electrophoresis mobility shift assay (EMSA) was used to detect interactions between DNA-binding proteins and DNA. Throughout this study biotin-labelled probes were used directly to visualise protein-DNA complexes. Primer pairs containing 5' biotin tags were used to amplify DNA sequence of interest. In this study the DNA-binding proteins IHF, CRP and OmpR were tested for their ability to bind DNA sequences; the conditions used for each protein are described:

The interaction of IHF and DNA was investigated as previously described (Yang and Nash, 1995). Purified IHF (a kind gift from Prof Steven Goodman) was stored in storage buffer containing 600 mM KCl, Tris-Cl pH 7.4, 10% (v/v) glycerol at -80°C. IHF was diluted to the desired concentration as a 10X stock in storage buffer. Typically 20 µl reactions were set up containing the following: 0.5 ng of biotin-labelled DNA, 0.5X TBE (45 mM Tris-borate, 1.25 mM EDTA, pH 8.3), 500 µg/ml of bovine serum albumin (BSA), 10% (v/v) glycerol, 60 mM KCl and 1 µg polydeoxyinosinic-deoxycytidylic acid (poly(dI.-dC)) and increasing concentrations of IHF at 1X final concentration. Reactions were incubated at room temperature for 20 min prior to loading onto 5% non-denaturing acrylamide gels (see section 2.8.1).

Purified His-tagged CRP was stored in 200 mM KCl, 40 mM Tris pH 8.8, 20% (v/v) glycerol, CRP was diluted in storage buffer to the desired concentration as 10X stock. CRP-DNA binding reactions were set up as previously described (Cameron and Redfield, 2008). 30 µl reactions were set up on ice containing 0.5 ng of biotin-labelled DNA, 0.2X TBE, 12mM Tris pH 8.8, 40 mM KCl, 3% (v/v) glycerol, pH 8.8, 500 µg/ml of BSA, 1 µg of poly(dI.-dC), 2 mM dithiothreitol and 200 µM cyclic adenosine monophosphate (cAMP). Reactions were incubated at room temperature for 20 min prior to loading onto 5% non-denaturing acrylamide gels (see section 2.8.1).

Purified His-tagged OmpRD55E from plasmid pET21-*ompR*(D55E) (Cameron and Dorman 2012) was used to test OmpR binding to DNA sequences. This mutation changes codon 55 from GAT to GAA; this changes the aspartate residue to a glutamate. This mutation mimics aspartyl-phosphate and activates the protein (Lan and Igo, 1998;

Cameron and Dorman, 2012). The binding reactions were carried out as described for CRP however no cAMP was added to the binding reaction.

2.8.1 Electrophoresis through non-denaturing polyacrylamide gel

The interaction of protein and DNA were examined using non-denaturing acrylamide gels. 15 µl of each binding reaction was loaded directly onto gels with no loading dye as this may interfere with protein-DNA interactions. Empty lanes were loaded with bromophenol blue at 2.5 mg/ml in 80% (v/v) glycerol to track migration of the DNA.

IHF-DNA binding reactions were added to gels routinely made up in 15 ml volumes as follows: 5% (v/v) acrylamide, 2% (v/v) glycerol, 17.5 µl of N, N, N', N'-Tetramethylethylenediamine (TEMED), 0.5X TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA pH 8.3) and 0.75 mg/ml ammonium persulfate. Gels were run in 0.5X TBE buffer at 90-100 V.

CRP-DNA binding reactions were loaded in gels containing 5% (w/v) acrylamide, 2% (v/v) glycerol, 17.5 µl of TEMED, 0.2X TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA pH 8.3), 0.75 mg/ml ammonium persulfate and 200 µM cAMP. Gels were run in 0.2X TBE containing 200 µM cAMP at 90-100 V. OmpRD55E-DNA binding reactions were treated as described for CRP (above), without the addition of cAMP in the gels or running buffer.

2.8.2 Electrophoretic transfer of DNA and proteins

Following electrophoresis gels were electroblotted onto Biodyne Nylon 0.45 µm membrane (PALL) using Bio-Rad Mini Trans-Blot cell in 0.2-0.5X TBE buffer at 30 V for 1 h. Membranes were then UV cross-linked twice for 150 s using GS Genelinker (Bio-Rad) and developed using the Chemiluminescent Nucleic acid Detection module (Thermo-scientific) (see section 2.8.3).

2.8.3 Chemiluminescent detection of DNA probes

The Chemiluminescent Nucleic Acid Detection module (Thermo-Scientific) was used to visualise biotin-labelled DNA after electrophoresis and transfer. This kit uses

streptavidin conjugated with horseradish peroxidase (HRP). Streptavidin serves to tightly bind biotin-labelled DNA. Conjugation of streptavidin with HRP allows DNA to be detected in the presence of luminol. Luminol is oxidised by HRP and emits blue light as it decays.

Membranes were blocked in 20 ml of blocking buffer for 20 min at room temperature with gentle rocking. 66 μ l of stabilised streptavidin-horseradish peroxidase was then added to the blocking buffer and incubated for a further 20 min. The membrane was washed for 20 min; the wash buffer was replaced every 5 min. The membrane was incubated with substrate equilibrium buffer for 5 min followed by incubation with equal volumes of luminol and oxidising reagents for 5 min. The membrane was then exposed to autoradiography film to detect the light emitted signal (see 2.14.3).

2.9 5' Rapid amplification of cDNA ends (RACE)

RACE was used to determine the 5' ends of transcripts based on a strategy described previously (Bensing *et al.*, 1996; Urban and Vogel, 2009). Primary transcripts in bacteria carry a 5' triphosphate whereas processed transcripts carry 5' monophosphates. To distinguish primary transcripts from that which are processed the enzyme tobacco acid pyrophosphatase (TAP) is used. TAP hydrolyses between the α and β phosphate groups to generate 5' monophosphates. Using RNA ligase, the 3' hydroxyl group of an RNA adapter is ligated to the 5' monophosphate of all transcripts. This is followed by first-strand cDNA synthesis using random primers. Transcripts of interest are amplified by PCR using an adaptor-specific primer with a nested gene specific primer. TAP treatment is expected to yield specific or strongly enhanced signal where the adaptor has been ligated to the primary transcript. However, ligation of the adaptor to processed transcripts will also be amplified by PCR. PCR products are then gel extracted, cloned into vector of choice and transcription start sites are determined by DNA sequencing. The protocol described in the following sections is described in detail elsewhere (Urban and Vogel, 2009).

2.9.1 Tobacco acid pyrophosphatase treatment

RNA was extracted from cultures and DNase treated as described (see section 2.6.3). Total RNA was denatured at 90°C for 1 min and cooled on ice for 5 min. 10 µg of RNA was brought to a volume of 87.5 µl with nuclease-free H₂O. To this, 10 µl of 10X TAP buffer and 0.5 µl of RNasin (20 U/µl Promega) were added. The reaction was then split into two tubes (49 µl each); to one tube 1 µl of TAP (Epicentre, 10 U/µl) was added, to the other, 1 µl of RNase-free H₂O was added. Both tubes were incubated at 37°C for 30 min. 300 pmol of RNA oligonucleotide (Table 2.8) was added to each tube.

2.9.2 Phenol:chloroform extraction

The RNA samples from section 2.9.1 was brought up to a volume of 100 µl with nuclease-free H₂O and added to a 2 ml PLG tube (Phase Lock Heavy, 5Prime, VWR). The sample was mixed by shaking for 30 s followed by centrifugation at 13,000 rpm, 12 min at 15°C. The upper aqueous phase was then transferred to a nuclease-free microfuge tube (Sarstedt). Approximately 300 µl of ethanol containing 0.3 M sodium acetate (pH 5.7) was added and RNA was precipitated for 1 h at -80°C. The sample was then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was removed and the pellet was washed with 350 µl 70% ethanol followed by centrifugation for 13,000 rpm 10 min at 4°C. The supernatant was removed and the pellet was air-dried and resuspended in 9.5 µl of nuclease-free H₂O. The sample was incubated at 65°C for 5 min with vortexing in between.

2.9.3 RNA adapter ligation

RNA was denatured at 90°C for 5 min and cooled on ice for 5 min. The sample was collected by short centrifugation and kept on ice where possible. To the RNA sample, 10.5 µl RNA-ligation mix was added, this contained the following: 2 µl dimethyl sulfoxide (DMSO 100%, New England Biolabs), 2 µl 10X RNA ligation buffer, 4 µl T4 RNA ligase (40 U New England Biolabs), 0.5 µl of RNasin (20 U/µl Promega) and 2 µl RNase-free H₂O was added. The sample was then incubated for 12 h overnight at 17°C. RNA was precipitated (see section 2.9.2) and resuspended in 10 µl of nuclease-free H₂O.

2.9.4 First-strand cDNA synthesis

cDNA synthesis was carried out using the Superscript™ II kit (Invitrogen). 2 µg of RNA was mixed with 100 pmol of random hexamer primers and adjusted to 10 µl with nuclease-free H₂O in a 500 µl microfuge tube. RNA and random hexamers were denatured at 65°C for 5 min. To this, 9 µl RT mix was added containing the following: 4 µl 5X RT buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, 2 µl 10 mM dNTP mix, 5 µl nuclease-free H₂O, 2 µl 0.1 M dithiothreitol (DTT) and 1 µl of RNasin was added. This was mixed gently and the sample collected by short centrifugation. The sample was then incubated at 25°C for 10 min. 1 µl of reverse transcriptase was added and the tubes were placed in a PTC-200 Peltier Thermal Cycler (MJ Research) and with the following cycle:

41°C, 15 min

50°C, 15 min

55°C, 15 min

60°C, 15 min

85°C, 5 min

The sample was cooled to 37°C and 1 µl (5 U) of RNase H was added. The tubes were returned to the thermo cycler and incubated at 37°C for 20 min and stored at -20°C.

2.10 DNA transformation

Transformation is the process of introducing free DNA into bacterial cells. As *E. coli* and *Salmonella* are not naturally competent, strategies must be used to make cells competent and deliver DNA. Throughout this study electro-and chemically-competent cells were used.

2.10.1 Preparation of electrocompetent cells

250 µl of the recipient strain was diluted from an overnight culture into 25 ml fresh L-broth in a 250 ml flask. Cells were grown to OD₆₀₀ ~0.5 at 37°C and 200 rpm. The culture was chilled on ice for 15 min after which cells were centrifuged at 4,000 rpm for 8 min at 4°C. The supernatant was removed and cells were resuspended in 25 ml of chilled sterile ddH₂O and incubated on ice for 30 min. Cells were centrifuged at 4,000

rpm for 8 min at 4°C and the pellet resuspended in 25 ml chilled sterile ddH₂O. This step was repeated twice more. The final pellet was resuspended in 1 ml of chilled sterile ddH₂O and added to a pre-chilled microfuge tube. Cells were centrifuged at 6,000 rpm for 5 min at 4°C, the pellet was then resuspended in 1 ml chilled sterile ddH₂O; this wash step was repeated twice more. Cells were washed thoroughly to remove any trace of media that could lead to arcing of the samples during electroporation. The final pellet was resuspended in 250 µl chilled sterile ddH₂O and used for electroporation as described in section 2.10.2.

2.10.2 Electroporation of electrocompetent cells

Electroporation uses an electric field pulse to induce transient pores into the cell membrane allowing the uptake of free DNA. On ice 50-500 ng of DNA was added to pre-chilled electroporation cuvette (Cell Projects) with a gap-width of 2 mm. To this 50 µl of electrocompetent cells were added. Using a Gene Pulser chamber (Bio-Rad) an electrical pulse of 2.5 kV from the 25 µF capacitor with a resistance setting of 200 Ω was delivered ~4-5 msec. 1 ml of pre-warmed L-broth was then added to the cuvette and cells were incubated at 37°C and 200 rpm for at least 1 h to allow for phenotypic expression. Cells were then centrifuged at 13,200 rpm for 2 min at room temperature and the supernatant removed. Cells were resuspended in residual liquid and plated on L-agar containing appropriate selective antibiotic. As a control electrocompetent cells were plated onto L-agar the containing selective antibiotic. Plates were incubated at 37°C overnight. Transformants were typically re-streaked on L-agar containing appropriate antibiotics.

2.10.3 Preparation of rubidium chloride competent cells

Cells were prepared based on methodologies described elsewhere (Hanahan *et al.*, 1991). 250 µl of the recipient strain was diluted from an overnight culture into 25 ml fresh L-broth in a 250 ml flask. Cells were grown to OD₆₀₀ ~0.5 at 37°C and 200 rpm. Cells were then centrifuged at 4,000 rpm for 8 min at 4°C. The pellet was resuspended in 10 ml TFB1 buffer (30 mM KOAc, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂, 15% (v/v) glycerol pH 5.8) and incubated on ice for 5 min. Cells were then centrifuged at 4,000 rpm for 8 min at 4°C. The pellet was then resuspended in 1 ml TFB2 buffer (10

mM MOPS pH 6.5, 75 mM CaCl₂, 10 mM RbCl, 15% (v/v) glycerol pH 6.5) and incubated for 1 h on ice followed by transformation (see section 2.10.4).

2.10.4 Transformation with rubidium chloride competent cells

100 µl of chemically competent cells were added to chilled microfuge tube on ice. 10-100 ng of DNA was added to the cells, mixed gently and incubated on ice for 30 min. Cells were then heat shocked at 42°C for 1 min followed by incubation on ice for 1 min. Heat shock opens pores in the cell membrane facilitating entry of DNA into cells. 1 ml of pre-warmed L-broth was then added to the cells and the culture was incubated at 37°C and 200 rpm for at least 1 h to allow phenotypic expression. Cells were then centrifuged at 13,200 rpm for 2 min at room temperature and the supernatant removed. Cells were resuspended in residual liquid and plated on L-agar containing appropriate selective antibiotic. As a control electrocompetent cells were plated onto L-agar the containing selective antibiotic. Plates were incubated at 37°C overnight. Transformants were typically re-streaked on L-agar containing appropriate antibiotics.

2.11 Transduction

Transducing bacteriophage particles are formed in donor bacterial cells during phage development. There are two types of transducing particles distinguished by the DNA that they carry. Generalised transducing particles carry host DNA whereas specialised transducing particles carry both host and viral DNA sequences on the same DNA molecule (Masters, 1996). Generalised transduction packages host DNA into phage heads and upon infection of recipient cells delivers this packaged DNA into the recipient cells. The DNA can recombine into recipient chromosome where extensive homology permits homologous recombination (Masters, 1996).

The bacteriophage P22 and P1 were used in this study to package DNA from *Salmonella* and *E. coli*, respectively, for generalised transduction. The P22 phage can package approximately 44 kb of DNA (Casjens and Hayden, 1988), however P1 has a larger capacity of approximately 70-95 kb (Sternberg, 1990). Bacteriophage P22 binds to the O-antigen receptor of lipopolysaccharide on the outer membrane of the cell (Baxa *et al.*, 1996) and injects double-stranded DNA into the bacterial host. A P22 derivative

P22 HT105/1 *int*-201 was used in this study (Schmieger, 1972). This high transducing phage (HT) contains a nuclease with low specificity for Pac sites, thereby increasing the likelihood that bacterial host DNA will be packaged. This phage also harbours a mutation in the *int* gene that is required for phage integration into host DNA, thus preventing lysogeny.

The P1 receptor on the host cell is a terminal glucose of lipopolysaccharide (LPS) (Franklin, 1969). A P1 *vir* derivative was used here as the *vir* mutation prevents phage from generating P1 lysogens (circular phage molecules that are self-replicating) among transductants (Sternberg and Hoess, 1983).

2.11.1 Preparation of P22 lysates

Overnight cultures of donor strains were used to inoculate 10 ml broth with 100 µl of culture and appropriate antibiotics. Cells were grown at 37°C and 200 rpm to OD₆₀₀ 0.1. 10 µl of P22 phage stock was added to the culture (approximately 10⁸ p.f.u/ml). The culture was kept at 37°C and 200 rpm for 4 h after which 250 µl of chloroform was added to the culture to kill bacterial cells. This was mixed for 10 min at room temperature followed by centrifugation at 4,000 rpm for 20 min to pellet cell debris. The supernatant was transferred to a clean 15 ml falcon tube and a few drops of chloroform were added. The lysate was stored at 4°C.

2.11.2 P22 transduction

The recipient strain was grown overnight at 37°C and 200 rpm. The donor P22 lysate was diluted to 10⁻² using L-broth. Transductions were set up in microfuge tubes containing 200 µl final volume made up of 100 µl of P22 lysate at the appropriate dilution and 100 µl of the recipient strain. As a control 100 µl of neat P22 stock was incubated with 100 µl of L-broth to test for the presence of bacterial contamination. 100 µl of recipient culture was incubated with 100 µl of L-broth to test if the recipient strain was capable of growth on the appropriate antibiotic prior to transduction. The transduction mix and controls were incubated statically at 37°C for 1 hr. These mixes were then plated on L-agar containing appropriate antibiotics to select for transductants and incubated overnight at 37°C. Transductants were picked from plates where the

lowest dilution of P22 was used and re-streaked onto green agar containing appropriate antibiotics (see section 2.2.4). Genotypes were confirmed by PCR (section 2.6.2).

2.11.3 Preparation of P1 lysates

P1 lysates of donor strains were made as outlined previously (Thomason *et al.*, 2007). Donor strains were grown overnight in 4 ml of L-broth containing appropriate antibiotics at 37°C and 200 rpm. Cultures were then diluted 1 in 100 into fresh L-broth containing 5 mM CaCl₂ and 0.2% glucose at 37°C and 200 rpm to an OD₆₀₀ 0.4-0.6. 100 µl of P1 *vir* (~10⁹ phage/ml) was added to the cultures. Cultures were kept at 37°C and 200 rpm for 1-2 h (cell debris usually visible). 250 µl of chloroform was added to the cultures and mixed for 10 min with gentle mixing. Cultures were then centrifuged at 13,200 rpm for 5 min to pellet cell debris. The supernatant was then added to a fresh tube and the lysate was stored at 4°C.

2.11.4 P1 transduction

Recipient strains were grown overnight in 4 ml L-broth at 37°C and 200 rpm. 1.5 ml of the overnight culture was added to a microfuge tube and centrifuged at 13,200 rpm for 2 min at room temperature. The supernatant was removed and the pellet was resuspended in 750 µl P1 salts solution containing 10 mM CaCl₂ and 5 mM MgSO₄. 100 µl of these cells were incubated with 1, 10 and 100 µl of the P1*vir* donor lysate and incubated at 37°C for 30 min. 100 µl of cells and 100 µl of P1 lysate were incubated separately as controls for presence of resistance marker in the recipient and bacterial contamination of the donor lysate respectively. 1 ml of L-broth containing 200 µl of 1 M sodium citrate was added to each transduction mix including the controls. Transductions were incubated for 1 h at 37°C and 200 rpm. Cultures were centrifuged at 13,200 rpm for 2 min at 4°C to pellet the cells. The supernatant was removed and the pellets were resuspended in 50 µl of L-broth and plated onto L-agar plates containing 5 mM sodium citrate and appropriate antibiotics. Plates were incubated overnight at 37°C. Transductants were picked from plates that used the lowest volume of P1 lysate and re-streaked onto L-agar plates containing 5 mM sodium citrate and appropriate antibiotics. Genotypes were confirmed by PCR (section 2.6.2).

2.12 Protein preparation

2.12.1 Over-expression and purification of His-tagged CRP

E. coli DH10B containing plasmids pQE30*crp* and pREP4 were used to purify N-terminal His-tagged CRP. The vector pQE30 (Qiagen) contains a promoter-operator element consisting of the coliphage T5 promoter and the *lac* operator sequence. The plasmid pREP4 constitutively expresses the *lac* repressor that binds to the operator sequences of T5 promoter and represses transcription. IPTG (isopropyl- β -D-thiogalactoside) is used to bind and inactivate the *lac* repressor protein thereby permitting expression from the T5 promoter.

DH10B pQE30 pREP4 was cultured overnight at 37°C and 200 rpm in 4 ml of L-broth with carbenicillin at 50 μ g/ml and kanamycin at 25 μ g/ml. Cells were diluted 1 in 100 into 100 ml of L-broth in a 250 ml flask containing antibiotics as above. Cells were grown to OD₆₀₀ ~0.5 before IPTG was added at final concentration of 1 mM. The bacterial cells were allowed to grow for another 4 h before harvesting. CRP was purified as previously described (Peekhaus and Conway, 1998).

Briefly, cultures were centrifuged at 4,000 x g at 4°C for 20 min. Pellets were routinely stored at -20°C overnight. Pellets were thawed on ice for 15 min. Cells were resuspended in 10 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8) containing lysozyme at 2 mg/ml for 30 min on ice. Cells were sonicated with Soniprep 150 (Sanyo) on setting 5; 10 s bursts with 10 s rest was performed 6 times on ice followed by centrifugation at 13,200 rpm for 30 min at 4°C. 6 ml of cleared lysate was then mixed with 1.5 ml of 50% Ni-NTA (Nickel Nitrilo-triacetic Acid, Qiagen) slurry and incubated on a rotating wheel for 60 min at 4°C.

The following steps were carried out at 4°C. The lysate-slurry mix was then added to polypropylene column (Qiagen) and the nickel resin bed was allowed to settle (~5 min). The lysate was then allowed to pass through the column. The column was washed with 8 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8). The bound protein was eluted from the column in 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8. The fractions were analysed for the presence CRP by 12% (v/v)

acrylamide SDS-PAGE (see section 2.12.2-2.12.3). Fractions containing CRP were concentrated and desalted using an Amicon Ultra centrifugal filter device (Millipore) with a molecular weight cut off of 10,000 daltons. CRP was resuspended in storage buffer containing 20% glycerol, 40 mM Tris, 200 mM KCl pH 8.8. The protein was aliquoted into 50 μ l volumes for single use and stored at -80°C.

2.12.2 Preparation of samples for SDS-PAGE

Samples were prepared for analysis by SDS-PAGE as described previously (Laemmli, 1970). Samples were harvested and normalised to OD₆₀₀ 0.2 (e.g. 1 ml of cells at OD₆₀₀ 0.2), cells were centrifuged at 13,200 rpm for 2 min and the supernatant was removed. The pellet was then resuspended in 25 μ l 1X laemmli buffer (Sigma) and brought to a final volume of 50 μ l with ddH₂O. Laemmli buffer contains SDS that coats positively charged proteins with a negative charge allowing protein to be separated based on size. Laemmli buffer also contains β -Mercaptoethanol that reduces intra and inter-molecular disulfide bonds of the proteins to allow proper separation and bromophenol blue as a tracker dye and glycerol for loading the samples (Laemmli, 1970). Samples were then heated to 95°C for 5 min before running in SDS-PAGE or stored at -20°C.

2.12.3 Preparation of SDS-PAGE gels

Proteins were separated by discontinuous polyacrylamide gel electrophoresis as described previously (Laemmli, 1970; Maniatis *et al.*, 1982) This discontinuous buffer system uses gels with different pH composition and acrylamide concentrations to separate out negatively charged proteins based on their size. The upper stacking gel contains Tris-Cl (pH 6.8) and low concentration of acrylamide, while the lower resolving gel contains Tris-Cl (pH 8.8) and a higher concentration of acrylamide.

The gel is run in a buffer containing Tris-glycine (pH 8.3). The chloride ions in the stacking gel form a leading edge of moving ions while slower glycine molecules from the buffer form the trailing edge. Between these migration boundaries is an area of low conductivity that moves the proteins behind the chloride ions in a tight band up to the resolving gel. The higher pH in the resolving gel negatively charges the glycine molecules resulting in faster migration of the glycine molecules and loss of the

migration boundary. The proteins enter the resolving gel of higher acrylamide concentration and are separated by size.

Samples were typically separated on a 12% (v/v) acrylamide resolving gel. Resolving gels were routinely made up to 5 ml volume containing 12% (v/v) acrylamide, 0.1% SDS, 0.375 M Tris-HCl (pH 8.8), 1 mg/ml ammonium persulfate and 2 μ l TEMED.

Stacking gels were made to 5 ml volume containing 6% (v/v) acrylamide, 0.1% SDS, 0.125 M Tris-HCl (pH 6.8), 1 mg/ml ammonium persulfate and 5 μ l of TEMED. Gels were run in buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 at 90-100 V.

2.12.4 Transfer of proteins to nitrocellulose membranes

Following electrophoresis, gels were electroblotted onto Protran® nitrocellulose transfer membrane (Whatman) using Bio-Rad Mini Trans-Blot cell. Proteins were transferred in buffer containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. Transfer was carried out at 100 V for 1 h.

2.13 Western blot

2.13.1 Immunological detection of proteins

Western blotting also known as immunoblotting was used to identify specific proteins of interest that have been immobilised on nitrocellulose membrane after transfer (see section 2.12.4). Following a blocking step, which reduces non-specific binding of the primary antibody to the membrane, the membrane is incubated with a primary antibody (poly- or monoclonal) that was raised against the antigen of interest. The primary antibody is then removed and the membrane is washed before incubating with a secondary antibody. The secondary antibody is enzyme conjugated and reactive towards the primary antibody. In this study the enzyme conjugated to the secondary antibody was horseradish peroxidase (HRP), this is necessary for signal detection. The secondary antibody is then removed and the membrane is washed again and incubated with the enzyme substrate. The following protocol was carried out throughout this study.

Membranes were blocked overnight at 4°C in 20 ml of 2.5% (w/v) dried semi-skimmed milk powder (Marvel) in PBS (Phosphate saline buffer) containing 0.05% Tween®-20. Tween®-20 was added as a surfactant to minimise background created by non-specific interactions between the blocking reagent and the target. The blocking buffer was removed and the membrane was incubated for 1 h with the primary antibody that was diluted in 5% dried semi-skimmed milk powder in PBS. The following dilutions were used for each primary antibody:

Anti-FLAG® (F3165, Sigma):	1 in 10,000
Anti-IHF (a kind gift from Prof. Steven Goodman):	1 in 10,000
Anti-DnaK (1.0mg/ml, Enzo life sciences):	1 in 200,000

The primary antibody was then removed and the membrane was washed for 1 h with 20 ml of PBS containing 0.05% Tween®-20; the buffer was replaced every 15 min. The membrane was incubated with the appropriate secondary antibody diluted in 5% dried semi-skimmed milk powder in PBS. The following dilutions were used for each secondary antibody:

Goat anti-mouse IgG HRP (Millipore)	1 in 10,000
Goat anti-rabbit IgG HRP (Millipore)	1 in 10,000

The membrane was incubated for 1 h with the secondary antibody. The secondary antibody was then removed and the membrane was washed for 1 h with 20 ml of PBS containing 0.05% Tween®-20, the buffer was replaced every 15 min. The membrane was then incubated with equal volumes of luminol and oxidising reagents (Pierce) for 5 min before visualising using by autoradiography (see section 2.14.3).

2.14 Visualisation and quantification of proteins

2.14.1 Coomassie blue protein stain

After electrophoresis gels were stained in Coomassie Protein Staining solution overnight. Staining solution was prepared by mixing 2.5 g Coomassie Brilliant Blue G-250 in 1 litre solution of the following; 100 ml glacial acetic acid, 450 ml methanol, 450

ml ddH₂O. Gels were destained in the solution above without the addition of Coomassie Brilliant Blue G-250. Destaining solution was replaced every 20-30 min until protein was clearly visible in the gel.

2.14.2 Bradford assay

The Bradford assay uses the spectral properties of Coomassie Brilliant Blue G-250 to estimate the amount of protein in a solution (Bradford, 1976). Coomassie dye is in a low pH solution, this keeps the dye in its cationic form absorbing light at 465 nm. Coomassie blue dye preferentially binds to basic and aromatic amino acid residues. Upon binding of the dye to protein the dye shifts to its anionic form absorbing light at 585 nm. The increase in optical absorption at 595 nm is monitored by spectrophotometry (Fazekas de St Groth *et al.*, 1963; Bradford, 1976).

Protein Assay Dye Reagent Concentrate (Bio-Rad) was used in this assay. Bovine serum albumin (BSA) (Pharmacia, Biotech) was used as a reference protein for total protein quantification. BSA was serially diluted in phosphate buffer saline (PBS) to a final volume of 100 µl in microfuge tube. Each tube was set up in duplicate. 900 µl of Protein Assay Dye Reagent Concentrate was added and mixed thoroughly. The mix was incubated at room temperature for 5 min and absorbance was measured at OD₅₉₅ using a spectrophotometer.

2.14.3 Autoradiography

Autoradiography was used to visualise chemiluminescent emissions derived from horseradish peroxidase oxidation of luminol (western or EMSA analysis). Hyperfilm MP (Amersham Biosciences) film was used in all cases. When photon emissions strike silver halide crystal in the film, the crystal adsorbs energy and releases an electron. This electron is attracted to a positively charged silver ion forming an atom of metallic silver in developer solution. After an appropriate exposure time, the film was placed in a tray containing Kodak LX-24 X-ray developer for 2 min. The film was washed briefly in tap water and fixed in Kodak Industrex liquid fixer for a further 2 min. The fixer serves to remove any unreacted silver halides from the film by conversion into soluble silver thiosulphate. Developed films were rinsed in tap water.

2.15 Chromatin immunoprecipitation and microarray analysis

Chromatin immunoprecipitation and microarray analysis (ChIP-on-chip) was carried out to determine the association of OmpR with DNA in *Salmonella* Typhimurium and *E. coli* (see chapter 5). RNAP binding at the *ompR* promoter was also investigated herein. Experimental conditions used to investigate the RNAP binding are described in section 2.2.6.

This process involves the fixation of live cells with formaldehyde to cross-link protein-protein and protein-DNA interactions in the cells. This provides a ‘snapshot’ of protein binding across the entire genome. Cells are lysed and DNA sonicated to approximately 500 bp. Cellular material is immunoprecipitated with antibody specific to the protein of interest. Immunoprecipitation selectively enriches the sample. Genomic DNA that has not been selectively immunoprecipitated is used as an ‘input’ sample to control of non-specific enrichment of ChIP samples. ChIP and input DNA are then fluorescently labelled, hybridised to microarrays and detected in an array scanner. This procedure is described in detail below based on the work described previously (Dillon *et al.*, 2010).

2.15.1 Harvesting cells for chromatin immunoprecipitation (ChIP)

Experiments were carried out as described in section 2.2.6. OD₆₀₀ 15 units of cells (i.e. 15 ml of cells at OD₆₀₀ 1.0) were harvested for each ChIP procedure. Cells were harvested by centrifugation at 4,000 rpm for 8 min at room temperature and resuspended in 50 ml of pre-warmed PBS (37°C) in a 250 ml flask.

2.15.2 Fixation

DNA-protein and protein-protein interactions were cross-linked by adding formaldehyde (37%, Sigma). 1,351 µl of formaldehyde was added drop-wise to a final concentration of 1%. Samples were cross-linked at room temperature with gentle stirring for 3 min. 3.425 ml of ice-cold 2 M glycine was added to a final concentration of 0.125 M with gentle stirring for 5 min at room temperature. Glycine is used to terminate the cross-linking reaction. Cells were centrifuged at 4,000 rpm for 8 min at 4°C and the supernatant removed.

2.15.3 Cell lysis

Cells were kept on ice where possible. The lysis and dilution buffer described below were made fresh each time. 2 protease inhibitor tablets (Roche) were dissolved in 25 ml ddH₂O and used as a solvent for each buffer. The pellet was re-suspended in 0.6 ml of lysis buffer containing 50 mM Tris-HCl, 10 mM EDTA, 1% SDS and incubated on ice for 10 min.

2.15.4 Sonication

1.4 ml dilution buffer containing 20 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.01% SDS was added to the cells and transferred to 15 ml BD Falcon™ tube. Chromatin was sonicated on ice using Sanyo MSE Soniprep 150 sonicator. Samples were pulsed 8 times for 30 s (amplitude 10 microns) and 1 min rest between each pulse. The sonicated material was transferred to a 2 ml microfuge tube and spun at 13,200 rpm for 10 min at 4°C. Supernatant was removed and added to 15 ml BD Falcon™ and 1 ml dilution buffer added. Samples can be stored at -80°C at this point until needed.

2.15.5 Immunoprecipitation

The chromatin material was precleared by adding 50 µl of normal rabbit immunoglobulin G (IgG, 1 mg/ml, Millipore). Samples were incubated for 1 h at 4°C on a rotating wheel. 100 µl of homogenous protein G-agarose (Roche) was added to the precleared chromatin and the samples were incubated for 3-5 h at 4°C on a rotating wheel. Samples were centrifuged at 4,000 rpm for 2 min at 4°C to pellet the protein G-agarose beads and the supernatant was used to set up two immunoprecipitation (IP) conditions in 2 ml microfuge tubes. An aliquot of 200 µl chromatin was removed and stored at -20°C to be used as input sample for hybridisation. The remaining chromatin was used to set up two immunoprecipitation reactions as follows:

1. Experimental IP- 1,350 µl chromatin and 3.3 µg/µl of anti-FLAG antibody (Sigma).

or

1. Experimental IP- 1,350 µl chromatin and 10 µl anti-β' RNAP (W0023, Neoclone).

2. Mock IP- 1,350 μ l chromatin and 10 μ g of normal mouse IgG (Millipore).

Samples were incubated overnight on a rotating wheel at 4°C. Samples were centrifuged at 13,000 rpm for 5 min at 4°C and the supernatant was transferred to 2 ml microfuge tubes. 50 μ l of protein G-agarose was added to each sample and incubated at 4°C for 3 h. The samples were centrifuged at 7,500 rpm for 2 min at 4°C to pellet the protein G-agarose beads. The supernatant was removed and the protein G-agarose beads were carefully washed. For each wash, the wash buffer was added; the samples were vortexed briefly and were centrifuged at 7,500 rpm for 2 min at 4°C. The samples were left to stand on ice for 1 min before removing the supernatant. The washes were carried out in the following sequence:

1. The beads were washed twice with 750 μ l of cold IP wash buffer 1 containing 20 mM Tris-HCl (pH 8.1), 50 mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS. The beads were transferred to a 1.5 ml microfuge tube after the first wash.
2. The beads were washed once with 750 μ l of cold IP wash buffer 2 containing 10 mM Tris-HCl (pH 8.1), 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% deoxycholic acid.
3. The beads were washed twice with 750 μ l of ice-cold TE buffer containing 10 mM Tris base (pH 8) and 1 mM EDTA.

2.15.6 DNA elution

DNA-protein-antibody complexes were eluted from the protein G-agarose beads by adding 225 μ l of IP elution buffer containing 100 mM NaHCO₃ and 1% SDS. The beads were resuspended in IP elution buffer, briefly vortexed and centrifuged at 7,500 rpm for 2 min at room temperature. The supernatant was collected in fresh 1.5 ml microfuge tubes. The bead pellets in the original tubes were resuspended in 225 μ l of IP elution buffer again and briefly vortexed and centrifuged at 7,500 rpm for 2 min. Both elutions were combined in the same tube and stored at -20°C.

2.15.7 Reversal of cross-links

IP and input samples were thawed on ice. 0.1 µl of RNase A (10 mg/ml, ICN Biochemicals) and 16 µl of 5 M NaCl (to a final concentration of 0.3 M) was added to the input sample. Similarly, 0.2 µl of RNase A and 27 µl of 5 M NaCl (to a final concentration of 0.3 M) was added to each IP sample. All samples including the input DNA were incubated at 65°C for 6 h to reverse cross-links. 9 µl of Proteinase K (10 mg/ml, GibcoBRL) was added to each sample and incubated overnight at 45°C (samples at this point can be stored at -20°C until needed).

2.15.8 DNA extraction

2 µl of yeast tRNA (5mg/ml, Invitrogen) was added to each sample followed by 250 µl of phenol (pH 8, Sigma) and 250 µl of chloroform. Samples were centrifuged at 13,000 rpm for 5 min at room temperature. The aqueous layer was collected in fresh 1.5 ml centrifuge tubes and 500 µl of chloroform was added. The samples were vortexed and centrifuged at 13,000 rpm for 5 min at room temperature. The aqueous layer was transferred to a 2 ml microfuge tube.

5 µg of glycogen (5 mg/ml, Roche), 1 µl of tRNA (5 mg/ml, Invitrogen) and 50 µl of 3 M NaAc (pH 5.2) was added to each sample and mixed well. The DNA was precipitated with 1,375 µl of 95% ethanol and incubated at -80°C for 1 h. The samples were centrifuged at 13,000 rpm for 20 min at 4°C. The DNA pellets were washed with 500 µl of ice-cold 70% ethanol and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was removed and the samples were air-dried for 10-15 min. DNA pellets of IP samples were resuspended in 50 µl nuclease-free H₂O. Input DNA samples were resuspended in 100 µl of nuclease-free H₂O. Samples were incubated at 37°C for 1 h.

2.15.9 Labeling of DNA for microarray

The BioPrime Array CGH kit (Invitrogen) was used to fluorescently label DNA for ChIP microarray experiments. 100 µl volume IP labelling reactions were set up as follows: 40 µl 2.5X random primers, 40 µl of IP DNA, 6.5 µl nuclease-free H₂O, this was heated to 100°C for 10 min and chilled on ice for 5 min. To this sample, 10 µl of

10X dNTP mix (1mM dCTP, 2mM dGTP, 2mM dTTP, 2 mM dATP), 1.5 µl of Cy3/Cy5-dCTP (23 nmol GE Healthcare) and 2 µl exo-Klenow (40 U/µl) was added. Reactions were carried out at 37°C overnight in the dark. 15 µl of stop buffer was added to each sample to terminate the reaction. 200 ng of input DNA was labelled as described above. Samples were purified using Qiagen MinElute PCR purification kit as per manufacturer's instructions.

2.15.10 Hybridisation and scanning of microarrays

Samples were prepared for hybridisation as follows: 50 µl 2X Hi-RPM Hybridisation buffer (Agilent), 25 µl ChIP DNA, 5 µl input DNA, 10 µl 10X GE blocking Agent (Agilent) and 10 µl nuclease-free H₂O. Samples were added to a microfuge tube, vortexed briefly and collected by short centrifugation spin.

100 µl was added to an array on the microarray slides produced by Oxford Gene Technology. These slides were formatted as tiled arrays and each slide contained 4 x 44 K identical arrays. These tiling path arrays contained probes spanning the entire genome and were composed of 60-mer oligonucleotide probes that were printed directly onto the slide. The annotation of each probe by its gene name and genome co-ordinate was performed by Dr Karsten Hokamp. The arrays were sealed with the appropriate gasket slide and loaded into the Agilent Microarray Hybridisation Chamber Kit (G2534A). Microarrays were hybridised for 24 h at 65°C in a hybridization oven (Agilent).

Slides were washed in wash buffer 1 followed by wash buffer 2 as described below. 200 ml of buffer was used for each wash step. Wash buffers were made up as 1 L solutions and subsequently filter sterilised using 0.22 µm pore membrane (Nalgene) as follows:

Wash buffer 1: 1.4 L ddH₂O, 0.6 L 20X SSC (Sigma), 1 ml 10% Triton.

Wash buffer 2: 994.5 L ddH₂O, 5 ml 20X SSC (Sigma), 500 µl 10% Triton.

The slide was removed from the hybridisation oven and prised apart submerged in wash buffer 1 that was pre-warmed to 60°C. The slide was then carefully placed into a wash

dish in wash buffer 1 at 37°C for 10 min with stirring, followed by washing in wash buffer 1 at room temperature for 10 min.

The slide was then washed in wash buffer 2 at room temperature for 5 min with stirring; this was followed by a second wash in fresh wash buffer 2 for 5 min again. Slides were dried and scanned on Agilent High-Resolution C scanner. Each microarray is scanned at two wavelengths (Cy5; 635 and Cy3; 532 nm). The median fluorescence intensities for both channels and for each spot is acquired by Agilent Feature Extraction Software version 10.5.1.1. This software calculates the background fluorescence for each spot and as such these values were used to calculate the background subtracted ChIP/input ratio. The data was median-normalised by calculating the median fluorescence for each channel (Cy3 or Cy5) and using a scaling factor to ensure the median of the data set was equal to 1. This method was used to adjust for unequal starting quantities of RNA, uneven signal intensity or differences in dye incorporation during labelling that may have occurred. The ChIP/input ratio was calculated as it was customary practice to swap the fluorescent (Cy3 and Cy5) dyes used to label the ChIP and input samples. At least two control 'mock' experiments were performed using normal mouse IgG antibody at the immunoprecipitation step (see section 2.15.5), this control was carried out during the experimental ChIP reaction to identify any non-specific binding; The mock/input ratio was calculated. The median $\log_2$ ratio for each independent biological replicate was calculated. To correct for non-specifically enriched peaks, the mock median $\log_2$ ratio was subtracted from each ChIP median $\log_2$ ratio. The $\log_2$ ratios were used in the ChIPOTle programme (2.15.11).

2.15.11 ChIPOTle v1.0

The ChIPOTle programme (Buck *et al.*, 2005) was used to analyse and define peaks of enrichment in the ChIP-chip data sets. ChIPOTle was set to analyse the chromosome in 500 bp segments and move along the chromosome in 125 bp steps. Each segment is referred to as a 'window' and 500 bp was chosen as the chromosome was sonicated into approximately 500 bp fragments. The average $\log_2$ ratio of all regions encompassed in a window was calculated. The window was moved downstream in 125-bp intervals and the average $\log_2$ ratio was calculated for each new window, this was repeated iteratively

for the whole chromosome. This is referred to as a sliding window and sliding average was used to define binding sites as peaks. The p value was set to $p < 0.001$.

2.15.12 Graphpad prism and Microsoft Excel

Unless otherwise stated, numerical data were analysed using Microsoft Excel 2011 or Prism 5.

Chapter 3 Expression of *ompR* at low pH in *Salmonella* Typhimurium and *Escherichia coli*.

3.1 Introduction

S. Typhimurium and *E. coli* are exposed to a wide variety of stressful environments during their life cycle inside the human host. These conditions include high temperature, low pH, osmotic shock, exposure to cationic antimicrobial peptides, bile, and changes in levels of anaerobiosis. Of these stressful conditions bacteria frequently encounter fluctuations in pH (Bearson *et al.*, 1997; Rychlik and Barrow, 2005; Ramos-Morales, 2012). Once inside the gastrointestinal tract of the host the bacteria enter the acidic stomach which can be as low as pH 1.7 in fasted healthy humans (Dressman *et al.*, 1990); this acidic environment is one of the first host defences against ingested bacteria. Additionally, *S. Typhimurium* experiences low pH inside host cells. Within host phagocytes *Salmonella* exists inside an intracellular vacuole called the *Salmonella*-containing vacuole (SCV) (Finlay and Brumell, 2000). This vacuole undergoes acidification between pH 4 and pH 5 within 1 h of formation (Rathman *et al.*, 1996). Despite these assaults both *Salmonella* and *E. coli* are adept at surviving low pH; in particular *E. coli* can survive pH 2 for up to 2 hours (Gorden and Small, 1993; Small *et al.*, 1994). Both species have evolved a variety of mechanisms (AR and ATR systems) that enable them to survive low pH, maintain pH homeostasis and subsequently cause infection. These acid resistance (AR) systems include the decarboxylase(s)/antiporter systems which require specific supplementation for their induction e.g. lysine for CadA/B. The acid tolerance response (ATR) systems are induced in minimal media at low pH and require no supplementation (discussed in section 1.6.8) (reviewed by Ramos-Morales, 2012).

Studies directly comparing the ability of *S. Typhimurium* and *E. coli* to tolerate low pH have revealed that *S. Typhimurium* is less acid-tolerant than *E. coli* (Lin *et al.*, 1995). When challenged at pH 3.5 *S. Typhimurium* cells die rapidly. However this is circumvented when *Salmonella* is first pre-adapted to a mildly acidic pH (e.g. pH 4.3) followed by challenge at pH 3 (Lin *et al.*, 1995). To survive acid challenge *S. Typhimurium* employs both the log and the stationary phase ATRs that are dependent upon the growth phase in which the cells are challenged with low pH (Audia *et al.*, 2001; Foster, 2004; Ramos-Morales, 2012).

The OmpR protein was identified as a regulator of the stationary phase ATR in *S. Typhimurium* (Bang *et al.*, 2000). Expression of *ompR* and *envZ* is induced at low pH and mutations in the *ompR* gene result in an acid sensitive phenotype (Bang *et al.*, 2002). The OmpR protein is the response regulator of the OmpR/EnvZ two-component system (TCS). The OmpR/EnvZ system is a prototypical TCS best characterised in *E. coli* where OmpR reciprocally activates and represses expression of the porin genes *ompF* and *ompC* in response to changes in osmolarity (Alphen and Lugtenberg, 1977; Slauch *et al.*, 1988; Slauch and Silhavy, 1989). The cytoplasmic domain of EnvZ senses changes in osmolarity (Wang *et al.*, 2012). Activation of the EnvZ leads to autophosphorylation followed by transfer of the phosphoryl group to its cognate response regulator OmpR (Wang *et al.*, 2012). Phosphorylated OmpR (OmpR-P) is required for the expression of OmpF and OmpC in conditions of both high and low osmolarity. The total levels of OmpF and OmpC proteins remain constant in response to osmolarity. However, the ratio of OmpF and OmpC is differs at high and low osmolarity (reviewed by Pratt and Silhavy, 1995). At low osmolarity the OmpF protein is predominantly expressed, whereas at high osmolarity the OmpC protein is predominantly expressed. This difference in ratio is determined by the phosphatase and kinase activity of EnvZ that controls the levels of OmpR-P in the cell (reviewed by Pratt and Silhavy, 1995). Phosphorylated OmpR has an enhanced DNA-binding activity, enabling OmpR to modulate expression of the *ompF* and *ompC* genes accordingly (discussed in section 1.6.2) (Head *et al.*, 1998; Feng *et al.*, 2004).

The identification of OmpR as a regulator of the stationary phase ATR highlights the importance of OmpR in acidic conditions in *S. Typhimurium*. Recent studies have also identified OmpR as an important regulator of acid resistance in *E. coli* (Stincone *et al.*, 2011). Thus, there is mounting evidence identifying OmpR as an important regulator of pH response in both *S. Typhimurium* and *E. coli*. However, it is unknown if *ompR* in *E. coli* is induced by low pH similar to *S. Typhimurium*. It is apparent from a comparative study that these species possess distinctive acid tolerance levels (Lin *et al.*, 1995). Therefore, understanding the effect of low pH on *ompR* expression in both species will contribute to our understanding the role of OmpR in pH survival in both species and promoting virulence in *S. Typhimurium*. This chapter describes an investigation of the effect of low pH on *ompR* expression in both *E. coli* and *S.*

Typhimurium and aims to identify the important factors contributing to *ompR* expression in low pH.

3.2 Results

3.2.1 Growth of *S. Typhimurium* and *E. coli* in EG-minimal medium

Prior to investigating *ompR* gene expression in both SL1344 (*S. Typhimurium*) and CSH50 (*E. coli*) it was necessary to first establish appropriate growth conditions to investigate the effect of low pH on *ompR* expression. These conditions were chosen based on the original work describing *ompR* gene expression at low pH in *S. Typhimurium* (Bang *et al.*, 2002).

It was necessary to develop a growth medium suitable for both SL1344 and CSH50. E-minimal medium supplemented with glucose (EG-minimal medium, pH 7.2) and 0.5 mM L-histidine was used as a chemically defined medium. L-histidine was added to promote growth of SL1344, which carries a mutation in the *hisG* gene. Proline and thiamine were added to the medium to fulfil the growth requirements of CSH50, which carries mutations in the *proA/B* and *thi* genes respectively. To analyse the effect of proline and thiamine on growth of SL1344 in EG-minimal medium, growth of SL1344 was monitored with and without the addition of proline (100 µg/ml) and thiamine (1 µg/ml) as described in section 2.2.7 (Fig. 3.1). Growth of CSH50 in the presence of proline (100 µg/ml) and thiamine (1 µg/ml) was also monitored (Fig. 3.1). The mean doubling time of SL1344 was calculated as 61 min both in the presence and absence of proline and thiamine. Therefore, growth of SL1344 was unaffected by these supplementations. The doubling time of CSH50 was slightly faster at 59 min. Therefore, the auxotrophy of both SL1344 and CSH50 was overcome without impeding the growth of either strain. This defined medium was subsequently used in the following experiments.

3.2.2 *ompR* gene expression at low pH in SL1344 and CSH50

Previous work has shown that *ompR* transcript levels in *S. Typhimurium* strain UK-1 were upregulated at acidic pH (Bang *et al.*, 2000; Bang *et al.*, 2002). Bang *et al.* reported *ompR* expression increased approximately 3.7 fold at pH 4.4 using *lacZ* reporter fusions to the *ompR* gene expressed ectopically from the *putPA* locus

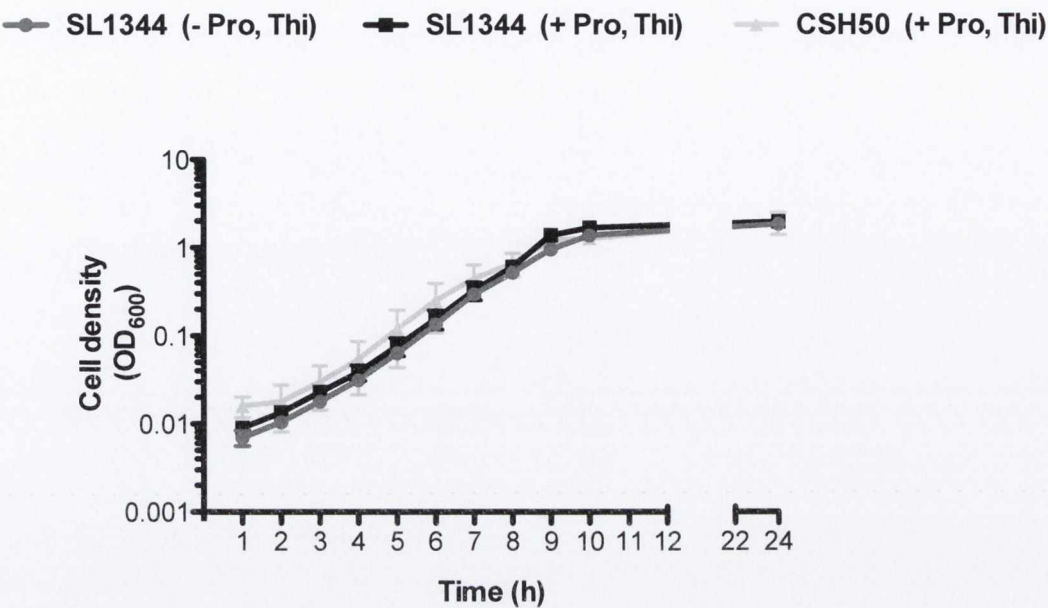


Fig. 3.1. Growth curve of SL1344 and CSH50 in EG-minimal medium (pH 7.2) supplemented with proline and thiamine. Overnight cultures of SL1344 and CSH50 were equalised to an optical density (OD₆₀₀) of 0.001. Cultures were grown in EG-minimal medium supplemented with (+Pro, Thi) or without (-Pro, Thi) proline and thiamine at a final concentration of 100 µg/ml and 1 µg/ml respectively. Cultures were grown at 37°C with constant agitation (200 rpm) in a rotary shaker. Optical density (OD) measurements at 600 nm were taken at various time points. The mean of 3 independent biological replicates is shown; standard deviation is represented as error bars.

(Bang *et al.*, 2002). The present study employed qPCR to assess the effect of low pH on *ompR* expression in both SL1344 and CSH50. Using qPCR, *ompR* expression was measured from its native position on the chromosome in both species. Strains were cultured as described in section 2.2.6 and samples taken after 90 min at pH 7 and pH 4.5. Primer pairs specific to the *ompR* gene in SL1344 (*ompR_RT_S.e_F* and *ompR_RT_S.e_R*; see Table 2.8) and the *ompR* gene in CSH50 (*ompR_E.c_RT_F* and *ompR_E.c_RT_R*; see Table 2.8) were used. Expression levels were normalised to the reference gene *gmk* (SL1344; *gmk_F_Se* and *gmk_R_Se*, CSH50; *gmk_F_E.c* and *gmk_R_E.c*; see Table 2.8) as previous studies in *S. Typhimurium* have shown it is unaffected by low pH in EG-minimal medium (Muller *et al.*, 2009).

The data show *ompR* gene expression levels increased approximately 2.7 fold in SL1344 at pH 4.5 (Fig. 3.2, A). Interestingly, in CSH50 the mean level of *ompR* gene expression in CSH50 did not change in acidic conditions. Additionally, OmpR protein levels were measured by western blotting (section 2.13). SL1344 carrying the 3x FLAG epitope fused to the C-terminal domain of OmpR was used to report OmpR protein levels (Cameron and Dorman, 2012). The 3x FLAG epitope was fused to OmpR in CSH50 in an identical manner (see section 5.2.1). Samples were taken after 90 min at pH 7 and pH 4.5. In SL1344, OmpR protein levels were more abundant at pH 4.5 compared to pH 7. OmpR levels in CSH50 were relatively unaffected by acidic pH (Fig. 3.2, B).

These results indicate that *ompR* is differentially expressed in SL1344 and CSH50. *ompR* expression in SL1344 was upregulated in acidic conditions and this was also observed at the protein level. In contrast, *ompR* gene expression in CSH50 was unaffected by acidic pH at both the RNA transcript and protein level. These results prompted the question; what factor(s) contribute to the species-specific differences in *ompR* gene expression at low pH?

