The Role of the BipA GTPase in

Salmonella enteritidis

By

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ABSRACT

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The BipA protein is a novel GTPase of the ribosome-binding GTPase superfamily that is highly conserved in eubacterial and plants. Recent studies suggest that it regulates virulence-associated processes in enteropathogenic *Escherichia coli*, raising the question of whether it has a more general role in the pathogenesis of enteric bacteria. To address this issue, a *bipA* null mutant of *Salmonella enteritidis* has been constructed, validated, and characterised in *in vitro* and *in vivo* assays.

In vitro, the mutant showed no significant growth defects but positively regulated the expression of several surface appendages including SEF14, SEF17 and flagella. Consistent with the flagella results, BipA also increased the cell motility of *S. enteritidis*. Conversely, BipA was shown to negatively regulate the expression of SEF21 and another fimbrial system, possibly the plasmid encoded fimbriae (PEF). Growth of the mutant in cultured macrophages was assessed using a gentamicin resistance assay, where a decrease in its survival, relative to the parent strain, was observed. Moreover, the mutant had an impaired response to oxidative stress.

In vivo studies were used to compare the invasion/colonisation characteristics of the *bipA* null mutant with those of the wild type parent strain. Using a one day old chick model, only a slight reduction in the number of CFU for the mutant was found in the liver and spleen compared to the wild type strain. However, studies with the mouse model of infection indicated that the *bipA* mutant is not significantly attenuated relative to the parent strain.

Taken together, these results suggest that the BipA GTPase plays a more significant role in survival within the environment than in the host.

Analysis of the genes downstream of *bipA* uncovered a unique 5.8kb region found only in specific *Salmonella* serovars. This region was found to have a lower GC content, compared to that of the normal *Salmonella* chromosome, which suggested that it had been acquired by horizontal gene transfer and hence might encoded pathogenicity-related components

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CHAPTER 1

1 Introduction

1.1 *Salmonellae*

Salmonella is a global pathogen and no population is spared. Members of the *Salmonellae* are named after the pathologist D.E. Salmon who first isolated *S. choleraesuis* from porcine intestine. *Salmonellae* belong to the family of Enterobacteriaceae and are gram-negative, peritrichously flagellated and faculatively anaerobic bacilli. The *Salmonella* genus contains at least 2,463 serotypes that are traditionally distinguished by the Kauffmann-White scheme (Kaufmann, 1952) and each serotype has been given their own species name. However, with the advent of DNA studies, *Salmonella* have now been reclassified as one species, *S. enterica*, that is divided into six distinct subgroups (Popoff and Le Minor, 1992). Accordingly, *S. enteritidis* should be referred to as *S. enterica* serovar Enteritidis. It was however proposed that for simplicity each *Salmonella* strain should be reported with its common serotype name (Euzeby, 1999). *Salmonellae* are widely dispersed in nature, being found in the gastrointestinal tracts of domesticated and wild animals, reptiles, birds and insects. They are effective commensals, as well as being pathogens that cause a spectrum of diseases in man and animals (Farmer and Kelly, 1991).

1.1.1 *Salmonella* serotypes and host range diversity

Salmonella serotypes can be classified according to their adaptation to human and animal hosts. *Salmonella typhi* and *Salmonella paratyphi* cause enteric fever in humans and higher primates only and have no other known hosts. *Salmonella dublin* and *Salmonella choleraesuis* cause systemic infection and gastroenteritis in calves and swine respectively. *Salmonella gallinarum* and *Salmonella pullorum* cause systemic disease in avian hosts. Species such as *Salmonella*

enteritidis and *Salmonella typhimurium* are able to cause disease in a wide range of animals and infection in humans can lead to gastroenteritis (Isaacson, 1998). *S. typhimurium* infection in mice, which mimics the systemic diseases caused by *S. typhi* in humans, is used as a model of systemic *Salmonella* infections as well as a general model system to study host-pathogen interaction at the molecular level. *S. enteritidis* is the subject of this thesis and will be discussed in more detail in the next section. Regardless of the symptoms it is thought that all types of *Salmonella* need to enter and survive within host cells in order to provoke disease (Slauch *et al.*, 1997).

1.1.2 *Salmonella enteritidis* – economic cost and control

In the last 20 years *S. enteritidis* has become the single most common cause of food poisoning in the UK and abroad (Baumler *et al.*, 2000). *S. enteritidis* is particularly adept at infecting and persisting within chicken flocks without causing visible disease and as a result detection and treatment are made difficult. *S. enteritidis* is therefore most commonly found in chicken carcasses, eggs and egg products. Government and public concerns about the increase in *S. enteritidis Salmonellosis* initiated investigations into both the causes and economic costs of these infections. It has been suggested that the recent increase in the rise of *S. enteritidis* infections may be the result of modern poultry farming practices. The mass production chicken farms allow the rapid spread of sub-clinical *Salmonella* infections (Pignato *et al.*, 1996). The cost of human *Salmonellosis* in England and Wales is ambiguous as it is thought that only 1 in 100 cases are reported. However, a conservative estimate for the cost of *S. enteritidis* infections in 1993 was suggested to be between £224 and £321 million (Roberts and Sockett, 1994).

The control of *Salmonella* in poultry has been implemented using several different methods including, antibiotic treatments, vaccination, and competitive exclusion. Antibiotic treatment is one of the least attractive methods for controlling *Salmonella* infections due to its expense and

the alarming increase in the appearance of antibiotic-resistant bacteria. This developed resistance is due to the increased use of antibiotics combined with the ability of the bacteria to develop resistance although this resistance is thought to confer a cost on the bacteria. Consequently, one strategy to reduce these resistant bacteria would be to decrease the use of these antibiotics which would result in the counter-selection against these strains. However, studies carried out by Bjorkman *et al.* (1998) indicate that this may not be the case as avirulent antibiotic-resistant bacteria rapidly accumulated second-site compensatory mutations that restored virulence and fitness without the loss of the resistance. This suggested that in an antibiotic free environment these mutations will allow the resistant bacteria to survive and compete successfully with sensitive strains (Bjorkman *et al.,* 1998).

Several different vaccines have been used to provide farmed flocks with immunity to *Salmonella* infection. Vaccination with live avirulent *Salmonella* can induce long-term protection against colonisation and invasion of *Salmonella* within intestinal, visceral and reproductive tract tissues. Also, colonisation of the yolk, albumen, and egg shells is prevented (Wilson *et al.,* 1990; Cooper *et al.,* 1994a; Hassan and Curtiss, 1997). Vaccination is however not full proof because at least 7-10 days are required for the stimulation of the acquired immune response and studies have shown that chicks are most susceptible to *Salmonella* infection for up to four days post hatch (Hinton *et al.,* 1989; Humphrey *et al.,* 1991; Nakamura *et al.,* 1993).

Rantala and Nurmi (1973) found that it was possible protect young chicks from *Salmonella* infection by the oral administration of caecal material derived from normal adult chickens. Bacteria from the natural microflora are thought to competitively exclude *Salmonella* which results in a reduction in the invasion of the bacteria. As a prevention of *Salmonella* infection many farms now exploit this method of competitive exclusion.

1.1.3 Epidemiology and disease state of *Salmonella* infections

Salmonella serovars cause a broad spectrum of diseases which range from self-limiting gastroenteritis to the more serious systemic disease typhoid fever. Recent estimates from the World Health Organisation (WHO) suggest that the incidence of non-typhoidal *Salmonellosis* is increasing worldwide. Serovars responsible for non-typhoidal disease account for approximately 1.3 billion incidences of acute gastroenteritis per year, and of these infections, approximately 3 million result in death (Pang *et al.,* 1995). In contrast, typhoid fever infections are decreasing and approximately 16.6 million typhoid cases per year result in 600,00 deaths (Pang *et al.,* 1995).

1.1.3.1 Gastroenteritis

Gastroenteritis due to *Salmonella* is often mild and self-limiting but can be severe and often fatal in the young, the elderly and severely immunocompromised patients (Salyers and Whitt, 1994). The most common source for a non-typhoidal infection in humans is through contaminated food via the faecal/oral route. Surprisingly, as little as 10^5-10^6 *Salmonella* need to be ingested to cause an infection and symptoms normally appear within 6-24 hours and generally last up to 7 days. They include fever chills, vomiting and nausea followed by abdominal cramps and diarrhoea (fluid secretion). The mechanisms by which *Salmonella* causes diarrhoea are poorly understood. However, *Salmonella*-induced fluid secretion is believed to be partly due to an influx of polymorphonuclear cells (PMNs) into infected mucosa (Wallis *et al.*, 1986). Wallis *et al.* (1989) found that infections with bacterial strains that did not exhibit this fluid secretion failed to trigger this influx. Cytotoxins and enterotoxins have also been shown to cause fluid accumulation *in vitro*. *S. typhimurium* encodes a cholera-like enterotoxin gene, *Stn*, which elicited fluid secretion when cloned into *E. coli* (Prasad *et al.,* 1992). *S. typhimurium* also produces a cytotoxin, Cyx, that has been liked to the induction of watery, sometimes bloody, diarrhoea (Reitmeyer *et al.,*

1986; Libby *et al.*, 1990). Additionally, given the inflammatory nature of diarrhoea the endotoxin, lipopolysaccharide (LPS), may contribute to diarrhoea (Finlay, 1994).

1.1.3.2 Typhoid fever

Typhoid fever and para-typhoid fever are severe systemic diseases caused by *S. typhi* and *S. paratyphi* respectively. The disease is usually initiated due to contaminated food or water via the faecal/oral route and generally as many as 10^6-10^9 organisms are needed to cause infection in healthy humans (Hornick *et al.* 1970). Symptoms of systemic disease normally include high fever, a flushed appearance, anorexia and other symptoms such as chills, convulsions and delirium can also occur. The infectious bacteria penetrate through to the intestine via the M cells and Peyer's patches (mechanism discussed in section 1.2.1) to the mesenteric nodes where they are able to infect mononuclear cells such as neutrophils and macrophages (mechanism discussed in section 1.2.3). After infection of the mesenteric lymph nodes they spread systemically, infecting the spleen, liver and bloodstream in large numbers (Miller *et al.* 1995b). The endotoxin activity of bacterial LPS can stimulate host cells to produce cytokines which results in fever and shock. Observations carried out by Hornick *et al.* (1970) showed that healthy volunteers injected with theses endotoxins produced symptoms of fever and abdominal pain similar to those seen in a typhoidal infection. Finally, the bacteria progress from the liver into the gall bladder and are shed into the intestine, where severe inflammation and ulceration of the Peyer's patches can result in death.

Salmonella is considered to be an important facultative intracellular pathogen and the mechanism of pathogenesis is discussed below.

1.2 *Salmonella* as a pathogen

Interest in bacterial pathogenesis has increased dramatically in recent years due to the emergence of new pathogens, antibiotic resistance and the lack of effective therapeutics. As a result,

knowledge in this field has increased and research into pathogens such as *Salmonella, Shigella, Yersinia* and *Listeria* have led the way in providing fresh insights into the molecular and cellular mechanisms of microbial pathogenesis. It is now clear that many pathogens share common mechanisms of interaction with the host, but each species has also evolved unique approaches to exploit host processes (Finlay & Cossart, 1997).

Salmonella is an intracellular pathogen that has the capacity to adhere to and cross the intestinal mucosal barrier, where it then invades and replicates within a restricted number of host cell types (Isaacson, 1998).

1.2.1 Bacterial Adhesion and Invasion of Phagocytic cells

Once *Salmonella* have passed through the stomach and survived the gastric barrier they will colonise the intestinal epithelial. The intestine provides several barriers to infection such as competition from natural microflora, osmotic stress, low oxygen levels, bile salts, pancreatic enzymes, secretory IgA antibodies and physical barriers including natural shedding of the gut lining and peristalsis (Finlay, 1995). Not only must the bacteria overcome these defences it must also be able to attach itself to the intestinal lining.

The precise site of entry for *Salmonella* is still controversial and appears to depend on the *Salmonella* serovar and the host it is infecting (McGovern and Slavutin, 1979). In humans the primary site of infection for non-typhoidal *Salmonellae* is in the distal part of the small intestine (Aleekseev *et al.,* 1960). At this entry site the *Salmonellae* will preferentially adhere to and invade specialised epithelial cells called M cells. M cells are found overlaying the lymphoid tissues of the Peyer's patches. They are phagocytic cells responsible for the uptake of antigenic components from the lumen and presenting them to the underlying cells of the immune system (Siebers and Finlay, 1996).

The best understood mechanism of adherence to host cells is the attachment of proteinaceous bacterial surface appendages known as fimbriae. Examination of the *S. typhimurium* genome identified twelve chaperone-usher-dependent fimbrial operons (Edwards *et al.*, 2000). However, only a few of these operons have been characterised and these include type 1 fimbriae (Collinson *et al.*, 1996b), long polar fimbriae (LPF) (Baumler and Heffron, 1995), plasmid encoded fimbriae (PEF) (Friedrich *et al.*, 1993), thin aggregative fimbriae (curli) (Sukupolvi *et al.*, 1997), the putative fimbrial gene cluster, *saf* (Folkesson *et al.*, 1999) and the novel *S. typhimurium* fimbrial operon *stfACDEFG* (Emmerth *et al.*, 1999). Type 1 fimbriae (encoded by *fimAICDHF* operon), which mediate mannose-sensitive agglutination of erythrocytes (Duguid *et al.*, 1966), are probably involved in the initial adherence. LPF (encoded by *lpfABCDE* operon) mediate adherence to murine Peyer's patches (Bäumler *et al.*, 1996) and PEF (encoded by *pefBACD* operon on the 90 kb virulence plasmid) mediate adhesion to murine small intestine (Bäumler *et al.*, 1996). In contrast to *E. coli*, where the adhesive function of certain fimbriae to enterocytes has been described, the specific role of fimbriae in *Salmonella* pathogenesis is poorly defined (Dibb-Fuller *et al.*, 1999). It is not clear whether these determinants just facilitate productive contact between the host and the bacteria or whether they play a direct role in bacterial entry.

Upon interaction with host cells, *Salmonella* elicit membrane ruffles in the apical membranes of M cells which results in the uptake of bacteria in membrane bound vesicles (Francis *et al.*, 1993; Jones *et al.*, 1993). Invasion of M cells rapidly leads to cellular destruction and the bacterial invasion of enterocytes or the dissemination of the bacteria into deeper tissues (Clark *et al.*, 1994; Jones and Falkow, 1994b; Jensen *et al.*, 1998). Invasion and destruction of M cells is essential in *Salmonella* virulence as mutants defective in these processes are avirulent (Kohbata *et al.*, 1986; Penheiter *et al.*, 1997).

The systems involved in M cell adherence, invasion and destruction are complex and they include the alternative sigma factor RpoS and the *inv* locus (encoded on *Salmonella* Pathogenicity Island 1; SPI-1) (Clark *et al.,* 1996; Nickerson and Curtiss, 1997; Clark *et al.,* 1998). Also, recent research has shown that that *S. typhimurium* DNA adenine methylase (Dam) mutants are attenuated for virulence and are defective in M cell invasion and destruction. Dam is thought to play a role in the regulation of Type III secretion genes encoded on SPI-1 (Garcia-del Portillo *et al.,* 1999). In addition, the *slyA* gene, originally identified as gene required for virulence and survival in macrophages (Libby *et al.,* 1994), is essential in cytotoxic destruction of M cells and the subsequent survival in the Peyer's patches, but not in colonisation or invasion (Daniels *et al.,* 1996; Watson *et al.,* 1999).

Once the bacteria have penetrated into deeper tissue they either replicate and establish a local infection or are taken up by macrophages that carry the bacteria via the lymphatic system to the liver and spleen to establish a systemic infection. The bacteria can also progress to the gall bladder and from there are shed into the intestine, where inflammation and ulceration of the Peyer's patches results in diarrhoea (Siebers and Finlay, 1996).

M cells are the most likely sites of entry for the bacteria because *Salmonellae* have developed the ability to survive the onslaught of macrophages (Jones, 1997; Slauch *et al.,* 1997). Invasion genes encoded in SPI-1 are required for the invasion of M cells (Galan and Curtiss, 1989; Clark *et al.,* 1998). However, SPI-1-deficient *S. typhimurium* strains, when inoculated orally, are still virulent in mice (Galan and Curtiss, 1989; Jones and Falkow, 1994a). This suggests that the bacteria are able to disseminate from the gastrointestinal tract without invading M cells or localising Peyer's patches. Recent evidence suggests that *Salmonella* also use a second route to penetrate across the intestinal barrier. Vazquez-Torres *et al.,* (1999) proposed that the bacteria are able to invade specific CD18-expressing phagocytes that reside in the gastrointestinal tract. The transmigration of the phagocytes across normal tissue barriers allows the bacteria to be

transported through the gastrointestinal tract into the blood stream where they can be carried to the liver and spleen (Vazquez-Torres *et al.*, 1999).

1.2.2 Bacterial Invasion of Non-Phagocytic cells

Salmonellae are also capable of inducing normal non-phagocytic epithelial cells to internalise bacteria. This type of invasion is an important feature of its pathogenicity as it enables the bacteria to reach deeper tissues where it can avoid host defence mechanisms. The entry mechanisms are complex and involve an intimate interaction between the bacteria and the host cell (Figure 1-1a). This interaction initiates a series of complex signal transduction processes that result in actin rearrangement and polymerisation at the host cell membrane and an accumulation of cytoskeletal proteins at the point of entry. These cytoskeletal rearrangements induce membrane ruffling and macropinocytosis that ultimately result in the engulfment of the bacteria in a membrane bound vacuole (Francis *et al.*, 1992) (Figure 1-1b & c). The membrane ruffling is, however, transient as once the bacterium is engulfed the architecture of the epithelial cells returns too normal (Francis *et al.*, 1992). Inside the vacuole the bacteria replicate and different vacuoles may coalesce to form large spacious vacuoles carrying numerous bacteria (Figure 1-1d). The intracellular trafficking of these vacuoles differs from that of other internalised particles as they appear to subvert the classical endocytic pathway (Garcia-del Portillo and Finlay, 1995). However, very little is known about the mechanism of how *Salmonella* alters this intracellular trafficking. Following trancytosis, *Salmonella* reach the basolateral surface of the epithelial membrane where they enter the reticuloendothelial system and are taken up by macrophages.

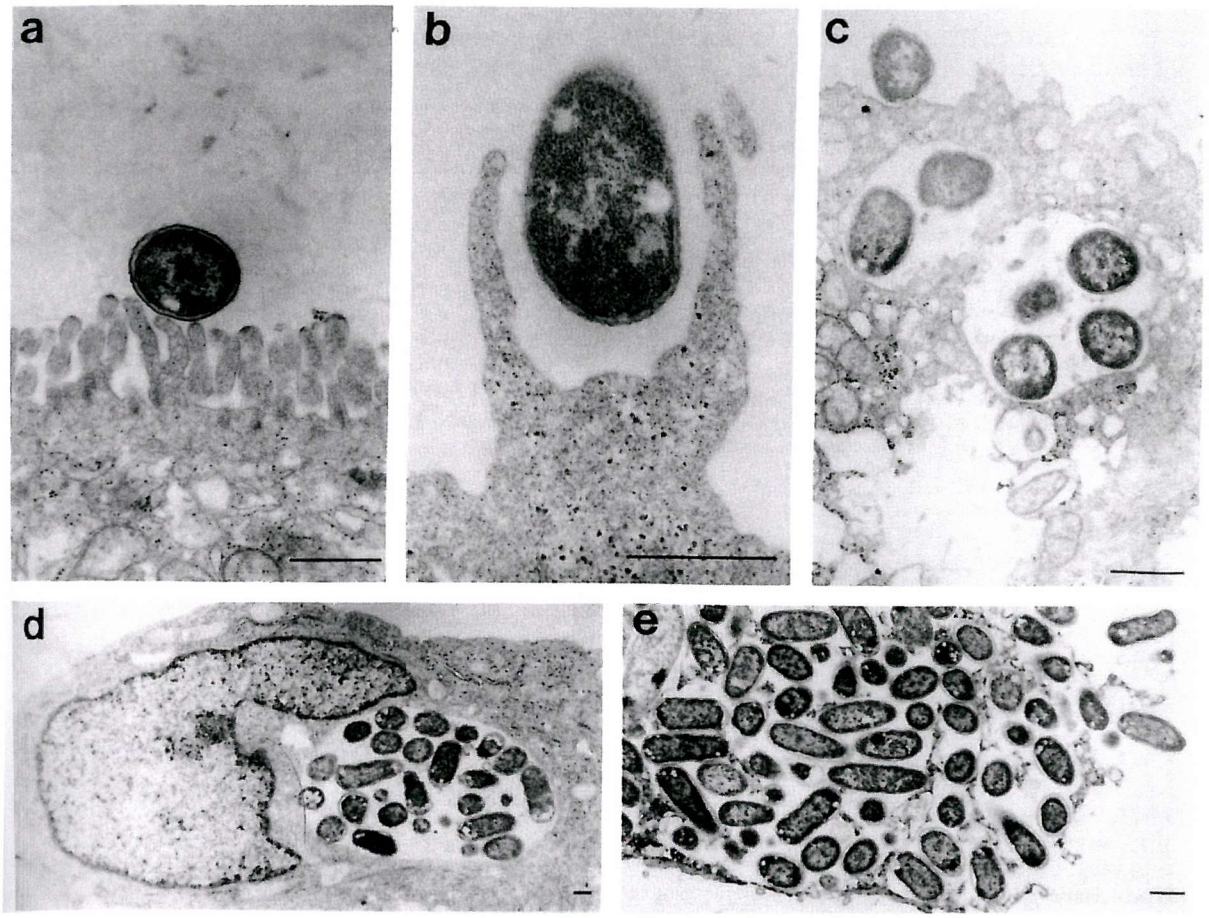


Figure 1-1 ***S. typhimurium*** **interactions with polarized human intestinal epithelial cells**

a. Bacterial adherence to microvilli on the apical surface. **b.** Engulfment and internalization of the bacterium. **c.** Bacteria inside vacuoles within the epithelial cell (1 hr post infection). **d.** Vacuoles containing bacteria coalesce and the bacteria multiply within the vacuole (12 hrs post infection). **e.** Release of the organisms occurs (24 hrs post infection). The bar represents 1 μm. (Picture obtained from Finley *et al.*, 1991)

1.2.3 Macrophage survival

Once the bacteria have crossed the epithelial barrier they will encounter macrophages that line the lymph nodes; these macrophages are the first effective barrier to prevent further spread. *Salmonella* must be able to survive within these macrophages to cause systemic disease as mutants defective in intracellular survival are avirulent in the mouse model of infection (Fields *et al.,* 1986). Macrophages possess both oxygen-dependent and -independent killing mechanisms to kill internalised bacteria. The primary oxygen-dependent mechanisms include the production of toxic oxygen molecules such as superoxide, hydrogen peroxide, and hydroxyl radicals within the phagosome. The oxygen-independent mechanisms include the acidification of the phagosome, the secretion of digestive enzymes and antibacterial cationic proteins within the phagosome (Beaman and Beaman, 1984; Elsbach and Weiss, 1985).

The internalisation of *Salmonella* into macrophages occurs either by conventional phagocytosis or by macropinocytosis. Phagocytosis leads to the bacteria being engulfed by the membrane and the formation of a tightly adherent phagosome (Finlay and Falkow, 1997), whereas macropinocytosis is characterised by the formation of membrane ruffles leading to the engulfment of the bacteria in spacious phagosomes (Alpuche-Aranda *et al.,* 1994). Once the bacteria are enclosed within the phagosomes the environment within these vacuoles becomes progressively acidic. It has been reported that *Salmonella* can significantly delay the acidification of the phagosome (Alpuche *et al.,* 1992). Also following engulfment the bacteria are normally exposed to toxic enzymes that are released when the lysosome fuses with the phagosome. Research has shown that *Salmonella* are able to inhibit this phagosome-lysosome fusion (Buchmeier and Heffron, 1991). Additionally, recent studies have shown that the bacteria are able to avoid exposure to the NADPH oxidase-dependent respiratory burst (discussed in section 1.3.4) as SPI-2 interferes with the trafficking of the oxidase-containing vesicles to the

phagosome (Vazquez-Torres *et al.,* 2000). It follows therefore that all these properties may contribute to the survival of *Salmonellae* within macrophages and in virulence.

The ability of the bacteria to adapt to and/or modify the phagosome is controlled by the two component regulatory locus, *phoP/phoQ* (Miller *et al.,* 1989b). PhoQ is a transmembrane protein that senses changes in the external environment and exhibits histidine kinase activity. When *Salmonellae* are engulfed by macrophages PhoQ senses the low Mg^{2+} and Ca^{2+} concentrations within the phagosome and subsequently autophosphorylates via a histidine kinase activity (Garcia *et al.,* 1996). The phosphate group is subsequently transferred to an Asp residue on PhoP and phosphorylated PhoP acts as a transcriptional activator (Groisman *et al.,* 1989; Volz, 1993) that positively and negatively regulates a subset of virulence genes, *pags* (*phoPQ*-activated genes) and *prgs* (*phoPQ*-repressed genes) (Miller *et al.,* 1989b). Miller and Mekalanos (1990a) reported that both constitutive and null mutants of the *phoP* regulon were attenuated for virulence and survival within macrophages. This suggested that a balanced expression of the *phoPQ*-activated and -repressed genes is needed for bacterial survival and propagation within the macrophage (Miller and Mekalanos, 1990a).

The *phoP/phoQ* locus is also involved in resistance to host antimicrobial peptides, regulation of the ATR and invasion through the host cell membrane (Fields *et al.,* 1989; Groisman *et al.,* 1992a; Behlau and Miller, 1993; Bearson *et al.,* 1998).

Several host cell responses and transduction pathways are triggered following *Salmonella* adhesion and invasion. These are discussed in more detail below.

1.2.4 Mucosal Immune Responses to *Salmonella* Invasion

One of the hallmarks of nontyphoidal *Salmonella* infections is the massive influx of neutrophils (polymorphonuclear leukocytes, PMNs) in both the large and small intestines that cause an inflammatory response that leads to symptoms of gastroenteritis (McCormick *et al.,* 1995).

Studies indicate that when the bacteria make intimate contact with the host a series of signal transductions are initiated which results in the secretion of cytokines from epithelial cells (McCormick *et al.,* 1993). These cytokines are thought to play an important role in the recruitment and trafficking of PMNs across intestinal epithelial cells to the site of bacterial infection (Miller and O'Byrne, 1995a). When bacterial contact is made the pro-inflammatory cytokine interleukin-8 (IL-8) is expressed at the basolateral surface of the epithelial cells and directs PMN migration through the lamina propria (Hobbie *et al.,* 1997). In contrast, the pathogen-elicited epithelial chemoattractant (PEEC) is expressed from the apical cell surface and directs PMN migration across the epithelial monolayer to the intestinal lumen (McCormick *et al.,* 1998). Even though the precise details of this mechanism are poorly understood it is thought that adherence to host epithelial cells and subsequent protein synthesis in both bacterial and epithelial cells is required to produce this phenomenon (McCormick *et al.,* 1993; McCormick *et al.,* 1995) . However, *Salmonella* invasion does not play a key role in this trafficking as blocking *Salmonella* invasion does not reduce PMN migration (Gewirtz *et al.,* 1999).

1.2.5 Transduction Pathways Triggered by Bacterial Adhesion and Invasion

A number of studies have shown that the host cell plays a role in *Salmonella* internalization as addition of specific actin microfilament polymerization inhibitors, Cytochalasins B and D, prevent the formation of membrane ruffles and the subsequent invasion. This suggests that an intact host cell cytoskeleton is needed for bacterial internalization (Bukholm, 1984). These host cell responses are likely to be the result of signal transduction triggered by *Salmonellae* when they adhere to the epithelial cells (Eckmann *et al.,* 1993).

In cultured epithelial cells *S. typhimurium* adhesion has been shown to trigger an increase in intracellular Ca^{2+}. Calcium ions activate a number of actin-binding proteins that are involved in the disassembly of actin filaments (Stossel, 1993). Therefore, an increase in calcium ions would

result in the increase of free actin monomers available for use in the assembly of new cytoskeletal structures. Strains containing mutations in invasion genes no longer show the Ca^{2+} flux or actin rearrangements (Pace *et al.*, 1993). Ruschkowski *et al.* (1992) found that invasion of *S. typhimurium* was coupled with an increase in inositol phosphate and that addition of chelators of intracellular Ca^{2+}, but not extracellular Ca^{2+}, inhibited *S. typhimurium* invasion. These results suggested that bacterial uptake is facilitated by *S. typhimurium* activation of host cell phospholipase C activity to form inositol phosphate which subsequently stimulates the release of intracellular calcium stores (Ruschkowski *et al.*, 1992).

Although the understanding of the signalling events that control cytoskeletal rearrangements and Ca^{2+} flux is incomplete, *Salmonellae* do have similarities to other bacteria that elicit both the cytoskeletal reorganisation and induction of gene expression. Central to this regulation are the small GTPases, *Rac*, *Rho*, and *Cdc42* (Nobes and Hall, 1999) that regulate actin-based structures involved in cell motility, cytokinesis, phagocytosis and intracellular transport processes (Finlay & Falkow, 1997). *Salmonella* entry appears to be *Cdc42*-dependent (Chen *et al.*, 1996) but *Rac*- and *Rho*-independent (Jones *et al.*, 1993).

1.2.6 Genetic Bases of *Salmonella* Entry

The molecular genetic bases of *Salmonella* invasion are very complex and involve many different genes from different loci. There are at least five main loci that are responsible for the expression of most of the determinants involved in *Salmonella* pathogenicity. These virulence gene clusters are referred to as pathogenicity islands, with each island assigned a SPI designation (*Salmonella* Pathogenicity Island).

1.2.6.1 SPI-1

SPI-1 (~ 40 kb) is located at 63 min on the *Salmonella* chromosome (Mills *et al.*, 1995). Most of the genes in SPI-1 encode a type III secretion system that is involved in protein secretion and

invasion of intestinal M cells and epithelial cells (Collazo and Galan, 1997). Type III secretion systems enable effector proteins to be translocated across the inner and outer membranes of the bacterium into the target host cell (Gauthier and Finlay, 1998; Hueck, 1998).

In vitro experiments show that SPI-1 is essential for *S. typhimurium* invasion of the intestinal epithelial cells and the lysis of macrophages. *In vivo*, SPI-1 mutants are not attenuated when inoculated intraperitoneally and are only moderately attenuated when administered orally (Galan and Curtiss, 1989; Jones and Falkow, 1994a) . This suggests that SPI-1 is primarily involved with the intestinal stage of the disease, which may include the initial killing of the first macrophages encountered after penetration of the epithelium. The ability of SPI-1 deficient strains being able to cause systemic infection without invading M cells, or localising Peyer's patches, may now be explained by the alternative route of invasion of *Salmonella* through the CD18-expressing phagocytes (Vazquez-Torres *et al.,* 1999).

1.2.6.2 SPI-2

SPI-2 (~ 25 kb) is located at 31 min on the *Salmonella* chromosome and encodes a second type III secretion system. This secretion system is required for intramacrophage survival and seems to play an auxiliary role in the development of systemic disease (Ochman *et al.,* 1996; Shea *et al.,* 1996).

In contrast to SPI-1, SPI-2 mutants are dramatically attenuated when administered systemically or orally. The SPI-2 mutants colonise the Peyer's patches, but are unable to spread to the mesenteric lymph nodes, liver or spleen (Cirillo *et al.,* 1998). Similarly, *in vitro* experiments show that within macrophages SPI-2 mutants fail to replicate to the same extent as wild type strains (Cirillo *et al.,* 1998; Hensel *et al.,* 1998) . These experiments suggested that SPI-2 is necessary during the systemic phase of the disease.

1.2.6.3 SPI-3

SPI-3 (~ 17 kb) is located at 82 min on the *Salmonella* chromosome and encodes a high-affinity Mg^{2+} uptake system that may be required for survival within macrophage phagosomes (Blank-Potard & Groisman, 1997). The island encodes two genes, *mgtCB*, which are required for intramacrophage survival and virulence in mice (Blanc-Potard and Groisman, 1997; Blanc-Potard *et al.,* 1999). The transport of magnesium at low Mg^{2+} conditions within the macrophage is carried out by these encoded proteins, although their function as Mg^{2+} transporters is still controversial (Moncrief and Maguire, 1998; Smith *et al.,* 1998). These genes form an operon that is regulated by the two-component regulatory system PhoP/Q (Soncini *et al.,* 1996).

1.2.6.4 SPI-4

SPI-4 (~ 27 kb) is located at 92 min on the *Salmonella* chromosome and is thought to be involved with survival in murine macrophages and toxin secretion (Wong *et al.*, 1998). Consistent with this are reports that show several *Salmonella* serovars are cytotoxic to macrophages (Chen *et al.,* 1996; Guilloteau *et al.,* 1996; Lindgren *et al.,* 1996; Monack *et al.,* 1996). It has been proposed that SPI-4 is involved in the secretion of a cytotoxin that causes apoptosis of *S. typhimurium* infected macrophages (Wong *et al.,* 1998). Previous work carried out by Baumler and co-workers (1994) identified a *S. typhimurium* locus involved in survival within macrophages and this locus maps within SPI-4 (Baumler *et al.,* 1994). The main function of SPI-4 remains to be determined.

1.2.6.5 SPI- 5

SPI-5 (~ 7.5 kb) is located at 20 min on the *Salmonella* chromosome and is thought to contribute to enteric but not to systemic *Salmonellosis* as it encodes proteins required for recruitment of neutrophils and fluid secretion (Wood *et al.,* 1998). Mutations in proteins encoded by SPI-5, for example, PipA (pathogenicity island encoded protein), PipB, PipD or SopB (*Salmonella* outer

protein) display a marked attenuation in secretory responses in a bovine ligated ileal loop model of enteritis, but have minimal effect on the systemic disease of *Salmonella* (Wood *et al.*, 1998). It is thought that the secreted effector protein, SopB, is translocated into the host where it causes an inflammatory response and fluid secretion in infected ileal mucosa (Galyov *et al.*, 1997a).

1.3 Stresses encountered by *Salmonella*

Salmonella infections are initiated when the bacteria are ingested by a suitable host via contaminated food or water. To survive and cause disease, *Salmonellae* must express a variety of gene products to adapt to environments inside and out of the host.

Outside the host the bacteria are often present in water at low temperatures, low osmotic strengths, neutral pH and low concentrations of organic nutrients. Inside the host the organisms are faced with the acidic environment of the stomach, intestinal bile salts, low oxygen levels, low iron levels, abundant organic nutrients and competition with resident microflora for nutrients and space. The bacteria are also engulfed by macrophages where they encounter low pH, nutrient limitations, and various antimicrobial peptides. Several of these stresses will be considered individually below.

1.3.1 Starvation stress response

One of the most common stresses encountered by *Salmonella* is starvation for phosphate, carbon and nitrogen sources (Harder and Dijkhuizen, 1983). In response to this starvation *Salmonella* undergo several genetic and physiological changes as a result of the starvation stress response (SSR). Mud-directed *lac* operon fusions identified eight starvation-inducible loci (*sti*A-H) that were transcriptionally regulated by nutrient starvation conditions (Spector *et al.*, 1988). The regulation of these loci are very complex and involve the alternative sigma factor σ^{38} (also known as σ^{s}) and the cyclic AMP receptor protein (CRP) (Lange and Hengge-Aronis, 1991; Spector and Cubitt, 1992; O'Byrne and Dorman, 1994). CRP negatively regulates the genes *stiA*,

stiC and *stiD* and is cAMP dependent, whereas the CRP-mediated negative control of *stiB* is cAMP independent. This suggests that CRP acts alone, or with a signal molecule other than cAMP, to cause repression of the *stiB* locus (Spector and Cubitt, 1992). The σ^s alternative sigma factor positively regulates *stiA*, and *stiC*, but negatively regulates *stiB* (Fang *et al.*, 1992).

1.3.2 Iron stress

Salmonella require iron for their growth, and this requirement is no less stringent when the bacteria are within the host. However, the host sequesters much of the free iron as it is tightly bound by high-affinity, iron-binding, glycoproteins such as lactoferrin and transferrin (Otto *et al.*, 1992). Moreover, in response to infection, cytokines within the host mediate an increase in transferrin that further limits the availability of iron (Beutler and Cerami, 1987). As well as surviving iron-limitations in the host, *Salmonella* must also survive in nature where most of the iron is not freely available, as it is insoluble at neutral or alkaline pH in the form of ferric hydroxide.

In response to iron stress the bacteria secrete high-affinity iron chelators, known as siderophores, to scavenge iron from the host (Aznar *et al.*, 1989). These compounds are able to bind iron with an even higher affinity than host iron-binding proteins and can therefore effectively compete with transferrin and lactoferrin. Siderophores are also able to solubilize iron from mineral complexes in the environment (Neilands, 1981). *S. typhimurium* utilise a catechol type of siderophore, enterobactin (enterochelin), which is synthesised from proteins encoded on the *ent* gene cluster. Enterochelin is secreted into the environment where it binds to iron forming a ferric-enterochelin complex that is transported back into the cell. The internalised ferric-enterochelin complex is then cleaved to release the iron molecule inside the bacteria (Braun *et al.*, 1991). The outer membrane pores are not large enough for the complex to diffuse through, so a series of proteins, Fep A-G, TonB, ExbB, and ExbD are required for transport (Bell *et al.*,

1990; Braun *et al.*, 1991). TonB connects the outer and inner membrane and the proteins ExbB and D bind to form a complex with TonB (Skare and Postle, 1991). TonB also interacts with the outer membrane receptor FepA to form a gated pore to allow translocation of the complex. The periplasmic protein, FepB, passes the complex to the inner membrane proteins FepGDC where the iron is cleaved by enterochelin esterase (Raupach *et al.*, 1999)

The regulation of iron uptake (Fe(II)) is mediated by a DNA-binding protein known as Fur (Ferric uptake regulator). Fur is considered to be a negative regulator that senses an increase in intracellular iron and as a result represses the genes involved in iron uptake (Hantke, 1987). The Fur protein uses ionic Fe(II) as a corepressor and binds to a specific DNA sequence (the Fur box) located in the promoter region of the iron acquisition genes to repress their transcription (Hantke, 1982; Bagg and Neilands, 1987).

1.3.3 Acid-Tolerance Response

During its life cycle, *Salmonella* is exposed to a variety of acidic conditions in the environment (pond water, acid rain, faecal matter, and decaying organic matter), during the infection process (extreme low pH in the stomach, volatile fatty acids present in the intestine and faeces) and in the phagocytic vesicle of the macrophage (Lin *et al.*, 1995; Bearson *et al.*, 1997; Jones, 1997).

Survival within these acidic environments requires the adaptive acid tolerance response (ATR), which is characterized by the induction of several *Salmonella* proteins upon exposure to mildly acidic conditions. These induced proteins protect the bacterium from death under severe acid challenge. The ATR response was first discovered when it was found that *S. typhimurium* had the ability to survive extreme low pH (pH 3.0 to 4.0) if first adapted to mild pH (pH 5.5 to 6.0) (Foster and Hall, 1990). Studies have shown that when the pH drops below about 4.3 *Salmonella* synthesise an array of at least 67 new Acid Shock Proteins (ASP) that are involved in both log-phase and stationary-phase systems (Foster, 1991; Foster, 1993).

The regulatory genes involved in log-phase acid tolerance include σ^s, (encoding an alternative sigma factor), *fur* (encoding the major iron regulator) and *phoPQ*, (encoding the two-component regulatory system) (Loewen and Hengge-Aronis, 1994; Hall and Foster, 1996; Hengge-Aronis, 1996; Bearson *et al.,* 1997; Bearson *et al* , 1998). The stationary-phase ATR also exhibits σ^S-dependent or, -independent systems (Lee *et al.,* 1994; Lee *et al.,* 1995). Very little is known about the regulation of the σ^S-independent system except recent studies have shown that the regulatory gene, *ompR*, is involved in the stationary-phase ATR (Bang *et al.,* 2000).

Induction of acid tolerance provides cross-protection to heat, oxidative stress, and osmotic stress. However, none of these stresses induce acid tolerance (Lee *et al.,* 1995; Leyer and Johnson, 1997)

1.3.4 The oxidative-stress response

Oxidative stress occurs when pathogenic bacteria encounter elevated levels of reactive oxygen species such as superoxide anions (O^{2-}), hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2). These reactive species can occur as a by-product of normal aerobic metabolism (Fridovich, 1983) or are generated by redox-cycling compounds (e.g. paraquat or quinones). Redox-cycling agents catalyse the flow of reducing equivalents to oxygen which divert electrons unproductively to oxygen within the cell (Kappus and Sies, 1981). Oxygen is also converted to potent cytotoxic oxidants during the phagocytic respiratory burst (Parkinson and Gabig, 1988; Babior, 1992; De Groote *et al.,* 1997):

Superoxide formation

$NADPH + 2O_2 \rightarrow 2O_2^{-\cdot} + H^+ + NADP^+$ (in presence of NADPH oxidase)

Hydrogen peroxide formation

$2O_2^{-\cdot} + H^+ \rightarrow H_2O_2 + O_2$ (in presence of superoxide dismutase)

Hypochlorous acid formation

$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$ (in presence of myeloperoxidase)

Hydroxyl radical formation

$2O_2^{-\cdot} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2$

The toxic effects of oxidative stress lead to DNA, protein and membrane damage. In *Salmonella* two regulons have been recognised to be involved in the adaptive resistance to oxidative stress. The *oxyR* gene mediates the response to increased levels of hydrogen peroxide whereas the two-gene locus *soxRS* mediates the response to increased levels of superoxide (reviewed in Demple, 1991).