3.2.3 Comparison of the *ompR/envZ* operon in SL1344 and CSH50

The *ompR* and *envZ* genes are organised as a polycistronic operon, historically referred to as the *ompB* locus (Sarma and Reeves, 1977). Expression of both genes is

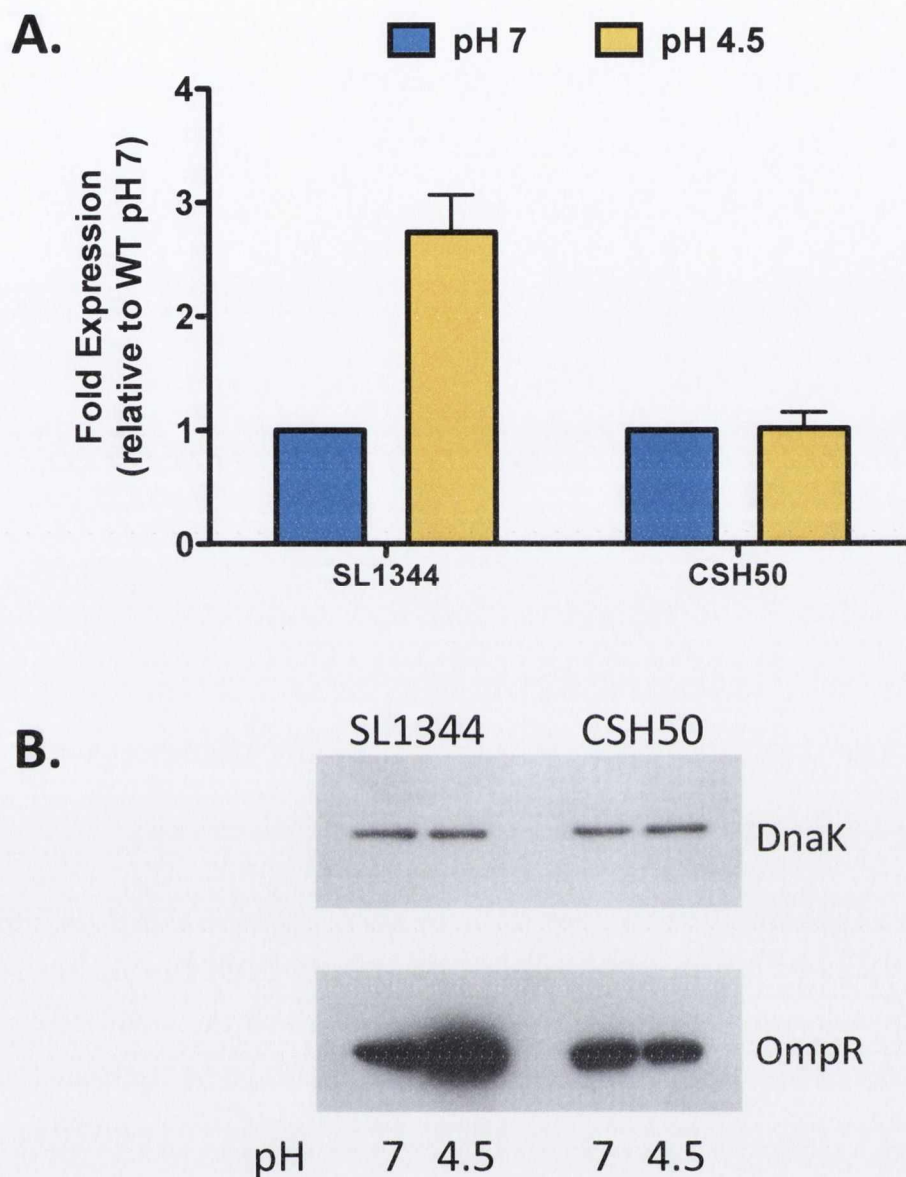


Fig. 3.2. *ompR* gene expression and OmpR protein levels are insensitive to low pH in CSH50. **A.** Quantitative PCR measurements of *ompR* transcripts levels were made in SL1344 (*S. Typhimurium*) and CSH50 (*E. coli*), after 90 min in EG-minimal media at pH 7 (blue) and pH 4.5 (yellow). Transcript levels are expressed in arbitrary units relative to pH 7. The mean from at least 3 independent biological replicates is shown. Error bars denote standard deviations. **B.** OmpR protein was detected by western blotting in SL1344 (*ompR*:3xFLAG) and CSH50 (*ompR*:3xFLAG). Samples were taken after 90 min in EG-minimal media at pH 7 and pH 4.5. Anti-FLAG antibody was used to detect the FLAG epitope and anti-DnaK was used as a loading control. A representative blot from three independent replicates is shown.

dependent on the promoter region upstream of *ompR*. Bang *et al.* identified a transcription start site (TSS) in the *ompR* regulatory region of *S. Typhimurium* that was active at low pH (Bang *et al.* 2002). The *ompR* gene was found to be positively autoregulated by the OmpR protein, requiring the cognate sensor kinase EnvZ for this autoinduction (Bang *et al.*, 2002). It was rationalised that these factors are important for the upregulation of *ompR* in *S. Typhimurium* at low pH. Therefore, any differences in these factors in *E. coli* may determine the species-specific pattern of *ompR* expression at low pH. Comparative analysis of these factors was carried out. Bioinformatics was used to compare the nucleotide sequence of the *ompR* regulatory region and the amino acid sequence of OmpR and EnvZ from both *S. Typhimurium* and *E. coli*.

The alignment program ClustalW (Larkin *et al.*, 2007) was used to compare approximately 300 bp upstream of the *ompR* start codon from both species. Initial analysis comparing the *ompR* promoter region from *S. Typhimurium* strains SL1344 and LT-2 with *S. Typhi* strains CT18 and TY2 found no difference in the nucleotide sequence. Similarly, comparing the *ompR* promoter sequence of *E. coli* strains: MG1655, CFT073, O157:H7 str. EDL933, O157:H7 str. Sakai and *S. flexneri* 2a strains 2457T and str. 302 revealed 100% identity across all strains. The *ompR* promoter nucleotide sequence of SL1344 and MG1655 were chosen to represent *S. Typhimurium* and *E. coli* respectively. These sequences were compared using ClustalW. The *ompR* regulatory region shared 88% nucleotide identity (Fig. 3.3) between both species. In addition to nucleotide differences, each species also possessed unique transcription start sites (TSSs). In *S. Typhimurium* five *ompR* TSSs have been described (Fig. 3.3, blue). TSS-4 was the first to be identified after cells were grown in LB medium (Liljestrom *et al.*, 1988). TSSs 1-3 were subsequently identified after cells were grown in minimal medium. TSS-1 was constitutively active at pH 8 and pH 4.4; while TSSs-2 and -3 were detected at pH 4.4 (Bang *et al.*, 2002). Kroger *et al.* identified TSS-5, this same study also detected TSS-3 (Kroger *et al.*, 2012).

In *E. coli* five *ompR* TSSs have been reported. All five TSSs in *E. coli* are affected by CRP; TSS-1 and TSS-4 are repressed by CRP while TSS-2 and TSS-3 are activated by CRP (Fig. 3.3, red) (Huang *et al.*, 1992). TSSs-1 and -2 were identified as IHF

repressed (Tsui *et al.*, 1991). TSS-5 was first identified using *in vitro* transcription assays (Mizuno *et al.*, 1982; Wurtzel *et al.*, 1982). These differences in *ompR* transcriptional profiles in both *S. Typhimurium* and *E.coli* may account for the differential expression of *ompR* at low pH.

A previous study has shown the EnvZ protein shares 95% amino acid sequence identity between *E. coli* and *S. Typhi* (Martinez-Flores *et al.*, 1999). The EnvZ amino acid sequence was compared from the following *Salmonella enterica* Typhimurium strains: LT-2, ST4/74, SL1344, 798, 14028S and UK-1. The EnvZ sequence from *Salmonella enterica* Typhi strains: Ty2, CT18 and ParaTyphi strain ATCC 9150 were also included in the analysis. ClustalW revealed the EnvZ amino acid sequence was 100% conserved in all strains except Typhi strains Ty2 and CT18; both strains had a single amino acid substitution (G354C). However, the EnvZ amino acid sequence from SL1344 was used as a representative sequence for all *S. Typhimurium* strains.

The EnvZ amino acid sequence from the following eighteen *E. coli* and two *Shigella flexneri* strains were also compared: MG1655, DH10B, D12, E2348/69, UTI89, W3310, BW2952, HS, ATCC8739, RM12579, IAI1, O26:H11 str. 11368, O157:H7 str. EDL933, O157:H7 str. Sakai, BL2-DE3, O104:H4 str. 2011C-3493, P12b, CFT073; *S. flexneri* strains 2a str. 2457T and str. 301. A total of 7 amino acid substitutions were found using ClustalW. The amino acid substitutions are as follows:

Substitution	Strain(s)
1. A25V	UTI89, E2348/69 and D12
2. P41L	BL21(DE3)
3. E329D	O26:H11 str. 11368
4. R383C	IAI1
5. G445S	O157:H7 str. Sakai
6. T446M	UTI89, E2348/69
7. T447I	O104:H4 str. 2011C-3493

In this study, the amino acid sequences of EnvZ from *E. coli* (MG1655) and *S. Typhimurium* (SL1344) were compared using ClustalW; revealing 95% amino acid identity sequence between species (Fig 3.4, B). Furthermore, those amino acids that

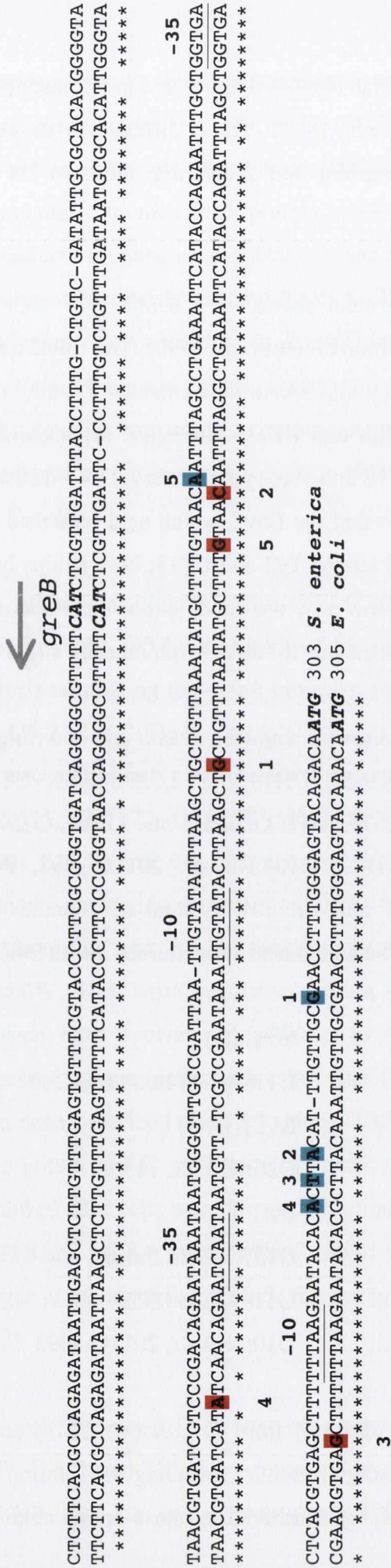
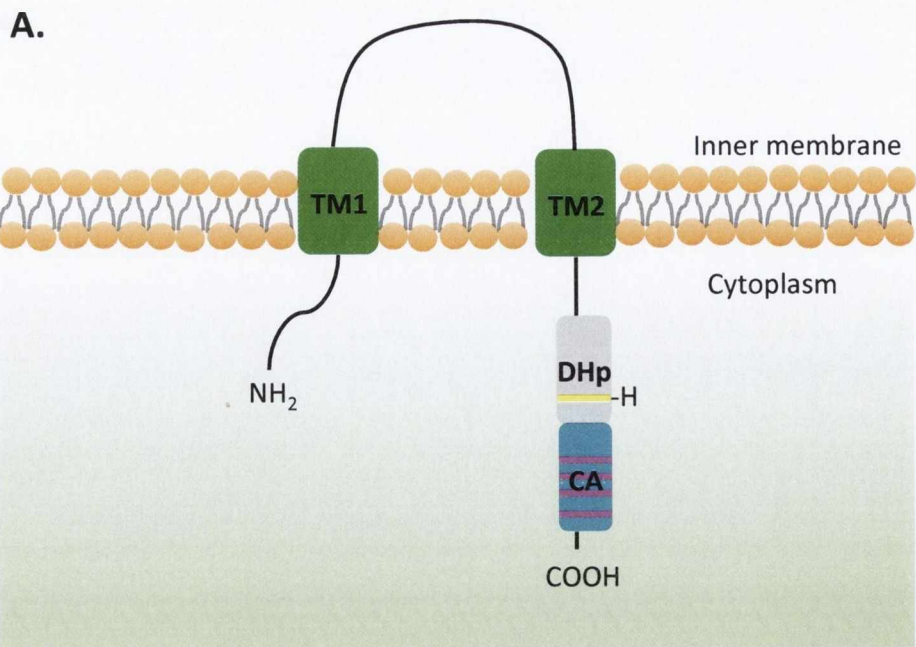


Fig. 3.3. Nucleotide sequence alignment of the *ompR* regulatory region of *S. Typhimurium* and *Escherichia coli*. Alignment of the *ompR* regulatory region of *S. enterica* (Top) and *E. coli* (Bottom) is shown. Conserved nucleotides are indicated by an asterisk (*). Transcription start sites (TSSs) for *E. coli* are highlighted in red and are in bold (Mizuno *et al.*, 1982; Wurtzel *et al.*, 1982; Tsui *et al.*, 1991; Huang *et al.*, 1992). TSSs in *S. enterica* are highlighted in blue and are in bold (Liljestrom *et al.*, 1988; Bang *et al.*, 2002). The ATG start codon for each species is in bold and italicised. The -10 and -35 for the *E. coli* TSS-1 and *S. enterica* TSS-3 are underlined. The start codon for the divergently transcribed *greB* gene is in bold and highlighted by a grey arrow.



B.

	TM 1	
<i>E. coli</i>	MRRLRFSRSSFARTLLLIIVTLFASLVTTYLVVINFAILPSLQQFNKVLAYEVRMLMTD	60
<i>S. ent</i>	MRRMRFSRSSFARTLLLIIVTLFVSLVTTYLVVINFAILPSLQQFNKVLAYEVRMLMTD	60
	:**	
<i>E. coli</i>	KLQLEDGTQLVPPAFRREIYRELGISLYSNEAAEEAGLRWAQHYESFLSHQMAQQLGPT	120
<i>S. ent</i>	KLQLEDGTQLVPPAFRREIYRELGISLYTNEAAEEAGLRWAQHYESFLSHQMAQQLGPT	120
	*****:*****	
	TM 2	
<i>E. coli</i>	EVRVEVNKSSPVVWLKTWLSPIWVRVPLTEIHQGDFSPLFRYTLAIMLLAIGGAWLFIR	180
<i>S. ent</i>	EVRVEVNKSSPVVWLKTWLSPIWVRVPLTEIHQGDFSPLFRYTLAIMLLAIGGAWLFIR	180
	*****:*****	
<i>E. coli</i>	IQNRPLVDLEHAALQVGKGIIPPLREYGASEVRSVTRAFNHMAAGVKQLADDRLLMAG	240
<i>S. ent</i>	IQNRPLVDLEHAALQVGKGIIPPLREYGASEVRSVTRAFNHMAAGVKQLADDRLLMAG	240
	*****:*****	
<i>E. coli</i>	VSHDLRTPLTRIRLATEMMSEQDGYLAESINKDIEECNAIIIEQFIDYLRITGQEMPMEAD	300
<i>S. ent</i>	VSHDLRTPLTRIRLATEMMGEEEDGYLAESINKDIEECNAIIIEQFIDYLRITGQEMPMEAD	300
	*****:*****	
	N box	
<i>E. coli</i>	LNAVLGIVIAAESGYEREIETALYPGSIQVIMHPLSIKRAVANMVVNAARYNGNWKVSS	360
<i>S. ent</i>	LNSVLGEVIAAESGYEREINTALQAGSIQVIMHPLSIKRAVANMVVNAARYNGNWKVSS	360
	:**:***	
	G1 box F box G2 box	
<i>E. coli</i>	GTEPNRAWFQVEDDGPGLAPEQRKHLFQPFVRGDSARTISGTGLGLAIVQRIVDNHNHGM	420
<i>S. ent</i>	GTESHRAWFQVEDDGPGLKPEQRKHLFQPFVRGDSARSTSGTGLGLAIVQRIIDNHNHGM	420
	:**:*****:*****	
<i>E. coli</i>	ELTGSERGGLSIRAWLPVPVTRAQGTKEG	450
<i>S. ent</i>	EIGTSERGGLSIRAWLPVPVARVQGTKEA	450
	*:*****:*	

Fig. 3.4. Structure and amino acid sequence alignment of the EnvZ proteins from *S. Typhimurium* and *Escherichia coli*. **A.** Schematic representation of the EnvZ protein. Conserved regions of the histidine kinase family are marked by pink and yellow boxes and refer to the corresponding sequence in part B. Domains are labelled as follows: TM (Transmembrane region, green), CA (catalysis-assisting and ATP-binding, blue), DHp (dimerization and histidine phosphotransferase domain, grey). **B.** The amino acid sequences of EnvZ of *E. coli* (Top) and *S. enterica* (Bottom) are shown. The alignment was carried out using ClustalW version 2 (Larkin *et al.*, 2007). Conserved

residues are denoted by an asterisk (*), a colon (:) indicates conservation between groups with strongly similar properties and a full stop (.) indicates conservation between groups with weakly similar properties. Regions are coloured based on the corresponding locations in the illustration above (A). The HAMP (histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and phosphatases) linker is underlined which also marks the beginning of the cytoplasmic domain.

```

enterica      MQENYKILVVDDDMRLRALLERYLTEQGFQVRSVANAEQMDRLLTRESFHLMVLDLMLPG 60
coli          MQENYKILVVDDDMRLRALLERYLTEQGFQVRSVANAEQMDRLLTRESFHLMVLDLMLPG 60
*****

enterica      EDGLSICRRLRSQSNPMPIIMVTAKGEEVDRIVGLEIGADDYIPKPFNPPELLARIRAVL 120
coli          EDGLSICRRLRSQSNPMPIIMVTAKGEEVDRIVGLEIGADDYIPKPFNPPELLARIRAVL 120
*****

enterica      RRQANELPGAPSQEEAVIAFGKFKLNLGTREMFREDEPMPLTSGEFAVLKALVSHPREPL 180
coli          RRQANELPGAPSQEEAVIAFGKFKLNLGTREMFREDEPMPLTSGEFAVLKALVSHPREPL 180
*****

enterica      SRDKLMNLAGREYSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVPDGSKA 239
coli          SRDKLMNLAGREYSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVPDGSKA 239
*****

```

Fig. 3.5. Amino acid sequence alignment of the OmpR proteins from in *S. Typhimurium* and *Escherichia coli*. Sequence alignment of the OmpR proteins of *S. enterica* and *E. coli*. This alignment was carried out using ClustalW version 2 (Larkin *et al.*, 2007). Conserved residues are denoted by an asterisk (*), the amino acid sequences are identical.

differed were identical to those described in *S. Typhi* (Martinez-Flores *et al.*, 1999). Of the 20 amino acid differences, 15 were located in the CA (catalysis-assisting and ATP-binding) domain (Fig. 3.4, B (blue)) (Martinez-Flores *et al.*, 1999). The CA domain binds ATP and catalyses auto-phosphorylation of EnvZ at a conserved histidine found in the DHP (dimerization domain and phosphotransferase) domain (Fig. 3.4 grey) (Yang and Inouye, 1991; Qin *et al.*, 2000). In addition the CA domain also contains highly conserved regions (G1, G2, F and N boxes) (Fig. 3.4 pink) that are found in all members of the histidine kinase family (Swanson *et al.*, 1994; Tanaka *et al.*, 1998; Dutta and Inouye, 2000). Interestingly, the amino acids that differed in EnvZ were localised to the regions outside of these conserved boxes (Fig. 3.4, B) (Martinez-Flores *et al.*, 1999). The effect of these amino acid changes on the pH sensitivity of OmpR/EnvZ system is unknown. However, these differences could affect the ability of EnvZ to transmit signals and communicate to the OmpR protein during acidic conditions.

OmpR was not implicated as a factor contributing to the differential expression of the *ompR* gene at acidic pH as the OmpR proteins shared 100% amino acid identity between both species (Fig. 3.5) (Martinez-Flores *et al.*, 1999). This analysis included comparison of the following strains: *S. Typhimurium* SL1344, *S. Typhi* str. CT18 and TY2, *S. flexneri* 2a str. 2457T and str. 302, *E. coli* str. MG1655, CFT073, O157:H7 str. EDL933, O157:H7 str. Sakai. Consequently, the contributions of both the *ompR* regulatory region and the EnvZ protein to the pH sensitivity of *ompR* were tested.

3.2.4 Strategy to generate strain CSH50 *PompR* *S. enterica*

It was important to test if the pH sensitivity of the *ompR* gene in *S. Typhimurium* was hard-wired into the *ompR* regulatory region. To do this the *ompR* regulatory region of SL1344 was introduced in place of the *ompR* regulatory region of CSH50 generating the strain CSH50 *PompR* *S. enterica*. It was hypothesised that if the *ompR* regulatory region of SL1344 was responsible for the pH sensitivity of *ompR* transcription then it would stimulate *ompR* expression in CSH50 at low pH. Strain CSH50 *PompR* *S. enterica* was created using the λ Red recombination system (Datsenko and Wanner, 2000), this strategy is described below and illustrated in Fig. 3.6.

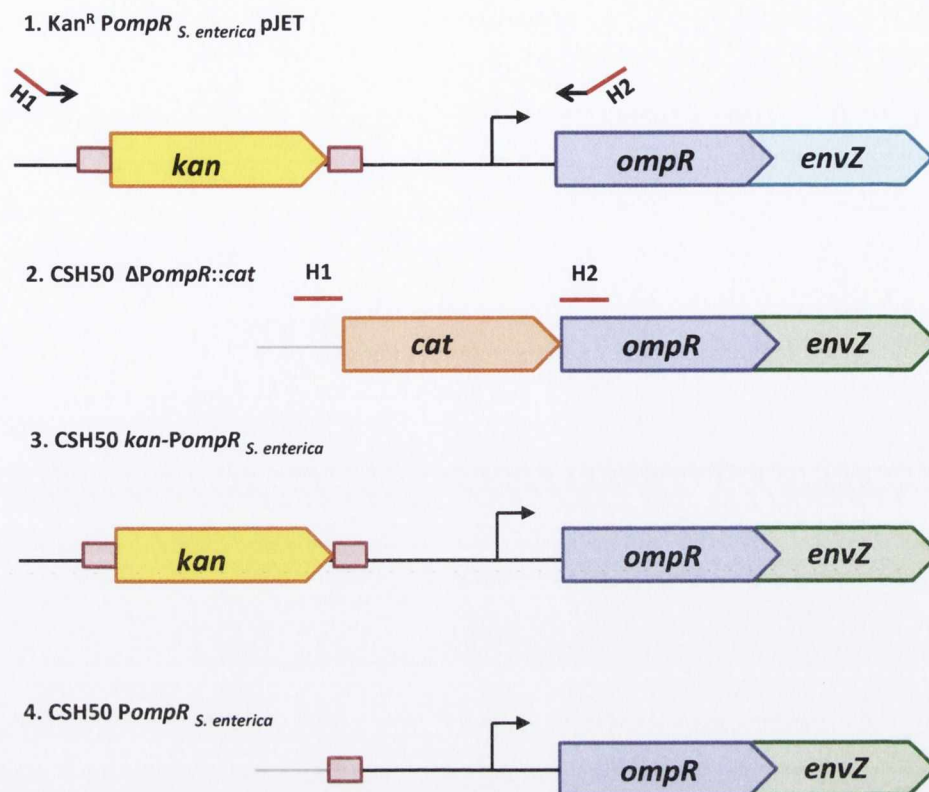


Fig. 3.6. Strategy for integrating the *ompR* promoter of *S. Typhimurium* in place of the native *ompR* promoter in *E. coli*. 1. Primers (*Pompr*_int_E.c_F and *Pompr*_int_E.c_R, see Table 2.8) were used to amplify the SL1344 *ompR* promoter and the upstream kanamycin resistance marker from the plasmid *Pompr*_{*S. enterica*} pJET. These primers (bent, dual-coloured arrows) used were 60 bp in length; 20 bp flanked the regions to be amplified and the remaining 40 bp (red) were complementary to the integration sites in CSH50 (H1 and H3 in step 2). 2. CSH50 Δ *Pompr*::*cat* was made by replacing 302 bp upstream of the *ompR* start codon in CSH50 with the *cat* gene (conferring chloramphenicol resistance) using primers *Pompr*_KO_E.c_F and *Pompr*_KO_E.c_R (Table 2.8). The PCR product containing the *kan* gene upstream of the *ompR* promoter of SL1344 was electroporated into CSH50 Δ *Pompr*::*cat*. 3. After the recombination event, the *kan* gene and *ompR* promoter of SL1344 was placed upstream of the *ompR* gene in CSH50 (CSH50 *Pompr* *S. enterica*). 4. CSH50 *Pompr*_{*S. enterica*} was created by removing the *kan* gene using the plasmid pCP20 leaving a single 40 bp FRT site (pink box). For simplicity, only a single transcription start site is shown (bent black arrow).



Fig. 3.7. Confirmation of CSH50 *PompR*_{S. enterica} by PCR. **A.** Schematic representing the PCR primers sets used to confirm integration of the *PompR*_{S. enterica} in place of the native *ompR* promoter in CSH50. Primer sets are denoted by black and red arrows. The red primer set included primers complementary upstream of the *kan* gene and the *ompR* gene of CSH50 (*PompR*_int_E.c_F and *ompR*_E.c_RT R, Table 2.8). The expected PCR product size for the mutant and wild type was 2,071 bp and 566 bp, respectively. The black primer set included primers complementary to upstream of the *kan* gene and a second primer complementary to the *ompR* gene of CSH50 (*PompR*_int_E.c_F and *ompR*_conf_E.c_R, Table 2.8). The expected PCR product size of the mutant and wild type was 2,642 bp and 1,137 bp respectively. **B.** Gel image representing the results of both PCR sets. In each case wild type CSH50 (WT) was used as a control and 3 mutants were screened (1-3). M: 1 kb DNA ladder. Products were analysed on a 1% TAE agarose gel. For simplicity, only a single transcription start site is shown (bent blue arrow).

As the *ompR* regulatory region of both SL1344 and CSH50 share 88% DNA sequence identity, it was necessary to first delete the *ompR* regulatory region in CSH50. This was carried out to avoid unwanted homologous recombination occurring between the *ompR* regulatory regions of both strains. To do this the *cat* gene (conferring chloramphenicol resistance) was amplified from plasmid pKD3 using primers *PompR_KO_E.c_F* and *PompR_KO_E.c_R* (Table 2.8). This produced a linear DNA fragment with flanking regions of homology to the *ompR* regulatory region of CSH50. The *cat* gene was integrated into CSH50 using the λ Red recombination system (Datsenko and Wanner, 2000). This deleted 302 bp upstream of the *ompR* start codon creating CSH50 Δ *PompR::cat* (Fig. 3.6, 2). The λ Red recombination system (Datsenko and Wanner, 2000) was then used to integrate the *ompR* regulatory region of SL1344 into CSH50 Δ *PompR::cat* as follows: primers (*PompR_int_E.c_F* and *PompR_int_E.c_R*; see Table 2.8) were used to amplify the *ompR* regulatory region and upstream *kan* gene from plasmid *PompR* *S. enterica* pJET (construction of this plasmid is described in section 4.2.1). This plasmid encodes the *kan* gene conferring kanamycin resistance and the *ompR* regulatory from SL1344. The *kan* gene is flanked by FRT sites that were later used to remove the *kan* gene after integration onto the chromosome. PCR amplification with *PompR_int_E.c_F* and *PompR_int_E.c_R* produced a linear DNA fragment flanked by DNA sequence homologous to regions adjacent to the *cat* gene. Integration of this DNA into CSH50 Δ *PompR::cat* resulted in the replacement of the *cat* gene with the *ompR* regulatory region from SL1344 and upstream *kan* cassette (Fig. 3.6, 3). Transformants were screened by checking resistance to kanamycin and sensitivity to chloramphenicol on L-agar plates containing either chloramphenicol or kanamycin. Subsequently, correct integration was confirmed by PCR (Fig. 3.7) and by DNA sequencing (GATC Biotech).

Primer sets used to confirm correct integration are illustrated in Fig. 3.7 (A). A forward primer complementary to the region upstream of the kanamycin cassette and a reverse primer specific to the *ompR* gene in CSH50 were used (Fig. 3.6, A (black arrows)). PCR performed with these primers amplified a product approximately 2,642 bp and 1,137 bp in size in the transformants and wild type (control) respectively (Fig. 3.6, B). PCR using the primer specific to the upstream region of the *kan* gene and a different primer specific to the *ompR* gene (Fig. 3.6 A, red arrows) was also carried out. This amplified a product 2,071 bp and 566 bp in size in transformants and wild type (control)

respectively (Fig. 3. 7, B). Correct integration was confirmed by DNA sequencing of these PCR products (GATC Biotech). The mutant was P1 transduced into a fresh background (CSH50) and the kanamycin cassette was then removed using the plasmid pCP20 (Datsenko and Wanner, 2000) and screened for sensitivity to kanamycin (Fig. 3.6, 4).

3.2.5 Strategy to generate CSH50 *ompB* *S. enterica*

As described in section 3.2.3, the EnvZ proteins of *S. Typhimurium* and *E. coli* are 95% identical (Martinez-Flores *et al.*, 1999). It was important to test whether the pH response of *ompR* expression in *S. Typhimurium* was as a result of these amino acid differences. To do this, the *ompB* locus (*ompR/envZ* genes) of CSH50 was replaced by the *ompB* locus of SL1344 creating strain CSH50 *ompB* *S. enterica*. It was rationalised that if the pH sensitivity of *ompR* in SL1344 was attributable to EnvZ, the introduction of SL1344 EnvZ into CSH50 should stimulate upregulation of *ompR* in CSH50. As the OmpR protein is 100% conserved at the amino acid level between SL1344 and CSH50; thus, any observed differences in CSH50 *ompB* *S. enterica* can be credited to EnvZ and not OmpR.

A similar strategy, as described in section 3.2.4, was used to create CSH50 *ompB* *S. enterica*. Firstly, the *ompB* locus of CSH50 was replaced by the *cat* gene to avoid unwanted homologous recombination occurring when the SL1344 *ompB* locus was introduced into CSH50. Primers *ompB::cat_E.c_F* and *ompB::cat_E.c_R* were used to PCR amplify the *cat* gene from plasmid pKD3, producing a linear DNA fragment with homology flanking the *ompB* locus in CSH50. Using the λ Red recombination system the *ompB* locus was replaced by the *cat* gene (Fig. 3.8, 2) (Datsenko and Wanner, 2000). The *kan* gene was then introduced downstream of the *ompB* locus in SL1344. The primers *ompB_kan_S.e_F* and *ompB_kan_S.e_R* (Table 2.8) were used to amplify the *kan* gene from plasmid pKD4. PCR amplification generated a linear DNA product with homology to DNA sequence downstream of the *ompB* locus in SL1344. Using the λ Red recombination system (Datsenko and Wanner, 2000) the *kan* gene was inserted downstream of the *ompB* locus on the chromosome of SL1344 creating SL1344 *ompB-kan* (Fig. 3.8, 1). This conferred resistance to kanamycin and was used as a selective marker for integration of the SL1344 *ompB* locus into CSH50.

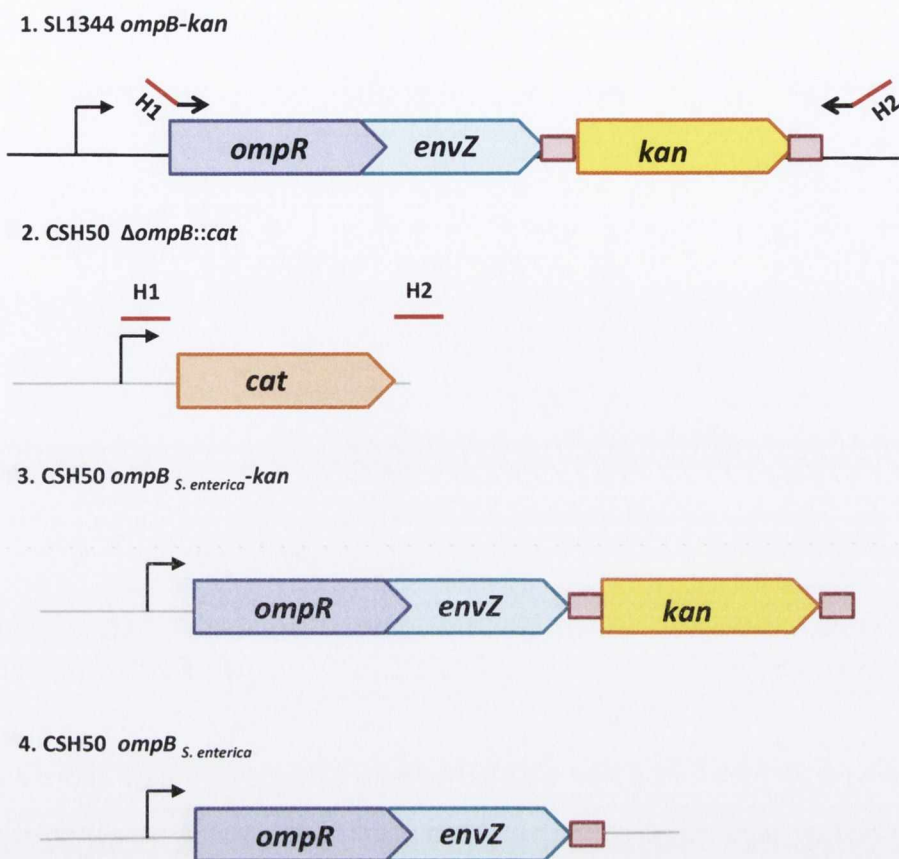


Fig. 3.8. Strategy used to integrate the *ompR/envZ* locus of *S. Typhimurium* in place of the native *ompB* locus in *E. coli*. 1. The primers used to amplify the *ompB*-*kan*^R locus from SL1344 *ompB*_{*S. enterica*} *kan*^R are shown as bent, dual-coloured arrows. These primers contained 20 bp complementary to the regions to be amplified and another 40 bp (red) that are complementary to the integration sites in CSH50 (H1 and H2 in step 2). 2. The *ompB* locus of CSH50 was replaced by the *cat* gene creating strain CSH50 $\Delta ompB::cat$. The PCR product from step 1 containing the *ompB*-*kan* locus was electroporated into CSH50 $\Delta ompB::cat$. 3. Following recombination the *ompB*-*kan* locus of SL1344 was introduced in place of the native *ompB* locus in CSH50. 4. CSH50 *ompB*_{*S. enterica*} was created by removing the *kan* gene using the plasmid pCP20, leaving a single FRT behind (pink box).

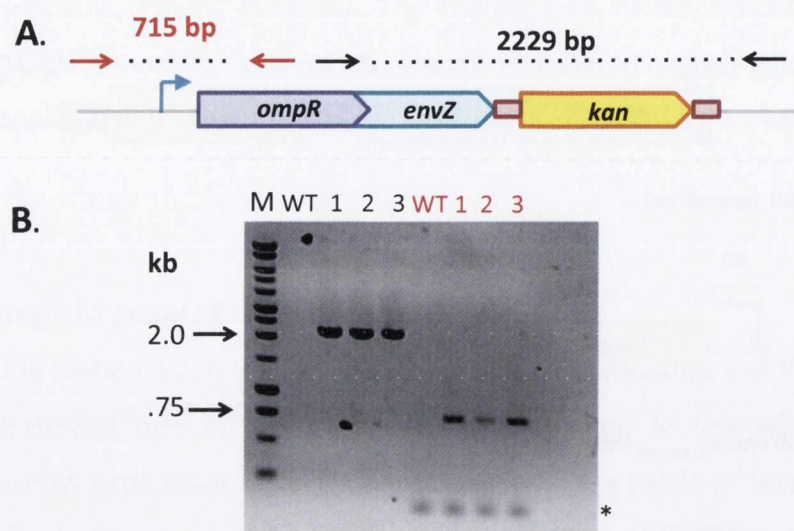


Fig. 3.9. Confirmation of CSH50 *ompB*_{S.enterica}-*kan* by PCR. **A.** Schematic representing the PCR sets used to confirm integration of the *ompB*_{S.enterica}-*kan* in place of the native *ompB* locus in CSH50. Primer sets are denoted by black and red arrows. The red primer set included primers complementary to the *ompR* promoter of CSH50 and the *ompR* gene of SL1344 (*PompR*_KO_E.c_F and *ompR*_S.e_R, see Table 2.8). The expected PCR product size was 715 bp. The black primer set included primers complementary to the *ompR* gene of SL1344 and downstream of the CSH50 *ompB* locus (*ompR*_S.e_F and *ompB*_E.c_R, see Table 2.8). The expected PCR product size was 2,229 bp. **B.** Gel image representing the results of both PCR sets. In each case wild type CSH50 (WT) was used as a control and 3 mutants were screened (1-3). As these primer sets target both CSH50 and SL1344 DNA sequence no product was expected in WT lane. M: 1 kb DNA ladder. Products were analysed on a 1% TAE agarose gel. Asterisk denotes possible primer dimers.

Finally, the *ompB-kan* locus of SL1344 *ompB-kan* was amplified using primers *ompR_F* and *kan_R* (Table 2.8). This PCR product had flanking DNA sequences homologous to regions adjacent to the *cat* gene in CSH50 $\Delta ompB::cat$. This product was then integrated into CSH50 $\Delta ompB::cat$ by λ Red recombination; this introduced the SL1344 *ompB-kan* locus downstream of the native *ompR* promoter in CSH50. This resulted in the *cat* gene and introduction of the *ompB-kan* locus. As such, transformants were screened for resistance to kanamycin and sensitivity to chloramphenicol. Integration was confirmed by PCR (Fig. 3.9, B) and DNA sequencing (GATC biotech). Primers used for PCR confirmation are illustrated in Fig. 3.9, A. Two primer sets were used; primer set one (Fig. 3.9, A (red)) was complementary to the *ompR* promoter of CSH50 and the *ompR* gene of SL1344; the expected product size was approximately 715 bp. Primer set two (Fig. 3.9, A, (black)) was complementary to the *envZ* gene of SL1344 and downstream of the *ompB* locus in CSH50; the expected product size was approximately 2,229 bp. As both primer sets target both SL1344 and CSH50 DNA sequence no product was expected using CSH50 DNA template. The mutant was then moved into a fresh background by P1 transduction and the *kan* gene was removed using the plasmid pCP20 (Fig. 3.8, 4) and screened for sensitivity for kanamycin.

3.2.6 The pH sensitivity of *ompR* expression determined by the regulatory region

After generation of CSH50 *PompR S. enterica* and CSH50 *ompB S. enterica ompR* expression was examined. Strains were cultured in EG-minimal medium as described in section 2.2.6; samples were taken after 90 min and prepared for analysis by qPCR. Primers specific to *ompR* in SL1344 (*ompR_RT_S.e_F* and *ompR_RT_S.e_R*; see Table 2.8) were used to measure *ompR* expression in SL1344 and CSH50 *ompB S. enterica*, as both strains carry the *ompR* gene of SL1344. Primers specific to *ompR* in CSH50 (*ompR_E.c_RT_F* and *ompR_E.c_RT_R*; see Table 2.8) were used to measure *ompR* expression in CSH50 and CSH50 *PompR S. enterica*, as both strains carry the native *ompR* gene.

ompR expression in CSH50 *ompB S. enterica* was 1.2 fold higher at pH 4.5 relative to pH 7; this difference was not statistically significant (Fig. 3.10). This inferred that the *ompB* locus, and therefore the EnvZ protein of SL1344, was not accountable for upregulating

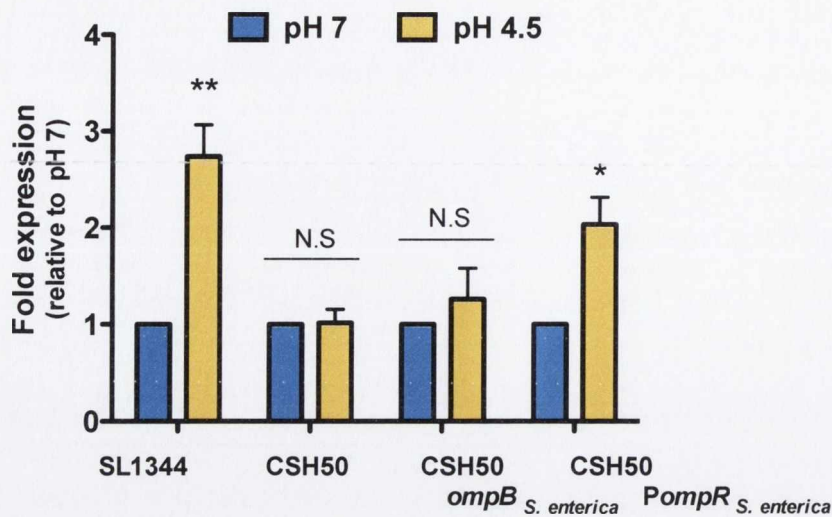


Fig. 3.10. The *ompR* promoter of *S. enterica* is responsible for *ompR* expression at low pH. *ompR* transcript levels were measured in SL1344, CSH50, CSH50 containing the *ompB* locus from SL1344 (CSH50 *ompB* *S. enterica*) and CSH50 harbouring the *ompR* promoter from SL1344 (CSH50 *PompR* *S. enterica*). Samples were taken after 90 min in EG-minimal media at pH 7 (blue) and pH 4.5 (yellow). Transcript levels are expressed relative to WT at pH 7. The mean of 3 independent biological replicates is shown. Error bars denote standard deviation. Student's *t*-test were performed comparing samples at pH 7 and pH 4.5, ** $p < 0.001$, * $p < 0.05$, N.S not statistically significant.

ompR at low pH in SL1344. However, *ompR* expression in CSH50 *PompR* *S. enterica* was 2 fold higher at pH 4.5; this difference was statistically significant. This indicates that the introduction of the *ompR* regulatory region from SL1344 into CSH50 stimulated upregulation of *ompR* at low pH. Furthermore, CSH50 *PompR* *S. enterica* harbours the native CSH50 EnvZ protein. Therefore, the induction of *ompR* at low pH can occur in the presence of EnvZ from CSH50, but is dependent on the presence of the regulatory region from SL1344. It can be concluded that the species-specific differences in *ompR* expression is determined by the *ompR* regulatory region. However, it is unknown what features of the regulatory region are responsible.

3.2.7 Identification of *ompR* transcription start sites in SL1344 and CSH50

Results thus far implicated the *ompR* regulatory region as the major contributing factor to *ompR* induction at low pH in *S. Typhimurium*. However this prompts a further question; what features of the *ompR* promoter cause this? As described in section 3.2.3, the *ompR* regulatory regions of *E. coli* and *S. Typhimurium* share 88% identity at the nucleotide sequence level. Furthermore, both species use different TSSs (Liljestrom *et al.*, 1988; Tsui *et al.*, 1991; Huang *et al.*, 1992; Bang *et al.*, 2002). A previous study in *S. Typhimurium* strain UK-1 identified an acid-inducible *ompR* TSS (Bang *et al.*, 2002). However, the TSSs active in *E. coli* under the same acidic conditions have not been identified. It was hypothesised that pH insensitivity of the *E. coli* *ompR* promoter may be due to a difference in the TSSs. For this reason 5' RACE was used to identify the TSSs expressed in both SL1344 and CSH50 in the conditions used in this study.

Strains were grown in EG-minimal medium, as described in section 2.2.6. After 90 min at pH 7 and pH 4.5 samples were harvested for RNA extraction and analysis by 5' RACE (see section 2.9). PCR was then performed on cDNA samples using adapter-specific (RNA oligonucleotide A4; see Table 2.8) and *ompR* specific primer (RACE_*ompR*; see Table 2.8). This primer is complementary to a region of conserved nucleotide sequence in both species. PCR products were then separated on a 3% agarose gel (Fig. 3.11). Analysis in SL1344 revealed PCR products less than 200 bp in size (Fig. 3.11). The major product (Fig. 3.11, 1) was identified by DNA sequencing as the same acid-inducible TSS described by Bang *et al.* in *S. Typhimurium* strain UK-1 (See TSS-3 (blue) in Fig. 3.3.) (Bang *et al.*, 2002). Bang *et al.* observed this TSS after 2 h at pH 4.4

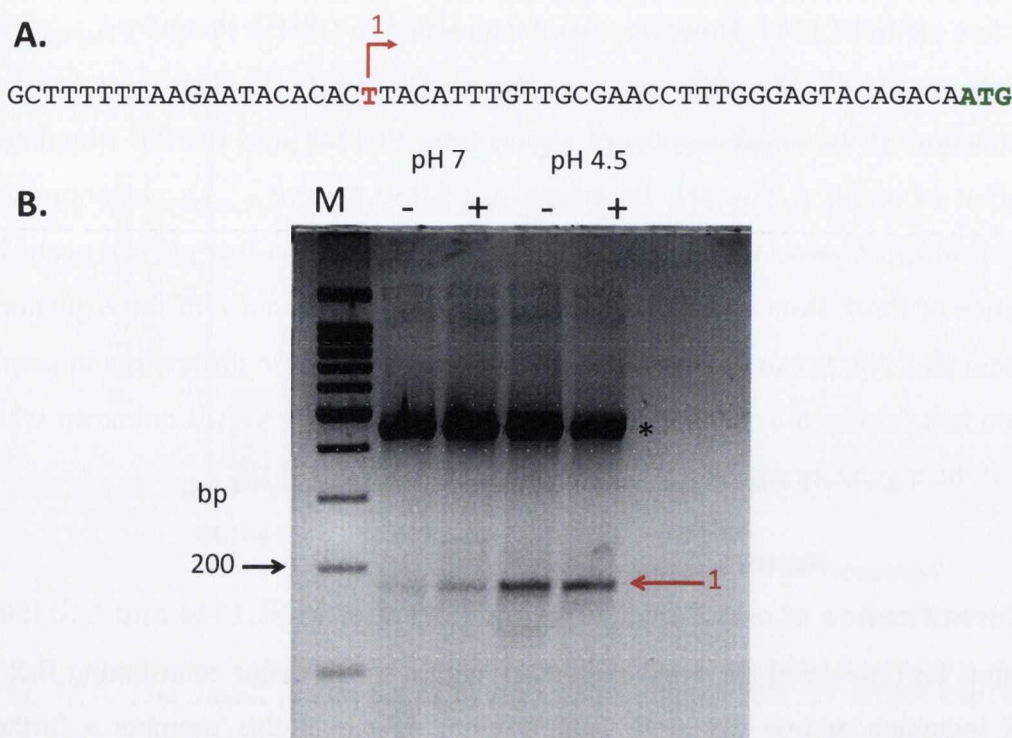


Fig. 3.11. Identification of *ompR* transcription start sites in SL1344. **A.** Location of the primary product (1) extracted from the gel is shown as bent arrow. The *ompR* start codon ATG is highlighted in green. **B.** Gel electrophoresis of 5' RACE-amplified *ompR* cDNA ends were analysed on a 3% agarose gel. RNA was extracted after 90 min in EG minimal medium at pH 7 and pH 4.5. Each sample was split in two; one was treated (+) and one was untreated (-) with tobacco acid pyrophosphatase and ligated with an RNA linker. This RNA was reverse-transcribed into cDNA. PCR was performed on each cDNA sample with RNA oligonucleotide A4 and RACE_*ompR* primers; see Table 2.8. The PCR product of interest is denoted by a red arrow (number 1). Product 1 was gel extracted and cloned directly into the pJET plasmid and at least five clones were sequenced. M: 100 bp ladder. Asterisk denotes non-specific PCR product; this product was sequenced and shared 100% identity with 23S ribosomal RNA product as determined using BLAST (Basic Local Alignment Search Tool).

A.

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AAGCTGCTGTTTAAATATGCTTTGTAACAATTAGGCTGAAATTCATACCAGATTTAGCTGGTGACG
      1
AACGTGAGCTTTTTTAAGAATACACGCTTACAAATTGTTGCGAACCTTTGGGAGTACAAACAATG
                                2
  
```



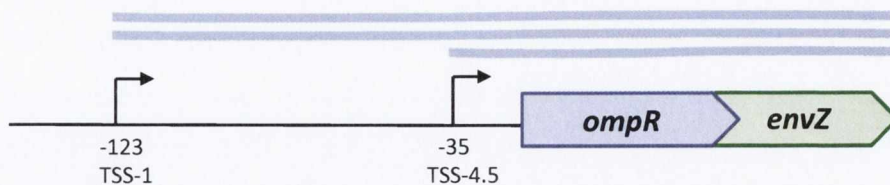
Fig. 3.12. Identification of *ompR* transcription start sites in CSH50 and CSH50 *PompR* *S. enterica*. **A.** The location of the primary products (1 and 2) in the *ompR* regulatory region are shown as bent arrows. **B.** Gel electrophoresis of 5' RACE-amplified *ompR* cDNA ends were analysed on a 1% agarose gel. RNA was extracted CSH50 *PompR* *S. enterica* after 90 min in EG minimal medium at pH 7 and pH 4.5. TAP-treated samples are shown. PCR was performed on each cDNA sample with linker-A4 and *ompR*-specific primers. PCR products 1 and 2 (red arrows) were gel extracted and cloned directly into the pJET plasmid. M: 100 bp ladder.

(Bang *et al.*, 2002). However, in this study the same TSS was present in samples harvested at both pH 7 and pH 4.5 after 90 min. It is unknown why Bang *et al.* observed this TSS only at low pH. This may be attributed to the strain background used in their study (UK-1) or this TSS may not be active at pH 8, as used in their study (Bang *et al.*, 2002). These results revealed *ompR* expression depends largely on the same TSS at pH 7 and pH 4.5 and expression is enhanced from this TSS at pH 4.5. In CSH50, transcription was detected from two TSSs at pH 7 (Fig. 3.12, A). Notably, a single TAP treated product was observed at pH 4.5. DNA sequencing was used to identify the two TSSs expressed at pH 7 (Fig. 3.12, B, 1+2). At pH 7 the primary enriched product in the TAP-treated samples was TSS-1, which was first identified by Tsui *et al.* in *E. coli* (Tsui *et al.*, 1991). The second TSS was active at pH 7 and pH 4.5. This was identified as the same acid-inducible TSS present in SL1344 (Bang *et al.*, 2002), hereafter TSS-4.5. Notably, TSS-4.5 was expressed at pH 7 in both species. However, TSS-1 was also expressed in CSH50. At pH 4.5 TSS-4.5 was the primary TSS active in both species. TSS-4.5 is, therefore, the first common *ompR* TSS active in both *S. Typhimurium* and *E. coli*. 5' RACE was also carried out in CSH50 *PompR S. enterica* under the same conditions; interestingly, the *ompR* regulatory region of SL1344 maintained its natural TSS profile when moved into a new genetic background (Fig. 3.12, C). This supports the qPCR data presented in Fig. 3.10, A, which shows *ompR* expression in CSH50 *PompR S. enterica* displayed a pH sensitive response.

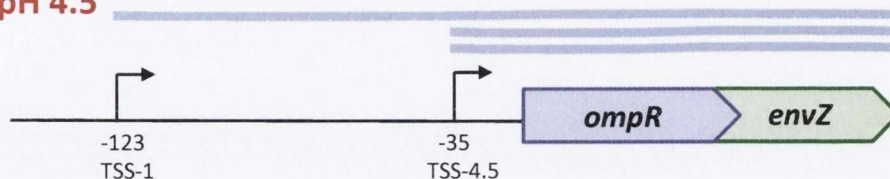
Combining analysis using 5' RACE and the qPCR data from Fig. 3. 10 (A) a working model is predicted (Fig. 3.13). CSH50 maintains steady a state of *ompR* expression at pH 7 and pH 4.5. To do this CSH50 employs two predominant TSSs, TSS-1 and TSS-4.5. At pH 7 both TSSs are active, however at pH 4.5 transcription shifts to TSS 4.5. Expression from TSS-4.5 at pH 4.5 may increase to preserve steady levels of *ompR* expression. In SL1344, *ompR* expression depends primarily on TSS-4.5 at both pH 7 and pH 4.5. Gene expression analysis in Fig. 3.2 showed transcription increased from TSS-4.5 at pH 4.5. Therefore, the *ompR* TSSs profile and their activity differs between species; the next question was what factors are responsible for the difference in *ompR* TSSs?

1. CSH50

pH 7

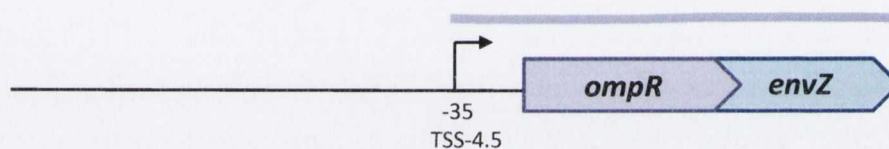


pH 4.5



2. SL1344

pH 7



pH 4.5

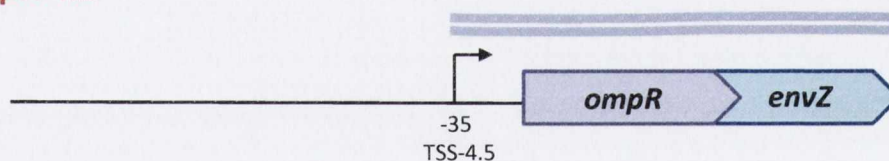


Fig. 3.13. Schematic of transcription from predominant transcription start sites (TSSs) in *ompR*. Based on qPCR data and 5' RACE analysis a working model of *ompR* expression at pH 7 and pH 4.5 is shown. **1.** CSH50 maintains a steady level of *ompR* expression at both pH 7 and pH 4.5 using primarily TSS-1, however transcription is also detected from TSS-4.5. At pH 4.5 expression switches to TSS-4.5 which may upregulate expression levels in order to maintain a steady state of gene expression observed in Fig. 3.10. **2.** In SL1344 TSS-4.5 is the primary TSS active at pH 7 and pH 4.5. Expression is also upregulated at pH 4.5. Positions are described relative to that start codon (ATG, +1). Purple lines illustrate mRNA transcripts.

3.2.8 The *ompR* regulatory region in *E. coli* is more susceptible to strand separation than *S. Typhimurium*

Evolutionary changes in the promoter architecture of *ompR* could account for the presence of TSS-1 in *E. coli* and its absence in *S. Typhimurium* under the conditions used in study. Differences in *cis*-acting factors such as regulatory protein binding sites may account for the species-specific difference in TSS activity. The ability of SL1344 *ompR* regulatory region to maintain its pattern of expression in an *E. coli* background argues that some inherent feature unique to the *ompR* promoter in SL1344 accounts for the absence of TSS-1.

An additional *cis*-acting feature that could influence the presence or absence of transcriptional activity is the susceptibility of the *ompR* regulatory region to DNA supercoiling (Bang *et al.*, 2002; Cameron and Dorman, 2012). DNA supercoiling is considered a global regulator of gene expression (Smith, 1981; Peter *et al.*, 2004). Supercoiling can introduce torsional tension in the DNA that can be relieved by strand separation. This stress-induced strand separation can influence cellular processes such as transcription and DNA replication that require separation of the double helix (Benham, 1993). Certain stretches of DNA are more energetically prone to strand separation than others, these are known as stress induced duplex destabilisation (SIDDD) regions. These regions are usually A+T rich and succumb to strand separation when placed under superhelical stress (Benham, 1993). The *ompR* regulatory region in both SL1344 and CSH50 were calculated to be 58% and 62% A+T rich respectively. The *ompR* gene in *S. Typhimurium* is sensitive to changes in supercoiling such that DNA relaxation is known to activate *ompR* transcription (Bang *et al.*, 2002). Moreover SIDDD regions have been identified in the *ompR* promoter in SL1344 (Cameron and Dorman, 2012). Cameron and Dorman hypothesise that OmpR binding to this SIDDD site may transmit this region of destabilisation downstream to the promote strand separation, and the initiation of transcription (Cameron and Dorman, 2012).

It was rationalised that comparing the SIDDD profiles of the *ompR* regulatory region of both *S. Typhimurium* and *E. coli* may reveal why TSS-1 is not active in *S. Typhimurium* and why TSS-4.5 is more active in *S. Typhimurium*. The web-based program WebSIDDD was used to predict regions of destabilisation (Bi and Benham,

2004). Previous work used the *ompR* promoter cloned into an expression vector to measure *ompR* expression and therefore also used this DNA sequence to predict SIDD regions (Cameron and Dorman, 2012). However, in this study *ompR* expression was measured from its native position on the chromosome, therefore the DNA sequence pertaining to the chromosomal location of *ompR* was used in WebSIDD.

The SIDD profiles display the free energy (Gx) required to denature any base pair; the less free energy needed to denature base pairs the increased likelihood that a SIDD site will be predicted (Bi and Benham, 2004). The DNA sequence of the *ompR* regulatory region in *E. coli* was predicted to be more prone to stress-induced duplex destabilisation than *S. Typhimurium* (Fig. 3.14). This is not surprising given its higher A+T content compared to *S. Typhimurium*. Three SIDD regions were predicted in the *ompR* regulatory region of both *E. coli* and *S. Typhimurium*. Region 1 overlaps the acid-inducible TSS-4.5 (+1) (+94 to -63, relative to +1), notably, in *E. coli* this SIDD site was extended from +94 to -87. Region 2 ranged from approximately -146 to -194 (relative to +1). In *S. Typhimurium* the lowest G(x) value predicted in this region was 6.9, however in *E. coli* this was 3.6 indicating that this region is predicted to be more destabilised in *E. coli* and thus more susceptible to strand separation. Region 3 ranges from approximately -290 to -240 (relative to +1); this region is also more destabilised in *E. coli*, however this difference was not as striking as in region 2 (lowest G(x)= 4.7 and 3.3 in *S. Typhimurium* and *E. coli* respectively).

It was clear that the SIDD profiles for the *ompR* regulatory region from both species were unique. These profiles indicated these regions have different susceptibilities to strand separation and subsequently transcription. This may provide evidence as to why different TSSs are active in the *ompR* regulatory region. In *E. coli* the highly destabilised SIDD region 2 is located 59 bp upstream of TSS-1 that was detected only in *E. coli* at pH 7. It has been shown previously that transcription factors such as IHF and Fis can bind to SIDD regions upstream of promoters (Sheridan *et al.*, 1998; Opel *et al.*, 2004). These proteins can suppress the tendency of DNA in this region to become single-stranded by transmitting this energy to the next susceptible stretch of DNA. These proteins can therefore indirectly promote transcription in this manner (Sheridan *et al.*, 1998; Opel *et al.*, 2004).

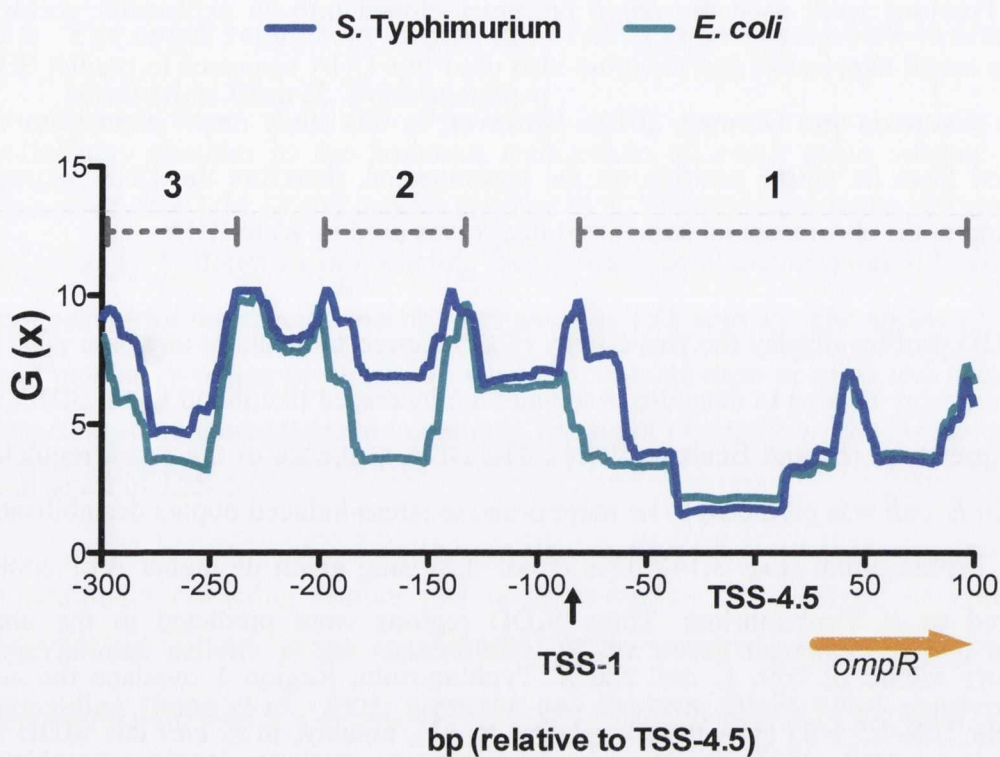


Fig. 3.14. SIDD profile for the *ompR* promoter region in *S. Typhimurium* and *Escherichia coli*. SIDD profiles for *S. Typhimurium* and *E. coli* are shown as purple and blue traces respectively. Regions of destabilisation predicted by WebSIDD are numbered 1-3 (Bi and Benham, 2004). $G(x)$ is expressed as kcal/mol/bp as a function of bp location (x). The location of each bp is shown relative to the transcription start site TSS-4.5. The location of TSS-1 present in CSH50 is shown. Orange arrow denotes the location of the *ompR* gene.

Speculatively, this may be the manner in which transcription is activated at TSS-1 in *E. coli* and may account for why this weakly destabilised region in *S. Typhimurium* does not promote transcription in this region. Indeed as this TSS was not activated even when the *S. Typhimurium ompR* regulatory region was introduced in *E. coli* suggests some intrinsic factor and/or protein binding is involved in TSS-1 activation in *E. coli*. Alternatively, species-specific differences supercoiling levels, together with the difference in potential to undergo strand separation may be a contributing factor in the unique TSS profiles. Global supercoiling levels are investigated in chapter 5.

Susceptibility to DNA supercoiling may also account for why TSS-4.5 is less active in *E. coli*. The SL1344 *ompR* promoter retained its TSS profile and was pH sensitive in an *E. coli* background, suggesting the regulatory factors influencing the increased activity of TSS-4.5 are likely to be present in *E. coli*. However, the intrinsic nature of the *ompR* promoter *E. coli* (i.e. susceptibility to supercoiling changes or differences in nucleotide sequence) may account for the decreased activity of TSS-4.5. SIDD region 1 that overlaps TSS-4.5 was quite similar in *E. coli* and *S. Typhimurium*, however this region was extended in *E. coli*. This extended SIDD region in *E. coli* may negatively affect activation of TSS-4.5.

3.3 Discussion

Microbial responses to acidic pH are of crucial importance. Understanding the mechanisms used by bacteria to survive low pH is essential to understanding how organisms persist and cause infection in the human host. Organisms that are more acid-tolerant are more capable of surviving and causing disease. Although acidic pH is a common threat faced by *E. coli* and *S. Typhimurium* in the acidic stomach of the host, these species vary in both their ability and mechanisms employed to tolerate low pH (Lin *et al.*, 1995). OmpR has been identified in both *S. Typhimurium* and *E. coli* as an important regulator required in acidic conditions, such that, *ompR* mutants in both species are sensitive to low pH (Bang *et al.*, 2002; Stincone *et al.*, 2011). An OmpR orthologue (HP0166) in *Helicobacter pylori*, a Gram-negative bacterium that colonises the stomach, has been identified an important player in the acid response (Bury-Mone *et al.*, 2004). Furthermore, OmpR in *Yersinia pseudotuberculosis* was identified as an important factor required for acid survival (Hu *et al.*, 2009). This highlights the importance of the OmpR/EnvZ TCS in the adaptation to acidic conditions across species.

Although OmpR has been identified as a common factor required by *E. coli* and *S. Typhimurium* to tolerate acidic pH, the results presented show *ompR* expression is not conserved between species. These experimental results identified the *ompR* regulatory region as a crucial factor determining the differential expression of *ompR* at low pH. Both SL1344 and CSH50 promote transcription from a shared TSS at pH 4.5; however, from measuring *ompR* gene expression and OmpR protein levels it can be deduced that TSS-4.5 is more active in SL1344.

These experimental results add to an emerging view that orthologous genes are often subject to differential expression (Osborne *et al.*, 2009). Differential expression patterns are thought to promote species diversity. This can also be achieved by introducing functional changes in transcription factors and their target promoters (Perez and Groisman, 2009). These variations may enable bacteria to respond to new stimuli, integrate new genes into their pre-existing regulatory network or occupy new niches (Winfield and Groisman, 2004; Perez and Groisman, 2009). It is tempting to speculate that differential expression of *ompR* at low pH is as a result of changes in the OmpR

regulon between species. Upregulation of *ompR* at low pH may have occurred to enable *S. Typhimurium* adopt a pathogenic lifestyle in the acidified *Salmonella*-containing vacuole (SCV). *ompR* mutants are attenuated for virulence and defective in macrophage killing (Dorman *et al.*, 1989; Lindgren *et al.*, 1996). Furthermore, OmpR directly binds and activates expression of *ssrA* (Lee *et al.*, 2000; Feng *et al.*, 2003) part of the SsrA/B two-component system that co-ordinates the expression of SPI-2 that is required for intracellular replication, survival inside macrophages and establishment of systemic infection (Ochman *et al.*, 1996; Shea *et al.*, 1996; Cirillo *et al.*, 1998). The environmental signal sensed by SsrA is currently unknown; however, *ssrA* gene expression is induced at low pH inside macrophages in an OmpR-dependent manner (see Fig. 3.15) (Lee *et al.*, 2000).

The acquisition of SPI-2 has provided *Salmonella enterica* with the necessary tools for niche expansion (Ochman *et al.*, 1996; Shea *et al.*, 1996; Cirillo *et al.*, 1998). SPI-2 allows *Salmonella* to survive inside the acidified SCV, a unique lifestyle not exploited by *E. coli*. As such OmpR has facilitated this step via activation of *ssrA* (Lee *et al.*, 2000). It is therefore hypothesised that upregulation of *ompR* in acidic pH may occur out of necessity to communicate the acidic conditions of the vacuolar compartment to *ssrA* and in turn activate SPI-2. A model whereby *ompR* modifies its transcriptional output in acidic conditions as a result of acquisition of SPI-2 is an attractive one. This argument could be strengthened by examining *ompR* expression in *Salmonella bongori* species, a species that lacks SPI-2 (Fookes *et al.*, 2011). However, it is conceivable that upregulation of *ompR* in acidic conditions may occur in *Salmonella* regardless of the presence of SPI-2 and OmpR has simply been co-opted into regulating *ssrA*. Nonetheless, it is plausible that differential expression of *ompR* has occurred in *S. Typhimurium* as a consequence of pathoadaptation.

Studies in the commensal *E. coli* K-12 derivative BW25113 identified a requirement for the OmpR protein to tolerate low pH. Stincone *et al.* reported a decrease in *ompR* expression at pH 5.5 after 15 min (Stincone *et al.*, 2011). It should be noted these experiments were performed using LB medium and as such these results are not directly comparable to experiments performed herein. Nonetheless, Stincone *et al.* have identified OmpR as an important potential regulator of pH response in *E. coli*. As a

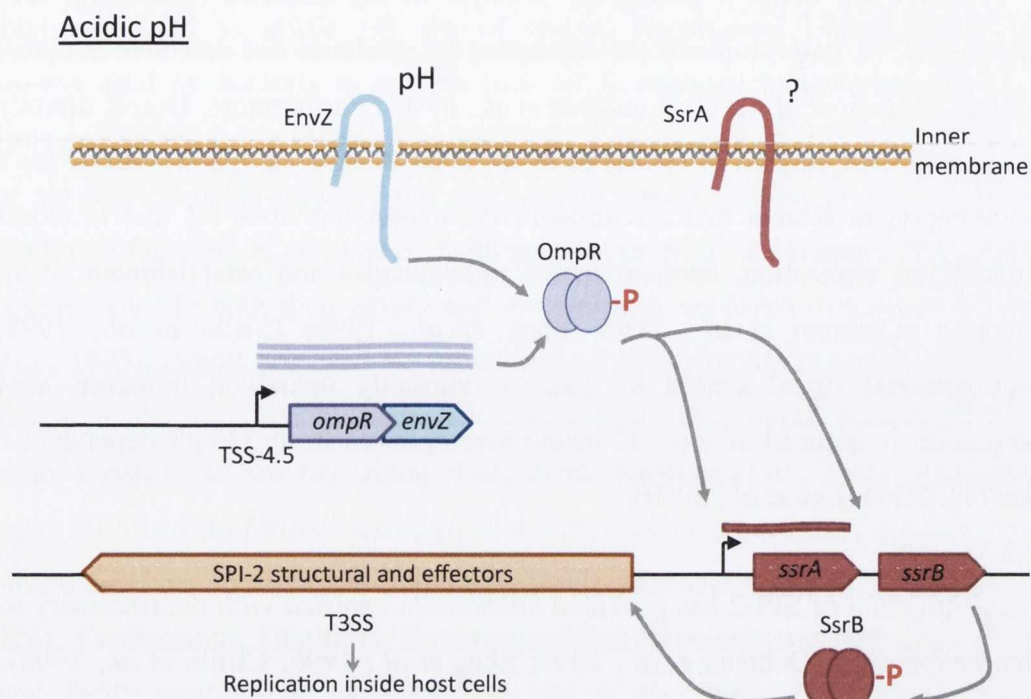


Fig. 3.15. Model of activation of SPI-2 by OmpR inside host cells. The OmpR/EnvZ system responds to acidic conditions. EnvZ is required to phosphorylate OmpR (OmpR-P) but however it is currently not known if EnvZ senses changes in pH. *ompR* expression is activated from TSS-4.5 resulting in increased OmpR levels. OmpR-P binds and directly activates *ssrA/B* expression. The SsrB protein subsequently activates expression of genes encoded in SPI-2 promoting the expression genes coding for a type 3 secretion system (T3SS) and its effector proteins. This T3SS is required for replication inside macrophages (Adapted from Lee *et al.* 2000).

result of the decrease in *ompR* expression, the authors hypothesised that OmpR normally represses genes required for acid survival; after acid shock OmpR derepresses such genes and promotes survival (Stincone *et al.*, 2011). Therefore, OmpR emerges as a key protein required by both the pathogenic bacterium *S. Typhimurium* and the commensal bacterium *E. coli* in acidic conditions and *ompR* gene expression levels may be tailored for the requirements of either species.

Overall, *ompR* gene expression did not change in CSH50 under the conditions tested here. However, the *ompR* TSS profile in CSH50 clearly shifted from activation of TSS-1 and TSS-4.5 at pH 7 to TSS-4.5 at pH 4.5. This unique *ompR* transcription profile observed in CSH50 may be attributable to predicted SIDD profiles shown. The identification of a highly destabilised SIDD region upstream of TSS-1 in *E. coli* could account for the variations in TSS profile between CSH50 and SL1344 at pH 7. Regulatory proteins mediate activation of transcription by transmission of destabilised regions to neighbouring DNA sequences. To date the IHF, Fis and OmpR proteins have been described in such roles (Sheridan *et al.*, 1998; Opel *et al.*, 2004; Cameron and Dorman, 2012). IHF binds to three sites in the *ompR* promoter in *E. coli*. IHF binds and represses transcription of TSS-1 and TSS-2 by inhibiting RNA polymerase binding to promoters (Tsui *et al.*, 1991). As a repressor of *ompR* expression IHF is an unlikely candidate for activation of TSS-1 through the mechanism proposed herein. The Fis and OmpR proteins are required to activate transcription of *ompR* in SL1344; these proteins bind directly to the *ompR* regulatory region and reciprocally occupy the *ompR* promoter in response to changes in DNA supercoiling (Cameron and Dorman, 2012). Therefore, Fis and OmpR are likely candidates for the activation of TSS-1 in CSH50 at pH 7 however binding of these proteins to the *ompR* regulatory region in *E. coli* has not been tested, however, this does provide scope for future work.

The results in this chapter have identified the *ompR* gene is differentially expressed in *E. coli* and *S. Typhimurium* in response to pH. This work provides additional insights into the importance differential expression of orthologous genes, which has previously been shown to contribute to the evolution of species diversity (Osborne *et al.*, 2009). Although the significance of *ompR* expression in acidic conditions was discussed here, a key question emerges from this study; what factors of the *ompR* regulatory region are

responsible for the increase in *ompR* expression at low pH? This is investigated in the next chapter.

**Chapter 4 Regulation of the *ompR* gene in *Salmonella*
Typhimurium at low pH**

4.1 Introduction

The OmpR/EnvZ two-component system of *S. Typhimurium* is orthologous to the OmpR/EnvZ system in *E. coli* which is best known for sensing changes in osmolarity (see section 1.6.2) (Slauch and Silhavy, 1989). In *E. coli*, OmpR reciprocally activates and represses the expression of the porin-coding genes, *ompF* and *ompC*, in response to changes in osmotic pressure (Alphen and Lugtenberg, 1977). Unlike its osmo-responsive function in *E. coli* the *ompR* gene in *S. Typhimurium* is not osmo-regulated but instead is induced by acid shock (Bang *et al.*, 2002). Furthermore, results previously presented (section 3.2.2) show the osmoregulated *ompR* gene in *E. coli* is not induced by acid shock. Thus, these orthologous systems appear to respond differently to various stimuli and, as discussed in chapter 3, the *ompR* regulatory region was identified as the defining feature responsible for differential expression of *ompR* in *S. Typhimurium* and *E. coli* at acidic pH.

Studies in *S. Typhimurium* have shown that the *ompR* gene is subject to positive autoregulation. At low pH phosphorylated OmpR (OmpR-P) binds to four sites in the *ompR* regulatory region and stimulates *ompR* expression (see Fig. 4.1) (Bang *et al.*, 2002; Cameron and Dorman, 2012). It is thought that acidic pH induces DNA relaxation at the *ompR* promoter; consistent with this proposal is the observation that treating cells with the drug novobiocin (a DNA gyrase inhibitor that promotes DNA relaxation) stimulates *ompR* expression (Bang *et al.*, 2002; Cameron and Dorman, 2012). The physiological relevance of gene induction by DNA relaxation is apparent from a study by Ó Cróinín *et al.* that showed DNA relaxed inside host macrophage and this was accompanied by increased activity of SPI-2 master regulator *ssrA* (Ó Cróinín *et al.*, 2006). Notably, expression of *ssrA* in macrophages was found to be dependent on OmpR (Lee *et al.*, 2000). Under non-inducing conditions *ompR* expression is repressed by the global regulator H-NS (Bang *et al.*, 2002). H-NS is an important DNA-binding protein that typically exerts a negative effect on gene expression (see section 1.7.4) (reviewed by Dorman, 2007). H-NS mediated repression of genes can be relieved by local remodelling of DNA and/or by the action DNA binding proteins such as Fis and OmpR. A recent study has shown Fis binds directly to the *ompR* promoter in *S. Typhimurium* (Cameron and Dorman, 2012). Cameron and Dorman found a reciprocal relationship between Fis and OmpR occupancy of the *ompR* promoter; as DNA relaxed

OmpR occupancy increased with the concomitant decrease in Fis occupancy (Cameron and Dorman, 2012). It had been proposed that DNA relaxation in acidic conditions and increased OmpR-P binding to relaxed DNA may alleviate H-NS repression and induce increased levels of *ompR* transcription (Bang *et al.*, 2000; Cameron and Dorman, 2012).

Studies of *ompR* regulation in *E. coli* have been conducted. These show *ompR* expression is subject to regulation by the site-specific DNA binding proteins IHF and CRP (Tsui *et al.*, 1991; Huang *et al.*, 1992). *ompR* expression is negatively affected by the integration host factor (IHF) that occupies three sites in the *ompR* promoter (see Fig. 4.1) (Tsui *et al.*, 1991). Transcription assays performed *in vitro* found that IHF specifically inhibited transcription from TSS-1 (see Fig. 4.1, TSS numbered 1, red) and inhibition was only observed when purified IHF protein was added before RNA polymerase (RNAP) suggesting that IHF prevents RNAP binding to the *ompR* promoter (Tsui *et al.*, 1991). In contrast *ompR* transcription was both repressed and activated by the cAMP receptor protein (CRP). CRP binds to a single binding site in the *ompR* promoter (see Fig. 4.1) and mediates repression of two proximally located promoters (Fig. 4.1, TSS-4 and TSS-1 in red) with the concomitant activation of two distally located promoters (Fig. 4.1, TSS-5 and TSS-2 in red) (Huang *et al.*, 1992). As discussed in chapter 3 the *S. Typhimurium* *ompR* regulatory region has diverged from its *E. coli* counterpart with a 12% difference in nucleotide sequence (see section 3.2.3). Hitherto, the effect of IHF and CRP on acid-induction of *ompR* in *S. Typhimurium* has not been tested. It was therefore hypothesised that IHF and CRP might contribute to the acid-induction *ompR* in *S. Typhimurium*.

The experiments described in this chapter aim to identify the major factors responsible for regulating *ompR* in *S. Typhimurium* at low pH by testing those factors that have been shown to regulate *ompR* in *E. coli*. This study aims to provide a detailed model of acid-induction of *ompR* in *S. Typhimurium*. Understanding the mechanism of *ompR* regulation may offer insights into how *ompR* governs the virulent phenotype and promotes survival of *S. Typhimurium* in acidic conditions.

1.1 Results

1.1.1 Construction of Kan^R *PompR* *S. enterica* pJET

To accurately assess the role of site-specific DNA binding proteins IHF and CRP on *ompR* expression it was necessary to inactivate their binding sites in their native locations on the chromosome. The following strategy was developed to allow the desired mutations to be initially introduced into the *ompR* regulatory region on a plasmid by site-directed mutagenesis (SDM, see section 2.7.2). The mutated regulatory regions were then integrated onto the chromosome in place of the native *ompR* promoter and the effect of these mutations on *ompR* expression measured using qPCR. Firstly, the *ompR* regulatory region was amplified from the SL1344 chromosome using primers *ompR*_F_pJET and *ompR*_R_pJET (Table 2.8). This produced 346 bp DNA fragment containing 302 bp upstream and 44 bp downstream of the *ompR* start codon. This fragment was then cloned directly into the linearised pJET plasmid creating *PompR* *S. enterica* pJET (Fig. 4.2, A).

To select for integration of the *ompR* regulatory region into the chromosome it was necessary to introduce a selectable marker into *PompR* *S. enterica* pJET. The *kan* gene from the plasmid pKD4 was chosen as the flanking FRT sites enabled its removal after introduction onto the chromosome. The *kan* gene was amplified using primers PstI_pKD4_F and XhoI_pKD4_R (Table 2.8) yielding a 1,501 bp fragment. This fragment was digested with *Pst*I and *Xho*I restriction enzymes. The plasmid *PompR* *S. enterica* pJET was digested with the same restriction enzymes and the PCR fragment was ligated into the linearised plasmid by its compatible cohesive ends (Fig. 4.2, B). This generated the plasmid Kan^R *PompR* *S. enterica* pJET (Fig. 4.2, C) and is hereafter referred to as *PompRpJET* (Table 2.7). The sequence of the *PompRpJET* plasmid was confirmed by DNA sequencing (GATC Biotech) and was suitable for mutagenesis of the *ompR* regulatory region by SDM.

1.1.2 IHF binds to the IHF-I and IHF-III binding sites in *ompR* promoter

Previous work in *E. coli* has shown the site-specific DNA binding and bending protein IHF binds to three sites in the *ompR* regulatory region (IHF-I, IHF-II and IHF-III, see Fig. 4.1) and represses transcription (Tsui *et al.*, 1991). IHF binding to the *ompR*

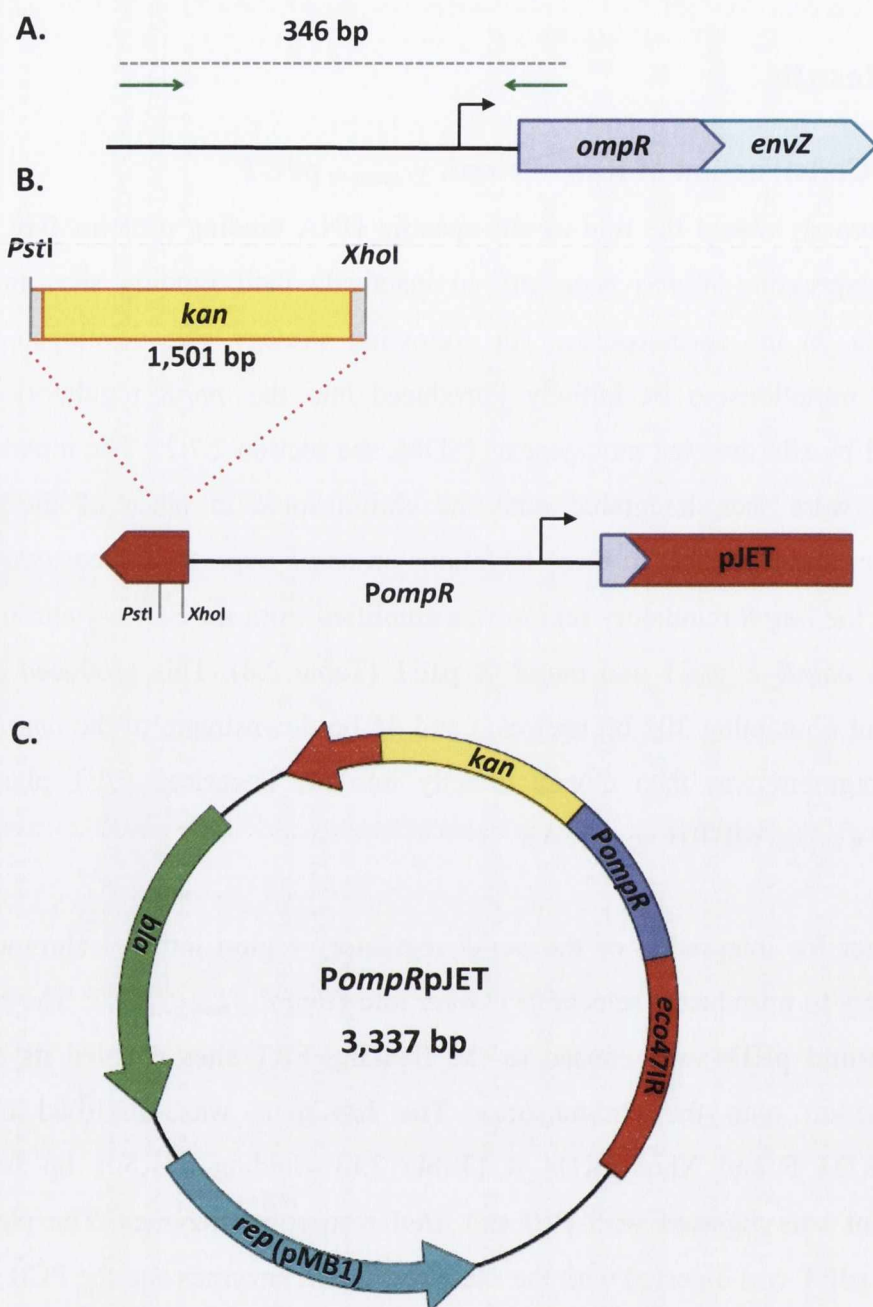


Fig. 4.2. Construction of the plasmid *PomRpJET*. **A.** A 346 bp fragment encompassing the *ompR* regulatory region from SL1344 was PCR-amplified using primers *ompR_F_pJET* and *ompR_R_pJET* (Table 2.8). This product was cloned into the *pJET* plasmid, creating *PompR S. enterica pJET*. **B.** The *kan* gene conferring resistance to kanamycin was PCR amplified from the plasmid *pKD4* using primers *PstI_pKD4_F* and *XhoI_pKD4_R* (Table 2.8). This amplicon and the plasmid *PompR S. enterica pJET* were restriction digested with the enzymes *PstI* and *XhoI*. **C.** The digested products were ligated via compatible overhangs creating the plasmid *PomRpJET*.

promoter in *S. Typhimurium* and its contribution to *ompR* regulation in acidic conditions has not been described. Analysis of the *ompR* regulatory region in *S. Typhimurium* identified two strong matches to the IHF consensus sequence (Fig 4.3, B) IHF-I and IHF-III (Hales *et al.*, 1994). These sites were identified in the same location as IHF-I and IHF-III in *E. coli*, so the nomenclature has been conserved to avoid confusion (Fig. 4.1). The equivalent IHF-II site identified in *E. coli* had a poor match in *S. Typhimurium* to the IHF consensus sequence as only 5 of 13 bp were conserved (Fig. 4.3, A). IHF binding to the *ompR* regulatory region was investigated; firstly site directed-mutagenesis was carried out on the IHF-I and IHF-III sites. Key nucleotides required for IHF binding were mutated (Hales *et al.*, 1994) and the ability of purified IHF protein to bind to these sites was examined *in vitro* using EMSA analysis (section 2.8) with purified IHF protein (a kind gift from Prof. Steven Goodman).

The IHF-I binding site was mutated using primers IHF-I_SDM_F and IHF-I_SDM_R (Table 2.8) and the plasmid *PompRpJET* as template. These primers introduced 3 bp substitutions into the IHF-I site generating the plasmid *PompR* IHF-I⁻ pJET (Fig. 4.3, B). The IHF-III site was mutated in the same manner using primers IHF-III_SDM_F and IHF-III_SDM_R (Table 2.8) generating the plasmid *PompR* IHF-III⁻ pJET (Fig. 4.3, B). Finally, both IHF-I and IHF-III were mutated on the same template; the IHF-I site on the plasmid *PompR* IHF-III⁻ pJET was mutated using primers IHF-I_SDM_F and IHF-I_SDM_R (Table 2.8) this generated *PompR* IHF-I⁻ IHF-III⁻ pJET.

Mutation of the IHF-I and IHF-III sites introduced *Bce*AI and *Hpy*991 restriction enzyme cut sites into the plasmids *PompR* IHF-I⁻ pJET and *PompR* IHF-III⁻ pJET respectively. Plasmids were isolated from transformants after SDM and screened for correct mutations by digestion of the plasmids with *Bce*AI or *Hpy*991 restriction enzymes. These digestion patterns were then compared to those of unmutated plasmids digested with the same enzymes (Fig. 4.3, C). Mutations were subsequently confirmed by DNA sequencing (GATC, Biotech).

The wild-type and mutant sequences were then compared for their ability to bind purified IHF protein by EMSA analysis (see section 2.8). 340 bp biotinylated DNA probes (Fig. 4.4, A) containing either wild-type or mutant IHF binding sites were amplified using primers bio_ompR_F and bio_ompR_R (Table 2.8) and the plasmid

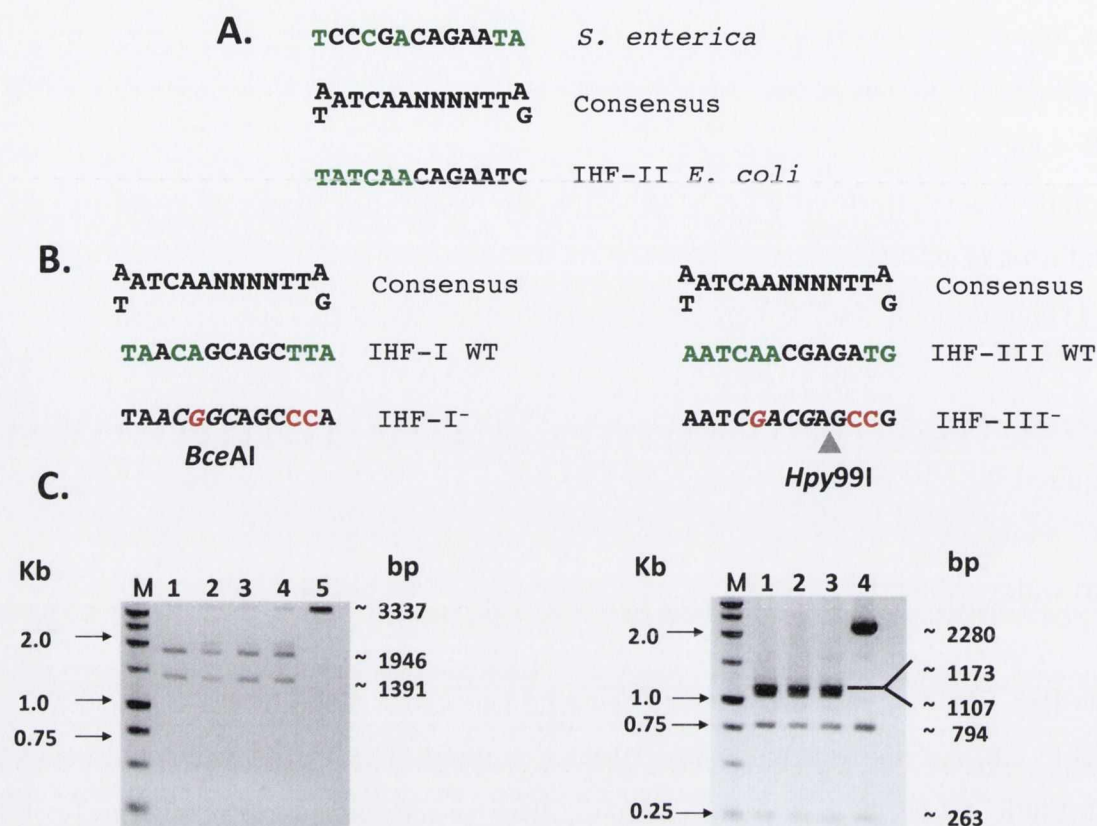


Fig. 4.3. Inactivation of the IHF-I and IHF-III binding sites. A+B. The DNA sequence of the IHF bindings sites are shown aligned with the consensus sequence for IHF binding sites (Hales *et al.*, 1994), matches are shown in green. **B.** The base pair substitutions made in IHF-I and IHF-III are shown in red. These mutations introduced restriction enzyme sites *BceAI* and *Hpy99I* respectively; the restriction enzyme recognition sequences are italicised. The *Hpy99I* cut site is highlighted (grey triangle); *BceAI* cuts outside the IHF-I⁻ sequence and is therefore not shown. **C.** Gel images of plasmid digestions are shown. On the left, M: 1 kb DNA ladder, 1-4 *PompR S. enterica* IHF-I⁻ pJET, 5 *PompR S. enterica* pJET. On the right, M: 1 kb DNA ladder, 1-3 *PompR S. enterica* IHF-III⁻ pJET, 4 *PompR S. enterica* pJET. Products were analysed on a 1% TAE agarose gel.

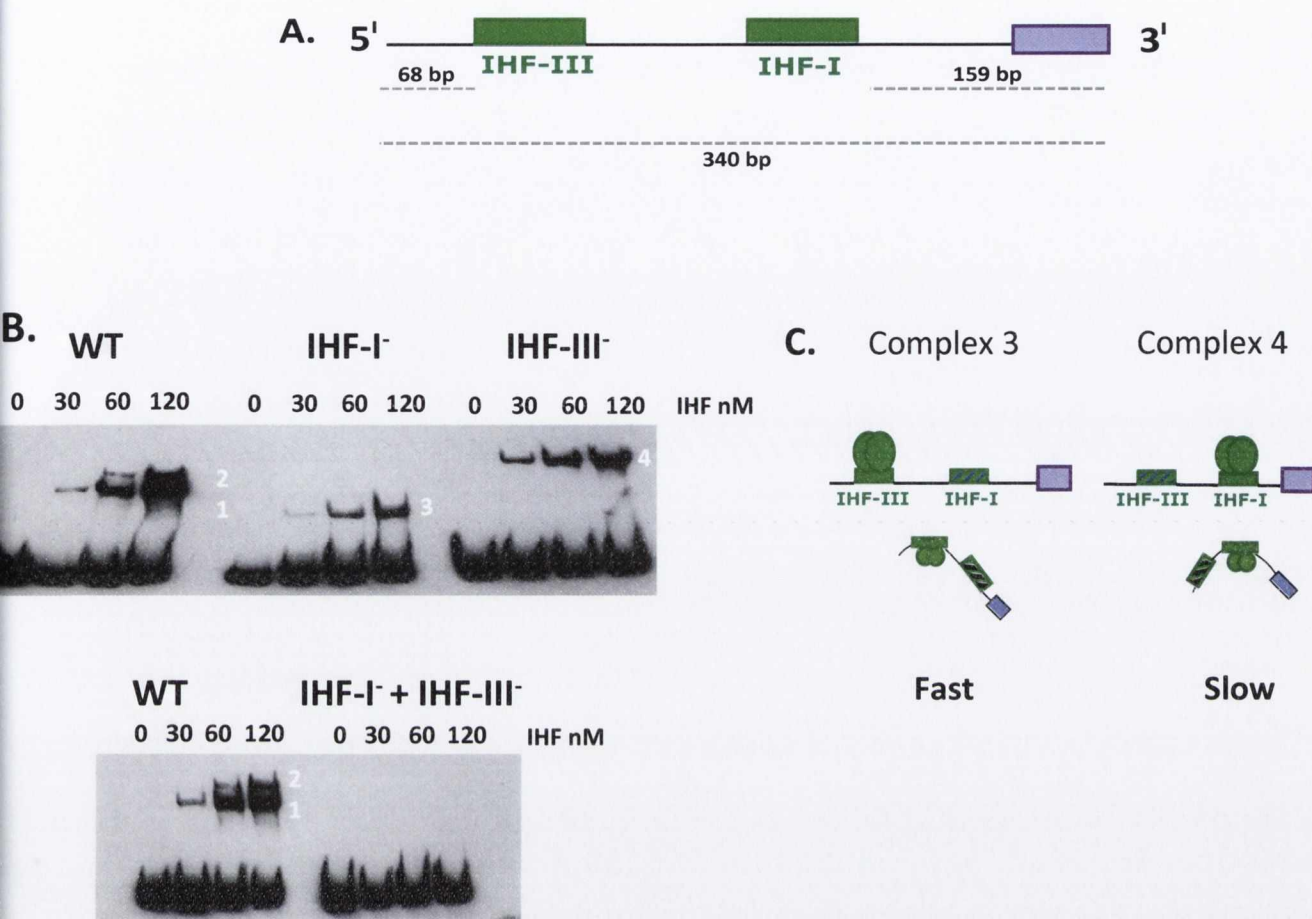


Fig. 4.4. EMSA analysis of IHF binding to the *ompR* regulatory region. **A.** An illustration of the 340 bp biotinylated probe encompassing the *ompR* regulatory region. Distances from the IHF binding sites (green boxes) to the end of the probe are shown. The purple box denotes the part of the *ompR* open-reading frame encompassed in the probe. **B.** The *ompR* probe was incubated with increasing concentrations of IHF protein. IHF formed two complexes with the wild-type (WT) DNA sequence (complex 1 + 2). IHF formed a single complex when incubated with the IHF-I⁻ or IHF-III⁻ mutant sequence (complex 3+4 respectively). Mutations in both the IHF-I and IHF-III sites abrogated IHF binding to DNA under the concentrations tested (IHF-I⁻ + IHF-III⁻). **C.** Predicted protein-DNA complexes and migration speeds are shown. Pairs of green circles denote IHF dimers. Green boxes denote the WT binding sites, hatched boxes denote mutated binding sites. Purple boxes denote part of the *ompR* open-reading frame contained in the DNA probe.

series *PompRpJET*, *PompR* IHF-I⁻ pJET, *PompR* IHF-III⁻ pJET and *PompR* IHF-I⁻ IHF-III⁻ pJET as template.

The EMSA results revealed that IHF formed two complexes with the wild-type *ompR* regulatory region sequence (Fig. 4.4, B). Mutations in either the IHF-I or IHF-III binding sites compromised IHF binding; IHF formed a single complex with IHF-I⁻ and IHF-III⁻ mutant sequences indicating IHF was capable of occupying the unmutated sites independently (Fig. 4.4, B, IHF-I⁻, IHF-III⁻). This was also clear from the different migration patterns observed as a result of DNA bending by IHF (Fig 4.4, C). Mutation of the IHF-I site restricted IHF binding to the intact IHF-III site located at the 5' end of the DNA probe. IHF binding and bending at this site resulted in a DNA-protein complex capable of traveling faster through the gel matrix (Fig. 4.4, C, 'Fast') (Thompson and Landy, 1988). Similarly, mutation of the IHF-III site restricted IHF binding to the intact IHF-I site located in the centre of the DNA probe. Bending by the IHF protein at this site resulted in a DNA-protein complex that migrated slower through the gel matrix (Fig. 4.4, C, 'Slow') (Thompson and Landy, 1988). However, neither singly occupied complex migrated in the same manner as complex 1 or 2 as observed in the WT (Fig 4.4, B). This indicated that a third IHF site was occupied in the WT; thus, complex 1 represented two IHF dimers bound to DNA and complex 3 represented three IHF dimers bound to DNA resulting in an unique and distinct migration pattern. Interestingly, combining the IHF-I and IHF-III mutant binding sites on the same DNA molecule (IHF-I⁻ + IHF-III⁻) had abrogated the ability of IHF to bind the mutant sequence. Thus, in the absence of intact IHF-I and IHF-III sites IHF did not bind to the *ompR* promoter under the concentrations tested indicating the third IHF site was indeed a weak site and unable to recruit IHF independently. It was initially thought that the weak IHF site represented IHF binding to the poorly conserved IHF-II site, however DNase I footprinting revealed no protection of this region by IHF (Cameron, A.D.S unpublished data). Protection was observed downstream of the IHF-I site (-34 to -51 relative to the *ompR* start codon, ATG +1) and thus accounted for the migration patterns observed in Fig. 4.4 (B). However, a good match to the IHF binding site consensus sequence was not identified.

4.2.3 Integration of the mutated IHF binding sites into the chromosome *in vivo*

The *ompR* regulatory regions harbouring the mutated IHF binding sites were integrated into the chromosome to test the contributions of the IHF-I and IHF-III sites to *ompR* expression *in vivo*. In a similar strategy to that described previously (section 3.2.4), the SL1344 *ompR* regulatory region was replaced with the *cat* gene. Primers *PompR_S.e_KO_F* and *PompR_S.e_KO_R* (Table 2.8) were used to amplify the *cat* gene from the plasmid pKD3. Using the λ Red recombination method this PCR product replaced the *ompR* regulatory region (Fig. 4.5, A) (Datsenko and Wanner, 2000) and created SL1344 Δ *PompR::cat*.

The structure of SL1344 Δ *PompR::cat* was confirmed by PCR using primers *PompR_KO_conf_F* and *bio_ompR_F* (Table 2.8) (Fig. 4.5, B) and the presence of the correct sequence was confirmed by DNA sequencing (GATC, Biotech). The mutated *ompR* regulatory region from *PompR* IHF-I⁻ pJET was amplified and subsequently integrated into the chromosome in SL1344 Δ *PompR::cat* using the primers *PompR_S.e_int_F* and *PompR_S.e_int_R* (Table 2.8). As a result the *cat* gene was replaced by the *ompR* regulatory region containing the mutated IHF-I⁻ site (Fig. 4.5, A). The *ompR* regulatory regions containing the single and double IHF site mutations were introduced into the chromosome in the same manner through recombination with *PompR* IHF-III⁻ pJET and *PompR* IHF-I⁻ IHF-III⁻ pJET respectively. To confirm replacement of the *cat* gene with the kanamycin resistance gene, *ompR* promoter transformants were screened by testing their resistance to kanamycin and sensitivity to chloramphenicol. As a control the wild-type *ompR* regulatory region from *PompRpJET* was integrated into the chromosome in SL1344 Δ *PompR::cat* as described above. This strain was expected to behave as wild-type and designed to test if the integration process had spurious effects on *ompR* expression. Correct integration of the wild-type and mutated *ompR* regulatory regions was confirmed by PCR using primers *PompR_KO_conf_F* and *bio_ompR_R* (Table 2.8) (Fig. 4.5, C) followed by DNA sequencing (GATC, Biotech). The mutants were then introduced into a fresh genetic background (SL1344) by P22 transduction and the *kan* gene was removed using the plasmid pCP20.

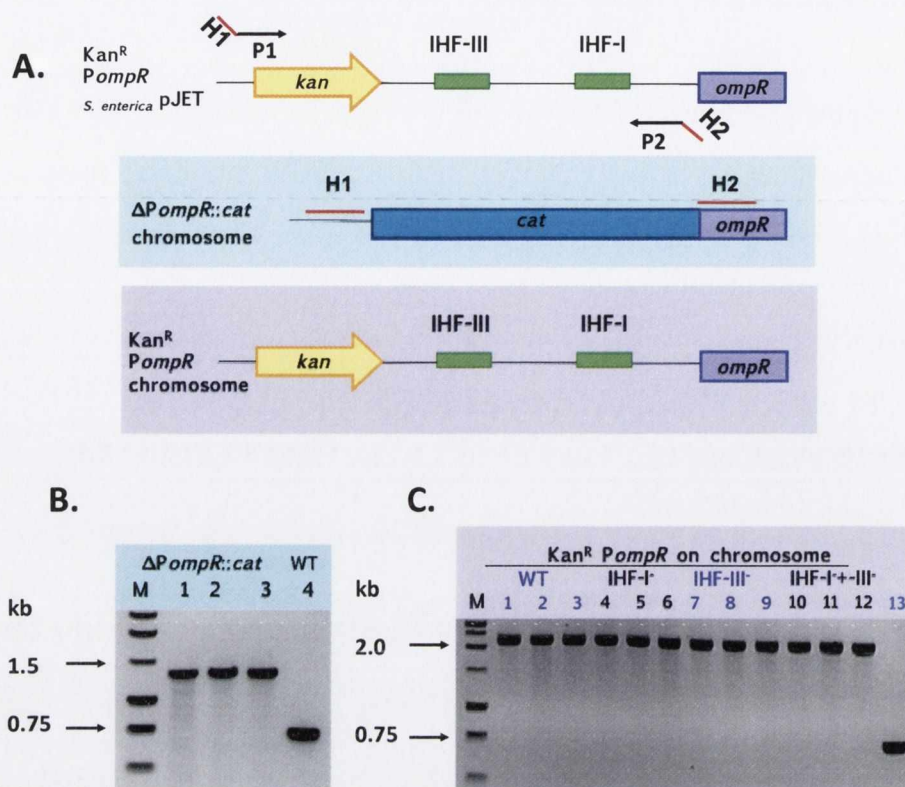


Fig. 4.5. Strategy for chromosomal integration of IHF binding site mutations. **A.** The *ompR* regulatory region was PCR amplified from the plasmid *Pompr*pJET using primers specific to sites P1 and P2 (*Pompr*_S.e_int_F and *Pompr*_S.e_int_R, Table 2.8). This PCR product had flanking regions of homology (H1 and H2) specific for DNA sequence in Δ *Pompr::cat*. Using the λ -Red recombination system (Datsenko and Wanner, 2000) the *cat* gene in Δ *Pompr::cat* was replaced by the *ompR* regulatory region. **B.** Primers flanking the *ompR* regulatory region (*Pompr*_KO_conf_F and *bio_ompR_R*, Table 2.8) were used to confirm integration of *cat* gene in place of the native *ompR* regulatory region. M: 1 kb ladder, 1-3 Δ *Pompr::cat* (~1,307 bp), 4 wild-type (WT, ~720 bp) DNA. **C.** The same primers (*Pompr*_KO_conf_F and *bio_ompR_R*) were used to confirm replacement of the *cat* gene in Δ *Pompr::cat* with the kanamycin resistant *ompR* regulatory region. M: 1 kb ladder, 1-3 (wild-type) WT *Kan^R Pompr*, 4-6 *Kan^R Pompr* IHF-I⁻, 7-9 *Kan^R Pompr* IHF-III⁻, 10-12 *Kan^R Pompr* IHF-I⁻ + IHF-III⁻, 13 WT (wild-type native *ompR* promoter on the SL1344 chromosome).

In addition to the IHF binding site mutations an *ihfA* mutant was constructed to test the effect of IHF on *ompR* expression. IHF is a heterodimeric protein with its subunits encoded by two unlinked genes *ihfA* (*himA*) and *ihfB* (*himD*, *hip*) (Drlica and Rouvière-Yaniv, 1987). The *ihfA* mutant was created using primers *ihfA_KO_F* and *ihfA_KO_R* (Table 2.8) to amplify the *cat* gene from the plasmid pKD3. This amplified a 1,014 bp DNA fragment with regions of homology flanking the *ihfA* gene. This PCR product was introduced into SL1344 using the λ Red recombination system and replaced the *ihfA* gene with the *cat* gene (Fig. 4.6, A). The structure of the SL1344 $\Delta ihfA::cat$ was confirmed by PCR (Fig. 4.6, B) using primers that flanked the *ihfA* gene (*ihfA_KO_conf_F* and *ihfA_KO_conf_R*, Table 2.8) and the correct sequence was confirmed by DNA sequencing (GATC, Biotech). The $\Delta ihfA::cat$ mutation was moved into a fresh genetic background (SL1344) by P22 transduction. The ability of $\Delta ihfA::cat$ to produce IHF heterodimers was tested by western blot analysis using IHF polyclonal antibody. Samples were harvested from overnight cultures grown in L-broth at 37°C and 200 rpm on a rotary shaker. IHF was detected in the wild-type samples, however there was a reduced level of IHF detected in the $\Delta ihfA::cat$ mutant. As the polyclonal antibody detects both subunits of IHF (IHF- α and IHF- β) it was rationalised that the weaker band represents the IHF antibody binding to the remaining IHF- β subunit present in $\Delta ihfA::cat$.