1.3.5 Heat-shock response

When the bacterial cells are exposed to temperatures above optimal growth several heat shock proteins (HSPs) are expressed. A phenomenon, known as thermotolerance has been demonstrated in *S. typhimurium*. Exposing cells to a primary heat shock at 48°C enhances the resistance of stationary phase *S. typhimurium* to heating at 55°C (Bunning *et al.*, 1990; Mackey and Derrick, 1990). Many HSPs are produced in response to other stress factors. However, only acid and starvation produce cross-protection to heat.

Many of the genes associated with *S. typhimurium* thermotolerance have not been identified although it is thought that the system is analogous to the *E. coli* system. In *E. coli* at least 30 heat-shock proteins form a regulon that is temperature-dependent and requires the alternative sigma factor, σ^{32} (RpoH). The HSPs include the chaperones GroEL, GroES, DnaK, DnaJ, the stress protein GrpE and the housekeeping RNA polymerase sigma factor σ^{70} (RpoD) (Yura *et al.*, 1993). These chaperones and cooperating proteins bind to heat denatured proteins to prevent further degradation and to facilitate in the refolding of the protein (Parsell and Lindquist, 1993).

Other HSPs identified include proteins involved in proteolysis (Lon and Clp proteases) and Htr proteins involved in high-temperature resistance (Delaney *et al.*, 1993).

At high temperatures the induction of the HSP regulon requires the release of σ^{32} from DnaK/DnaJ so that the sigma factor is free to activate target genes and to allow the binding of DnaKJ to denatured proteins. The resulting binding of DnaKJ increases the concentration of free σ^{32} as less DnaKJ is available to bind to and inactivate σ^{32} (Lipinska *et al.*, 1989). In *S. typhimurium* the genes *dnaJ*, *dnak*, *lon*, *htrA* and *htrB* have been identified (Chatfield *et al.*, 1992; Rutz *et al.*, 1992; Raupach *et al.*, 1999).

1.3.6 Osmotic-Shock Response

Salmonella will experience osmotic stress inside and out of the host. A change in medium osmolarity will cause either an inward pressure (high external osmolarity) or an outward pressure (low external osmolarity) on the cell. Turgor pressure (minimum outward pressure) of the cell must be maintained for bacterial survival. If the external osmolarity increases compatible solutes (K^+, proline, glycine-betaine, glutamate or trehalose) are transported into the cell to counterbalance the increase in external pressure. This adaptive response allows the bacteria to maintain a constant volume over a wide range of external osmolarity (Foster & Spector, 1995).

The osmotic-shock response is controlled by the two-component regulatory system OmpR/EnvZ. EnvZ is a transmembrane protein that senses high osmolarity and phophorylates transcriptional regulator, OmpR (Csonka and Hanson, 1991). OmpR/EnvZ inversely regulates the major outer membrane porins, OmpC and OmpF. Inside the gut, where osmolarity is high, the expression of OmpC is favoured compared to outside the host where OmpF expression is favoured. The smaller pore size of OmpC could aid the exclusion of harmful molecules, such as bile salts, found in the gut (Csonka and Hanson, 1991). Studies showed that *S. typhimurium ompR*$^-$ strains were dramatically attenuated in the mouse model when inoculated orally and intravenously,

which suggests a role for OmpR in virulence (Dorman *et al.,* 1989). In contrast, strains harbouring mutations in both *ompC* and *ompF* were attenuated only via the oral route, suggesting a protective role for these proteins in the gut (Chatfield *et al.,* 1991).

1.3.7 Resistance to Cationic Peptides

Salmonella will be exposed to a variety of antimicrobial peptides and proteins found within secretions of mucosal epithelia, neutrophil granules, and macrophage phagosomes. These antimicrobial factors include azurocidin (CAP37) cryptidins, defensins, lysozyme, and Bactericidal/Permeability-Increasing protein (BPI or CAP57) (Shafer *et al.,* 1984; Hovde and Gray, 1986; Gabay *et al.,* 1989; Joiner *et al.,* 1989; Ouellette and Lualdi, 1990; Stolzenberg *et al.,* 1997). Other antimicrobial factors include melittin, magainins, polymixin B (PM), protamine sulphate, and polylysine (Vaara and Vaara, 1983; Vaara, 1992; Storm *et al.,* 1977).

The mechanism of action for most of these antimicrobial agents appears to be similar. These agents bind to the lipopolysaccharides present in the bacterial membrane via unsubstituted, negatively charged phosphyl groups of lipid A. This binding causes permeabilization of the outer and inner membranes, thereby rendering it permeable to a range of hydrophobic compounds (Vaara, 1992). In contrast, protamine has been found to have an antibacterial effect without causing cell lysis or cytoplasmic membrane permeabilization by disrupting energy transduction and inhibiting nutrient uptake (Aspedon and Groisman, 1996).

Genetic loci implicated in the resistance to these defences include eight *sap* (sensitivity to antimicrobial peptides) loci, the *bipA* locus (discussed in section 1.4.2) and the two-component regulatory systems *pmrA/pmrB* and *phoP/phoQ* (Vaara and Vaara, 1981a; Vaara *et al.,* 1981b; Groisman *et al.,* 1992a; Roland *et al.,* 1993; Guo *et al.,* 1997)

1.3.7.1 *sap* loci

Screening for mutants that were hypersensitive to the cationic peptide protamine originally identified the *sap* loci (Groisman *et al.,* 1992b). Of the eight *sap* loci identified the *sapABCDF* operon encodes a new member of the ABC transporter family that has been previously implicated in the transport of K^+ ions across the cytoplasmic membrane. The SapABCDF transporter is thought to transport cationic peptides into the cell for degradation by cellular proteases (Parra-Lopez *et al.,* 1993). Alternatively, the transporter may be a sensory protein that activates the relevant peptide-resistant determinants. Similarly, the *sapG* locus also identified was shown to encode components of a low-affinity K^+–transport system. Strains harbouring mutations in *sapG* and either *sapJ* or *sapABCDF* were as sensitive as *sapG* single mutants, which suggested that *sapG* may regulate *sapJ* and *sapABCDF* which then mediate the transport of cationic peptides (Parra-Lopez *et al.,* 1994).

1.3.7.2 Two component regulatory systems *pmrA/pmrB* and *phoP/phoQ*

The two-component regulatory system *pmrA/pmrB* (polymyxin resistant loci) regulates the resistance of several antimicrobial peptides including polymixin, azurocidin, BPI, polylysine and protamine (Roland *et al.,* 1993b). Polymyxin resistant mutants exhibit an increase in the level of phosphate substitutions on the LPS and a high degree of attachment of aminoarabinose and palmitate to lipid A. These alterations reduce the net negative charge on the LPS thereby decreasing the affinity of the positively charged cationic peptides onto the bacterial surface (Vaara and Vaara, 1981a; Vaara *et al.,* 1981b; Guo *et al.,* 1997). Activation of *pmrA/pmrB* loci is induced by mild acidic conditions and by the two-component regulatory system, PhoPQ (Fields *et al.,* 1989; Miller *et al.,* 1990b Groisman *et al.,* 1992a; Groisman *et al.,* 1997).

1.4 The Discovery of BipA and its Possible Involvement in virulence

Since *Salmonella* must avoid or inactivate cationic antimicrobial peptides the question of the mechanisms involved arises. Such studies with *S. typhimurium* have uncovered a novel GTPase that is up-regulated sevenfold when the bacteria are exposed to BPI, a cytotoxic host defense component produced by human granulocytes (Qi *et al.,* 1995). Since this GTPase is the subject of this thesis, it is appropriate to now review what is known about this protein and the gene that it encodes. This novel protein, termed BipA (BPI-inducible protein A), is involved in many virulence-associated processes including antibacterial resistance, EPEC cytoskeletal rearrangements and secretion of EPEC effector proteins involved in a type III secretion system (Farris *et al.,* 1998; Barker *et al.,* 2000; Grant, unpublished results). BipA has also been shown to interact with ribosomes (Owens, R., unpublished results)

1.4.1 Sequence Homology of BipA

Sequence analysis of the EPEC *bipA* gene revealed that BipA shares substantial sequence similarity to the ribosomal binding proteins, elongation factor G (EF-G) and tetracycline resistance proteins, TetQ/TetM (Farris *et al.,* 1998) (Figure 1-2). Stretches of conserved sequences are found throughout BipA although the strongest similarities are found in the amino-terminal third of the protein, which include regions that define a guanine nucleotide-binding pocket (residues 12-20, 74-83 and 128-131). In addition, BipA shares sequence similarity with domain IV of EF-G (residues 486-677), which is believed to interact with ribosomes (Nissen *et al.,* 1995). Interestingly, in the non-pathogenic strain, *E. coli* K-12, there are only two sites were the sequence is different (Ile→Thr at position 609 and Val→Ala at position 617) and these sites also map to domain IV. Initial studies have shown that BipA does interact with ribosomes and that BipA GTPase activity is stimulated in the presence of ribosomes (Owens, R., unpublished results).

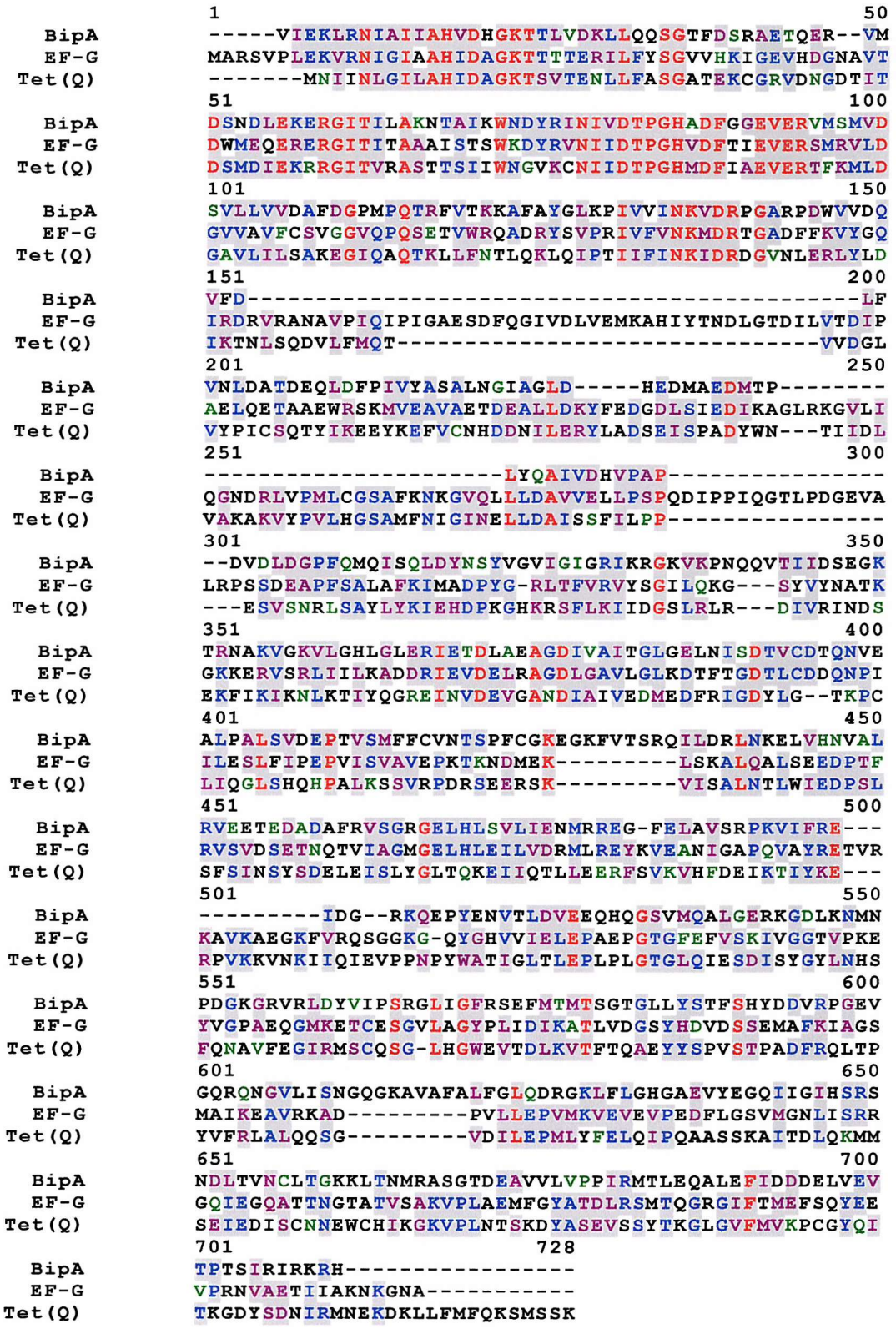


Figure 1-2 Alignment of BipA with EF-G and TetQ

The proteins were identified using the gapped BLAST algorithm to search the SWISSPROT database and aligned using Omiga 1.1.3. Residues that are identical in all three proteins or in just two of the proteins are highlighted in red or blue shading respectively. Residues that are or conserved in ≥ two of the sequences or weakly similar are highlighted in magenta and green respectively.

1.4.2 Involvement of BipA in Resistance to BPI and BPI-derived Peptides

BPI specifically binds to the surfaces of gram-negative bacteria via the lipid A component of LPS (Gazzano-Santoro *et al.,* 1992). Following attachment to LPS, BPI disrupts the outer membrane and permeabilises the membrane to a range of hydrophobic compounds which ultimately results in cell lysis. Studies also suggest that BPI selectively activates certain phopholipases A2 (mediate phospholipid hydrolysis) and peptidoglycan-degrading enzymes (Forst *et al.,* 1987; Elsbach and Weiss, 1993). The N-terminal fragment of BPI exhibits the bactericidal and anti-endotoxin properties of the holoprotein (Gray and Haseman, 1994; Little *et al.,* 1994). Peptides containing this region retain significant antibacterial action and elicit all the effects of the holoprotein (H.C. Barker., unpublished results).

Studies have shown that stationary phase cells of *Salmonella* and *E. coli* can be rescued from the cytotoxic effects of a BPI-derived peptide if either formate or succinate is present. This protective effect requires the oxidation of these organic acids and a specific part of the respiratory chain may be the primary target for BPI. More importantly, BipA appears to participate in the formate protection effect in stationary phase cells, but its mode of action is unknown (Barker *et al.,* 2000).

1.4.3 Involvement of BipA in EPEC Virulence

Enteropathogenic *E. coli* (EPEC) is a diarrhoeagenic *E. coli* often associated with mortality and morbidity in developing countries (Gomes *et al.,* 1989; Donnenberg and Kaper, 1992). Its ability to adhere to epithelial host cells and cause cytoskeletal rearrangements that result in the formation of attaching and effacing lesions (AE) is essential to its pathogenicity (Moon *et al.,* 1983; Knutton *et al.,* 1987). Attached EPEC trigger a series of signal-transduction pathways that result in the tyrosine phosphorylation of a membrane protein known as Tir or Hp90. This protein is then integrated into the host membrane and associates directly with an EPEC adhesin, intimin

(Rosenshine *et al.*, 1992; Kenny *et al.*, 1997). These interactions lead to cytoskeletal nucleation and the formation of cup-like projections known as pedestals (Knutton *et al.*, 1989). More developed pedestals, known as pseudopods, can elevate the bacteria more than 10μm above the surface of the host cell (Rosenshine *et al.*, 1996). The EPEC-induced pedestals are rich in polymerized actin and also contain α-actin, myosin light chain, erzin and talin (Finlay *et al.*, 1992c). Recent studies have uncovered a pathogenicity island, the locus of enterocyte effacement (LEE), that is involved in signal transduction and pedestal formation (McDaniel *et al.*, 1995). This gene cluster encodes secreted proteins involved in signal transduction (e.g. EspA, EspB and EspD) and proteins involved in the delivery of these secreted proteins into host cells. Other proteins also encoded on the LEE locus include intimin and Tir. The LEE locus regulates intimate adhesion, tyrosine phosphorylation of Tir and actin accumulation beneath the bacteria (Frankel *et al.*, 1998).

Further studies with EPEC BipA suggest that, in this pathogen at least, the protein directly or indirectly regulates the complex cytoskeletal rearrangements induced in host cells by adherent bacteria (Farris *et al.*, 1998). Farris *et al.* (1998) found that when HeLa cells were infected with EPEC strain MAR001 they exhibited normal microvilli destruction, formation of pedestals, and actin accumulation. However, an isogenic *bipA* mutant strain failed to trigger cytoskeletal rearrangements, although adherence to most host cells was observed and in some cases thickening of microvilli was seen. This defect was restored when a high-copy-number plasmid containing *bipA* was introduced into the mutant cells. In fact EPEC-associated pseudopods and a 40% increase, compared to the wild type, in actin accumulation was observed.

Also recent evidence has shown that *bipA*$^-$ mutants of EPEC do not express several proteins encoded in the LEE locus as the expression of intimin, Tir, and EspABD are down regulated in the EPEC BipA mutant (Grant, A.J., unpublished results).

1.4.4 BipA negatively regulates flagella-mediated cell motility in EPEC

Many wild-type strains of *E. coli* and *Salmonella* are motile, as they are able to produce surface locomotive appendages known as flagella (Macnab, 1976). Flagella are slender, rigid structures, which are usually 15-20 nm in diameter and 10-20 μm long. The bacterial flagellum is composed of three parts, the filament which extends from the cell surface to the tip, the basal body which is embedded in the cell and the hook, a short curved segment which links the filament to its basal body. The filament is a hollow, rigid cylinder constructed of a single protein called flagellin. (Silverman, 1980; Macnab, 1992). The synthesis and control of flagella is a complex process involving at least 40 genes (Aizawa, 1996; Macnab, 1996). The importance of motility as a virulence factor is becoming increasingly apparent. Several co-workers have suggested that motility is important as it enables the bacteria to swim towards nutrient rich environments or away from toxic environments. Also, in the intestine, the bacteria are able swim across the mucus layer where they are able to adhere and colonise the host epithelial cells (Ottemann and Miller, 1997)

Interestingly, Farris and co-workers (1998) found that EPEC BipA negatively regulates flagellin and flagella-mediated motility. Two-dimensional gels showed that, compared to the wild type strain, flagellin expression was 4-fold higher in the BipA$^-$ strain and 2.8-fold lower in the transcomplemented strain. In addition, the BipA$^-$ strain was hypermotile compared to the parent or transcomplemented strain. It is thought that BipA regulates other components associated with flagella, as it is unlikely that the increased motility in *bipA*$^-$ deficient cells is solely due to the increased expression of flagellin (Farris *et al.*, 1998).

Taken together, the previous observations in sections 1.4.1 to 1.4.4 suggest that BipA is a global regulatory protein that controls a number of virulence-related processes in EPEC and this therefore raises the question about its role in *Salmonella* pathogenesis.

1.5 Aims of Work

Given the potential role of *Salmonella* BipA in virulence this study set out to achieve the following aims:

1. To construct a *bipA* null mutant of *Salmonella*
2. To investigate the role of *bipA* in the regulation of surface appendages involved in adherence
3. To investigate the role of *bipA* in the adhesion and invasion of host epithelial cells and macrophages
4. To investigate the role of *bipA* in stress responses such as the ATR and the oxidative stress response
5. To investigate the role of *bipA* in *Salmonella* pathogenicity
6. To investigate the flanking regions of *bipA*

In view of the upsurge in the incidence of *S. enteritidis* in gastrointestinal disease (Baumler *et al.*, 2000) it was decided to carry out these studies with this serovar. It should be noted, however, that a further *bipA* null mutant in *S. typhimurium* was constructed in parallel with the *S. enteritidis* studies (Kinsella, N., White, A.L, and O'Connor, C.D., unpublished results). Therefore, the properties of this mutant will also be described where they are relevant to the studies on *S. enteritidis* described here.

CHAPTER 2

2 Materials and Methods

2.1.1 Strains and culture conditions

All bacterial strains used in this study are given in Tables 2.1 to 2.4. Bacteria were routinely cultured in Luria-Bertani (LB) or minimal medium containing M9 salts (media compositions are given in Table 2-5). HeLa cells and RAW macrophage-like cells were obtained from the National Cell Culture Collection [Porton, UK] and grown at 37°C, 5% CO_2 in DMEM medium [Gibco BRL] supplemented with 10% fetal calf serum. When needed kanamycin was used at a concentration of 25 μg per ml, ampicillin was used at a concentration of 25-50 μg per ml, and chloramphenicol was used at a concentration of 10 μg per ml.

To prepare inocula for *in vivo* studies several colonies were picked into LB broth and grown at 37°C for 16h with orbital shaking at 225 rpm and serial dilutions prepared in phosphate buffered saline (PBS), pH 7.2 (composition given in Table 2-5).

Table 2-1 *Salmonella* serovars

Serotype	Group	Somatic
S. agona	B	4,12
S. derby	B	1,4,12
S. typhimurium PT104	B	4,5,12
S. heidelberg	B	4,5,12
S. montevideo	C1	6,7
S. hadar	C2	6,8
S. dublin	D1	9,12
S. enteritidis PT4	D1	9,12
S. pullorum	D1	1,9,12
S. binza	E2	3,15
S. kedougou	G2	13,23
S. arizona 61:k:1,5,7	SIII	35,

Table 2-2 The *E. coli* Reference Collection (ECOR)

The ECOR was established in 1984 by Howard Ochman and Robert K. Selander (*J. Bacteriol.* 157:690-693) The O:H serotypes were determined in 1985 by the *E. coli* reference Centre at Penn State under the direction of Richard A. Wilson

Isolate	Group	O	H	Host	Locale	Clinical	Original
EC01	A	ON	HN	human	USA (Iowa)	healthy	RM74A
EC02	A	ON	H32	human	USA (N.Y.)	healthy	STM1
EC03	A	O1	NM	dog	USA (Mass.)	healthy	W1R1(a)
EC04	A	ON	HN	human	USA (Iowa)	healthy	RM39A
EC05	A	O79	NM	human	USA (Iowa)	healthy	RM60A
EC06	A	ON	NM	human	USA (Iowa)	healthy	RM66C
EC07	A	O85	HN	orangutan	USA (Wash.)*	healthy	RM73C
EC08	A	O86	NM	human	USA (Iowa)	healthy	RM77C
EC09	A	ON	NM	human	Sweden	healthy	FN98
EC10	A	O6	H10	human	Sweden	healthy	ANI
EC11	A	O6	H10	human	Sweden	UTI(C)	C97
EC12	A	O7	H32	human	Sweden	healthy	FN59
EC13	A	ON	HN	human	Sweden	healthy	FN10
EC14	A	OM	HN	human	Sweden	UTI(P)	P62
EC15	A	O25	NM	human	Sweden	healthy	FN3
EC16	A	ON	H10	leopard	USA (Wash.)*	healthy	RM191F
EC17	A	O106	NM	pig	Indonesia	healthy	RM200Q
EC18	A	O5	NM	Celebese ape	USA (Wash.)*	healthy	RM210F
EC19	A	O5	HN	Celebese ape	USA (Wash.)*	healthy	RM210J
EC20	A	O89	HN	steer	Bali	healthy	RM213I
EC21	A	O121	HN	steer	Bali	healthy	RM213K
EC22	A	ON	HN	steer	Bali	healthy	RM215C
EC23	A	O86	H43	elephant	USA (Wash.)*	healthy	RM183E
EC24	A	O15	NM	human	Sweden	healthy	FN33
EC25	A	ON	HN	dog	USA (N.Y.)	healthy	MS1
EC26	B1	O104	H21	infant	USA (Mass.)	healthy	LL
EC27	B1	O104	NM	giraffe	USA (Wash.)*	healthy	RM24J
EC28	B1	O104	NM	human	USA (Iowa)	healthy	RM52B
EC29	B1	O150	H21	kangaroo rat	USA (Nev.)	healthy	RM3A
EC30	B1	O113	H21	bison	Canada	healthy	RM10A
EC31	E	O79	H43	leopard	USA (Wash.)*	healthy	RM12
EC32	B1	O7	H21	giraffe	USA (Wash.)*	healthy	RM28
EC33	B1	O7	H21	sheep	USA (Calif.)	healthy	RM56C
EC34	B1	O88	NM	dog	USA (Mass.)	healthy	WIR2(a)
EC35	D	O1	NM	human	USA (Iowa)	healthy	RM42B
EC36	D	O79	H25	human	USA (Iowa)	healthy	RM77B
EC37	E	ON	HN	marmoset	USA (Wash.)*	healthy	RM44B
EC38	D	O7	NM	human	USA (Iowa)	healthy	RM75A
EC39	D	O7	NM	human	Sweden	healthy	FN104
EC40	D	O7	NM	human	Sweden	UTI(P)	P60
EC41	D	O7	NM	human	Tonga	healthy	T44
EC42	E	ON	H26	human	USA (Mass.)	healthy	DAR1
EC43	E	ON	HN	human	Sweden	healthy	FN36
EC44	D	ON	HN	cougar	USA (Wash.)*	healthy	RM189I

EC45	B1	ON	HM	pig	Indonesia	healthy	RM201C
EC46	D	O1	H6	ape	USA (Wash.)*	healthy	RM202F
EC47	D	OM	H18	sheep	New Guinea	healthy	RM211C
EC48	D	ON	HM	human	Sweden	UTI(C)	C90
EC49	D	O2	NM	human	Sweden	healthy	FN90
EC50	D	O2	HN	human	Sweden	UTI(P)	P97
EC51	B2	O25	HN	infant	USA (Mass.)	healthy	DD
EC52	B2	O25	H1	orangutan	USA (Wash.)*	healthy	RM73A
EC53	B2	O4	HN	human	USA (Iowa)	healthy	RM33B
EC54	B2	O25	H1	human	USA (Iowa)	healthy	RM64A
EC55	B2	O25	H1	human	Sweden	UTI(P)	FN4
EC56	B2	O6	H1	human	Sweden	healthy	P106
EC57	B2	ON	NM	gorilla	USA (Wash.)*	healthy	RM71B
EC58	B1	O112	H8	lion	USA (Wash.)*	healthy	RM185S
EC59	B2	O4	H40	human	USA (Mass.)	healthy	SIL8
EC60	B2	O4	HN	human	Sweden	UTI(C)	C89
EC61	B2	O2	NM	human	Sweden	healthy	FN83
EC62	B2	O2	NM	human	Sweden	UTI(P)	P69
EC63	B2	ON	NM	human	Sweden	healthy	FN21
EC64	B2	O75	NM	human	Sweden	UTI(C)	C70
EC65	B2	ON	H10	Celebese ape	USA (Wash.)*	healthy	RM202I
EC66	B1	O4	H40	Celebese ape	USA (Wash.)*	healthy	RM209I
EC67	B1	O4	H43	goat	Indonesia	healthy	RM217T
EC68	B1	ON	NM	giraffe	USA (Wash.)*	healthy	RM224H
EC69	B1	ON	NM	Celebese ape	USA (Wash.)*	healthy	RM45EM
EC70	B1	O78	NM	gorilla	USA (Wash.)*	healthy	RM70B
EC71	B1	O78	NM	human	Sweden	ABU	ABU84
EC72	B1	O144	H8	human	Sweden	UTI(P)	P68

Abbreviations: ON,HN= non-typeable with standard antisera; NM=non-motile strain; * indicates isolate from zoo animal; ABU = asymptomatic bacteriuria; UTI = symptomatic urinary tract infection, (C=acute cystitis), (P=acute pyelonephritis

Table 2-3 Other bacterial species

Strain	Reference
Citrobacter spp.	VLA Weybridge
Citrobacter freundii	VLA Weybridge
Edwardsiella tarda	VLA Weybridge
Enterobacter cloacae	VLA Weybridge
Enterobacter fergusonii	VLA Weybridge
Enterobacter hafina	VLA Weybridge
Enterobacter omnigenus	VLA Weybridge
E. coli (O9:K30;K99,F41)	VLA Weybridge
E coli (O101:K99,F41)	VLA Weybridge
E. coli (O8:K87, K88ab)	VLA Weybridge
E. coli (O8:K87, K88ac)	VLA Weybridge
E. coli (O149:K99,K88)	VLA Weybridge
E. coli (O9:K103,987p)	VLA Weybridge
Klebsiella	VLA Weybridge
Proteus vulgaris	VLA Weybridge
Serratia spp.	VLA Weybridge
Serratia marcescens	VLA Weybridge

Table 2-4 Strains used in cloning experiments

Strain	Reference
E. coli JM109 (λ*pir*)	VLA Weybridge
E. coli S-17 (λ*pir*)	VLA Weybridge

Table 2-5 Media composition

Media	Composition
Luria-Bertani Broth (LB) *	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, in distilled water, pH7.2
Luria-Bertani Agar (LB Agar)	LB with the addition of 1.5% Bacto agar. In the case of motility plates, tryptone was used (per litre: 10g tryptone, 5g NaCl, 0.1g thymine and 0.35% agar).
Brilliant Green Agar (BGA) *	1% (w/v) proteose peptone, 0.3% (w/v) yeast extract, 1% lactose, 1% (w/v) sucrose, 0.5% (w/v) NaCl, 0.008% (v/v) phenol red, 0.00125% (v/v) brilliant green and 1.5% Bacto agar, in distilled water.
Colonisation Factor Antigen Agar (CFA) *	1% (w/v) casamino acids, 0.15% (w/v) yeast extract, 0.005% (w/v) $MgSO_4$, 0.0005% (w/v) $MnCl_2$ and 1.5% Bacto agar, in distilled water. When required, Congo red was added to CFA to a concentration of 0.01% (w/v).
Minimal medium (MM)	0.2% (w/v) glucose and M9 salts (0.6% (w/v) Na_2PO_4, 0.3% (w/v) KH_2PO_4, 0.1% (w/v) NaCl) in distilled water. MM base was sterilised by autoclaving and cooled before the addition of separately autoclaved 1mM $CaCl_2$ and 10mM $MgCl_2$.
Heart Infusion Broth (HIB) *	0.5% (w/v) beef heart infusion solids, 1% (w/v) proteose peptone, 0.5% (w/v) NaCl, 0.2% (w/v) glucose, 0.25% (w/v) disodium phosphate in distilled water at a pH of 7.4.
Phosphate Buffered Saline (PBS) *	0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na_2HPO_4, 0.02% KH_2PO_4 in distilled water at a pH of 7.2.
SOC medium (SOC)	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgSO_4$, 10mM $MnCl_2$, and 20mM glucose, in distilled water.

*BGA, CFA, HIB, LB, PBS were supplied by Oxoid in powdered or tablet form.

2.1.2 Chemicals and Reagents

All chemicals and reagents used in this study were, wherever possible, Analar grade and are listed below in alphabetical order followed by the supply company in brackets.

Acetic acid [Fisher Scientific], Acrylamide [Bio-Rad], ammonium persulphate [Bio-Rad], ampholines [Amersham Pharmacia Biotech] and antibiotics (ampicillin, kanamycin, gentamicin, and chloramphenicol) [Sigma]. Bromophenol blue [Sigma], CHAPS [Amersham Pharmacia Biotech], 2'-deoxynucleotides (dNTPs) [Amersham Pharmacia Biotech] and dithiothreitol (DTT) [Sigma]. Ethanol, ethylene-diamine-tetra-acetic acid, disodium salt (EDTA) [Sigma], glycerol [Fisher Scientific], D-(+)-glucose [Sigma] and hydrochloric acid (HCl) [Fisher Scientific]. Idoacetamide [Sigma], IPG-Buffer pH 4-7L [Amersham Pharmacia Biotech], orange G [Sigma], methanol [BDH chemicals], β-mercaptoethanol PMSF [Sigma] and propan-2-ol (isopropanol) [BDH chemicals]. Phenol [BDH chemicals], proteinase K [Gibco BRL Life technologies], sodium dodecyl sulphate (SDS) [BDH Chemicals] and sucrose [Sigma]. TEMED (N,N,N',N'- Tetramethylethylenediamine [Sigma], Tris base [Sigma], Triton X 100 [Sigma], and Urea [BRL].

2.1.3 Extraction and purification of plasmid DNA

Plasmid DNA was extracted from transformed cells using the PerfectPrep plasmid DNA extraction kit [5'→ 3'] and the kit was used according to the manufacturer's instructions. Plasmid DNA was also extracted using the Qiaprep spin miniprep kit [Qiagen] according to the manufacturer's instructions

2.1.4 Extraction and purification of total genomic DNA

Bacterial cultures were grown for 16 hours at 37°C in 100 ml LB, cells were pelleted by centrifugation and resuspended in 5 ml TE buffer (0.1% (v/v) Tris-HCl, 0.2% (v/v) 0.5M EDTA

pH 8.0 in distilled water). Proteinase K (1 ml of a 50 μg/ml solution in distilled water) and SDS (1 ml of 10% (w/v) solution in water) were added to the cell suspension and the mixture incubated at 65°C for 1 hour. The clear lysate was cooled to room temperature and extracted three times with an equal volume of phenol:chloroform 1:1 using Phase Lock ™ gel [5'→ 3'] to aid phase separation, according to the manufacturer's instructions. The final, aqueous extract was added to three times the volume of 100% ethanol at room temperature, and carefully mixed by inversion to precipitate the DNA. The DNA was removed from the solution by spooling with a glass rod, and washed once in 70% (v/v) ethanol in water, before being allowed to air-dry for 10 minutes. The DNA was then resuspended in 3 mls TE buffer and stored at 4°C.

2.1.5 Purification and Quantification of DNA from Agarose gels

Agarose gel electrophoresis was carried out as essentially described in Sambrook *et al* (1989). DNA fragments were recovered from excised agarose bands using the Sephaglass ™ Band Prep kit [Amersham Pharmacia Biotech] according to the manufacturer's instructions. The concentration of DNA in a solution was approximately quantified by comparing it to known concentrations of salmon testes DNA [Stratagene]. Serial dilutions of the salmon testes DNA solution, and the DNA solution to be quantified were made, and 5 μl of each dilution was mixed with 0.1 μm/ml ethidium bromide and pipetted onto the screen of a UV transilluminator emitting at 302nm. A visual comparison of brightness was made and used to estimate the concentration of purified DNA.

2.1.6 Restriction endonuclease digestion of DNA

Restriction endonucleases were purchased from either Promega Corporation or New England Biolabs [NEB]. 0.1-0.5 μg of DNA or purified restriction fragments were incubated with 2 units of each appropriate restriction enzyme in a final volume of 20-50 μl at 37°C (or 25oC when appropriate) for a minimum of 2 hours.

2.1.7 Ligation of DNA

"Ready to go ™" T4 DNA ligase was purchased from Amersham Pharmacia Biotech and ligations were performed according to the manufacturer's instructions.

2.1.8 Transformation

Electro-competent *E. coli* and *Salmonella* bacterial cells were prepared according to the Bio-Rad protocol. Initially, 500 µl of an overnight broth of bacteria was subcultured into 30 mls of LB broth and incubated at 37°C until the cells reached an OD_{600nm} of approximately 0.4-0.5 (mid-log phase). The cells were harvested by centrifugation at 6000 g (Beckman 8x50ml rotor) for 10 minutes and the pellet was washed in 10ml of ice-cold, sterile, HPLC-grade water. The cells were recentrifuged and the pellet resuspended in 300-500 µl of ice-cold water. 40 µl of competent cells were mixed with approximately 200 ng of DNA and left on ice for 2 minutes. The mixture was placed into an electroporation cuvette with 1mm gap [Bio-Rad] and pulsed in a Bio-Rad Gene Pulser at a potential of 1.8kV, a capacitance of 25µF and a resistance of 200Ω. Immediately after electroporation, the cells were transferred to 1 ml of SOC and incubated with gentle agitation at 37°C for 1 hour. Aliquots were plated onto agar containing the appropriate antibiotics and incubated overnight at 37°C.

2.1.9 Polymerase Chain Reaction (PCR)

DNA sequences were amplified using PCR as essentially described in Sambrook *et al.* (1989). Magnesium chloride solution, dNTPs, 10x*Taq* DNA polymerase reaction buffer and *Taq* DNA polymerase [Amersham Pharmacia Biotech] were all used in the PCR. Oligonucleotide primers were based on published DNA sequences found in the EMBL database, and were synthesized by Oswel (Southampton, U.K). Reaction volumes of 50 µl typically consisted of 10x*Taq* DNA polymerase reaction buffer (5 µl), 100mM dNTP solution (5 µl), 50 pmol of each primer (1 µl of a 50 nmol/ml solution), molecular biology grade water (33 µl) and *Taq* DNA polymerase (0.5

μl). The PCR reactions were conducted using a Perkin Elmer thermal cycler and a typical cycling programme consisted of 35 cycles amplification of denaturation at 95°C for 1.5 min, annealing at 56°C for 1.5 min and elongation at 72°C for 2 min. The annealing temperatures were raised to increase specificity when needed and the elongation time was adjusted by an increase or decrease of approximately 1min per 1kb of product. Following amplification the reactions were stored at 4°C.

2.1.10 DNA manipulations

Cloning was carried out as essentially described by Sambrook *et al.* (1989). The *S. enteritidis bipA* gene, plus the promoter region, was amplified using primers AJG001 and ALW21 (primer sequences given in Table 2-6). The PCR product (2243 bp) was then cloned into the plasmid vector pCR2.1 using the Original TA Cloning Kit [Invitrogen] according to the manufacturer's instructions. Incorporation of the desired PCR fragment was confirmed by restriction analysis of plasmid DNA.

To knock out the *bipA* gene of S1400 the region around the 3' end was PCR amplified from chromosomal DNA using primers ALW1 and ALW2. The amplified fragment (644 bp) was cloned into the suicide vector, pERFORMC (Allen-Vercoe *et al.*, 1997) as a *SalI- SmaI* fragment and transformed into JM109(λpir) cells to give plasmid pAW2. Transformants were selected using the chloramphenicol marker in the suicide vector. Several restriction digests were carried out on pAW2 to check that the correct fragment had been cloned. The 5' end of *bipA* was then amplified using the primer pair ALW3 and ALW4. A stop codon was inserted into primer ALW3 (underlined in Table 2-6) so that BipA would not be expressed as BipA has been found to be toxic to some cells. The amplified fragment (735 bp) was cloned into plasmid pAW2 as a *SacI-PmeI* fragment. The construct was transformed into JM109(λpir) cells to give plasmid pAW3. Again, restriction digests were carried out to check that the correct fragment had been

cloned. A kanamycin resistant cassette was cut from plasmid pDOC70 (O'Connor, unpublished results) as a *HincII* fragment to yield blunt ends and ligated into *PmeI*-cut pAW3. Ligation of the cassette destroys the *PmeI* sites, therefore the ligation mix was incubated with *PmeI* to ensure that only the vector containing the kan^r cassette would be present in circular form and hence be transformed. Selection was made jointly for kanamycin and chloramphenicol at their respective working concentrations. The construct, designated pAW4, was transformed into JM109(λpir) cells.

2.1.11 Mobilisation of pAW4 into wild type S1400

To mobilise pAW4 into *S. enteritidis* S1400/94 (recipient strain) the plasmid was transformed into an auxotrophic mobilising strain S-17(λpir) (donor strain). The recipient and transformed donor strains were grown overnight at 37°C in 3 ml LB containing antibiotics were appropriate. Both cultures were pelleted, washed with 0.9% NaCl and resuspended in 100 µl LB broth. They were then mixed together and placed onto a 0.2 µm sterile filter overlaid on a LB agar plate. The cells were incubated for 4-6 hours at which time the filter was removed from the plate, submerged in sterile 1xM9 salts solution (10 mls), and vortexed vigorously for 1 minute. The filter was removed and the cells were spread onto glucose minimal medium plates containing the appropriate antibiotics and incubated at 37°C for 2 days. A kanamycin-resistant, chloramphenicol-sensitive transconjugant was selected in which the native chromosomal *bipA* gene had been replaced by homologous recombination with the *bipA::kan* construct with subsequent loss of the chloramphenicol marked suicide vector. The genotype of the *bipA::kan* mutant was confirmed by colony dot blot, Southern hybridisation and PCR.

2.1.12 Cloning flanking regions of *bipA*

To clone the flanking regions of *bipA*, pAW4 was mobilised into *S. enteritidis* S1400/94 and a kanamycin-resistant, chloramphenicol-resistant transconjugant (single crossover event) was

selected. Chromosomal DNA was then prepared and digested with an enzyme that cuts outside of *bipA, MluI.* The digested DNA was religated and transformed into S-17(λ*pir*). Therefore the resulting plasmid, pAW5, contained the flanking regions of *bipA*, which could then be sequenced

2.1.13 DNA sequencing

DNA sequencing of the cloned *S. enteritidis bipA* was performed using primers AJG001, ALW4, ALW7, ALW21, ALW23, and ALW24. The resulting *bipA* sequence was compared to the sequence obtained from pYL98 (*S. enteritidis bipA* cloned into pBR322, Y. Li., unpublished results. EMBL accession no. AJ276889).