4.2.4 The effect of IHF on *ompR* expression at low pH

Before testing the contribution of IHF on *ompR* expression in acidic conditions it was necessary to first test if the IHF protein was expressed in these conditions. IHF protein levels were measured by western blot analysis using IHF polyclonal antibody, IHF was present at equal levels after 90 min in EG-minimal media at both pH 7 and pH 4.5 (Fig. 4.7, C). Next qPCR was used to measure the effect of IHF on *ompR* expression in strains harbouring mutations in IHF binding sites and in SL1344 $\Delta ihfA::cat$. The *gmk* gene was used as a reference gene (primers *gmk_F* and *gmk_R*, Table 2.8).

Results show the integration process had no effect on *ompR* expression as the control (WT *PompR*, see Fig 4.7, A) behaved like wild-type (WT). *ompR* transcript levels in IHF- I^- were very mildly upregulated at pH 4.5 (*t*-Test, $p < 0.05$) indicating IHF

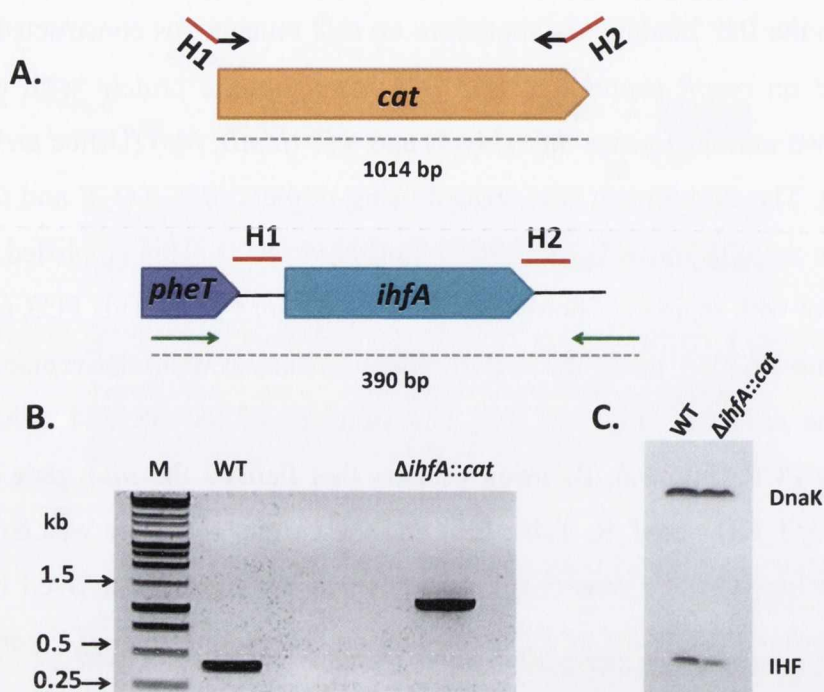


Fig. 4.6. Construction of SL1344 $\Delta ihfA::cat$. **A.** Primers *ihfA_KO_F* and *ihfA_KO_R* (Table 2.8) were used to amplify a 1,014 bp fragment encompassing the *cat* gene from the plasmid pKD3. This generated a PCR product flanked by homologous DNA sequence (H1 and H2) specific to regions bordering the *ihfA* gene. This PCR product was introduced into SL1344 using the λ -Red recombination system (Datsenko and Wanner, 2000) and the *ihfA* gene was replaced by the *cat* gene. **B.** Primers complementary to regions outside the *ihfA* gene, *ihfA_KO_conf_F* and *ihfA_KO_conf_R*, (Table 2.8; denoted by green arrows) were used to confirm the structure of $\Delta ihfA::cat$. M: 1 kb DNA ladder, wild-type (WT, ~391 bp), $\Delta ihfA::cat$ (~ 1,105 bp). **C.** Western blot analysis of IHF protein levels in wild-type (WT) and $\Delta ihfA::cat$ using polyclonal IHF antibody. Anti-DnaK was used as a loading control. Samples were taken from overnight cultures grown in 4 ml L-broth at 37°C and 200 rpm in a rotary shaker.

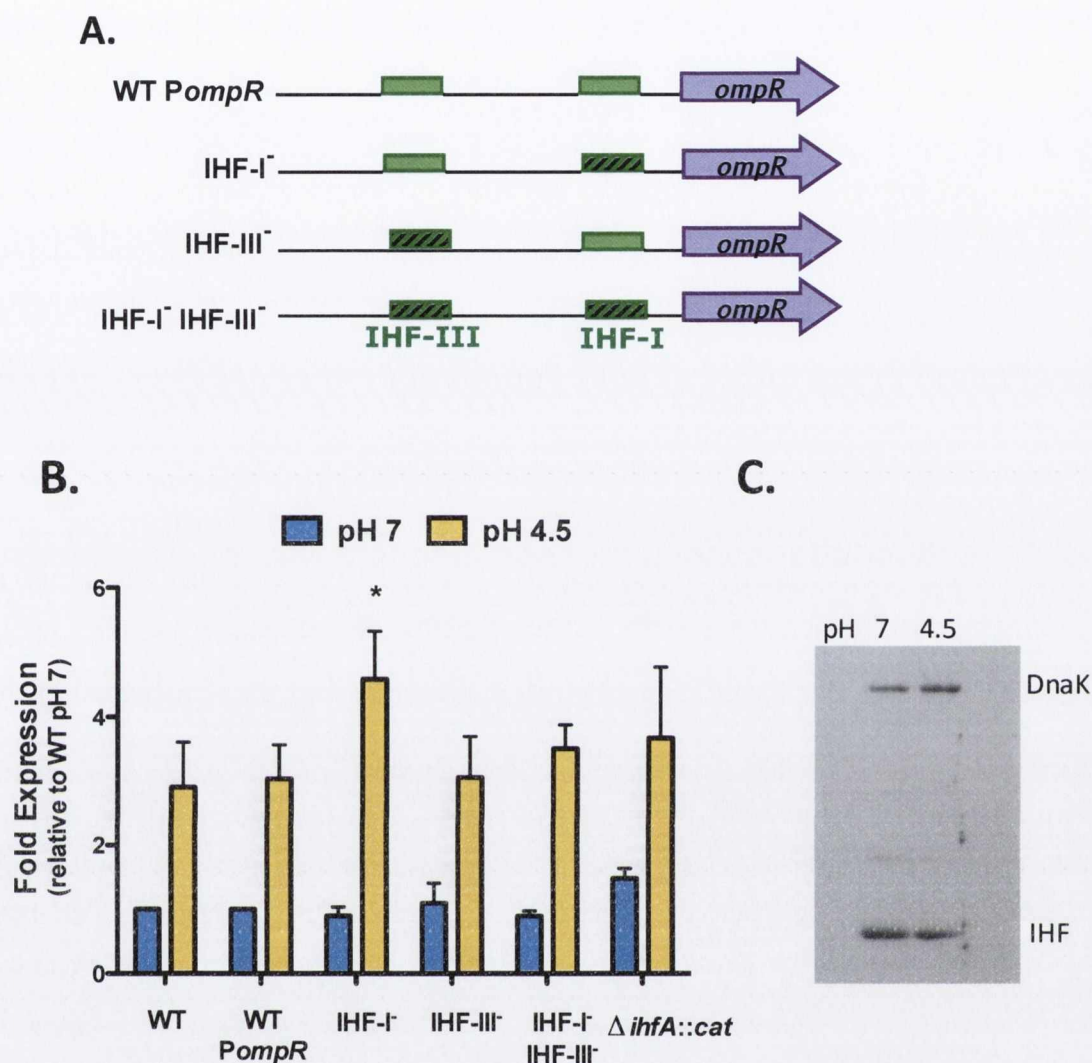


Fig. 4.7. The effect of IHF binding site mutations on *ompR* expression. **A.** Structure of the *ompR* regulatory regions in mutants tested. Green boxes represent wild-type IHF binding sites; hatched boxes denote mutated IHF binding sites. **B.** Quantitative PCR measurements of *ompR* transcript levels after 90 min in EG-minimal media at pH 7 and pH 4.5. *ompR* gene expression was measured in wild-type (WT), positive control (WT *Pompr*) *ihfA::cat* and mutants illustrated in part A. Mean levels of expression from at least three independent biological replicates are shown. Standard deviation is represented as error bars. Student's *t*-Test was performed * $p < 0.05$ ($p = 0.042$). **C.** Western blot analysis of IHF proteins levels in wild type SL1344 using polyclonal antibody against IHF protein. Antibody against DnaK was used as a loading control. Samples were taken after 90 min in EG-minimal media at pH 7 and pH 4.5.

repressed *ompR* expression via the IHF-I binding site (Fig. 4.7, B). Interestingly, *ompR* gene expression did not change in single IHF-III⁻ mutant or the double mutant IHF-I⁻ + IHF-III⁻ compared to wild type (Fig. 4.7, B). These results indicated the mild derepression observed in the IHF-I⁻ mutant was dependent on an intact IHF-III site and for this reason derepression was not observed in the single (IHF-III⁻) double mutant (IHF-I⁻+IHF-III⁻) respectively. Therefore IHF binding to the IHF-I site mediated *ompR* repression however maximum levels of *ompR* derepression required an intact IHF-III site. The IHF-III site is located upstream of the TSS-4.5 (-182 to -194 relative to TSS-4.5, +1); therefore it is hypothesised that the IHF-III site plays an indirect and perhaps architectural role in *ompR* transcription by DNA bending, thus facilitating contact between distantly bound proteins with the *ompR* promoter.

As expected at pH 4.5 *ompR* expression in SL1344 $\Delta ihfA::cat$ resembled that observed in the double mutant IHF-I⁻+IHF-III⁻. *ompR* expression at pH 7 in SL1344 $\Delta ihfA::cat$ was very mildly upregulated, this may be an indirect effect of the *ihfA* mutant as this pattern was not observed in strains with mutated IHF binding sites. Alternatively this may suggest a role for the IHF-II site in regulation of *ompR* at pH 7. These experimental results revealed a dual role for IHF regulation of *ompR* expression; IHF negatively affected *ompR* expression via the IHF-I site and in the absence of an intact IHF-I site IHF exerted a positive affect via the IHF-III site. In *E. coli* the IHF-I site was also responsible for repressing *ompR* and IHF mediated repression of *ompR* by inhibiting RNA polymerase binding to the *ompR* promoter (Tsui *et al.*, 1991). However, in *S. Typhimurium* the IHF-I site is located upstream of the acid-inducible TSS-4.5 promoter. Therefore IHF binding and bending at this site may inhibit the activity of positive regulators acting at *ompR* (investigated in the section 4.2.6). Overall IHF occupancy of the IHF-I and IHF-III sites served to finely tune *ompR* expression in acidic conditions.

4.2.5 The OmpR-II site is critical for *ompR* expression

OmpR is an archetypal response regulator transcription factor that is phosphorylated by its cognate sensor kinase EnvZ at an aspartate residue (D55) (Delgado *et al.*, 1993). Phosphorylated OmpR (OmpR-P) has an increased affinity for DNA and is capable of both activating and repressing transcription at target genes (Head *et al.*, 1998; Feng *et*

al., 2004). Using OmpR binding sites present in the *ompF* and *ompC* promoters studies identified OmpR binds to a 20 bp sequence composed of two 10 bp subsites, within which the motif GXXAC was identified as important for OmpR binding (Harlocker *et al.*, 1995; Yoshida *et al.*, 2006). Previous work has shown in *S. Typhimurium* *ompR* is positively autoregulated and OmpR binds to four sites in the *ompR* regulatory region (Bang *et al.*, 2002; Cameron and Dorman, 2012). However, it is not known which of these OmpR binding sites are important for *ompR* induction in acidic conditions. DNase I footprinting from two independent studies reported the OmpR-II binding site is the primary OmpR binding site (Bang *et al.*, 2002; Cameron and Dorman, 2012); however, its contribution to *ompR* gene expression has not been tested. In this study the contribution of the OmpR-II site to *ompR* expression was investigated.

Key nucleotides in the OmpR-II site were mutated using SDM (see section 2.7.2). These nucleotides were chosen based on the work of Yoshida *et al.* who identified important residues for OmpR binding (Yoshida *et al.*, 2006). Using the primers OmpR-II_SDM_F and OmpR-II_SDM_R (Table 2.8) 5 bp substitutions were introduced into the OmpR-II site on the plasmid *PompRpJET* (Fig. 4.8, A) creating the plasmid *PompR* OmpR-II⁻ pJET. Mutation of the OmpR-II site was confirmed by DNA sequencing (GATC, Biotech). The wild-type and mutant sequences were compared for their ability to bind OmpR by EMSA analysis (section 2.8). A 340 bp biotinylated DNA probe containing the OmpR-II⁻ binding site was amplified using primers bio_ompR_F and bio_ompR_R (Table 2.8). The protein OmpRD55E (Cameron and Dorman 2012) was used in the EMSA analysis; this protein was His-tagged and contained a glutamate residue at position 55 which mimicked constitutive phosphorylation thus enhancing the affinity of OmpR for DNA (Lan and Igo, 1998; Cameron and Dorman, 2012).

As observed by Bang *et al.* OmpR occupied the wild-type (WT) *ompR* promoter sequence in a stepwise manner (Bang *et al.*, 2002); four complexes were resolved (Fig 4.8, B, a-d) and approximately 50% of the DNA was shifted at 0.75 μ M OmpR. In the presence of OmpR-II⁻ mutant DNA sequence complex 'a' did not form, this complex was the first complex to form with WT DNA. OmpR formed only three complexes with the OmpR-II⁻ mutant sequence and required approximately 2-fold more OmpR protein (1.5 μ M) to shift approximately 50% of the DNA. Therefore, OmpR had a reduced

A. AATATGCTTTGTAACAATTT OmpR-II WT
 AATTTGCTTTATATGCATTT OmpR-II⁻

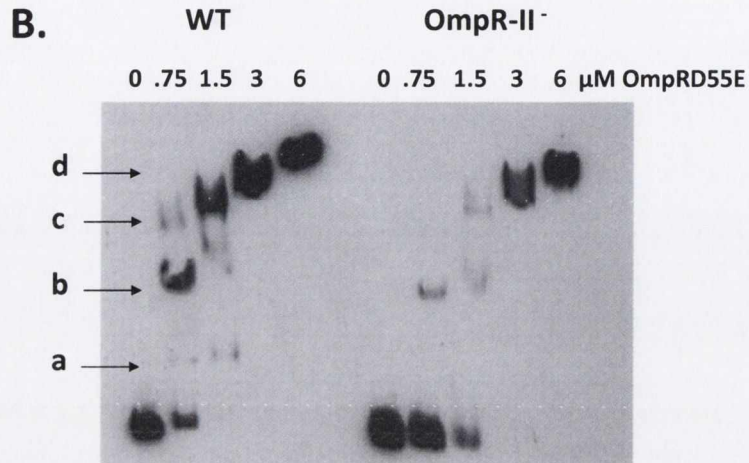


Fig. 4.8. Mutation of the OmpR-II binding site. **A.** The DNA sequence of the OmpR-II binding site in the *ompR* regulatory region aligned with the mutated OmpR-II site (OmpR-II⁻). Key bases for OmpR binding are shown in green (Yoshida *et al.*, 2006) and base pair substitutions introduced in this study are shown in red. The GXXACA motif is underlined. **B.** Binding of OmpRD55E to the wild-type (WT) *ompR* regulatory region formed four complexes (a-d). OmpRD55E protein formed three complexes with the mutated OmpR-II site (b-d).

affinity for DNA harbouring the mutated OmpR-II site. However, stepwise occupancy of the OmpR-II⁻ sequence still occurred and complexes ‘b-d’ (Fig 4.8, B) maintained the same migration patterns compared to WT. Thus, OmpR-P could occupy the OmpR-II⁻ site and the next highest affinity site co-operatively (see complex ‘b’ Fig. 4.8), however OmpR could not occupy OmpR-II⁻ alone (see complex ‘a’ Fig. 4.8, present in WT and absent in OmpR-II⁻).

4.2.6 The effect of the OmpR-II⁻ site on *ompR* expression

The *ompR* regulatory region from *PompR* OmpR-II⁻ pJET was integrated onto the SL1344 chromosome *in situ*, as described for the IHF mutants in section 4.2.3. *ompR* gene expression was quantitatively measured by qPCR after 90 min in EG-minimal medium at pH 7 and pH 4.5. In the OmpR-II⁻ mutant background *ompR* expression was reduced 3-fold at pH 7 and 14-fold at pH 4.5 compared to wild-type (Fig. 4.9, A). This indicated the OmpR-II site plays a crucial role in the activation of *ompR* expression. This may be directly or by negatively affecting co-operative binding of OmpR-P to the remaining OmpR binding sites. Importantly, the induction of *ompR* was dependent on the OmpR-II site as expression was more severely affected at pH 4.5 compared to pH 7. Thus, the OmpR-II site was identified as the critical factor behind the acid-induction of *ompR*. In addition *ompR* expression was measured in an $\Delta envZ::kan$ mutant. Results in Fig. 4.9 (A) showed *ompR* expression was no longer acid-inducible in the $\Delta envZ::kan$ mutant. This confirms earlier studies demonstrating phosphorylation of OmpR (OmpR-P) by EnvZ is required for *ompR* induction in acidic conditions (Bang *et al.*, 2002).

Previous results (Fig. 4.7, B) demonstrated the IHF protein is a very mild repressor of *ompR* expression and mediated its effect through the IHF-I binding site. The IHF-I binding site is located adjacent to the OmpR-II binding site (see Fig. 4.1), therefore IHF may exert an enhanced repressive effect in the OmpR-II⁻ mutant. To test this hypothesis the IHF-I⁻ and OmpR-II⁻ mutated binding sites were combined on the same DNA molecule. To do this the primers IHF-I_SDM_OmpR-II_F and IHF-I_SDM_OmpR-II_R (Table 2.8) were used to introduce the IHF-I⁻ binding site onto *PompR* OmpR-II⁻ pJET. Mutation of both sites on plasmid *PompR* IHF-I⁻ OmpR-II⁻ pJET was confirmed by DNA sequencing (GATC Biotech). The *ompR* regulatory region from *PompR* IHF-I⁻ OmpR-II⁻ pJET was integrated into the chromosome *in situ* as described in section

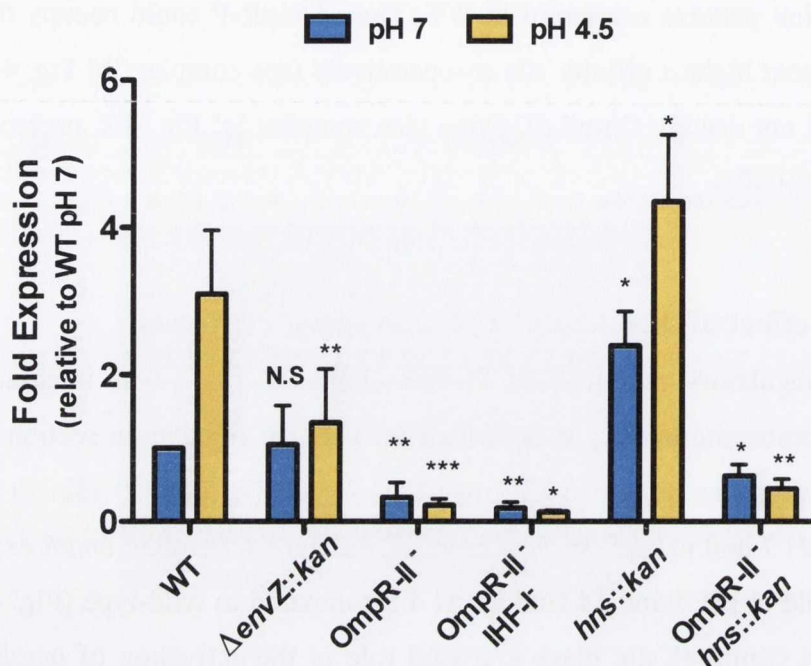


Fig. 4.9. Control of *ompR* expression in SL1344. Quantitative PCR measurements of *ompR* transcript levels after 90 min in EG-minimal media at pH 7 and pH 4.5. *ompR* expression was measured in wild-type (WT), $\Delta envZ::kan$, *PompR* OmpR-II⁻ (OmpR-II⁻), *PompR* OmpR-II⁻ IHF-I⁻ (OmpR-II⁻ IHF-I⁻), *hns::kan* and *PompR* OmpR-II⁻ *hns::kan* (OmpR-II⁻ *hns::kan*). Mean levels of expression from at least three independent biological replicates are shown. Standard deviation is shown as error bars. Students *t*-Tests were performed; in each case the mutant was compared to WT at the same pH (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, N.S not statistically significant).

4.2.3. The effect of the IHF-I⁻ OmpR-II⁻ double mutant on *ompR* expression was measured after 90 min in EG-minimal media at pH 7 and pH 4.5 using qPCR (Fig. 4.9). The results showed mutations in both the IHF-I and OmpR-II binding sites further decreased *ompR* levels compared to the OmpR-II⁻ single mutant; *ompR* expression was 5-fold lower at pH 7 and approximately 24-fold lower at pH 4.5 compared to WT. These results showed the IHF-I site was not responsible for the decreased expression observed in the single OmpR-II⁻ mutant (Fig. 4.9); instead these results indicated a requirement for OmpR binding to the OmpR-II site even in the absence of the repressor IHF. However, these results also indicated that an additional repressor acted at *ompR* and repressed expression more tightly in the presence of defective IHF-I and OmpR-II binding sites.

An earlier study has shown the global repressor H-NS represses *ompR* expression (Bang *et al.*, 2002). More recently it has been shown that H-NS binds to the *ompR* promoter albeit weakly (Cameron and Dorman, 2012). This weak interaction may explain why H-NS binding to *ompR* was not detected in a previous study that investigated genome-wide H-NS binding in *S. Typhimurium* using ChIP-on-chip analysis (Lucchini *et al.*, 2006). This prompted examination of *ompR* expression in the absence of a functional H-NS protein and OmpR-II⁻ site. SL1344 *hns::kan* harbours a kanamycin resistance cassette inserted at nucleotide 252 in the *hns* gene and as a result expresses a truncated form of the H-NS protein (Falconi *et al.*, 1991; Hinton *et al.*, 1992). SL1344 *hns::kan* expresses the first 84 amino acids encompassing the H-NS oligomerisation domain but lacks the C-terminal DNA binding domain (Ueguchi *et al.*, 1997; Dorman *et al.*, 1999). The *hns::kan* mutation was moved into SL1344 *PompR* OmpR-II⁻ by P22 transduction and *ompR* expression was investigated.

The results in Fig. 4.9 (A) show *ompR* expression was derepressed in SL1344 *hns::kan* confirming earlier study that demonstrated H-NS repressed *ompR* expression (Bang *et al.*, 2002). *ompR* expression was measured in the *hns::kan* and OmpR-II⁻ double mutant, these results showed *ompR* levels were approximately 2-fold higher compared to OmpR-II⁻ but still lower than WT. Therefore, the OmpR-II site was still required to activate *ompR* expression in the absence of H-NS, indicating OmpR acted as both an anti-repressor and activator of the *ompR* gene. These results raised the question how does OmpR-II⁻ participate in the activation of *ompR* expression?

4.2.7 RNA polymerase binding to the *ompR* promoter

OmpR binding to the OmpR-II site may activate transcription by enhancing RNAP binding at the *ompR* promoter. Previous studies have shown OmpR required the α C-terminal domain (α -CTD) of RNAP to activate transcription at the *ompC* gene, indicating OmpR activates transcription by making direct contact with the α -CTD of RNAP (Igarashi *et al.*, 1991; Slauch *et al.*, 1991). The OmpR-II site is located upstream of the -35 hexameric element of the TSS-4.5 *ompR* promoter, and so OmpR binding to this site may recruit RNAP to the promoter and activate transcription.

To investigate this hypothesis analysis of RNAP binding to the *ompR* promoter was tested using chromatin immunoprecipitation (ChIP; section 2.15) quantitative PCR with antibody specific to the β subunit of RNAP (W0023 Neoclone; see section 2.15.5). After 15 min at pH 4.5 cells were treated with rifampicin to trap RNAP at promoters; rifampicin inhibits bacterial growth by binding upstream of the active site in RNAP, preventing synthesis of RNA longer than 2-3 nucleotides (Campbell *et al.*, 2001). 15 min was chosen to 'capture' RNAP binding to the promoter. This was the earliest time point *ompR* transcript was detected by Bang *et al.* (2000). After 20 min treatment with rifampicin the cells were fixed using formaldehyde, which cross-linked all protein-DNA interactions *in vivo*. Samples were processed for ChIP analysis (described in section 2.15) and RNAP-enriched DNA was immunoprecipitated using antibody against the β subunit of RNAP. For control purposes a 'mock' immunoprecipitation was performed using normal mouse antibody IgG. Primers *PompR_ChIP_S.e_F* and *PompR_ChIP_S.e_R* (Table 2.8) were used to test RNAP binding to the *ompR* promoter.

The results presented in Fig 4.10 (B) show RNAP binding to the wild-type *ompR* promoter was significantly more enriched than the mock control, indicating RNAP was bound at the *ompR* promoter under the conditions tested. Mutation of the OmpR-II binding site led to a reduction in RNAP binding that was not significantly different to the mock control, this indicated the OmpR-II⁻ mutant led to a reduction in levels of RNAP binding. As ChIP quantifies protein binding for genomic regions approximately 500 bp in size it was reasonable to assume that RNAP bound at other *ompR* promoters could be detected during this analysis. Therefore, as a control, RNAP levels were measured in the same manner in an Δ *ompB* mutant that still carries the *ompR* promoter

A.

-35

GTGACT

TTGACA

-10

AAGAAT

TATAAT

TSS4.5

TACACTTACATT

TTCATACCAGAATTTGCTGACACGTGAGCTTTTTT

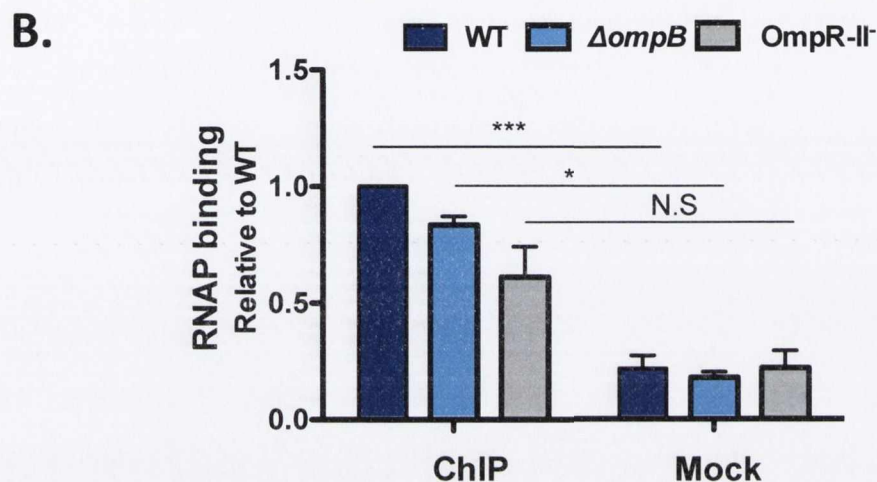


Fig. 4.10. RNA polymerase binding to the *ompR* promoter. **A.** Alignment of the -10 and -35 elements (underlined and bold) of the TSS-4.5 (red) with the consensus σ^{70} sequence. Matches are highlighted in green. **B.** Quantitative measurements of RNA polymerase (RNAP) binding determined by chromatin immunoprecipitation (ChIP) using antibody against the β subunit of RNAP. Samples were taken in wild-type (WT), $\Delta ompB$ and *PompR* OmpR-II⁻ (OmpR-II⁻) backgrounds. Rifampicin was added to samples after 15 min at pH 4.5 in EG-minimal medium. After 20 min treatment with rifampicin cells were processed for ChIP analysis (see section 2.15). Expression levels were normalised to input DNA. Mean levels of expression and standard deviation from three independent biological replicates are shown. Mock ChIP using non-specific immunoglobulin IgG antibody was used as a negative control. Student's *t*-Tests were performed; in each case the ChIP sample was compared to the mock sample (* $p < 0.05$, *** $p < 0.001$, N.S, not statistically significant).

but lacks the *ompR/envZ* open-reading frames. Surprisingly, RNAP binding in the $\Delta ompB$ mutant was similar to WT (Fig. 4.10, B). These results suggest RNAP binding to the *ompR* promoter occurred independently of OmpR. The proposed -10 and -35 elements for the TSS-4.5 promoter were good matches to the σ^{70} -10 and -35 consensus sequences (Fig. 4.10, A) (Shultzaberger *et al.*, 2007) and so the σ^{70} subunit of RNAP may bind the promoter region independently of OmpR. However the qPCR data in Fig. 4.9 demonstrate a requirement for the OmpR-II site to activate transcription. Therefore RNAP may recognise the TSS-4.5 promoter independently. OmpR may act post-recruitment of RNAP to facilitate *ompR* expression, perhaps by making direct contact with the α CTD (Igarashi *et al.*, 1991; Slauch *et al.*, 1991) and facilitate the transition from closed to open complex formation.

The results showed RNAP binding decreased in the presence of a defective OmpR-II site; however in an $\Delta ompB$ mutant these levels were comparable to wild-type. This suggests that the OmpR protein can bind elsewhere in the OmpR-II⁻ mutant and negatively affect RNAP binding. The OmpR-I site overlaps the -10 element of the TSS-4.5 promoter (see Fig. 4.1) and is thus a likely candidate for impeding RNAP binding, accounting for the decrease in RNAP binding in the OmpR-II⁻ mutant. Future work investigating the contribution of the OmpR-I site on *ompR* expression and RNAP binding may determine if OmpR both positively and negatively affects *ompR* expression.

OmpR may activate transcription at *ompR* by making direct contact with the α -CTD of RNAP thus facilitating the transition from a closed to an open transcription initiation complex at the TSS-4.5 promoter. Alternatively, as proposed by Cameron and Dorman, OmpR binding to the OmpR-II site may mediate activation by transmission of a supercoiling sensitive SIDD region (overlapping the OmpR-II site) to the downstream RNA polymerase binding site and facilitate transcription initiation by assisting in DNA strand separation (Cameron and Dorman, 2012).

4.2.8 CRP binds the *ompR* regulatory region in SL1344

The CRP protein is a global regulator of transcription. CRP in the presence of the allosteric effector cyclic-AMP (cAMP; a sugar-level signalling molecule) binds in the

proximity of target promoters and activates transcription by enhancing RNAP binding to promoters (reviewed by Ebright, 1993; Busby and Ebright, 1997; Busby and Ebright, 1999). CRP binds as a homodimer to a 22 bp consensus sequence (CRP half site, 5'-A₁A₂A₃T₄G₅T₆G₇A₈T₉C₁₀T₁₁) and introduces a 80-90° bend into the DNA (Schultz *et al.*, 1991). The CRP protein is a dual regulator of *ompR* expression in *E. coli* (Huang *et al.*, 1992). Work carried out *in vitro* revealed that CRP repressed two proximal TSSs and simultaneously activated two distal TSSs; overall, CRP mediated a positive effect on *ompR* expression *in vivo* (Huang *et al.*, 1992). Contrary to previous work suggesting the CRP-I binding site present in *E. coli* was severely compromised in *S. Typhimurium* (Bang *et al.*, 2002) a good match to the CRP site was identified in work carried out here (Fig. 41.). For this reason the effect of CRP on *ompR* expression in SL1344 was investigated.

Prior to investigating the presence of the CRP-I site *in vitro* it was first necessary to purify the CRP protein for use in EMSA analysis. The *crp* open reading frame from SL1344 was PCR amplified using primers *crp*_pQE30_F and *crp*_pQE30_R (Table 2.8). This PCR fragment and the His-tag vector pQE30 were digested with *Bam*HI and *Hind*III restriction enzymes. The PCR fragment and the pQE30 vector were ligated by their compatible cohesive ends (Fig. 4.11, A); this generated the plasmid pQE30*crp* (Table 2.7). CRP binds DNA via its C-terminal domain that contains the characteristic helix-turn-helix DNA-binding motif (Berg and von Hippel, 1988) and for this reason, the 6xHis tag was placed at the N-terminus of CRP. His-tagged CRP protein was purified (described in section 2.12.1) and samples taken throughout the purification process were analysed on a 12% SDS-PAGE gel stained with coomassie blue stain (Fig. 4.11, B). Purified His-tagged CRP migrated as a monomer and was the correct predicted size (~24 kDa) (Fig. 4.11, B, lane 'CRP').

EMSA analysis of CRP binding to the *lacZ* promoter of *E. coli* was carried out as a positive control to test the DNA binding activity of purified His-tagged CRP. The primers Bio_*lacZ*_F and Bio_*lacZ*_R (Table 2.8) were used to amplify a 228 bp biotinylated probe using *E. coli* CSH50 DNA as template. This probe encompassed two previously characterised CRP binding sites (Schmitz, 1981; Xiong *et al.*, 1991; Tutar and Harman, 2006). The results showed that the His-tagged CRP protein formed two

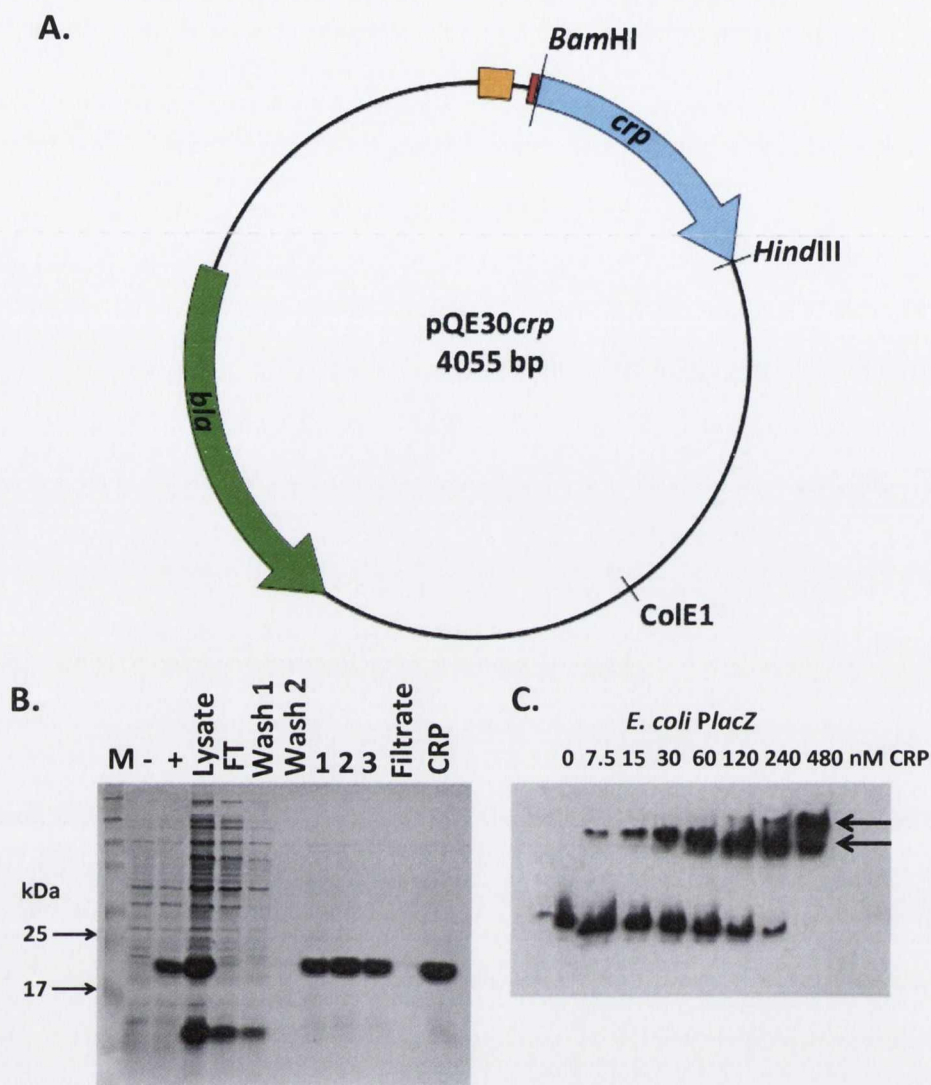


Fig. 4.11. Overexpression, purification and DNA binding of His-tagged CRP. **A.** The *crp* gene was PCR amplified using primers *crp*_pQE30_F and *crp*_pQE30_R (Table 2.8) and SL1344 DNA as template. The PCR fragment and the vector pQE30 were digested with *Bam*HI and *Hind*III restriction enzymes. Fragments were ligated together by their compatible cohesive ends creating the plasmid pQE30*crp*. The orange box denotes the IPTG-inducible T7 promoter, the red box denotes the x6 His tag. **B.** Analysis of CRP purification on 12% SDS polyacrylamide gel. The protein was purified using a nickel-nitriloacetic acid column. M: 175 kDa Protein maker (NEB), with (+) or without (-) IPTG, FT Flow through, 1-3 CRP eluted from the column. **C.** CRP binding to the promoter region of the *lacZ*. Arrows denote the two protein-DNA complexes formed.

A. TAACGTGATCGTCCCGACAGAA CRP-I WT
AAATGTGATCTAGATCACATTT Consensus
TAAC**TTATT**CGTCCCTATAGAA CRP-I⁻

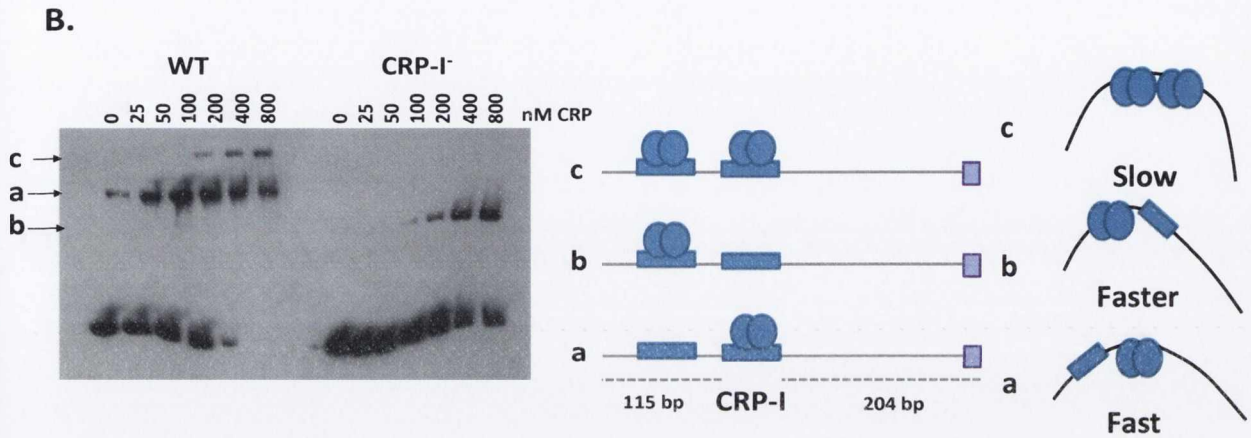


Fig. 4.12. Inactivation of the CRP-I site. **A.** The DNA sequence of the CRP-I site aligned with the CRP consensus sequence (matches are in green and the core sequence is underlined). The base substitutions introduced into CRP-I are in red. **B.** Binding of CRP to the *ompR* regulatory region. CRP formed three complexes with wild-type (WT) *ompR* regulatory sequence (a-c). The predicted protein-DNA complexes are illustrated on the right. The blue box denotes the CRP binding sites. Pairs of blue circles denotes CRP dimers, purple box denotes start of the *ompR* gene. Mutation of CRP-I (CRP-I⁻) resolved complex 'b' alone, complex 'b' represents CRP binding to a putative second site in the *ompR* regulatory region. Based on the migration speed of complex 'b' (see illustrations on right) and DNase I footprinting analysis (Cameron, A.D.S unpublished data) the second site may be located upstream of CRP-I.

DNA-protein complexes with the *lacZ* probe (Fig. 4.11, C). Thus the purified His-tagged CRP protein was capable of binding DNA. As a result CRP binding to the *ompR* regulatory region was investigated using this tagged CRP protein. SDM was carried out on the CRP-I site in the *ompR* regulatory region. Important bases required for CRP binding (Dickson *et al.*, 1977) and additional mutations were introduced into the core sequence (Fig 4.12, A) using the primers CRP-I_SDM_F and CRP-I_SDM_R (Table 2.8) and the template *PompRpJET*. Mutagenesis of the CRP-I site was confirmed by DNA sequencing (GATC, Biotech). The primers bio_ompR_F and bio_ompR_R (Table 2.8) were used to amplify a 340 bp biotinylated DNA probe containing the *ompR* regulatory sequence from the plasmid *PompR* CRP-I pJET. The wild-type (WT) and mutant (CRP-I⁻) *ompR* regulatory sequences were compared by EMSA analysis for their ability to bind CRP. CRP formed three DNA-protein complexes with the WT *ompR* sequence (Fig 4.12, B, a-c). Complex 'a' represented a single CRP dimer bound to the high affinity CRP-I site. As expected, this complex did not form after mutation of CRP-I site (Fig. 4.12, illustration a, 'Fast'). However, CRP formed complex 'b' with CRP-I⁻ mutant and this complex was also weakly resolved with WT DNA sequence. Thus, complex 'b' indicated there was a second CRP site in the *ompR* regulatory region bound by a single CRP dimer. Complex 'c' represented CRP bound to both CRP sites, this complex migrated the slowest and was not resolved in CRP-I⁻ (Fig. 4.12, illustration b, 'Slow'). To measure the affinity of CRP for the *ompR* promoter, binding curves were generated using data from densitometric analysis of the CRP EMSAs (Fig. 4.13, A). Apparent dissociation constants (K_{app}) were calculated for CRP binding to WT and CRP-I⁻ DNA using non-linear regression analysis for two-site and one-site respectively (Prism software, version 5.0). CRP's affinity for total DNA was calculated as $K_{app}=119$ nM. Mutations in the CRP-I site severely reduced CRP's affinity for *ompR* DNA ($K_{app}= >800$ nM) indicating CRP had a weak affinity for the second CRP site. As CRP did not shift 50 % of the CRP-I⁻ DNA under the concentrations tested the dissociation constant could not be accurately calculated and is thus described as >800 nM.

Attempts were made to experimentally verify the presence of the putative second CRP binding site. As complex 'b' in Fig. 4.12 migrated the fastest suggested that the putative CRP site was located towards the end of the DNA probe. Bioinformatic analysis identified a good match to the CRP consensus sequence adjacent to the CRP-I site (see Fig. 4.1 italicised and underlined). Furthermore, DNase I footprinting identified the

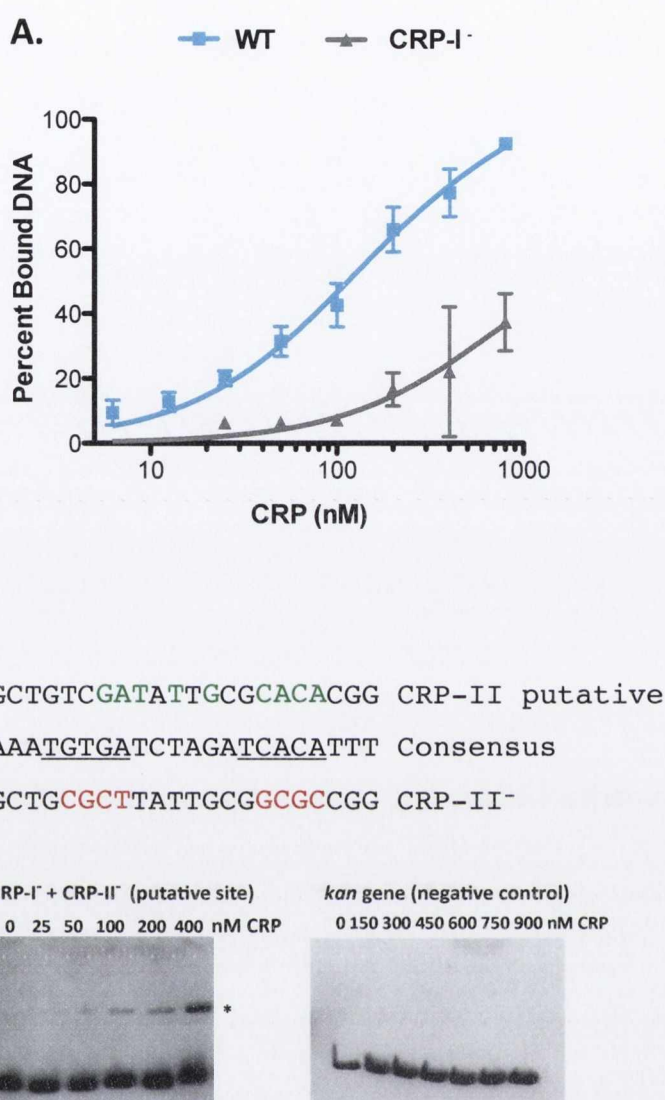


Fig. 4.13. Mutational analysis of CRP the putative CRP-II site and CRP binding curve. **A.** Binding curves were fitted to the mean data obtained from densitometric analysis of at least two independent bandshift blots. CRP binding to wild-type *ompR* sequence ($K_{app}=119\text{nM}$, blue) and CRP binding to *ompR* sequence with mutated CRP-I site ($K_{app} = >800\text{ nM}$, grey). Standard deviation is shown as error bars. **B.** Putative CRP-II site aligned with the CRP consensus sequence. Matches to the consensus sequence are in green; the core CRP sequence is underlined. The substitutions introduced are in red **C.** CRP binding to the *ompR* regulatory sequence harbouring mutations in the CRP-I and putative CRP-II site. Asterisk denotes protein-DNA complex resolved. CRP binding to the *kan* gene (negative control).

region of protection by CRP extended upstream of the CRP-I site (Cameron, A.D.S, unpublished data). Interestingly, a similar extended region of protection by CRP was observed in *E. coli* by Huang, *et al.* however this was not further investigated by the authors (Huang *et al.*, 1992). SDM was carried out using primers CRP-II_SDM_F and CRP-II_SDM_R to mutate the second putative CRP site alongside the CRP-I⁻ site on plasmid *PompRpJET* (Fig. 4.13, B). The *ompR* regulatory region encompassing both the CRP-I⁻ and putative CRP-II⁻ site was amplified with biotinylated primers *bio_ompR_F* and *bio_ompR_R* (Table 2.8). EMSA analysis showed CRP binding was unaffected by mutations in the putative second CRP binding site (Fig. 4.13, C). Furthermore, CRP did not specifically bind the negative control (*kan* gene; contains no CRP binding sites, verified by Cameron A.D.S) under the concentrations tested. The *kan* negative control was amplified using biotinylated primers *kan_bio_F* and *kan_bio_R* (Table 2.8) and the plasmid pKD4 encoding the *kan* gene was used as template. These results indicate CRP interacts specifically albeit weakly with a second CRP in the *ompR* region.

4.2.9 The construction of SL1344 Δ *crp*

To test the effect of CRP on *ompR* expression a *crp* mutant was constructed. The primers *crp_KO_F* and *crp_KO_R* (Table 2.8) were used to PCR amplify the *cat* gene from the plasmid pKD3. This PCR amplicon had flanking regions of homology to the *crp* gene. Using the λ Red recombination method this PCR fragment was introduced into SL1344 and replaced the *crp* gene with the *cat* gene by homologous recombination. The Δ *crp::cat* mutation was moved into a fresh genetic background (SL1344) by P22 transduction. The plasmid pCP20 was used to remove the *cat* gene by the flanking FRT sites generating the strain SL1344 Δ *crp* (Fig. 4.14, A). Correct construction of Δ *crp::cat* and Δ *crp* mutants were confirmed by PCR using primers *crp_KO_conf_F* and *crp_KO_conf_R* (Table 2.8) (Fig. 4.14, B). The correct sequence was confirmed by DNA sequencing (GATC, Biotech).

Sugar fermentation tests were carried out to confirm phenotypically the Δ *crp* mutant as described in section 2.2.8. Enteric bacteria preferentially consume glucose as a source of energy in a CRP independent manner. The protein III^{Glc} promotes uptake of glucose and transfers a phosphate group to glucose. In its unphosphorylated form III^{Glc} inhibits permeases for non-glucose carbon sources. In the absence of glucose the III^{Glc} protein

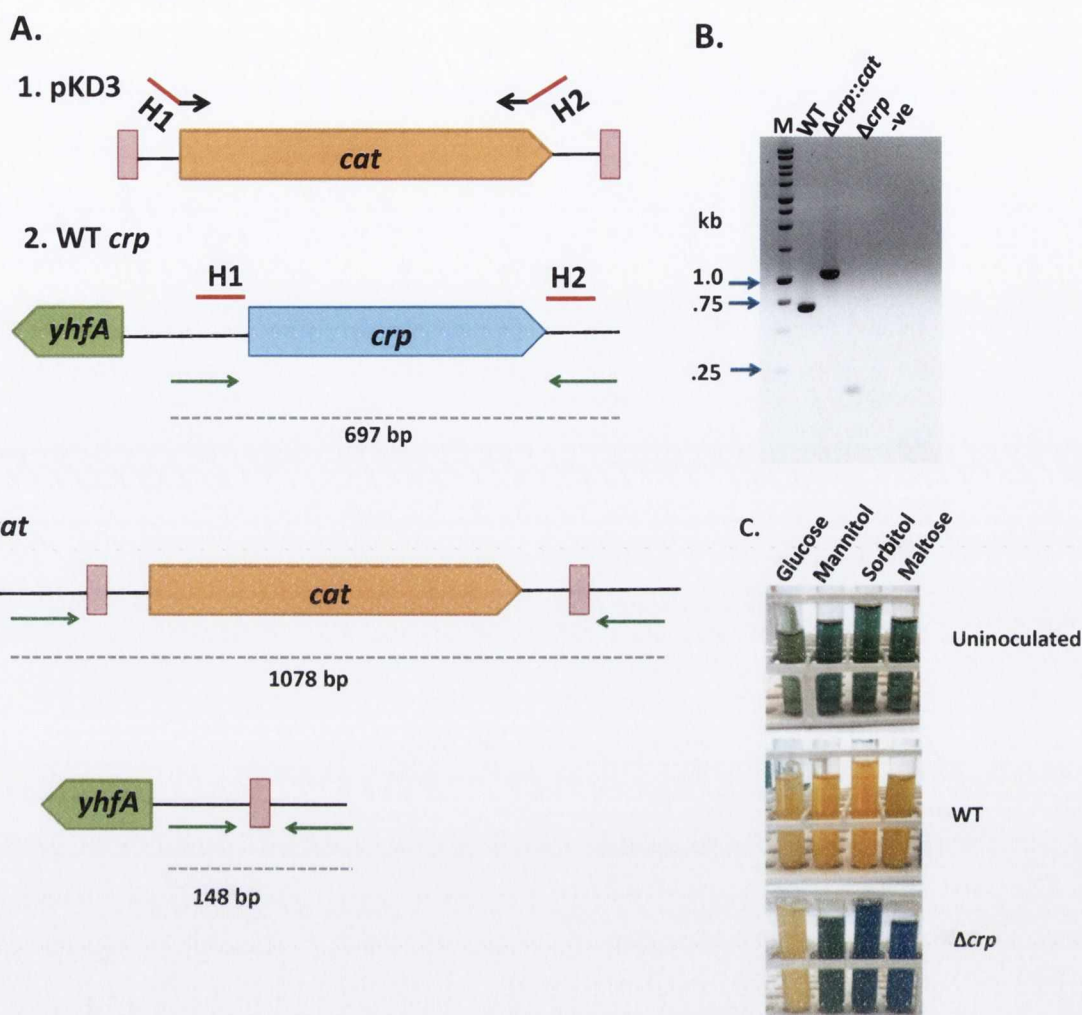


Fig. 4.14. Construction and confirmation of SL1344 Δcrp . **A.** (1) Primers were used to amplify the *cat* gene from plasmid pKD3. This generated a linear DNA fragment flanked by homologous sequence matching regions adjacent to the *crp* gene (2; H1 and H2). Using the λ red recombination system the *crp* gene was replaced with the *cat* gene (3). SL1344 Δcrp was created after the *cat* gene was excised by its FRT sites (red boxes) using the plasmid pCP20. **B.** The structure of SL1344 $\Delta crp::cat$ and SL1344 Δcrp was confirmed by PCR using primers that flanked the native *crp* gene. M: 1 kb ladder (promega), wild-type *crp* (WT; ~697 bp), $\Delta crp::cat$ (~1078 bp), Δcrp (~148 bp), -ve (negative; no template control). **C** Phenotypic confirmation of SL1344 Δcrp by sugar fermentation tests. Test tubes containing a peptone broth with glucose, mannitol, sorbitol or maltose were inoculated with wild-type (WT) or SL1344 Δcrp (Δcrp). Cultures were grown for 18 h at 37°C and 200 rpm in a rotary shaker. Yellow colour change indicates fermentation, No colour change (blue/green) indicates no fermentation.

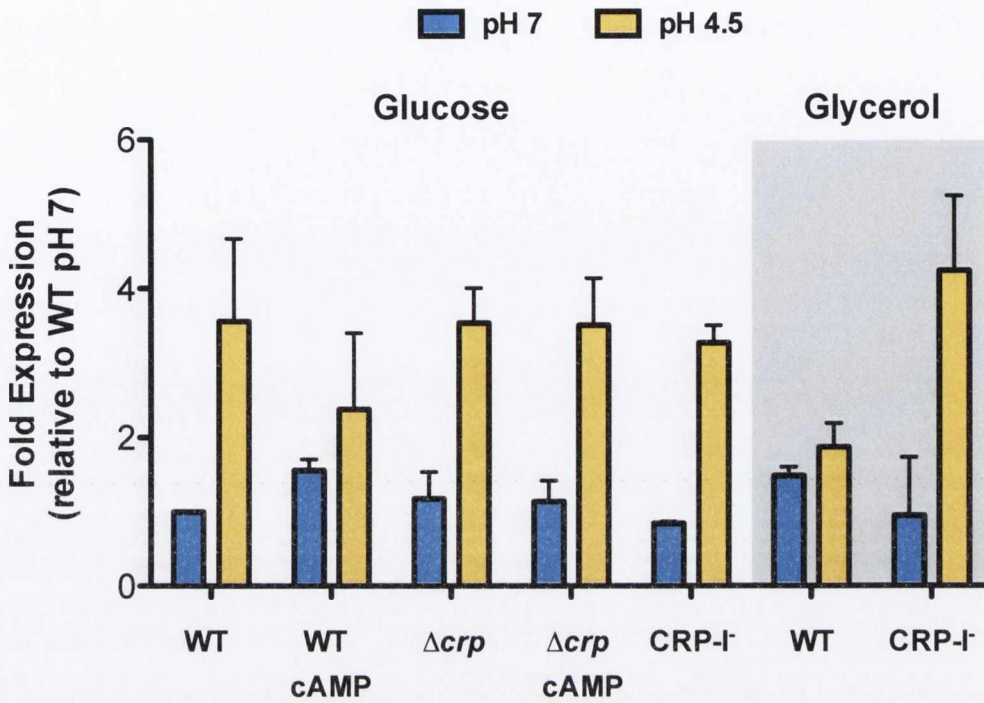
remains phosphorylated and no longer inhibits these permeases (reviewed by Magasanik and Neidhardt, 1987). In addition phosphorylated III^{Glc} activates membrane-bound adenylate cyclase, catalysing the synthesis of cAMP and converting CRP into its active, DNA-binding form. CRP activates genes encoding transporters and catabolic enzymes for non-glucose sugars, therefore CRP enables bacteria to ferment non-glucose carbon sources (e.g. glycerol, galactose, maltose) (reviewed by Deutscher, 2008). Therefore, in the absence of *crp* cells are unable to ferment these sugars, yet maintain the ability to ferment glucose. The ability of the Δcrp mutant to ferment alternative carbon sources was tested. The results in Fig. 4.14 (C) demonstrated the Δcrp mutant could not ferment any sugar tested other than glucose; this was due to the CRP dependency of the operons responsible for utilisation mannitol, sorbitol or maltose. These results confirm phenotypically the construction of SL1344 Δcrp mutant strain.

4.2.10 The effect of CRP on *ompR* expression

Earlier studies have reported *ompR* expression was not affected by CRP (Bang *et al.*, 2002). Indeed in this present study *ompR* expression was unaffected in the Δcrp mutant (Fig. 4.15, A). However this was tested in EG-minimal medium which contains glucose and citrate as carbon sources. It was rationalised that intracellular levels of cAMP would be low in the presence of glucose and therefore CRP may not be active in this medium. Therefore, CRP may not affect *ompR* expression under these conditions. CRP protein levels were analysed by western blot analysis in SL1344 *crp::x3FLAG*. This strain was constructed using a modified version of the λ Red recombination method (Uzzau *et al.*, 2001). The primers *crp::FLAG_F* and *crp::FLAG_R* (Table 2.8) were used in conjunction with the plasmid pSUB11 and a 3xFLAG epitope was fused to the C-terminus of CRP (section 2.7.5). CRP levels were then monitored using antibody against the FLAG epitope.

The results in Fig 4.15 (B) showed that CRP was present in EG-minimal medium at equal levels at pH 7 and pH 4.5. However, as cAMP levels were predicted to be low it was likely that CRP was not in its active DNA-binding form. To elevate cAMP levels and activate cAMP-CRP complexes exogenous cAMP was added to EG-minimal medium at 2 mM final concentration. In the presence of exogenous cAMP *ompR* was mildly upregulated at pH 7 and downregulated at pH 4.5, implicating CRP as regulator

A.



B.

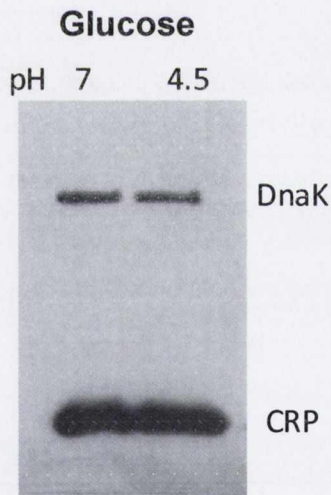


Fig. 4.15. The effect of CRP on *ompR* expression. **A.** *ompR* transcript levels were measured in E-minimal medium containing glucose or glycerol after 90 min at pH 7 and pH 4.5. Exogenous cAMP was added at a final concentration of 2 mM. Mean levels of expression were calculated from at least three independent biological replicates. Standard deviation is shown as error bars. **B.** Western blot analysis of CRP protein levels in SL1344 *crp::x3FLAG* in EG-minimal medium at pH 7 and pH 4.5. CRP protein levels were detected using an Anti-FLAG antibody. DnaK protein detected by an anti-DnaK antibody was used as a loading control.

of *ompR*. As a control to test if cAMP was mediating this affect via CRP, *ompR* expression was measured in SL1344 Δcrp with exogenous cAMP. As expected no difference in *ompR* transcript levels were observed, indicating cAMP was mediating this affect via CRP (Fig. 4.15, A). The effect of CRP on *ompR* transcription was further investigated using E-Glycerol minimal medium. This medium was identical to EG-minimal medium except it contained glycerol instead of glucose at 0.4% final concentration. Growth on glycerol is known to elevate intracellular cAMP and thus activate cAMP-CRP complexes (Notley-McRobb *et al.*, 1997). However, Δcrp mutants cannot grow on glycerol as a sole carbon source; the *glpFK* operon encodes proteins required for glycerol utilisation (Lin, 1976; Zhao *et al.*, 1994) and transcription of the *glp* operon is strongly dependent on the cAMP-CRP complex (Freedberg and Lin, 1973; Weissenborn *et al.*, 1992).

The Δcrp mutant was tested for its ability to grow on E-minimal agar containing 0.4% glycerol. As expected the Δcrp mutant was unable to grow on glycerol as a sole carbon source (Fig. 4.16). Therefore, to assess the effect of CRP on *ompR* expression the *ompR* regulatory region harbouring the CRP-I⁻ binding site from *PompR* CRP-I⁻ pJET was introduced onto the SL1344 chromosome *in vivo* as described in section 4.2.3. In the presence of glucose the CRP-I⁻ binding site mutation behaved similarly to wild-type (Fig. 4.16, A), re-emphasising that CRP had no effect on *ompR* expression in the presence of glucose. In the presence of glycerol wild-type *ompR* expression levels were different to that observed in glucose; transcript levels were insensitive to changes in pH. CRP is known to affect DNA supercoiling, CRP positively regulates *gyrA* (encoding the A subunit of DNA gyrase; required to introduced negative supercoils into DNA) expression (Gomez-Gomez *et al.*, 1996). Therefore, it is plausible to consider CRP may increase levels of negative DNA supercoiling and prevent induction of *ompR* by DNA relaxation at low pH. However, the CRP-I⁻ mutant resulted in upregulation of *ompR* expression in acidic conditions and restored the pH sensitivity of *ompR* observed in the presence of glucose. Therefore, during carbon starvation (i.e. growth on glycerol) the insensitivity of *ompR* to acidic pH was a direct result of CRP binding and repressing *ompR* expression. These results demonstrate CRP acts as a dual regulator of *ompR* expression; activating expression at pH 7 and repressing expression at pH 4.5 to maintain steady levels of *ompR* expression at both pH values. It is unknown how CRP mediates this differential effect on *ompR*. *ompR* expression may be driven from

different TSSs at pH 7 and pH 4.5 allowing CRP to differentially regulate expression from the CRP-I site. CRP may also modulate binding of other regulators and in turn differentially regulate *ompR* expression. Thus, CRP regulation of *ompR* in *S. Typhimurium* requires further exploration to understand how CRP exerts this dual effect on *ompR* levels.



Fig. 4.16. CRP mutant on E-Glycerol minimal agar plate. Visual comparison of wild-type (WT) and SL1344 Δcrp growth on glycerol as a sole carbon source. Strains were streaked onto E-minimal agar plate containing glycerol 0.4% and incubated at 37°C. The image was captured after 24 h and no growth was recorded for the mutant after 3 days at 37°C.

4.3 Discussion

The aims of this chapter were to identify major contributing factors influencing *ompR* expression in *S. Typhimurium* at low pH. To achieve these aims, a detailed molecular biological study of *ompR* regulation at low pH was carried out.