The cloned region downstream of *bipA* was sequenced using primers ALW10, ALW11, ALW12, ALW13, ALW14, ALW15, ALW16, ALW17, ALW19, and ALW20. The sequencing reactions were analysed on an automated ABI377 sequencing instrument [Oswell, Southampton, U.K.].

Table 2-6 Oligonucleotide sequences of primers

Primer	Oligonucleotide sequence 5'-3'
ALW1	AAAAAAAGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTGCGTTAACACCTCGCCGTTCTGCG
ALW2	AAAAAAAGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATCTAGATTTCTTACCGGTCAGGCAGTTTAC
ALW3	AAAAAAAAAGTTTAAACGAGCTCTGAATCGAAAATTTGCGTAACATCGCCATC
ALW4	AAAAAAAAACCCGGGTTTAAACAGGTCAACGTCAGGCGCCGGAAC
ALW7	TAAACTGCCTGACCGGTAAGAAAC
ALW10	GAAATGCTACGTTTAGCC
ALW11	GGCTAAACGTAGCATTTC
ALW12	AGAAAGAGACGCCGCCAA
ALW13	CGAAATGAAAGCTCGGCG
ALW14	CTTTCATACTGCGACAGC
ALW15	ATATTTTCCTGGCGAGGC
ALW16	CTAAAGCTGACGTTCTCC
ALW17	AAAAAAGGATCCGGGCGTCGATATAGCGTA
ALW19	ACGGTTTAGCAGCGTTCC
ALW20	ATGTTTGCAGGTTCGACG
ALW21	CTCGAGAGCTTTCATTTCGGCAGG
ALW23	TAGGTAAAGTGCTGACGC
ALW24	CAGAAGAACATAGACACG
ALW25	GCATCGACGGATTACTGA
ALW26	ACGAATCAGCATCACAGC
ALW27	CCAGGTGATTCAGCAATG
AJG001	AAAAAATCAGCATGCGGACATACTTTAACTCTCCT

2.1.14 Preparation of DNA probes for hybridisation

DNA probes were radioisotopically labelled with ^{32}P using a 'Ready to Go' dCTP labelling kit [Pharmacia] following the manufacturer's recommendations. Hybridisation and post-hybridisation washes were carried out using the RapidHyb (Amersham) system according to the manufacturer's protocols. High and low stringency hybridisations were performed by washing at 75 °C and 65 °C respectively.

2.1.15 Colony Dot Blots

A hybond-N nylon membrane (Amersham) was overlaid onto LB agar and the appropriate strains were streaked onto the membrane and incubated overnight. The cells were lysed by placing the membrane onto a piece of blotting paper soaked in 5% SDS for 5 minutes. The membrane was placed onto on a new piece of blotting paper soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 7 min and then onto a piece of blotting paper soaked in neutralising solution (1M Tris, 1.5M NaCl, pH7.4) for 10 min. To remove cell debris the membrane was dipped into beaker of 2xSSC (20xSSC: 17.53% (w/v) NaCl, 8.82% (w/v) sodium citrate, pH7) for a few seconds prior to being briefly blotted colony side down on blotting paper. The dipping in SSC and blotting was repeated. A positive control (1 µl probe in 9 µl distilled water) was denatured and the product spotted onto a marked place on the membrane. The DNA on the nitrocellulose was then fixed by incubation in an 80°C oven for 2hrs. The membrane was probed with a radioactively labelled DNA probe.

2.1.16 Southern hybridisation

Southern hybridisation was carried out as essentially described by Sambrook *et al.* (1989). Total genomic DNA was extracted, digested with the appropriate enzyme and the DNA fragments

resolved by gel electrophoresis. The DNA fragments were transferred to a nitrocellulose membrane and probed with the appropriate radioactively labelled DNA probe.

2.1.17 Western blotting

Proteins were resolved on SDS PAGE gels (Laemmli, 1970) and electroblotted on to nitrocellulose membranes using a "Mini-Trans Blot Electrophoresis Transfer Cell" [BioRad]. Following transfer the reactive sites on the nitrocellulose were blocked with 2% (w/v) skim milk in 10 mM Tris.HCl-0.9% (w/v) NaCl (Tris-NaCl pH7.4) for 1 hr. The nitrocellulose was incubated overnight with a 1:400 dilution of anti-BipA polyclonal antibodies and subsequently washed three times in blocking buffer. The nitrocellulose was incubated with anti-chicken antibody conjugated to Horse Radish Peroxidase (1:4000 dilution) [Promega] and washed three times in Tris-NaCl buffer. After incubation the immunoreactive bands were visualised by using a chemiluminescent substrate [Super Signal, Pierce].

The BipA antibody was obtained from the immunisation of chickens with 0.5 mg/ml $(His)_6$BipA. Antibodies were produced in the egg yolk and extracted by ammonium sulphate precipitation (Farris *et al.*, 1998.).

2.2 *In vitro* studies

2.2.1 Motility Test

Sterile needles were used to inoculate motility plates with bacterial samples. The plates were incubated for 16 hours at 37°C after which time the diameter size of each bacterial sample was noted.

2.2.2 "Lacy" colony formation test

Bacteria were inoculated into 3 mls of LB and allowed to grow overnight at 37°C with orbital shaking at 225 rpm. A 1 µl drop of overnight culture was placed onto a dried CFA plate and the

plate was incubated overnight at 37°C. Following this incubation period the plate was sealed with Nescofilm [Fisher Scientific] (to prevent drying out) and incubated at 25°C for 7days. The formation of convoluted ("lacy") colony after this time was considered indicative of SEF17 expression.

2.2.3 Transmission Electron Microscopy (TEM)

If grown in broth, the sample was pelleted by centrifugation and resuspended in 1xPBS. A Formavar-carbon coated electron microscopy grid [VLA, Weybridge] was then floated onto a droplet (50 μl) of the bacterial suspension for fifteen minutes. The grid was then blotted on filter paper, and stained with 2% (v/v) phosphotungstic acid [Agar Accessories]. When the cultures were grown on agar, nylon membrane was placed over the agar and the grids placed on the membrane. Several colonies were streaked across the grids and the plates were left to grow under the conditions required. To wash the grids, the grids were placed on to a droplet of 1xPBS for 5 minutes and blotted on filter paper, this wash was repeated three times. The grids were stained as before. Grids were viewed using a Philips CM10 transmission electron microscope. For optimal expression of the different surface appendages several different conditions were used to grow the cultures for TEM:

1. On cfa agar at 25°C the cultures were grown for 72 hrs
2. On cfa agar at 37°C the cultures were grown for 72 hrs
3. In cfa broth at 37°C the cultures were grown for 48 hrs
4. In Heart Infusion Broth (HIB) at 37°C the cultures were grown for 48 hrs
5. On LB-G agar at 25°C the cultures were grown for 24 hrs
6. On LB-G agar at 37°C the cultures were grown for 24 hrs

2.2.4 Log Phase Acid Tolerance Assay

The procedure used to measure acid tolerance response is essentially described by Foster and Hall (Foster and Hall, 1990). Bacteria were inoculated into 10 ml LB and grow overnight at 37°C. The culture was then diluted 1 in 40 (1 ml into 39 ml) into fresh LB pH7.2. The culture was grown to mid-log phase (OD_{600nm} of approximately 0.4-0.5) and diluted 1 in 40 (1 ml in 39 ml) into LB pH7.2 and LB pH5, to give nonadapted and adapted cultures respectively, and grown for 1 hour at 37°C. Following this incubation both cultures were then diluted 1 in 40 (1 ml in 39 ml) into LB pH3 and incubated with shaking at 37°C. Samples were taken from both the adapted and nonadapted cultures at several different time points. Serial dilutions were then plated and, after incubation overnight at 37°C the number of cfu/ml were calculated.

2.2.5 Oxidative stress assay

1ml of an overnight culture was diluted into 39 ml fresh LB. The culture was grown to mid-log phase at which time 10 ml of the culture was added to two universals. To one universal hydrogen peroxide [Sigma] was added to give a final concentration of 0.3 μm (34 μl of 10^{-2} hydrogen peroxide solution) whereas in the other universal only distilled water (34 μl) was added to give adapted and unadapted cultures respectively. Both samples were incubated shaking at 37°C for 1 hour after which hydrogen peroxide was added to both universals to give a final concentration of 30 μm (34 μl hydrogen peroxide solution). The cultures were incubated with shaking at 37°C and samples were taken at given time points and the number of viable bacteria was determined by plating serial dilutions.

2.2.6 Whole cell ELISA method

All bacterial samples were pelleted by centrifugation and resuspended in the same volume of carbonate buffer (0.1M) pH9.6 [VLA, Weybridge]. The top well of a Maxisorb microtitre plate [Nunc] was coated with the bacterial suspension (100 μl) and doubling dilutions of the

suspension in carbonate buffer were performed in subsequent wells. The plate was dried overnight at 37°C and subsequently washed twice with PBS-Tween. The reactive sites were blocked with 200 µl of 3% Marvel™ [Sigma] in PBS (v/v) and incubated shaking for 1 hour at 37°C after which the plate was washed twice with PBS-Tween. The required monoclonal antibody (diluted 1/1000 in conjugate buffer) was added to each well and incubated for a further hour shaking at 37°C and subsequently washed four times with PBS-Tween. Anti-mouse Ig peroxide (100 µl)(diluted 1/5000 in conjugate buffer) [Amersham] was added to each well and incubated shaking at 37°C for 1 hour. The plate was washed four times with PBS-Tween and twice with PBS. TMB (tetramethylbenzidine) substrate [Cambridge Veterinary Sciences] and TMB buffer were mixed (1:1) and added to each well and incubated at room temperature for 5 minutes, after which H_2SO_4 (100 µl)(10% v/v) was added to each well to stop the reaction. Absorbencies were read on a spectrophotometer at 450nm.

For optimal expression of different surface appendages several different conditions were used to grow the cultures for the ELISAs:

1. In cfa broth at 25°C the cultures were grown for 72 hrs
2. In cfa broth at 37°C the cultures were grown for 48 hrs
3. In LB-G broth at 25°C the cultures were grown for 24 hrs
4. In LB-G broth at 37°C the cultures were grown for 24 hrs

Each assay was performed at least twice and the standard *t* test was used for statistical analysis. The *t* test was based on the pooled variance from the analyses of variance. A p value < 0.05 indicated a significant difference at the 5% level.

2.2.7 Two dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed essentially as described previously (Gorg *et al.*, 1988; Qi *et al.*, 1995; Qi *et al.*, 1996; O'Connor *et al.*, 1997; Adams *et al.*, 1999).

2.2.7.1 Preparation of whole cell extracts

10 ml of an overnight culture was added to 990 ml of LB and grown to an OD_{600nm} of approximately 0.4-0.5. The cells were pelleted by centrifugation at 7000 rpm (Beckman 8x50 ml rotor) for 10 minutes. The pellet was washed in an equal volume of 0.9% NaCl (w/v) and respun at 7000 rpm (Beckman 8x50 ml rotor) for 20 minutes. The cells were resuspended in freshly made lysis solution (400 µl Triton X 100, 400 µl β-mercaptoethanol, 400µl ampholines, 5 mM PMSF, in a final volume of 16 ml in distilled water) and sonicated using an MSE Soniprep 150. Following sonication urea was added to a final concentration of 9M and the sample stored in aliquots at -70°C.

2.2.7.2 Preparation of Soluble and insoluble fractions

The sample was prepared as before (section 2.2.7.1), however no Urea or Triton was used in the lysis buffer. After sonication the sample was centrifuged at 1400 rpm (Beckman 8x50 ml rotor) for 10 minutes to remove cell debris and the supernatant respun at 16000 rpm (Beckman 8x50ml rotor) for 60 minutes. The supernatant (soluble fraction) was removed and the desired amount of urea (0.54 g/ml) and Triton X 100 (25 µl/ml) were added to the sample. The pellet ('cell envelope' fraction) was resuspended in 500 µl of lysis buffer containing urea and triton.

2.2.7.3 IPG phor strip rehydration and first dimension run

The appropriate volume of sample to give approximately 4mg of protein was mixed with rehydration solution (2.4g Urea, 0.1 g CHAPS, 100 µl IPG-Buffer pH 4-7, 15 mg dithiothreitiol (DTT), few grains of Orange G and 5ml of distilled water) to give a final volume of 360 µl. The

sample was added to an immobilised pH gradient strip (pH4-7) [Amersham-Pharmacia] and the strip rehydrated for 12 hours prior to electrophoresis.

2.2.7.4 Equilibration of immobiline drystrips

The strip was first equilibrated for 10 minutes in equilibration solution (10 ml 0.5M Tris-HCl pH6.8, 36 g Urea, 30 ml Glycerol, 1 g SDS made up to 100ml with distilled water) containing DTT (25 mg) and then for ten minutes in equilibration solution containing idoacetamide (0.45 g plus a few grains of bromophenol blue).

2.2.7.5 Second dimension run

Electrophoresis of the strip was carried out using 12-14% gradient gels [Amersham-Pharmacia] on a Pharmacia Mulitiophor II Electrophoresis unit according to the manufacturer's instructions.

2.2.7.6 Gel Analysis

Gels were visualised by coomassie blue staining and digitised as previously described (Qi *et al.*, 1995). Images were analysed using the Phoretix-2D software v. 5.1 [Phoretix] and imported to Adobe Photoshop v. 5.0 for annotating and printing. Only proteins that were reproducibly induced or repressed at least twice fold in at least two separate experiments were noted as significant.

2.2.8 Tyrosine Phosphorylation Assay

An overnight culture was diluted 1 in 30 (1 ml into 29 ml) into fresh LB and grown at 37°C to mid-log phase. The cells were pelleted by centrifugation and washed twice in 1 x kinase buffer (50 mM Tris-HCl (pH 7.5, 1 mM Na2EGTA (pH 8.0), resuspending in 30 ml the first time and in 10 ml buffer the second. The cells were sonicated using an MSE Soniprep 150 and spun at 5000 rpm (Beckman 8x50 ml rotor) for 10 minutes to remove unbroken cells. The supernatant

was respun in fresh sterile tubes at 20,000 rpm (Beckman 8x50 ml rotor) for 30 minutes to pellet the membranes. The supernatant was left on ice and the glassy membrane pellet resuspended in 1 x kinase RB (1 ml) and left on ice. The protein extract (membrane or supernatant) (10 µl) was added to a microcentrifuge tube to which [γ -^{32}P] ATP (1 µl) [3000 Cimmol^{-1}; Amersham] and 1 x kinase RB (8 µl) was added. The reaction was incubated at 37°C for 15-30 minutes after which 2 x FSB (1.0 ml 0.5 M Tris-HCl, 800 µl Glycerol, 1.6 ml 10% SDS, 400 µl β-mercaptoethanol, 200 µl 0.05% (w/v) bromophenol blue, 4.0 ml distilled water) (20 µl) was added. The sample was boiled and run on a 13% SDS-PAGE gel. The gel was dried and exposed to film.

2.2.9 Measurement of invasion by gentamicin resistance

The conditions used were essentially those described by Elsinghorst (1994). HeLa cells were seeded to a density such that after an overnight incubation (37°C / 5% CO_2) a 95-100% confluent monolayer was obtained. Confluent monolayers contained between 4 and 6 x 10^5 cells in each well of a 24-well plate. Three wells were used for each test.

For the adhesion assay, HeLa cell monolayers were washed twice with DMEM (no additions) and fresh DMEM was applied to a volume of 1 ml prior to infection. Wild type S1400/94 and the *bipA::kan* mutant were incubated statically overnight. A sample of culture was added to give a multiplicity of infection of approximately 10 bacteria per cell. After infection the plate was gently shaken to distribute the inoculum and incubated for 2 hrs at 37°C / 5% CO_2. The monolayers were washed six times with DMEM (no additions) to remove bacteria that had not adhered to the cells and then disrupted with 1 ml 0.1% Triton X-100 in PBS. The supernatant was then removed and the number of adherent bacteria determined by plating out serial dilutions.

Invasion assays were performed in duplicate 24-well plates. After allowing the bacteria to adhere with the monolayer for 2 hrs, wells were washed three times with DMEM (no additions) before adding 2 ml of DMEM containing 100 µg/ml gentamicin to the wells. The plates were incubated

for 2 hrs at 37°C / 5% CO_2. After this incubation the monolayers ware washed six times with PBS to remove the gentamicin and incubated for 5 min with 1 ml 0.1% Triton X-100 in PBS. The supernatant was removed and bacterial invasion determined by plating serial dilutions. Each adhesion and invasion assay was performed at least twice (three wells per test).

2.2.10 Invasion of Macrophages

Bone-marrow derived macrophages from CBA or B6 mice (obtained from Thelma Biggs, Southampton University) and RAW macrophage-like cells were grown in DMEM containing 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamicin. Monolayers of macrophages for bacterial invasion were prepared by seeding $2x10^6$ macrophages into each well of a 12-well plate. After allowing the bacteria to adhere with the monolayers for 2 hrs, wells were washed twice with DMEM (no additions) and fresh DMEM was applied (1ml per well) prior to infection. The monolayers were infected with bacteria at a 10:1 multiplicity of infection. After infection the plate was gently shaken for 5 minutes to distribute the inoculum and incubated for 2 hrs (37°C / 5% CO_2). After incubation the monolayers were washed three times with DMEM (no additions) to remove bacteria that had not adhered to the cells. Fresh DMEM supplemented with 100 μg/ml of gentamicin was added to the monolayers and incubated for 2 hrs to kill any bacteria that had not invaded. The monolayers were washed twice with PBS and lysed in 1ml 0.1% Triton X-100. Serial dilutions were plated on LB agar medium and, following overnight incubation at 37°C, the number of survivors were determined.

2.3 *In vivo* studies

2.3.1 Chickens dosing regimes

30 newly hatched White Leghorn chicks (specific-pathogen-free) were divided into two groups comprising of fifteen birds each, which were housed in two separate isolators. Chicks were dosed orally with 0.1 ml culture containing $5x10^4$ cfu (Allen-Vercoe *et al.*, 1999) and delivered

into the crop by gavage tube (Cooper *et al.*, 1994b). Each group of birds was dosed with either *S. enteritidis* S1400/94 or *S. enteritidis bipA::kan*. The chicks were permitted feed and water *ad libitum*, observed three times daily and killed *in extremis*. At 6 hours, 24 hours and 48 hours post inoculation, five birds were selected at random from each group and liver, spleen and caecal counts of *Salmonellas* were performed.

2.3.2 Enumeration of *Salmonellae* in organ homogenates

Birds were killed by cervical dislocation and whole liver, spleen and caecum were removed at post mortem, placed in individual sterile vessels and homogenised in PBS (pH 7.2) for 1 minute. The viable count in liver, spleen and caecum was determined by plating decimal dilutions made in sterile PBS on LB with or without kanamycin. Samples of organ homogenates were enriched for *Salmonellae* in Selenite-F broth (CM395/L121, Oxoid) with incubation for 24h at 37°C followed by subculture on to brilliant green agar, BGA (CM329, Oxoid).

2.3.3 Mice dosing regimes

135 6-week old BALB/c mice were randomly divided into groups of 5 animals per box. Six boxes of mice were infected, by the intraperitoneal (i.p) route, with one serial dilution in the range 10^1 cfu to 10^6 cfu of *Salmonella typhimurium* SL1344. This was repeated for *Salmonella typhimurium* SL1344 *bipA::cat*. Six boxes of mice were infected, by the oral route, with one serial dilution in the range 10^4 cfu to 10^9 cfu of *Salmonella typhimurium* SL1344. This was repeated for *Salmonella typhimurium* SL1344 *bipA::cat*. The three remaining boxes were left as controls. Each group of mice were kept separate, with food and water was permitted *ad libitum*, and any mouse *in extremis* was killed. Survival over a period of sixteen days was recorded.

CHAPTER 3

3 Production and preliminary characterisation of a *S. enteritidis bipA* null mutant

3.1 Introduction

EPEC BipA has been shown to be involved in several processes associated with pathogenesis (Farris *et al.,* 1998), therefore one of the aims of this study was to identify if *Salmonella enteritidis* pathogenesis is affected by BipA. To study its role in pathogenesis and other processes a *bipA* knockout mutant was constructed in the strain *S. enteritidis* S1400/94. The approach usually used to construct a knock out mutant is by insertional inactivation of the target gene. This method relies on the gene being cloned into a vector so that a selectable marker can be inserted within the gene to render it inactive. However, although PCR amplification of full-length *bipA* was readily achieved, repeated experiments to clone the fragment into many different vectors proved unsuccessful. As a result the approach taken was to PCR amplify only the 3' and 5' ends of *bipA* and insert both fragments into a suicide vector. A selectable marker could then be inserted in between the two fragments. Several different techniques, including PCR, colony dot blots, southern hybridisation and western blotting, were used to confirm the construction of the mutant. Interestingly, further into my studies, the full-length *bipA* was eventually cloned into the vector pCR2.1. This proved very useful as the clone could then be used for sequence analysis studies. This is important because, for example, low sequence conservation between different organisms could account for the different phenotypes observed when comparing the role of a protein in two different species.

Like all other organisms, environmental temperature will profoundly affect the growth of the microorganism. *Salmonella* and *E. coli* are able to survive within a temperature range of approximately 10-45°C. Above or below these temperatures key proteins become denatured and

growth stops altogether. Little is known about the role of *S. enteritidis bipA* so it was important to establish if this gene affected such basic growth characteristics. Such studies are also important because in the case of *E. coli*, it has been shown that if you knock out BipA the organism becomes temperature sensitive at 25°C (A.J. Grant, unpublished results).

Previous studies have also showed that EPEC BipA undergoes tyrosine phosphorylation (Farris *et al.,* 1998). Therefore, to carry out initial comparisons of the *S. enteritidis* BipA and EPEC BipA, experiments were carried out to determine if *S. enteritidis* BipA undergoes phosphorylation.

3.2 RESULTS

3.2.1 Cloning and Sequencing of *S. enteritidis bipA* gene

The *bipA* gene of *S. enteritidis* strain S1400 was cloned and sequenced to determine the degree of sequence conservation of *S. enteritidis bipA* with *S. typhimurium bipA* and *E. coli bipA*. The full-length *bipA,* including the promoter region, was amplified using primers AJG001 (complementary to start of *glnA*) and ALW21 (complementary to the region just 5' to the stop codon of *bipA*). The resulting amplified fragment was then cloned into the vector, pCR2.1, to give plasmid pAW5 (Figure 3-1).

3.2.2 Sequence analysis of *bipA*

Sequencing was carried out from the plasmid clone, pAW5 and this sequence was compared to the sequence obtained from pYL98 (*S. enteritidis bipA* cloned into pBR322, Y. Li., unpublished results. EMBL accession no. AJ276889). No differences were seen between the two sequences, suggesting that the sequence obtained from pAW5 is correct.

Comparison of *bipA* gene sequences of *S. typhimurium* and *S. enteritidis* revealed ten base differences between the two sequences (Figure 3-2). However, only one of these base differences is not a silent mutation (Figure 3-3). At residue 266, the asparagine amino acid in *S. enteritidis* is substituted for an aspartic acid in *S. typhimurium* (GAT→ AAT). Thus, the two proteins are almost identical.

Alignment of the *S. enteritidis* and EPEC *bipA* genes show that the sequences are more divergent towards the 3' end of *bipA*. There are two main areas where the sequences diverge more compared to the rest of the alignment; these areas include nucleotides 1051 to 1163 (80.3% homologous) and nucleotides 1929 to 3007 (82.0% homologous). However, overall the *S. enteritidis bipA* sequence is 90% homologous to the EPEC *bipA* sequence, suggesting that BipA is a highly conserved protein.

3.2.3 Construction of a *bipA* null mutant of *S. enteritidis* S1400/94

As a prerequisite to studying the role of BipA protein in *S. enteritidis*, a knockout mutant was constructed using allelic replacement. The 5' and 3' portions of the *S. enteritidis bipA* gene were isolated by PCR using primers based on the sequence of the *bipA* gene of EPEC strain MAR001. The resulting amplified DNA fragments were then cloned sequentially into the suicide vector pERFORMC to generate plasmid pAW3. The construct was then modified by insertion of a 1.4 kb cassette, carrying a Km^r gene, between the 5' and 3' fragments to generate plasmid pAW4 (Figure 3-5). pAW4 was mobilised into S1400/94 to enable allelic exchange between the chromosomal *bipA* gene and the insertionally inactivated form present on the plasmid (Figure 3-6). Candidate *bipA::kan* colonies were screened for the loss of the wild type gene by PCR, colony dot blots, Southern hybridisation and Western blotting.

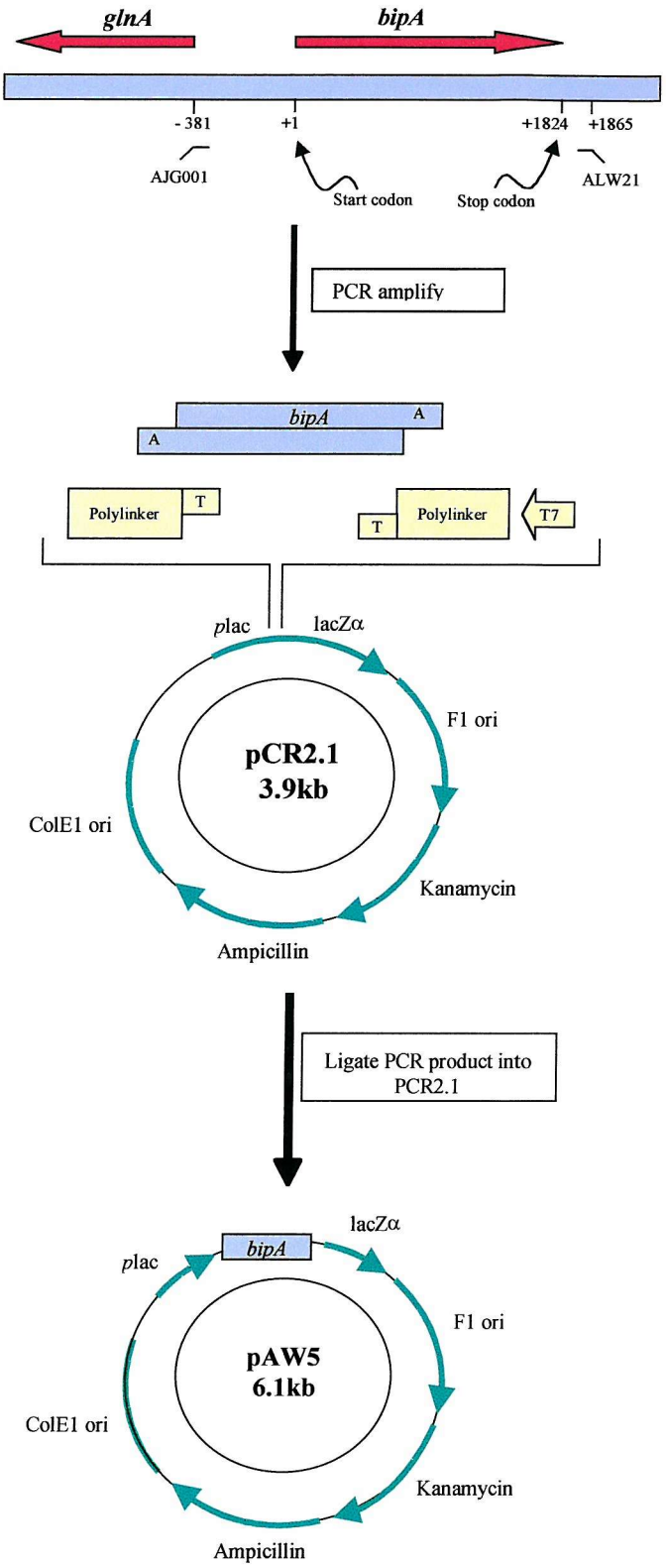
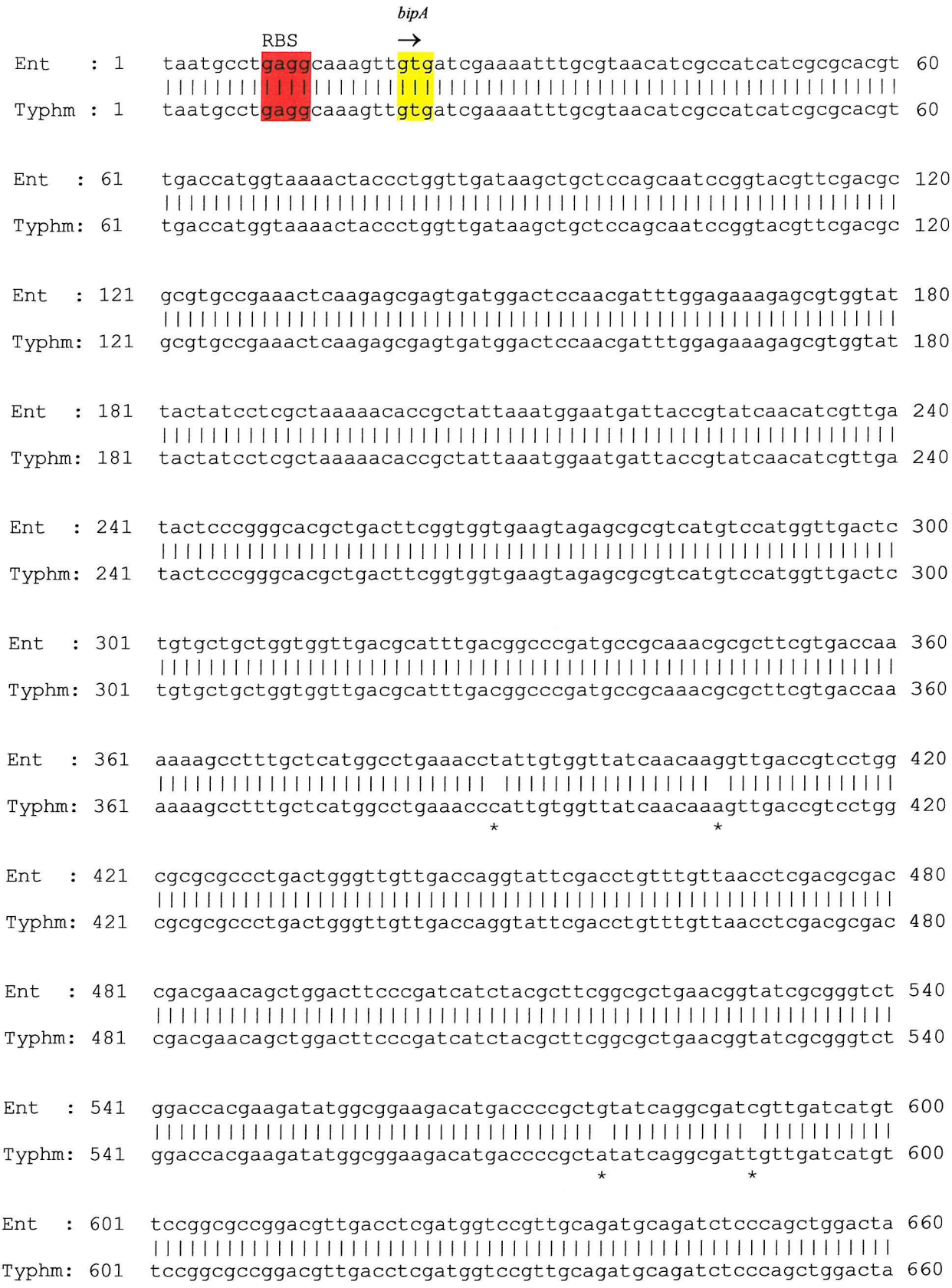


Figure 3-1 A flow diagram that outlines the cloning of *bipA* of *S. enteritidis* S1400

Following PCR amplification of the *bipA* gene and its promoter region the DNA fragment was inserted into pCR2.1 using the Original TA Cloning Kit. This works on the principle that *Taq* polymerase has a non template-dependent activity, which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The vector supplied in the kit has single deoxythymidine (T) residues that therefore allow the PCR insert to ligate efficiently with the vector

Figure 3-2 Alignment of the nucleotide sequences of *S. enteritidis* and *S. typhimurium*



```
Ent  : 661  caacaactatgttggcgttatcggcattggccgtatcaaacgcggcaaagtgaagccgaa 720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Typhm: 661  caacaactatgttggcgttatcggcattggccgtatcaaacgcggcaaagtgaagccgaa 720

Ent  : 721  ccagcaggtcactatcatcgatagtgaagggaaaacccgtaacgcgaaagtaggtaaagt 780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Typhm: 721  ccagcaggtcactatcatcgatagtgaagggaaaacccgtaacgcgaaagtaggtaaagt 780

Ent  : 781  gctgacgcatctgggtctggagcgtatcgacagcgatatcgccgaagcgggcgatatcat 840
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 781  gctgacgcatctgggtctggagcgtatcgacagcaatatcgccgaagcgggcgatatcat 840
                                         *

Ent  : 841  tgcgatcaccggtctgggcgagctgaacatttccgacaccatctgcgacccgcagaacgt 900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Typhm: 841  tgcgatcaccggtctgggcgagctgaacatttccgacaccatctgcgacccgcagaacgt 900

Ent  : 901  tgaagcgctgccggcgttgtccgttgatgagccgaccgtgtctatgttcttctgcgttaa 960
            ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Typhm: 901  tgaagcgctgccggcgctgtccgttgatgagccgaccgtgtctatgttcttctgcgttaa 960
                          *

Ent  : 961  cacctcgccgttctgcggtaaagaaggtaagtttgtgacttctcgtcagattcttgaccg 1020
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Typhm: 961  cacctcgccgttctgcggtaaagaaggtaagtttgtgacttctcgtcagattcttgaccg 1020

Ent  : 1021 tctgaacaaagagctggtgcataacgtggcgctgcgcgttgaagaaaccgaagatgcgga 1080
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Ent  : 1081 tgcgttccgtgtatccggtcgtggcgaactgcacctgtccgtgctgattgaaaatatgcg 1140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Ent  : 1201 tcgtaaacaagagccgtacgaaaacgtgacgctggacgtcgaagagcagcaccaggggtc 1260
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Ent  : 1261 tgtcatgcaggcgctgggcgagcgtaaaggcgacctgaaaaacatgaatccggacggtaa 1320
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Typhm: 1261 tgtcatgcaggcgctgggcgagcgtaaaggcgacctgaaaaacatgaatccggacggtaa 1320

Ent  : 1321 aggccgcgtacgtctcgactacgtgatcccaagccgtgggctgattggtttccgttcaga 1380
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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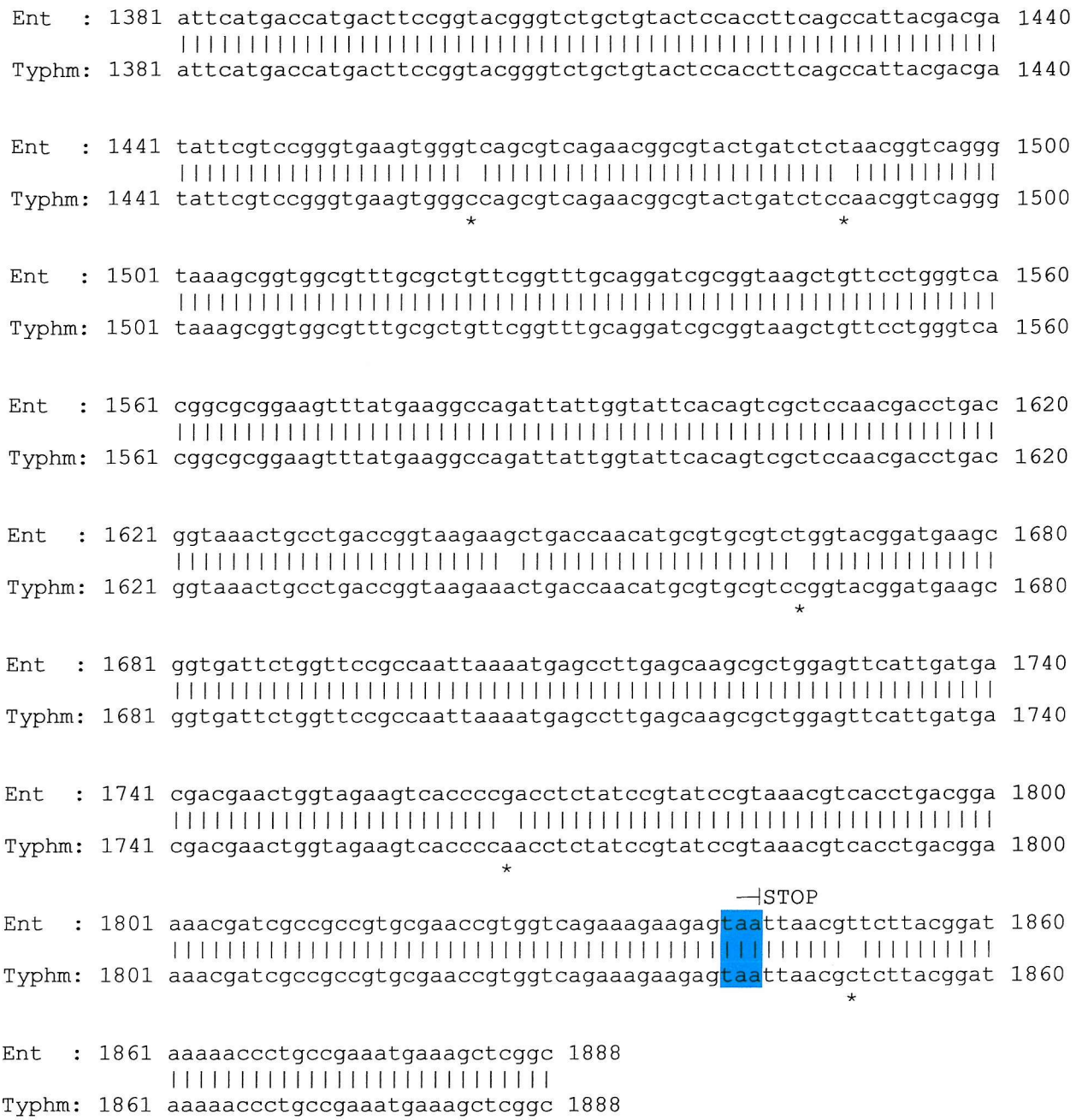


Figure 3-2 shows an alignment of the nucleotide sequences of *S. enteritidis* S1400 and *S. typhimurium* SL1344. The *S. typhimurium* sequence was obtained from the NCBI database (accession no. AJ276889). An asterisk highlights the sequence differences between the two strains. The yellow and blue highlighted sequences represent the start and stop codons of *bipA* respectively. The red highlighted sequences represent the ribosomal binding sites. The alignment shows that the sequences are very similar and only differ in 10 nucleotides.

```
Ent  : 1   MIENLRNIAIIAHVDHGKTTLVDKLLQQSGTFDARAETQERVMDSNDLEKERGITILAKN 60
           MIENLRNIAIIAHVDHGKTTLVDKLLQQSGTFDARAETQERVMDSNDLEKERGITILAKN
Typhm: 1   MIENLRNIAIIAHVDHGKTTLVDKLLQQSGTFDARAETQERVMDSNDLEKERGITILAKN 60

Ent  : 61  TAIKWNDYRINIVDTPGHADFGGEVERVMSMVDSVLLVVDAFDGPMPQTRFVTKKAFAHG 120
           TAIKWNDYRINIVDTPGHADFGGEVERVMSMVDSVLLVVDAFDGPMPQTRFVTKKAFAHG
Typhm: 61  TAIKWNDYRINIVDTPGHADFGGEVERVMSMVDSVLLVVDAFDGPMPQTRFVTKKAFAHG 120

Ent  : 121 LKPIVVINKVDRPGARPDWVVDQVFDLFVNLDATDEQLDFPIIYASALNGIAGLDHEDMA 180
           LKPIVVINKVDRPGARPDWVVDQVFDLFVNLDATDEQLDFPIIYASALNGIAGLDHEDMA
Typhm: 121 LKPIVVINKVDRPGARPDWVVDQVFDLFVNLDATDEQLDFPIIYASALNGIAGLDHEDMA 180

Ent  : 181 EDMTPLYQAIVDHVPAPDVDLDGPLQMQISQLDYNNYVGVIGIGRIKRGKVKPNQQVTII 240
           EDMTPLYQAIVDHVPAPDVDLDGPLQMQISQLDYNNYVGVIGIGRIKRGKVKPNQQVTII
Typhm: 181 EDMTPLYQAIVDHVPAPDVDLDGPLQMQISQLDYNNYVGVIGIGRIKRGKVKPNQQVTII 240

Ent  : 241 DSEGKTRNAKVGKVLTHLGLERIDSDIAEAGDIIAITGLGELNISDTICDPQNVEALPAL 300
           DSEGKTRNAKVGKVLTHLGLERIDS+IAEAGDIIAITGLGELNISDTICDPQNVEALPAL
Typhm: 241 DSEGKTRNAKVGKVLTHLGLERIDSNIAEAGDIIAITGLGELNISDTICDPQNVEALPAL 300

Ent  : 301 SVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVALRVEETEDADAFRVSG 360
           SVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVALRVEETEDADAFRVSG
Typhm: 301 SVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVALRVEETEDADAFRVSG 360

Ent  : 361 RGELHLSVLIENMRREGFELAVSRPKVIFREIDGRKQEPYENVTLDVEEQHQGSVMQALG 420
           RGELHLSVLIENMRREGFELAVSRPKVIFREIDGRKQEPYENVTLDVEEQHQGSVMQALG
Typhm: 361 RGELHLSVLIENMRREGFELAVSRPKVIFREIDGRKQEPYENVTLDVEEQHQGSVMQALG 420

Ent  : 421 ERKGDLKNMNPDGKGRVRLDYVIPSRGLIGFRSEFMTMTSGTGLLYSTFSHYDDIRPGEV 480
           ERKGDLKNMNPDGKGRVRLDYVIPSRGLIGFRSEFMTMTSGTGLLYSTFSHYDDIRPGEV
Typhm: 421 ERKGDLKNMNPDGKGRVRLDYVIPSRGLIGFRSEFMTMTSGTGLLYSTFSHYDDIRPGEV 480

Ent  : 481 GQRQNGVLISNGQGKAVAFALFGLQDRGKLFLGHGAEVYEGQIIGIHSRSNDLTVNCLTG 540
           GQRQNGVLISNGQGKAVAFALFGLQDRGKLFLGHGAEVYEGQIIGIHSRSNDLTVNCLTG
Typhm: 481 GQRQNGVLISNGQGKAVAFALFGLQDRGKLFLGHGAEVYEGQIIGIHSRSNDLTVNCLTG 540

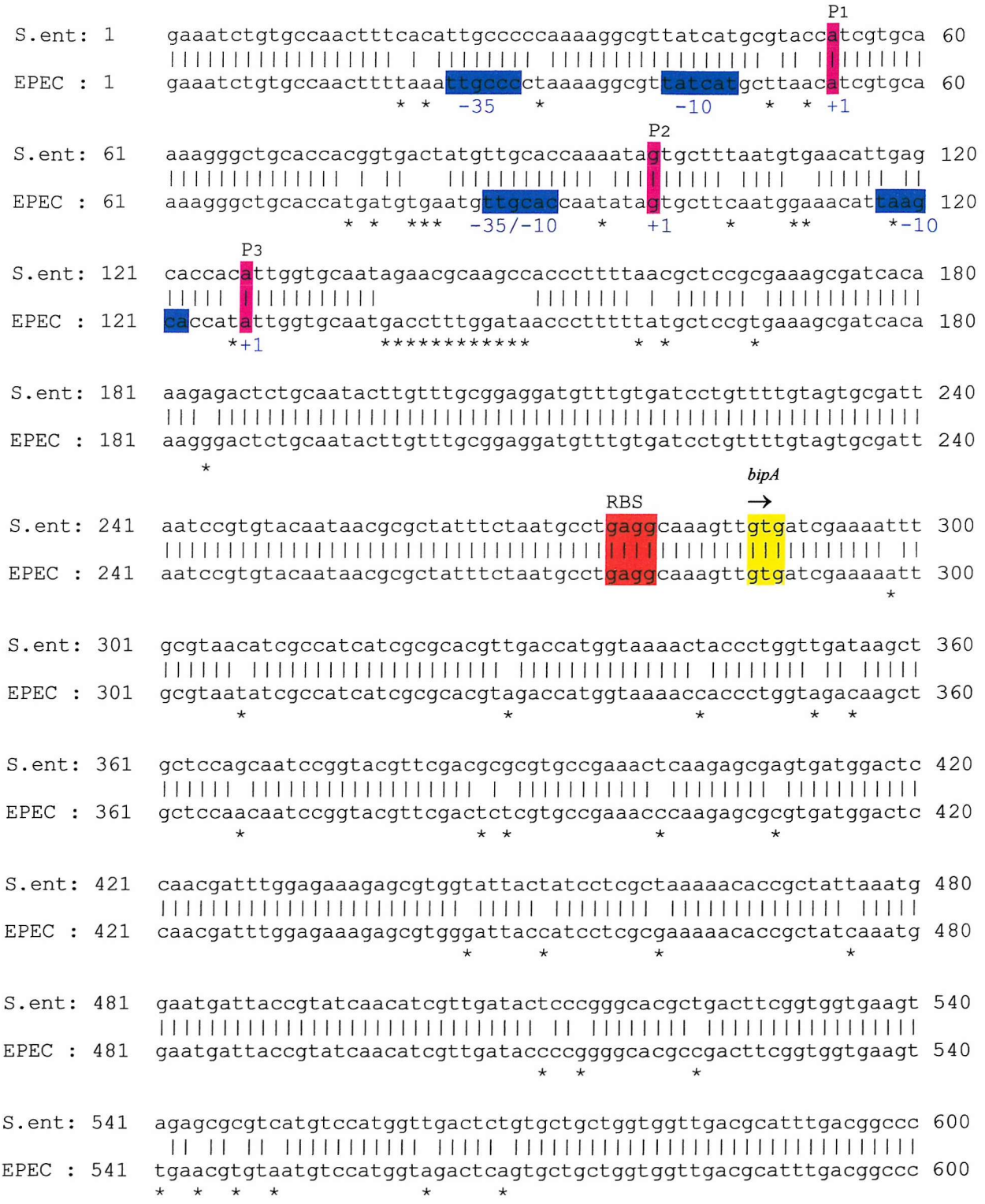
Ent  : 541 KKLTNMRASGTDEAVILVPPIKMSLEQALEFIDDDELVEVTPTSIRIRKRHLTENDRRRA 600
           KKLTNMRASGTDEAVILVPPIKMSLEQALEFIDDDELVEVTPTSIRIRKRHLTENDRRRA
Typhm: 541 KKLTNMRASGTDEAVILVPPIKMSLEQALEFIDDDELVEVTPTSIRIRKRHLTENDRRRA 600

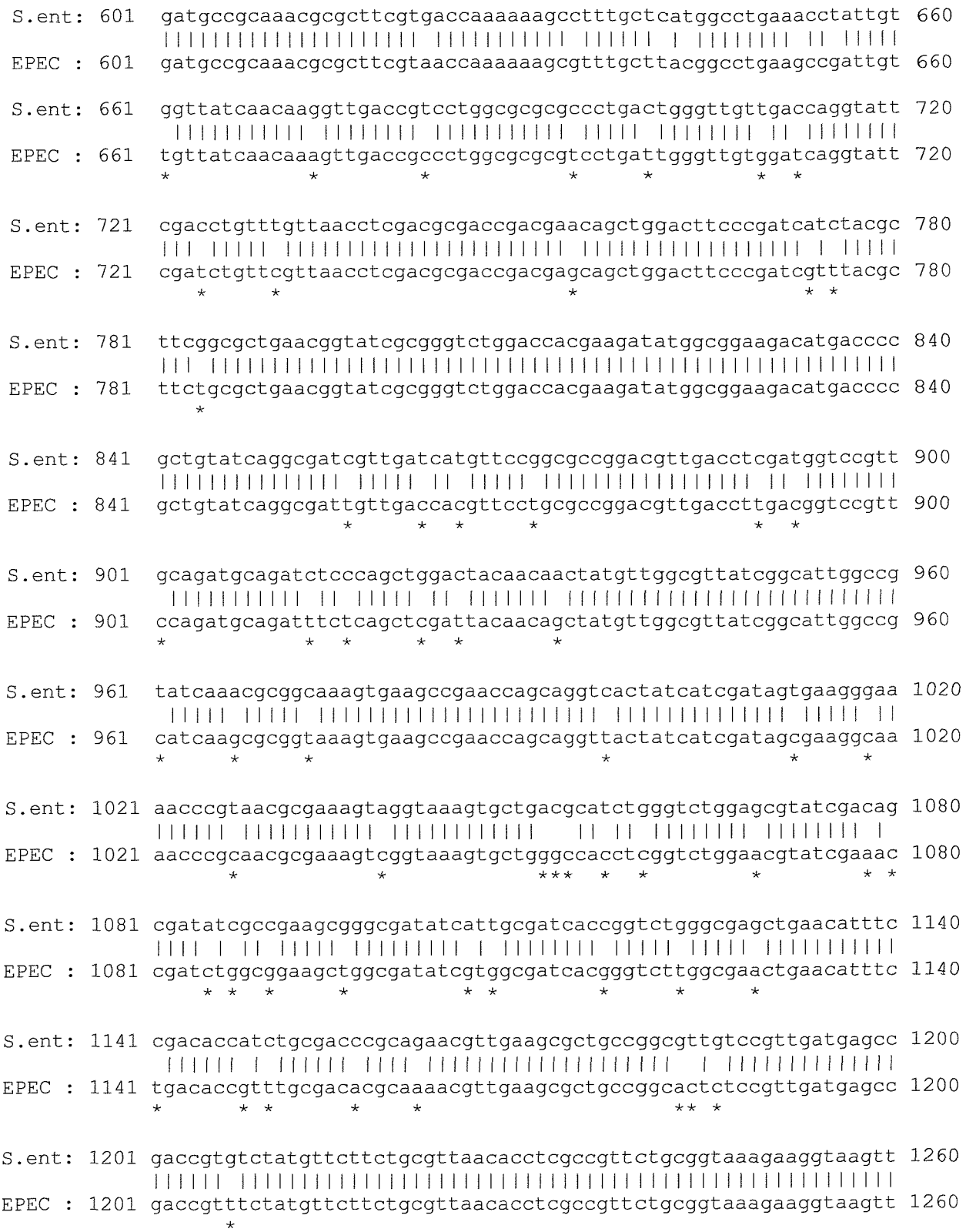
Ent  : 601 NRGQKEE 607
           NRGQKEE
Typhm: 601 NRGQKEE 607
```

Figure 3-3 Protein alignment of *S. enteritidis* and *S. typhimurium* BipA

The *S. enteritidis* and *S. typhimurium* BipA proteins only differ in one protein (highlighted in blue). At residue 266, the asparagine amino acid in *S. enteritidis* is substituted for an aspartic acid in *S. typhimurium* (GAT→ AAT).

Figure 3-4 **Alignment of the nucleotide sequences of *S. enteritidis* and EPEC strain E2348/69**

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EPEC : 601 gatgccgcaaacgcgcttcgtaaccaaaaaagcgtttgcttacggcctgaagccgattgt 660
S.ent: 661 ggttatcaacaaggttgaccgtcctggcgcgcgccctgactgggttgttgaccaggtatt 720
EPEC : 661 tgttatcaacaaagttgaccgccctggcgcgcgtcctgattgggttgtggatcaggtatt 720
S.ent: 721 cgacctgtttgttaacctcgacgcgaccgacgaacagctggacttcccgatcatctacgc 780
EPEC : 721 cgatctgttcgttaacctcgacgcgaccgacgagcagctggacttcccgatcgtttacgc 780
S.ent: 781 ttcggcgctgaacggtatcgcgggtctggaccacgaagatatggcggaagacatgacccc 840
EPEC : 781 ttctgcgctgaacggtatcgcgggtctggaccacgaagatatggcggaagacatgacccc 840
S.ent: 841 gctgtatcaggcgatcgttgatcatgttccggcgccggacgttgacctcgatggtccgtt 900
EPEC : 841 gctgtatcaggcgattgttgaccacgttcctgcgccggacgttgaccttgacggtccgtt 900
S.ent: 901 gcagatgcagatctcccagctggactacaacaactatgttggcgttatcggcattggccg 960
EPEC : 901 ccagatgcagatttctcagctcgattacaacagctatgttggcgttatcggcattggccg 960
S.ent: 961 tatcaaacgcggcaaagtgaagccgaaccagcaggtcactatcatcgatagtgaagggaa 1020
EPEC : 961 catcaagcgcggtaaagtgaagccgaaccagcaggttactatcatcgatagcgaaggcaa 1020
S.ent: 1021 aacccgtaacgcgaaagtaggtaaagtgctgacgcatctgggtctggagcgtatcgacag 1080
EPEC : 1021 aacccgcaacgcgaaagtcggtaaagtgctgggccacctcggtctggaacgtatcgaaac 1080
S.ent: 1081 cgatatcgccgaagcgggcgatatcattgcgatcaccggtctgggcgagctgaacatttc 1140
EPEC : 1081 cgatctggcggaagctggcgatatcgtggcgatcacgggtcttggcgaactgaacatttc 1140
S.ent: 1141 cgacaccatctgcgacccgcagaacgttgaagcgctgccggcgttgtccgttgatgagcc 1200
EPEC : 1141 tgacaccgtttgcgacacgcaaaacgttgaagcgctgccggcactctccgttgatgagcc 1200
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EPEC : 1321 acgcgtagaagaaaccgaagacgccgatgcgttccgcgtttctggtcgtggcgaactgca 1380

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EPEC : 1681 gtactccaccttcagccactacgacgacgtacgtccgggtgaagtgggtcagcgtcagaa 1740

S.ent: 1741 cggcgtactgatctctaacggtcagggtaaagcggtggcgtttgcgctgttcggtttgca 1800
EPEC : 1741 cggcgtactgacctctaacggtcagggtaaagcggcagcgttcgcgctgttcggtctgca 1800

S.ent: 1801 ggatcgcggtaagctgttcctgggtcacggcgcggaagtttatgaaggccagattattgg 1860
EPEC : 1801 ggatcgcggtaagctgttcctcggtcacggtgctgaagtttacgaaggtcagattatcgg 1860

S.ent: 1861 tattcacagtcgctccaacgacctgacggtaaactgcctgaccggtaagaagctgaccaa 1920
EPEC : 1861 tattcacagccgctctaacgacctgactgtaaactgcctgaccggtaagaaactgaccaa 1920
```

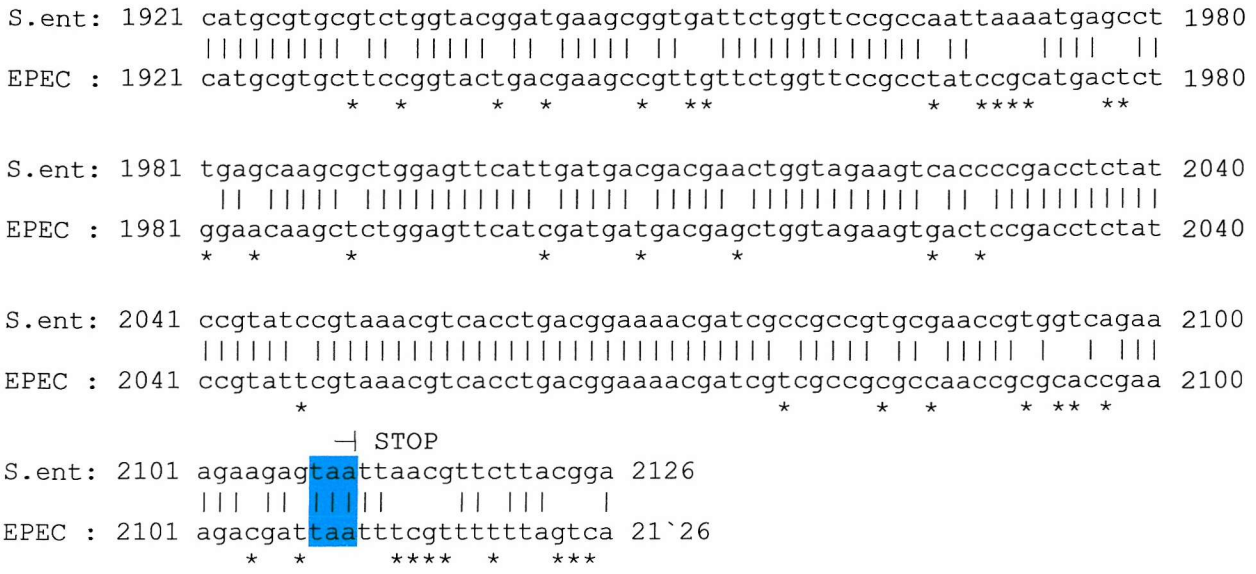
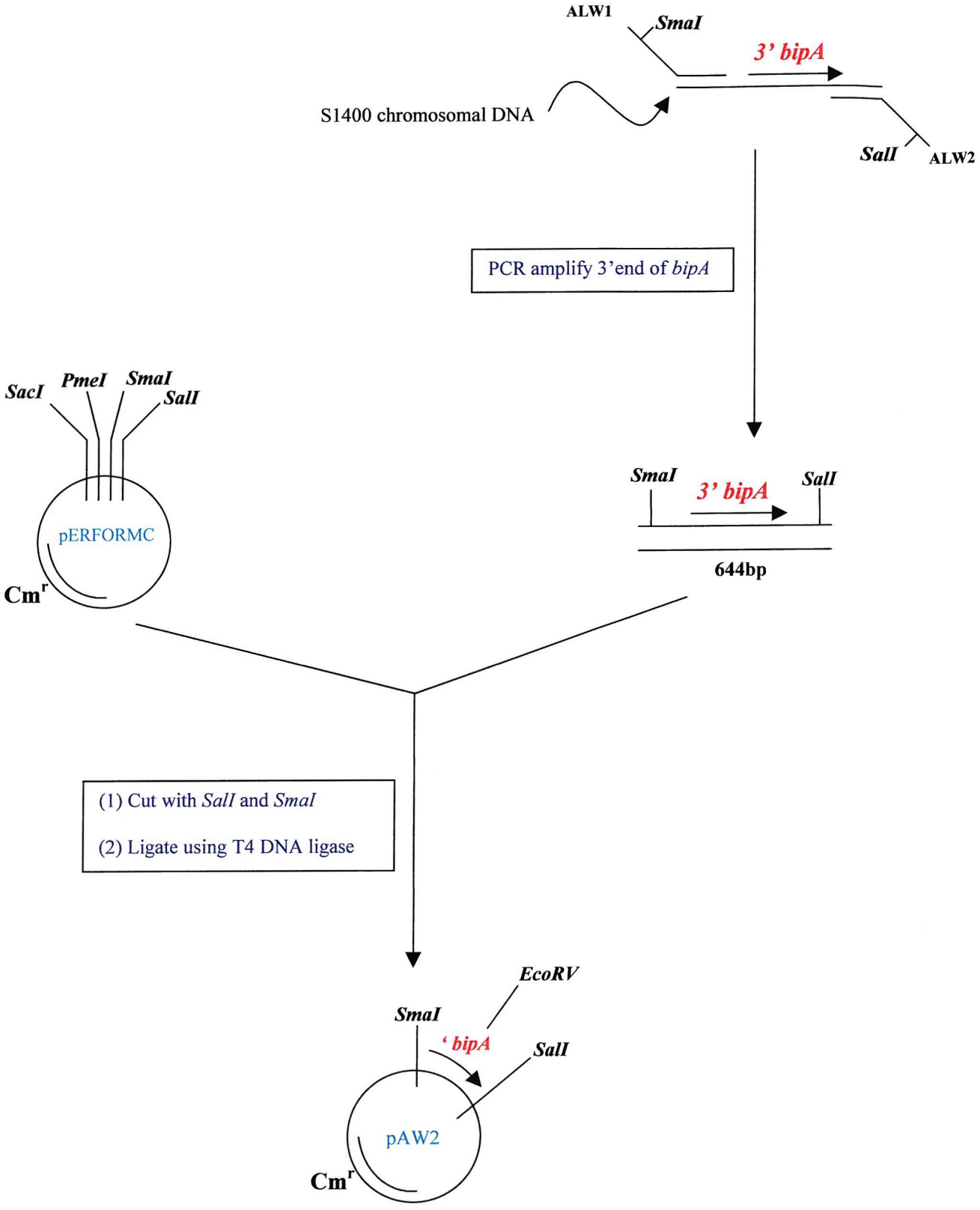
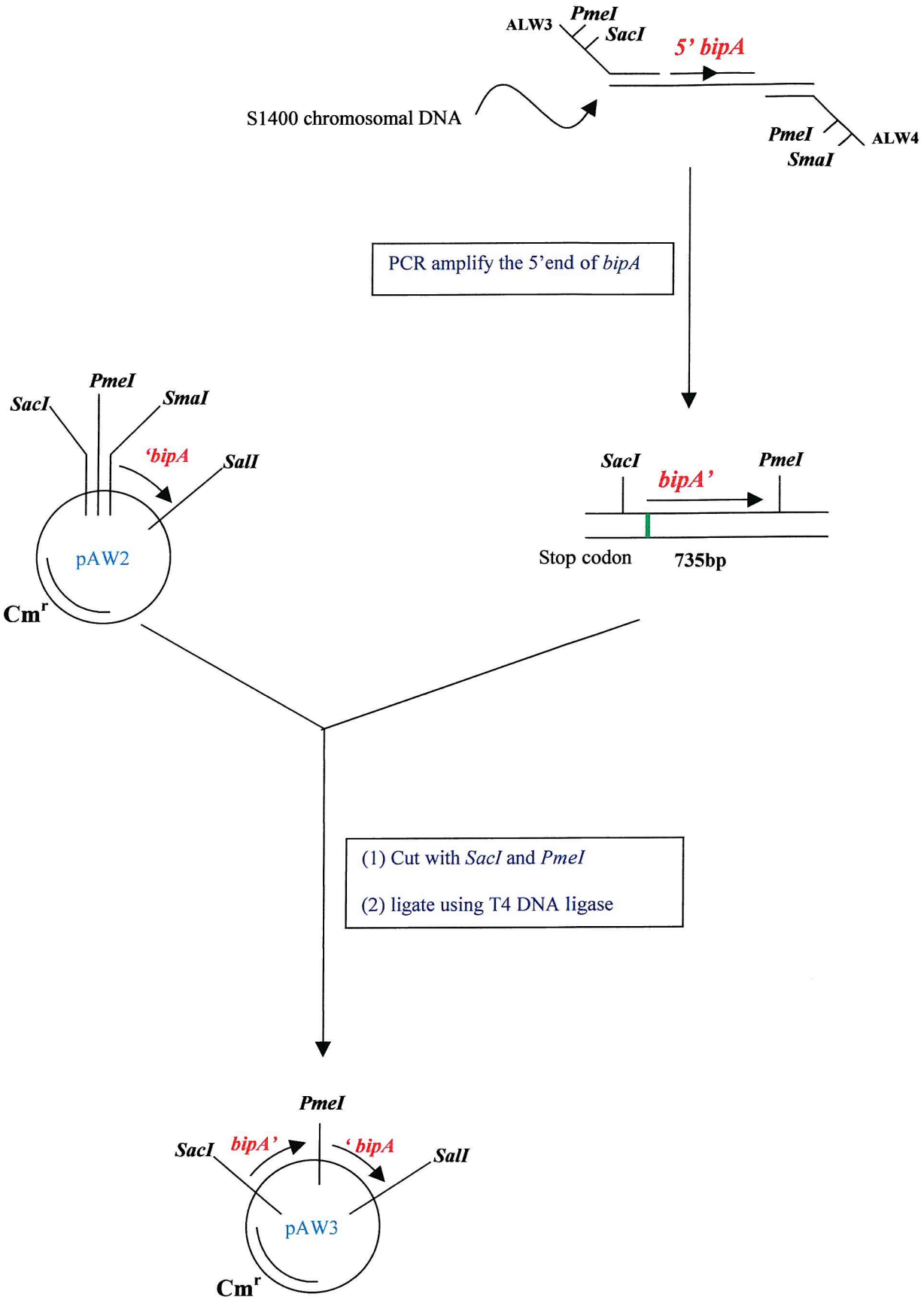


Figure 3-4 shows an alignment of the nucleotide sequences of *S. enteritidis* S1400 and that of the enteropathogenic *E. coli* strain E2348/69. The E2348/69 sequence was obtained from the NCBI database (accession no. AJ278218). An asterisk highlights the sequence differences between the two strains. The yellow and blue highlighted sequences represent the start and stop codons of *bipA* respectively. The red highlighted sequences represent the ribosomal binding sites. The alignment shows that the sequences are more divergent towards the 3' end of *bipA*. However, overall the sequences are very similar suggesting that BipA is a highly conserved protein. Transcript mapping using the EPEC strain E2348/69 has show that in *E. coli bipA* there are three potential promoters, P1, P2 and P3 (S. Payot., unpublished data). The potential transcription starts (+1) are highlighted in pink, whereas the potential RNA polymerase recognition (-35) and binding sites (–10) are highlighted in blue.

Figure 3-5 Construction of plasmid designated pAW4




ALW3
PmeI
SacI
5' bipA
S1400 chromosomal DNA
PmeI
SmaI
ALW4
PCR amplify the 5'end of bipA
SacI
PmeI
SmaI
'bipA
SalI
pAW2
Cm^r
SacI
bipA'
PmeI
Stop codon
735bp
(1) Cut with SacI and PmeI
(2) ligate using T4 DNA ligase
PmeI
SacI
bipA'
'bipA
SalI
pAW3
Cm^r

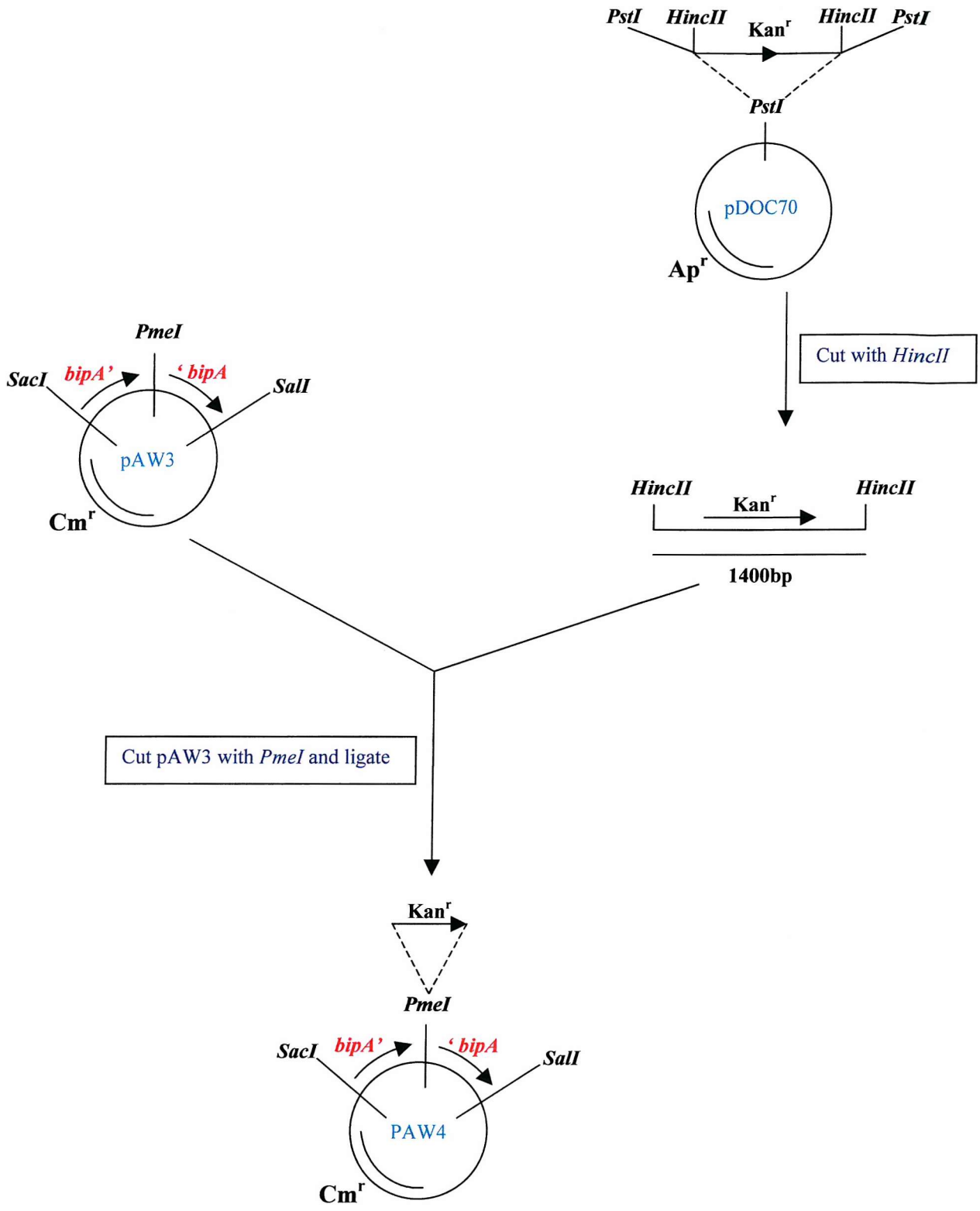


Figure 3-5 outlines the construction of a plasmid pAW4. To knock out the *S. enteritidis bipA* gene the 5' and 3' portions of *bipA* were isolated by PCR and cloned sequentially into the suicide vector pERFORMC to generate plasmid pAW3. The construct was then modified by insertion of a 1.4 kb cassette, carrying a Km^r gene, between the 5' and 3' fragments to generate pAW4.

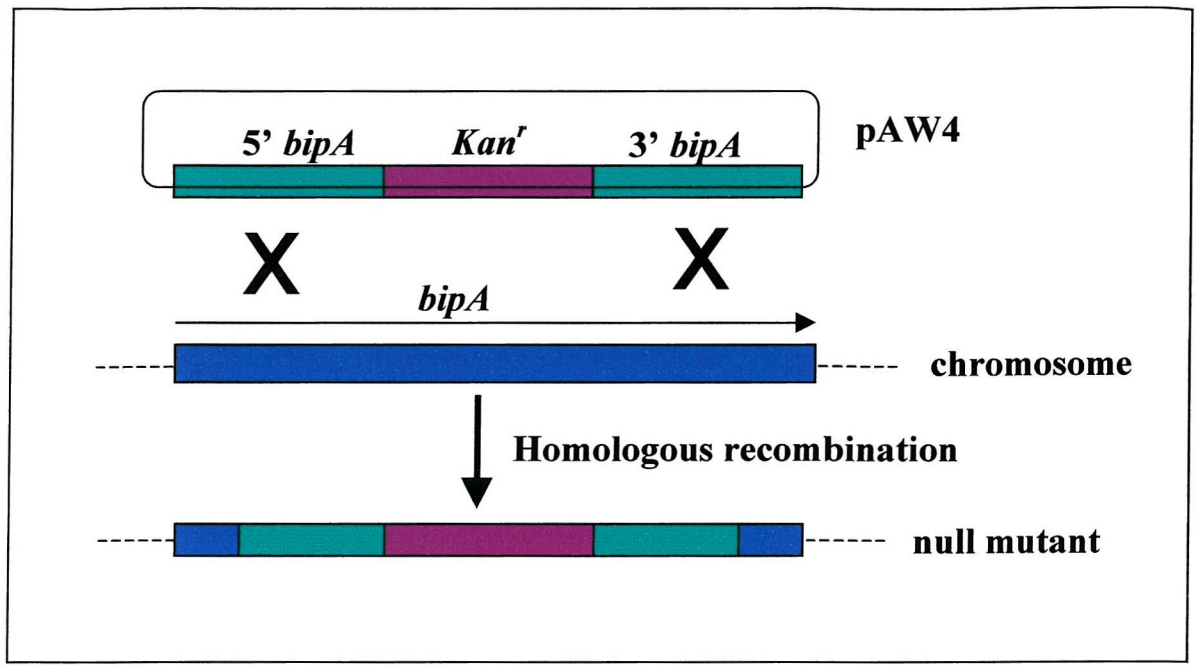


Figure 3-6 Construction of a *bipA* null mutant of *S. enteritidis* S1400/94

pAW4 was mobilised into S1400/94 to enable homologous recombination to occur between the chromosomal *bipA* gene and the insertionally inactivated form present on the plasmid. Double crossover recombination events were selected for by selection of kanamycin-resistant, chloramphenicol-sensitive colonies

3.3 Characterisation of putative null mutant

3.3.1 Colony blot hybridisation

Three 'putative' null mutants were screened for the insertion of the kanamycin cassette by colony dot blots. Wild type *S. enteritidis* and three 'putative' mutants were grown overnight on a membrane overlaid on a LB plate. The cells were then lysed *in situ* and the DNA probed. The presence of the kanamycin cassette was confirmed by probing with a radiolabelled *HincII* fragment derived from the kanamycin cassette (Figure 3-7) and the loss of the suicide vector was confirmed by probing with radiolabelled pERFORMC DNA (data not shown).

3.3.2 PCR analysis of the *bipA* genes in *S. enteritidis* S1400 and S1400 *bipA::kan* strains

Primers ALW2 and ALW3 will amplify full-length *bipA*. The primer pair was therefore used to amplify the *bipA* region from wild type *S. enteritidis*, three putative *bipA::kan* mutants and plasmid pAW4. In contrast to the wild type strain, which generated a 1.63 kb fragment, the three putative mutants each yielded a 3.03 kb PCR product, as did the positive control pAW4 (Figure 3-8). This would be expected if the wild type *bipA* gene had been replaced by the *bipA::kan* allele as the upshift of 1.4 kb is due to the insertion of the kanamycin cassette.

3.3.3 Southern Hybridisation of DNA from S1400 and putative *bipA::kan* mutant

Southern hybridisation experiments were carried out to confirm that homologous recombination had occurred in a putative *bipA::kan* strain. Total genomic DNA was extracted from *S. enteritidis* S1400/94 and one isogenic *bipA*$^-$ derivative. The genomic DNA was digested to completion using *MluI*, resolved by agarose gel electrophoresis, transferred to nylon membranes and hybridised with a probe consisting of the *bipA* gene. Analysis of the resulting autoradiogram showed that an approximately 9.50 kb *MluI* fragment hybridised with the *bipA* probe in S1400

genomic DNA. In contrast, an approximately 10.9 kb fragment was found in the putative *bipA::kan* mutant (Figure 3-9). This is also consistent with replacement of the wild type *bipA* gene on the chromosome with the *bipA::kan* allele as the 1.4 kb increase in the *MluI* fragment of the mutant confirms the presence of the kanamycin cassette.

3.3.4 Western Blotting

Whole cell extracts from S1400 and a putative null mutant were run on a SDS PAGE gel and transferred to a nylon membrane prior to probing with anti-BipA antibodies. No expression of the BipA protein could be detected in the sample of the mutant extract although a 72 kDa band, consistent with that of BipA, was readily detected in the wild type sample (Figure 3-10).

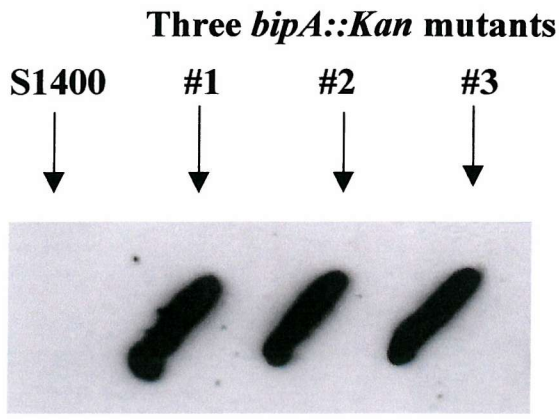


Figure 3-7 Autoradiogram of colony dot blot Hybridisation

Three candidate *bipA::kan* transconjugants that were screened for the insertion of the kanamycin cassette into *bipA* by colony dot blots. The insertion of the kanamycin cassette was confirmed by probing with a radiolabelled DNA fragment of the kanamycin cassette. Wild type S1400/94 was used as a negative control. Lane 1: S1400/94, lane 2: *bipA::kan* #1, lane 3: *bipA::kan* #2, lane 4: *bipA::kan* #3

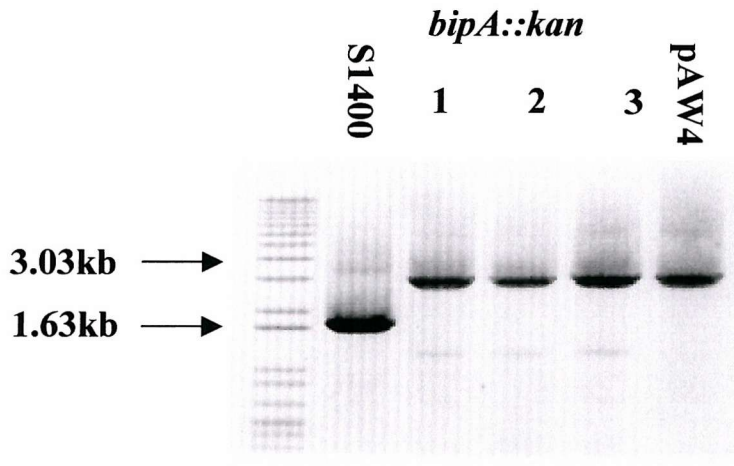


Figure 3-8 PCR amplification of the *bipA* region from S1400 and putative *bipA::kan* mutant cells

Further characterisation of the three mutants was carried out using PCR and genomic DNA from the relevant strains. The insertion of the kanamycin cassette was confirmed using primers ALW2 and ALW3 that amplify full-length *bipA*. Lane 1: S1400/94, lane 2: *bipA::kan* #1, lane 3: *bipA::kan* #2, lane 4: *bipA::kan* #3, lane 5: pAW4.

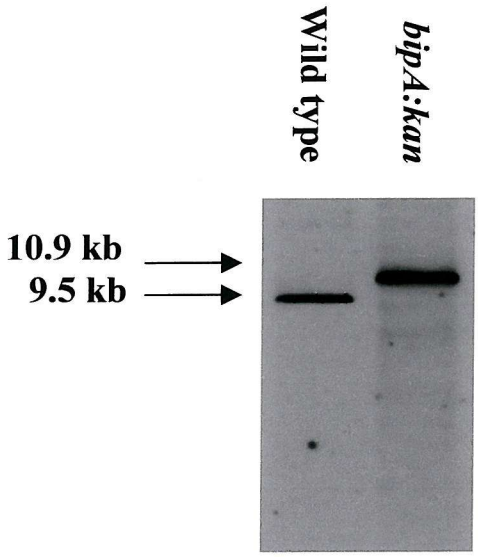


Figure 3-9 Southern Hybridisation of DNA from S1400 and putative *bipA::kan* mutant

MluI-digested chromosomal DNA from the parental and *bipA::kan* #3 strains were analysed by southern hybridisation using a radiolabelled full length *bipA* fragment as probe. Lane 1: S1400/94, Lane 2: *bipA::kan* #3.

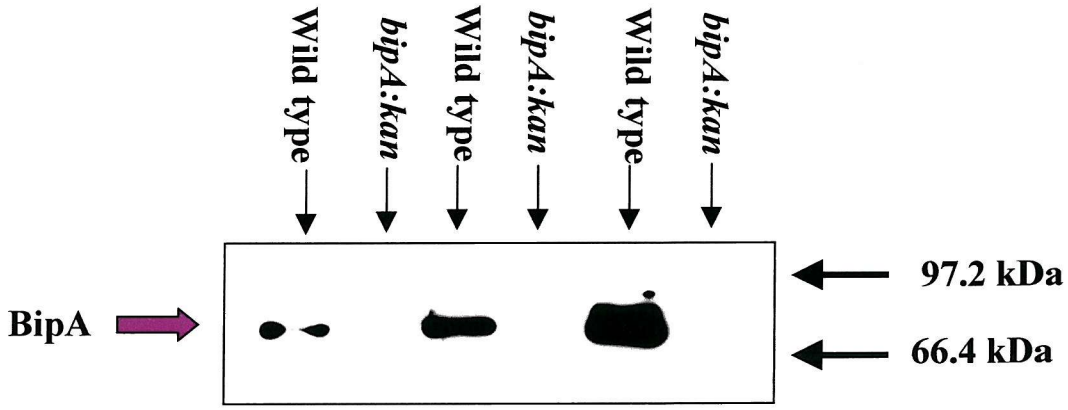


Figure 3-10 Western Blotting of whole cell extracts from S1400 and a putative *bipA::kan* mutant

Proteins from S1400/94 and the null mutant (#3) were separated by a SDS PAGE gel and transferred to a nylon membrane that was probed with anti-BipA antibodies. Increasing amounts of whole cell extracts from the WT and putative null mutant strain were loaded from left to right on the gel.

3.4 *In vitro* characterisation of general features/properties of the *bipA::kan* mutant of *S. enteritidis*

From the experiments above it was concluded that a *bipA::kan* mutant had been successfully constructed. This was therefore taken for further study. The first set of experiments was to characterise the role of *bipA* in *S. enteritidis* by determining if the gene affected basic growth characteristics or conferred auxotrophy. Secondly, previous immunoblotting experiments indicated that the BipA protein of EPEC is tyrosine phosphorylated (Farris *et al.,* 1998). Eukaryotic tyrosine phosphorylation is common and pivotal in the regulation of cell division and development, it is however uncommon in prokaryotes, and its biological significance is unclear (Zhang, 1996). It was therefore of interest to determine if *S. enteritidis* BipA undergoes phosphorylation.

3.4.1 Growth characteristics of mutant relative to parent cells

To determine the base-line characteristics of the *bipA::kan* mutant relative to parent cells their growth rates in LB and minimal medium were compared. No significant differences were detected at 37°C when grown in either LB or minimal media (Figure 3-11) or at 30°C when grown on LB plates. This indicates that BipA does not regulate processes that are essential for growth under these conditions.