In *E. coli*, IHF binds to three sites in the *ompR* promoter and negatively affects *ompR* expression by inhibiting RNAP binding to the promoter (Tsui *et al.*, 1991). In this present study bioinformatic and EMSA analysis revealed the IHF-I and IHF-III sites identified in *E. coli* are conserved in *S. Typhimurium*. IHF bound to each site independently and did not bind to the *ompR* promoter when both sites were mutated. However, careful examination of the EMSA images indicated that a third weak IHF site was present in the *ompR* promoter and this was in agreement with DNase I footprints performed elsewhere which identified a region of protection from -34 to -51 relative *ompR* to the start codon (ATG +1) (Cameron, A.D.S, unpublished data). Examining the contribution of the IHF-I and IHF-III sites to *ompR* expression revealed that both sites were involved in fine-tuning *ompR* transcription in acidic conditions and a model for IHF regulation of *ompR* is proposed in Fig. 4.17. IHF acts as a mild repressor of *ompR* expression via the IHF-I site, inactivation of the IHF-I site indeed relieved this repression however the upstream IHF-III site was required for optimum levels of derepression. As neither the IHF-I nor IHF-III binding sites overlapped the TSS-4.5 promoter, IHF is thought to mediate this dual regulatory effect indirectly by introducing bends into the DNA that may affect the binding of other regulatory proteins. IHF binding to IHF-III may introduce an architectural change into the DNA that promotes interaction of regulatory proteins with RNAP to enhance *ompR* expression. Such examples of IHF-mediated negative and positive effects on transcription have been described previously. For instance, IHF binds to the *ompF* promoter in *E. coli* and is thought to indirectly repress *ompF* transcription by interfering with OmpR binding and activation of gene expression (Ramani *et al.*, 1992). At the *narGHJI* operon in *E. coli* IHF mediates an indirect positive effect on transcription, IHF is thought to introduce a bend in the *narG* promoter, which enhances the action of activators NarL and Fnr (Schroder *et al.*, 1993). The experimental results in this study indicate IHF plays a dual role at the *ompR* promoter in *S. Typhimurium* in acidic conditions. However, as the effect of IHF was mild, it was considered to act in concert with other regulators to fine

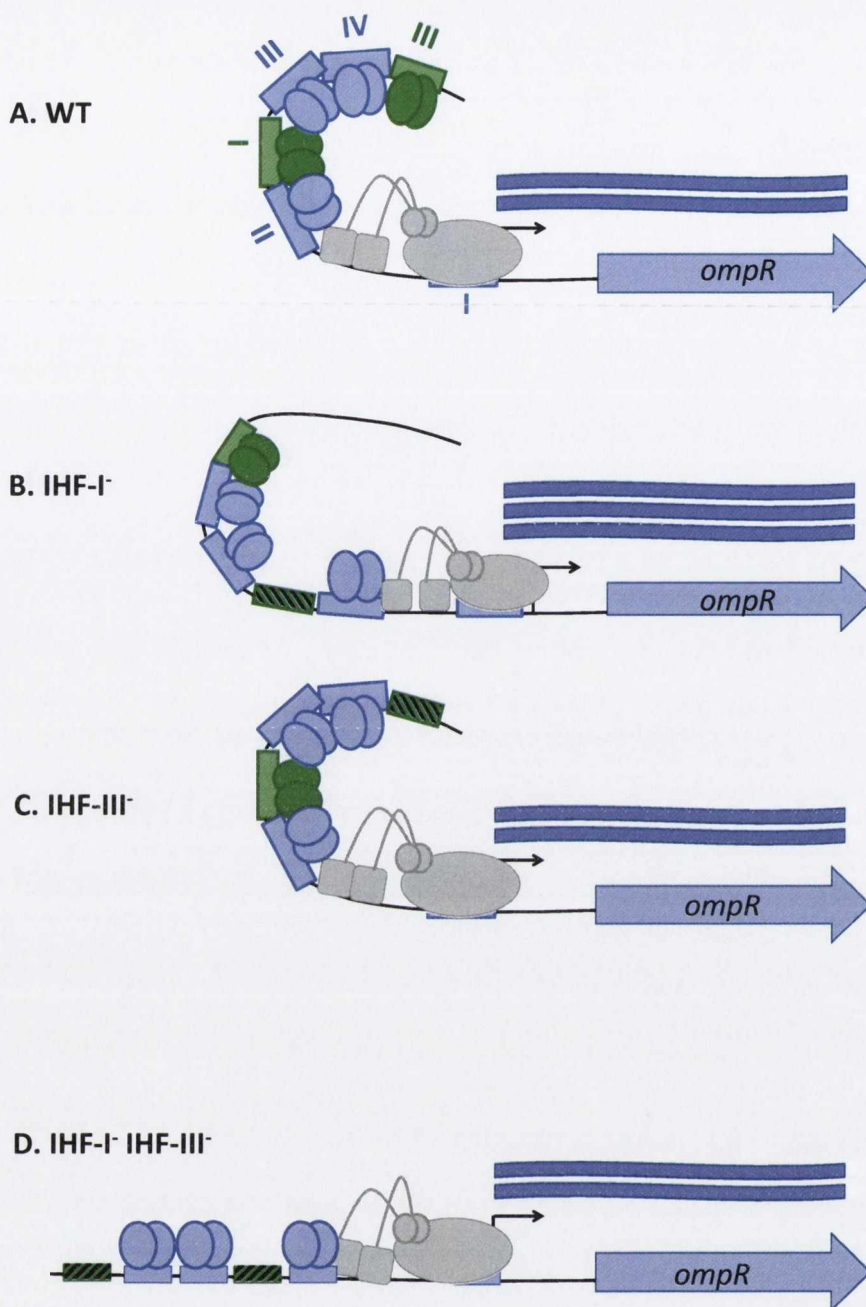


Fig. 4.17. Model of *ompR* regulation by IHF. IHF is a dual regulator of *ompR* expression. At low pH IHF (green circles) binds to at least two IHF binding sites IHF-I and IHF-III (green rectangles) to mediate control of *ompR* expression **B**. IHF mediates repression of *ompR* via the IHF-I site; in the presence of a defective IHF-I site (hatched box) *ompR* expression is upregulated. However, derepression of *ompR* requires an intact IHF-III site; *ompR* expression could not be derepressed when the IHF-III site was mutated alone **(C)** or in combination with the IHF-I site **(D)**. IHF may co-ordinate *ompR* expression by fine-tuning OmpR (pairs purple circles) and RNA polymerase (grey) binding through DNA bending. *ompR* gene purple arrow, mRNA purple lines, bent arrow TSS-4.5.

tune *ompR* expression. Therefore the contribution of other regulators that may act in concert with IHF was examined.

In *S. Typhimurium* OmpR binds to four sites in the *ompR* regulatory region and is required for autoinduction at low pH (Bang *et al.*, 2002; Cameron and Dorman, 2012). The OmpR-II site was proposed as the primary site due to its high affinity for OmpR and the presence of the GXXAC motif (Harlocker *et al.*, 1995; Bang *et al.*, 2002), however its effect on *ompR* expression was unknown. In this present study the OmpR-II site was mutated and its effect on *ompR* expression was examined. The result identified the OmpR-II site as a crucial factor for *ompR* expression at both pH 7 and pH 4.5, *ompR* expression was more severely affected at low pH highlighting the importance the OmpR-II site in the acid-induction of *ompR*. OmpR binding to the OmpR-II site may directly affect transcription or it may affect OmpR binding to the remaining OmpR binding sites in the promoter, this remains to be tested. As previously reported the acid-induction of *ompR* expression required the EnvZ protein (Bang *et al.*, 2002). Bang *et al.* found OmpR protein levels were no longer acid-induced in the absence of EnvZ and the alternative phosphodonor acetyl phosphate (Bang *et al.*, 2002). The results presented in this study also show *ompR* expression was no longer acid-inducible in an $\Delta envZ::kan$ mutant suggesting that *ompR* expression at pH 4.5 required phosphorylated OmpR and that EnvZ was the primary phosphodonor. Furthermore, *ompR* expression at pH 7 did not require EnvZ and therefore it is proposed that unphosphorylated OmpR may drive *ompR* expression at pH 7.

The possibility that IHF bound to the IHF-I site was responsible for the decrease in *ompR* expression in the OmpR-II⁻ mutant was examined. However, *ompR* expression in double IHF-I⁻ OmpR-II⁻ site mutant was further decreased. This indicated that repression by IHF was not accountable for the decrease in *ompR* expression in OmpR-II⁻ mutant. However, it was hypothesised that H-NS was responsible for this further decrease *ompR* levels. An earlier study has shown H-NS acts a negative regulator of *ompR* in *S. Typhimurium* (Bang *et al.*, 2002). The results presented in this study confirm earlier studies that have found that *ompR* was derepressed in an *hns* mutant. Interestingly, in the OmpR-II⁻ *hns* double mutant, *ompR* expression levels remained significantly lower than WT suggesting the OmpR-II site was required for OmpR

antagonism of H-NS and activation of *ompR* expression. However, is it also possible that *ompR* expression was repressed by OmpR occupying the remaining intact OmpR binding sites.

Previous studies suggest that OmpR activates expression by making direct contact with the α CTD of RNAP (Igarashi *et al.*, 1991). The α subunit of RNAP is required for promoter recognition, RNAP assembly and participates in direct protein-protein interactions with transcription factors (Ebright and Busby, 1995). Studies have shown the α CTD subunit of RNAP is required in order to activate expression from the *ompC* promoter. Furthermore suppressor mutations in the *rpoA* gene (encoding the α subunit) suggest that OmpR targets a region near amino acids 322-323 of the subunit (Slauch *et al.*, 1991). ChIP-qPCR performed in this present study revealed RNAP bound equally as well to the *ompR* promoter in an $\Delta ompB$ mutant, suggesting RNAP binds to the promoter independently of OmpR. However, OmpR was required to activate expression and therefore it is thought that OmpR activates transcription by enhancing open-complex formation. Interestingly, RNAP binding was reduced when the OmpR-II site was mutated; this was not observed in a $\Delta ompB$ mutant and therefore suggests that the OmpR protein is capable of hindering RNAP binding. Indeed the OmpR-I site overlaps the -10 element of the *ompR* TSS-4.5 promoter (Fig. 4.1). OmpR occupancy of this site may directly obstruct RNAP binding to the *ompR* promoter.

Bang *et al.* reported that *ompR* expression was derepressed in an *hns ompR* double mutant (Bang *et al.*, 2002), this present study aimed to identify the specific contribution of the OmpR-II site to *ompR* expression and as such found expression was decreased in the OmpR-II⁻ *hns* double mutant. From this work it is hypothesised that the presence of multiple OmpR binding sites in the *ompR* promoter enables OmpR to act as both an activator and a repressor of *ompR* expression perhaps via the OmpR-II and OmpR-I sites respectively, however, this has not been tested to date. In the presence of multiple OmpR binding sites OmpR is known to act as both an activator and repressor of target genes. In *E. coli* the OmpR dependent genes *ompF* and *ompC* are reciprocally regulated by OmpR, OmpR executes this reciprocal regulation by differential occupancy of binding sites in response to changes in osmolarity (Alphen and Lugtenberg, 1977). At low osmolarity OmpR occupies the

high affinity OmpR binding sites (F1-F2-F3) at the *ompF* promoter and activates transcription. At high osmolarity OmpR levels increase and OmpR occupies the low affinity F4 site responsible for repression of *ompF* but also occupies the C1-C2-C3 site that are responsible for activation of *ompC* (reviewed by De la Cruz and Calva, 2010). Thus, in the presence of multiple sites the OmpR protein can act as both an activator and repressor of gene expression and as such OmpR may mediate activation and repression of its own gene via the OmpR-II and OmpR-I sites respectively.

In summary from the results presented in this chapter and previous reports of *ompR* activation by DNA relaxation (Cameron and Dorman, 2012) the following model has been proposed for *ompR* expression in acidic conditions (Fig. 4.18.). Results presented in section 3.2.7 show at both pH 7 and pH 4.5 *ompR* is expressed primarily from TSS-4.5. Under non-inducing conditions (pH 7) expression is repressed by H-NS. As expression was also reduced at pH 7 in the OmpR-II⁻ mutant but not in the $\Delta envZ$ mutant it can be deduced that unphosphorylated OmpR is also bound to the promoter region. In addition, a recent study has shown the Fis protein positively regulates *ompR* expression (Cameron and Dorman, 2012). Fis binding was affected by local DNA topology and behaved in the opposite manner to OmpR such that Fis binding at *ompR* decreased as DNA relaxed (Cameron and Dorman, 2012). Thus under the conditions used in this study Fis may act in conjunction with OmpR and H-NS at the *ompR* promoter to maintain low levels of *ompR* expression at pH 7. The role of Fis in *ompR* regulation at low pH requires investigation. Upon a shift to acidic pH EnvZ phosphorylates OmpR and DNA at the *ompR* promoter relaxes (Fig. 4.18, B). OmpR-P induces co-operative DNA binding and H-NS repression is thought to be alleviated by DNA relaxation and OmpR antagonism (Cameron and Dorman, 2012). OmpR and IHF act to fine-tune *ompR* transcript levels.

The second theory tested in this study hypothesised CRP was a regulator of *ompR* in *S. Typhimurium*. From the results presented in this study it can be concluded that CRP is indeed an important regulator of *ompR*. This shows *ompR* expression is affected by carbon starvation and is linked to the energy status of the cell. A good match to the CRP consensus sequence was identified and the presence of the CRP-I site was validated by EMSA analysis. Thus, the CRP-I binding, first identified in *E. coli* (Huang *et al.*, 1992), was found to be conserved in *S. Typhimurium* and capable

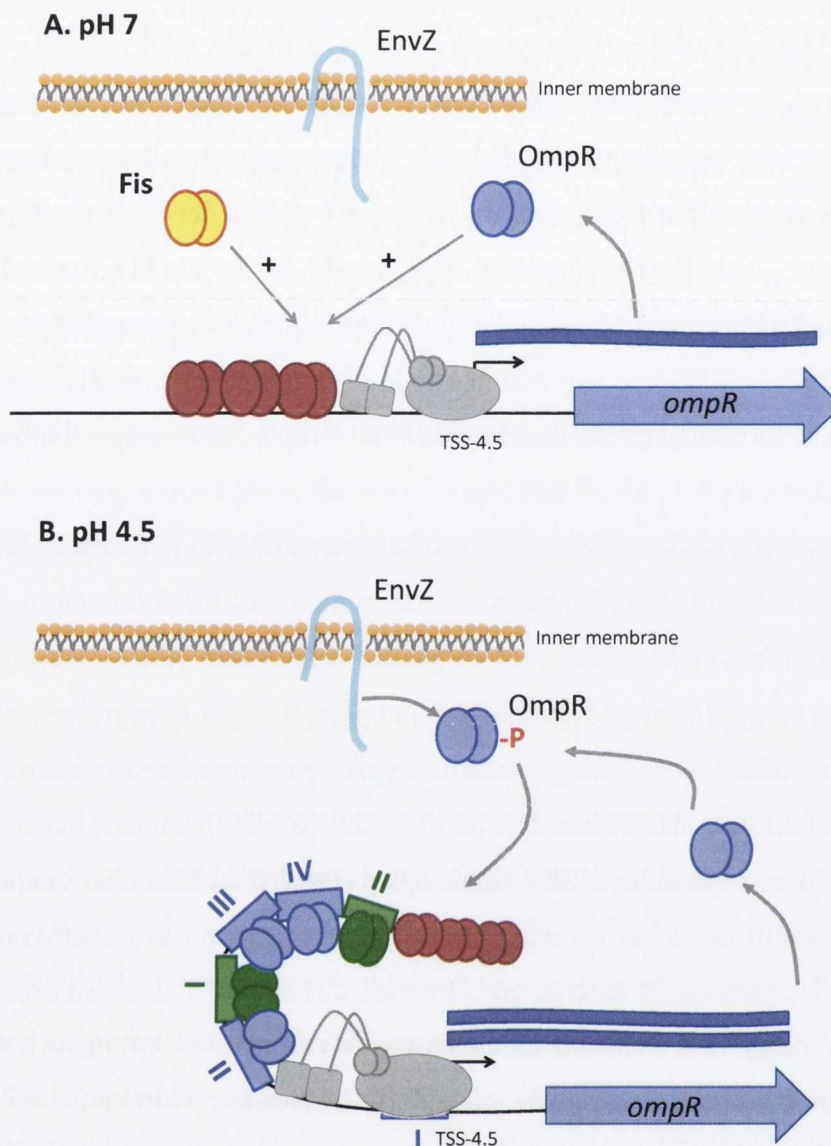


Fig. 4.18. Model for *ompR* activation at low pH. **A.** At pH 7 *ompR* expression is repressed by H-NS (red circles) by DNA bridging or stiffening. Unphosphorylated OmpR (purple circles) exerts a positive affect on *ompR* expression (+). It is hypothesised that the Fis protein (yellow circles) binds to the *ompR* promoter (see text) **B.** Upon a shift to pH 4.5 the local topology of DNA at *ompR* relaxes and H-NS binding is restructured. OmpR phosphorylation by EnvZ enhances OmpR's affinity for DNA. OmpR increases *ompR* expression from TSS-4.5 promoter specifically via the OmpR-II site. OmpR may directly contact the C-terminal domain of the RNA polymerase α subunit (grey squares). Alternatively, OmpR may promote open complex formation by transmitting energy from the locally destabilised region (SIDD region; see text) to the TSS-4.5 promoter region. Purple arrow denotes the *ompR* gene, purple lines denote *ompR* mRNA, RNA polymerase is grey and the bent arrow denotes the acid-induced transcription start site TSS-4.5. IHF is denoted by green dimers.

of binding CRP *in vitro*. Furthermore, the existence of a putative second CRP site was also reported. According to DNase I footprinting performed elsewhere (Cameron, A.D.S, unpublished data) this site was thought to be located upstream and adjacent to CRP-I (Fig. 4.1 italicised and underlined). Attempts to further validate the presence of this putative second CRP site by SDM analysis were not successful. Nevertheless, CRP's affinity for this site was >8 times lower than that of CRP-I, which raises the question of the physiological relevance of this site. This study found that a *crp* mutant had no effect on *ompR* expression when glucose was used as a sole carbon source. It was hypothesised that intracellular cAMP level would be low under these conditions and therefore active CRP levels were likely to be low also. Therefore, CRP was unlikely to regulate *ompR* expression under these conditions. However, artificially increasing cAMP levels resulted in a mild effect on *ompR* expression.

ompR expression was insensitive to changes in pH in conditions predicted to elevate active CRP levels (i.e. glycerol as a sole carbon source). This affect was found to be as a direct result of the CRP-I site; at pH 7 CRP activated *ompR* expression and at pH 4.5 CRP repressed *ompR* expression via the CRP-I binding site. Thus, it was concluded that CRP acts as a dual regulator of *ompR* expression in *S. Typhimurium*. However, the mechanism of how CRP mediates this affect is unclear. In *E. coli* CRP inhibits expression of two TSSs and activates the expression of another two TSSs (Huang *et al.*, 1992). The negatively regulated CRP TSSs are located in close proximity to the CRP binding site and as such the CRP site overlaps the -10 and -35 elements. The positively regulated TSSs are located further downstream (Huang *et al.*, 1992). In *E. coli* CRP binding to the *ompR* promoter may negatively affect RNAP binding to proximal promoters and facilitate RNAP binding to distal promoters (Huang *et al.*, 1992). It is unclear at this time how CRP regulates *ompR* in *S. Typhimurium*. Further analysis into which of the *ompR* TSSs are utilised in the presence of glycerol and those which are affected in the presence of a defective CRP-I site may offer insights into the dual regulation of *ompR* by CRP. Dual regulation by CRP is has been documented in examples outside of *ompR*. Such as the *gal* gene for galactose utilisation, CRP binds to a single site but possesses dual function by repressing the *gal* P1 promoter and activating the *gal* P2 promoter which is located just 5 bp upstream of P1 (Musso *et al.*, 1977).

Dual regulation of the *ompR* gene by CRP appears to be conserved in *S. Typhimurium* and *E. coli* however this is not the case in a related species *Yersinia pestis* where recently it has been shown that CRP does not regulate *ompR* (Gao *et al.*, 2011b). CRP bypasses regulation of the *ompR/envZ* operon and instead directly regulates OmpR target genes *ompF* and *ompC* (Gao *et al.*, 2011b). Thus, regulation of *ompR* in related species appears to be distinct. In *S. Typhimurium* *crp* mutants are attenuated for virulence (Curtiss and Kelly, 1987). However, *crp* expression is strongly repressed in *Salmonella* infected macrophages (Eriksson *et al.*, 2003). Therefore, the physiological relevance of *ompR* regulation by CRP may be apparent during macrophage infection; repression of *crp* expression inside macrophage may permit *ompR* induction in acidic condition and thus promote virulence and survival by activation of SPI-2 master regulator *ssrA* (Lee *et al.*, 2000).

Studies have shown the *ompR* gene is not osmo-sensitive in *S. Typhimurium*, instead *ompR* is activated by acidic pH during the stationary phase ATR and by DNA relaxation (Bang *et al.*, 2002; Cameron and Dorman, 2012). The results present here offer detailed analysis of *ompR* regulation in acidic pH. IHF protein levels are known to peak at the onset of stationary phase (Bushman *et al.*, 1985; Ditto *et al.*, 1994) and results presented here identified IHF as a regulator of *ompR* during the stationary phase ATR. These results add to previous work highlighting the importance of IHF in orchestrating pathogenicity in *S. Typhimurium* (Mangan *et al.*, 2006). This study has also highlighted the OmpR-II site as the major contributing factor required for H-NS antagonism and activation of *ompR* at low pH. In addition, *ompR* was shown to be directly regulated by the CRP protein in a dual manner. It is clear that factors previously shown to regulate *ompR* in *E. coli* (Tsui *et al.*, 1991; Huang *et al.*, 1992) are conserved in *S. Typhimurium* however the acid induction of *ompR* required multiple regulatory proteins that act in concert to co-ordinate optimal levels of expression. The ability of *ompR* in *S. Typhimurium* to respond to multiple signals such as acidic pH, DNA relaxation and carbon starvation reflects the importance of *ompR* expression under diverse conditions.

Chapter 5 ChIP-on-chip analysis of the OmpR regulon in *S.*
Typhimurium and *E. coli*

5.1 Introduction

Chromatin immunoprecipitation (ChIP) is an effective method to investigate protein-DNA interactions *in vivo*. Hybridisation of ChIP DNA to microarrays (ChIP-on-chip) enables protein-DNA interactions across an entire genome to be interrogated (reviewed by Wade *et al.*, 2007). Briefly, protein-DNA interactions are cross-linked using formaldehyde; cells are then lysed followed by sonication of chromatin into fragments typically 300-500 bp in size. The cross-linked protein-DNA complexes are immunoprecipitated using antibody specific for the protein of interest. The cross-links are reversed by heating followed by recovery of the ChIP-enriched DNA; this is fluorescently labelled and hybridised to a DNA microarray. Typically, genomic DNA or DNA immunoprecipitated by a non-specific antibody is fluorescently labelled and hybridised to the same array as a reference. This enables quantitative measurements of protein-DNA interactions to be made (reviewed by Wade *et al.*, 2007).

In this study, ChIP-on-chip was employed to ascertain the genome-wide distribution of OmpR and the impact of acidic pH on OmpR binding to DNA. The OmpR protein is 100% conserved at the amino acid sequence level in both *E. coli* and *S. Typhimurium*, thus enabling direct comparisons of OmpR binding in both organisms to be assessed. Previous work investigating global gene expression in *E. coli* using an *ompR/envZ* mutant identified 125 genes with altered expression patterns (Oshima *et al.*, 2002); indicating that OmpR has an effect on a large number of genes. Studies in *E. coli* and *S. Typhimurium* have demonstrated that OmpR binds and regulates a number of genes including the *ompF* and *ompC* genes that encode the major outer membrane porins OmpF and OmpC respectively (Alphen and Lugtenberg, 1977; Slauch and Silhavy, 1989). Other genes known to be directly regulated by OmpR include *fadL*, encoding an outer membrane protein involved in uptake of long-chain fatty acids (Higashitani *et al.*, 1993), *flhDC*, encoding the master regulatory complex for flagellar biogenesis (Shin and Park, 1995) and genes responsible for the production of curli fimbriae, *csgDEFG* (Prigent-Combaret *et al.*, 2001). OmpR also regulates expression of the *tpxB* gene that encodes a tripeptide permease (Gibson *et al.*, 1987; Goh *et al.*, 2004).

Salmonella is thought to have diverged from a common ancestor with *E. coli* ~100 million years ago (Ochman and Wilson, 1987; Doolittle *et al.*, 1996). While their

genomes display similarity in terms of gene content and gene order, divergence between these two species is apparent in the large genomic pathogenicity islands (SPIs), prophage and plasmids that have been acquired by the *Salmonella* species (McClelland *et al.*, 2001). OmpR binds to some of these horizontally-acquired targets in *S. Typhimurium*. For example, it activates expression of the horizontally acquired SPI-2 master regulator operon *ssrA/B*, SsrB then activates expression of structural genes for a T3SS complex and its effectors that are required for survival inside host macrophages (Lee *et al.*, 2000; Bang *et al.*, 2002; Feng *et al.*, 2004). More recently, a study has shown that OmpR binds directly to the SPI-1 *hilC* and *hilD* genes (Cameron and Dorman, 2012). These genes encode the regulators HilC and HilD respectively, which mediate the activation of the SPI-1 T3SS required for invasion of host epithelial cells (Cameron and Dorman, 2012). These previous studies have provided evidence that the OmpR regulon encompasses genes with broad functions inside the cell. Furthermore, OmpR binding to horizontally acquired genes present in *S. Typhimurium* yet absent from *E. coli* indicates the OmpR regulon has expanded since *S. Typhimurium* diverged from *E. coli*. Furthermore, it also shows that OmpR is a regulator both of genes in the core genome and of genes in the horizontally acquired genome.

The goal of this chapter is to map the genome-wide distribution of OmpR in *E. coli* and *S. Typhimurium* and to compare the two. In addition, this chapter investigates the influence of acidic pH on OmpR binding in both organisms and in doing so, aims to further the current knowledge of OmpR regulon in both organisms, while identifying species-specific OmpR regulons.

5.2 Results

5.2.1 Construction of *E. coli* CSH50 *ompR*::3xFLAG.

To map OmpR distribution in *E. coli* a 3xFLAG epitope tag was fused to the C-terminus of OmpR in strain CSH50. CSH50 *ompR*::3xFLAG was generated in an identical manner as SL1344 *ompR*::3xFLAG (Cameron and Dorman, 2012). This enabled comparative analysis of OmpR binding in both *E. coli* and *S. Typhimurium* respectively. CSH50 *ompR*::3xFLAG was created using a modified version of the λ Red recombination system (Uzzau *et al.*, 2001). The primers *ompR*::FLAG_E.c_F and *ompR*::FLAG_E.c_R (Table 2.8) were used to amplify the FLAG epitope and kanamycin resistance marker from the plasmid pSUB11 (see Fig. 5.1, A). The primers were designed with 40 bp of homologous DNA sequence to the 3' end of the *ompR* gene. However the *ompR/envZ* locus (*ompB*) has a four base pair overlap between the *ompR* stop codon and *envZ* start codon. The reverse primer (*ompR*::FLAG_E.c_R; Table 2.8) was designed to reinstate the natural *envZ* start codon to avoid disrupting translation initiation in *envZ*. Insertion of this DNA fragment by λ Red recombination removed the *ompR* stop codon and inserted the FLAG epitope fusion in-frame with the *ompR* gene. The *ompR*::3xFLAG mutation was transduced into a fresh background (CSH50) by P1 transduction and the kanamycin resistance cassette was removed using the plasmid pCP20.

Correct structure of the epitope fusion was confirmed by PCR using primers *ompR_conf_E.c_F* and *ompR_conf_E.c_R* (Table 2.8). These primers bind specifically upstream of the *ompR* gene and in the *envZ* open-reading frame respectively (see black arrows in Fig. 5.1, A). PCR amplification with these primers using wild-type CSH50 DNA template generated an amplicon ~892 bp in size (see Fig. 5.1, B). Using the same primers, PCR amplification was performed using DNA from CSH50 *ompR*::3xFLAG before and after the removal of the *kan* marker as template; this generated amplicons ~2,441 bp and ~1,048 bp in size respectively (Fig. 5.1, B). The correct DNA sequence was confirmed by DNA sequencing (GATC, Biotech). Expression of OmpR in CSH50 *ompR*::3xFLAG was confirmed by western blotting (Fig. 5.1, C). Samples were taken from overnight cultures grown in L-broth at 37°C at 200 rpm in a rotary shaker. Samples were equalised to OD₆₀₀ 0.2 and analysed by western blotting (section 2.13). OmpR was detected in CSH50 *ompR*::3xFLAG using antibody against the FLAG

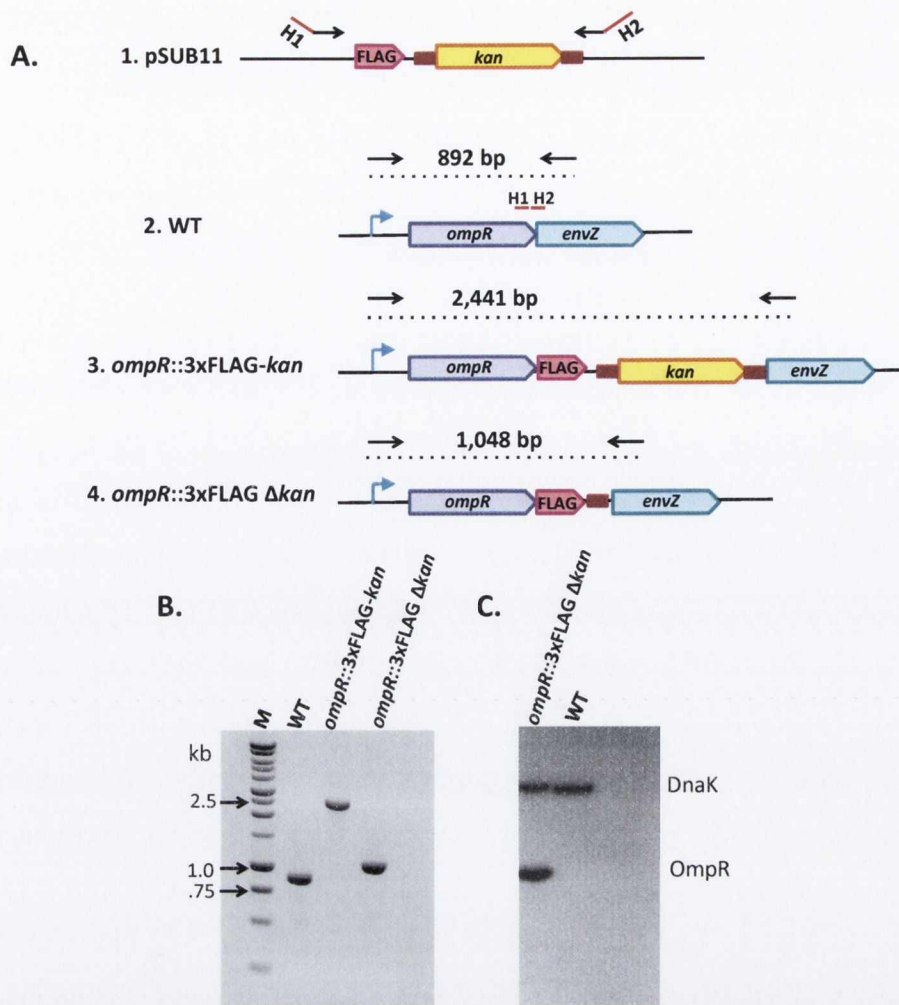


Fig. 5.1. Construction and confirmation of CSH50 *ompR::3xFLAG*. **A.** 1. The FLAG epitope sequence and the kanamycin resistance marker were amplified from plasmid pSUB11 using the primers *ompR::FLAG_E.c_F* and *ompR::FLAG_E.c_R* (Table 2.8, illustrated as bent, dual-coloured arrows). 2. This PCR-amplified product was flanked by 40 bp overhangs with DNA sequence homology (1. H1 and H1) to the 3' end of the *ompR* gene (2. H1 and H2). 3. This product was introduced in CSH50 by the λ Red recombination system (Uzzau *et al.*, 2001). 4. The *kan* gene was removed via the flanking FRT sites (red boxes) using the plasmid pCP20. Black arrows denote primer sets used for PCR confirmation (see part B) and the expected sizes of the amplicons are shown. **B.** Gel image representing the results of PCR using primers *ompR_conf_E.c_F* and *ompR_conf_E.c_R* (Table 2.8). Wild-type (WT) CSH50 DNA and DNA template from CSH50 *ompR::3xFLAG-kan* and CSH50 *ompR::3xFLAG Δkan* was tested. Products of expected size (as shown in part A) were obtained. M: 1 kb DNA ladder. **C.** Western blot analysis of OmpR expression in CSH50 *ompR::3xFLAG* and wild-type CSH50 (WT). Samples were taken from overnight cultures grown in 4 ml L-broth at 37°C and 200 rpm in a rotary shaker. Anti-FLAG antibody for the FLAG epitope and anti-DnaK was used as a loading control.

epitope and was the correct size (~30 kDa with the 3xFLAG epitope). Antibody against DnaK was used as a loading control. Wild-type CSH50 (WT) harbouring no epitope fusion was used as a negative control (Fig. 5.1, C).

5.2.2 Biological conditions and analysis of OmpR ChIP-chip data sets

To investigate the effect of acidic pH on OmpR binding in both *E. coli* and *S. Typhimurium* OmpR binding was mapped using ChIP-on-chip analysis. As shown in section 3.2.2 OmpR levels were upregulated in acidic pH in *S. Typhimurium* but not in *E. coli*. To measure the impact of acidic pH on OmpR binding in *S. Typhimurium* and *E. coli* the strains SL1344 *ompR::3xFLAG* and CSH50 *ompR::3xFLAG* were cultured as described in section 2.2.6. After 90 min in EG-minimal media at pH 7 and pH 4.5 DNA-protein interactions were cross-linked to generate a ‘snapshot’ of OmpR binding in both organisms. OmpR protein-DNA interactions were immunoprecipitated using anti-FLAG antibody and samples were processed as described in section 2.15. Three independent biological replicates were performed in both SL1344 and CSH50. Fluorescently labelled OmpR-enriched ChIP DNA and genomic non-immunoprecipitated DNA (input) from each biological replicate were co-hybridised to species-specific microarrays produced by Oxford Gene Technology. The intensity of fluorescence at each spot representing a 60-mer probe on the microarray was measured and the ChIP/input ratio for each biological replicate was calculated (see section 2.15.9-2.15.10).

The reproducibility of each data set at pH 7 and pH 4.5 in SL1344 and CSH50 was calculated using Pearson’s correlation coefficient (Excel, 2011) between all possible combinations of replicates (see Table 5.1). Pearson’s correlation coefficient quantifies the relationship between two variables, the correlation coefficient ranges from -1 to +1. A coefficient value of 0 means there is no relationship between the two variables; a value of -1 means there is perfect negative or inverse correlation while a value of 1 means there is a perfect positive correlation between two variables. The correlation coefficients calculated for the ChIP-chip data sets study ranged from 0.90-0.99 indicating there was a high positive relationship between replicates, thus the data was reproducible.

Table 5.1. Pearson’s correlation coefficient for ChIP data replicates

	<i>S. Typhimurium</i> (SL1344)		<i>E. coli</i> (CSH50)	
	pH 7	pH 4.5	pH 7	pH 4.5
Replicate 1 + 2	0.99	0.98	0.94	0.94
Replicate 2 + 3	0.99	0.99	0.95	0.90
Replicate 1 + 3	0.99	0.99	0.97	0.92

The median $\log_2$ ratios from three independent biological replicates at pH 7 and pH 4.5 in SL1344 and CSH50 were calculated. Two independent mock ChIP-on-chip experiments were performed in both SL1344 and CSH50 under the same experimental conditions using pre-immune antisera from mouse (normal mouse IgG antibody, Millipore). The mock/input ratio was calculated for each replicate and the median $\log_2$ ratio was then calculated. To remove non-specifically enriched peaks, the SL1344 mock median $\log_2$ ratio was subtracted from the SL1344 ChIP median $\log_2$ ratio. This was performed for the pH 7 and pH 4.5 datasets. The CSH50 ChIP datasets were treated in the same manner using the CSH50 mock median $\log_2$ ratios. The $\log_2$ ratios were used in the ChIPOTle programme (Buck *et al.*, 2005) to analyse and define peaks of OmpR enrichment using settings described in section 2.15.11; the P value threshold cut-off chosen was $p = 0.001$. Gene names were assigned to the probes on the arrays by Dr Karsten Hokamp.

5.2.3 Identification of OmpR targets in *S. Typhimurium* and *E. coli* using ChIP-on-chip

Analysis of genome-wide distribution of OmpR in both *E. coli* and *S. Typhimurium* revealed OmpR bound multiple sites across the entire chromosome in both organisms with multiple highly enriched peaks, see Fig. 5.2 and 5.3 respectively. In *E. coli* OmpR distribution appeared quite static and marginally affected by pH (Fig. 5.2). In contrast, OmpR appeared to occupy more sites at low pH in *S. Typhimurium* (Fig. 5.3). It should be noted that in *E. coli*, the microarray probes between genome coordinates 256375 and 371125 appeared to have no enrichment for OmpR binding after IgG normalisation (denoted by an asterisk in Fig. 5.2). This region may be more susceptible to non-specific binding by mouse IgG; the reason for this is unknown. Nonetheless, the purpose of removing IgG enriched peaks was to reduce background levels associated with non-specific interactions.

In SL1344 the ChIPOTle programme identified 85 significantly enriched OmpR peaks at pH 7, this increased to 225 peaks at pH 4.5 (Listed in Table S1. and S2). Further analysis revealed 61 OmpR-bound genes were bound at pH 7 and pH 4.5 (see Fig 5.4; genes are listed in Table 5.2). Therefore, 24 OmpR targets were bound uniquely at pH 7 and 164 OmpR targets were bound uniquely at pH 4.5 (see Fig. 5.4; genes are listed in

Tables 5.3-5.4 respectively). These data quantified the earlier observation; that OmpR binding increased in acidic conditions in *S. Typhimurium*.

In CSH50 67 OmpR-bound genes were identified at pH 7 and 49 at pH 4.5 (Listed in Table S3. and S4.). 28 OmpR-targets were bound at both pH 7 and pH 4.5 (see Fig. 5.4; genes are listed in Table 5.5). Therefore, 39 OmpR targets were bound uniquely at pH 7 and 21 OmpR targets were bound uniquely at pH 4.5 (see Fig 5.4; genes are listed in Table 5.6 and 5.7 respectively). These data indicate, in contrast to the pattern observed in SL1344, the number of OmpR-bound genes decreased in acidic conditions in *E. coli*. Previously characterised OmpR targets were amongst the genes bound by OmpR in this study; this validates the experimental approach undertaken herein. These targets are noted in Tables 5.2 to 5.7.

The first observation from these ChIP-chip data sets was the difference in numbers of genes targeted by OmpR at low pH in SL1344 and CSH50; OmpR bound to more genes at pH 4.5 in SL1344. This increase in DNA binding may be due to an increase in OmpR protein levels present in SL1344 in acidic conditions (see Fig. 3.2, B), which in turn may increase OmpR-P levels thus enhancing OmpR affinity for DNA (Aiba *et al.*, 1989; Huang and Igo, 1996; Head *et al.*, 1998). Total OmpR-P levels are modulated by the phosphatase function of EnvZ which dephosphorylates OmpR and therefore determines the output of the system (Igo *et al.*, 1989; Jin and Inouye, 1993). It should be noted that the OmpR-P/OmpR ratio under these conditions is unknown. An additional model which may account for this difference comes from a recent study by Cameron and Dorman (2012) describing the influence of DNA topology on OmpR binding to DNA (Cameron and Dorman, 2012). The model proposes phosphorylation of OmpR by EnvZ and relaxation of DNA increases OmpR binding and activity at target genes (Cameron and Dorman, 2012). Based on this model it was hypothesised herein that the global levels of DNA supercoiling at low pH may differ in both species and thus account for the difference in OmpR binding between SL1344 and CSH50.

5.2.4 DNA supercoiling differs at low pH in SL1344 and CSH50

To measure DNA supercoiling in *S. Typhimurium* and *E. coli* the reporter plasmid pUC18 was transformed into SL1344 and CSH50 respectively. SL1344 pUC18 and

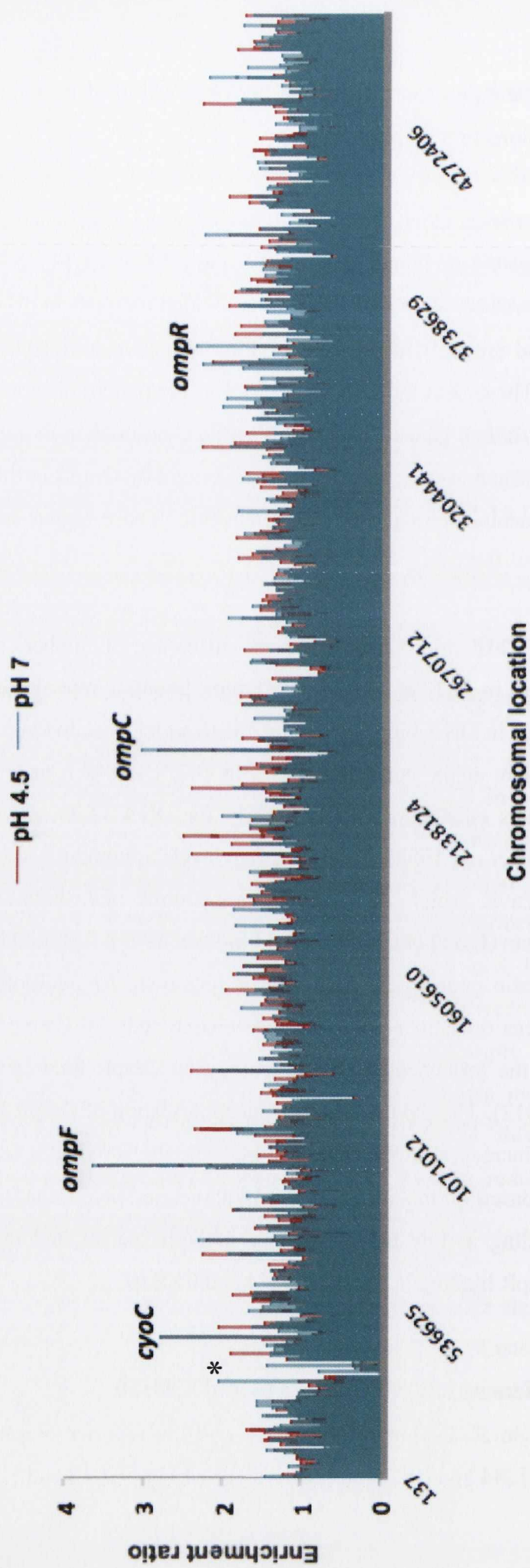


Fig. 5.2. Genome-wide distribution of OmpR in *E. coli*. Results from genome-wide analysis of OmpR binding in *E. coli* at pH 7 (blue) and pH 4.5 (red). OmpR-enrichment is shown on the y-axis and genome co-ordinates are shown on the x-axis. Genome coordinates 256375 to 371125 appeared to have no enrichment for OmpR binding after IgG normalisation (denoted by an asterisk), the reason for this is unknown.

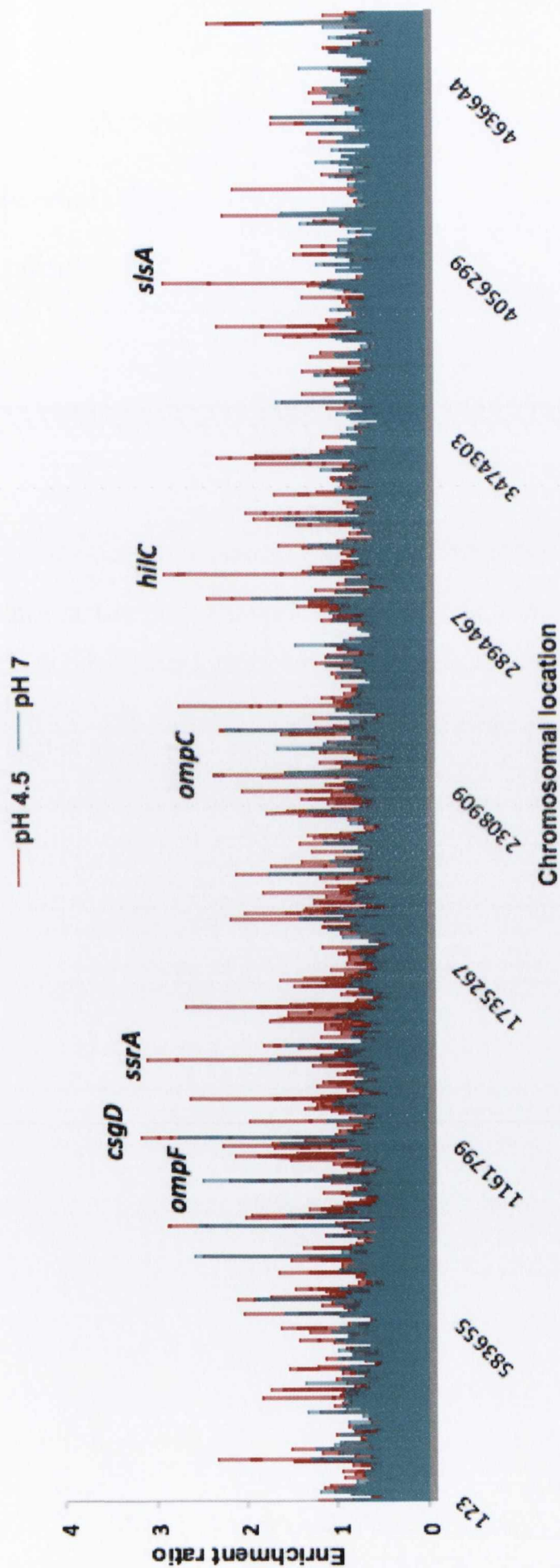


Fig. 5.3. Genome-wide distribution of OmpR in *S. Typhimurium*. Results from genome-wide analysis of OmpR binding in *S. Typhimurium* at pH 7 (blue) and pH 4.5 (red). OmpR-enrichment is shown on the y-axis and genome co-ordinates are shown on the x-axis.

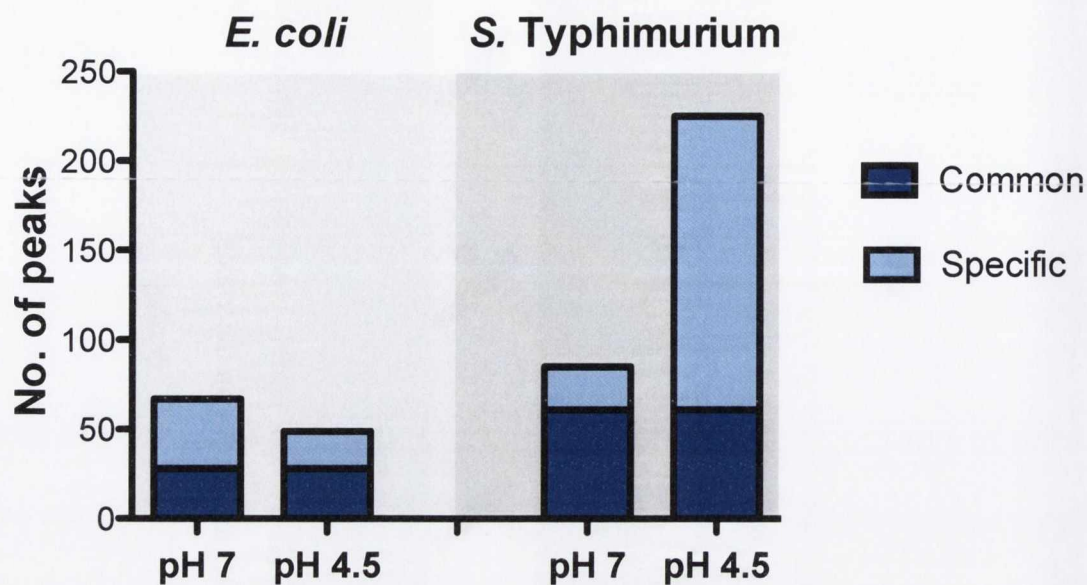


Fig. 5.4. Overlap between OmpR binding sites in *S. Typhimurium* and *E. coli*. Histogram illustrating the number of OmpR targets bound at pH 7 and pH 4.5 in *E. coli* and *S. Typhimurium*. Number of common targets, identified at both pH 7 and pH 4.5 is shown in navy. Number of specific targets at pH 7 or pH 4.5 is shown in light blue.

Table 5.2. OmpR targets at pH 7 and pH 4.5 in *S. Typhimurium*

	Gene name	Function	Peak Start	P-value
1	intergenic <i>ompF</i>	outer membrane protein F precursor	1047125	2.93492E-75
2	intergenic <i>ybiF</i>	threonine and homoserine efflux system	898250	8.81671E-68
3	intergenic <i>pgtC</i>	phosphoglycerate transport regulatory protein precursor	2509375	8.61387E-57
4	intergenic <i>cstA</i>	carbon starvation protein	660250	7.91272E-48
5	intergenic <i>tpdB</i>	tripeptide transporter permease	1484375	4.14796E-37
6	intergenic <i>mig-14</i>	transcriptional activator	2952000	1.01123E-36
7	STM2273	dehydratase	2374875	3.22856E-32
8	intergenic <i>prfC</i>	peptide chain release factor 3	4835875	3.23947E-30
9	intergenic <i>ompC</i>	outer membrane porin protein C	2364125	6.96701E-25
10	intergenic STM1994	inner membrane protein	2034500	6.4749E-24
11	<i>ygiR</i>	dehydrogenase	3412500	3.73206E-21
12	intergenic <i>fruR</i>	DNA-binding transcriptional regulator FruR	139000	1.01975E-20
13	intergenic <i>ssrA</i>	sensor kinase	1435500	3.00844E-19
14	SL1958	SL1958	2064625	6.61868E-17
15	<i>orgC</i>	hypothetical protein	3035750	1.05065E-15
16	intergenic <i>ftsI2</i>	penicillin-binding protein 3	1892625	3.22984E-15
17	STM2148	hypothetical protein	2239750	6.43407E-15
18	STM0557	inner membrane protein	614125	1.3941E-12
19	STM0859	transcriptional regulator	931625	2.71073E-11
20	intergenic <i>yhjD</i>	tRNA-processing ribonuclease	3803875	5.3099E-11
21	intergenic <i>marT</i>	transcriptional regulator	3980250	3.43877E-10
22	intergenic STM2745	inner membrane protein	2908250	8.6982E-10
23	intergenic <i>sopE2</i>	type III-secreted effector protein	1910125	9.58228E-10
24	STM0856	electron transfer protein alpha subunit	926750	1.80235E-09
25	intergenic <i>tatA</i>	twin arginine translocase protein A	4201875	4.1741E-09
26	intergenic <i>yjcC</i>	diguanylate cyclase/ phosphodiesterase	4523625	5.84383E-09
27	<i>asrA</i>	anaerobic sulfide reductase	2684500	3.05576E-08
28	intergenic <i>iroN</i> ; <i>pipB2</i>	outer membrane receptor FepA; secreted effector protein	2948875	3.13226E-08
29	<i>pyrG</i>	CTP synthase	3120750	3.55643E-08
30	intergenic <i>ycfH</i>	metallodependent hydrolase	1242000	4.69521E-08
31	<i>malG</i>	maltose transporter permease	4468500	5.44409E-08
32	<i>adrA</i>	diguanylate cyclase AdrA	437125	6.63038E-08
33	intergenic STM3117	lactoylglutathione lyase	3297125	8.67974E-08
34	STM1250	hypothetical protein	1291750	1.40207E-07
35	<i>yigF</i>	inner membrane protein	4180875	1.45078E-07
36	intergenic <i>cspA</i>	major cold shock protein	3857750	1.74203E-07
37	intergenic <i>prgH</i>	needle complex membrane protein	3040125	1.95664E-07

Table 5.2. cont.

38	STM2585	transposase-like protein	2729000	2.61048E-07
39	STM2626	replication protein 15-like	1063000	4.70898E-07
40	intergenic <i>ygjT</i>	tellurite resistance protein	3413875	9.06678E-07
41	<i>exbB</i>	biopolymer transport protein ExbB	3340875	1.50545E-06
42	intergenic <i>tatE</i>	twin arginine translocase protein E	693875	1.63279E-06
43	intergenic <i>aroP</i>	aromatic amino acid transporter	174875	1.84798E-06
44	<i>sopA</i>	secreted effector protein	2139625	2.46922E-06
45	<i>ylaC</i>	inner membrane protein	526375	3.21953E-06
46	<i>yeeI</i>	inner membrane protein	2079875	3.7995E-06
47	intergenic <i>yccT</i>	hypothetical protein	1125875	3.88843E-06
48	SL1928	SL1928	2040500	4.01849E-06
49	intergenic <i>lpxC</i>	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	157750	4.62095E-06
50	STM3052	outer membrane protein	3232125	5.79797E-06
51	<i>sseA</i>	secretion system chaperone protein	1440250	9.10E-06
52	intergenic <i>pmrD</i>	polymyxin resistance protein B	2409000	1.05239E-05
53	intergenic <i>putP</i>	major sodium/proline symporter	1170125	1.07895E-05
54	<i>ribD</i>	bifunctional diaminohydroxyphosphoribosylaminopyrimidine deaminase/5-amino-6-(5-phosphoribosylamino)uracil reductase	469500	1.38239E-05
55	intergenic <i>sseK2</i>	hypothetical protein	2229250	2.22568E-05
56	<i>ybdH</i>	hypothetical protein	664000	6.40874E-05
57	<i>idnD</i>	L-idonate 5-dehydrogenase	4749250	8.39547E-05
58	intergenic <i>atpI</i> ; <i>gidB</i>	F0F1 ATP synthase subunit I; 16S rRNA methyltransferase GidB	4102250	9.17841E-05
59	intergenic <i>yeaJ</i> ; <i>yeaH</i>	methyl-accepting chemotaxis protein; hypothetical protein	1317000	0.000157758
60	<i>sseG</i>	secreted effector protein	1445750	0.000192608
61	<i>iroN</i>	outer membrane receptor FepA	2948125	0.00069824

Table 5.3. OmpR targets at pH 7 in *S. Typhimurium*

	Gene name	Function	Peak Start	P-value
1	intergenic <i>csgD</i>	DNA-binding transcriptional regulator CsgD	1186125	1.04633E-79
2	intergenic <i>yqjA</i>	hypothetical protein	3415625	1.39541E-11
3	intergenic <i>ompR</i>	osmolarity response regulator	3681875	2.66603E-09
4	intergenic STM0509	outer membrane protein	570250	1.96631E-08
5	<i>fadL</i>	long-chain fatty acid outer membrane transporter	2500000	1.18962E-06
6	<i>thiH</i>	thiamine biosynthesis protein ThiH	4398250	1.31259E-06
7	<i>ccmA</i>	cytochrome c biogenesis protein CcmA	4039375	3.06033E-06
8	<i>mutY</i>	adenine DNA glycosylase	3290000	3.19383E-06
9	<i>yicH</i>	inner membrane protein	3964375	3.45368E-06
10	<i>hyaB2</i>	hydrogenase-1 large subunit	1568625	3.55873E-06
11	STM0291	RHS-like protein	334625	7.44819E-06
12	<i>uvrD</i>	DNA-dependent helicase II	4178375	9.98473E-06
13	<i>torD</i>	chaperone protein TorD	4041375	2.43246E-05
14	intergenic STM4506; <i>uxuR</i>	dienelactone hydrolase; DNA- binding transcriptional repressor UxuR	4781375	2.92363E-05
15	intergenic STM3796A	integral membrane protein	4020125	3.3947E-05
16	<i>sdhD</i>	succinate dehydrogenase cytochrome b556 small membrane subunit	797875	3.8455E-05
17	intergenic <i>yqgA</i> (tRNA-phe, SPI-13)	inner membrane protein	3296750	4.03753E-05
18	<i>tdcD</i>	propionate/acetate kinase	3429625	6.30107E-05
19	<i>ydeV</i>	autoinducer-2 (AI-2) kinase	4302125	7.65119E-05
20	STM3166	cation transporter	3347500	0.000143088
21	<i>cyoE</i>	protoheme IX farnesyltransferase	493000	0.000176383
22	<i>ccmF</i>	cytochrome c-type biogenesis protein	4036000	0.000224982
23	intergenic <i>hrpB</i>	ATP-dependent RNA helicase HrpB	218375	0.000351998
24	<i>bcfE</i>	fimbrial subunit	29750	0.000695714

Table 5.4. OmpR targets at pH 4.5 in *S. Typhimurium*

	Gene Name	Function	Peak Start	P-value
1	intergenic <i>csgF</i>	curli assembly protein CsgF	1185375	6.5349E-125
2	<i>yddG</i>	hypothetical protein	1610500	1.2966E-99
3	intergenic <i>acrD</i>	aminoglycoside/multidrug efflux system	2594500	5.75305E-62
4	<i>yeaJ</i>	methyl-accepting chemotaxis protein	1315125	3.09973E-53
5	<i>hyaC2</i>	Ni/Fe hydrogenase 1 b-type cytochrome subunit	1568375	1.90942E-46
6	intergenic <i>wrbA</i>	TrpR binding protein WrbA	1161875	2.65873E-42
7	intergenic <i>yfcZ</i>	hypothetical protein	2499625	4.80534E-42
8	intergenic <i>steB</i>	Secreted effector protein steB	1676125	4.60738E-38
9	intergenic <i>phoE</i>	outer membrane phosphoporin protein E	365000	1.11741E-37
10	intergenic <i>setB</i>	proton efflux pump	2306625	2.38844E-37
11	intergenic STM0509	outer membrane protein	570000	1.25656E-36
12	intergenic STM1109, STM1110	hypothetical protein, hypothetical protein	1153875	3.87327E-35
13	intergenic <i>dppA</i> , STM3631	dipeptide transport protein, xanthine permease	3837375	1.68775E-28
14	intergenic STM4054	periplasmic dicarboxylate-binding protein	4287375	1.0795E-27
15	<i>glnS</i>	glutaminyI-tRNA synthetase	748125	9.47786E-27
16	intergenic <i>stdA</i>	fimbrial-like protein	3213250	9.31117E-25
17	intergenic <i>ygjU</i>	serine/threonine transporter SstT	3415250	3.45454E-24
18	intergenic <i>nupG</i>	nucleoside transport	3291750	4.52519E-23
19	intergenic STM1575	transcriptional regulator	1615375	2.87822E-22
20	STM1089	inner membrane protein	1133250	1.90436E-21
21	intergenic <i>stcA</i>	fimbrial-like protein	2245125	5.08556E-21
22	intergenic <i>sfcA</i>	malate dehydrogenase	1604250	1.58002E-20
23	<i>galS</i>	DNA-binding transcriptional regulator GalS	2285625	1.64678E-20
24	intergenic <i>ybaJ</i>	hypothetical protein	528125	1.92175E-20
25	intergenic <i>napF</i>	ferredoxin-type protein	2357125	4.68879E-20
26	STM0295	hypothetical protein	339500	1.15007E-19
27	<i>znuA</i>	high-affinity zinc transporter periplasmic protein	1944500	6.94728E-19
28	intergenic <i>yccA</i> , <i>pipA</i>	hypothetical protein, pathogenicity island-encoded protein A	1131375	3.50136E-18
29	STM1650	hypothetical protein	1698500	3.53061E-18
30	intergenic STM0854	hypothetical protein	925250	1.57952E-17
31	<i>leuO</i>	leucine transcriptional activator	135250	3.28462E-17
32	intergenic <i>flhD</i>	transcriptional activator FlhD	1979625	5.95008E-17
33	intergenic <i>ydeI</i> ; STnc560	hypothetical protein; sRNA	1550500	6.78349E-17

Table 5.4. cont.

34	STM1877	amidohydrolase	1927500	7.70465E-17
35	<i>rfbP</i>	undecaprenol-phosphate galactosephosphotransferase/O- antigen transferase	2159375	1.56731E-16
36	intergenic <i>yhjK</i>	phosphodiesterase	3812875	3.10806E-16
37	intergenic <i>lrhA</i>	NADH dehydrogenase transcriptional repressor	2438750	6.26732E-16
38	STM3845	inner membrane protein	4073125	2.39151E-15
39	STM0761	fumarate hydratase	824375	9.24906E-15
40	<i>ycgN</i>	hypothetical protein	1867000	1.20512E-14
41	intergenic <i>rmbA</i>	hypothetical protein	3975125	1.80616E-14
42	intergenic <i>aas</i> , (<i>omrA</i> <i>omrB</i> sRNA)	bifunctional acyl-[acyl carrier protein] synthetase/2- acylglycerophosphoethanolamine acyltransferase	3191625	4.01493E-14
43	<i>pagO</i>	integral membrane protein	1917500	4.15717E-14
44	intergenic STM2406	oxidoreductase	2520750	5.6975E-14
45	intergenic <i>malP</i>	maltodextrin phosphorylase	3697875	5.98832E-14
46	intergenic <i>ybgS</i>	hypothetical protein	822750	1.05598E-13
47	STM1839	hypothetical protein	1893750	1.2232E-13
48	intergenic STM0858	dehydrogenase	929375	1.37025E-13
49	intergenic <i>ydhl</i>	inner membrane protein	1476375	1.43187E-13
50	STM1635	ABC-type polar amino acid transport system ATPase component	1681500	2.00602E-13
51	STM1554	coiled-coil protein	1587875	4.46491E-13
52	intergenic <i>leuL</i>	leu operon leader peptide	133875	9.88856E-13
53	intergenic STM2503	diguanylate cyclase	2618625	1.25116E-12
54	intergenic <i>bacA</i>	undecaprenyl pyrophosphate phosphatase	3392125	1.35624E-12
55	<i>ybdO</i>	transcriptional regulator	668125	8.36036E-12
56	intergenic <i>ydhO</i>	cell wall-associated hydrolase	1467625	1.30998E-11
57	<i>ybjM</i>	inner membrane protein	943625	1.5462E-11
58	<i>pqaA</i>	PhoPQ-regulated protein	1577875	1.61868E-11
59	intergenic <i>pykF</i>	pyruvate kinase	1416625	1.89797E-11
60	intergenic <i>yodD</i>	hypothetical protein	2026125	2.17804E-11
61	intergenic <i>yeaS</i>	leucine export protein LeuE	1307250	3.61552E-11
62	intergenic STM1861	STM1861	1915625	3.73874E-11
63	intergenic <i>hyaA2</i>	hydrogenase-1 small subunit	1571375	4.88836E-11
64	STM1130	N-acetylneuraminic acid mutarotase	1176875	5.97674E-11
65	<i>lysP</i>	lysine transporter	2296625	9.26318E-11
66	<i>rfbV</i>	abequosyltransferase	2165750	1.05248E-10
67	intergenic <i>nifJ</i> (sRNA MicC)	pyruvate-flavodoxin oxidoreductase	1702375	1.25176E-10
68	<i>ssaV</i>	secretion system apparatus protein SsaV	1451750	1.73114E-10

Table 5.4. cont.

69	intergenic STM1267	hypothetical protein	1306125	2.46036E-10
70	<i>ssrB</i>	transcriptional activator	1432875	2.85466E-10
71	intergenic <i>rumB</i>	23S rRNA methyluridine methyltransferase	953250	3.13375E-10
72	<i>rfaZ</i>	lipopolysaccharide core biosynthesis protein	3932250	4.56588E-10
73	<i>gst</i>	glutathionine S-transferase	1482750	7.47251E-10
74	intergenic <i>ompA</i>	outer membrane protein A	1118375	9.11463E-10
75	intergenic STM1287	arylsulfatase regulator	1321875	2.12422E-09
76	<i>orfX</i>	hypothetical protein	1136625	3.01329E-09
77	intergenic <i>sirA</i>	response regulator	1996500	3.01947E-09
78	intergenic <i>mipA</i>	MltA-interacting protein A	1321000	4.12265E-09
79	intergenic <i>ydiM</i> , <i>ydiL</i>	transporter, hypothetical protein	1401125	5.80852E-09
80	intergenic <i>fes</i>	enterobactin/ferric enterobactin esterase	643375	7.1006E-09
81	<i>ssrA</i>	sensor kinase	1434250	7.21845E-09
82	STM1653	membrane transporter of cations	1703375	8.7374E-09
83	STM0721	glycosyl transferase family protein	785625	9.19757E-09
84	<i>aroL</i>	shikimate kinase	440125	9.95302E-09
85	intergenic <i>yaiB</i>	hypothetical protein	436375	1.10493E-08
86	intergenic <i>ybgH</i> , <i>ybgI</i>	POT family transport protein, hydrolase-oxidase	777250	1.21756E-08
87	intergenic <i>pipB2</i>	secreted effector protein	2950125	1.41903E-08
88	<i>phsA</i>	thiosulfate reductase precursor	2138375	1.49249E-08
89	STM1637	inner membrane protein	1684250	1.53184E-08
90	<i>prgI</i>	needle complex major subunit	3038750	2.36251E-08
91	<i>rna</i>	ribonuclease I	679875	2.46509E-08
92	intergenic <i>cspB</i>	cold-shock protein	2036875	2.82985E-08
93	intergenic <i>cspH</i>	cold shock-like protein	1287500	2.91214E-08
94	intergenic <i>yohG</i>	multidrug resistance outer membrane protein MdtQ,	2268125	3.32957E-08
95	<i>rfe</i>	O-antigen polymerase	1367250	3.73774E-08
96	STM1910	penicillin-binding protein	1962000	3.80305E-08
97	intergenic <i>ycgL</i>	hypothetical protein	1868625	3.92751E-08
98	STM0723	ABC-type polysaccharide/polyol phosphate transport system ATPase component	787875	6.31711E-08
99	<i>pduF</i>	propanediol diffusion facilitator	2113125	6.32272E-08
100	intergenic <i>ydbH</i> , <i>ldhA</i>	hypothetical protein, D-lactate dehydrogenase	1696750	1.11855E-07
101	intergenic <i>gtgE</i>	hypothetical protein	1099500	1.23039E-07
102	<i>rfbM</i>	mannose-1-phosphate guanylyltransferase	2161750	1.44352E-07
103	<i>ydfJ</i>	STM1507	1541500	2.00924E-07
104	intergenic <i>ybiT</i>	ABC transporter ATPase	905750	2.01578E-07

Table 5.4. cont.

105	<i>siiC, siiB</i>	type-I secretion protein, methyl-accepting chemotaxis protein	4501875	2.10374E-07
106	<i>rfbK</i>	phosphomannomutase	2160875	2.75274E-07
107	STM4413	metallo-dependent hydrolase	4674625	3.35705E-07
108	intergenic <i>ychE</i>	hypothetical protein	1798625	4.05905E-07
109	intergenic <i>ftnB</i>	ferritin-like protein	1984625	4.12004E-07
110	<i>yfeK</i>	hypothetical protein	2548625	5.0346E-07
111	STM2762	inner membrane protein	2926125	5.54127E-07
112	STM2761	inner membrane protein	2925375	6.6367E-07
113	<i>stcC</i>	outer membrane protein	2242625	7.76331E-07
114	STM1548	S-adenosylmethionine/tRNA-ribosyltransferase-isomerase	1581875	7.9014E-07
115	<i>perM</i>	permease	2605500	8.33669E-07
116	<i>prgJ</i>	needle complex minor subunit	3038375	1.09211E-06
117	STM0765	cation transporter	827625	1.13784E-06
118	intergenic STM3116	cation transporter	3347250	1.3492E-06
119	<i>yciE</i>	hypothetical protein	1783500	1.47186E-06
120	<i>smf</i> , SL2696	SL2696	2878000	1.54641E-06
121	<i>ydiQ</i>	electron transfer flavoprotein YdiQ	1392000	2.04401E-06
122	<i>rfbN</i>	rhamnosyl transferase	2163250	2.2109E-06
123	<i>gogB</i>	hypothetical protein	2727375	2.21209E-06
124	<i>ybaL</i>	cation:proton antiport protein	551375	2.26998E-06
125	STM0726	glycosyl transferase family protein	791875	2.59376E-06
126	intergenic <i>ybiS, ybiT</i>	hypothetical protein, ABC transporter ATPase	904375	2.68768E-06
127	intergenic <i>tdcA</i>	DNA-binding transcriptional activator TdcA	3434375	2.95849E-06
128	intergenic <i>rhuM</i>	hypothetical protein	3974375	3.09422E-06
129	STM1543	transporter	1574875	4.08097E-06
130	<i>rspA</i>	dehydratase	1539000	4.43692E-06
131	<i>celB</i>	PTS system N,N'-diacetylchitobiose-specific transporter subunit IIC	1349625	4.45869E-06
132	<i>yajI</i>	hypothetical protein	468125	4.72326E-06
133	intergenic <i>mazG</i>	nucleoside triphosphate pyrophosphohydrolase	3122750	4.86472E-06
134	intergenic <i>yqeF</i>	acetyl-CoA acetyltransferase	1366625	7.89529E-06
135	STM1003	transcriptional regulator	1053000	1.14888E-05
136	<i>oafA</i>	O-antigen acetylase	2331250	1.25035E-05
137	intergenic <i>slyB</i>	outer membrane lipoprotein	1477625	1.29259E-05
138	<i>pagK</i>	PagK	1920750	1.49677E-05
139	intergenic STM1530	outer membrane protein	1564375	1.93265E-05
140	<i>siiE</i>	adhesin	4506125	2.18174E-05
141	intergenic <i>bcfA</i>	fimbrial subunit	25000	2.28236E-05

Table 5.4. cont.

142	<i>pphB</i>	serine/threonine-specific protein phosphatase 2	3071625	2.43564E-05
143	<i>proA</i>	γ -glutamyl phosphate reductase	367375	2.45733E-05
144	STM1128	sodium/glucose cotransporter	1173500	2.60369E-05
145	intergenic STM1131, STM1132	outer membrane protein, sialic acid transporter	1177625	3.50999E-05
146	<i>rfbG</i>	CDP glucose 4,6-dehydratase	2170250	3.9742E-05
147	STM1864	inner membrane protein	1918750	4.76943E-05
148	STM1123	hypothetical protein	1163375	5.53014E-05
149	<i>cysC</i>	adenylylsulfate kinase	3094875	6.09943E-05
150	<i>siiC</i>	putative type-I secretion protein	4501250	6.83167E-05
151	<i>siiA, siiB</i>	hypothetical protein, methyl-accepting chemotaxis protein	4499625	7.77676E-05
152	intergenic <i>mlc</i>	pts operon transcriptional repressor	1523125	0.000116947
153	<i>yeiE</i>	DNA-binding transcriptional regulator	2298250	0.000122028
154	<i>ybjP</i>	lipoprotein	961375	0.000122508
155	intergenic <i>srcA</i>	hypothetical protein	2230250	0.000159908
156	<i>sopD2</i>	secreted effector protein	1011000	0.00016784
157	intergenic <i>ampH, sbmA</i>	beta-lactam binding protein AmpH, transporter	429750	0.000204337
158	<i>envF</i>	envelope lipoprotein	1285500	0.000205437
159	<i>ybiJ</i>	hypothetical protein	889500	0.000241892
160	intergenic SL2116	SL2116	2231500	0.000344296
161	intergenic <i>mgtC</i>	Mg ²⁺ transport protein	3986625	0.000349836
162	intergenic <i>nuoA</i> , STM2329	NADH dehydrogenase subunit A, hypothetical protein	2437500	0.000402533
163	<i>sprB</i>	transcriptional regulator	3033875	0.000489051
164	intergenic <i>pntA</i>	NAD(P) transhydrogenase subunit alpha	1512500	0.000549416

Table 5.5. OmpR common targets at pH 7 and pH 4.5 in *E. coli*

	Gene name	Function	Peak Start	P-value
1	intergenic <i>ompF</i>	outer membrane porin 1a	985750	5.13321E-55
2	intergenic <i>ompC</i>	outer membrane porin protein C	2310500	1.94082E-39
3	intergenic <i>ompR</i>	DNA-binding response regulator in two-component regulatory system with EnvZ	3534375	9.27061E-30
4	intergenic <i>gltA</i>	citrate synthase	753750	1.40421E-18
5	<i>degQ</i>	serine endoprotease, periplasmic	3378875	1.53815E-17
6	intergenic <i>csgD</i>	DNA-binding transcriptional activator for <i>csgBA</i>	1102250	2.09856E-12
7	<i>ygeQ</i>	pseudo	2996500	1.59493E-11
8	<i>yhaC</i>	predicted protein	3266750	5.35476E-11
9	intergenic <i>ugpB</i>	glycerol-3-phosphate transporter subunit	3590250	6.77393E-11
10	intergenic <i>aer</i>	used signal transducer for aerotaxis sensory component/methyl accepting chemotaxis component	3217250	1.03826E-10
11	<i>yehA</i>	predicted fimbrial-like adhesin protein	2185750	3.06349E-10
12	intergenic (<i>pawZ</i>)	tRNA	2474375	1.13195E-08
13	<i>yibG</i>	conserved protein	3766500	2.22663E-08
14	<i>rhsA</i>	<i>rhsA</i> element core protein RshA	3764125	2.40051E-08
15	intergenic <i>aspA</i>	aspartate ammonia-lyase	4366500	2.82873E-08
16	<i>yqfA</i>	inner membrane protein, hemolysin III family HyIIII	3041000	3.09373E-08
17	<i>rnlA</i>	CP4-57 prophage; RNase LS	2763875	3.2448E-08
18	intergenic <i>chiP</i>	chitoporphin, uptake of chitosugars	707500	8.81618E-08
19	intergenic <i>yfgF</i>	cyclic-di-GMP phosphodiesterase, anaerobic	2626500	1.35156E-07
20	<i>yfdV</i>	predicted transporter	2487875	2.17302E-07
21	<i>yhiJ</i>	predicted protein	3629375	6.15016E-07
22	intergenic <i>rhtA</i>	threonine and homoserine efflux system	849375	6.28417E-06
23	<i>dapE</i> , <i>yffB</i>	predicted reductase, function unknown, ArsC family; low abundance protein	2589375	1.50444E-05
24	intergenic <i>yjiP</i>	inner membrane protein, H-NS- repressed, DUF1212 family	4601250	1.95948E-05
25	<i>cadB</i>	predicted lysine/cadaverine transporter	4357750	2.02733E-05
26	<i>yiaG</i>	predicted transcriptional regulator, HTH_CROC1 family	3717750	8.05065E-05
27	<i>yhdU</i>	predicted membrane protein	3410750	0.0001192
28	<i>yddJ</i>	predicted protein	1542500	0.000130803

Table 5.6. OmpR targets unique at pH 7 in *E. coli*

	Gene name	Function	Peak Start	P-value
1	<i>ytfJ</i>	predicted transcriptional regulator	4437000	1.69415E-36
2	<i>yihO</i>	predicted transporter	4062750	1.57392E-11
3	intergenic (<i>yibB</i>)	conserved protein, pfam09612 family	3791000	4.0519E-11
4	intergenic <i>mreB</i>	cell wall structural complex MreBCD, actin-like component MreB	3399125	1.93868E-10
5	<i>yjiT, yjiV</i>	pseudo	4571625	7.3348E-10
6	<i>ghrB</i>	glyoxylate/hydroxypyruvate reductase B	3715500	1.70435E-09
7	intergenic <i>exuR; yqjA</i>	DNA-binding transcriptional repressor, required, with yghB, for membrane integrity; inner membrane protein	3245500	1.9722E-09
8	<i>tmcA</i>	elongator methionine tRNA (ac4C34) acetyltransferase	2591750	2.09383E-09
9	intergenic <i>nudF</i>	ADP-ribose pyrophosphatase	3175875	9.75982E-09
10	<i>yhjB</i>	predicted two-component system response regulator, LuxR-like HTH domain, function unknown	3669625	2.0915E-08
11	intergenic (<i>ytfM</i>)	predicted outer membrane protein and surface antigen	4440125	5.50222E-08
12	intergenic <i>yjhl</i>	KpLE2 phage-like element; predicted DNA-binding transcriptional regulator	4523875	6.58562E-08
13	<i>yhcD</i>	predicted outer membrane subunit usher protein	3363250	8.07956E-08
14	<i>yjgH</i>	conserved protein, UPF0131 family	4470875	8.38055E-08
15	intergenic (<i>ycdF</i>)	conserved SAM-binding protein, DUF218 superfamily	1486125	5.82931E-07
16	<i>cld</i>	regulator of length of O-antigen component of lipopolysaccharide chains	2096125	6.78437E-07
17	<i>malE</i>	maltose transporter subunit	4244375	7.7989E-07
18	<i>mqsR</i>	GCU-specific mRNA interferase toxin of the MqsR-MqsA toxin-antitoxin system and biofilm/motility regulator	3166375	1.0087E-06
19	intergenic (<i>fimA</i>)	major type 1 subunit fimbrin (pilin)	4541000	1.52433E-06
20	<i>cadC</i>	DNA-binding transcriptional activator	4359125	1.84622E-06
21	<i>yicG</i>	inner membrane protein, UPF0126 family	3817125	1.89109E-06

Table 5.6. cont.

22	intergenic (<i>elfA</i>)	predicted fimbrial-like adhesin	997125	2.73502E-06
23	<i>gspD</i>	general secretory pathway component, cryptic	3454375	6.45415E-06
24	<i>arpB</i>	pseudo	1802875	6.6948E-06
25	<i>dgsA</i>	Global DNA-binding transcriptional repressor; autorepressor; required for anaerobic growth on glucosamine	1665750	7.28749E-06
26	intergenic (<i>dut</i>)	deoxyuridinetriphosphatase	3812500	9.24564E-06
27	<i>yafT</i>	lipoprotein	237625	1.00953E-05
28	<i>yjgX</i>	pseudo	4497750	1.18212E-05
29	<i>yeeJ</i>	probable adhesin	2047750	1.85241E-05
30	<i>murB</i>	UDP-N-acetylenolpyruvoylglucosamine reductase, FAD-binding	4170625	3.10579E-05
31	<i>yiaW</i>	inner membrane protein, DUF3302 family	3752375	3.57016E-05
32	<i>lsrK</i>	autoinducer-2 (AI-2) kinase	1597000	3.96963E-05
33	intergenic <i>ydjL</i>	predicted oxidoreductase, Zn-dependent and NAD(P)-binding	1859375	4.33571E-05
34	<i>hofO</i>	protein required for the utilization of DNA as a carbon source	3519250	4.74922E-05
35	<i>sstT</i>	sodium:serine/threonine symporter	3237875	8.82398E-05
36	<i>gcvB</i>	ncRNA	2941000	9.01607E-05
37	intergenic <i>acs</i>	acetyl-CoA synthetase	4285500	0.000418638
38	<i>arnB</i>	uridine 5'-(beta-1-threo-pentapyranosyl-4-ulose diphosphate) aminotransferase, PLP-dependent	2364750	0.000462656
39	intergenic <i>tsgA</i>	predicted transporter	3490625	0.00077745

Table 5.7. OmpR targets unique at pH 4.5 in *E. coli*

	Gene name	Function	Peak Start	P-value
1	intergenic <i>ytfJ</i>	predicted transcriptional regulator	4437000	1.3918E-18
2	<i>rseX</i>	ncRNA	2031625	2.41733E-16
3	intergenic <i>flhD</i>	DNA-binding transcriptional dual regulator with FlhC	1976000	9.35834E-13
4	<i>yibB</i>	conserved protein, pfam09612 family	3791000	3.36328E-12
5	intergenic <i>yjhL</i>	KpLE2 phage-like element; predicted phosphotransferase enzyme IIA component	4523750	2.53956E-11
6	intergenic <i>exuR</i>	DNA-binding transcriptional repressor	3245500	7.69054E-09
7	<i>uspC</i>	Universal stress protein C	1977625	1.70664E-08
8	<i>emrK</i>	EmrKY-TolC multidrug resistance efflux pump, membrane fusion protein component	2480625	2.56487E-08
9	<i>yqiH</i>	predicted periplasmic pilin chaperone (hypothetical protein)	3188375	2.79178E-07
10	<i>gtrS</i>	serotype-specific glucosyl transferase, CPS-53 (KpLE1) prophage	2467500	6.60377E-07
11	intergenic <i>csrD</i>	targeting factor for csrBC sRNA degradation	3399250	2.68673E-06
12	<i>ypjC</i>	pseudo	2781750	5.79742E-06
13	<i>emrY</i>	predicted multidrug efflux system	2479875	2.68722E-05
14	intergenic <i>yfcV</i>	predicted fimbrial-like adhesin protein	2454125	6.41049E-05
15	intergenic <i>lrhA alaA</i>	DNA-binding transcriptional repressor of flagellar, motility and chemotaxis genes,	2405125	6.7673E-05
16	<i>agaB</i>	N-acetylgalactosamine-specific enzyme IIB component of PTS	3282250	7.13185E-05
17	<i>yhiD</i>	predicted Mg ²⁺ transport ATPase, inner membrane protein	3653500	9.70061E-05
18	<i>yhiL</i>	pseudo	3632250	0.000128702
19	intergenic <i>queE</i>	7-carboxy-7-deazaguanine synthase; queosine biosynthesis	2903625	0.000157597
20	<i>yjbI</i>	pseudo	4249125	0.000162767
21	intergenic <i>ygeH</i>	predicted transcriptional regulator	2989750	0.000186502

CSH50 pUC18 were cultured as described in section 2.2.6 in the presence carbenicillin to select for the plasmid pUC18. After 90 min in EG-minimal media at pH 7 and pH 4.5 samples were prepared and plasmid extracted as described in section 2.5.1. DNA topoisomer distributions of extracted plasmids were analysed on TBE agarose gels containing 2.5 µg/ml of chloroquine (see section 2.6.5); at this concentration of the intercalator, more supercoiled molecules migrate faster in the gels. The results in Fig 5.5 A + B show a representative gel image and the corresponding densitometry plot from SL1344 and CSH50 respectively. In SL1344, DNA supercoiling levels were unchanged at pH 7 and pH 4.5 and this was true for plasmids isolated at a range of different pH levels (pH 8.0 to 4.0, see Fig. 5.5, C). In contrast, the DNA in CSH50 became more supercoiled at pH 4.5.

A previous study by Karem and Foster measured the effect of pH on DNA supercoiling in *E. coli* and *S. Typhimurium*; the authors found DNA in *E. coli* became more relaxed in LB broth at pH 6.3 compared to pH 7.5 (Karem and Foster, 1993). Additionally, the authors report that DNA was also more relaxed in *S. Typhimurium* (strain LT-2) using non-citrate mannose minimal media at pH 5 compared to pH 8 (Karem and Foster, 1993). The data in the present study is not directly comparable to that obtained by Karem and Foster due to differences with the culture conditions in their study (Karem and Foster, 1993). Nonetheless, to investigate if the difference in results were due to strain background the plasmid pUC18 was transformed into *S. Typhimurium* strain LT-2. Supercoiling analysis was then performed in EG-minimal media as described in section 2.6.5. Consistent with results obtained in SL1344 pUC18 supercoiling levels remained unchanged at pH 7 and pH 4.5 (Fig. 5.5, D). Under the conditions used in this study DNA supercoiling remained static in *S. Typhimurium*. Other aspects of culture conditions such as culture medium, levels of aeration and growth phase may account for the differences in DNA supercoiling results in *S. Typhimurium* obtained in this study compared to that found by Karem and Foster (1993).

This study found DNA supercoiling levels differed between SL1344 and CSH50 when exposed to identical culture conditions; DNA became more supercoiled in CSH50 in acidic conditions. This may have a negative effect on global levels of OmpR binding as DNA relaxation has been shown to enhance OmpR binding (Cameron and Dorman, 2012). This is an attractive hypothesis to account for the decrease in number of genes

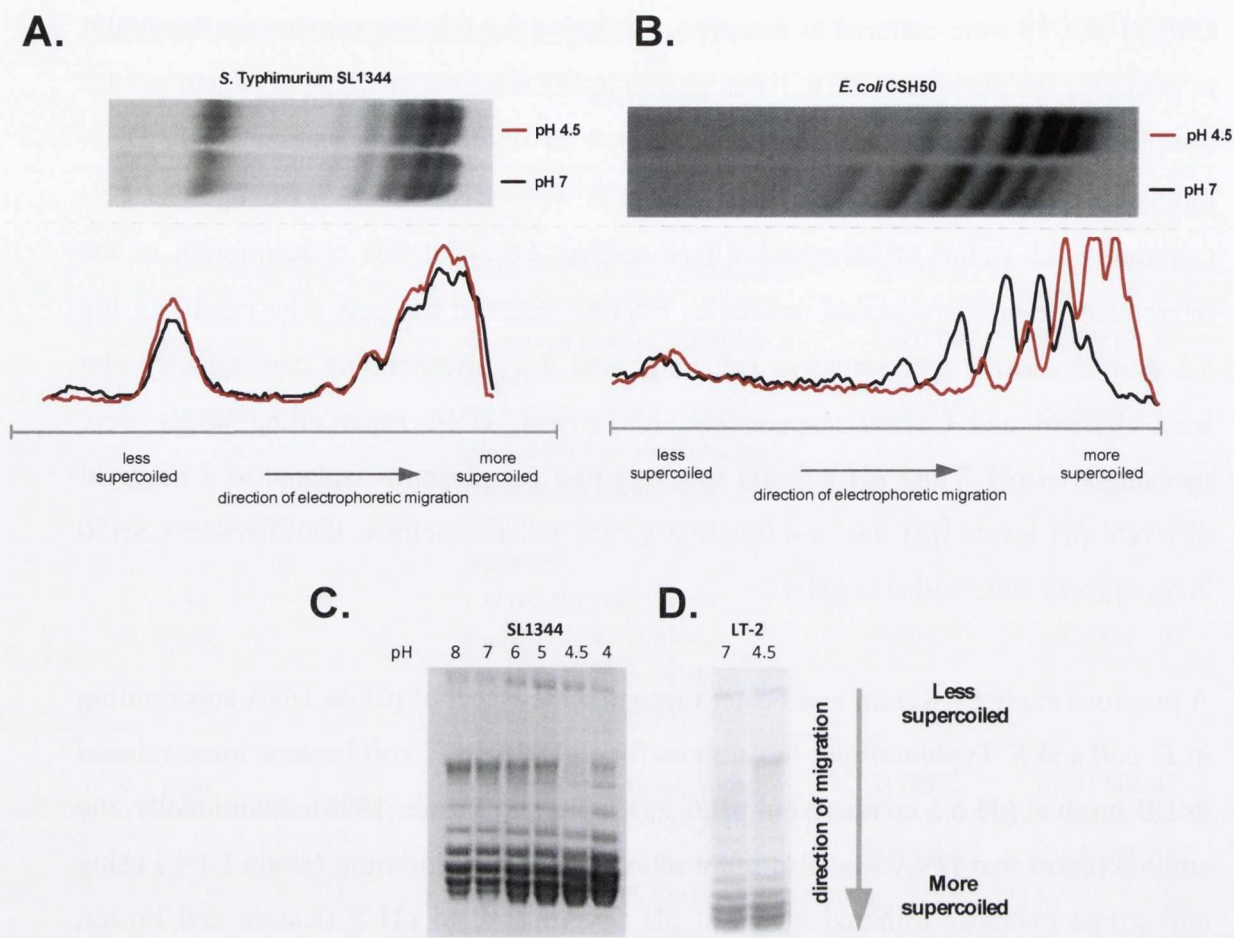


Fig. 5.5. The effect of pH on DNA supercoiling in *S. Typhimurium* and *E. coli*.

DNA supercoiling was monitored using the plasmid pUC18 in *S. Typhimurium* (SL1344) (**A**) and *E. coli* (CSH50) (**B**), plasmids were harvested after 90 min in EG-minimal media at pH 7 and pH 4.5 and analysed on a 1% TBE agarose gel containing 2.5 $\mu\text{g/ml}$ chloroquine. Representative gel images and the corresponding densitometry plots are shown. Black trace denotes pH 7 and red trace denotes pH 4.5.

C. Plasmids were harvested from *S. Typhimurium* (SL1344) after 90 min in EG-minimal media at a range of pH values (pH 8.0 to 4.0). Samples were analysed on a 1% TBE agarose gel containing 2.5 $\mu\text{g/ml}$ chloroquine. At this concentration more supercoiled plasmids run furthest down the gel.

D. Plasmids were harvested from *S. Typhimurium* (LT-2) after 90 min in EG-minimal media at pH 7 and at pH 4.5, and analysed on a 1% TBE agarose gel containing 2.5 $\mu\text{g/ml}$ chloroquine. At this concentration, more supercoiled plasmids run furthest down the gel.

bound by OmpR at pH 4.5 in CSH50. However, DNA supercoiling levels in SL1344 remained relatively static and unchanged in acidic pH and the number of OmpR targets increased at pH 4.5. This may be indicative of local relaxation of DNA at target genes, which is not apparent with global analysis using chloroquine gels. Therefore, local DNA relaxation coupled with an increase in OmpR protein levels may account for the global differences in OmpR binding between these organisms. The ratio of OmpR and OmpR-P must also be taken into account. Although not measured in this study, the ratio of OmpR/OmpR-P levels may change between pH 7 and pH 4.5 and may result in differences in OmpR binding in both species.

In addition to the clear difference in the number of OmpR targets between *S. Typhimurium* and *E. coli*, the ChIP-chip data sets revealed OmpR bound to unique targets in both species and there was limited overlap between OmpR binding in both species. This is discussed in the following sections.

5.2.5 OmpR targets *Salmonella* pathogenicity island 1 (SPI-1)

OmpR in *S. Typhimurium* has previously been shown to regulate genes within the horizontally acquired pathogenicity islands SPI-1 and SPI-2 (Lee *et al.*, 2000; Osborne and Coombes, 2011; Cameron and Dorman, 2012). SPI-1 and SPI-2 encode structural, effector and regulatory genes required to create T3SSs that enable invasion of epithelial cells and survival within host macrophages respectively (reviewed by Haraga *et al.*, 2008). Each island is equipped with genes encoding regulatory proteins that regulate expression of the SPI genes, however, expression is also governed by regulatory proteins encoded in the ancestral *S. Typhimurium* genome (Bajaj *et al.*, 1996; Lee *et al.*, 2000; Kelly *et al.*, 2004; Lim *et al.*, 2006; Mangan *et al.*, 2006; Navarre *et al.*, 2006). OmpR has been shown to regulate expression of *hilC* and *hilD*, which encode regulatory proteins HilC and HilD of SPI-1. (Lucas and Lee, 2001; Osborne and Coombes, 2011; Cameron and Dorman, 2012). HilC and HilD act together to activate expression of the *hilA* gene, which encodes the HilA protein that plays a central role in SPI-1 regulation by activating the *inv/spa* and *prg/org* operons encoded in SPI-1. HilA directly activates *invF* that encodes the InvF protein which activates expression of effector proteins encoded inside and outside the SPI-1 island (Bajaj *et al.*, 1995; Lostroh and Lee, 2001).

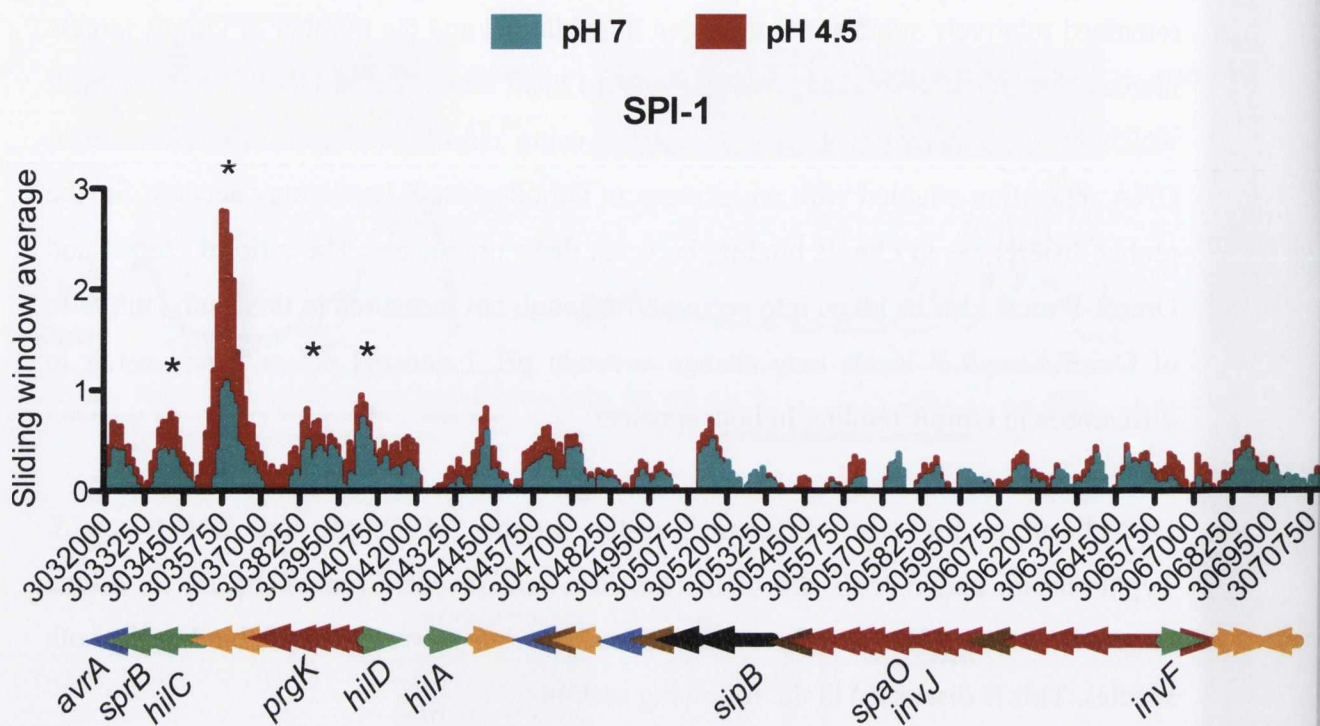


Fig. 5.6. Distribution of OmpR binding across *Salmonella* pathogenicity island 1 (SPI-1) at pH 7 and pH 4.5. OmpR enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005). Peaks identified as significant are denoted with an asterisk. Genome co-ordinates are shown on the x-axis. Below the x-axis are coloured arrows representing the genes within SPI-1, the location of the genes was determined using Jbrowse (Kroger *et al.*, 2012). Effector genes are purple, regulatory genes are green, genes of unknown function are orange, structural genes of the type 3 secretion apparatus are red, chaperone genes are brown and translocon genes are black (as adapted from Dillon *et al.*, 2010).

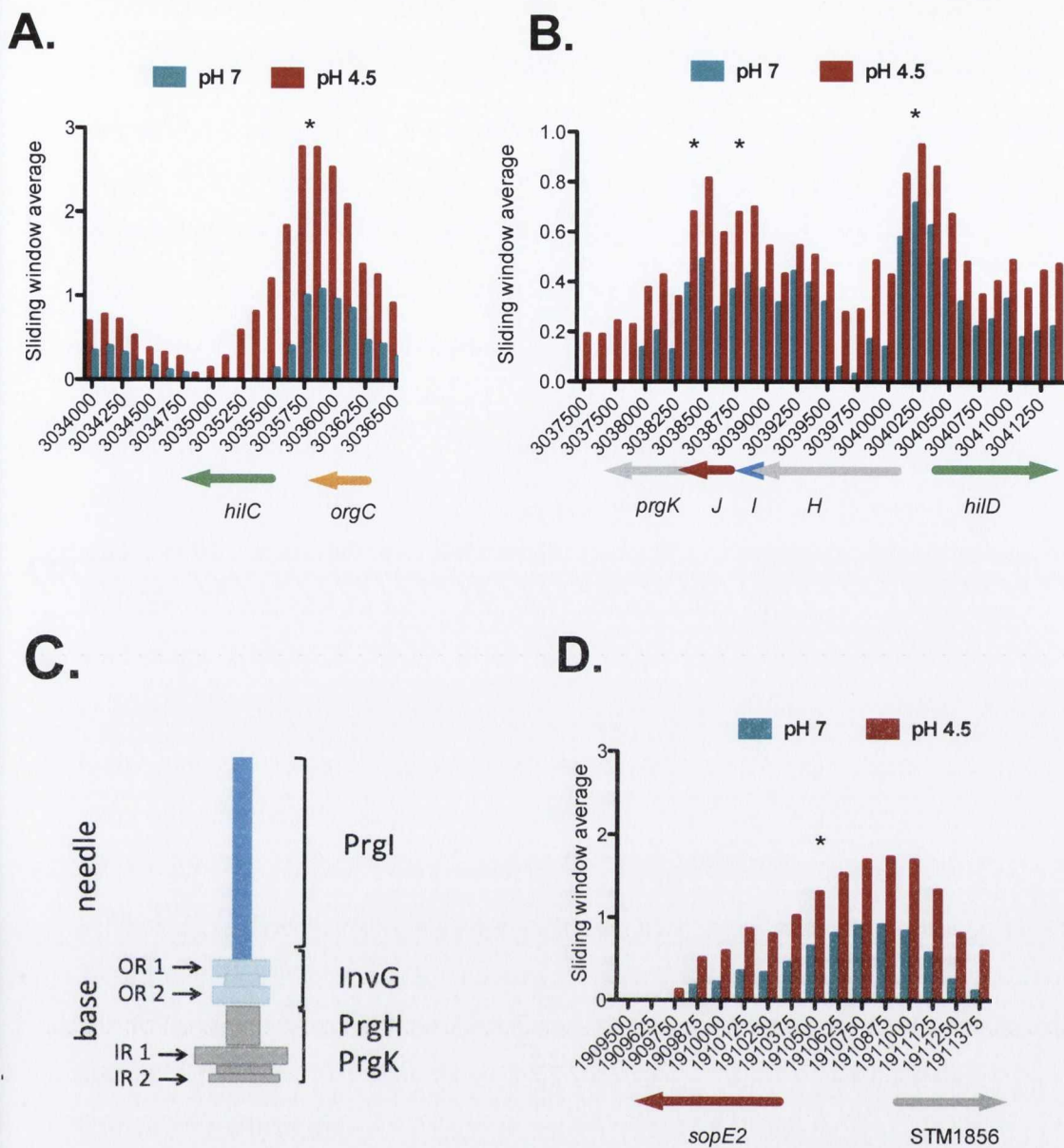


Fig. 5.7. OmpR enrichment at genes within, and regulated, by *Salmonella* pathogenicity island 1. **A.** OmpR binding at the *hilC*-*orgC* intergenic region. **B.** OmpR binding at the *prg* operon. **D.** OmpR binding at the *sopE2*-STM1856 intergenic region. OmpR enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005), significant peaks are denoted by an asterisk. Genome co-ordinates are on the x-axis and coloured arrows denotes the location of genes as determined using Jbrowse (Kroger *et al.*, 2012). **C.** A diagram depicting the structure of the SPI-1 type 3 secretion system. The needle complex is composed of the membrane embedded base and the needle filament. The base is composed of the inner-membrane-associated rings (IR 1 and IR 2) and the outer-membrane-associated rings (OR 1 and OR 2). The extracellular needle filament is docked to the base. The protein composition is shown on the right (as adapted from Marlovits *et al.*, 2004).

Peaks of OmpR enrichment were identified across the SPI-1 locus (Fig. 5.6). OmpR was most significantly enriched upstream of *hilC* at both pH 7 and pH 4.5; OmpR occupancy was clearly higher in acidic conditions (Fig. 5.7, A). These results identify that OmpR binds the *hilC* promoter *in vivo* and confirms recently published work (Cameron and Dorman, 2012). However, the results in Fig. 5.7 (A) showed OmpR preferentially binds to this region at low pH. OmpR was also enriched in the *prgH-hilD* intergenic region and within the *prgHIJK* operon (Fig. 5.7, B). The *prgHIJK* operon encodes four proteins (PrgH, PrgI, PrgJ and PrgK) that, along with the InvG protein, form the structural components of the SPI-1 needle complex (Kubori *et al.*, 1998). This structure is composed of a membrane-spanning base and a filamentous needle, which is used to secrete effector proteins from the bacterial cells into the host cells where they act to modify host cell functions (reviewed by Haraga *et al.*, 2008). The base is composed of four rings; two are associated with the outer membrane (OR1 and OR2) and are composed of the InvG protein. The remaining two are associated with the inner membrane (IR1 and IR2) and are composed of PrgH and PrgK proteins (see Fig. 5.7, C) (Kubori *et al.*, 2000). The needle structure is composed of a single protein PrgI (Kubori *et al.*, 1998); the final protein encoded in the *prg* operon is PrgJ, which is thought to be located within the base structure (Marlovits *et al.*, 2004). OmpR enrichment within the coding region of *prgJ* and *prgI* and upstream of the *prgH* gene (Fig. 5.7, B) indicated OmpR binding here may exert a regulatory affect on the expression of these structural genes, however this requires further investigation. OmpR binding within the *prgH-hilD* intergenic region was mildly increased at low pH (Fig. 5.7, B). This implicates OmpR as a potential regulator of *prgH* and/or of *hilD*, which is divergently transcribed. A recent study has shown OmpR binds and represses *hilD* expression (Cameron and Dorman, 2012). DNase I footprinting revealed OmpR protected a region within the *hilD* open reading frame but no protection was observed up to 300 bp upstream of the *hilD* start codon (Cameron and Dorman, 2012). Peaks of OmpR binding upstream of *hilD* and *prgH*, identified in this study, indicates OmpR may regulate expression of these genes under the conditions tested in this study. These results show OmpR not only targets regulatory genes encoded in SPI-1 (i.e. *hilC* and *hilD*) but also targets structural genes responsible for the formation of the T3SS needle complex. Thus, OmpR may have a more complex relationship with SPI-1 expression than previously known. Further investigation is required to test if OmpR regulates expression of these structural genes.

The hypothesis that OmpR is more intimately linked to SPI-1 than previously known is made more attractive by the finding that OmpR targeted SPI-1 effector proteins that are located outside the SPI-1 region. The data presented in Fig. 5.7 (D) show OmpR targets the *sopE2* gene encoding the SopE2 protein. SopE2 is one of several effector proteins that is encoded outside the SPI-1 locus and is important during intestinal stage of infection by *S. Typhimurium* (Raffatellu *et al.*, 2005) *sopE2* is located in the vicinity of phage remnants and is homologous to the SopE protein (Bakshi *et al.*, 2000). Both SopE and SopE2 are secreted into host cells and promote invasion by inducing membrane ruffling and actin cytoskeletal reorganisation via guanine nucleotide exchange with host Rho GTPases: Cdc42, Rac1 and RhoG (Hardt *et al.*, 1998a; Stender *et al.*, 2000; Haraga *et al.*, 2008). This study found OmpR was enriched at the *sopE2*-STM1856 intergenic region and was further enriched at low pH (Fig 5.7, D). OmpR also targeted *sopA* and *gtgE* (see Table 5.2 and 5.4), both of which code for effectors that are translocated by the SPI-1 T3SS and are located outside of the SPI-1 pathogenicity islands (Ho *et al.*, 2002; Brussow *et al.*, 2004). The demonstration of OmpR binding at genes both within the SPI-1 locus and those located outside the SPI-1 locus provides evidence that OmpR involvement in SPI-1 expression may be more complex than previously known. OmpR may potentially act as a regulator of these genes in a pH-dependent manner, however the effect of OmpR on their expression remains to be elucidated.

5.2.6 OmpR targets *Salmonella* pathogenicity island 2 (SPI-2)

Analysis of OmpR distribution across SPI-2 revealed several significantly enriched OmpR peaks (Fig. 5.8). OmpR was most significantly enriched in the *ssrA-ssaB* intergenic region and this enrichment was increased at pH 4.5 (Fig. 5.9, A). To investigate the effect of OmpR binding in this region, quantitative PCR measurements of *ssrA* expression were made in wild-type and an *ompR* mutant background. Culture conditions used were identical to that used for the ChIP experiments; samples were taken after 90 min in EG-minimal media at pH 7 and pH 4.5. The primers *ssrA_RT_F* and *ssrA_RT_R* (Table 2.8) were used to assess *ssrA* transcript levels. Results in Fig. 5.9 (B) revealed *ssrA* expression increased in at pH 4.5. Furthermore, *ssrA* expression at both pH 7 and pH 4.5 was dependent on OmpR. Therefore, at low pH, the increased levels OmpR occupancy at the *ssrA* promoter results in a concomitant increase in *ssrA*.

OmpR has previously been shown to activate expression of *ssrA* expression inside host macrophages and in acidified minimal medium (Lee *et al.*, 2000). This study confirms the OmpR-dependency of *ssrA* expression and provides evidence of increased OmpR binding at *ssrA* at low pH *in vivo*.

OmpR has previously been shown to activate expression of *ssrA* expression inside host macrophages and in acidified minimal medium (Lee *et al.*, 2000). This study confirms the OmpR-dependency of *ssrA* expression and provides evidence of increased OmpR binding at *ssrA* at low pH *in vivo*.

The results in Fig. 5.8 demonstrated OmpR binding extended beyond the *ssrA-ssaB* intergenic region. OmpR was enriched upstream of *sseA*; the *sseA* promoter controls expression of the genes within the effector/chaperone operon of SPI-2 (Tomljenovic-Berube *et al.*, 2010). The *sseA* gene encodes SseA; a virulence chaperone protein responsible for the secretion of SseB and SseD proteins, these proteins are components of the translocon, located at the tip of the needle complex (Zurawski and Stein, 2004). The data also identified OmpR binding peaks within the coding sequence of *sseG* and *ssaV*. OmpR binding within the effector gene, *sseG*, increased at pH 4.5; OmpR binding here may influence expression of the downstream structural operon beginning with *ssaG*. OmpR binding was also detected within the *ssaV* gene which forms an essential structural component of the T3SS. The effect of OmpR binding within the coding sequence of these genes remains to be investigated. As observed with SPI-1, OmpR targeted effector proteins located outside SPI-2 that are secreted by the SPI-2 T3SS. For example, OmpR was enriched upstream of the translocated effector *sseK2* Fig. 5.9 (C) (Kujat Choy *et al.*, 2004). Other OmpR-enriched effectors located outside of the SPI-2 locus included: *gogB*, *pipB2*, *sopD2* and *gtgE* (see Table 5.2 and 5.4) (Knodler *et al.*, 2003; Jiang *et al.*, 2004; Coombes *et al.*, 2005a). It should be noted *gtgE*, located in the bacteriophage Gifsy-2, is secreted by both the SPI-1 and SPI-2 T3SS (Ho *et al.*, 2002). OmpR enrichment at additional genes located within SPI-2 extends our knowledge of OmpR targets within this locus. As found with SPI-1, OmpR was also enriched at genes regulated by SPI-2 but located outside the SPI-2 locus. Thus, OmpR may co-ordinate SPI-2 and SPI-1 expression at additional levels beyond controlling the SPI master regulators *ssrA/B*, *hilC* and *hilD*.

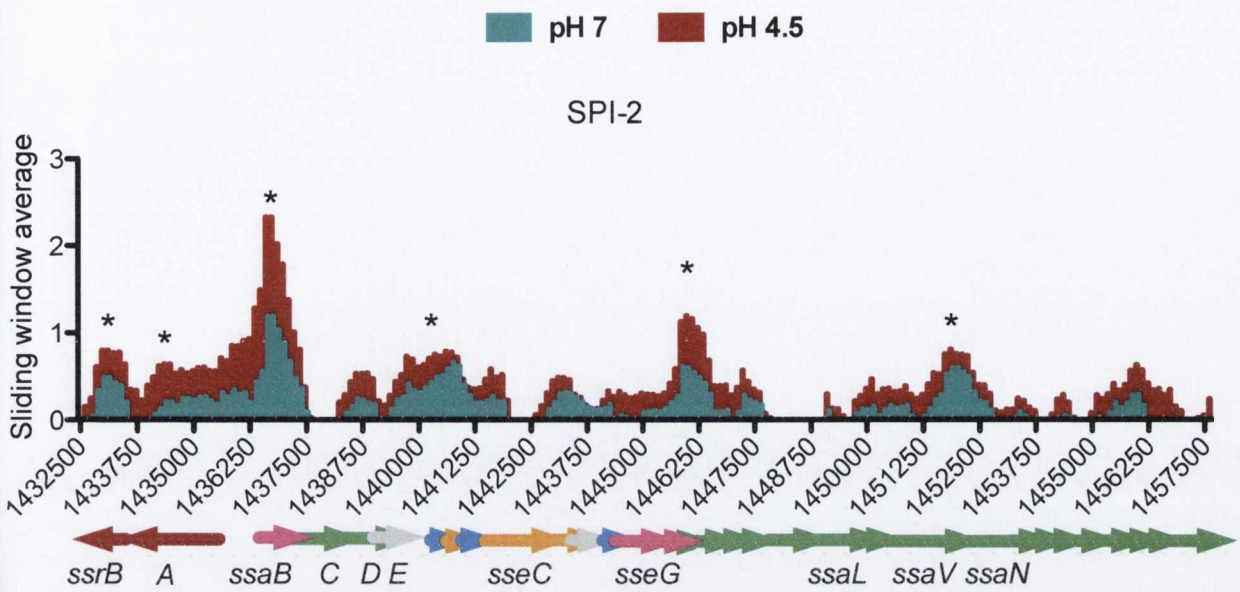


Fig. 5.8. OmpR enrichment at *Salmonella* pathogenicity island-2. A. OmpR enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005). Peaks identified as significant are denoted with an asterisk. Genome co-ordinates are shown on the x-axis. Coloured arrows below the x-axis represent the genes within SPI-2, the location of the genes was determined using Jbrowse (Kroger *et al.*, 2012). Genes are coloured based on their function: regulatory genes are red, effector genes are pink, structural genes are green, translocon genes are orange, chaperone genes are blue and grey genes are of unknown function.

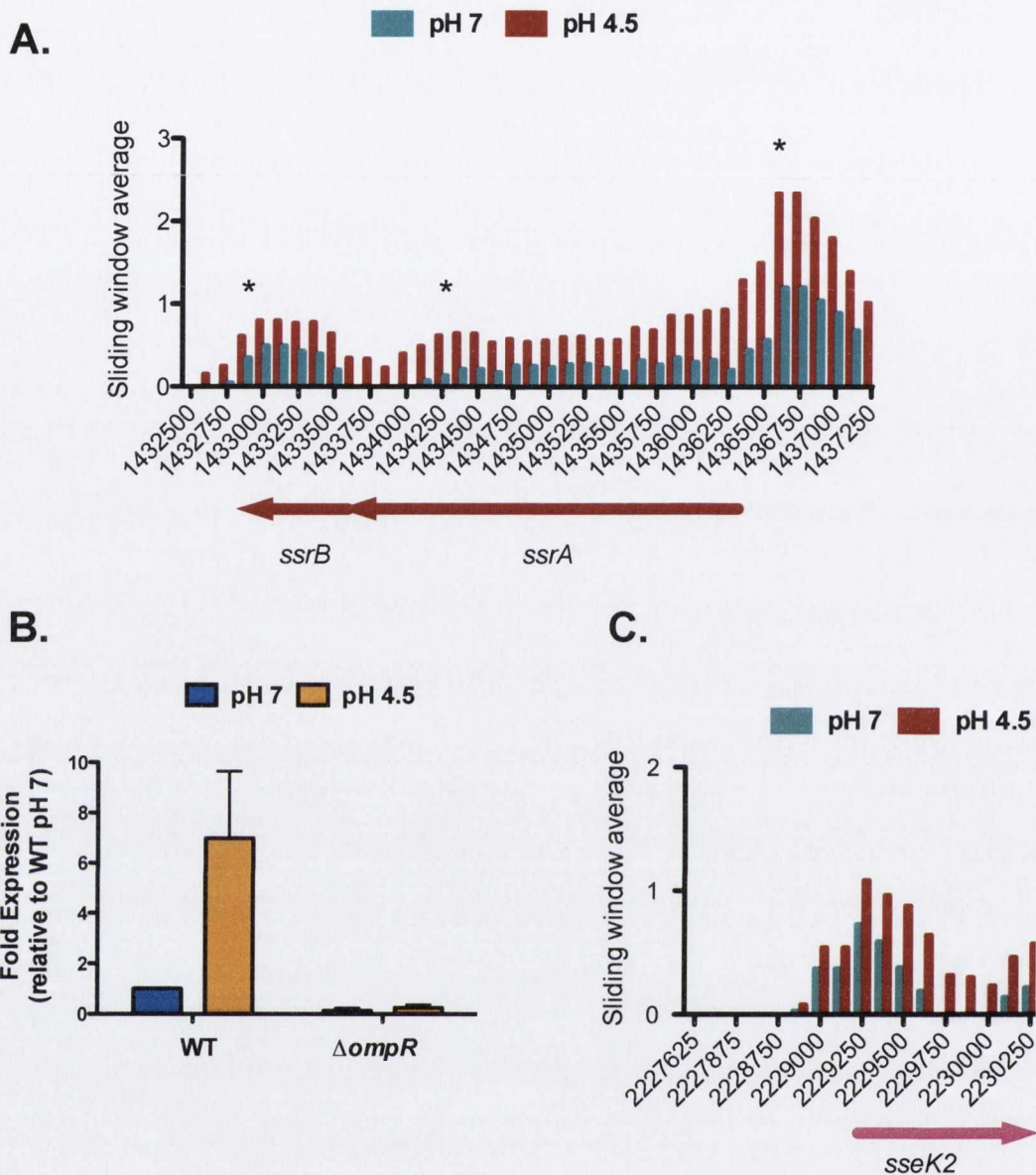


Fig 5.9. OmpR enrichment at genes within, and regulated, by *Salmonella* pathogenicity island 2. **A.** Detailed view of OmpR enrichment at the *ssrA/B* operon; pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005). Peaks identified as significant are denoted with an asterisk. Genome co-ordinates are shown on the x-axis. Coloured arrows below the x-axis represent the genes within SPI-2, the location of the genes was determined using Jbrowse (Kroger *et al.*, 2012). **B.** Quantitative PCR measurements of *ssrA* RNA levels in wild-type SL1344 (WT) and in SL1344 $\Delta ompR$ after 90 min in EG-minimal media at pH 7 (blue) and pH 4.5 (yellow). Transcript levels are expressed relative to WT. The mean and standard deviations from at least three independent biological replicates are shown. **C.** OmpR enrichment at the secreted effector *sseK2*.

5.2.7 OmpR targets *Salmonella* pathogenicity island 4 (SPI-4)

OmpR enrichment of horizontally acquired genes extended to regions outside SPI-1 and SPI-2. Specifically, this study identified peaks of OmpR enrichment within SPI-4. SPI-4 is a 24-kb pathogenicity island that harbours the *siiABCDEF* operon, which codes for a type 1 secretion system (T1SS) (Wong *et al.*, 1998; Morgan *et al.*, 2004; Gerlach *et al.*, 2007b). T1SSs consist of three conserved proteins that form a membrane-spanning channel, which selectively transports substrates across the bacterial envelope (reviewed by Delepelaire, 2004). The SPI-4 T1SS selectively transports the adhesin, SiiE, to the cell surface. *siiF* encodes the inner membrane ATPase, SiiF, which hydrolyses ATP providing energy for transport of SiiE. *siiD* encodes the periplasmic adaptor protein SiiD and *siiC* codes for the outer membrane protein. SiiA and SiiB are localised in the inner membrane; they are crucial for oral virulence in mice however, neither is required for SiiE secretion (See Fig. 5.10, B) (Gerlach *et al.*, 2007b). SiiE is a large (595 kDa) non-fimbrial adhesion, required for adhesion and invasion of polarised epithelial cells during the intestinal phase of infection (Morgan *et al.*, 2004; Gerlach *et al.*, 2008).

The results presented here showed that OmpR binds directly to SPI-4; specifically, OmpR was enriched within the coding sequence of the *siiA-E* genes (Fig. 5.10, A). OmpR binding was significantly enriched at pH 4.5; however, the effect of OmpR on SPI-4 expression under the conditions used in this study is unknown. Previous work has found SPI-4 expression is coordinated with that of SPI-1; activation of HilA leads to the activation of *sprB*, both of which are required to activate *siiA* expression (Saini and Rao, 2010). SPI-4 expression is repressed by H-NS; in the absence of H-NS *siiE* expression does not require HilA, suggesting HilA functions as a H-NS antagonist at SPI-4 (Main-Hester *et al.*, 2008). Importantly for this work, a study found inactivation of the *ompR/envZ* locus caused a reduction in *siiA* expression (Gerlach *et al.*, 2007a), indicating OmpR regulates expression of *siiA*.

The data presented here identified OmpR enrichment within both the SPI-1 and SPI-4 loci. As expression of these loci has been found to be coordinated (Main-Hester *et al.*, 2008), OmpR binding at these loci may function as a potential coordinator of their expression. As shown in Fig. 5.9 (A+B), OmpR binds and induces expression of the SPI-2 regulator *ssrA* under the conditions used in this study. It is tempting to speculate

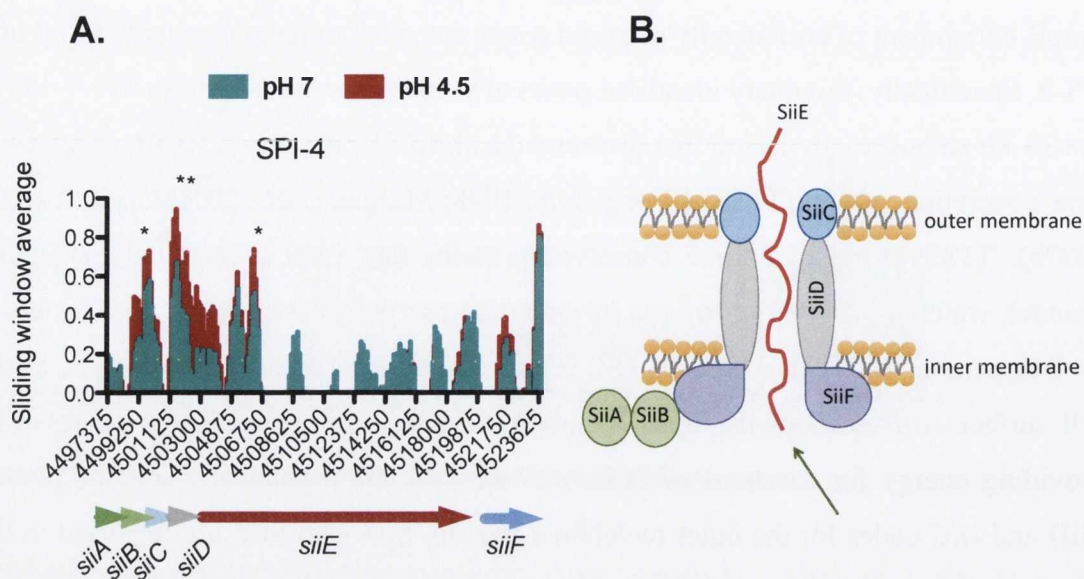