3.4.2 *S. enteritidis bipA* does not undergo tyrosine phosphorylation

The immunoblotting experiments showed that in EPEC a protein that migrates at approximately 80kDa cross-reacted with [γ-^{32}P] ATP, whereas no detectable band was seen in either the membrane extracts or soluble extracts of *S. enteritidis* (Figure 3-12). The 65kDa BipA protein migrates anomalously in SDS-polyacrylamide gels at 75kDa (S.Y. Qi and C.D. O'Connor, unpublished results). Therefore, these results may suggest that EPEC BipA undergoes

phosphorylation, but *S. enteritidis* BipA does not. However, further experiments would need to be carried out to determine this conclusively.

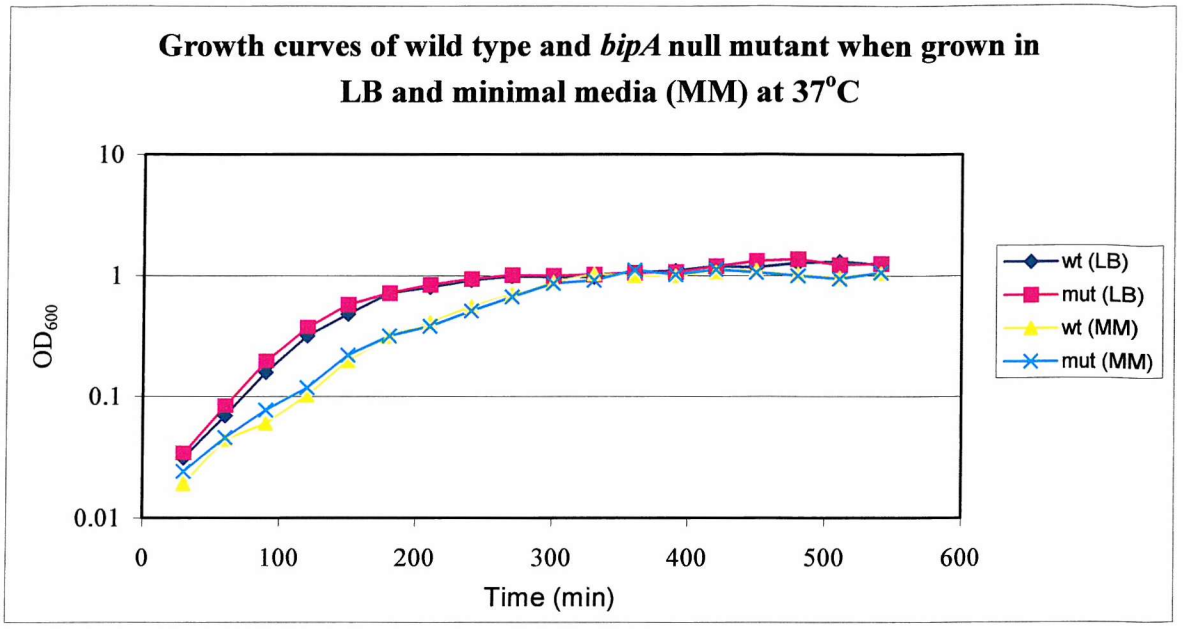


Figure 3-11 Growth characteristics of *bipA::kan* mutant relative to parent cells

Overnight cultures of S1400 and *bipA::kan* were inoculated into pre-warmed, fresh LB and minimal media (MM) (1 in 50 dilution) and grown at 37°C for ten hours. An OD_{600} was taken every thirty minutes to determine the growth rates of the wild type and mutant in both rich and minimal media.

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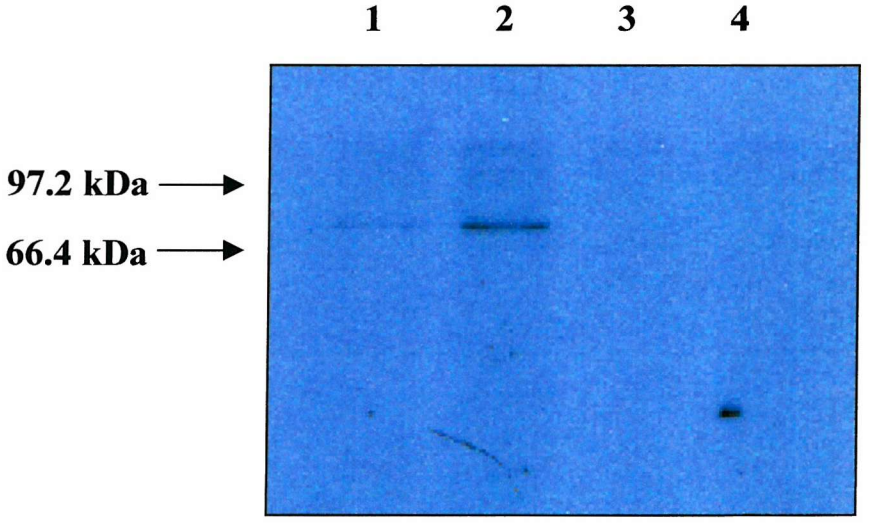


Figure 3-12 Cross-reaction of *S. enteritidis* and EPEC extracts with [γ^{32}P] ATP

S1400 and MAR001 were sonicated to give soluble and membrane extracts which were then incubated with [γ^{32}P] ATP. The extracts were then separated by SDS PAGE gel and the gel was dried and exposed to film. Equal amounts of each extract were loaded. Lane 1: MAR001 membrane extract, Lane 2: MAR001 soluble extract, Lane 3: S1400 membrane extract, Lane 4: S1400 soluble extract

3.5 Discussion

Very little is known about the role of BipA in *Salmonella*, therefore my main aim in this chapter was to first clone the *bipA* gene, for sequence comparisons and transcomplementation studies, and secondly to construct a *bipA* knockout mutant to allow detailed analysis of phenotypic changes. In this chapter, it has been established that the *S. enteritidis bipA* gene has been cloned and that a *bipA* knockout mutant has been constructed. Further studies with this knockout mutant have shown that BipA does not regulate processes that are essential for growth in LB or minimal media. It has also been shown that, unlike EPEC BipA, *Salmonella* BipA is not phosphorylated.

3.5.1 Cloning of *S. enteritidis bipA* gene

Repeated attempts to clone the *bipA* into several different vectors using restriction enzymes were not successful. The reason for this is unclear however partial fragments of *bipA* could be cloned into the suicide vector, pERFORMC. In this case a stop codon was engineered into the 5' fragment of *bipA* so that no BipA protein would expressed and therefore could not be deleterious to the cells. This meant that a knockout mutant could be constructed without having to clone the whole of *bipA*. As it is important to be able to transcomplement any phenotypic defects observed with a knockout mutant further strategies were used to try and clone *bipA*. Successful cloning of *bipA* was eventually achieved when a TA cloning approach was used. This relies on the knowledge that PCR amplification of DNA, when using *Taq* polymerase, results in deoxyadenosine (A) overhangs. The vector is therefore constructed with deoxythymidine (T) overhangs that will allow efficient ligation of the PCR insert without the use of restriction enzymes.

The insertion of a kanamycin cassette into *bipA* may have resulted in polar effects where the expression of genes downstream of *bipA* may have been affected. That is, the cassette may cause termination of transcripts initiated by the operon's promoter but the cassette itself does not

provide a promoter capable of expressing distal genes. This could only be avoided if the kanamycin cassette is designed with ribosomal binding sites and start codons at either end of the cassette so that when the cassette was inserted into the gene in either orientation the transcription of genes in an operon would not stopped. Unfortunately the cassette that was designed failed to be inserted into *bipA* and the resulting cassette that was cloned did not contain additional ribosomal binding sites or start codons. In addition, the kanamycin cassette used contained its own promoter and several studies (Hirsh *et al.,* 1986) have reported that the promoters of antibiotic resistance genes may affect the expression of distal genes (Kendrick and Reznikoff, 1988; Hirsh *et al.,* 1986) . However, the phenotypes seen with the mutant are assumed to be a result of the *bipA* mutation, but it is essential to be aware that the insertion of the cassette could cause polarity and misregulation.

3.5.2 Sequence analysis of *bipA*

The *S. typhimurium* BipA sequence differs from the *S. enteritidis* BipA sequence by only one residue (Asp→Asn). This base change does not map within domain IV of EF-G, which mimics the anti-codon arm of tRNA and is believed to interact with ribosomes (Nissen *et al.,* 1995; Rodnina *et al.,* 1997), or within the regions that define a guanine nucleotide-binding pocket. This suggests that the interaction of BipA with ribosomes will be the same in *S. typhimurium* and *S. enteritidis*.

3.5.3 *S. enteritidis bipA* is not phosphorylated

Until recently tyrosine phosphorylation was considered to be confined to eukaryotes (Levitzki and Gazit, 1995). However, it has now been reported in several species of bacteria (Freestone *et al.,* 1998). Previous studies have shown that *E. coli* BipA is tyrosine phosphorylated (Farris *et al.,* 1998). In contrast, this study indicates that *S. enteritidis* BipA is not phosphorylated. However, further work needs to be carried out to determine this conclusively. The reason for the

difference between the two species is unknown as the role for BipA phosphorylation remains to be determined.

CHAPTER 4

4 Phenotypic characteristics and virulence properties of *bipA* Null mutant

4.1 Introduction

In the previous chapter a *bipA* knockout mutant was constructed and characterised. In this chapter, using this *bipA* null mutant, the role of *bipA* in the regulation of fimbriae and flagella will be determined as these appendages have been implicated in the association between host cells and *Salmonella*.

4.1.1 Fimbriae

Fimbriae are considered to be virulence factors as they are thought to be involved in the initial adherence of *Salmonella* to host mucosal surfaces. *S. enteritidis* exhibit at least five different types of fimbriae including SEF21 (type 1 fimbriae), SEF14, SEF17, PEF and LPF.

4.1.1.1 Type 1 fimbriae

Type 1 fimbriae, which mediate mannose-sensitive agglutination of erythrocytes (Duguid *et al.*, 1966), have been shown to be involved in the adhesion to various tissue culture cell-lines (Tavendale *et al.*, 1983; Ernst *et al.*, 1990; Dibb-Fuller *et al.*, 1999) and animal tissues (Lillard, 1986; Lindquist *et al.*, 1987; Oyofo *et al.*, 1989; Craven *et al.*, 1992; Isaacson and Kinsel, 1992; Ewen *et al.*, 1997). However, other studies have failed to shown any role for type 1 fimbriae in the adhesion to various cell lines (Barrow *et al.*, 1992). Lockman and Curtis (1992) found that single mutants in either flagella or type 1 fimbriae were as virulent as the wild type strain in the murine typhoid model, but strains harbouring mutations in both flagella and type 1 were attenuated. These discrepancies may be due to the use of undefined mutants or to the lack

of knowledge about factors that influence *in vivo* fimbrial phase variation. Also, the use of different cell lines may explain these contradictory results as Baumler *et al.* (1996a) reported that type 1 fimbriae were involved in the adhesion to HeLa cells but not to Hep-2 and MDCK cells .

The *Salmonella* serovars Gallinarum and Pullorum exhibit host specificity for poultry and aquatic birds and are not able to cause disease in mammalian hosts. Gallinarum and Pullorum are unable to mediate mannose-sensitive hemagglutination and it was found that when these serovars expressed *S. typhimurium* type 1 fimbriae they exhibited a significant increase in adherence and invasiveness for mammalian cells. This suggested that these fimbriae are essential in adherence and invasion (Wilson *et al., 2000*). In contrast, recent studies using defined mutants have shown that type 1 fimbriae are not involved in the adhesion to chick kidney epithelial cells or to chick gut explants (Lee *et al.,* 1996; Allen-Vercoe and Woodward, 1999). The role for SEF21 in *S. enteritidis* therefore remains equivocal.

4.1.1.2 SEF14 fimbriae

The SEF14 fimbriae are restricted to *S. enteritidis* and other closely related group D *Salmonella.* Again there are conflicting results as to the role of SEF14 in pathogenesis. Evidence suggests that SEF14 is involved in the adherence of *S. enteritidis* to mouse epithelial cells and that the pre-treatment of mice with SEF14 antibodies protects mice from *S. enteritidis* infection (Peralta *et al.,* 1994). Also, Thiagarajan *et al.* (1996) have demonstrated that SEF14 mediates the attachment of *S. enteritidis* to chicken ovarian granulosa cells. In contrast, various other *in vitro* and *in vivo* studies suggested no role for SEF14 fimbriae in *Salmonella* pathogenesis (Thorns *et al.,* 1996; Ogunniyi *et al.,* 1997; Allen-Vercoe *et al.,* 1999; Allen-Vercoe and Woodward, 1999; Dibb-Fuller *et al.,* 1999; Rajashekara *et al.,* 2000). Studies carried out by Edwards *et al.* (2000) may explain this paradox as they revealed that subsequent to the colonisation and penetration of the intestinal barrier SEF14 fimbriae mediate interactions between the bacteria

and macrophages. Therefore, SEF14 may only be required for the systemic infection at stages beyond the initial colonisation of host epithelial surfaces.

4.1.1.3 SEF17 fimbriae

SEF17 fimbriae, analogues of the 'curli' fimbriae of *Escherichia coli*, are essential for the generation of 'convoluted' or 'lacy' colonies of *S. enteritidis* at 25°C on Colonisation Factor Agar (CFA) (Allen-Vercoe *et al.,* 1997). The elaboration of these convoluted colonies is associated with the ability of the strain to contaminate eggs (Petter, 1993). It is also associated with the ability to cause infection in mice and to its tolerance to certain environmental conditions including heat, acid and hydrogen peroxide (Humphrey *et al.,* 1996). In *S. typhimurium* the SEF17 homologue is thought to play a role in the adhesion and invasion of mouse tissues as *in vitro* work showed that SEF17 promotes interaction of the bacteria with mouse small intestine epithelial cells (Sukupolvi *et al.,* 1997). *In vivo* work showed that a *S. typhimurium* mutant which displayed a decrease in the expression of SEF17 caused a chronic infection in mice as opposed to the normal systemic infection (Sukupolvi *et al.,* 1997). Also, a *S. enteritidis* SEF17$^-$ strain showed a significant reduction in the adherence and invasion of cultured epithelial cells (Dibb-Fuller *et al.,* 1999). In contrast, Rajashekara and co-workers (2000) suggest that SEF17 did not enhance *S. enteritidis* internalisation of enterocytes or macrophages and that in SPF chickens the invasion, persistence and excretion of the bacteria was not affected by SEF17. Allen-Vercoe and Woodward (1999b) also found that *S. enteritidis* SEF17 defective strains adhered as well as the wild type strain to chick gut explants.

4.1.1.4 PEF fimbriae

Serotype Associated Plasmids (SAPs) have been identified in a number of *Salmonella* spp. and are thought to contribute to virulence. One locus on the plasmid has been identified as a fimbrial gene that elaborates fine, peritrichous, matted fimbriae, otherwise know as Plasmid Encoded

Fimbriae (PEF) (Friedrich *et al.,* 1993). The *pef* fimbrial operon mediates adhesion of *Salmonella typhimurium* to the murine small intestine and is necessary for fluid accumulation (Bäumler *et al.,* 1996). PEF may selectively bind to certain tissues as it has been demonstrated that even though PEF was involved in adhering to the murine small intestine, adhesion to epithelial cell lines such as Hela, Hep-2, Int-407, MDCK or murine Peyer's patch cells could not be seen (Bäumler *et al.,* 1996). It has been show that the *Salmonella* virulence plasmid enhances *Salmonella*–induced lysis of macrophages and influences inflammatory responses, therefore the SAP genes may only be required after the initial colonisation and invasion (Guilloteau *et al.,* 1996). The role of PEF in *S. enteritidis* is unclear, but it has been shown *in vivo* to be expressed within the chick (Woodward *et al.,* 1996).

4.1.1.5 LPF fimbriae

Long Polar Fimbriae (LPF) were discovered by Baumler and Heffron (1995) and were found to be long, fine fimbriae radiating from the poles of the bacteria and are thought to be involved in adhesion to murine Peyer's patches (Bäumler *et al.,* 1996). Very little is known about how the *lpf* operon contributes to the colonisation of the Peyer's patches.

4.1.2 Flagella

In *E. coli* flagella are one of the cell surface appendages that have been well defined and are thought to be responsible for bacterial motility. Bacteria are exposed to a wide variety of environments and in order to survive the bacteria must be able to swim away from or towards favourable or toxic environments respectively (Aizawa, 1996). Flagella are composed of three parts, the basal body, the curved hook and the filament. The synthesis of flagella is controlled by the expression of genes that are organised into a regulatory hierarchy of three classes. Each class is required for the expression of the next (Kutsukake *et al.,* 1990). The first class (class I) includes two master regulatory genes, *flhDC*, that when induced turn on expression of the

middle-class (class II) of genes that are required for the structure and assembly of the hook-basal body complex (Yokota and Gots, 1970; Komeda *et al.,* 1975). Included in the middle-class of genes is the *fliA* gene that encodes the alternative sigma factor, σ^{28}, required specifically for the transcription of the late class of genes (Kutsukake *et al.,* 1990; Ohnishi *et al.,* 1990). The late class (class III) includes genes that express flagellin, hook associated proteins (HAP) (Ikeda *et al.,* 1987) and genes that are responsible for chemotaxis and motility. Class III also encodes the anti-sigma factor, FlgM, which binds to FliA to prevent its association with RNA polymerase core enzyme. Following assembly of the hook-basal body complex, the flagellum-specific export apparatus secretes FlgM, therefore flagella assembly is effectively coupled with transcriptional regulation.

Flagella have been implicated in the survival within murine macrophages. Flagellated *S. typhimurium* survived longer than non-flagellated derivatives in an *in vitro* macrophage assay (Weinstein *et al.,* 1984). Also, non-motile *S. typhimurium* mutants were less able to survive within mouse macrophages and were significantly less virulent than the wild type *in vivo* (Fields *et al.,* 1986). Previous studies have also implicated the importance of motility in the invasion of host epithelial cells (Jones *et al.,* 1981; Khoramian-Falsafi *et al.,* 1990; Jones *et al.,* 1992). Until recently however it was unclear if it was due to a direct requirement for motility or whether flagellum-associated genes were required for entry. In the serovars *S. typhimurium* and *S. typhi*, Eichelberg and Galan (2000) introduced loss-of-function mutations in the three regulatory classes of flagellar genes and examined their effect on bacterial invasion and gene expression. It was found that knocking-out any flagella genes impaired the ability of *S. typhimurium* to invade tissue culture cells and induce macrophage cell death. These defects could however be reversed by a mild centrifugal force. Moreover, McCormick (1988) found that non-flagellated *S. typhimurium* mutants adhered to and invaded the mouse large intestine to the same extent as the parent strain. These results and previous results (Jones *et al.,* 1981) suggest that in *S.*

typhimurium motility *per se* is not required for entry into host cells. Motility may aid the entry process by facilitating the intimate contact between the bacteria and the host cell that is required for the delivery of effector proteins via the type III secretion system. In contrast, *S. typhi flhDC* and *fliA* mutants were unable to invade host epithelial cells and cause macrophage cytotoxicity even when a mild centrifugal force was applied (Eichelberg and Galan, 2000). Eichelberg and Galan (2000) also found a decrease in the expression of components of the SPI-1 encoded type III secretion system in the *S. typhi* flagella regulatory protein mutations (*flhDC*$^-$ and *fliA*$^-$). These results suggested that in *S. typhi* motility is not necessary for invasion but there is an overlap between regulatory mechanisms that control flagellar and type III secretion gene expression.

Using ELISAs and TEM the role of BipA in the regulation of surface appendages will be investigated in this chapter. Another technique to identify other proteins regulated by BipA is to compare the 2-D profiles of the *bipA* mutant and its parent strain.

4.1.3 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis is an alternative approach to transposon mutagenesis to identify and study proteins implicated in virulence. Two-dimensional gel electrophoresis is a technique that separates hundreds of polypeptides orthogonally by their isoelectric points and molecular masses (Qi *et al.,* 1996). There are several important reasons why protein identification is relatively simple in *Salmonella.* Firstly, *Salmonella* has a small genome compared to eukaryotic genomes as it is estimated to have coding potential of around 4000 gene products, which is approximately half the size of the smallest eukaryotic genomes. Secondly, due to the close evolutionary relationships between *Salmonella* and *E. coli,* proteins expressed by S*almonella* often have homologous sequences in *E. coli* and can therefore be reliably assigned. Thirdly, protein identification by N-terminal sequencing may be hindered by post-translational modifications, however these modifications in prokaryotes are relatively rare (O'Connor *et al.,*

1997). Taken together, these factors suggest that in the case of the *bipA* null mutant, proteomics can be used to identify what proteins are under the control of *bipA*.

This chapter first describes results obtained from a phenotypic analysis of the *bipA* null mutant on the expression of different fimbriae and flagella. It then details the results of the 2-D gels carried out.

4.2 RESULTS

Previous studies have shown that *bipA* negatively regulates flagella-mediated cell motility in the enteropathogenic *E. coli* strain MAR001 (Farris *et al.,* 1998). It was therefore of interest to investigate the expression of flagella and other surface appendages including SEF14, SEF17 and SEF21 in the *S. enteritidis bipA* null mutant. This was carried out using enzyme-linked immusorbent assays (ELISAs) and transmission electron microscopy (TEM). For optimal expression of the different surface appendages several different conditions were used to grow the cultures for the ELISAs and TEM (described in Materials and Methods).

4.2.1 The effect of BipA on the expression of SEF14

Compared to its parent strain, the expression of SEF14 in the *bipA* null mutant differed depending on the culture conditions. No difference in SEF14 expression was seen between the wild type and mutant when grown in cfa at either 25°C or 37°C (Figure 4-1). When grown in LB-G there was no difference at 25°C, however at 37°C the expression of SEF14 in the mutant was reduced by 40% ($p < 0.001$) compared to the wild type (Figure 4-2). A difference in possible SEF14 expression was seen under TEM when the strains were grown for 24 hrs on LB-G agar as the surface appendages seen were similar in appearance to SEF14 fimbriae seen by Thorns (1995). Compared to wild type very little SEF14 expression could be seen in the mutant (Figure 4-3).

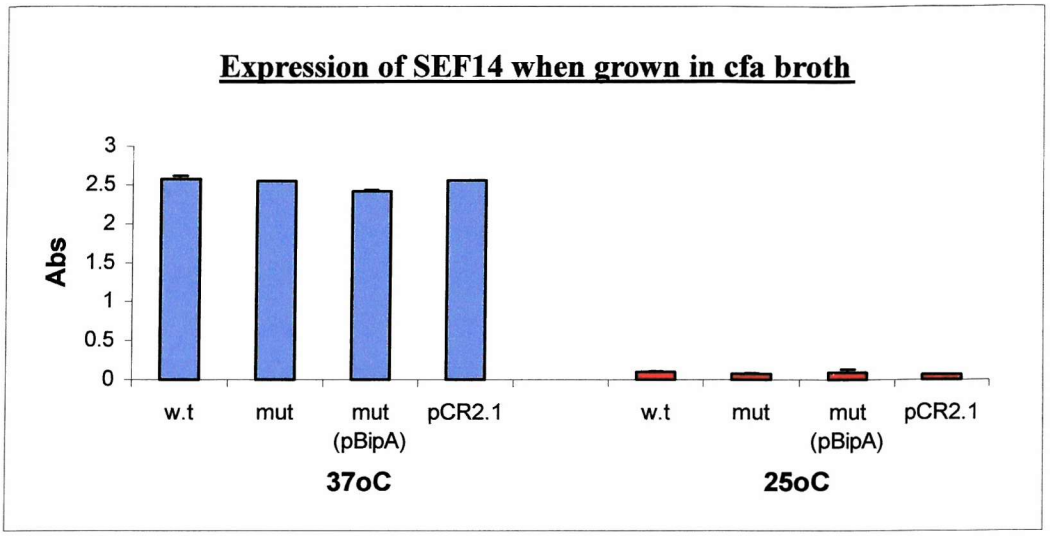


Figure 4-1 The effect of *bipA* on the expression of SEF14 when grown in cfa broth

The ELISA was carried out as described in Materials and Methods. Strains were grown at either 37°C or 25°C in cfa broth for 72hrs under oxygen-limiting conditions before the assay. The assay was carried out in triplicates using the same culture. This assay was then repeated using a fresh culture. w.t: S1400, mut: *bipA::kan* mutant, mut (pBipA): *bipA::kan* mutant transcomplemented with pBipA, pCR2.1: *bipA::kan* mutant transcomplemented with pCR2.1

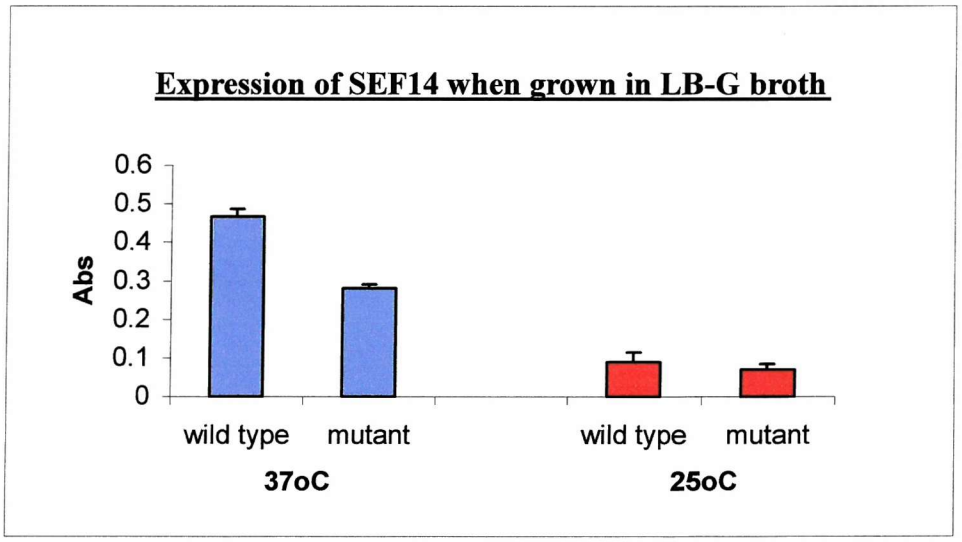
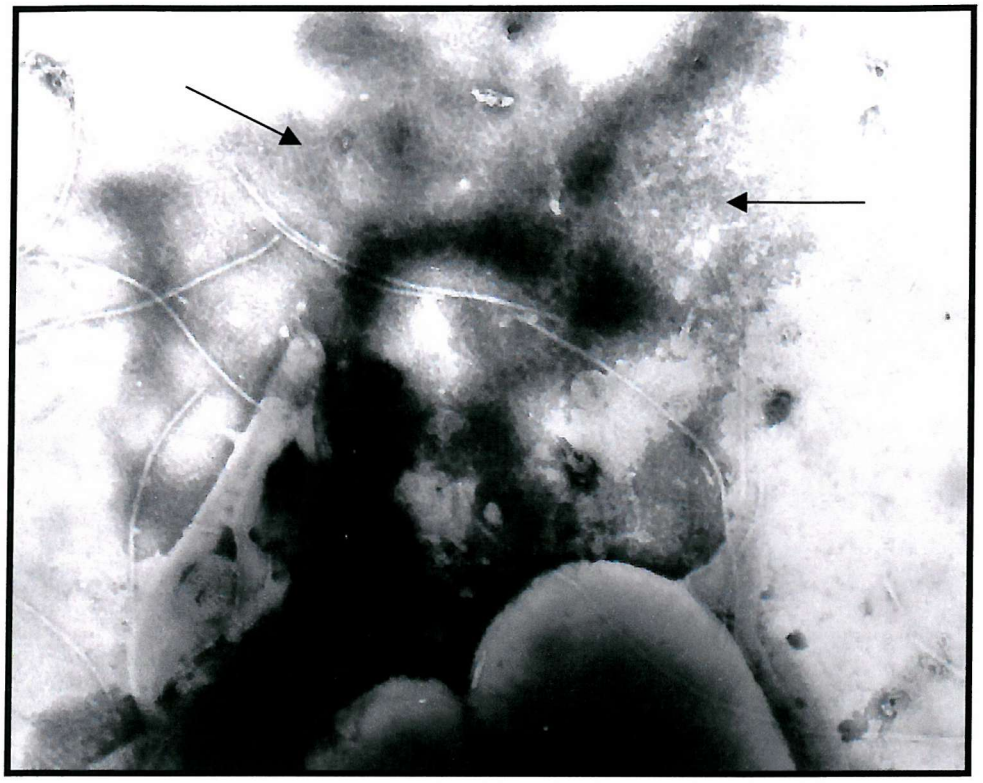


Figure 4-2 The effect of *bipA* on the expression of SEF14 when grown in LB-G broth

The ELISA was carried out as described in Materials and Methods. Strains were grown at either 37°C or 25°C in LB-G broth for 24hrs under oxygen-limiting conditions before the assay. The assay was carried out in triplicates using the same culture. This assay was then repeated using a fresh culture. Wild type: S1400, mutant: *bipA::kan* mutant.

Figure 4-3 Transmission Electron Micrographs of Wild type and Mutant when grown on LB-G agar at 37°C for 24hrs

Salmonella enteritidis negatively stained with phosphotungstic acid showing SEF14 fimbriae (arrows) in the wild type but no fimbrial organelles in the mutant. Both micrographs are at a magnification of 28.75K.



Wild Type

***bipA::kan* mutant**

4.2.2 The role of BipA in the expression of SEF17

When *S. enteritidis* is grown in cfa broth at 37°C BipA has a slight affect on the expression of SEF17 as a 30% reduction ($p < 0.05$) was seen in SEF17 expression in the mutant compared to the wild type. This defect was not however transcomplemented by the expression of BipA on a plasmid inserted into the mutant (Figure 4-4). TEM studies showed that when the strains were grown on cfa agar at 25°C for 72hrs there was a decrease in the expression of a particular fimbriae, possibly SEF17, in the mutant (Figure 4-6). The fimbriae seen were similar in appearance to those observed by Allen-Vercoe *et al.* (1997) . In contrast, when the strains were grown at 25 °C in cfa broth (Figure 4-4) or in LB-G broth at either 37 °C or 25 °C (Figure 4-5) there was no significant reduction in the expression of SEF17.

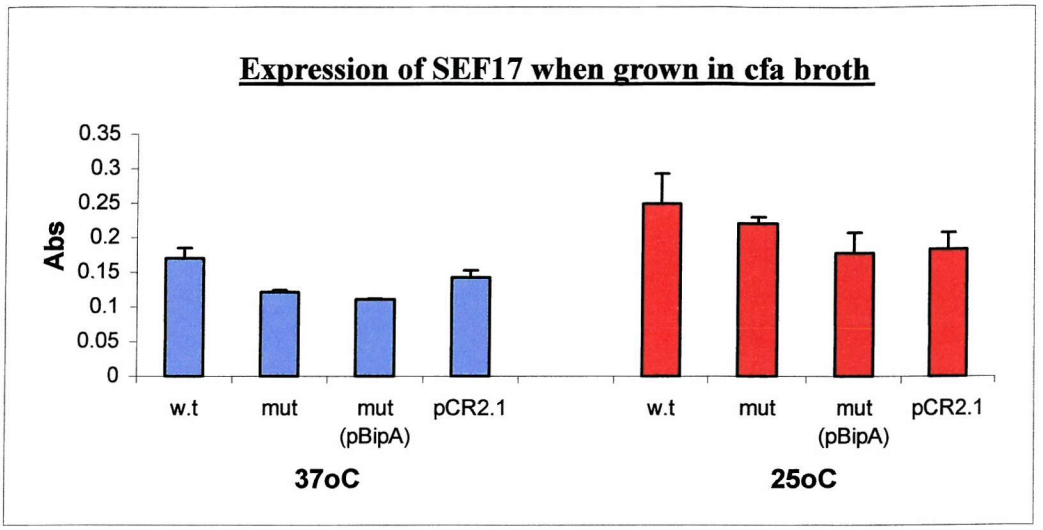


Figure 4-4 The effect of *bipA* on the expression of SEF17 when grown in cfa broth

Strains were grown at either 37°C or 25°C in cfa broth for 72hrs under oxygen-limiting conditions before the assay. The assay was carried out in triplicates using the same culture and the assay was then repeated using a fresh culture. w.t: S1400, mut: *bipA::kan* mutant, mut (pBipA): *bipA::kan* mutant transcomplemented with pBipA, pCR2.1: *bipA::kan* mutant transcomplemented with pCR2.1

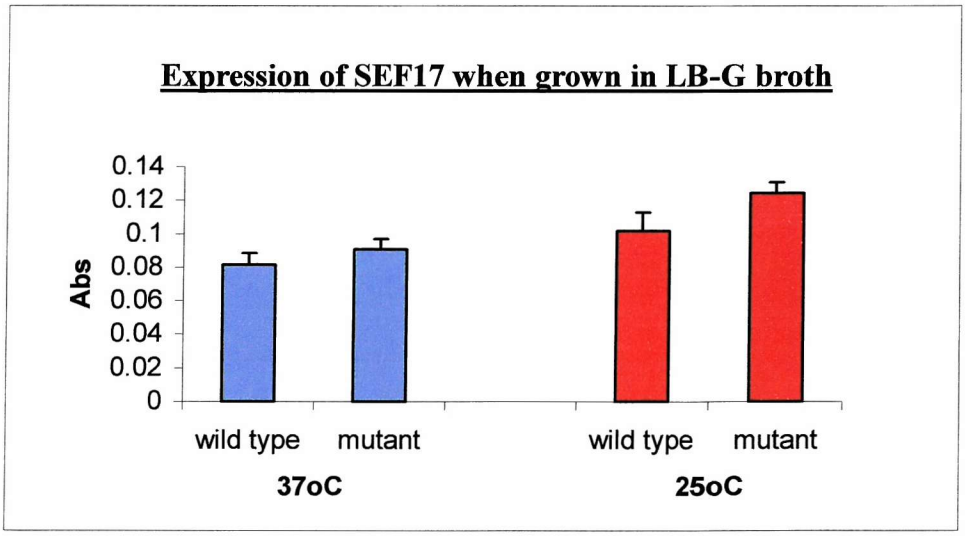
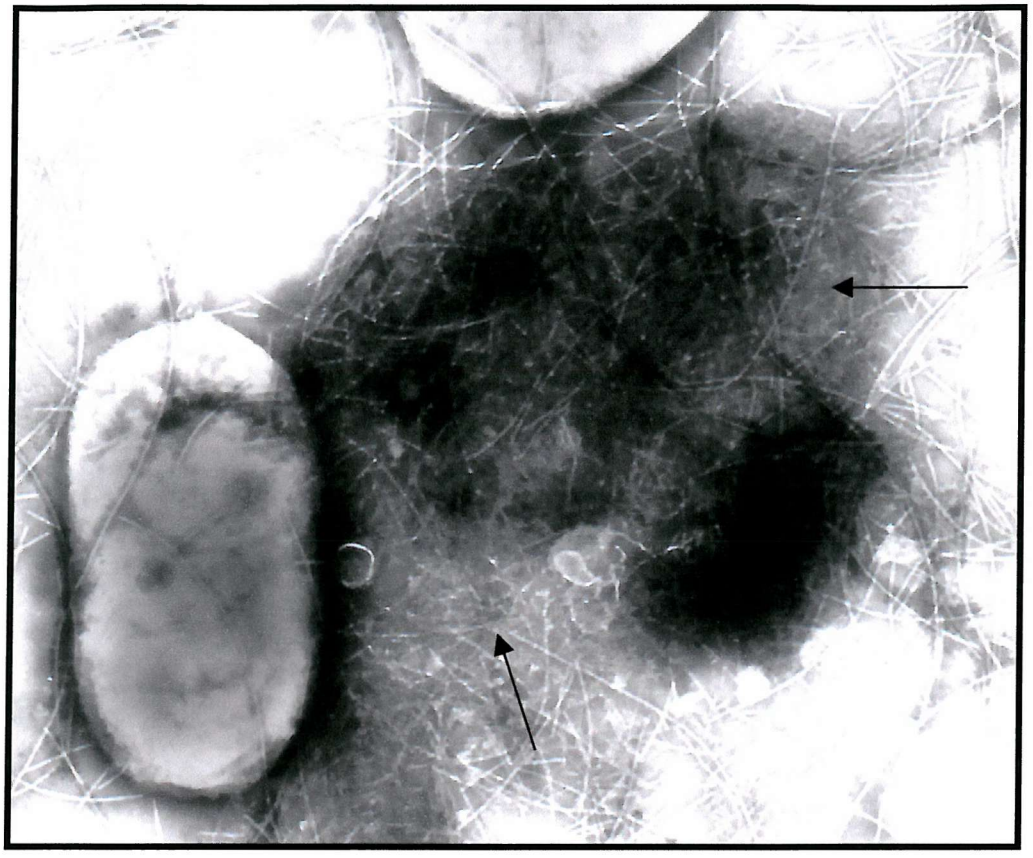


Figure 4-5 The effect of *bipA* on the expression of SEF17 when grown in LB-G broth

For 24hrs under oxygen-limiting conditions the strains were grown at either 37°C or 25°C in LB-G broth before the assay. The assay was carried out in triplicates using the same culture. This assay was repeated using a fresh culture. Wild type: S1400, mutant: *bipA::kan* mutant.

Figure 4-6 Transmission Electron Micrographs of Wild type and Mutant when grown on cfa agar at 25°C for 72hrs

Salmonella enteritidis negatively stained with phosphotungstic acid showing a certain fimbrial organelle, possibly SEF17. There is an increase in the amount of SEF17 in the wild type (arrows) compared to the mutant. Both micrographs are at a magnification of 28.75K.



Wild Type

***bipA::kan* mutant**

4.2.3 BipA negatively regulates the expression of SEF21

A significant difference was seen in the expression of SEF21 between the wild type and mutant. Figure 4-7 shows that *bipA* negatively regulates the expression of SEF21 as a 41% increase ($p < 0.05$) in the expression of SEF21 fimbriae was seen in the mutant when grown in cfa broth at 37°C. This defect was not restored if a plasmid containing full-length *S. enteritidis bipA* was inserted into the mutant. Surprisingly the defect was enhanced as the expression of SEF21, compared to the mutant, increased by a further 58% ($p < 0.01$). This increase was not due to the insertion of the plasmid as pCR2.1, containing no *bipA*, was transformed into the mutant and SEF21 expression was not affected (Figure 4-7). These findings were supported by TEM as the micrographs in Figure 4-9 show an increase in SEF21 expression in the mutant when grown in cfa broth for 48hrs at 37°C. Also the expression of SEF21 was affected to a lesser extent when the cultures were grown in LB-G at either 37 °C or 25 °C. There was an increase in the expression of SEF21 by 29% ($p = 0.05$) and 27% ($p < 0.05$) in the mutant at 37 °C and 25 °C, respectively (Figure 4-8). In contrast, the expression of SEF21 was not affected by *bipA* when the cultures are grown in cfa broth at 25°C ($p = 0.82$) (Figure 4-7).