Fig. 5.10. OmpR enrichment within *Salmonella* pathogenicity island 4 (SPI-4). **A.** OmpR enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005). Peaks identified as significant are denoted with an asterisk. Genome co-ordinates are shown on the x-axis. Below the x-axis are coloured arrows representing the genes within SPI-4, the location of the genes was determined using Jbrowse (Kroger *et al.*, 2012). **B.** An illustration of the SPI-4 type 1 secretion system (T1SS). Secretion of the adhesion SiiE (red). SiiF (purple) is an inner membrane transporter ATPase, SiiD (grey) is a periplasmic adaptor protein and SiiC is an outer membrane protein. The function of SiiA and SiiC proteins (green) is unclear, these proteins may be interaction partners and regulate the retention of SiiE (as adapted from Wagner *et al.*, 2011).

that OmpR enrichment across the SPI loci coordinates expression of virulence genes involved in intestinal colonisation and intracellular survival. Analysis of OmpR binding thus far has focussed on OmpR binding within horizontally-acquired genes required for infection and absent in *E. coli*. This study has identified OmpR binding at horizontally acquired genes extends beyond previously identified targets (Lee *et al.*, 2000; Osborne and Coombes, 2011; Cameron and Dorman, 2012) and as such provides additional compelling evidence that the OmpR regulon has evolved since the divergence of *Salmonella* and *E. coli*. Moreover, the evolution of the OmpR regulon in *S. Typhimurium* was also identified by integration of genes best characterised as part of the PhoP/Q TCS, as discussed in the following section.

5.2.8 OmpR targets overlap with the PhoP/Q regulon

Analysis of the ChIP-on-chip data in *S. Typhimurium* revealed OmpR was enriched at genes previously identified as part of the PhoP/Q regulon (Table 5.8); indicating an overlap between the OmpR and PhoP regulon. The PhoP/Q TCS governs expression of genes required for adaptation to conditions of low levels of extracellular divalent cations (e.g. Mg^{2+} , Ca^{2+} and Mn^{2+}) (reviewed by Groisman, 2001). PhoQ is a sensor kinase that modifies the phosphorylation state of its cognate response regulator PhoP, PhoP-P binds to DNA and regulates gene expression accordingly. The PhoP/Q TCS enables *S. Typhimurium* to sense the intracellular vacuolar environment and activate virulence genes. PhoP regulates a number of genes including genes required for magnesium transport, intramacrophage survival and resistance to antimicrobial peptides (reviewed by Groisman, 2001). In the presence of low levels of magnesium, PhoP-P activates expression of the *mgtA*, which codes for a magnesium transporter. In addition, in the presence of low Mg^{2+} concentrations and acidic pH, PhoP activates the *mgtCBR* operon. *mgtC* codes for an inner membrane protein involved in magnesium acquisition and is required for intramacrophage survival (Blanc-Potard and Groisman, 1997; Retamal *et al.*, 2009; Lee and Groisman, 2012a). *mgtB* codes for a magnesium transporter, MgtB, which transports Mg^{2+} from the periplasm to the cytoplasm (Snively *et al.*, 1991). *mgtR* encodes the peptide MgtR which promotes degradation of MgtC (Alix and Blanc-Potard, 2008). In this study OmpR was significantly enriched upstream of the *mgtC* gene (Fig. 5.11, A). To confirm OmpR binding within the *mgtC* promoter region EMSA analysis was performed. The biotinylated primers, P*mgtC*_F_EMSA and

Table 5.8. PhoP-regulated genes identified as OmpR-enriched

	Gene name	Function	Peak Start
1	intergenic <i>mgtC</i>	Mg ²⁺ transport protein	3986625
2	intergenic <i>mig-14</i>	transcriptional activator	2952000
3	intergenic <i>prgH</i>	needle complex inner membrane protein	3040125
4	intergenic <i>pmrD</i>	polymyxin resistance protein B	2409000
5	intergenic <i>pgtC</i>	phosphoglycerate transport regulatory protein precursor	2509375
6	intergenic <i>slyB</i>	Outer membrane lipoprotein	1477625
7	intergenic <i>yaiB</i> (<i>iraP</i>)	Hypothetical protein	436375
8	<i>pagO</i>	integral membrane protein	1917500
9	<i>pagK</i>	PagK	1920750
10	<i>prgJ</i>	needle complex minor subunit	3038375
11	<i>prgI</i>	needle complex major subunit	3038750
12	<i>pqaA</i>	PhoPQ-regulated protein	1577875
13	<i>ssrB</i>	transcriptional activator	1432875

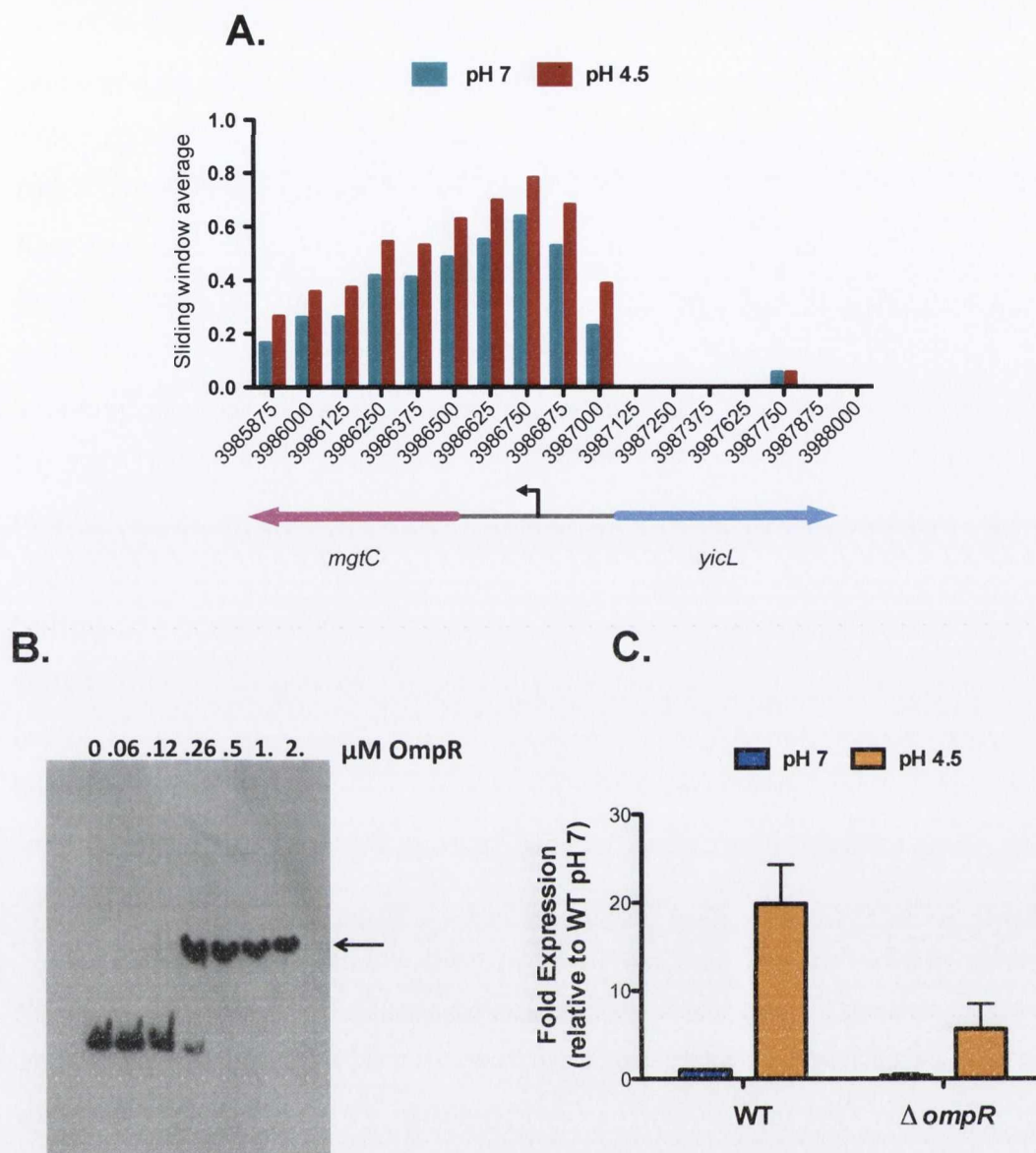


Fig. 5.11. OmpR regulates *mgtC* expression in *S. Typhimurium*. **A.** OmpR enrichment at the *mgtC* promoter is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005), enrichment at pH 7 is coloured teal and enrichment at pH 4.5 is red. Genome co-ordinates are shown on the x-axis, below, coloured arrows representing the divergently transcribed *mgtC* and *yicL* genes, the location of the genes and the *mgtC* transcription start site (bent arrow) was determined using Jbrowse (Kroger *et al.*, 2012). **B.** EMSA analysis of OmpR binding to the *mgtC* promoter. Increasing concentrations of OmpR were incubated with biotinylated probes encompassing the *mgtC* promoter; protein-DNA complex is highlighted with an arrow. **C.** Quantitative PCR measurements of *mgtC* expression in SL1344 wild-type (WT) and SL1344 $\Delta ompR$ after 90 min in EG-minimal media at pH 7 (blue) and pH 4.5 (yellow). Transcript levels are expressed relative to WT at pH 7. Mean levels of expression and standard deviation from at least three independent biological replicates are shown.

*PmgtC_R*_EMSA (Table 2.8), were used to PCR-amplify a 529 bp probe encompassing the *mgtC* promoter. This was incubated with increasing concentrations of OmpR protein (see section 2.8). Results in Fig. 5.11 (B) reveal OmpR formed a single protein-DNA complex with the *mgtC* probe; confirming the ChIP-chip results and indicating OmpR occupied a single binding site in the *mgtC* promoter. To investigate the effect of OmpR on *mgtC* expression quantitative measurements of *mgtC* transcripts were made using primers *mgtC_F_RT* and *mgtC_R_RT* (Table 2.8). Samples were taken in wild-type SL1344 (WT) and SL1344 *ompR* after 90 min in EG-minimal media at pH 7 and pH 4.5. Results presented in Fig. 5.11 (C) show *mgtC* expression was induced ~19 fold in acidic pH and was reduced ~3.5 fold in the *ompR* mutant. Therefore, OmpR binding at the *mgtC* promoter increased *mgtC* expression at low pH. Recent studies have identified *mgtC* expression is subject to transcriptional attenuation by two short open-reading frames (ORFs; *mgtM* and *mgtP*), encompassed in the *mgtC* intergenic region (Lee and Groisman, 2012a; Lee and Groisman, 2012b). The alternative RNA structures formed by these short ORFs are sensitive to intracellular levels of ATP and proline respectively; the alternative stem-loop structures formed in the *mgtC* leader mRNA influence transcription of the downstream ORFs *mgtC* and *mgtB*. High levels of ATP (as a result of acidic pH) and low levels of proline-charged tRNA (as a result of low levels of proline), act independently, and additively, to induce optimum levels of *mgtC* expression (Lee and Groisman, 2012a; Lee and Groisman, 2012b). In this present study *mgtC* was induced at low pH and high levels of proline; recall proline was added to meet the requirements of proline auxotroph CSH50 (see section 3.2.1). Thus, under these conditions OmpR activates transcription of *mgtC* (Fig. 5.11, C), subsequently, *mgtM* is predicted to adopt a stem-loop structure favouring *mgtC* expression (Low pH, high ATP), while *mgtP* adopts a stem-loop structure which negatively affects *mgtC* expression (High proline, high tRNA^{Pro}) (Lee and Groisman, 2012a; Lee and Groisman, 2012b).

OmpR binding was also discovered upstream of the PhoP-activated gene, *mig-14* gene. *mig-14* is induced by low Mg²⁺ concentration, acidic pH and in the presence of specific antimicrobial peptides such as polymyxin B, protegrin-1 and the cathelin-related antimicrobial-peptide, CRAMP. However, the mechanism underlying Mig-14 mediated antimicrobial resistance is not well understood. Additionally *mig-14* is activated inside macrophages and is required for persistence in the liver and spleen of chronically

infected mice (Valdivia and Falkow, 1997; Brodsky *et al.*, 2002; Brodsky *et al.*, 2005). In this study, OmpR was enriched in the *mig-14* promoter region at both pH 7 and pH 4.5 (Fig. 5.12, A). qPCR was used to investigate the effect of OmpR *mig-14* expression; using the primers *mig-14_F* and *mig-14_R* (Table 2.8) expression levels of *mig-14* were assessed after 90 min in EG-minimal media at pH 7 and pH 4.5. Results in Fig. 5.12 (B) revealed *mig-14* expression was lower at acidic pH. This is in contrast to a previous study that demonstrated *mig-14* was mildly induced at pH 5.8 (Brodsky *et al.*, 2002). Fig. 5.12 (B) shows *mig-14* was mildly reduced at pH 7 in the *ompR* mutant and unaffected at pH 4.5; indicating a requirement for OmpR to mediate *mig-14* expression at pH 7. Taken together, qRT and ChIP-chip results appear counter-intuitive; OmpR levels at *mig-14* were more enriched at pH 4.5, however, OmpR removing *ompR* from the system had no affect on *mig-14* expression at pH 4.5. This suggests that OmpR may bind to *mig-14* *in vivo*, however, OmpR may only mediate an affect on *mig-14* under non-acidic conditions.

The overlap between OmpR binding targets and PhoP-regulated genes included PhoP activated genes (*pag*), *pagK* and *pagO* (Belden and Miller, 1994) and PhoP repressed genes, *prgHIJK* (Table 5.8); OmpR binding at within in the *prgHIJK* operon was previously discussed in section 5.2.3. *pagK* and *pagO* are located within a phage remnant, which also contains the SPI-1 effector protein *sopE2* (McClelland *et al.*, 2001), however their role during infection is unknown (Belden and Miller, 1994). Based on the overlap between these two regulons, it was decided to investigate if OmpR regulated the *phoP* gene; a peak in OmpR binding was present in the *phoP* intergenic region; however, correction with the mock antibody removed this peak from the final data set. A recent microarray based study performed in an *ompR* mutant in *Yersinia pestis*, a facultative intracellular pathogen and causative agent of human plague, identified OmpR as a positive regulator of *phoP* expression. Furthermore, the authors identified a putative OmpR binding site upstream of the *phoP* start codon (-158 to -177 bp upstream of the *phoP* start codon) (Gao *et al.*, 2011a). It was hypothesised herein that OmpR may bind to the *phoP* promoter in *S. Typhimurium*; however, this interaction was relatively weak and thus was removed by mock correction. A role for OmpR at the *phoP* gene in *S. Typhimurium* was investigated using qPCR and EMSA analysis.

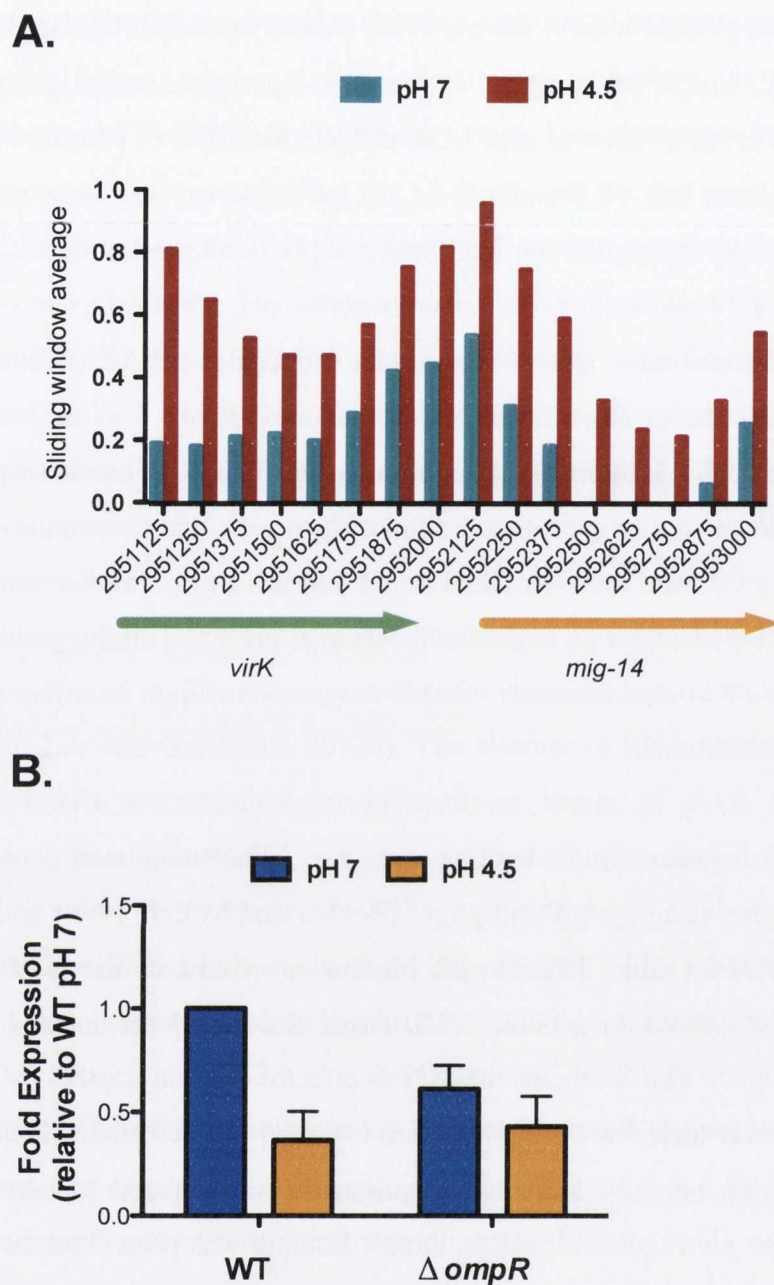


Fig. 5.12. OmpR binding and regulation of *mig-14* gene in *S. Typhimurium*. **A.** OmpR enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005). Genome co-ordinates are shown on the x-axis, below, coloured arrows representing *mig-14* and *virK* genes, the location of the genes was determined using Jbrowse (Kroger *et al.*, 2012). **B.** Quantitative PCR measurements of *mig-14* expression in SL1344 wild-type (WT) and SL1344 $\Delta ompR$ after 90 min in EG-minimal media at pH 7 (blue) and pH 4.5 (yellow). Transcript levels are expressed relative to WT at pH 7. Mean levels of expression and standard deviation from at least three independent biological replicates are shown.

Bioinformatic analysis of the *phoP* promoter in SL1344 identified two putative OmpR binding sites in the *phoP* promoter region (OmpR-I; -11 to -29 bp and OmpR-II; -61 to -80 bp, relative to the *phoP* start codon). To examine if OmpR bound to the *phoP* promoter, the biotinylated primers P*phoP*JET_F and P*phoP*JET_R (Table 2.8) were used to amplify a 213 bp product encompassing the *phoP* intergenic region. This was cloned into the linearised pJET vector and generated the plasmid P*phoP*JET. Site-directed mutagenesis was then performed using primers P*phoP*_SDM_F and P*phoP*_SDM_R (Table 2,8), which introduced 5 bp substitutions into the putative OmpR-I site (Fig. 5.13, A). Mutagenesis of the OmpR-I site was confirmed by DNA sequencing (GATC, Biotech). OmpR binding to wild-type *phoP* promoter (WT) and *phoP* promoter harbouring mutated OmpR-I site (OmpR-I⁻) was tested by EMSA analysis using purified OmpR protein. Results in Fig. 5.13 (B) showed OmpR formed a complex with WT DNA at 2 μ M OmpR, no complex was observed at 2 μ M with OmpR-I⁻ DNA, however, almost all DNA was shifted at 4 μ M. Compared to OmpR binding at *mgtC*, more OmpR protein was required to form protein-DNA complexes at the *phoP* promoter. This is in agreement with the earlier observation that OmpR has a weak affinity for the *phoP* promoter. However, the 5 bp mutations introduced into the OmpR-I⁻ site reduced the affinity of OmpR for *phoP* DNA, indicating OmpR specifically bound to the *phoP* promoter. The formation of additional protein-DNA complexes in the presence of the OmpR-I⁻ mutated binding site may represent OmpR binding to the putative OmpR-II site. A previous study in *S. Typhimurium* demonstrated H-NS binds to three sites in the *phoP* promoter and represses *phoP* expression (Fig. 5.13, A). Two of these sites overlap the putative OmpR binding sites identified herein. In addition, PhoP binds to the *phoP* promoter and positively autoregulates expression (Kong *et al.*, 2008). In this study, qPCR was used to test if OmpR regulates *phoP* expression under the conditions used herein. *phoP* expression was measured using primers *phoP*_S.e_RT_F and *phoP*_S.e_RT_R (Table 2.8) after 90 min in EG-minimal media at pH 7 and pH 4.5. Results presented in Fig. 5.13 (C) show *phoP* expression was reduced in an *ompR* mutant at both pH 7 and pH 4.5 suggesting OmpR activates *phoP* expression. *phoP* levels were upregulated in an *hns::kan* mutant confirming earlier studies that H-NS repressed *phoP* expression (Kong *et al.*, 2008).

To test if OmpR acts as both an activator and anti-repressor of H-NS, the *hns::kan* mutation was moved by P22 transduction into SL1344 Δ *ompR*; this generated the

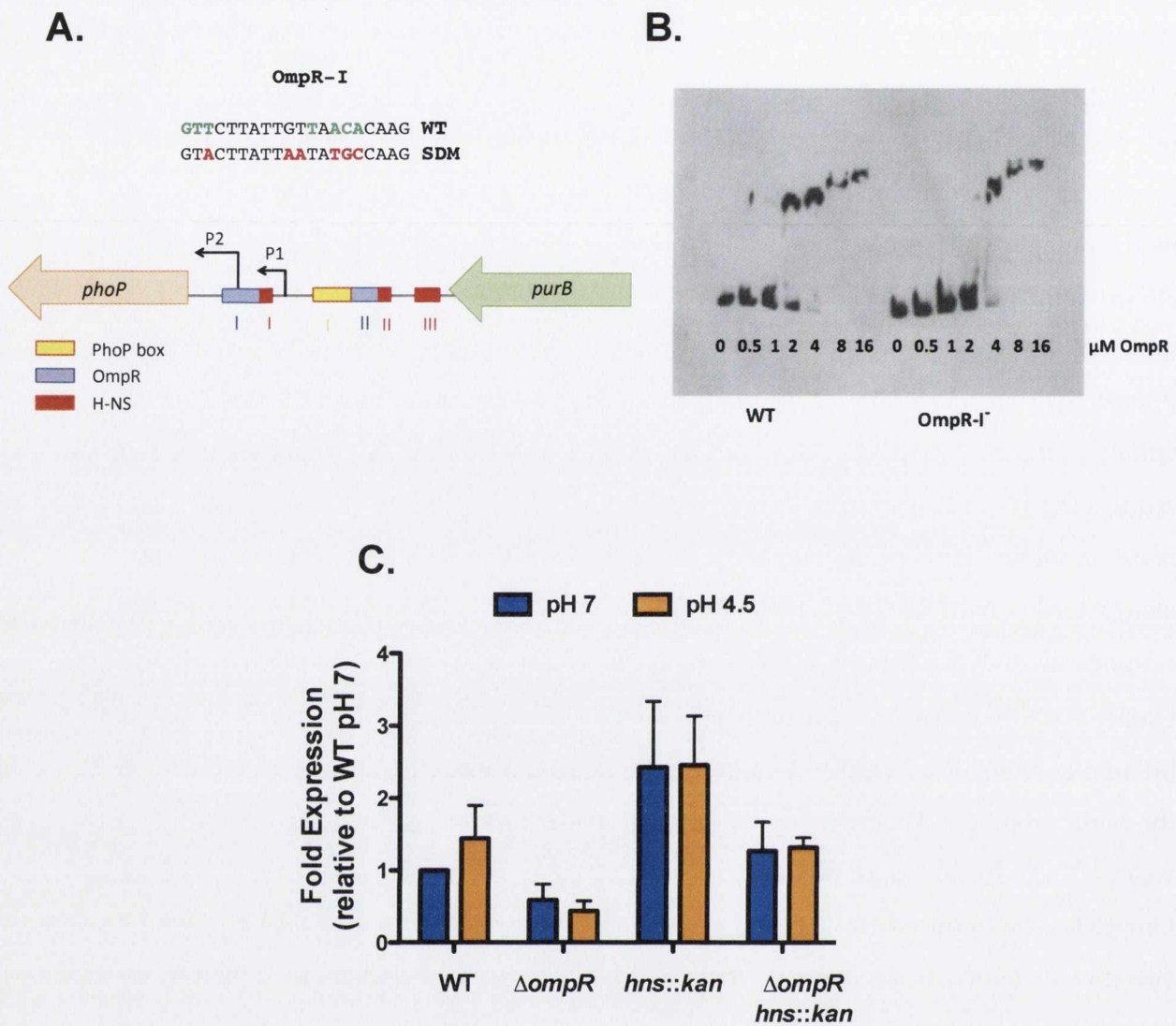


Fig. 5.13. OmpR regulates *phoP* expression in *S. Typhimurium*. **A.** Mutations introduced into the OmpR-I site by site-directed mutagenesis (SDM) are shown in red. Bases important for OmpR binding, as determined by Yoshida *et al.* (2006) are shown in green. Below is an illustration of the organisation of the *phoP-purB* intergenic region. Bent arrows denote P1 and P2 TSSs (Kong *et al.*, 2008) **B.** EMSA analysis of OmpR binding to the wild type *phoP* promoter (WT) and the *phoP* promoter containing a mutated OmpR-I site (OmpR-I⁻). **C.** Quantitative PCR measurements of *phoP* expression in wild-type SL1344 (WT), $\Delta ompR$, *hns::kan* and $\Delta ompR$ *hns::kan*. Samples were taken after 90 min in EG-minimal media at pH 7 (blue) and pH 4.5 (yellow). Transcript levels are expressed relative to WT at pH 7. Mean expression levels and standard deviation from at least three independent biological replicates are shown.

double mutant SL1344 $\Delta ompR$ $hns::kan$. Expression of *phoP* in the *ompR*, *hns* double mutant was higher than the $\Delta ompR$ single mutant suggesting OmpR antagonises H-NS at the *phoP* promoter. OmpR binding to the OmpR-I and OmpR-II sites, located adjacent to H-NS binding sites, may mediate H-NS antagonism of OmpR. However, *phoP* expression in the *ompR*, *hns* double mutant was not as high as observed in the $hns::kan$ single mutant, indicating a requirement for OmpR to reach optimum *phoP* expression in the absence of H-NS. Thus, OmpR may mediate H-NS antagonism and *phoP* activation via the OmpR-I and putative OmpR-II binding sites. Further work is required to validate the existence of the OmpR-II site and assess the direct contributions of both the OmpR-I and OmpR-II sites to *phoP* expression.

The PhoP/Q and OmpR/EnvZ systems are found across members of the *Enterobacteriaceae*. Results presented here identified an overlap between both regulons in *S. Typhimurium*; specifically, OmpR was enriched at multiple genes, best described as part of the PhoP regulon. Importantly, this overlap was only observed in *S. Typhimurium* and OmpR did not target PhoP-regulated genes in *E. coli*. Many of the genes described in Table 5.8 are not present in the commensal *E. coli* except *slyB* and bound *phoP*, both of which are present in *E. coli* and are PhoP-regulated (Minagawa *et al.*, 2003; Monsieurs *et al.*, 2005). OmpR may bind at the *slyB* and *phoP* genes in *E. coli* under other conditions not tested here. The overlap between these two regulons to include genes that are not present in *E. coli* suggests this integration of the OmpR and PhoP regulatory circuits may have arisen since *S. enterica* diverged from *E. coli*. Many of the genes are virulence associated; this indicates the evolution of the OmpR regulon to include members the PhoP regulon may have occurred out of necessity to regulate genes required for pathogenesis.

Thus far, unique OmpR targets in *S. Typhimurium* have demonstrated the evolution of the OmpR regulon. In the following section, OmpR regulation of unique targets in *E. coli* is discussed.

5.2.9 OmpR targets in *E. coli* –*cadBA* operon

Analysis of the of OmpR ChIP-chip data sets revealed OmpR targeted some genes in *E. coli* and did not target their homologues in *S. Typhimurium*. One notable example is the

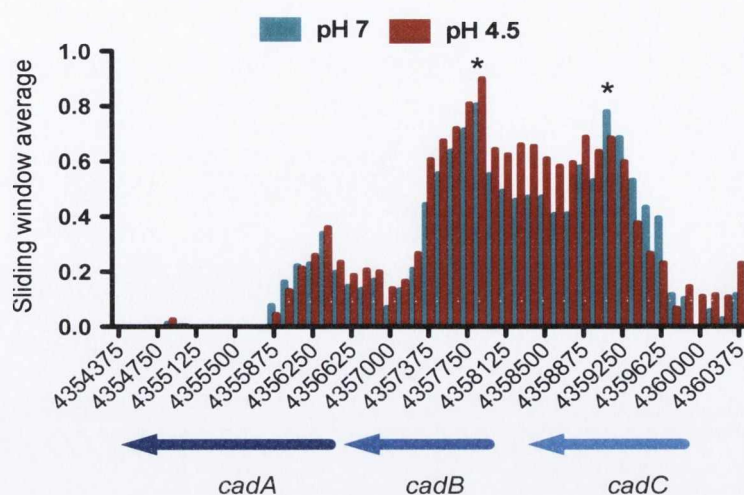
cad system, this system is one of several decarboxylases used by *E. coli* to survive acidic pH by maintaining pH homeostasis (reviewed by Ramos-Morales, 2012). The CadC protein, is a membrane localised activator of the *cadBA* operon in conditions of low pH and in the presence of lysine (Watson *et al.*, 1992; Neely and Olson, 1996). CadC senses low pH that induces conformational change in an intramolecular disulphide bond transforming CadC into a semi-active state (Tetsch *et al.*, 2011). CadC senses lysine by interplay with the lysine permease, LysP (Tetsch *et al.*, 2008). The CadA protein decarboxylates lysine to cadaverine, consuming an intracellular proton in the process. Cadaverine is then exported from the cell by the CadB antiporter in exchange for more lysine. CadB and CadA are encoded by the *cadB* and *cadA* genes respectively; these genes are organised in an operon and are activated by the CadC protein (Auger *et al.*, 1989; Meng and Bennett, 1992; Soksawatmaekhin *et al.*, 2004).

In this present study OmpR was enriched upstream of the *cadBA* operon at both pH 7 and pH 4.5 (Fig. 5.14, A), however, the effect of OmpR on *cadBA* expression is currently unknown. It should be noted that the culture conditions used in this study did not contain lysine, the co-factor required to induce the *cad* system. A previous study has shown if cells are grown in the presence of one stimulus (i.e. low pH or lysine), followed by the treatment with the second stimuli the *cadB* gene is maximally induced (Neely and Olson, 1996). Therefore, future work measuring *cadBA* expression in an *ompR* mutant at both pH 7 and pH 4.5, followed by treatment with lysine, may offer insights into the role of OmpR in regulating the *cadBA* operon. Speculatively, OmpR may regulate acid resistance in *E. coli* by binding and regulating expression of the *cadBA* operon. Interestingly, OmpR binding *cad* system was specific *E. coli*; the orthologous *cad* system in *S. Typhimurium* did not appear in the ChIP-chip data sets. This indicates OmpR may regulate acid-resistance differently between these two species.

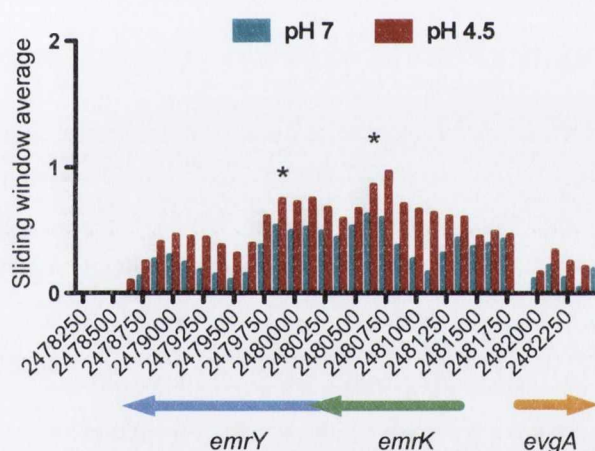
5.2.10 OmpR targets in *E. coli* at low pH

At low pH, OmpR was enriched at the *emrKY* operon that encodes the proteins EmrK and EmrY respectively (Fig. 5.14, B); *emrKY* codes for an efflux pump and shows sequence similarity to the EmrAB-TolC drug efflux system (Tanabe *et al.*, 1997). A recent study by Han *et al.* found the *emrK* and *emrY* genes were an important for lethal

A.



B.



C.

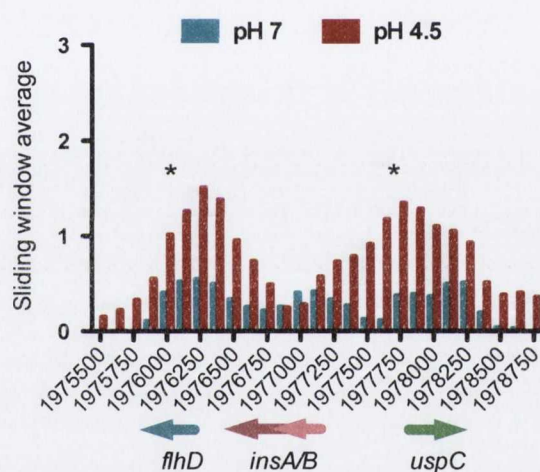


Fig. 5.14. OmpR enrichment at genes in *E. coli*. OmpR enrichment at the *cadBA* operon (A), the *emrKY* operon (B) and at *uspC* (C). Enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005) significant peaks are denoted by an asterisk. Genome co-ordinates are shown on the x-axis, below, coloured arrows representing genes, the location of the genes was determined using the Entrez Gene database at NCBI (Maglott *et al.*, 2011).

stress tolerance; *emrK* and *emrY* mutants were more susceptible than wild-type to mitomycin C, UV radiation and H₂O₂ treatment (Han *et al.*, 2010). An additional study has shown the EmrK/Y system mediated deoxycholate resistance in *E. coli*; deoxycholate is a bile acid prevalent in the host intestine (Nishino *et al.*, 2009). In addition, Matsumura *et al.* have shown *emrK* mutants have a reduced ability to form biofilms (Matsumura *et al.*, 2011). Microarray analysis performed found over-expression of the *ompR* gene in *E. coli* lead to a 2.5 fold increase in *emrK* expression indicating OmpR activated *emrK* expression. This same study found over-expression of *ompR* resulted in the up-regulation of many drug-exporter systems however, under the conditions used herein, OmpR was enriched only *emrKY* (Hirakawa *et al.*, 2003). Interestingly, at pH 4.5 OmpR was also enriched at the *alaA* (*yfbq*) gene, which encodes a putative aminotransferase; *alaA* was identified in the same study as *emrK* and *emrY* as an additional gene, which reduces lethal stresses in *E. coli*. Specifically, an *alaA* mutant was more sensitive to treatment with mitomycin C than wild type (Han *et al.*, 2010).

OmpR was enriched at the *uspC* gene at pH 4.5 (see Fig. 5.14, C). *uspC* encodes the universal stress protein C (UspC), it is one of six Usp proteins in *E. coli*. Usp orthologous have been identified in bacteria, eukaryotes and archaea (Gustavsson *et al.*, 2002). A comprehensive study of the Usp proteins in *E. coli* revealed *uspC* was induced in the stationary phase of growth and by multiple stresses; *uspC* mutants were sensitive to UV exposure (Gustavsson *et al.*, 2002). A second study found *uspC* mutants are rendered non-motile and devoid of flagella suggesting UspC mediates a positive affect on motility, this study also found *uspC* mutants displayed enhanced levels of fimbriae-mediated adhesion indicating UspC has a negative affect on adhesion. Thus, Nachin *et al.* hypothesised UspC promotes motility at the expense of adhesion (Nachin *et al.*, 2005).

This present study found, at low pH, OmpR was enriched at genes required to tolerate stressful environments; these genes may confer resistance to acidic pH among others. Further work is required to elucidate the expression patterns of these genes and the role of OmpR binding at these genes.

5.2.11 Genes targeted by OmpR common to *S. Typhimurium* and *E. coli*

Investigation of genes targeted by OmpR that are common to both SL1344 and CSH50 revealed there was limited overlap of OmpR binding between the two species. As shown in Table 5.9, OmpR bound 15 genes that are present in both organisms. The limited overlap suggests that the OmpR regulon has evolved and may have become tailored to meet the requirements of each organism. OmpR targets common to both species included surface expressed proteins (OmpF and OmpC) and appendages (curli and fimbriae). OmpR binding may regulate these genes as part of a generalised role for OmpR in both species.

Curli, also called thin aggregative fimbriae (Tafi in *S. Typhimurium*), are thinly-coiled proteinaceous fibers expressed on the cell surface (reviewed by Barnhart and Chapman, 2006). Curli are important for biofilm formation and promote adhesion to abiotic surfaces and to human cells by binding extracellular matrix proteins fibronectin, laminin and plasminogen (Olsen *et al.*, 1989; Romling *et al.*, 1998). OmpR targeted the *csgD* promoter in both SL1344 and CSH50; *csgD* codes for the CsgD transcriptional regulator responsible for activating genes involved in curli assembly. CsgD is an activator of *csgBA* operon that codes the major structural component CsgA and the nucleator protein CsgB. The *csgBA* genes are divergently transcribed from the *csgDEFG* operon which codes for the transcriptional activator CsgD and the curli export machinery. Expression of *csgD* is under the influence of many transcription factors including OmpR, IHF, RpoS, Crl and H-NS (reviewed in Barnhart and Chapman, 2006). A study in *E. coli* found OmpR binds upstream at position -49.5 relative to the *csgD* TSS and activates transcription at low osmolarity (Prigent-Combaret *et al.*, 2001). An additional study in *E. coli* found *csgD* expression is unchanged at pH 7 and pH 4.5, expression is dependent on the OmpR/EnvZ system at both pH, however, at pH 4.5 an additional regulator RtsA was required to activate expression (Ogasawara *et al.*, 2010). In *S. Typhimurium*, a study identified multiple OmpR binding sites in the *csgD* promoter and proposed OmpR acts as both an activator and repressor of *csgD* expression (Gerstel *et al.*, 2003). In the present study, OmpR was enriched in the *csgD-csgB* intergenic region at both CSH50 and SL1344 (Fig. 5.15, A + B, respectively).

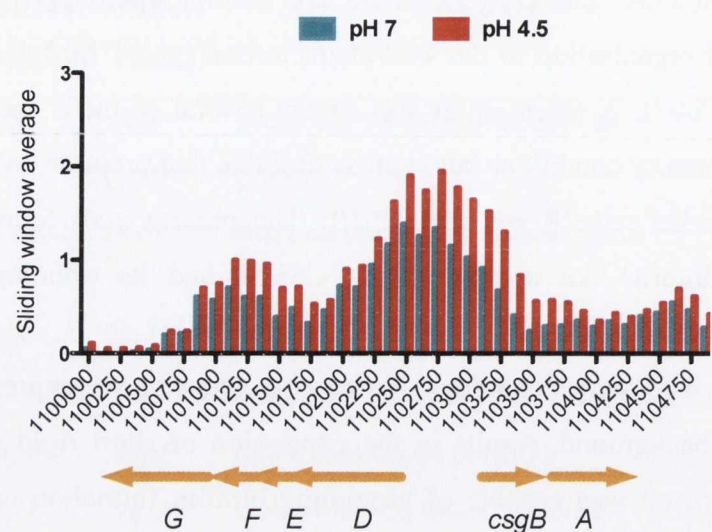
Table 5.9. OmpR common targets in *S. Typhimurium* and *E. coli*

	SL1344	CSH50	FUNCTION	SL1344	CSH50
	Name	Name		Peak	Peak
1	<i>bcfA</i>	<i>elfA</i> (<i>ycb</i>)	predicted fimbrial-like adhesin protein	25000	997125
2	STM02 91	<i>rhsA</i>	<i>rhsA</i> element core protein RshA	334625	3764125
3	<i>ybiF</i>	<i>rhtA</i>	threonine and homoserine efflux system	898250	849375
4	<i>ompF</i>	<i>ompF</i>	outer membrane porin 1a	1047125	985750
5	<i>csgD</i>	<i>csgD</i>	DNA-binding transcriptional activator for <i>csgBA</i>	1186125	1102250
6	<i>flhD</i>	<i>flhD</i>	DNA-binding transcriptional dual regulator with FlhC	1979625	1976000
7	<i>ompC</i>	<i>ompC</i>	outer membrane porin protein C	2364125	2310500
8	<i>lrhA</i> , <i>ala</i>	<i>lrhA</i> , <i>alaA</i>	DNA-binding transcriptional repressor of flagellar, motility and chemotaxis genes,	2438750	2405125
9	<i>ygjU</i>	<i>sstT</i>	sodium:serine/threonine symporter	3415250	3237875
10	<i>yqjA</i>	<i>exuR</i>	DNA-binding transcriptional repressor	3415625	3245500
11	<i>ompR</i>	<i>ompR</i>	DNA-binding response regulator in two-component regulatory system with EnvZ	3681875	3534375
12	<i>ydeV</i>	<i>lsrK</i>	autoinducer-2 (AI-2) kinase	4302125	1597000
13	STM19 94	<i>rseX</i>	ncRNA	2034500	2031625
14	STM25 03	<i>yfgF</i>	cyclic-di-GMP phosphodiesterase, anaerobic	2618625	2626500
15	<i>mle</i>	<i>dgsA</i>	Global DNA-binding transcriptional repressor; autorepressor; required for anaerobic growth on glucosamine	1523125	1665750

Amongst the common OmpR targets identified in Table 5.9, OmpR also targeted *fim*-like operons in both SL1344 and CSH50. There are several operons with strong sequence homology and organisation to the well-characterised type 1 fimbriae operon (Nuccio and Baumler, 2007). A recent study has shown several of these operons are poorly expressed in laboratory conditions but express fimbriae that promote adhesion to both abiotic and/or epithelial cells (Korea *et al.*, 2010). This present study found OmpR was enriched at the fimbrial-like operon *ycb* in CSH50 and its orthologous *bcf* (synonym *elfA*) operon in SL1344 (Fig. 5.16 A+B, respectively). In *E. coli* the *ycb* operon is cryptic under laboratory conditions; Korea *et al.* showed overexpression the *ycb* operon, in a Δfim background, results in the expression of short rigid fimbriae. Furthermore, the *ycb* operon was capable of promoting biofilm formation on abiotic surfaces (Korea *et al.*, 2010). OmpR enrichment was localised within the *ycbQ* and *bcfA* genes that encode the major fimbrial subunit of the *ycb* and *bcf* operons in CSH50 and SL1344 respectively (see Fig. 5. 16). A second OmpR binding peak was identified further downstream in the *bcfE* gene in SL1344. It is clear OmpR targeting the *ycb* and *bcf* is conserved between species, however the OmpR enrichment profile was not mirrored and was affected by pH. Thus, OmpR may mediate a species-specific effect on the expression of these operons, however, this remains to be examined. Given that expression of these genes is quite poor in *E. coli* elucidating a role for OmpR may prove difficult. OmpR binding to these fimbrial operons in both species hints at a broader role for OmpR in regulation of surface-appendage expression and surface colonisation of abiotic surfaces.

The well characterised OmpR-regulated genes, *ompF* and *ompC*, were identified in the OmpR ChIP-chip data sets for both organisms (Table 5.9). In *E. coli*, OmpR reciprocally regulates *ompF* and *ompC* expression in response to changes in osmotic pressure (Alphen and Lugtenberg, 1977). Reciprocal regulation is mediated by osmotic induced changes increases in OmpR levels and hierarchical occupation of OmpR binding sites in the *ompF* and *ompC* promoters (Harlocker *et al.*, 1995; Yoshida *et al.*, 2006). At low osmolarity, *ompF* is predominantly expressed, but upon a shift to high osmolarity *ompC* is predominantly expressed with the concomitant repression of *ompF* (Huang *et al.*, 1994). However reciprocal regulation of *ompC* is not conserved in *S. Typhi* and *S. Typhimurium*, as expression of *ompC* is constitutive in response to changes in osmolarity (Martinez-Flores *et al.*, 1999). Although expression in response

A. *E. coli*



B. *S. Typhimurium*

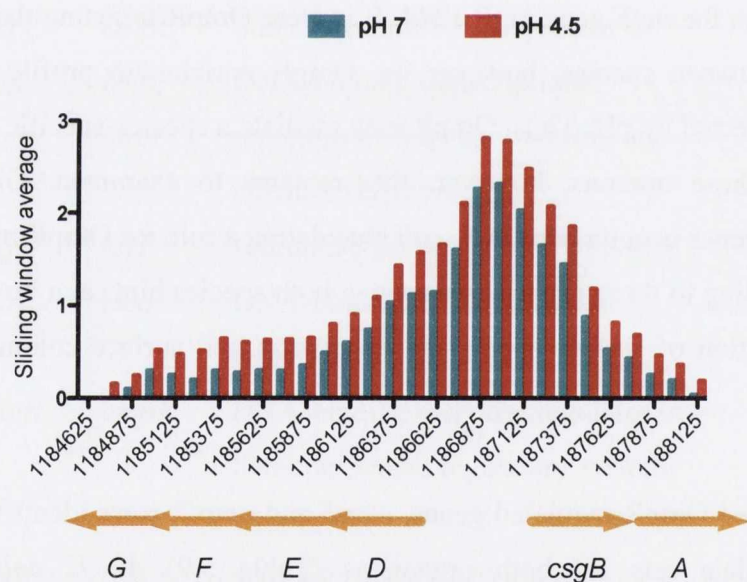


Fig. 5.15. OmpR binding at *csg* operon in *E. coli* and *S. Typhimurium*. OmpR binding at the *csg* operon in *E. coli* (A) and *S. Typhimurium* (B). Enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005). Genome co-ordinates are shown on the x-axis, the coloured arrows below represent genes. The location of these genes was determined using the Entrez Gene database at NCBI (Maglott *et al.*, 2011).

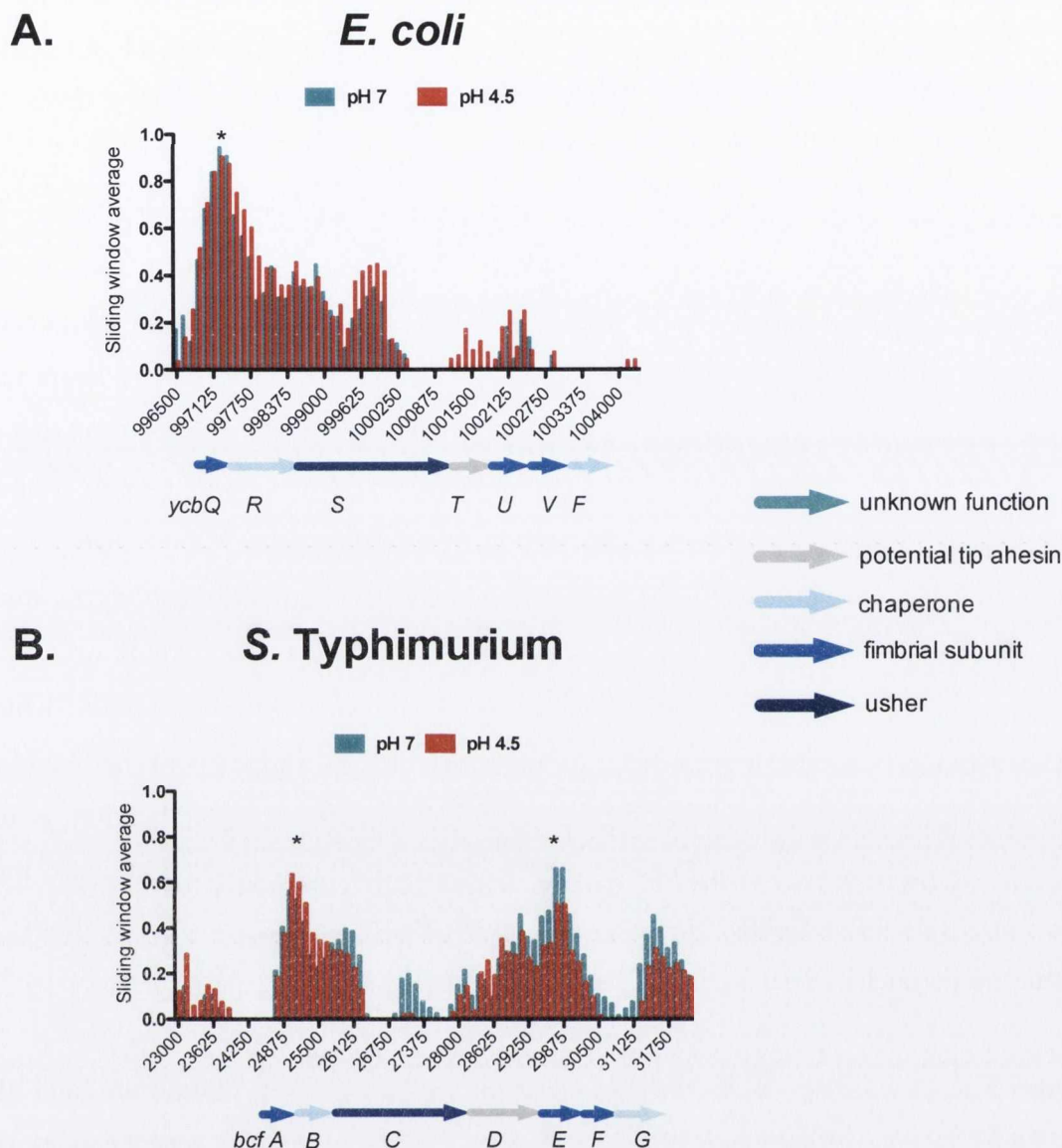


Fig. 5.16. OmpR binding at fimbriae-encoding operons in *E. coli* and *S. Typhimurium*. OmpR enrichment at the *ycb* operon in *E. coli* (**A**) and the orthologous *bcf* operon in *S. Typhimurium* (**B**). Enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005). Genome co-ordinates are shown on the x-axis, the coloured arrows below represent genes. The location of these genes was determined using the Entrez Gene database at NCBI (Maglott *et al.*, 2011) and Jbrowse (Kroger *et al.*, 2012). Key (right) shows predicted gene function (adapted from Korea *et al.*, 2010).

to osmolarity is not conserved between species, other studies have shown that *ompC* is induced by low pH in both *E. coli* and *S. Typhimurium* in an OmpR-dependent manner (Thomas and Booth, 1992; Bang *et al.*, 2000; Sato *et al.*, 2000).

In this present study OmpR enrichment at the *ompF* promoter was unaffected by changes in pH in either organism (Fig. 5.17, A). In *E. coli*, OmpR enrichment at the *ompC* promoter was unaffected by pH (Fig. 5.17, B, left), however, in *S. Typhimurium*, OmpR was enriched at low pH at *ompC* (Fig. 5.17, B, right). These data indicate that OmpR binding the *ompC* promoter is not conserved between these organisms and is pH-dependent in *S. Typhimurium*. Although previous studies have found *ompC* is acid-inducible in both species (Thomas and Booth, 1992; Bang *et al.*, 2000; Sato *et al.*, 2000), comparative analysis of *ompC* expression under the conditions used in this study is required to investigate the implications of differential levels of OmpR on *ompC* regulation. The *ompC* promoter also encodes the small non-coding RNA *micF*, which inhibits *ompF* translation by forming a 20-bp imperfect RNA duplex with the 5' end of *ompF* mRNA at the translation-initiation region (Mizuno *et al.*, 1984; Schmidt *et al.*, 1995). Therefore the pH-dependent, differential levels of OmpR binding at *ompC* may have implications for the post-transcriptional regulation of *ompF* in both *S. Typhimurium* and *E. coli*.

OmpR was also enriched at the *yedR-ompS1* region in *S. Typhimurium* and the equivalent *yedR-yedS* region in *E. coli* (Fig. 5.17, C). The *ompS1* gene encodes the quiescent OmpS1 porin, previous studies have shown OmpR directly regulates expression of the *ompS1* gene in *Salmonella* Typhi and Typhimurium (Flores-Valdez *et al.*, 2003). The sRNA, *rseX*, is encoded in this intergenic region; RseX targets *ompC* and *ompA* mRNA and is predicted to down-regulate their translation by binding to the region ribosome binding sites (Douchin *et al.*, 2006). In this study, OmpR enrichment at *rseX* was pH dependent; specifically, OmpR binding was higher at low pH in *E. coli* but unchanged in *S. Typhimurium* (Fig. 5. 17, C). It is tempting to speculate that OmpR binding to this location regulates expression of RseX and in turn post-transcriptionally regulates the expression of *ompC* and *ompA*. Furthermore, OmpR may differentially regulate RseX in both organisms in a pH-dependent manner. Further investigation is required to identify if OmpR regulates *rseX* expression, northern blot analysis

E. coli

S. Typhimurium

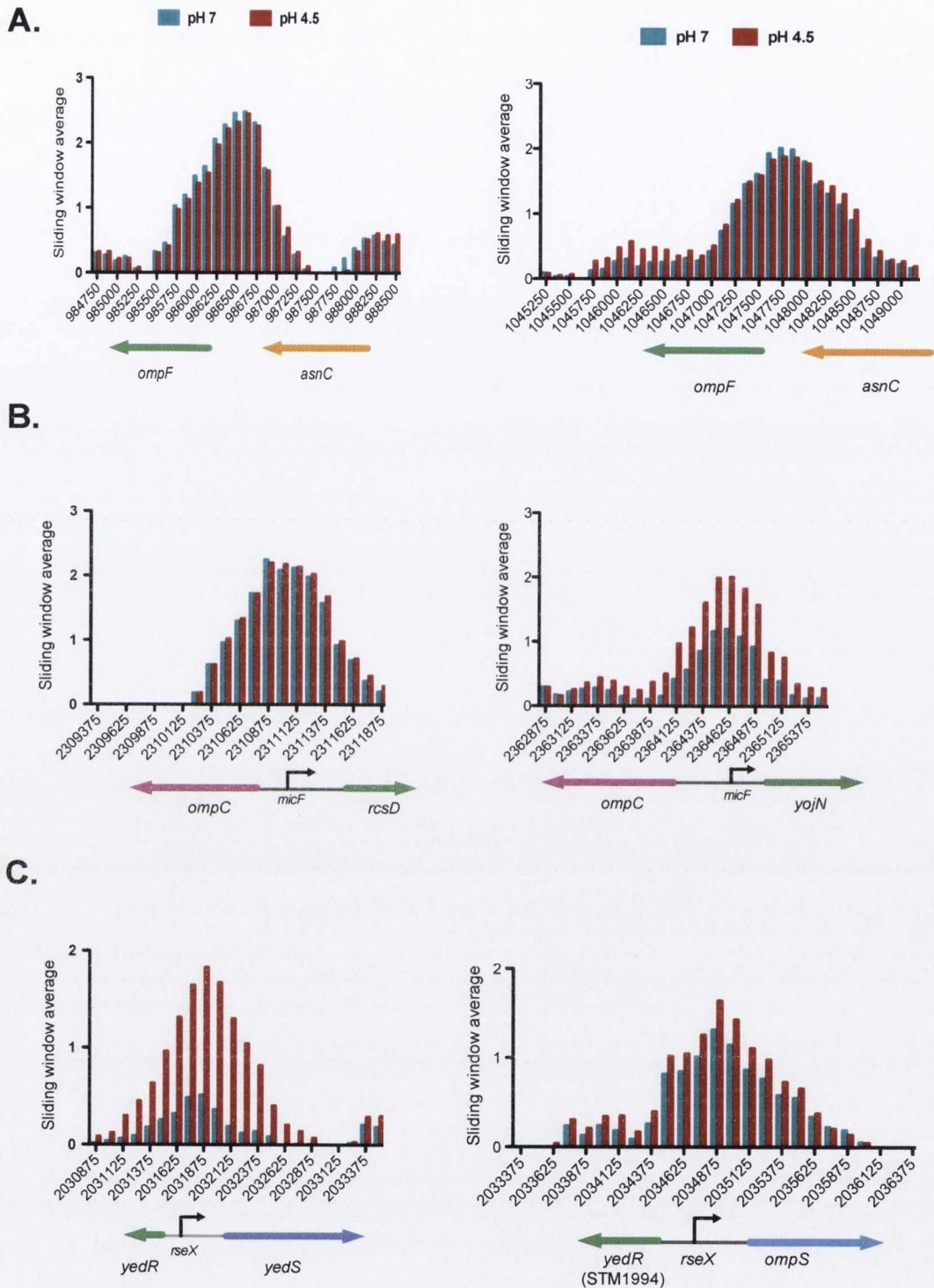


Fig. 5.17. Binding of OmpR at common targets in *E. coli* and *S. Typhimurium*. OmpR binding at *ompF* (A), *ompC* (B) and *rseX* (C) in *E. coli* (left panel) and *S. Typhimurium* (right panel). Enrichment at pH 7 (teal) and pH 4.5 (red) is shown on the y-axis as determined using ChIPOTle (Buck *et al.*, 2005). Genome co-ordinates are shown on the x-axis, coloured arrows below represent genes, the location of these genes was determined using the Entrez Gene database at NCBI for *E. coli* (Maglott *et al.*, 2011) and Jbrowse for *S. Typhimurium* (Kroger *et al.*, 2012).

investigating RseX levels in wild-type and in an *ompR* mutant background could help answer this question.

The data presented here revealed there was limited overlap between the OmpR regulon in the two organisms, suggesting that OmpR function has become specialised in each species. Genes that were targeted by OmpR in both bacteria included factors involved in general colonisation such as curli and fimbriae. OmpR was also enriched at genes involved in the regulation of chemotaxis and motility such as *flhD* and *lrhA* (See Table 5.9). *flhDC* encodes the master flagellar regulatory complex FlhDC which activates genes involved in chemotaxis and motility. Studies have shown that OmpR binds to the *flhDC* promoter at two sites and represses transcription (Shin and Park, 1995). OmpR was also enriched at the *lrhA-alaA* intergenic region. The *lrhA* gene encodes the LysR-type regulator, LrhA, which has a global and indirect effect on motility and chemotaxis by binding and repressing expression of *flhDC*; as such *lrhA* mutants are hypermotile (Lehnen *et al.*, 2002). OmpR was enriched at these genes at pH 4.5, suggesting that OmpR may co-ordinate the repression of motility at pH 4.5.

Furthermore, OmpR targeted genes encoding outer membrane proteins OmpF and OmpC and was bound at genes encoding regulators of their expression such as *ompR* and the sRNA *rseX*. From this data set, it appears that OmpR has a conserved regulon in both *E. coli* and *S. Typhimurium* involved in expression of surface-associated organelles. Although OmpR targeted a conserved set of genes, the location and enrichment of OmpR binding was not always conserved and in some cases was affected by pH, as was evident at *rseX* and *ompC* (Fig. 5.17, B+C). This suggests that OmpR may indeed target a small set of conserved targets under the conditions used here, however, OmpR may mediate a differential regulatory effect on their expression.

5.3 Discussion

The aims of this chapter were to identify the genome-wide distribution of OmpR in both *S. Typhimurium* and *E. coli* and investigate the influence of acidic pH on OmpR binding in both organisms. At the amino acid sequence level, OmpR is identical in both species thereby allowing direct comparisons of OmpR binding to be made. Using an epitope-tagged OmpR fusion, global analysis of OmpR binding to DNA was performed using ChIP-on-chip analysis.

This study has found that OmpR binds across the genomes of both SL1344 and CSH50, with multiple highly enriched peaks and numerous weaker peaks. In *E. coli*, although OmpR bound throughout the chromosome only 67 peaks were identified at pH 7, decreasing to 49 at pH 4.5. In contrast, OmpR binding increased in *S. Typhimurium* from 85 peaks at pH 7 to 225 peaks at pH 4.5. This coincides with earlier results presented in section 3.2.2 showing an increase in OmpR protein levels at pH 4.5 in *S. Typhimurium* but not in *E. coli*. In addition, a species-specific difference in DNA topology was also observed. DNA became more supercoiled at low pH in *E. coli*, corresponding with a decrease in the number of OmpR peaks. This result agrees with findings from a previous study that identified OmpR has an increased affinity for relaxed DNA (Cameron and Dorman, 2012). In *S. Typhimurium*, DNA topology was relatively static and was unaffected by pH. However, it is hypothesised that local DNA topology may become more relaxed and thus, local levels of DNA relaxation together with an increase in OmpR protein levels may account for the higher number of OmpR peaks in *S. Typhimurium*. This is further supported by the following observation in this study; *ssrA* expression increased at low pH with a concomitant increase in OmpR occupancy (Fig. 5.9). Previous studies have shown *ssrA* is induced by DNA relaxation (Ó Cróinín *et al.*, 2006; Cameron and Dorman, 2012) and this leads to an increase in OmpR occupancy (Cameron and Dorman, 2012). Thus, the results presented here agree with a model whereby total OmpR protein levels in conjunction with global and local DNA topology affect OmpR binding at acidic pH.

In addition to DNA topology, other factors such as genome content had a high impact on the OmpR regulon. Examination into the species-specific OmpR targets in *S. Typhimurium* revealed OmpR bound to horizontally acquired genes, scattered

throughout the chromosome. OmpR was enriched at genes within the SPI-1; specifically, at *hilC* and *hilD* which have previously been described as OmpR targets (Osborne and Coombes, 2011; Cameron and Dorman, 2012). OmpR enrichment was observed at the *prg* operon encoding structural genes for the SPI-1 needle complex and also at SPI-1 effector genes located outside the SPI-1 locus. OmpR was also enriched within the SPI-2 locus, specifically, at the regulatory genes *ssrA/B* that has previously been observed (Lee *et al.*, 2000; Feng *et al.*, 2003; Cameron and Dorman, 2012). OmpR enrichment extended to novel OmpR targets such as the chaperone and effector genes *sseA* and *sseG*, respectively and the structural gene *ssaV*. As observed with SPI-1 OmpR also targeted SPI-2 effectors located outside the SPI-2 locus. Enrichment extended to an additional pathogenicity island, SPI-4, which is co-ordinately regulated by SPI-1; both loci are thought to be complementary during the intestinal phase of infection (Main-Hester *et al.*, 2008). OmpR enrichment at both SPI-1 and -4 suggests OmpR may be involved in co-ordinating the expression of the virulence determinants encoded in these loci.