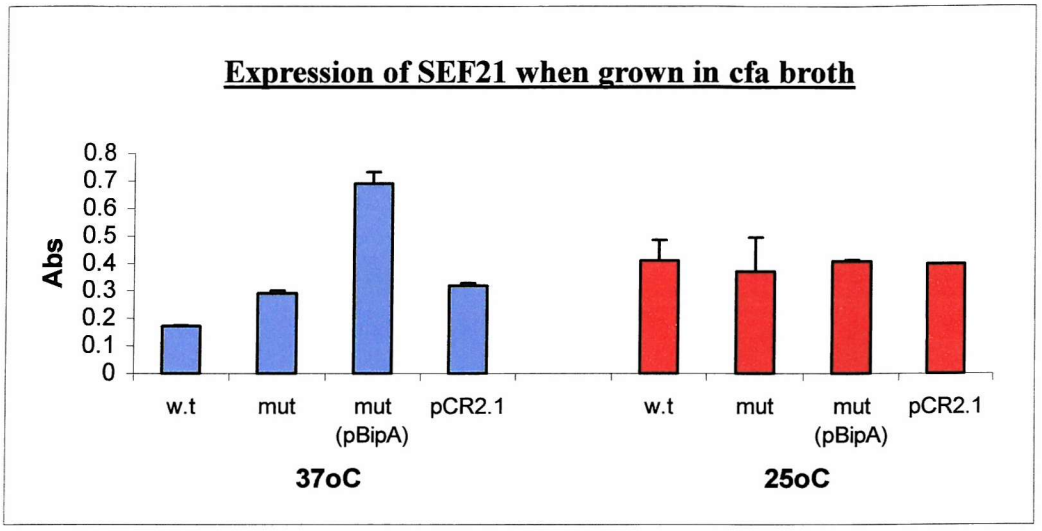


Figure 4-7 The effect of *bipA* on the expression of SEF21 when grown in cfa broth

The ELISA was carried out as described in Materials and Methods. For 72hrs under oxygen-limiting conditions the strains were grown at either 37°C or 25°C in cfa broth before the assay. The assay was carried out in triplicates using the same culture and the assay was then repeated using a fresh culture. w.t: S1400, mut: *bipA::kan* mutant, mut (pBipA): *bipA::kan* mutant transcomplemented with pBipA, pCR2.1: *bipA::kan* mutant transcomplemented with pCR2.1

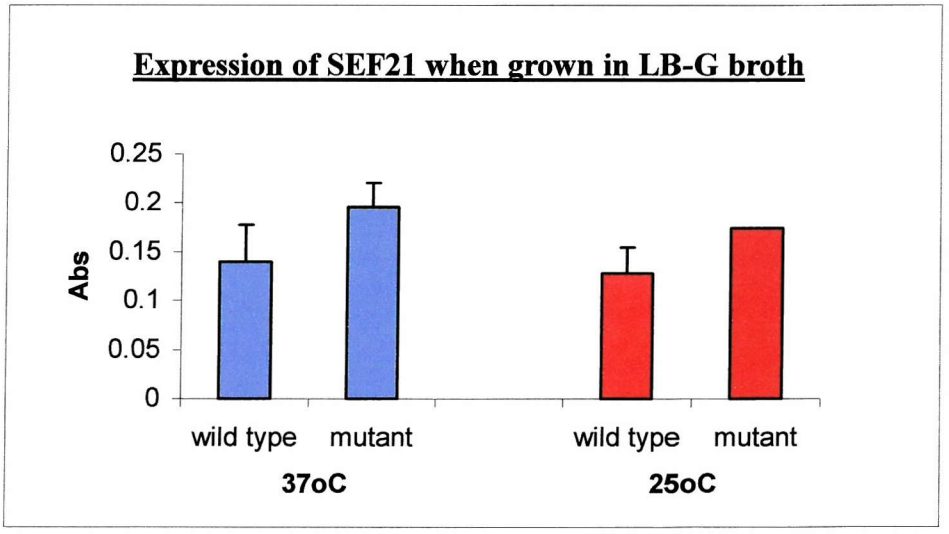
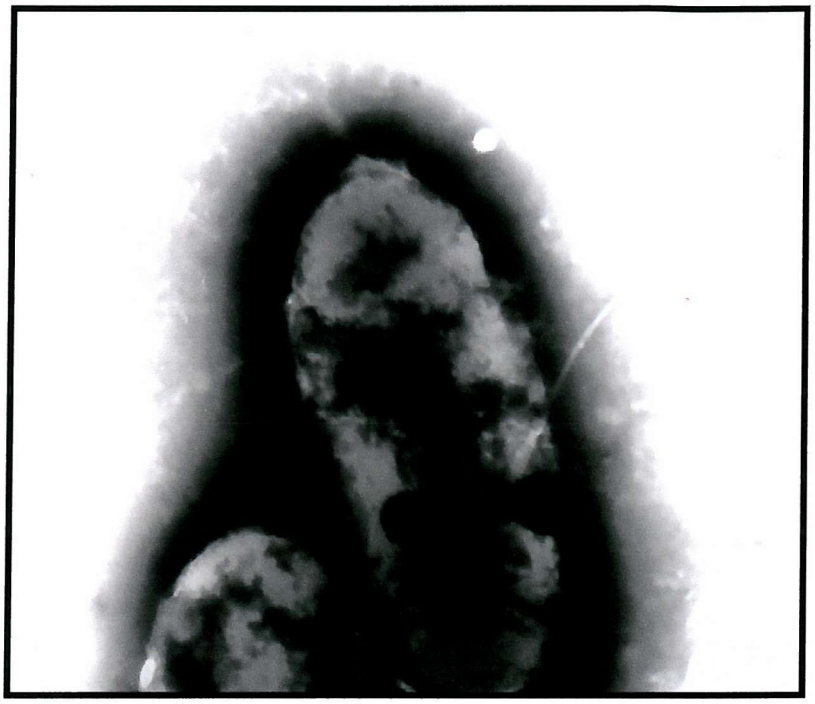


Figure 4-8 The effect of *bipA* on the expression of SEF21 when grown in LB-G broth

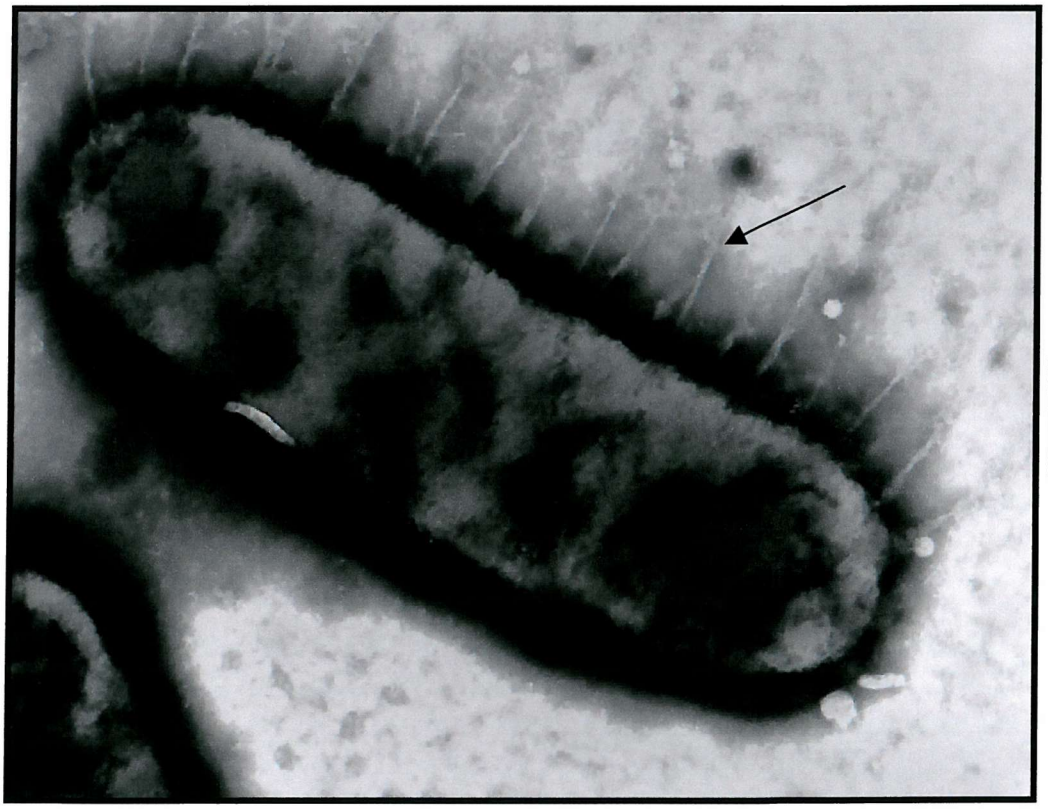
Strains were grown at either 37°C or 25°C in LB-G broth for 24hrs under oxygen-limiting conditions before the assay. The assay was carried out in triplicates using the same culture. This assay was then repeated using a fresh culture. Wild type: S1400, mutant: *bipA::kan* mutant.

Figure 4-9 Transmission Electron Micrographs of Wild type and Mutant when grown in cfa broth at 37°C for 72hrs

Salmonella enteritidis negatively stained with phosphotungstic acid showing SEF21. There is an increase in the amount of SEF21 in the mutant (arrow) compared to the wild type. Both micrographs are at a magnification of 28.75K.



Wild Type



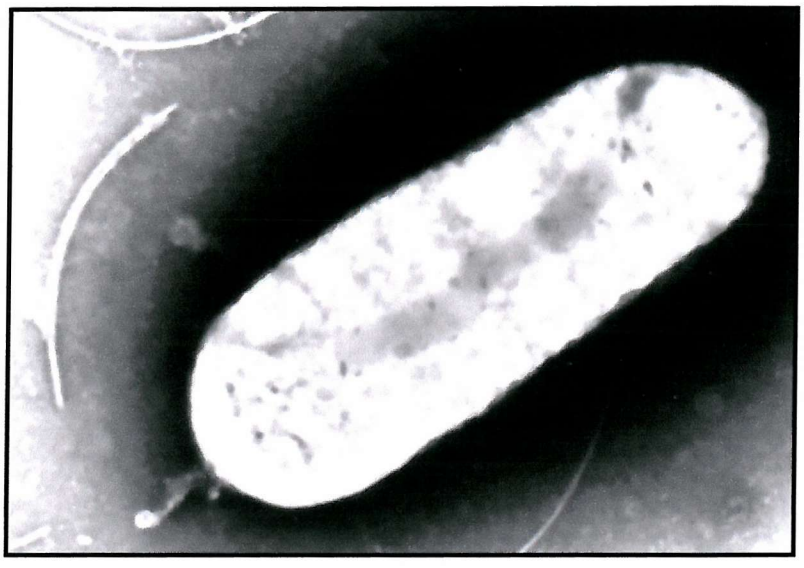
***bipA::kan* mutant**

4.2.4 The effect of *bipA* on other fimbrial organelles

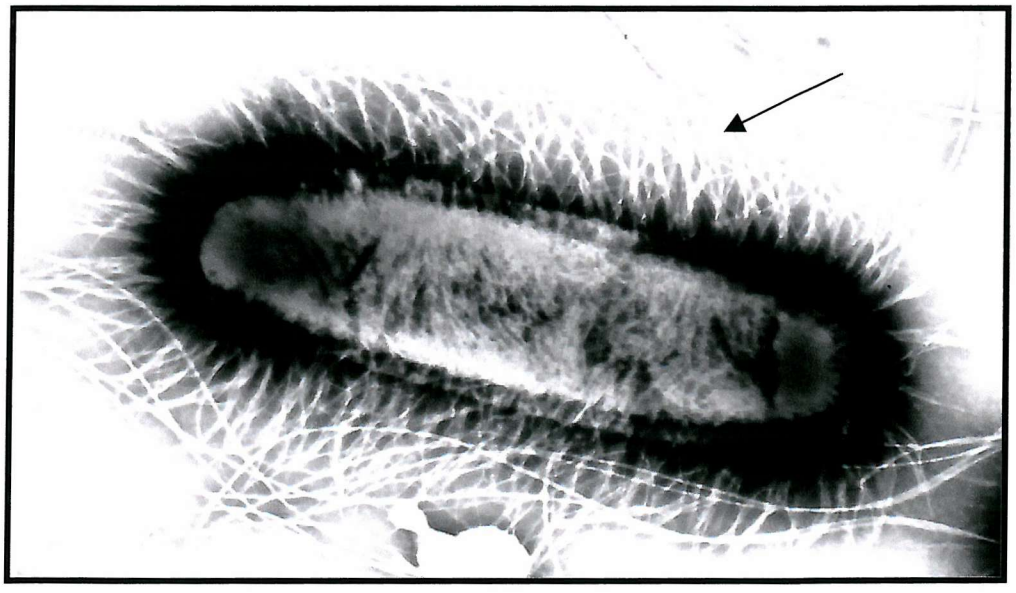
When *S. enteritidis* S1400, *S. enteritidis bipA::kan* and *S. enteritidis bipA::kan* (pBipA) were grown in cfa broth for 48 hrs at 37°C, the mutant and *S. enteritidis bipA::kan* (pBipA) both expressed two different type of fimbriae including SEF21 (section 4.2.3) and a unknown fimbriae, possibly PEF (Figure 4-10 & Figure 4-11). It is thought that the fimbriae are PEF because they share very similar morphology to the fimbriae seen by Woodward *et al.* (1996) when the *S. enteritidis pef* operon is expressed in *E. coli*. Again the transcomplemented strain seemed to enhance the defect as more bundles of the fimbriae can be seen in this strain (Figure 4-11). In the mutant and transcomplemented strains most cells were seen to exhibit SEF21 whereas the number of cells exhibiting possible PEF was approximately 1 in 10. These observations suggest that these cells alternate between PEF-fimbriated and non-PEF-fimbriated states. Further experiments would need to be carried out to determine the role of BipA in this possible phase variation.

Figure 4-10 Transmission Electron Micrographs of Wild type and Mutant when grown in cfa broth at 37°C for 72hrs

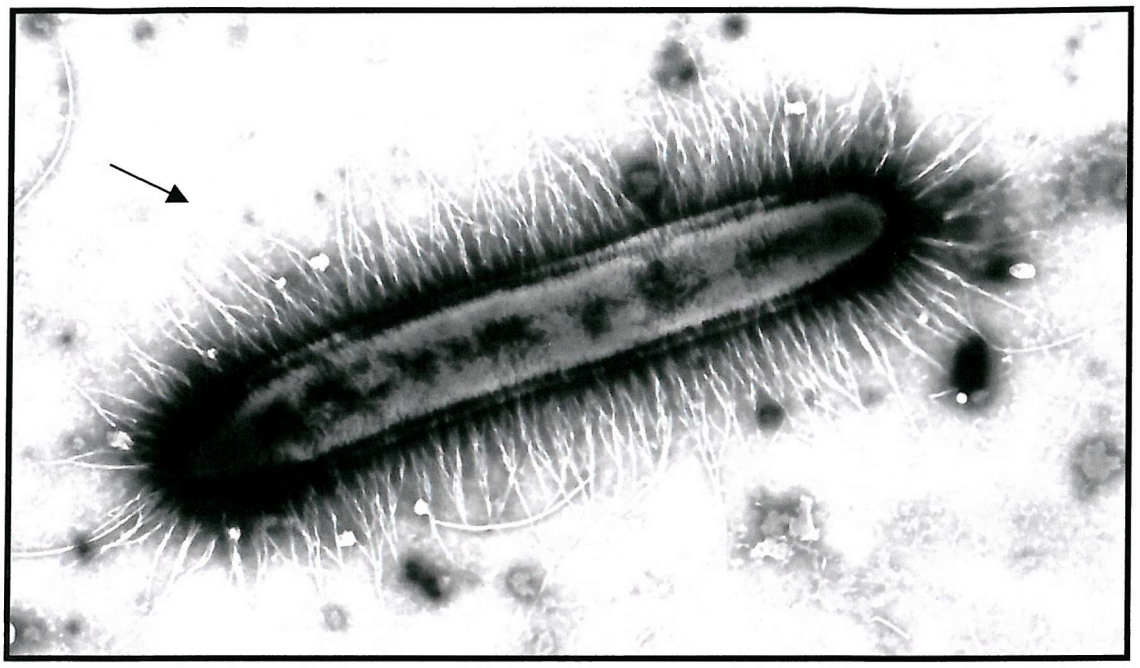
Salmonella enteritidis negatively stained with phosphotungstic acid showing a certain fimbrial organelle, possibly PEF. PEF only seems to be expressed in the mutant (arrows). Micrographs A and B are at a magnification of 38.75K. Micrographs C and D are at a magnification of 22.25K and 71.25K respectively. Micrograph D is the same bacteria as in micrograph E but at a higher magnification.



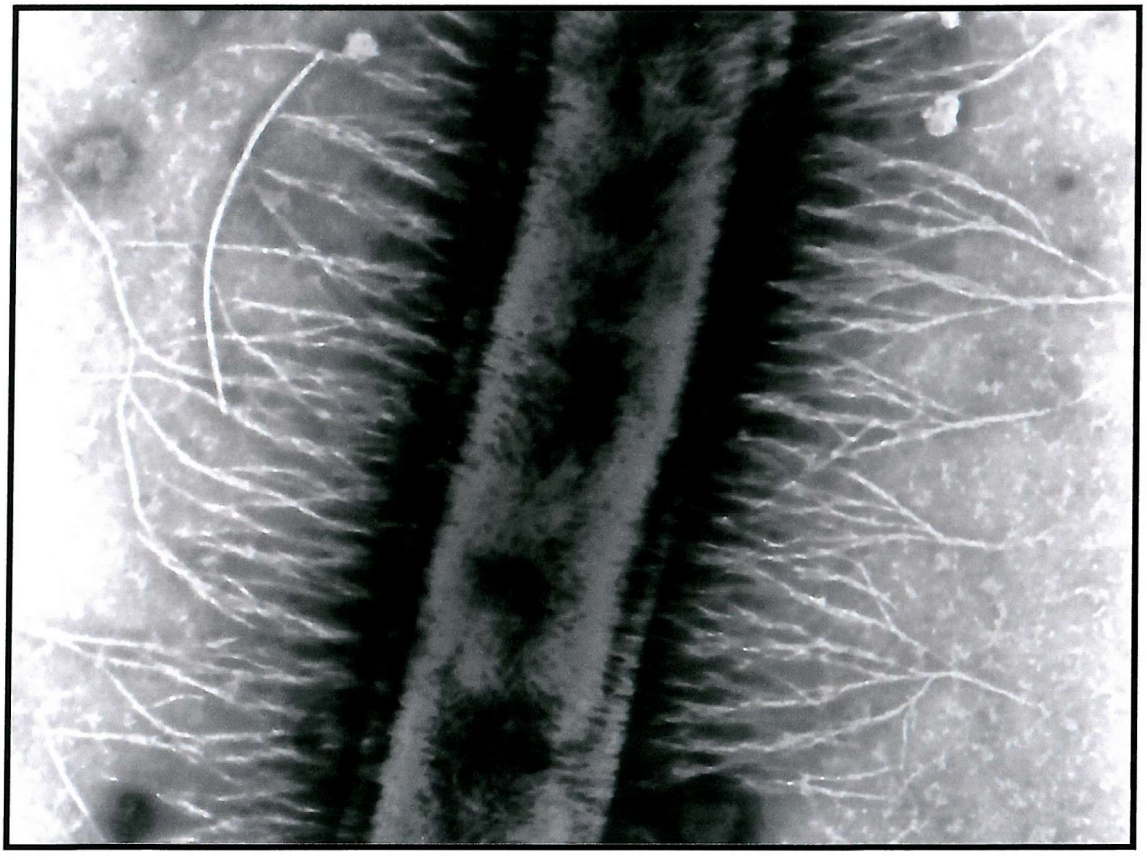
Wild Type (A)



***bipA::kan* mutant (B)**



bipA::kan **mutant (C)**



bipA::kan **mutant (D)**

Figure 4-11 Transmission Electron Micrographs of Mutant (pBipA) when grown in cfa broth at 37°C for 72hrs

Salmonella enteritidis mutant transcomplemented with pBipA negatively stained with phosphotungstic acid showing a certain fimbrial organelle, possibly PEF. PEF seems to be expressed in the mutant pBipA strain (arrows). Micrograph E is at a magnification of 52.5K.

***bipA::kan* mutant (pBipA) (E)**

4.2.5 BipA has a significant effect on the production of flagella

In *E. coli* BipA is known to negatively regulate flagella-mediated cell motility (Farris *et al.*, 1998). However, in the case of *S. enteritidis*, flagella expression seems to be positively regulated by *bipA*. A 55% reduction ($p < 0.05$) in flagella was seen when the *bipA* null mutant was grown in cfa broth for 72 hrs at 25°C. This defect was not transcomplemented by the insertion of pBipA into the mutant, on the contrary, the defect was enhanced as a further 62% reduction ($p < 0.05$) was seen compared to the mutant (Figure 4-12). At 37°C a 21% reduction ($p = 0.05$) was seen in the production of flagella in the mutant, this defect was also enhanced by the insertion of pBipA as a further 33% reduction ($p < 0.05$) was seen (Figure 4-12). When the cultures were grown in LB-G broth a slight, but not significant reduction (21%; $p = 0.07$), was seen when the cultures were grown at 25°C (Figure 4-13).

4.2.6 *S. enteritidis* S1400/94 *bipA::kan* is less motile than parental cells

The previous findings (section 4.2.5) showed that *S. enteritidis* BipA positively regulates flagella expression. It was therefore of interest to determine if this difference in flagella expression altered cell motility. Not surprisingly, the *bipA::kan* mutant was found to be less motile than the parent strain (Figure 4-14 A). Similar results were obtained with a *bipA::cat* mutant of *S. typhimurium* SL1344 (Kinsella, N., White, A.L., and O'Connor, C.D., unpublished results). Therefore, in contrast to the EPEC findings, the *bipA* gene of *Salmonella* spp. is a positive regulator of cell motility.

Again, when plasmid pAW5 (pCR2.1 containing full-length *bipA*) was transformed into the mutant cells the motility was not restored (Figure 4-14B) instead the defect was enhanced and the cells became less motile than the mutant cells. This transformation was also carried out using a plasmid (pBR322) containing full-length *S. typhimurium bipA* (pYL98, Li, Y., unpublished results) and again the defect was enhanced (Figure 4-15 B). The resulting phenotype

of both the transcomplemented strains was not due to the plasmid itself as when the cells were transformed with the vectors containing no *bipA* the motility of the cells remained the same as the mutant cells (Figure 4-14 C & Figure 4-15 C).

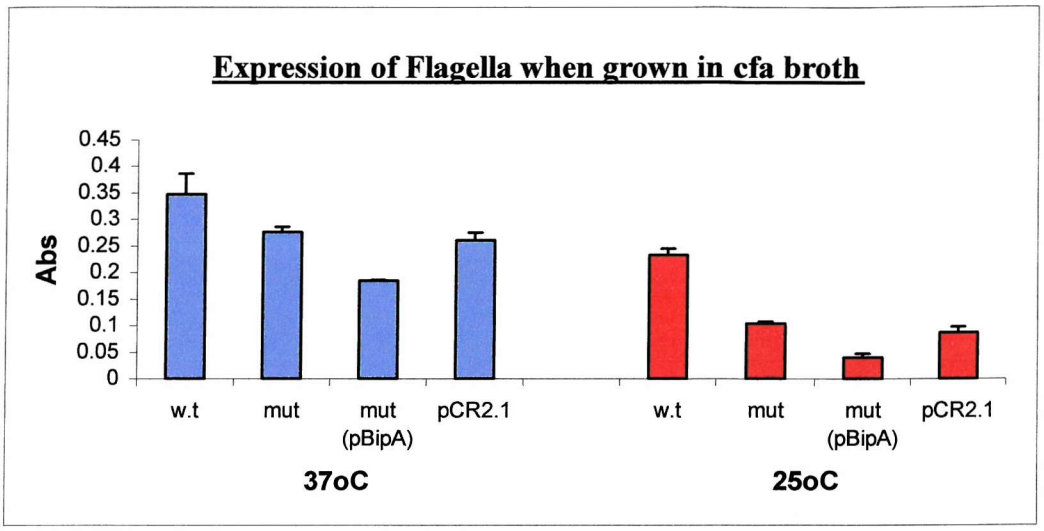


Figure 4-12 The effect of *bipA* on the expression of Flagella when grown in cfa broth

For 72hrs the strains were grown statically at either 37°C or 25°C in cfa broth. The assay was carried out in triplicates using the same culture. This assay was repeated using a fresh culture. w.t: S1400, mut: *bipA::kan* mutant, mut (pbipA): *bipA::kan* mutant transcomplemented with pbipA, pCR2.1: *bipA::kan* mutant transcomplemented with pCR2.1

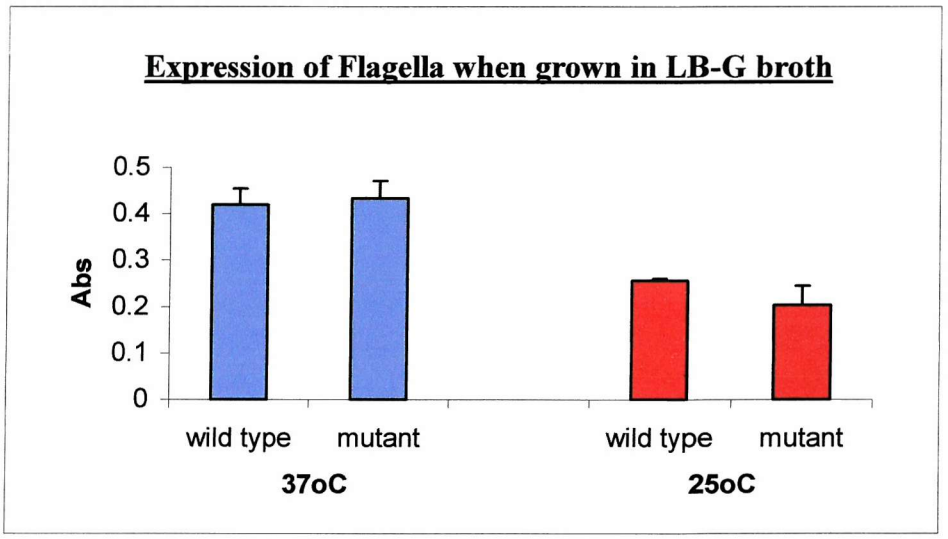


Figure 4-13 The effect of *bipA* on the expression of Flagella when grown in LB-G broth

Strains were grown statically at either 37°C or 25°C in LB-G broth for 24hrs. The assay was carried out in triplicates using the same culture. This assay was repeated using a fresh culture. Wild type: S1400, mutant: *bipA::kan* mutant.

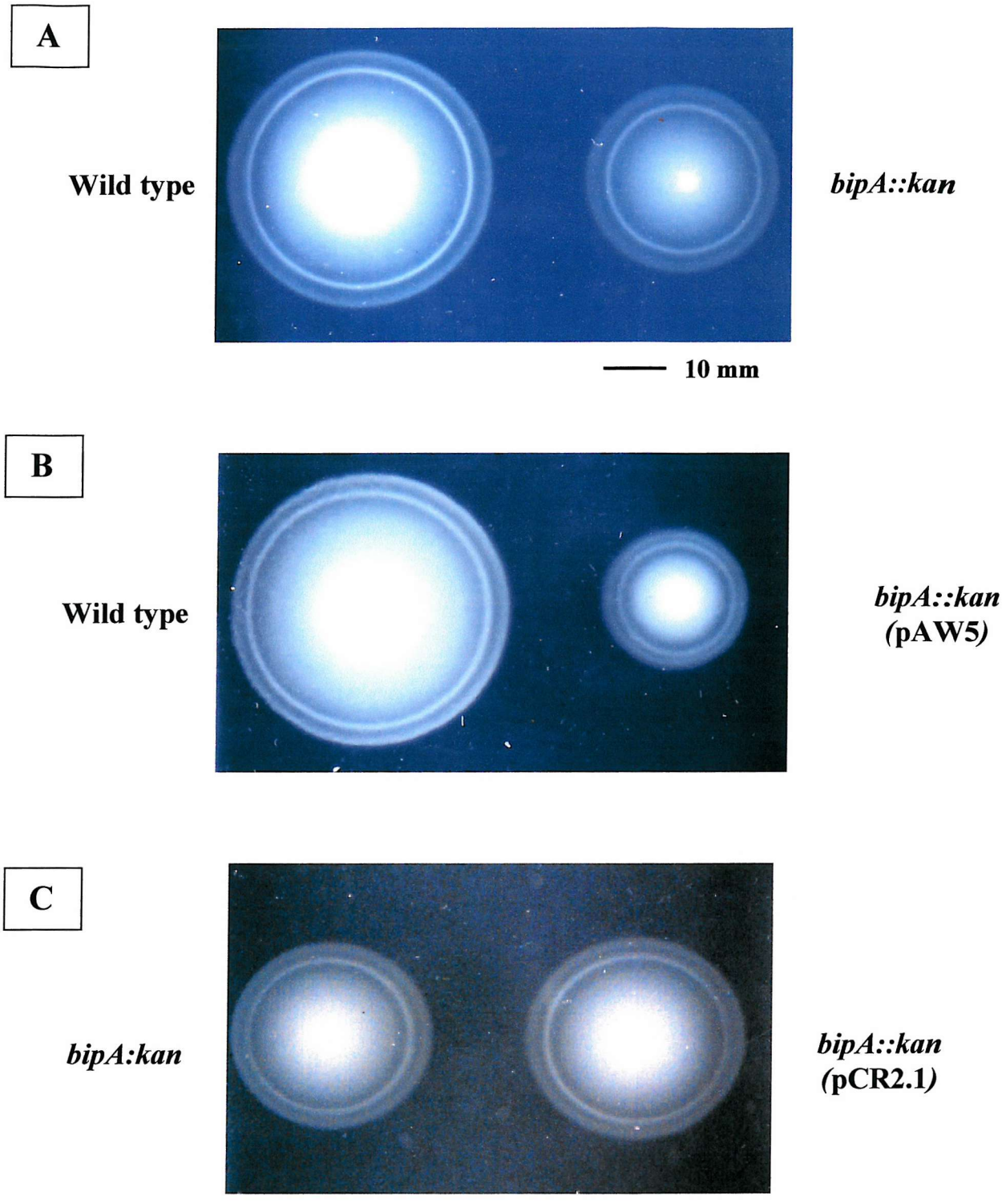


Figure 4-14 Effect of BipA on motility

Typical results obtained in motility tests. Mid-exponential phase cultures were inoculated into motility agar and incubated overnight at 37°C. Plate A represents motility of S1400 (left) and *bipA::kan* (right). Plate B represents motility of S1400 (left) and *bipA::kan* transformed with pAW5 (right). Plate C represents motility of *bipA::kan* (left) and *bipA::kan* (pCR2.1) (right)

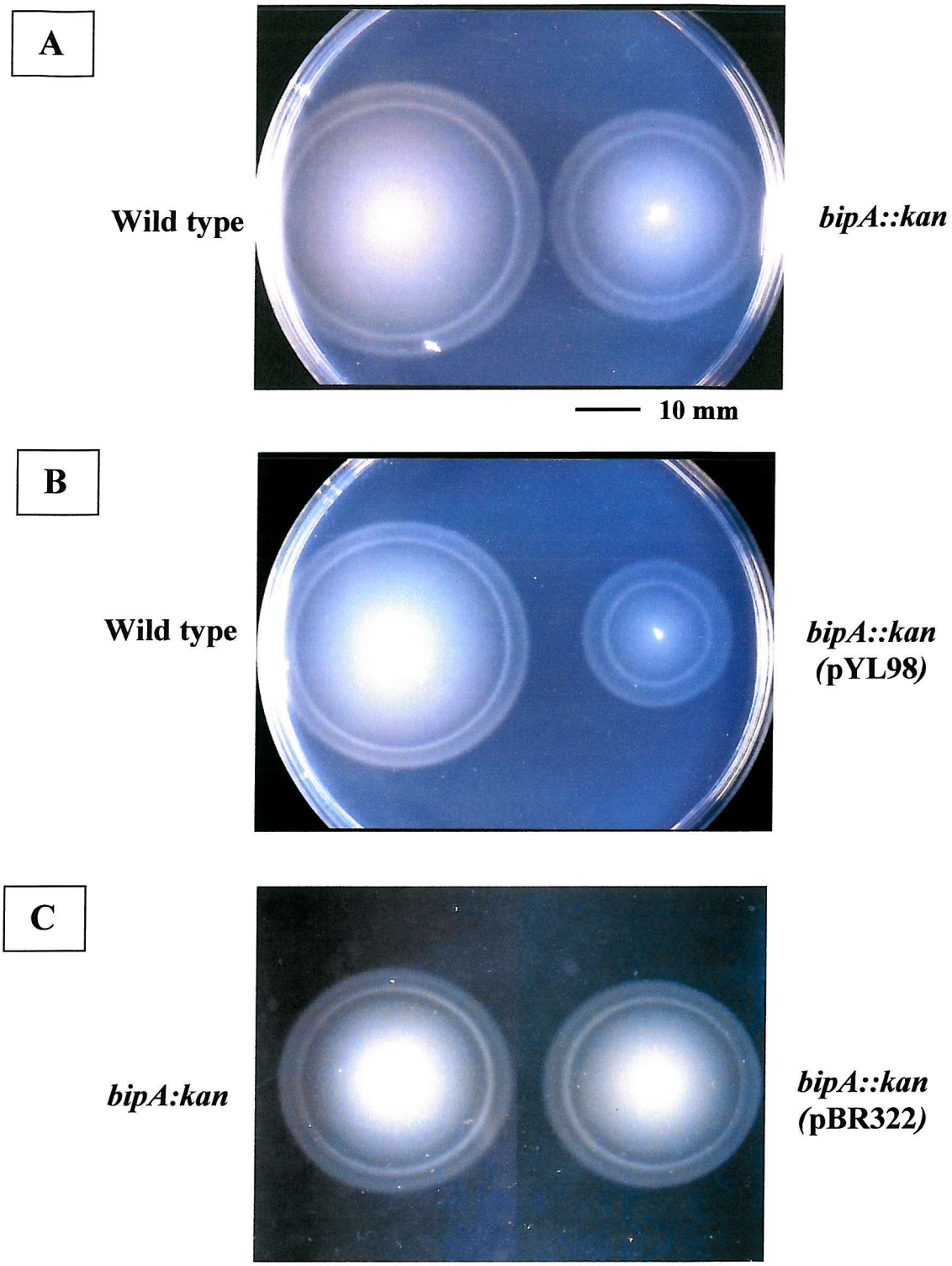


Figure 4-15 Effect of BipA on motility

Typical results obtained in motility tests. Mid-exponential phase cultures were inoculated into motility agar and incubated overnight at 37°C. Plate A represents motility of S1400 (left) and *bipA::kan* (right). Plate B represents motility of S1400 (left) and *bipA::kan* transformed with pYL98 (right). pYL98 contains full length *S. typhimurium* gene cloned into pBR322. Plate C represents motility of *bipA::kan* (left) and *bipA::kan* (pBR322) (right).

4.2.7 Comparing the protein profiles of wild type and *bipA* null mutant

Figure 4-16 shows the protein profiles obtained with whole cell lysates of *S. enteritidis* wild type and *S. enteritidis bipA* mutant. Spot changes were analysed by densitometry using the Phoretix-2D software v. 5.1 [Phoretix]. Spot volumes were quantified relative to three internal standards (spots circled in blue in Figure 4-1), as the level of these proteins were unaffected between the wild type and *bipA* mutant protein profiles. Only proteins that were reproducibly induced or repressed at least twice fold were considered to be valid.

When comparing the two profiles only one spot difference was seen (circled in red in Figure 4-16). The expression of this protein was absent in the mutant profile. This protein was found to have a pI of 5.26 and a molecular weight of 75kDa, which is indicative of the BipA protein. BipA is a 65.4Kda protein that migrates autonomously on 2D gels. When this spot was excised and identified by mass spectrometry it showed a 35% peptide match to BipA (S. Payot., unpublished results). This was also the only difference seen when the soluble membrane fraction proteins profiles of the wild type and mutant were compared. The loss of BipA expression in mutant whole cell and soluble fractions would be expected, as expression of BipA should not be seen in the knockout mutant.

Although the only difference seen in the two protein profiles was the loss of the BipA protein, the role of BipA in the regulation of a large number of proteins can be ruled out as many of the spots have already been identified in *S. typhimurium* (N. Kinsella., unpublished results), which has a very similar protein profile to *S. enteritidis*. For example, there was no difference in the ribosomal proteins L12 and L7 (spots 1 and 2, respectively) and since it is known that many ribosome components are co-regulated this suggests that other ribosome components are also not affected by loss of BipA. Similarly, several glycolytic enzymes have been identified (pyruvate dehydrogenase E2 and E1, enolase, pyruvate kinase, fructose bisphosphate aldolase,

phosphofructokinase and phosphoglycerate kinase; spots 3,4,5,6,7,8 and 9, respectively) so it is unlikely that BipA affects at least these stages of glycolysis. Also, several citric acid cycle enzymes have been identified, including succinyl CoA synthetase (spot 10), fumarase (spot 11) and malate dehydrogenase (spot 12), which suggests that BipA plays no role in these stages of the citric acid cycle. The heat shock proteins GroEL (spot 13) and DnaK (spot 14) have also been identified, therefore BipA is unlikely to affect the binding of these proteins to heat denatured proteins.

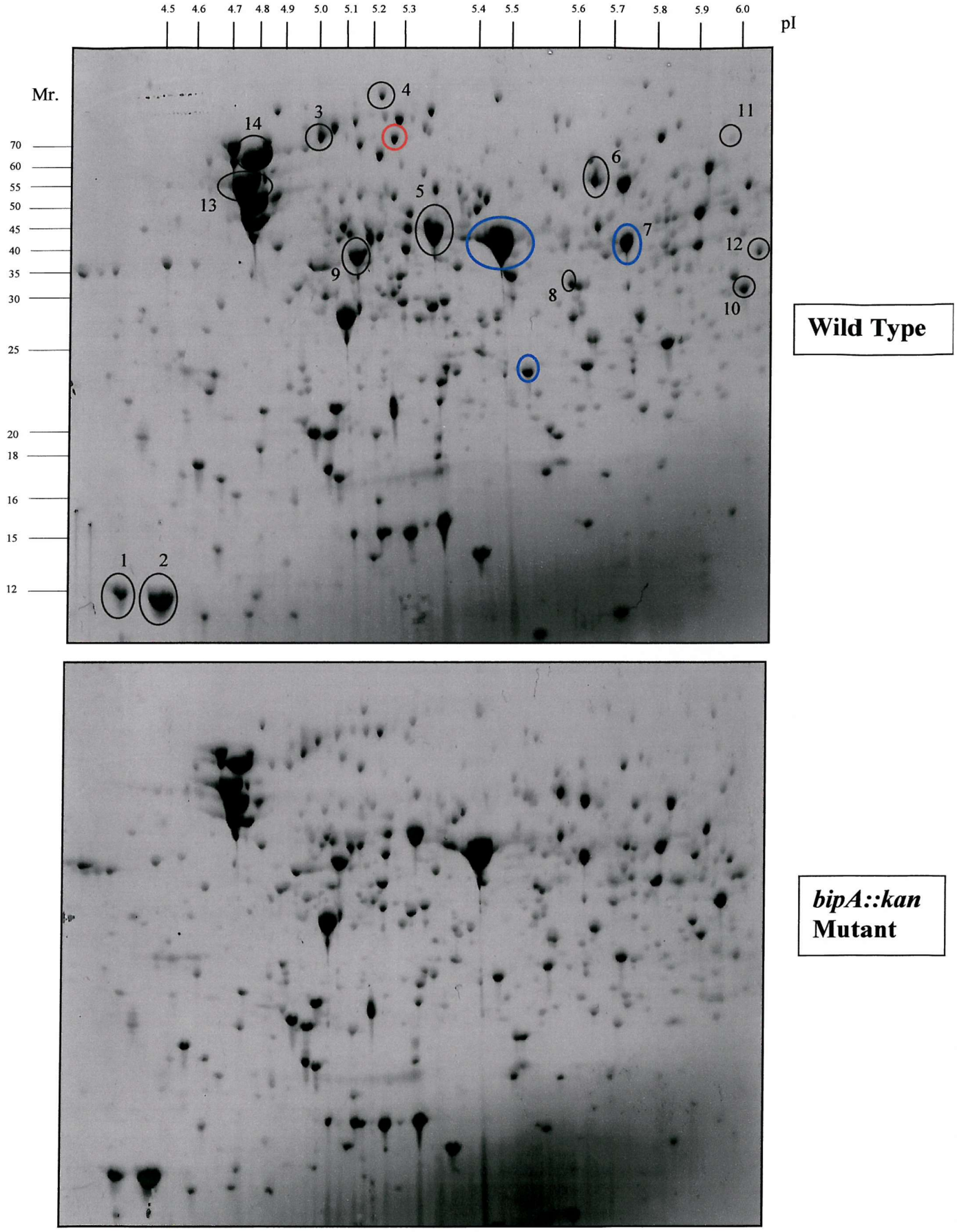


Figure 4-16 Two-Dimensional Gel profiles of Wild Type and Mutant

Protein profiles obtained with whole cell lysates of *S. enteritidis* wild type and *S. enteritidis bipA* mutant. Red circle: BipA, blue circles: internal standards and the black numbered circles: proteins identified in *S. typhimurium*

4.3 Discussion

In this chapter it has been established that BipA regulates one or more fimbrial systems in *Salmonella enteritidis*. BipA positively regulates SEF14 when grown in either LB-G broth at 37°C or grown on LB-G agar at 37°C. BipA also seems to positively regulate the expression of SEF17 when grown on cfa agar at 25°C or in cfa broth at 37°C. In contrast, BipA negatively regulates the expression of SEF21 and another fimbriae, possibly PEF, when grown in cfa broth at 37°C. It has also been established that the only difference seen in the protein profiles of *S. enteritidis* wild type and *S. enteritidis bipA* null mutant is the loss of the BipA protein in the mutant.

4.3.1 BipA positively regulates SEF14

It is interesting that BipA is involved in the regulation of fimbriae that are restricted to *S. enteritidis* and other closely related group D *Salmonella*. Investigation into how *bipA* regulates SEF14 may provide an insight into the unique aspects of virulence that distinguish these *Salmonella* serovars. The biogenesis and translocation of SEF14 is specified by the *sefABCD* operon (Clouthier *et al.*, 1993). *SefA* and *SefD* encode the major subunit and the putative adhesin of SEF14, respectively. *SefB* encodes a chaperone that is responsible for the translocation of *sefA* and *sefC* encodes the usher (Clouthier *et al.*, 1993; Turcotte and Woodward, 1993). The regulatory gene that activates the transcription of these genes, *sefR*, is located next to the *sefD* gene (Edwards *et al.*, 2000). TEM and ELISA studies showed that there is a reduction in the expression of SEF14 in the mutant. BipA may be involved in the regulation of *sefR*. Hence, further work could involve investigating the expression of this gene and other genes encoded on the *sefABCD* operon.