At low pH, OmpR occupancy increased at targets located within SPIs, highlighting the importance of pH as an environmental signal for OmpR binding. Enrichment of OmpR at multiple pathogenicity loci that confer specific traits reflects the complexity of the relationship between OmpR and pathogenicity. This also sheds light on why *ompR* mutants are attenuated for virulence (Dorman *et al.*, 1989; Yoon *et al.*, 2009). Horizontally acquired genes can provide bacteria with novel traits and functions (reviewed by Juhas *et al.*, 2009; Boto, 2010), the biological significance of OmpR binding to these genes tells us; (i) the OmpR regulon in *S. Typhimurium* has evolved since its divergence from *E. coli*, (ii) OmpR has co-opted xenogeneic genes into its regulon, (iii) OmpR enables the bacterium to exploit the traits encoded by horizontally acquired genes through transcriptional regulation, as demonstrated by activating *ssrA* expression. A recent study has shown OmpR acts as an effective H-NS antagonist when assisted by local changes in DNA topology (Cameron and Dorman, 2012). H-NS is known to bind and silence the expression of xenogeneic DNA in *S. Typhimurium* (Navarre *et al.*, 2006); thus, OmpR amongst other proteins (e.g. HilA, SsrB and SlyA) can relieve H-NS mediated repression facilitating the expression of these genes under certain circumstances (Stoebel *et al.*, 2008; Cameron and Dorman, 2012). However, further work is required to investigate the role of OmpR at the novel OmpR targets

identified here and to investigate the interplay between OmpR and other transcription factors in fine-tuning SPI gene expression in *S. Typhimurium*.

An additional observation from these data was OmpR bound to several gene targets within the PhoP regulon in *S. Typhimurium*. Furthermore, OmpR bound, albeit weakly, to the *phoP* promoter and positively affected *phoP* expression. By directly regulating expression of *phoP*, OmpR could mediate an affect throughout the PhoP regulon whilst directly targeting a select few PhoP-regulated genes. OmpR bound the *mgtC* promoter and positively effected expression, particularly at low pH. *mgtC* encodes an inner membrane protein required for growth in low Mg^{2+} concentrations, intramacrophage survival and is not present in *E. coli* (Blanc-Potard and Groisman, 1997). Interestingly, of the PhoP regulated genes bound by OmpR, only *slyB* and *phoP* have orthologues in *E. coli*, neither of which was targeted by OmpR in *E. coli*. Moreover, the remaining genes that were targeted by OmpR are involved in virulence; thus OmpR may have integrated PhoP-regulated genes into its regulon as part of an adaptation to a pathogenic lifestyle. This may be true for other pathogenic organisms as OmpR was recently found to positively affect *phoP* expression in the pathogen *Yersinia pestis* (Gao *et al.*, 2011a). In *Yersinia pestis*, OmpR was identified as an important regulator for mediating resistance to phagocytosis and survival at low pH (Gao *et al.*, 2011a). Integration of bacterial regulatory networks, in particular those that are controlled by TCS, may allow target genes to respond to multiple environmental stimuli sensed by the TCS (Wang *et al.*, 2011). The biological significance of OmpR binding to *phoP* and PhoP targets may ensure appropriate expression of virulence genes in response to multiple environmental cues. For instance, the OmpR/EnvZ TCS is activated by low pH and low-magnesium levels activate PhoP/Q TCS, *S. Typhimurium* experiences both conditions inside host macrophages (Rathman *et al.*, 1996; Deiwick *et al.*, 1999; Rychlik and Barrow, 2005).

In *E. coli*, OmpR was enriched at the *cad* decarboxylase system, which acts to maintain pH homeostasis by removing intracellular H^+ from the conversion of lysine to cadaverine (Foster, 2004). OmpR binding to this locus highlights a potential route whereby OmpR may contribute to acid resistance in *E. coli*, however the link between OmpR binding expression of the *cad* genes requires further investigation. The *cad* locus may confer acid resistance on some organisms, however, in some pathogenic *E. coli* and *Shigella* an intact *cad* locus confers a pathogenic disadvantage (Maurelli *et al.*, 1998).

The *cadA* gene was identified as an anti-virulence gene; these are genes that are detrimental to a pathogenic lifestyle and are therefore selectively inactivated by the pathogen (Maurelli, 2007). In enteroinvasive *E. coli* and the closely related *Shigella* species the *cadC* and *cadA* genes, respectively, have been inactivated since their evolution from the commensal *E. coli* K-12 (reviewed by Maurelli, 2007). Studies have shown that the production of cadaverine has a negative effect on the pathogenesis of *Shigella flexneri*; specifically cadaverine attenuated influx of polymorphonuclear leucocytes across intestinal epithelium in a tissue culture model (McCormick *et al.*, 1999). Sequence analysis in four *Shigella* species revealed distinct genetic rearrangements of the *cad* region in each species indicating these mutations have occurred by convergent evolution (reviewed by Maurelli, 2007). Thus, OmpR also targets genes that in some related pathogenic species have been inactivated out of necessity to pursue a pathogenic lifestyle. OmpR in *E. coli* also targeted genes which are required for survival under various stresses: *emrKY*, *alaA* and *uspC*. The effect of OmpR binding at these genes is currently unknown. However, OmpR was not enriched at their orthologues in *S. Typhimurium*. Therefore, these genes represent part of the species-specific OmpR regulon identified under the conditions tested in this study.

Investigation of the core set of OmpR-regulated genes in both species identified only a small number of OmpR targets shared by both. Common genes that were targeted were involved in the expression of surface-associated organelles such as curli fimbriae, flagellae and the outer membrane proteins OmpF and OmpC. Novel targets included operons encoding fimbrial-like proteins *ycb* (Korea *et al.*, 2010) and sRNA *rseX* that is known to effect expression of *ompA* and *ompC* (Douchin *et al.*, 2006). These targets suggest OmpR shares a core function between both organisms under the conditions tested. However, OmpR binding was not mirrored at each gene, which suggests OmpR may differentially regulate these genes to meet species-specific requirements. Alternatively, these genes may be subject to species-specific regulation by other factors impacting OmpR binding as a result.

Limited overlap OmpR of enriched genes between these organisms highlights the evolution of the OmpR regulon towards more specialised function to meet the requirements of each organism. Similar results were identified in a comparative study investigating the RovA regulon in *Yersinia pestis* and *Yersinia enterocolitica* (Cathelyn

et al., 2007). The RovA protein is highly conserved between both species; 97% amino acid identity (Cathelyn *et al.*, 2007). The RovA regulon has little overlap between these species; many of the RovA targets in one species have no orthologue in the other species (Cathelyn *et al.*, 2007). Thus, the RovA and OmpR proteins are highly conserved between these species, yet each regulon has evolved since the divergence of these species resulting in a limited shared regulon. This also illustrates that changes in transcription factors is not necessarily a prerequisite for regulon evolution. However, the evolution of some regulons is driven by changes in orthologous proteins, as observed for the sensor kinase PmrB which is part of the PmrA/B TCS.

The PmrA/B TCS senses low Fe^{3+} and activates genes involved in polymyxin B resistance in *S. Typhimurium* and *E. coli* (Wosten *et al.*, 2000; Chen *et al.*, 2011). In *S. Typhimurium*, polymyxin B resistance is also induced in low Mg^{2+} concentrations by the PhoP/Q TCS. Low Mg^{2+} levels induce expression of the PmrD protein in a PhoP-dependent manner (Kox *et al.*, 2000). PmrD binds to phosphorylated PmrA (PmrA-P) and protects it from the phosphatase activity of the sensor kinase PmrB. This leads to activation of genes involved in polymyxin B resistance in low Mg^{2+} conditions (Kato and Groisman, 2004). In *E. coli* PmrD expression is also induced in low Mg^{2+} , however, this does not lead to polymyxin B resistance (Winfield and Groisman, 2004). This species-specific trait is due to difference in amino acid composition in PmrB affecting the phosphatase activity of the PmrB protein in both species (Chen *et al.*, 2011). In *S. Typhimurium*, PmrB has reduced phosphatase activity which leads to increased PmrA-P levels and this activates genes involved in resistance to polymyxin B (Chen *et al.*, 2011). In *E. coli*, PmrB has increased phosphatase activity, which reduces PmrA-P levels, in turn, genes involved in polymyxin B resistance in low Mg^{2+} conditions are not activated. Thus, evolutionary changes in the PmrB protein lead to an expansion in the PmrA regulon in *S. Typhimurium* (Chen *et al.*, 2011).

A previous study examining the PhoP regulon in *S. Typhimurium* and *Yersinia pestis* found only three genes were PhoP-regulated in both species (Perez *et al.*, 2009). Additional genes were identified that were directly PhoP regulated in one species but were indirectly regulated by PhoP in the other; in this case, the overall ‘logic’ for these genes is conserved but the pathway of regulation has changed (Perez *et al.*, 2009). Therefore, one cannot rule out the possibility that the OmpR regulon in *S. Typhimurium*

and *E. coli* may share more overlap in the form of genes that are indirectly regulated by OmpR. Furthermore, OmpR may target genes under a certain condition in one species and target the same genes under an alternative condition in another species. Accordingly, investigations into OmpR binding under alternative conditions are recommended.

In many cases binding of a transcription factor to DNA may not result in a transcriptional effect; in some cases the transcription factor may be bound to a gene that is not expressed under the conditions tested, or there may be unidentified genes or sRNAs located in this region (Wade *et al.*, 2007). Alternatively, the transcription factor may be performing other cellular functions not involving transcription, as is observed with nucleoid-associated proteins (NAPs) (Wade *et al.*, 2007). NAPs are generally characterised as DNA binding proteins, usually in high abundance, that function in chromosome organisation and may be involved in other cellular processes such as recombination, transcription and replication (reviewed by Luijsterburg *et al.*, 2006; Dillon and Dorman, 2010). The observation of OmpR binding peaks throughout the entire chromosome is reminiscent of that observed for the global regulator CRP. Grainger *et al.* used ChIP-on-chip to map CRP targets in *E. coli* and found CRP bound to many weaker sites across the entire chromosome (Grainger *et al.*, 2005). This is in contrast to transcription factors such as FNR and MelR that bind to a limited number of targets; specifically, MelR occupies only a single site (Grainger *et al.*, 2005; Grainger *et al.*, 2007). As such, Grainger *et al.* hypothesised that CRP, may be involved in organisation of the bacterial genome in addition to acting as a conventional transcription factor (Grainger *et al.*, 2005). A recent study highlighted parallels between OmpR and CRP that may enable these proteins to act as NAPs involved in chromosome organisation (Cameron and Dorman, 2012). Both OmpR and CRP are estimated at 3,500 and 3,000 molecules/cell respectively (Berg and von Hippel, 1988; Cai and Inouye, 2002); the high abundance of these proteins may facilitate their activity as transcription factors and perhaps as organisers of the genome structure (Cameron and Dorman, 2012). Unlike other NAPs such as Fis, H-NS and IHF, both OmpR and CRP are subject to modification i.e. phosphorylation and binding allosteric effector cAMP respectively. These proteins may bind throughout the chromosome, however, modifications which alter the DNA binding activity of both proteins may drive protein binding to specific-target promoters (Cameron and Dorman, 2012). It is tempting to

speculate the numerous OmpR peaks found throughout the chromosome in this study provide evidence in support of OmpR having a broad influence on chromosome organisation and function.

In conclusion, the results presented here expand current knowledge of members of the OmpR regulon in both *S. Typhimurium* and *E. coli*. This study has found low pH may be a major influencing factor affecting global levels of OmpR binding. Based upon results presented here and elsewhere (Cameron and Dorman, 2012) it is hypothesised that changes in DNA topology, OmpR protein levels and genome content are important factors driving changes in the OmpR regulon. This study found there were few shared members of the OmpR regulon between these organisms. Members of the core OmpR regulon are associated with the regulation and expression of surface-associated organelles, whilst members of the species-specific regulon included genes involved in pathogenesis in *S. Typhimurium* and stress-management in *E. coli* (Fig. 5.18). Of particular interest is the finding that many OmpR targets have no orthologues in *E. coli*; indicating the OmpR regulon has evolved since the divergence of these species. Different members of bacterial regulons are thought to contribute to the development of species-specific traits and lifestyles (Perez *et al.*, 2009). Thus, the co-option of virulence genes into the OmpR regulon identifies a key role for OmpR in determining the success of *S. Typhimurium* as a pathogen.

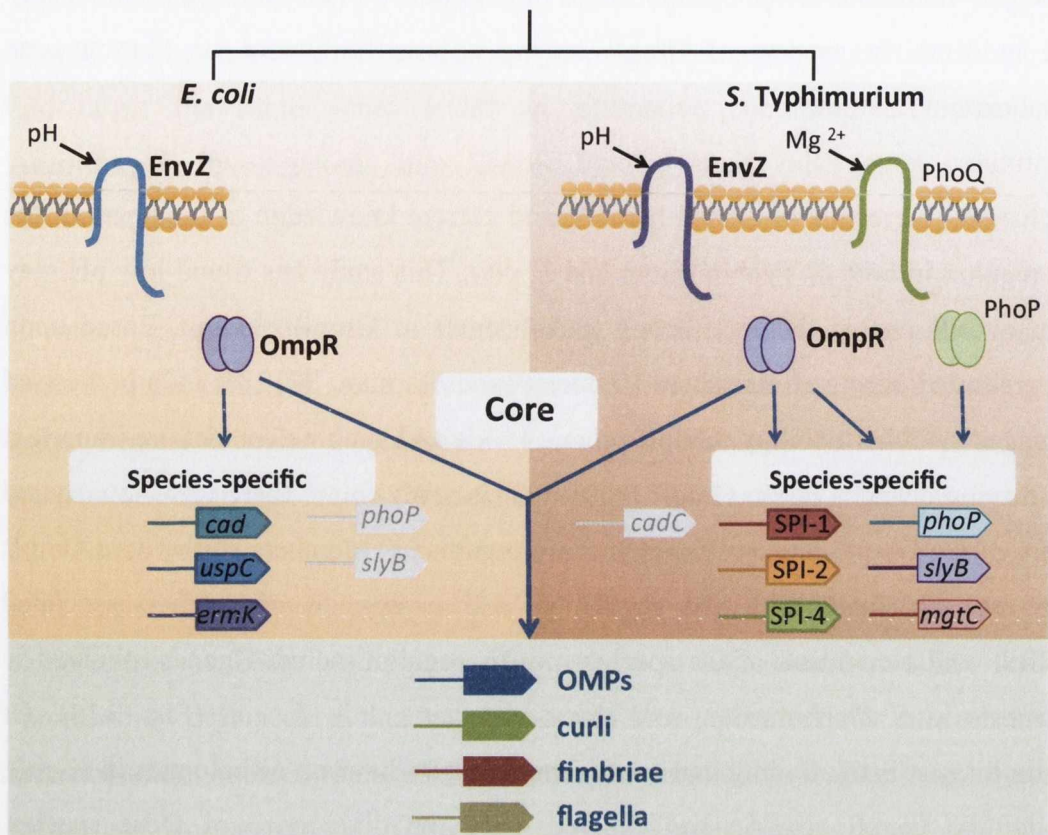


Fig. 5.18. Summary of the OmpR binding in *S. Typhimurium* and *E. coli*. OmpR (purple pairs of circles) binds to a species-specific regulon in both *E. coli* and *S. Typhimurium*, examples of which are shown. In *E. coli*, OmpR binds to genes involved in acid resistance and stress resistance. In *S. Typhimurium*, OmpR binds to pathogenicity islands SPI-1, -2, and -4, in addition OmpR binds to genes regulated by PhoP including the *phoP* gene itself. The genes identified as the core OmpR regulon, bound by OmpR in both species, were surface-associated organelles and proteins. Outer membrane proteins OMPs.

Chapter 6 General discussion

6.1 Discussion

Two-component systems are bacterial signalling pathways that involve a combination of protein-level reactions. In many cases these systems regulate gene expression to elicit a response to changing conditions (McAdams *et al.*, 2004; Capra and Laub, 2012). Typically, related organisms rely on orthologous systems to sense and respond to these conditions. However, in closely related species these orthologous systems can be differentially expressed in response to the same stimulus (Perez and Groisman, 2009). Studies in *E. coli* have shown the OmpR/EnvZ TCS senses changes in osmolarity (Alphen and Lugtenberg, 1977; Sarma and Reeves, 1977; Slauch *et al.*, 1988; Wang *et al.*, 2012). However, more recent studies showed that the orthologous OmpR/EnvZ system in *S. Typhimurium* is not activated by changes in osmolarity but instead *ompR/envZ* expression is activated by low pH (Bang *et al.*, 2000; Bang *et al.*, 2002). Therefore, the aims of this study were to investigate the effect of low pH on *ompR* expression in both organisms. Detailed analysis of *ompR* regulation in *S. Typhimurium* was also conducted followed by genome-wide analysis of OmpR binding in both *S. Typhimurium* and *E. coli* using ChIP-on-chip technology. The results and interpretations of this work were discussed in chapters 3, 4 and 5; the key findings and future work following on from this investigation will be discussed in this concluding chapter.

At low pH the *ompR/envZ* operon is differentially expressed in *S. Typhimurium* and *E. coli*; using qPCR and western blot analysis this study found *ompR* gene expression and OmpR protein levels were upregulated at low pH in *S. Typhimurium* as previously observed by Bang *et al.* (2000). In contrast, *ompR* gene expression and OmpR protein levels in *E. coli* were insensitive to acidic pH. Bioinformatic analysis comparing both organisms identified a 12% difference in the nucleotide composition of the *ompR* regulatory region and a 5% difference in the amino acid composition of EnvZ. The OmpR protein is 100% conserved at the amino acid level. To understand the genetic basis of *ompR* induction at low pH in *S. Typhimurium*, genetic re-wiring experiments were performed. Replacing the *ompR* promoter in *E. coli* with the *ompR* promoter from *S. Typhimurium* imparted pH sensitivity to the *ompR* gene in *E. coli*. Thus, the *ompR* promoter in *S. Typhimurium* was identified as the major contributing factor responsible for upregulation of *ompR* at low pH. Using 5' RACE it was demonstrated that the same

ompR TSS was active at low pH in both organisms (TSS-4.5); coupled with gene expression analysis it was deduced that TSS-4.5 was more active in *S. Typhimurium* at pH 4.5. TSS-4.5 was active in both organisms at pH 7; in *E. coli*, a second TSS was mapped upstream of TSS-4.5 to the same location as the previously identified TSS-1 in *E. coli* (Tsui *et al.*, 1991). When the *S. Typhimurium ompR* promoter was moved into *E. coli* it retained the *S. Typhimurium ompR* TSS profile and this was coupled with upregulation of *ompR* expression at low pH, although levels did not reach those observed in the wild type *S. Typhimurium* background. This highlights the fact that species-specific difference in *ompR* expression is due to the evolutionary changes in the *ompR* promoter, in particular the difference in TSS profile.

It is currently not clear what factors are responsible for the difference in *ompR* TSSs; this may be explained by differences in the *trans*-acting regulatory proteins that affect transcription. Alternatively, or indeed additively, *cis*-acting factors, such as protein binding sites or SIDD sites that assist in strand separation and thus, transcription initiation may also be responsible (Benham, 1993). Investigation into the SIDD sites present in the *ompR* regulatory region in both species predicts the *ompR* promoter in *E. coli* harbours regions that are more likely to undergo strand separation compared to *S. Typhimurium*. Specifically, the DNA upstream of TSS-1 is highly destabilised in *E. coli* and a second region downstream of TSS-1, encompassing TSS-4.5, is predicted to be further destabilised compared to *S. Typhimurium*. Furthermore, the global analysis of DNA supercoiling in both organisms showed DNA topology in *E. coli* is more supercoiled at low pH, compared to *S. Typhimurium*, which remained relatively unchanged. Given the *ompR* gene is sensitive to DNA supercoiling levels, it is hypothesised that the species-specific supercoiling differences, together with the difference in potential to undergo strand separation may be a contributing factor determining the location and strength of these TSSs in the *ompR* regulatory region of both organisms.

S. Typhimurium and *E. coli* share similar genetic regulatory mechanisms and display large regions of gene synteny (Fookes *et al.*, 2011), however, the results herein show that these organisms display differences in global supercoiling levels when both are exposed to low pH. An earlier study has shown that the supercoiling levels in these bacteria also differ in response to growth phase and osmotic pressure (Cameron *et al.*,

2011). DNA topology is responsive to a wide variety of environmental conditions such as temperature, osmotic pressure and pH (Goldstein and Drlica, 1984; Dorman *et al.*, 1988; Higgins *et al.*, 1988; Karem and Foster, 1993). Many genes respond to changes in supercoiling levels, this is thought to relay important information about the environment to the cell (Peter *et al.*, 2004). Thus, closely related organisms with different supercoiling levels have the potential to express orthologous supercoiling-sensitive genes differently. This can have detrimental effects on gene expression if genes are moved into new genetic backgrounds with different superhelical set points and that respond differently to environmental conditions (Cameron *et al.*, 2011). Indeed, transferring the *ompR* promoter from *S. Typhimurium* upstream of the *E. coli ompR* gene resulted in pH sensitivity of *ompR*, however the level of expression did not reach that observed in the wild-type *S. Typhimurium* background (Fig. 3.10). This may be the result of the species-specific supercoiling differences in the locale of the *ompR* gene.

Moving the *ompR/envZ* operon from *S. Typhimurium* downstream of the *ompR* regulatory region in *E. coli* was not sufficient to upregulate *ompR* expression in *E. coli* at low pH (Fig. 3.10). This again highlights the importance of the *S. Typhimurium ompR* regulatory region in the pH sensitivity of *ompR*. These results also indicate the differences in the EnvZ protein in *S. Typhimurium* are not responsible for the differential expression of *ompR*. However, *ompR* expression in *E. coli* was pH sensitive under the control of the *S. Typhimurium ompR* promoter; thus, demonstrating that the native EnvZ protein in *E. coli* is capable of functioning in the activation of *ompR* expression at low pH. Furthermore, as demonstrated in *S. Typhimurium* using an $\Delta envZ$ mutant (Fig. 4.9), the EnvZ protein is required for *ompR* expression at low pH. Therefore these results indicate that the EnvZ protein in both species is capable of participating in the activation of *ompR* at low pH. This raises questions for future work; how does EnvZ sense changes in pH? Does EnvZ sense changes in pH inside or outside the cell? A recent study has demonstrated using amide hydrogen/deuterium exchange mass spectrometry (HDXMS) that the EnvZ protein in *E. coli* senses osmolarity through its cytoplasmic domain (Wang *et al.*, 2012). Specifically, high osmolarity stabilises the four-helix bundle containing the His²⁴³ residue required for autophosphorylation as well as phosphor-transfer to OmpR (Wang *et al.*, 2012). EnvZ may use the cytoplasmic domain to sense changes in pH and may do so by directly sensing an influx of H⁺ in acidic conditions. This hypothesis remains to be tested and future work using

techniques such as HDXMS may further our understanding of environmental sensing by EnvZ and offer insights into how sensor kinases integrate multiple signals into their sensing repertoire.

One of the important aims of this work was to investigate *ompR* regulation at low pH in *S. Typhimurium*. The roles of IHF and CRP in *ompR* expression in *E. coli* was previously described (Tsui *et al.*, 1991; Huang *et al.*, 1992); their effect on *ompR* expression in *S. Typhimurium* was previously unknown. This study found the IHF-I and IHF-III binding sites in the *ompR* promoter were conserved in *S. Typhimurium*. The IHF-I site was responsible for mild repression of *ompR* expression at low pH, however, derepression of *ompR* required the IHF-III site. In *E. coli*, the IHF-I site overlaps the TSS-1 promoter and IHF represses expression from this site by inhibiting RNAP binding (Tsui *et al.*, 1991). In *S. Typhimurium* the IHF-I and IHF-III sites are located upstream of TSS-4.5; it is thought IHF may regulate *ompR* expression in a different manner than *E. coli* by modulating DNA topology and introducing DNA bends in the *ompR* promoter which finely-tune *ompR* expression. As OmpR is a regulator of the stationary-phase ATR, the IHF protein is well suited as a regulator of *ompR* expression; IHF levels are known to peak at the transition from exponential growth to stationary phase (Bushman *et al.*, 1985; Ditto *et al.*, 1994). Thus, IHF may function as a growth stage reporter for *ompR* expression during the stationary phase ATR.

This work has also found that the CRP binding site is conserved in the *ompR* promoter in *S. Typhimurium*. Using glycerol as a carbon source to elevate cAMP levels, activating CRP, resulted in similar levels of *ompR* expression at both pH 7 and pH 4.5. This pattern of expression is facilitated by the CRP-I site; CRP binding here positively regulates *ompR* expression at pH 7 and negatively regulates expression at pH 4.5. As a metabolic sensor CRP may play an important role in coupling *ompR* expression with the energy state of the cell and external environment. Repression of *ompR* expression during growth on a poor carbon source at low pH may be an adaptive mechanism by the cell to downregulate the expression of OmpR activated genes at low pH. Furthermore, results from this study and work performed elsewhere (Cameron, A.D., unpublished results), indicates there is a second CRP binding site in the *ompR* promoter. The physiological relevance and the mechanism underlying CRP regulation, in particular the contribution of the second putative site requires further investigation.

The *ompR* gene in *S. Typhimurium* is positively autoregulated by the OmpR protein, which binds to four sites in the *ompR* promoter (Bang *et al.*, 2002; Cameron and Dorman, 2012). A key finding that has emerged from this work is the importance of the OmpR-II site for *ompR* expression at both pH 7 and pH 4.5. Mutations in this site had detrimental effects on *ompR* expression, in particular at pH 4.5. This site plays an important role in the antagonism of H-NS mediated repression and is required in the absence of H-NS to activate *ompR* expression. ChIP-qPCR experiments found RNAP binding to the *ompR* promoter did not require the OmpR protein. However, as the OmpR-II site is required for *ompR* expression it is hypothesised that this site may act post-recruitment of RNAP. Currently two mechanisms of transcription activation have been proposed for OmpR; the OmpR-II site may facilitate OmpR contact with the α CTD of RNAP or facilitate OmpR-mediated transmission of superhelical energy encompassed in the SIDD site to the downstream acid-inducible promoter (Igarashi *et al.*, 1991; Slauch *et al.*, 1991; Cameron and Dorman, 2012). The results presented in this study also found in the absence of an intact OmpR-II site, RNAP binding to the *ompR* promoter was reduced compared to an $\Delta ompB$ mutant (Fig. 4.10). This indicates that in the absence of an intact OmpR-II site, OmpR binding elsewhere in the promoter negatively affects RNAP binding. The OmpR-I site overlaps the -10 element of the TSS-4.5 promoter; this site is well placed to affect RNAP binding. OmpR is known to act as both an activator and repressor of the porin genes *ompF* and *ompC*; several studies have shown OmpR employs a hierarchical arrangement of OmpR binding sites to reciprocally regulate *ompF* and *ompC* expression (Rampersaud *et al.*, 1994; Head *et al.*, 1998; Yoshida *et al.*, 2006). Multiple OmpR binding sites have been identified in the *csgD* and *ssrA* promoter where OmpR has been proposed to act as both an activator and repressor of gene expression (Feng *et al.*, 2003; Gerstel *et al.*, 2003). In other cases, such as the *tppB* promoter, only a single OmpR binding site is present (Goh *et al.*, 2004). To date, four OmpR binding sites have been identified in the *ompR* promoter (Bang *et al.*, 2002; Cameron and Dorman, 2012); analysis of their individual and co-operative contribution to *ompR* expression provides scope for future studies.

This study also aimed to identify the genome-wide distribution of OmpR in both *S. Typhimurium* and *E. coli* and to investigate the influence of low pH on OmpR binding in both organisms. OmpR was enriched across the genome in both species, but perhaps the most striking difference was the increase in the number of OmpR peaks at low pH in

S. Typhimurium (Fig. 5.4). This was in contrast to the number of OmpR peaks in *E. coli* which was reduced at low pH. These results coincide with species-specific differences in DNA topology and OmpR protein levels at low pH. The former supports the model by Cameron and Dorman (2012) showing OmpR occupancy increases as DNA relaxes. In *S. Typhimurium*, it may be argued that although DNA is not globally affected by pH it was affected more locally. For example, in this study *ssrA* expression was induced and had an increased level of OmpR occupancy at low pH. This is in agreement with earlier studies that demonstrated *ssrA* expression and OmpR occupancy of the *ssrA* promoter increases when DNA is relaxed following inhibition of DNA gyrase with the drug novobiocin (Ó Cróinín *et al.*, 2006; Cameron and Dorman, 2012). These results raise the question of the importance of DNA topology in the evolution of bacterial regulons? DNA supercoiling serves to compact of the bacterial chromosome into the cell; however, it is also has a global role in environmental signalling and gene expression (Peter *et al.*, 2004). As discussed earlier species-specific differences in DNA supercoiling levels can impart different expression patterns on orthologous supercoiling-sensitive genes (Cameron *et al.*, 2011). It is tempting to speculate that DNA supercoiling differences between closely related species also influences binding of regulatory proteins, such as OmpR and as such influences the ‘tendency’ of genes to be targeted by OmpR and recruited into the OmpR regulon. The second factor which may account for the higher number of OmpR peaks in *S. Typhimurium* is the OmpR protein levels. It is intuitive that an increase in OmpR protein levels at low pH agrees with an increase in the number of OmpR enriched peaks. Following on from these results, there are future questions which may prove more difficult to uncover; is the increase in OmpR levels in *S. Typhimurium* an optimisation strategy by the cell to meet the regulatory requirements of new gene networks such as horizontally acquired genes? Or have increased OmpR levels at low pH been co-opted by genes in *S. Typhimurium*? It was speculated in chapter 3 that upregulation of *ompR* at low pH may have occurred as a pathoadaptive mechanism to facilitate the expression of pH sensitive genes such as *ssrA* encoded on SPI-2. Investigation into *ompR* expression in additional *Salmonella* species such as *S. bongori*, may help elucidate the evolutionary significance of *ompR* expression at low pH.

Investigation of the members of the OmpR regulon in both species revealed there is little overlap between members of the OmpR regulon in *S. Typhimurium* and *E. coli*.

There were only 15 OmpR targets shared between both species, many of which were previously characterised as OmpR-regulated genes such as *ompF*, *ompC*, *ompR*, *csgD*, and *flhD*. Novel OmpR targets included the orthologous fimbrial-like operons *bcf* and *ycb* of *E. coli* and *S. Typhimurium* respectively. Despite the limited overlap, the shared OmpR regulon was composed of several genes which either regulate or are themselves surface-expressed organelles involved in motility, chemotaxis, biofilm formation and porin expression. In each organism OmpR targeted different sets of genes; many of the OmpR targets present in *S. Typhimurium* have no orthologues in *E. coli*. For example, OmpR targeted virulence genes within the *Salmonella* pathogenicity islands, many of which were previously uncharacterised as OmpR targets. At low pH, OmpR occupancy increased at targets located within SPIs, highlighting the importance of pH as an environmental signal for OmpR binding. These results also suggest that the OmpR regulon has expanded since the divergence of these species. This study has also highlighted a previously unrecognised relationship between the OmpR and PhoP regulons in *S. Typhimurium*; specifically, OmpR targets some members of the PhoP regulon. Many of these targets, with the exception of *phoP* and *slyB*, are absent from *E. coli*; this indicates the incorporation of these PhoP-regulated genes into the OmpR regulon has also occurred after the divergence of these species. This study has thus far shown OmpR has a regulatory effect on *phoP*, *mgtC* and *mig-14* expression; while further work is required to establish the role of OmpR at these genes, many of them are involved in determining the virulent phenotype of *S. Typhimurium*. The integration of the OmpR and PhoP regulatory circuits may allow target genes to respond to multiple environmental signals (i.e. low pH and Mg^{2+}). This may enable the cell to execute a virulence programme and adapt to multiple environmental signals present in the host milieu.

Bacteria display a high degree of plasticity in their genome content, this is due prevalence of horizontal gene transfer amongst bacteria (McAdams *et al.*, 2004; Perez and Groisman, 2009). By introducing new genes into existing genomes, horizontal gene transfer facilitates evolutionary innovation and adaptation. For cells to benefit from newly acquired genes they must be successfully integrated into the cells regulatory network (McAdams *et al.*, 2004). This typically requires the ancestral regulatory proteins to govern the expression of newly acquired genes and generally, as a result, horizontally acquired genes undergo more complex regulation than native genes (Price

et al., 2008). This may reflect the need to preserve the fitness strategies of the host by preventing detrimental expression of incoming genes (Perez and Groisman, 2009). Certain factors make the OmpR protein suitable to bind and regulate horizontally acquired DNA. To date, searches for an OmpR consensus sequence using the OmpR binding sites in the *ompF* and *ompC* promoters have identified a GXXAC motif (Harlocker *et al.*, 1995; Yoshida *et al.*, 2006). Analysis using additional sites from other OmpR regulated genes revealed a highly degenerate, yet A+T-rich, OmpR binding motif (Vuong and Misra, 2011). Furthermore, OmpR displays low specificity for DNA (Rhee *et al.*, 2008). Typically, horizontally acquired DNA is richer in A+T composition compared to the core genome; this is thought to recruit the H-NS protein that binds A+T-rich yet-highly-degenerate DNA sequences. Regulatory proteins with relatively degenerate target specificities are more likely than site-specific regulators to ‘recognise’ horizontally acquired genes and integrate them into global regulatory networks (Navarre *et al.*, 2007). OmpR is therefore well placed to act as a regulator of horizontally acquired DNA and this may serve to explain why OmpR has been recruited into regulating horizontally acquired virulence genes in *S. Typhimurium*. The low specificity of OmpR for DNA may also explain the observation of numerous weak peaks of OmpR binding detected throughout the genome in both *S. Typhimurium* and *E. coli*. Degenerate sequences and genome-wide binding allows OmpR to act as a global regulator of transcription. The presence of weaker sites scattered throughout the chromosome supports the view that OmpR has NAP-like properties (Cameron and Dorman, 2012). One of the shared functions of NAPs is their ability to impart structure and organisation to the chromosome (reviewed by Dillon and Dorman, 2010). Results from DNase I footprints of OmpR binding to the *ompR* promoter suggest OmpR binds to DNA and constrains a supercoiled like state (Cameron and Dorman, 2012). Although these characteristics provide a good basis that OmpR may function as a NAP, more evidence is required. Future research using single-molecule experiments, such as atomic force microscopy and optical tweezers, may be used to investigate the role of OmpR as a chromosome-shaping protein. In addition, many NAPs also act as transcriptional regulators (reviewed by Dillon and Dorman, 2010). The DNA binding properties of OmpR can be modulated through covalent modification (i.e phosphorylation) that increases the DNA binding affinity of OmpR (Head *et al.*, 1998; Feng *et al.*, 2003). OmpR may modulate its function between structural and regulatory roles through phosphorylation (Cameron and Dorman, 2012). Future work exploiting OmpR mutants

that mimic constitutively phosphorylated OmpR and unphosphorylated OmpR could be used to investigate if covalent modification modulates the NAP-like function of OmpR in the cell.

The work has shown the OmpR regulon has evolved since the divergence of *E. coli* and *Salmonella* from a common ancestor. Since this divergence, the OmpR regulon displays a propensity toward facilitating pathogenesis in *S. Typhimurium*. By continuing to improve our understanding of *ompR* regulation and the role of the OmpR/EnvZ TCS in pathogenesis this work could contribute to the development of improved therapeutic or preventive treatments. The absence of TCSs in humans has prompted research towards the development of inhibitors targeting these systems (Gotoh *et al.*, 2010). TCSs that perform essential functions and those that control virulence are of particular interest (Gotoh *et al.*, 2010). Targeting TCSs that control virulence draws appeal as it may be used as an alternative approach to antimicrobial therapy. Alternative strategies that inhibit virulence rather than bacterial growth may apply milder evolutionary pressure for the development of resistance (Cegelski *et al.*, 2008; Rasko and Sperandio, 2010). Given the importance of *ompR* for virulence in *S. Typhimurium* (Dorman *et al.*, 1989), the OmpR/EnvZ TCS is an attractive potential drug target. By understanding regulation of *ompR* there is a potential to control virulence without killing pathogenic bacteria. As the OmpR/EnvZ system is not essential for survival, targeting this system may apply less evolutionary pressure, or at least slow down the rate of resistance evolution. A recent study has found the compound obacunone, a limonoid found in citrus fruits, downregulates virulence in *S. Typhimurium* in an *envZ* dependent manner (Vikram *et al.*, 2012). The authors suggest that obacunone may serve as a lead compound for the development of antivirulence strategies against *S. Typhimurium* (Vikram *et al.*, 2012). By downregulating virulence bacteria are less able to colonise and infect the host, such drugs may be developed for use in combination with other antimicrobials in an effort to extend the potential lifespan of these drugs (Rasko and Sperandio, 2010).

To summarise, this study has extended our current knowledge on *ompR* expression and regulation at low pH and using ChIP-on-chip has identified additional members of the OmpR regulon. The OmpR protein is conserved in both species, despite this, there is limited overlap between the OmpR regulon in both *E. coli* and *S. Typhimurium*, with many different regulon members in *S. Typhimurium* having no orthologue in *E. coli*.

This bears striking similarities with other regulators such as RovA and PhoP. The RovA regulon in *Yersinia enterocolitica* and *Yersinia pestis* have limited overlap; many of the remaining targets are not shared and are species-specific (Cathelyn *et al.*, 2007). Likewise, the PhoP response regulator targets numerous genes in *S. Typhimurium* that have no orthologues outside the genus *Salmonella* (Perez *et al.*, 2009). This work has added to our understanding of how the evolutionary changes in orthologous systems can have an impact on gene expression and bacterial regulons. The adaptive capacity of bacteria is reflected in their ability to alter gene expression patterns and regulon members in response to the same external stimulus and changes in genome content. Comparative studies will continue to improve our understanding of the extent to which bacterial regulons are conserved across species and factors that drive these evolutionary changes.

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