The role of SEF14 in pathogenesis is controversial. Recent studies carried out by Edwards *et al.* (2000) suggest that the putative SEF14 adhesion subunit is essential for full virulence of *S.*

enteritidis in vivo and that the adhesin is required for either the uptake or survival in peritoneal macrophages. In conjunction with these results, it has been found that the *bipA* mutant shows reduced invasion/survival in macrophages (section 5.2.2), which could be a result of the reduction in SEF14 expression.

4.3.2 BipA positively regulates SEF17

Multicellular behaviour in pathogenic bacteria can be expressed in several different forms such as biofilm formation, cell clumping, swarming or fruiting body development (Shapiro, 1998). A multicellular morphotype (rdar), characterised by the expression of thin aggregative fimbriae and long-range cell-cell interactions, has been identified in *S. typhimurium* (Romling *et al.,* 1998). *Salmonella* produce thin aggregative fimbriae (known as SEF17 in *S. enteritidis*) which are composed of polymerised AgfA fimbrin proteins (Collinson *et al.,* 1996a). The *agfBAC* operon is responsible for the expression of these fimbrial subunit proteins (Collinson *et al.,* 1996a) and the transcriptional regulator, AgfD, positively regulates this operon (Hammar *et al.,* 1995). Studies have shown that a *S. typhimurium agfD* mutant lacked both aggregative fimbriae and long-range intracellular adhesion, whereas an *agfBA* mutant only lacked the thin aggregative fimbriae. A putative transmembrane protein, AdrA (*agfD* regulated gene), was found to be AgfD dependent and AdrA mutant cells were shown to express thin aggregative fimbriae, but did not exhibit long-range intercellular adhesion. An *agfBA* and *adrA* double mutant was shown to exhibit similar characteristics to those of the single *agfD* mutant. Therefore the *agfD* regulates at least two independent pathways contributing to the multicellular morphotype in *S. typhimurium* (Romling *et al.,* 2000). TEM and ELISA studies showed a reduction in the expression of SEF17 in the mutant. Therefore, it could be considered that BipA may be involved in the regulation of *agfD* or that BipA protein may interact with the fimbrial subunit proteins. If the BipA protein is involved in the regulation of *agfD* it would be interesting to look at the morphotype of the *bipA* mutant by scanning electron microscopy.

4.3.3 BipA negatively regulates the expression of SEF21

Type 1 fimbriae, also know as SEF21 in *S. enteritidis*, are encoded on the *fim* operon similar to that of *E. coli* (Clegg *et al.,* 1985; Clegg *et al.,* 1987; Feutrier *et al.,* 1988). This operon encodes at least four different subunits of type 1 fimbriae, including the major subunit, FimA, and the three minor subunits FimF, FimG and FimH. In *S. typhimurium* the regulation of *fimA* is controlled by a number of ancillary *fim* genes, including *fimZ*, *fimY*, *fimW*, and *fimU* (Swenson and Clegg, 1992). *fimZ* and *fimY* are transcriptional regulators where as *fimU* encodes an arginine tRNA molecule that is know to affect both *S. typhimurium* and *S. enteritidis* type 1 fimbrial expression (Swenson *et al.,* 1994; Clouthier *et al.,* 1998; Tinker and Clegg, 2000). It would be interesting to investigate if *bipA* alters the expression of any of these ancillary genes as the *bipA* knock out mutant exhibited higher levels of SEF21 expression. It is however unlikely that *bipA* would affect the expression of *fimU* as recent studies have shown that *fimU* simultaneously controls the production of SEF21 and SEF14 (Clouthier *et al.,* 1998). Therefore knocking out *bipA* should have the same effect on both these fimbriae i.e. both fimbriae would be positively regulated or negatively regulated by *bipA*. This is not the case as SEF21 is negatively regulated by *bipA* (section 4.2.3) whereas SEF14 is positively regulated by *bipA* (section 4.2.1).

4.3.4 BipA negatively regulates the expression of another fimbriae, possibly PEF

The PEF fimbrial operon mediates adhesion to murine small intestine and is necessary for fluid accumulation (Bäumler *et al.,* 1996). In *S. typhimurium* the *pefBACD* operon encodes a major subunit PefA, an usher, PefC, a chaperone PefD and a regulator PefB (Bäumler *et al.,* 1997). In the *bipA* null mutant the expression of PEF increases, therefore, it would be interesting to investigate if BipA is involved in negatively regulating any of the genes encoded on the *pef* operon or if BipA interacts with any of these proteins to prevent PEF synthesis. Future studies

could also involve comparing the fluid accumulation and murine small intestine adhesion of *S. enteritidis* wild type and *S. enteritidis bipA* mutant.

Recent evidence has shown that in *S. typhimurium* the leucine-responsive regulatory protein (Lrp) and DNA adenine methylase (Dam) are required for PEF transcription. In contrast, the histone-like protein (H-NS) and the stationary-phase sigma factor (RpoS) represses PEF transcription (Nicholson and Low, 2000). Freestone *et al.* (1998) reported that in certain conditions H-NS expression is increased in the *E. coli* MAR001 *bipA*$^-$ strain. The effect of *bipA* on H-NS expression *in S. typhimurium* and *S. enteritidis* has yet to be established. It would be interesting if *Salmonella* H-NS expression was positively regulated by BipA as this would tie in with the PEF results seen here.

4.3.5 BipA positively regulates the expression of flagella

The regulation of flagella is complex and involves the expression of genes that are organised in a hierarchy of three classes (Kutsukake *et al.,* 1990). In *S. enteritidis* it was found that in certain conditions *bipA* positively controls the expression of flagella. The BipA GTPase may interact with some component of the flagella system or it may be involved in the regulation of flagella biosynthesis. However, because the regulation and biosynthesis of flagella involves the expression of more than 50 genes it would be a laborious task to find where in the three classes *bipA* possibly exerts its effect.

The role of flagella in the survival within murine macrophages is equivocal. Several studies have shown no role for flagella in pathogenesis (Lockman and Curtiss, 1990; Lockman and Curtiss, 1992b). In contrast, non-flagellated *S. typhimurium* mutants showed a reduction in macrophage survival and were less virulent *in vivo* (Weinstein *et al.,* 1984; Fields *et al.,* 1986). Further studies in this thesis have shown that a loss of BipA results in a decrease in macrophage survival/invasion (section 5.2.2), this may be due to a reduction in flagella expression.

4.3.6 BipA positively regulates cell motility

The high degree of sequence conservation between *S. enteritidis*, *S. typhimurium*, and EPEC *bipA* (section 3.2.2) would suggest that BipA has a similar role in all three species, however this is not the case for motility. In contrast to EPEC findings (Farris *et al.*, 1998) *Salmonella* negatively regulates motility. This decrease in motility could be due to a reduction in flagella expression seen in the *bipA* null mutant (section 4.3.5).

Interestingly, this motility defect is not transcomplemented with plasmids that contain either the *S. enteritidis*, *S. typhimurium* or EPEC *bipA* gene. The EPEC *bipA* seems to be redundant in *S. enteritidis bipA::kan* cells as the defect remains the same, however the defect is enhanced when *bipA::kan* cells are transformed with a plasmid carrying a copy of either the *S. enteritidis* or *S. typhimurium bipA* gene. These findings suggest that in *Salmonella* the regulation of motility is controlled specifically by *Salmonella* BipA and that its level is critical. To investigate if a specific concentration of BipA is essential to the regulation of motility, *bipA* would need to be cloned into an arabinose inducible plasmid where you could control the amount of *bipA* that is being expressed. If the motility defect were restored at a specific concentration of *bipA*, but at no other concentration of *bipA*, you would be able to define this phenotype seen with the transcomplemented strain as a titration effect.

4.3.7 Comparing the protein profiles of wild type and *bipA* null mutant

The almost identical profiles of the wild type strain and the *bipA* mutant strain suggested that BipA is not involved in 1) the regulation of several ribosomal proteins 2) in several stages in the glycolytic or citric acid cycle pathways or 3) in the refolding of heat shock denatured proteins.

CHAPTER 5

5 The role of BipA in other processes implicated in virulence

5.1 Introduction

In the previous chapter studies showed that BipA plays a role in the regulation of numerous fimbriae and flagella-mediated cell motility. As these appendages have been implicated in virulence it therefore seemed logical to continue to look at the involvement of BipA in other virulence associated properties.

5.1.1 Bacterial adhesion of the intestine

Once *Salmonella* cells have passed through the stomach and survived the gastric barrier they will colonise the intestinal epithelial. Fimbriae are the best understood mechanism of adherence, although the fimbriae actually involved in adherence are equivocal (details in Chapter 4). The precise site of entry within the intestine is still controversial, however studies in *S. typhimurium* suggest that although these bacteria are able to penetrate normally non-phagocytic epithelial cells, M cells are the preferred target in this pathogen (Jones and Falkow, 1996). Binding to M cells is thought to be mediated by the *lpf* operon, though the host receptor for this adhesin is unknown (Bäumler *et al.,* 1996). Interestingly, despite the striking similarities in invasion loci and mechanism between *S. typhi* and *S. typhimurium*, *S. typhi* was shown to use the cystic fibrosis transmembrane conductance regulator (CFTR) as a receptor (Pier *et al.,* 1998) whereas *S. typhimurium* was not (Mills and Finlay, 1994).

5.1.2 Bacterial Invasion of the intestine

Invasive enteric bacteria must pass through the intestinal epithelium in order to establish infection. A type III secretion system (TTSS) encoded on *Salmonella* Pathogenicity Island 1

(SPI1) is required for the invasion of eukaryotic cells (Darwin and Miller, 1999; Schechter and Lee, 2000). Many bacterial pathogens use type III secretion systems to deliver virulence factors into the host cell to interfere with or subvert normal host cell signalling. The regulation of SPI1 gene expression is very complex and it has been shown *in vitro* that *S. typhimurium* invasion of host cells is regulated by many factors including oxygen, osmolarity and growth phase of the bacteria (Collazo and Galan, 1997). However, the principal factor in induction of the secretion system was found to be a change in the pH of the culture medium from acidic to mildly alkaline. These differing conditions would be experienced *in vivo* as the bacteria move from the acid environment of the stomach to the mildly alkaline mucus of the small intestine, where they cause disease (Daefler, 1999).

Several genes have been implicated in the cytoskeletal rearrangements involved in bacterial engulfment. Proteins encoded on the *sip* (Salmonella Invasion Protein) operon in SPI-1 (Figure 5-1) have different effects on the invasion of cultured epithelial cells. Mutations in *sipB*, *sipC* and *sipD* profoundly effect invasion whereas inactivation of *sipA* only has a subtle effect (Collazo and Galan, 1997). SipB, SipC and SipD are involved in the translocation of effector proteins whereas SipA is an effector protein. Recent evidence suggests that SipA is an actin-binding protein that contributes to host cell actin cytoskeletal rearrangements. SipA binds directly to actin and decreases its critical concentration for polymerization. SipA also inhibits depolymerization of actin filaments. These activities facilitate bacterial uptake as they result in the spatial localization and more pronounced outward extension of the *Salmonella*-induced membrane ruffles (Zhou *et al.,* 1999). Recent evidence suggests that SipC may also have an effector function in host cells as the amino-terminus of the protein was found to mediate condensation ('bundling') of actin filaments which is necessary for bacterial engulfment (Hayward and Koronakis, 1999). A different translocated effector protein, SopE (*Salmonella* outer proteins), stimulates cytoskeletal reorganization by acting as a guanyl-nucleotide-exchange

factor on Rho GTPase proteins such as Cdc42 and Rac which regulate actin cytoskeleton (Hardt *et al.,* 1998).

SptP (*Salmonella* protein tyrosine phosphatase) encoded on SPI-1 (Figure 1-1), is also involved in host membrane cytoskeletal rearrangements. Following bacterial engulfment the host membrane returns to normal (Finlay *et al.,* 1991) and recent evidence suggests that this may be mediated by SptP as it acts as a GTPase-activating protein (GAP) which promotes inactivation of Rac-1 and Cdc42 (Fu and Galan, 1999). Translocation of SptP requires the function of the secreted proteins, SipB, SipC and SipD, as strains carrying mutations in any of the genes failed to translocate SptP (Fu and Galan, 1998).

In contrast to the belief that SPI-1 invasion genes are essential to bacterial infection of the host mucosa, Murray and Lee (2000) have found that invasion genes are not required for *S. typhimurium* to breech the intestinal epithelium. A transcriptional activator encoded on SPI-1, HilA, regulates the expression of SPI-1 invasion genes (Eichelberg and Galan, 1999). A strain harbouring a mutation in *hilA* was recovered in lower numbers from infected mice compared to the parent strain and a strain where SPI-1 had been deleted. These results suggested that only *hilA* is necessary for intestinal colonisation (Murray and Lee, 2000).

A gene encoded on SPI-5, SigD (Salmonella invasion gene) (Figure 5-1), has also been implicated in invasion (Hong and Miller, 1998). SigD was originally identified in *S. dublin* as SopB which is an inositol phosphate phosphatase that mediates inflammation and fluid secretion in the intestinal mucosa (Galyov *et al.,* 1997).

5.1.3 Macrophage invasion and survival

Following invasion of host cells the bacteria are enclosed within a *Salmonella*-containing vacuole (SCV) where they are able to replicate and survive the formidable defences such as bactericidal cationic peptides and a decrease in pH (Elsbach and Weiss, 1985). The bacteria

eventually kill the host cell where they are released in to the extracellular medium so that they can infect other cells (Dunlap *et al.,* 1991; Richter-Dahlfors *et al.,* 1997). The ability of *Salmonella* to survive and replicate in host cells is essential for virulence as mutants defective in replication within the macrophage and cell lines are avirulent in animals (Fields *et al.,* 1986; Leung and Finlay, 1991). Several genes encoded on the pathogenicity islands have been implicated in the survival and lysis of macrophages.

SPI-2 encodes a second type III secretion system and mutants defective in SPI-2 fail to proliferate to the same level as wild-type strains in mouse macrophages and human epithelial cell lines (Cirillo *et al.,* 1998; Hensel *et al.,* 1998). Expression of these genes is regulated by the two-component regulatory systems PhoPQ and SsrA/SsrB (secretion system regulators; encoded on SPI-2) (Figure 5-1) (Cirillo *et al.,* 1998; Deiwick *et al.,* 1999). Further studies into this type III secretion system have shown that OmpR regulates SsrA/SsrB as early transcription of *ssrA*, after entry into macrophages, is most efficient in the presence of OmpR. Furthermore, the purified OmpR protein binds directly to the *ssrA* promoter (Lee *et al.,* 2000).

Genes encoded on SPI-3 and SPI-4 are also involved in macrophage survival and lysis. Two genes encoded on SPI-3, *mgtCB*, are involved in intramacrophage survival (Blanc-Potard *et al.,* 1999) whereas genes encoded on SPI-4 are thought to be involved in the secretion of a cytotoxin that causes macrophages lysis (Wong *et al.,* 1998).

Recent evidence has shown that SipB induces macrophage lysis by binding to the pro-apoptotic protease caspase-1. Caspase-1 activity is essential for macrophage cytotoxicity as macrophages lacking caspase-1 are not susceptible to *Salmonella*-induced lysis (Hersh *et al.,* 1999). Recent evidence has also shown that contrary to earlier results, *S. typhimurium* can lyse macrophages by a mechanism distinct from apoptosis and this mechanism is dependent on a on a subset of caspases and SipB (Watson *et al.,* 2000).

A recent report showed that *S. enterica* SEF14 fimbrial mutations were unable to survive in activated macrophages, indicating a role for the fimbriae in the attachment and/or invasion of macrophages (Edwards *et al.*, 2000).

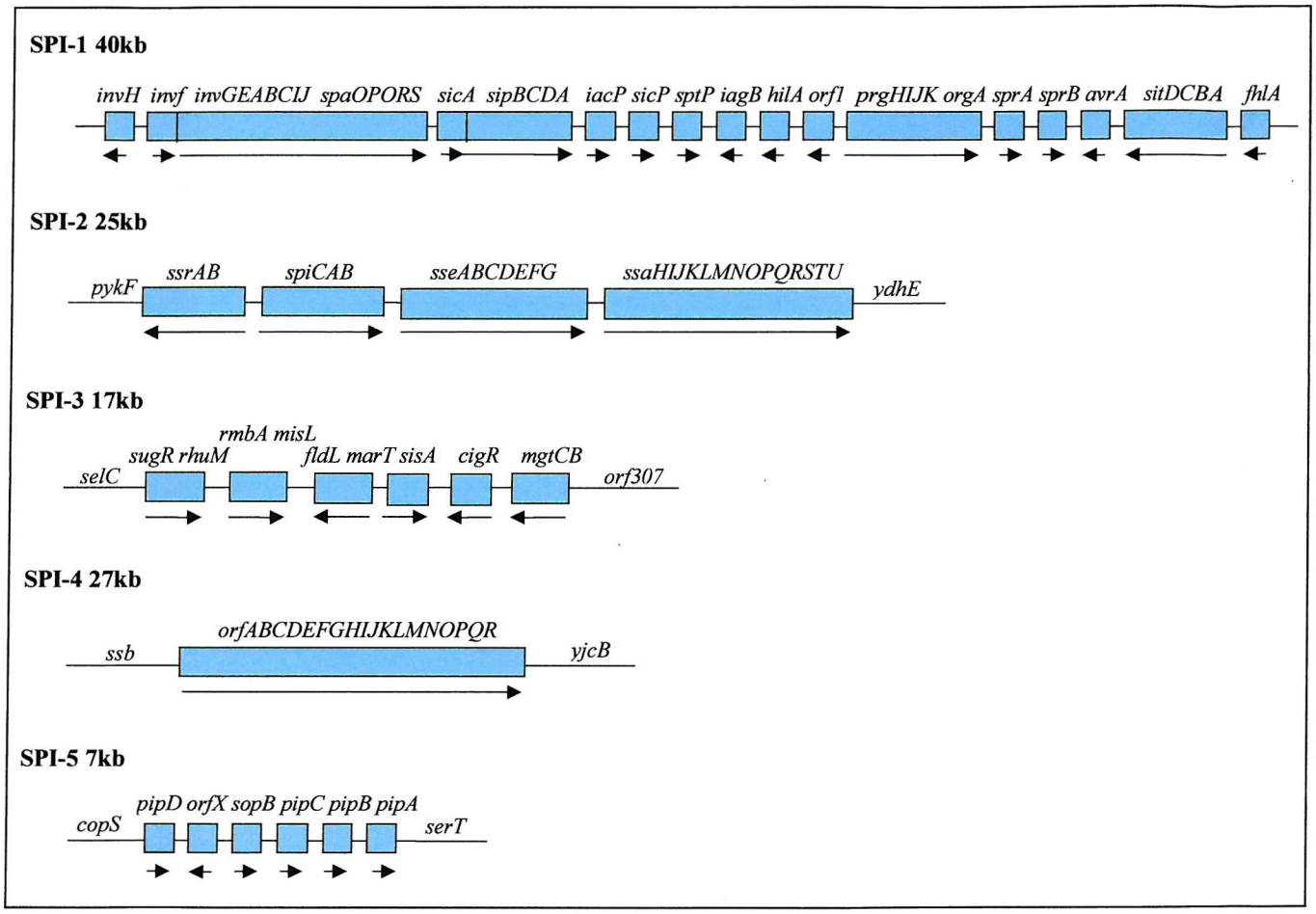


Figure 5-1 Genetic organisation of the *Salmonella* pathogenicity islands

The boxes indicate the genes encoded on the pathogenicity islands whereas the arrows indicate the direction of transcription. Diagram modified from Marcus *et al.* (2000). There are likely to be other 'islands' and 'islets', these are however the best characterised ones currently.

5.1.4 Acid-Tolerance response

During pathogenesis *Salmonella* encounter a variety of potentially lethal acid stress conditions. Following bacterial invasion of macrophages the *Salmonella* are exposed to a progressively acidic environment (pH 3-4) produced by various metabolic activities within the phagosome. *In vitro Salmonella* would not be able to survive such low pH, however *in vivo Salmonella* are able to survive within these acidic conditions due to the adaptive acid tolerance response (ATR) (Foster and Hall, 1990). Two major ATR systems have been identified in *Salmonella* and they are classified by the growth phase at which each becomes induced.

5.1.4.1 Log Phase Acid Tolerance

Three global regulators involved in exponential acid tolerance response include the alternative sigma factor σ^S encoded by *rpoS*, the major iron regulatory protein Fur, and the two-component regulatory system PhoPQ (Loewen and Hengge-Aronis, 1994; Hall and Foster, 1996; Hengge-Aronis, 1996; Bearson *et al.,* 1998). Mutations in any of these regulators conferred an acid-sensitive phenotype (Foster and Moreno, 1999). Also further work has shown that the Ada protein (involved with the adaptive response of *E. coli* to alkylating agents) is involved with resistance, but its role in ATR is unknown (Hakura *et al.,* 1991).

The σ^S protein is an essential transcription factor needed for the survival of *Salmonella* after entry into stationary phase. However, is it now recognised as a crucial factor in many stress responses (Loewen and Hengge-Aronis, 1994; Hengge-Aronis, 1996). Exponential phase acid tolerance mechanisms can be classified as either a σ^S -dependent or, -independent. Recent findings have shown that when log phase cells are exposed to acid they exhibit increased levels of σ^S which in turn stimulates at least 8 Acid Shock Proteins (ASPs) (Lee *et al.,* 1995) . RpoS is needed to sustain this acid tolerance response as RpoS mutants (defective in σ^S production) are

only transiently able to induce ATR (Lee *et al.,* 1995; Wilmes-Riesenberg *et al.,* 1997). The mouse virulence gene, *mviA* (*m*ouse *vi*rulence), is thought to negatively regulate the expression of σ^S. MivA is a 38-kDa protein which has significant sequence homology to the response regulatory family of bacterial transcriptional regulators (Bearson *et al.,* 1996).

The σ^S -independent systems involve the two global regulators Fur and PhoPQ. In contrast to iron regulation Fur is thought to positively regulate acid tolerance as mutants defective in *fur* repressed the expression of ≥ 8 ASPs. The role of Fur in acid tolerance is independent to its role in iron regulation (Hall and Foster, 1996; Foster and Moreno, 1999). The PhoPQ regulatory system is involved in survival in inorganic acid (low pH). Two-dimensional gel electrophoresis showed that ≥ four ASPs were missing from an acid-adapted $PhoP^-$ strain. PhoP was one of these ASPs, but the identities of the other three are at present unknown (Bearson *et al.,* 1998).

5.1.4.2 Stationary Phase Acid tolerance

Stationary phase growing cells also exhibit σ^S-dependent or, -independent ATR systems. The σ^S-dependent system is not induced by acidity because levels of σ^S are already increased due to entry into stationary phase, however these increased levels do improve acid tolerance (Lee *et al.,* 1994; Lee *et al.,* 1995). In contrast, the σ^S-independent system is induced by acid and ≥ 15 ASPs are activated (Lee *et al.,* 1994; Foster, 1991). Very little is known about this system except that Bang and co-workers (2000) have recently shown that the regulatory protein OmpR plays an integral role in controlling the expression of genes needed for acid-induced stationary phase acid tolerance. The production of OmpR is increased when the cells are exposed to acid and phosphorylated OmpR can trigger the expression of genes needed for an acid-induced stationary-phase acid tolerance (Bang *et al.,* 2000).

5.1.5 The oxidative-stress response

Salmonella are often exposed to the reactive oxygen species superoxide (O^{2-}) and hydrogen peroxide (H_2O_2) in the non-host environment (Kappus and Sies, 1981) and within the cell (normal aerobic metabolism) (Fridovich, 1983). These reactive species are lethal as they can damage DNA, proteins, and membranes. In response *Salmonella* have various regulons that produce numerous proteins that will either avert or repair the eventual oxidative damage. For example, the OxyR protein mediates hydrogen peroxide-inducible expression of ≥ nine proteins, whereas the two-gene locus *soxRS* controls the induction of at least fifteen other proteins in response to superoxide and nitric oxide (Farr and Kogoma, 1991).

In *E. coli* functional OxyR or SoxRS regulons are essential for macrophage survival as they are required for resistance to cytotoxic oxidants during the phagocytic respiratory burst (Christman *et al.,* 1989; Wu and Weiss, 1991). In contrast, *S. typhimurium* OxyR or SoxRS mutants are as virulent as the parental strain, which suggests that *Salmonella* have acquired an alternative strategy to avoid exposure to high concentrations of toxic phagocyte-derived oxidants (Fang *et al.,* 1997; Taylor *et al.,* 1998). Recent evidence suggests that, in *Salmonella*, SPI-2 interferes with the trafficking of oxidase-containing vesicles to the phagosome as SPI-2 mutants were only able to survive in the absence of the NADPH oxidase-dependent respiratory burst (Vazquez-Torres *et al.,* 2000).

5.1.5.1 Function of OxyR protein

OxyR is a transcriptional regulator that activates genes involved in the production of the peroxide-destroying enzymes catalse (*katG*), glutathione reductase (*gorA*) and the NADPH-dependent alkyl hydroperoxidase (*ahpFC*). KatG catalyses the reaction where hydrogen peroxide is converted to oxygen and water. Glutathione reductase maintains a pool of reduced glutathione, which as result maintains the reduced state of the cellular proteins. The hydroperoxidase

converts the lipid hydroperoxides (consequence of H_2O_2 damage) into corresponding non-toxic alcohols (Halliwell and Gutteridge, 1984; Storz *et al.*, 1987).

5.1.5.2 Function of SoxRS regulon

SoxR is a transcriptional regulator that when oxidised dramatically increases the production of a second transcriptional activator, SoxS (Li and Demple, 1994). SoxS then activates the transcription of various genes including *sodA* (Mn-containing superoxide dismutase), *nfo* (DNA-repair enzyme endonuclease IV), *zwf* (glucose-6-phosphate dehydrogenase), *micF* (posttranscriptionally decreases production of OmpF), *fpr* (NADPH ferredoxin oxidoreductase), *acrAB* (efflux pump), *acn* (aconitase), *fumC* (oxidation-resistance fumerase), and *nfsA* (nitroreductase A) (Pomposiello and Demple, 2000).

This chapter describes the studies to determine if the *bipA* mutant is compromised in its ability to invade epithelial and macrophage cells. Additionally, it reports the role of BipA in the acid tolerance response and the oxidative stress response.

5.2 RESULTS

Detailed studies in EPEC indicate that mutants defective for BipA, although capable of adhering to host epithelial cells, lose the capacity to trigger the cytoskeletal rearrangements that are found in epithelial cells infected with wild type EPEC. In marked contrast enhanced expression of BipA results in actin accumulation beneath the bacteria and the formation of EPEC-associated pseudopods (Farris *et al.*, 1998). BipA also appears to act as virulence regulator associated with the control of bacterial cell motility and resistance to antibacterial effects of host defence proteins. The question therefore has arisen as to whether *bipA* also plays a role in processes implicated in *Salmonella* pathogenesis. To address this issue, the involvement of *bipA* in the invasion of HeLa cells and macrophages *in vitro* was studied and the involvement of *bipA* in the acid tolerance response and the resistance to oxidative stress was also studied.

5.2.1 The role of BipA in the adhesion and invasion of HeLa cells

Invasion of *Salmonella* into host epithelial cells is essential for its virulence (Kohbata *et al.*, 1986; Penheiter *et al.*, 1997). In view of this finding the adhesion and invasion characteristics of the *bipA::kan* mutant were investigated. Studies with the HeLa cells showed that *bipA::kan* mutant cells adhere to and invade these cells to the same extent as wild type cells (Figure 5-2).

5.2.2 BipA plays a role in the invasion and survival in macrophages

Another important step in *Salmonella* infection is bacterial entry and survival in macrophages (Fields *et al.*, 1986; Gahring *et al.*, 1990). The intramacrophage survival of wild type *S. enteritidis* and BipA$^-$ *S. enteritidis* was consequently investigated using different types of macrophages. Two types of bone marrow derived mouse macrophages were used, CBA and B6, and one RAW macrophage-like cell line was used. CBA macrophages are resistant to bacterial survival due to their allele Nramp$^+$ (natural resistance-associated macrophage protein), whereas B6 macrophages are non-resistant to bacterial survival due to their Nramp$^-$ genotype. All three

macrophage assays showed that the *bipA::kan* mutant had reduced survival relative to wild type cells (Figure 5-3, Figure 5-4 and Figure 5-5). A 2.9, 2.2 and 1.74 fold decrease was seen in CBA, B6 and RAW macrophages, respectively. BipA therefore seems to play a role in macrophage invasion and/or survival, at least *in vitro*.

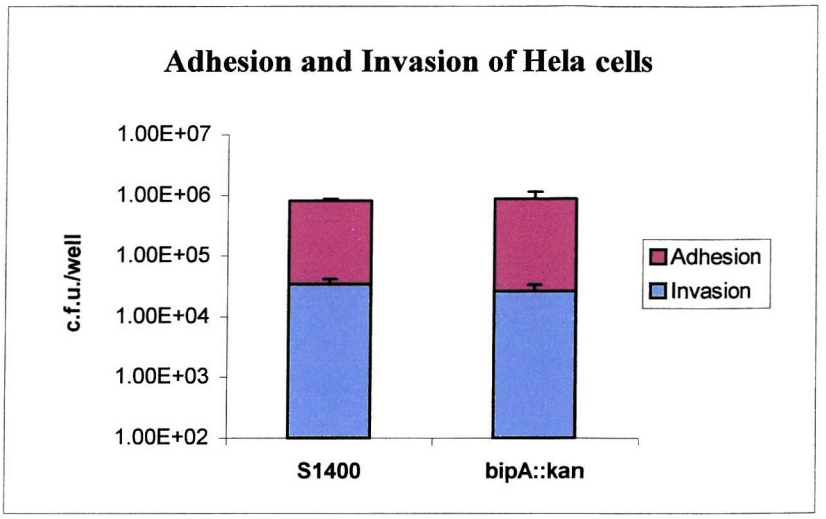


Figure 5-2 Analysis of the role of BipA on the invasion and survival of *S. enteritidis* in HeLa cells

HeLa cells were infected with S1400 or its *bipA::kan* derivative using a 1:10 ratio. The adhesion and invasion were measured as described in Materials and Methods. The values plotted are representative of three assays carried out. Strains were grown in LB under oxygen-limiting conditions before the assay.

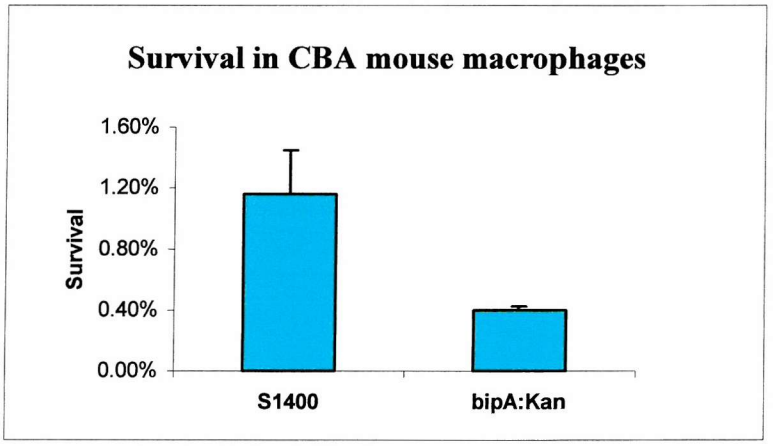


Figure 5-3 Effect of BipA on the invasion and survival of *S. enteritidis* in CBA macrophages

Bone marrow macrophages from CBA mice were collected and propagated as described in Materials and Methods. S1400 or S1400 *bipA::kan* were then added at a m.o.i of 10 and the number of gentamicin resistant bacteria was determined after 4 hours. The survival values are reported as the percentage of the bacterial inoculum surviving gentamicin treatment.

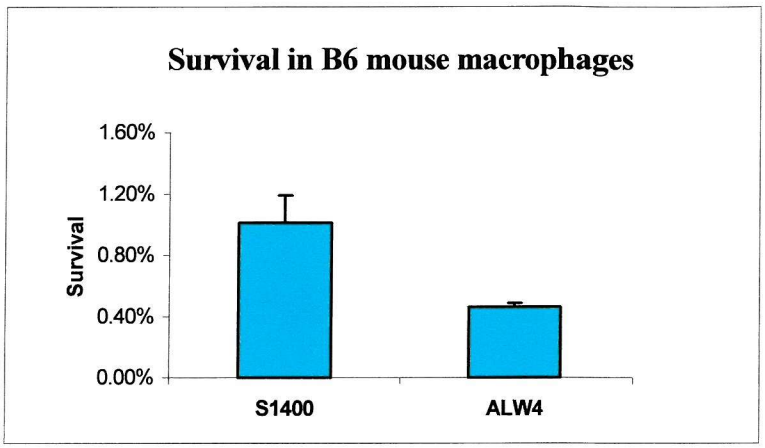


Figure 5-4 Effect of BipA on the invasion and survival of *S. enteritidis* in B6 macrophages

Bone marrow macrophages from B6 mice were infected with S1400 or S1400 *bipA::kan* mutant at a m.o.i. of 10 and the number of gentamicin resistant bacteria was determined after 4 hours. The survival values are reported as the percentage of the bacterial inoculum surviving gentamicin treatment

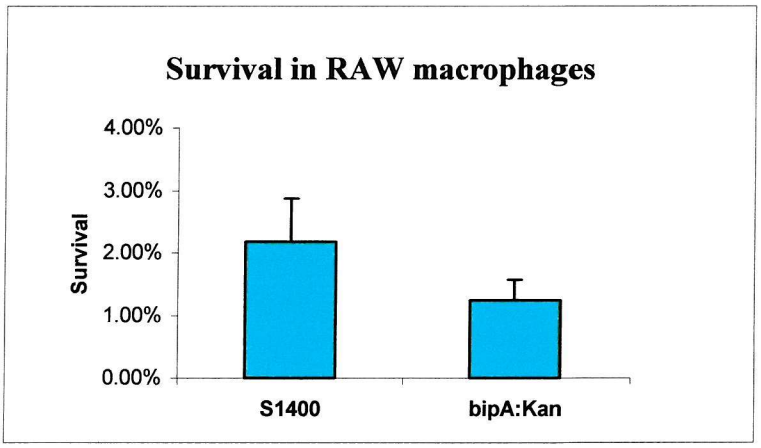


Figure 5-5 Effect of BipA on the invasion and survival of *S. enteritidis* in RAW-like macrophages

RAW macrophage-like cells were infected with S1400 or S1400 *bipA::kan* mutant at a m.o.i. of 10 and the number of gentamicin resistant bacteria was determined after 4 hours. The survival values are reported as the percentage of the bacterial inoculum surviving gentamicin treatment.

5.2.3 The effect of *bipA* on *S. enteritidis* acid tolerance

The acid tolerance response of *Salmonella* is fundamental to survival at low pH. When the bacteria are engulfed by macrophages they must survive the low pH within the phagosome (Bearson *et al.,* 1997). A reduction in macrophage survival was seen with the *bipA* mutant strain compared to the wild type (section 5.2.2). It was therefore of interest to investigate the effect of BipA on the acid tolerance response.

Studies into the acid tolerance of wild type and *bipA* mutant, when grown to mid-exponential phase, showed that both strains exhibited normal acid tolerance. Both experiments showed that the pre-adapted strains continued to survive for 60 minutes after exposure to pH 3 whereas the un-adapted strains died after only 10 minutes (Figure 5-6 & Figure 5-7)

5.2.4 The effect of *bipA* on *S. enteritidis* oxidative stress

Salmonella are often exposed to toxic oxidants in a variety of situations including the natural environment (Kappus and Sies, 1981; Fridovich, 1983). Very little studies have been carried out to determine the role of BipA in the non-host environment. Therefore, it was of interest to investigate the effect of BipA on *S. enteritidis* response to oxidative stress

The oxidative stress assays show that BipA affects the survival of *S. enteritidis* when exposed to hydrogen peroxide. Compared to the wild type cells the survival of both adapted and un-adapted *bipA::kan* cells is reduced (Figure 5-8 & Figure 5-9). After 5 minutes of exposure to hydrogen peroxide a 100-fold decrease was seen in the survival of unadapted mutant cells compared to the wild type. At 15 and 30 minutes the survival of wild type cells remained almost level, but were unable to survive after 90 minutes. In comparison, no mutant cells were able to survive after 30 minutes of exposure. Pre-adapted wild type cells were able to survive for longer, compared to pre-adapted mutant cells, when exposed to a high concentration of hydrogen peroxide. Throughout the time course the wild type cfu remained almost level whereas a steady decrease

was seen in the mutant and at 60 minutes a 1000-fold difference was seen in the survival of the mutant cells compared to the wild type cells (Figure 5-8 & Figure 5-9).

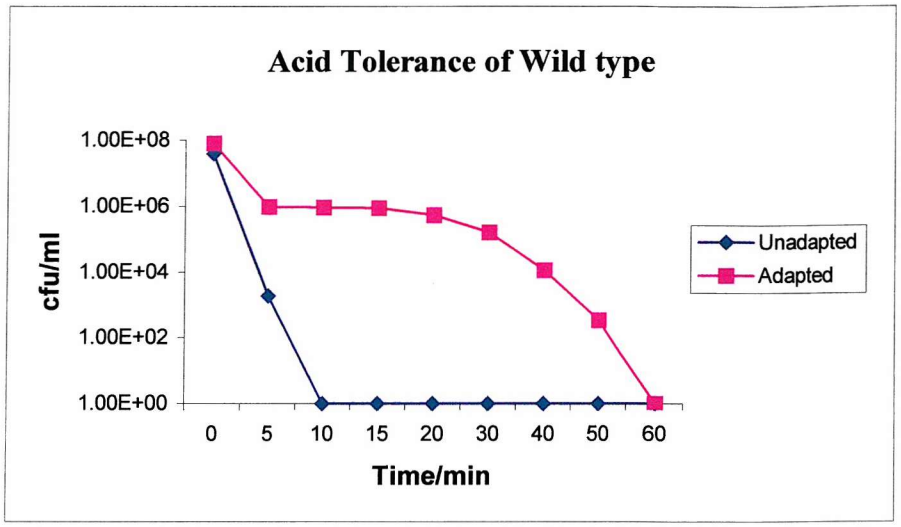


Figure 5-6 Acid tolerance response of wild type

A mid-log phase culture was diluted into LB pH7.2 and LB pH5 to give un-adapted and adapted cultures respectively. Both cultures were incubated for 1 hour at 37°C. Following incubation both cultures diluted into LB pH3 and incubated at 37°C. Samples were taken from both the adapted and un-adapted cultures at several different time points. Serial dilutions were then plated to establish bacterial survival.

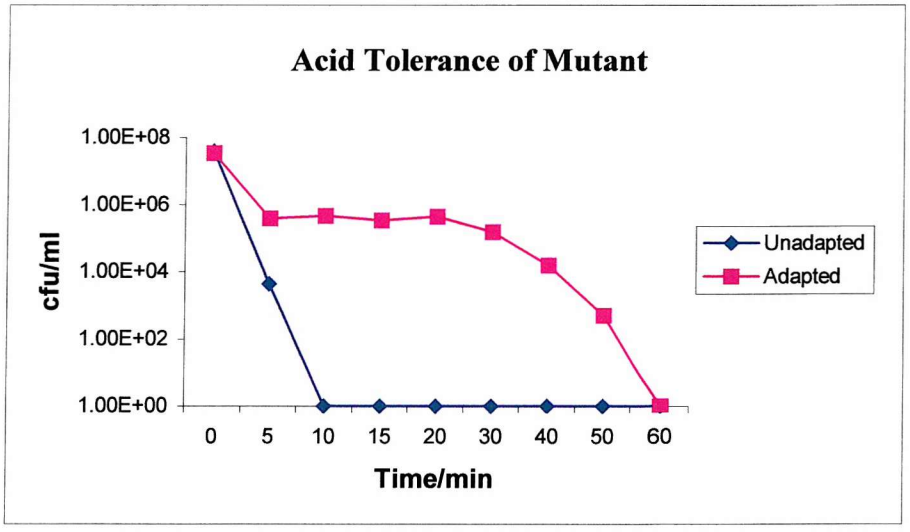


Figure 5-7 Acid tolerance response of wild type

A mid-log phase culture was diluted into LB pH7.2 and LB pH5 to give un-adapted and adapted cultures respectively. Both cultures were incubated for 1 hour at 37°C. Following incubation both cultures diluted into LB pH3 and incubated at 37°C. Samples were taken from both the adapted and un-adapted cultures at several different time points. Serial dilutions were then plated to establish bacterial survival.

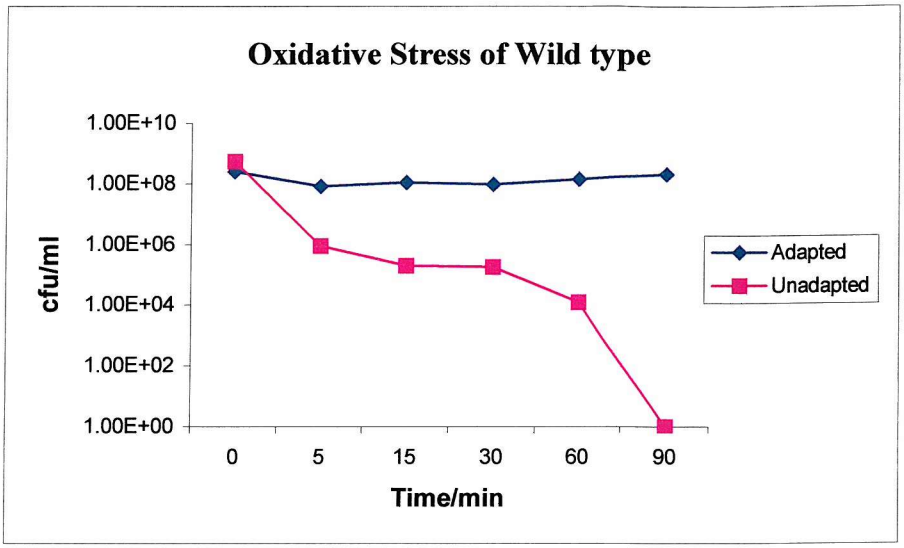


Figure 5-8 Oxidative stress of wild type

A mid-log phase culture was added to two universals. To one universal H_2O_2 was added to give a final concentration of 0.3μm whereas in the other only distilled water was added to give adapted and un-adapted cultures respectively. Both cultures were incubated for 1 hour at 37°C after which hydrogen peroxide was added to both universals to give a final concentration of 30μm. The cultures were incubated with shaking at 37°C. Samples were taken at different time points and the concentration of viable bacteria was determined by plating serial dilutions.

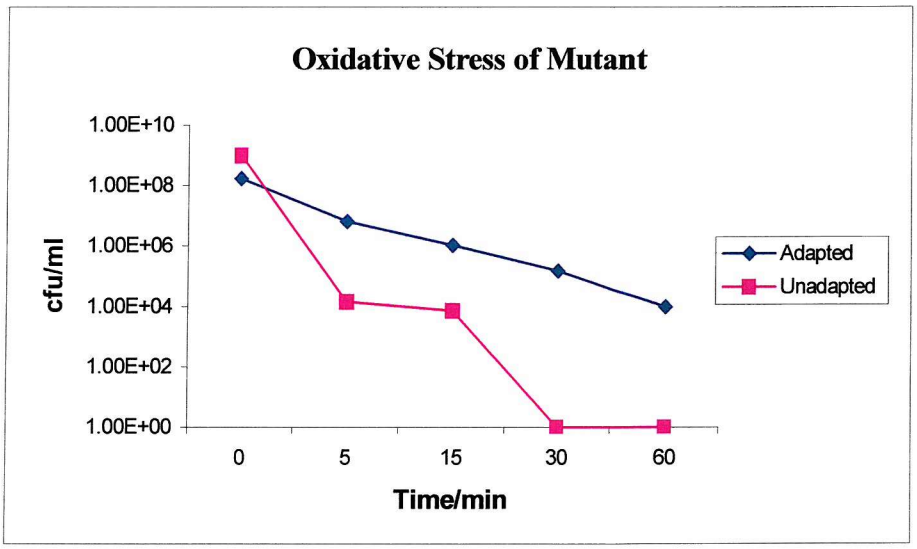


Figure 5-9 Oxidative stress of Mutant

A mid-log phase culture was added to two universals. To one universal H_2O_2 was added to give a final concentration of 0.3μm whereas in the other only distilled water was added to give adapted and un-adapted cultures respectively. Both cultures were incubated for 1 hour at 37°C after which hydrogen peroxide was added to both universals to give a final concentration of 30μm. The cultures were incubated with shaking at 37°C. Samples were taken at different time points and the concentration of viable bacteria was determined by plating serial dilutions.

5.3 Discussion

5.3.1 The contribution of BipA to invasion and survival in host cells *In Vitro*

The invasion of host epithelial cells and survival within macrophages is essential for the virulence of *S. typhimurium* (Fields *et al.,* 1986; Galan, 1996). *In vitro* studies have shown that SPI-1 encodes type III secretion system that is involved in protein secretion and invasion of host cells (Collazo and Galan, 1997). Also, a second type III secretion system is encoded on SPI-2 and this system is involved in bacterial entry and replication within macrophages (Cirillo *et al.,* 1998; Hensel *et al.,* 1998). Ongoing studies with EPEC BipA have shown that it is involved in the regulation of a type III secretion system in *E. coli* (Grant, A. unpublished results). Since it is presumed that *Salmonella* uses similar mechanisms during infection it was of interest to explore the role of BipA in *S. enteritidis* invasion and survival in host cells.

The HeLa cell results suggest that BipA has no effect on the invasion of host epithelial cells. The results of intracellular survival do suggest, however, that BipA may play a role in the invasion and/or survival in macrophages. The *bipA* mutant showed a small (three-fold) reduction in intracellular survival. Further experiments could be carried out to establish whether this is due to decreased invasion or a reduction in survival. The experiment would be repeated as before, but samples would be taken at several different time points after infection, not just after 2 hrs. If the number of bacteria recovered after 24 hrs was less than that recovered after 6 hrs it would suggest that BipA is involved with survival within the macrophage. However, if the number recovered at 24hrs was similar or slightly higher than at 6hrs it would suggest that BipA is involved in the initial penetration of macrophages. Also, in view of these results it would be interesting to see if BipA affects the biosynthesis or secretion of effector proteins encoded by the SPI-1 and particularly the SPI-2 pathogenicity islands.

The induction of SPI2 gene expression is regulated by the two-component regulatory systems PhoPQ and SsrAB (Cirillo *et al.,* 1998; Deiwick *et al.,* 1999). Furthermore, OmpR activates expression of *ssrA* soon after *Salmonella* enters the macrophage (Lee *et al.,* 2000). Therefore, if BipA did affect the expression of SPI-2 effector proteins it would also be interesting to look at the effect of BipA on the expression of OmpR, PhoP and PhoQ to determine where on the regulatory cascade it exerts its effect. Other pathogenicity islands, including SPI-3 and SPI-4, have been implicated in intramacrophage survival and secretion of cytotoxins (Wong *et al.,* 1998; Blanc-Potard *et al.,* 1999). Again *bipA* may be involved in the regulation of these genes and it would be of interest to look at the effect of *bipA* on the expression of the genes encoded on these pathogenicity islands, particularly *mgtC* and *mgtB* as they are involved in macrophage survival.

A recent report indicated a role for fimbriae in the attachment and/or invasion of macrophages as it showed that *S. enteritidis* SEF14 fimbrial mutants were unable to survive in activated macrophages (Edwards *et al.,* 2000). The results obtained in this chapter may support this hypothesis as previous results have shown that the expression of SEF14 fimbriae is down regulated in the *bipA* mutant (section 4.2.1). Therefore the reduction in macrophage survival in the mutant may be due to a reduction in SEF14 expression.

5.3.2 The contribution of BipA to acid tolerance and oxidative stress

Salmonella have evolved systems such as the acid tolerance response and the oxidative stress response to evade host defences such as low pH and reactive oxygen species. Within macrophages bacteria are faced with an acidic environment. The previous intramacrophage survival results suggested that BipA may play a role in the invasion and/or survival in macrophages (section 5.2.2). Consequently it seemed logical to investigate the role of BipA on acid tolerance and oxidative stress.

The acid tolerance results suggest that BipA does not affect the acid tolerance of *S. enteritidis*. The oxidative stress results do suggest, however, that BipA is involved in this response as both the un-adapted and pre-adapted mutant cells did not survive as well as the wild type cells. Similar results have been found in *S. typhimurium* (G. Howell, unpublished results). It would be valid to look at the expression of the genes encoded on the SoxRS and OxyR regulons that control the response of *Salmonella* to oxidative stress.

CHAPTER 6

6 Animal studies

6.1 Introduction

As discussed in chapters 4 and 5, *in vitro* studies have shown that BipA may be involved in several properties associated with *Salmonella* virulence including invasion and survival in macrophages. It followed therefore that the extent of attenuation in the one-day old chick model and the BALB/c mouse model of infection would be investigated using the *bipA* null mutant.

6.1.1 One-day old chick model

S. enteritidis phage type (PT) 4 is invasive in young chicks (Hinton *et al.*, 1989) and studies have shown that chickens generally become more resistant to *Salmonella* infection with age (Humphrey *et al.*, 1991). Hinton *et al.* (1989) found that if chicks were dosed 24 hours after hatching an invasive disease of the reticuloendothelial system was seen. However, if chicks were dosed at 7 days the chicks showed a huge reduction in invasive *S. enteritidis* infection and by 20 days the chicks appeared resistant to infection (Humphrey *et al.*, 1991).

The development of the natural gut microflora may account for the resistance to infection in the older chick (Coloe *et al.*, 1984). No bacteria could be detected in the small intestine, caecum or large intestine of one-day old SPF chicks, however 3 days after hatching significant levels of faecal streptococci and coliforms were isolated from all sites. By 40 days the flora had stabilised to consist predominantly of faecal streptococci, *Escherichia coli*, *Bacteroides* spp. and *Lactobacillus* spp. These species conferred increased resistance to the establishment of *Salmonella* infection (Coloe *et al.*, 1984) as the formation of this mat of microflora prevented the adherence of *Salmonellae* to the mucosa of the caecum (Soerjadi *et al.*, 1982). Results to confirm this resistance have shown that it is possible to protect young chicks from *Salmonella*

infection by the oral administration of caecal material derived from normal adult chickens (Rantala and Nurmi, 1973).

The change in the pathogenesis of *Salmonella* in different aged chicks may also be explained by the increase in the functional activity of the chicken heterophil (Wells *et al.,* 1998). In poultry one of the primary cells in the innate immune response to early bacterial invasion by *Salmonella* is the heterophil. By studying the phagocytic and bactericidal activities of heterophils from chickens during the first 7 days post-hatch, Wells *et al.* (1998) found that from 1 to 4 days after hatching there was no significant difference in the activity of the heterophils whereas by day 7 the activity had doubled. Therefore, chicks are most susceptible to *Salmonella* infection during the first 4 days post-hatch. The age response of chicks to infection may also be influenced by the development of the gut-associated lymphoid tissues (Jeurissen *et al.,* 1989).

Because chicks are most susceptible to *Salmonella* infection at an early age, the invasion properties of the wild type strain and the BipA mutant strain were compared using chicks dosed at 24 hours post-hatch. In this model the extent of bacterial invasion is estimated by counting the numbers of bacteria that have invaded the liver, spleen and caecum.

6.1.2 Persistence of *S. enteritidis*

Even though chickens generally become more resistant to *Salmonella* infection with age, the bacteria are still able to persist within the caeca (Humphrey *et al.,* 1991). The roles of fimbriae and flagella in the adherence and colonisation of the caeca are ambiguous. Thorns *et al.* (1996) showed that SEF14 fimbriae alone do not appear to play a significant role in the adhesion/invasion of *S. enteritidis* host cells. However, compared to the parent strain, the excretion of a strain harbouring a SEF14 mutation was reduced in the first week following infection. This difference was not seen between 2 and 7 weeks after infection. Therefore, the role of SEF14 may only be significant in the first few weeks post infection. Moreover, a significant

reduction in caecal colonisation and faecal shedding was observed in hens inoculated with a strain that lacked SEF14 and SEF21 fimbriae compared to hens inoculated with strains that expressed one or both these fimbriae (Thiagarajan *et al.,* 1996). In contrast, more recent studies using well-defined afimbriate and aflagellate mutants have shown that flagella, but not fimbriae, play a role caecal colonisation (Allen-Vercoe *et al.,* 1999a).

Many of these studies identified the extent of caecal colonisation by counting bacterial numbers taken from cloacal swabs. In this study the role of BipA in caecal colonisation and faecal shedding was investigated using this method.

6.1.3 Mouse model of infection

Different *Salmonella* species infect different hosts. For example *S. typhi* cases typhoid fever in humans whereas *S. typhimurium* causes mild gastroenteritis in humans but induces a systemic disease in mice that is similar to typhoid fever. Studies have shown that when murine ligated loops are infected with *S. typhimurium* the bacteria cause systemic disease by preferentially invading and destroying the specialised M cells of the Peyer's patches (Jones *et al.,* 1994b). The infection of a mouse with *S. typhimurium* is the best characterised animal model for studying human typhoid fever.

This chapter describes the studies to determine if the *bipA* mutant is attenuated in either the chick or mouse model of infection.

6.2 RESULTS

6.2.1 BipA$^-$ *S. enteritidis* cells are less invasive to the livers and spleens of one day-old SPF chicks

The invasion/colonisation characteristics of the mutant were compared with the wild type strain by the counting the number of bacteria recovered from livers, spleens and caeca of day old chickens at 6 hours, 24 hours and 48 hours post inoculation (Figure 6-1 & Figure 6-2).

In experiment 1, an analysis of colonisation/cell invasion of a day old chick model showed that a reduced number of CFU in the liver and spleen were recovered relative to the wild type. At 6 hours post inoculation, on average, 700 CFU were recovered from the livers of chicks inoculated with the wild type. In contrast, no *bipA::kan* cells were recovered from the livers of chicks inoculated with the mutant, even after enrichment of the organ homogenates. However, at 24 hours and 48 hours the invasion of wild type and mutant cells was almost identical (Figure 6-1. A). No *Salmonellae* were recovered from the spleen at 6 hrs in either the mutant or wild type. At 24 hours and 48 hours invasion of the spleen was greater in the wild type compared to the mutant, these differences were however not statistically significant (Figure 6-1. B).

In experiment 2, the analysis of bacterial invasion showed that no *Salmonellae* were recovered from the liver at 6 hours post inoculation in either the wild type or mutant. At 24 hours a 3.4 fold decrease was seen in the number of *bipA::kan* cells recovered from the livers compared to the wild type cells. After 48 hours the number of wild type cells and the mutant cells recovered was not significantly different (Figure 6-2. A). In the spleen at 6 hours post inoculation no *Salmonellae* were recovered from chicks inoculated with either the wild type or mutant. At 24 hours a 3.1 fold reduction was seen in the number of mutant cells compared to the wild type cells. After 48 hours the number of bacteria recovered from both groups was almost identical (Figure 6-2. B).

The caeca counts in both experiments showed an initial reduction in the number of mutant cells compared to the wild type. At 6 hours post inoculation a 4.4 and 21 fold reduction was seen in the number of mutant cells recovered from the caecum in experiments 1 and 2, respectively. After 6 hours no difference was seen in the numbers of bacteria recovered from either the wild type or mutant in both experiments 1 and 2 (Figure 6-1. C & Figure 6-2. C).

6.2.2 Persistence of wild type and mutant

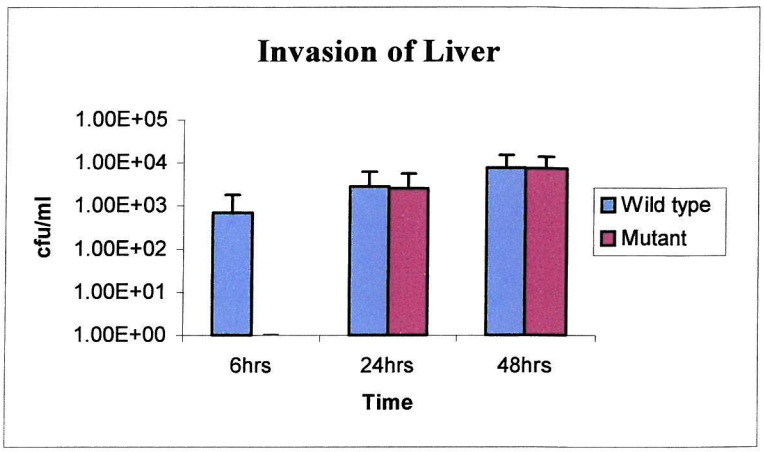
A semi-quantative method first used by Williams-Smith and Tucker (1975) was applied to determine the persistence of the wild type strain and mutant strain in the chick caeca. In this experiment 12 one-day-old SPF White Leghorn chicks were divided into two groups and inoculated with either the wild type strain or the *bipA* mutant strain (10^5 cfu/ml). Post inoculation, a cloacal swab was taken every week from each bird and the extent of shedding was noted by the amount of colonies seen after plating out the swab onto a BGA plate. The chick was considered to be shedding heavily if 200 or more colonies were seen, medium shedding was considered when 200-1 colonies were seen and low shedding was considered if the colonies were only detected after enrichment in selenite broth.

Figure 6-3 shows that the persistence of the wild type strain and mutant strain is different in the one-day-old chick animal model. The graph shows that at day one S1400 was able to colonise the caeca more efficiently than the mutant as 40% of the chicks infected with wild type were shedding heavily compared to only 20% when infected with the mutant. Also, only heavy and medium shedding was seen with the wild type whereas heavy, medium and low shedding was seen with the mutant. At day 7 80% wild type birds were shedding heavily compared to only 40% in the mutant birds. At day 14 the chicks infected with the wild type began to clear the infection and only 17% of the chicks remained to shed heavily. However, chicks infected with the mutant were not able to clear the infection and the percentage of the birds shedding heavily

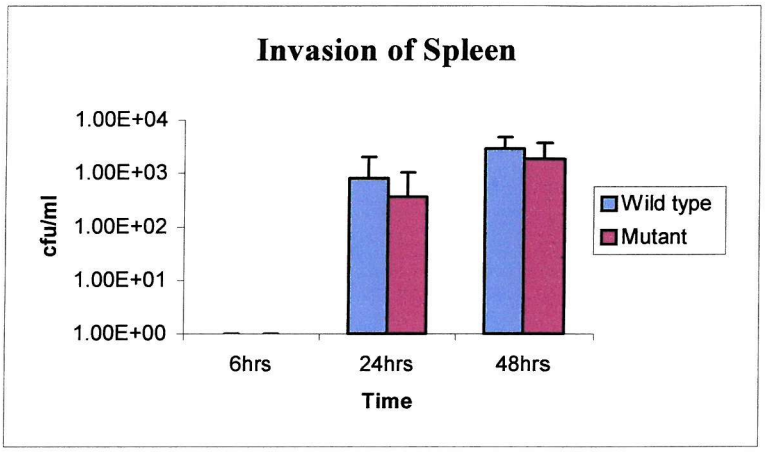
increased to 60%. At day 21 the wild type chicks became re-infected from the environment and thus an increase in the number of birds heavily shedding was seen. In the case of the mutant the chicks began to clear the infection and a decrease was seen in the number of bird shedding heavily. At day 28 the wild type birds started to clear the second infection and only medium, low and clear birds were seen. This was similar to the mutant birds except that they were only clearing the first infection and had not yet been re-infected. These results suggest that initially mutant cells are not able to colonise the caeca as efficiently as the wild type cells. However, once the bacteria have colonised the caeca the mutant cells seem more able to persist.

6.2.3 *S. typhimurium* SL1344 *bipA::cat* is not attenuated in the mouse model

The effect of the null mutant on virulence in the mouse model was also examined. 120 BALB/c mice were divided into two groups, one group was inoculated orally and the other by the intraperitoneal route. The i.p dose ranged from 10^1 cfu to 10^6 cfu, whereas the oral dose ranged from 10^4 cfu to 10^9 cfu. Surprisingly in both experiments the *bipA* null mutant was as virulent as the wild type. (Figure 6-4 & Figure 6-5)



A



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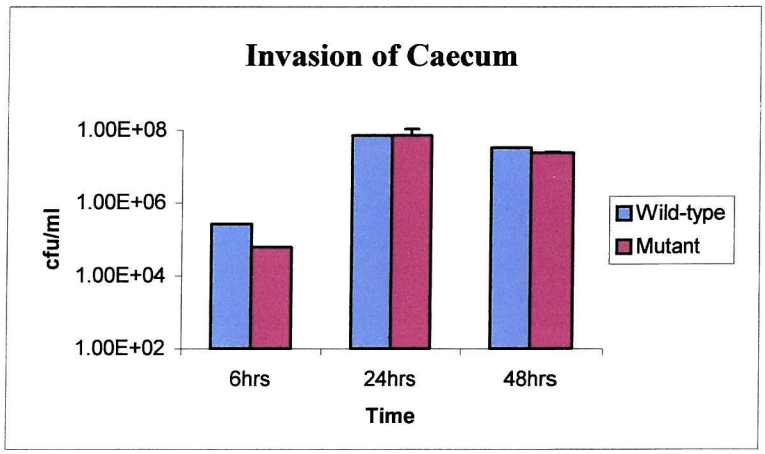
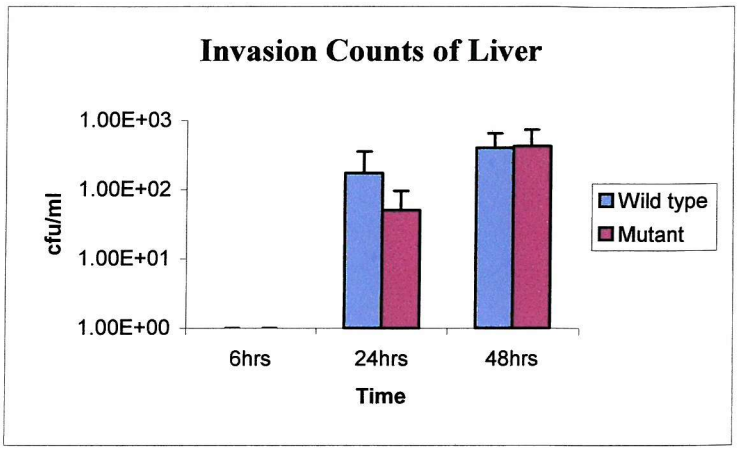


C

Figure 6-1 Experiment 1: Effect of BipA on the invasion of *S. enteritidis* in the livers, spleens and caeca of day old SPF chicks

Chicks were dosed orally with $5x10^4$ cfu of either S1400 or S1400 *bipA::kan*. Chicks were sacrificed at 6, 24, and 48 hours post-inoculation and their spleens, livers and caeca were removed and homogenised. Following homogenisation dilutions were plated on LB supplemented with or without kanamycin and incubated overnight at 37°C. A. invasion of the liver, B. invasion of the spleen and C. invasion of the caecum.



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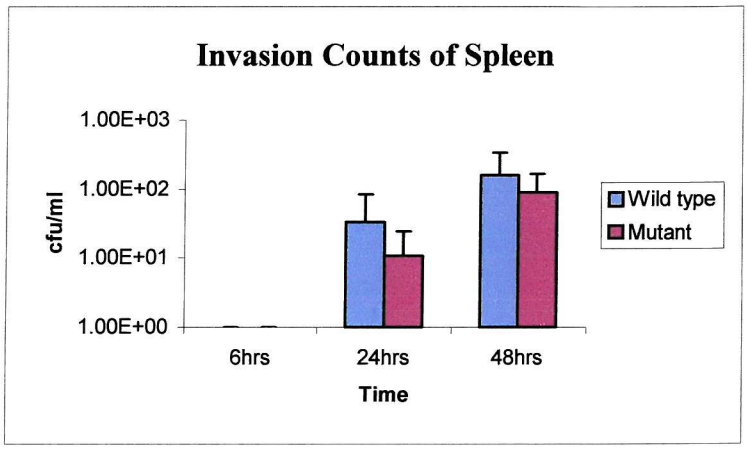

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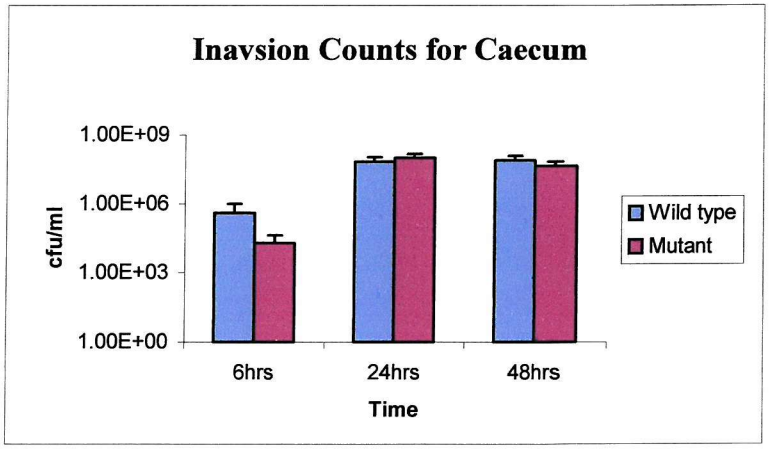
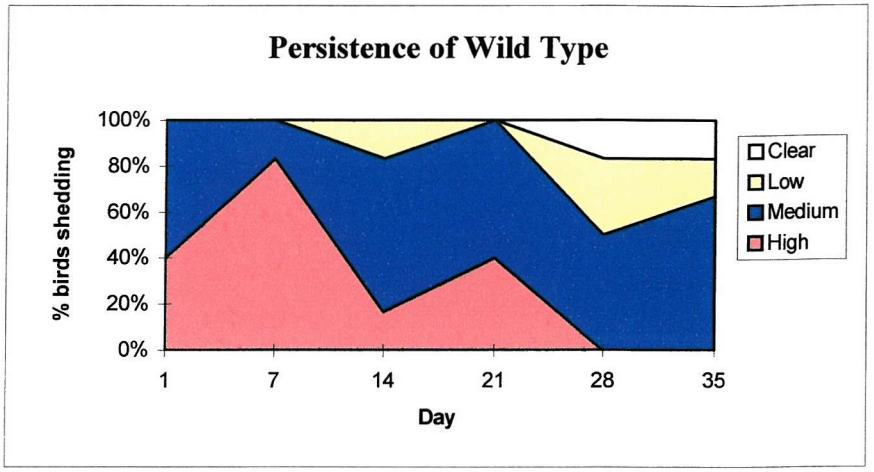


C

Figure 6-2 Experiment 2: Effect of BipA on the invasion of *S. enteritidis* in the livers, spleens and caeca of day old SPF chicks

Chicks were dosed orally with $5x10^4$ cfu of either S1400 or S1400 *bipA::kan*. Chicks were sacrificed at 6, 24, and 48 hours post-inoculation and their spleens, livers and caeca were removed and homogenised. Following homogenisation dilutions were plated on LB supplemented with or without kanamycin and incubated overnight at 37°C. A. invasion of the liver, B. invasion of the spleen and C. invasion of the caecum.



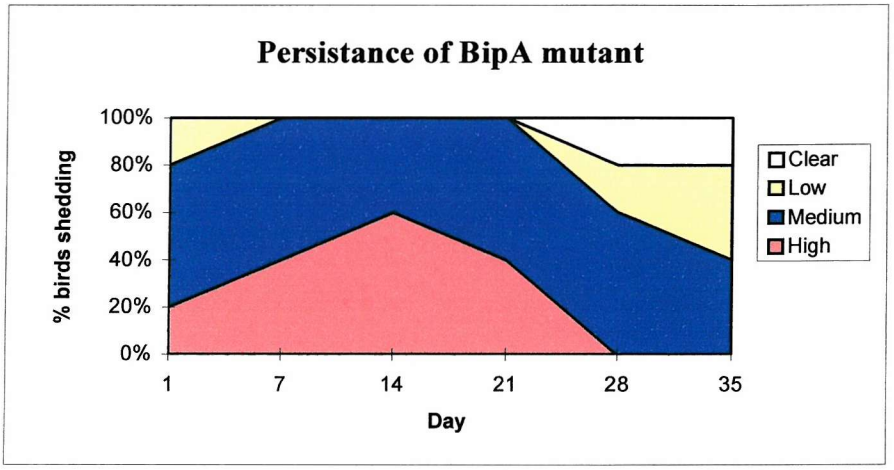


Figure 6-3 The persistence of wild type and BipA mutant

Cloacal swabs were taken every week post inoculation from chicks infected with either wild type strain or mutant strain. When streaking out the swab 200 or more colonies on a plate was considered to be heavy shedding by the chick, between 1-200 colonies was considered medium whereas colonies detected only after selenite enrichment was considered low.

SL1344

Mice	Dose	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Deaths
A1	1.0E+04								X				X		2\5
A2	1.0E+05														0\5
A3	1.0E+06					X	X	X				XX			5\5
A4	1.0E+07					XXX		X	X						5\5
A5	1.0E+08				XX	X	XX								5\5
A6	1.0E+09			X		XXX	X								5\5

SL1344 *bipA::cat*

Mice	Dose	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Deaths
B1	1.0E+04									X					1\5
B2	1.0E+05							X				X		XXX	5\5
B3	1.0E+06					XX	X	X	X						5\5
B4	1.0E+07					XXXX	X								5\5
B5	1.0E+08				X	XX		XX							5\5
B6	1.0E+09			X	X	X	X	X							5\5

Figure 6-4 Oral inoculation of BALB/c mice with SL1344 and SL1344 *bipA::cat* strains

60 BALB/c mice were divided into two groups and inoculated orally with either the wild type strain or mutant strain. The oral dose ranged from 10^4 cfu to 10^9 cfu. Survival over a period of sixteen days was recorded.

SL1344

Mice	Dose	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Deaths
A1	1.0E+01					XX	XX		X						5\5
A2	1.0E+02					XX	XX		X						5\5
A3	1.0E+03				XXX	XX									5\5
A4	1.0E+04			XXX	X				X						5\5
A5	1.0E+05		X	XXXX											5\5
A6	1.0E+06	X	XXX	X											5\5

SL1344 *bipA::cat*

Mice	Dose	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Deaths
B1	1.0E+01					XXXX								X	5\5
B2	1.0E+02					XXXX					X				5\5
B3	1.0E+03			XX	XXX										5\5
B4	1.0E+04			XXX				XX							5\5
B5	1.0E+05	X	XXX	X											5\5
B6	1.0E+06	XX	X	XX											5\5

Figure 6-5 IP inoculation of BALB/c mice with SL1344 and SL1344 *bipA::cat* strains

60 BALB/c mice were divided into two groups and inoculated by the IP route with either the wild type strain or mutant strain. The IP dose ranged from 10^1 cfu to 10^6 cfu. Survival over a period of sixteen days was recorded.

Note: The crosses indicate how many mice have died on that day. The highlighted grey cells indicate that all of the mice from that group have died and the last column gives the total number of mice that have died compared to the number of mice that were originally in each group.

6.3 Discussion

6.3.1 Day-old chick model

The response of SPF chickens to *S. enteritidis* infection is dependent on the age of the bird that is being dosed (Humphrey *et al.,* 1991). Invasion of the liver and spleen is seen with chicks dosed orally within 24 hrs of hatching, whereas by 7 days invasion is reduced significantly and by 20 days the chickens appear resistant to infection. One-day old chicks were therefore used to investigate the colonisation and invasion potential of the *bipA* mutant.

Experiments using the day-old chick model suggest that BipA is involved in the initial colonisation and invasion of the reticuloendothelial system. Experiment 1 showed that in the chicks dosed with the *bipA* mutant a reduced number of bacteria, compared to the wild type, were recovered from the liver at 6 hrs. In experiment 2, at 6 hrs post inoculation no *Salmonellae* were recovered from any of the chicks dosed. However, at 24 hrs a decrease was seen in the number of *bipA::kan* cells recovered from the livers compared to the wild type cells. Both experiments suggested that when you knock out BipA a slight reduction in the initial colonisation and invasion of host liver cells will be seen. It was only in experiment 2 that, at 24 hrs after infection, a reduction in the initial colonisation of the *bipA::kan* cells in the spleen was seen. This result may be artefactual, however it is consistent with the trend that cells which do not express BipA are slightly less invasive. This trend was also seen in the colonisation/invasion of mutant cells within the caeca as the number of mutant cells recovered from the caeca at 6 hrs was reduced in both experiments.

The initial reduced number of *bipA::kan* cells recovered from the liver, spleen and caeca may be due to a delay in colonisation/invasion of the *bipA::kan* cells or to a reduction in bacterial viability. However, further studies would be required to address this issue directly. Interestingly, these findings correlate with the *in vitro* studies as the differences seen in the invasion of the

wild type and mutant with macrophages (section 5.2.2) may be one of the contributing factors to the initial delay of invasion of the *bipA::kan* cells in the liver and spleen.

Several experiments have shown that when chicks are exposed to *S. enteritidis* within 24 hours of hatching they exhibit long-tem intermittent shedding at least until 28 weeks after contact (Nakamura *et al.,* 1993; Gast and Holt, 1998; Holt *et al.,* 1999). Chicks dosed with *S. enteritidis* wild type showed high levels of intermittent shedding, whereas chicks dosed with *S. enteritidis* *bipA*$^-$ initially showed slightly lower levels of shedding. After several weeks of contact however the mutant strain, compared to the wild type, became slightly more persistent and the chicks were less able to clear the infection. The roles of flagella and fimbriae in caecal colonisation are undetermined. However, studies have shown that SEF14 fimbriae may only play a role in colonisation in the first few weeks after infection (Thorns *et al.,* 1996). Interestingly, previous studies in this thesis (section 4.2.1) have shown that SEF14 is down regulated in the *bipA* null mutant. This phenotype could account for the initial decrease seen in caecal colonisation within the first few weeks after infection. SEF21 have also been implicated in the persistence of *S. enteritidis* (Thiagarajan *et al.,* 1996). Studies in this thesis have shown that SEF21 expression is increased in the *bipA* mutant (section 4.3.3). This phenotype could account for the increased persistence seen with the mutant several weeks after infection.

6.3.2 Mouse Animal Model

The BALB/c mouse, which contracts a typhoid-like disease when orally infected with *S. typhimurium*, is the best characterised animal model for studying the importance of *Salmonella* interactions with intestinal cells. The *in vivo* experiments carried out with the *S. typhimurium* *bipA* mutant showed that BipA has very little effect on *Salmonella* virulence in the mouse model. However, this does not conclusively rule out a role for BipA in *Salmonella* pathogenicity as controversial studies carried out by Vazquez-Torres *et al.,* (1999) suggest that *Salmonella* has

two entry routes through the gut epithelium. If this is the case then *bipA* may only be knocking out one of the routes of invasion, which may explain why the *bipA* mutant is still able to cause systemic disease. This hypothesis has also been used for experiments carried out with SPI-1-deficient *S. typhimurium* strains as these mutants are able to cause lethal infection in mice without invading M cells or localising in Peyer's patches (Galan and Curtiss, 1989; Jones and Falkow, 1994a; Baumler *et al.*, 1997b). Invasion genes encoded in SPI-1 are required for the invasion of M cells (Galan and Curtiss, 1989; Clark *et al.*, 1998), therefore this suggests that the bacteria use an alternative strategy to disseminate from the gastrointestinal tract.

It would be interesting to investigate the effect of BipA in the bovine model of gastroenteritis as it is often overlooked that the mouse model is a systemic disease model whereas the bovine is a diarrhoea model. BipA may only play a role in one of these diseases. For example, SirA (regulates genes within SPI1) is not a virulence factor in *S. typhimurium*-infected mice, yet SirA mutants are dramatically attenuated in the bovine model of gastroenteritis (Ahmer *et al.*, 1999).

CHAPTER 7

7 Analysis of the flanking regions of *bipA*

7.1 Introduction

Very little is known about the downstream region of *bipA* in different eubacteria. In this chapter bioinformatic and *in vitro* analysis will be carried out to firstly examine the differences in the flanking regions of *bipA* in several different species and secondly to investigate this region more thoroughly in several different *Salmonella* serotypes.

7.2 The genus *Salmonella enterica*

The current basis for *Salmonella* classification and identification has been a serological scheme initiated by White and elaborated by Kauffmann (1952). The Kauffmann-White scheme is based on the variations in O (somatic) and H (flagellar) antigens which provide each *Salmonella* serotype (serovars) with its own unique antigenic combination. As described in Chapter One, there are currently 2,463 serotypes of *Salmonella* (Popoff *et al.,* 2000). *Salmonella* serovars identified by this scheme have traditionally been given their own species name and are therefore designated by italicised Latin binomials (e.g. *S. typhimurium* and *S. enteritidis*). However, the actual phylogenetic relationships within a the *Salmonella* genus have been reclassified by multilocus enzyme electrophoresis (MLEE) and nucleotide sequence analysis (Boyd *et al.*, 1996). The genus is now distinguished by two species, *S. bongori* and *S. enterica* (Reeves *et al.*, 1989), where *S. enterica* is divided into six distinct subgroups (Popoff and Le minor, 1992).

7.2.1 Evolution of *Salmonella*

The genetic maps of *Salmonella* and *E. coli* K12 are highly conserved and both genomes are similar with respect to base composition, chromosome size and the order, orientation and

spacing of genes (Bachmann, 1990; Sanderson *et al.,* 1995). However, several large differences in the lengths of gene intervals between the two genomes have been observed (Krawiec and Riley, 1990). These differences are known as loops and their sizes can range from 20 to 70 kb (Krawiec and Riley, 1990). It is thought that these loops may contribute to the major physiological and biochemical differences between *E. coli* and *Salmonella* and are therefore part of the evolutionary process of these two genera from a common ancestor some 100-175 million years ago (Ochman and Wilson, 1987; Riley and Krawiec, 1987; Krawiec and Riley, 1990; Groisman *et al.,* 1992c).

7.2.2 Pathogenicity Islands

Salmonella have been implicated in an array of diseases and sequences found to be specific to this species often encode virulence attributes not seen in closely related benign microorganisms such as *E. coli* K-12. Most virulence genes are encoded on pathogenicity islands specific to *Salmonella* and are highly conserved between different serotypes. SPIs are defined as gene clusters that have a lower G+C content (between 37% and 47%) than the normal *Salmonella* G+C content (52-54%). SPIs are also often inserted into tRNA genes. These pathogenicity islands are therefore thought to have been acquired by horizontal gene transfer from phage or plasmid of unknown origin (Marcus *et al.,* 2000).

Genes within pathogenicity islands are often responsible for establishing specific interactions with the host and are required for bacterial virulence in a given animal model. At least five pathogenicity islands have been identified. SPI1 encodes a type III secretion system involved with protein secretion and invasion (Mills *et al.,* 1995; Galan, 1996). SP12 encodes a second type III secretion system required for later stages of systemic disease including macrophage survival (Ochman *et al.,* 1996; Shea *et al.,* 1996). SPI3 is also essential for macrophage survival and is involved in the ability of the bacteria to grow in Mg^{2+} limiting conditions (Blanc-Potard

and Groisman, 1997). SPI4 is involved in toxin secretion and macrophage survival (Wong *et al.*, 1998) whereas SPI5 is more important for gastroenteritis rather than systemic *Salmonellosis* (Wood *et al.*, 1998).

Although complete genomic sequence for any *Salmonella* has yet to be published, perusal of the available information suggests that additional pathogenicity islands and islets remain to be discovered. With this in mind, it was therefore decided to undertake an analysis of the genetic regions flanking the *bipA* gene in *Salmonella*.

7.3 RESULTS

7.3.1 Bioinformatic analysis: Analysis of genes that flank *bipA*

Pathogenic bacteria often need to regulate the expression of multiple genes in response to a particular signal. As a result, the genes can be organised in an operon where a single promoter transcribes all the genes as part of a single transcript. Often virulence genes are clustered in the same region to form an operon. For example, flagella and fimbriae genes are organised within discrete but different operons. Previous studies have shown that *bipA* is involved in regulating several virulence properties in *Salmonella* and *E. coli*. Very little research has been carried out to analyse the genes that flank *bipA*. Consequently, it was of interest to investigate, in different eubacteria, if *bipA* is part of an operon. Using the full length *S. enteritidis bipA* sequence (sequence obtained in Chapter 3) BLAST searches were carried out in the NCBI database and figures 7-1 and 7-2 show the organisation of *bipA* in several different eubacteria. The organisation of the genes in each species shows that genes that flanking *bipA* are not conserved. Hence, *bipA* does not seem to be part of an operon nor is it consistently associated with any gene. It is interesting, however, that a unique 5.8kb region is found downstream of *Salmonella bipA* and that the three genes found in *E. coli*, *yihL*, *yihM* and *yihN*, are replaced by this unique region (Figure 7-1).

7.3.2 Analysis of 5.8kb unique region found downstream of *Salmonella* bipA

A recent hypothesis on microbial pathogenicity suggests that horizontal gene transfer drives the evolution of virulence in *Salmonella*. This diversification has given rise to highly flexible pathogens that are able to colonize new niches and extend their host range (Baumler, 1997a). Baumler (1997a) suggests that tracing the record of these horizontal gene transfers could provide clues to the virulence factors that contribute to the formation of new pathovars. In *Salmonella* horizontal gene transfer is often associated with a change in the normal 52% G+C content. Using

Omiga 1.1.3 software the G+C content of this 5.8kb unique region was found to be 48.11% which suggested that this region of DNA has been acquired from horizontal gene transfer. As a result, the region was analysed in more detail to determine the putative open reading frames (ORFs) and to determine the putative proteins that may be expressed. Figure 7-3 shows that in *S. typhimurium* the 5.8kb region contains five possible ORFs. However, translation of these ORFs and subsequent BLAST analysis gave only one match to a protein. ORF4 shared 40% sequence identity to the oxygen-independent coproporphyrinogen III oxidase (coprogen oxidase) in *Synechocystis* spp.

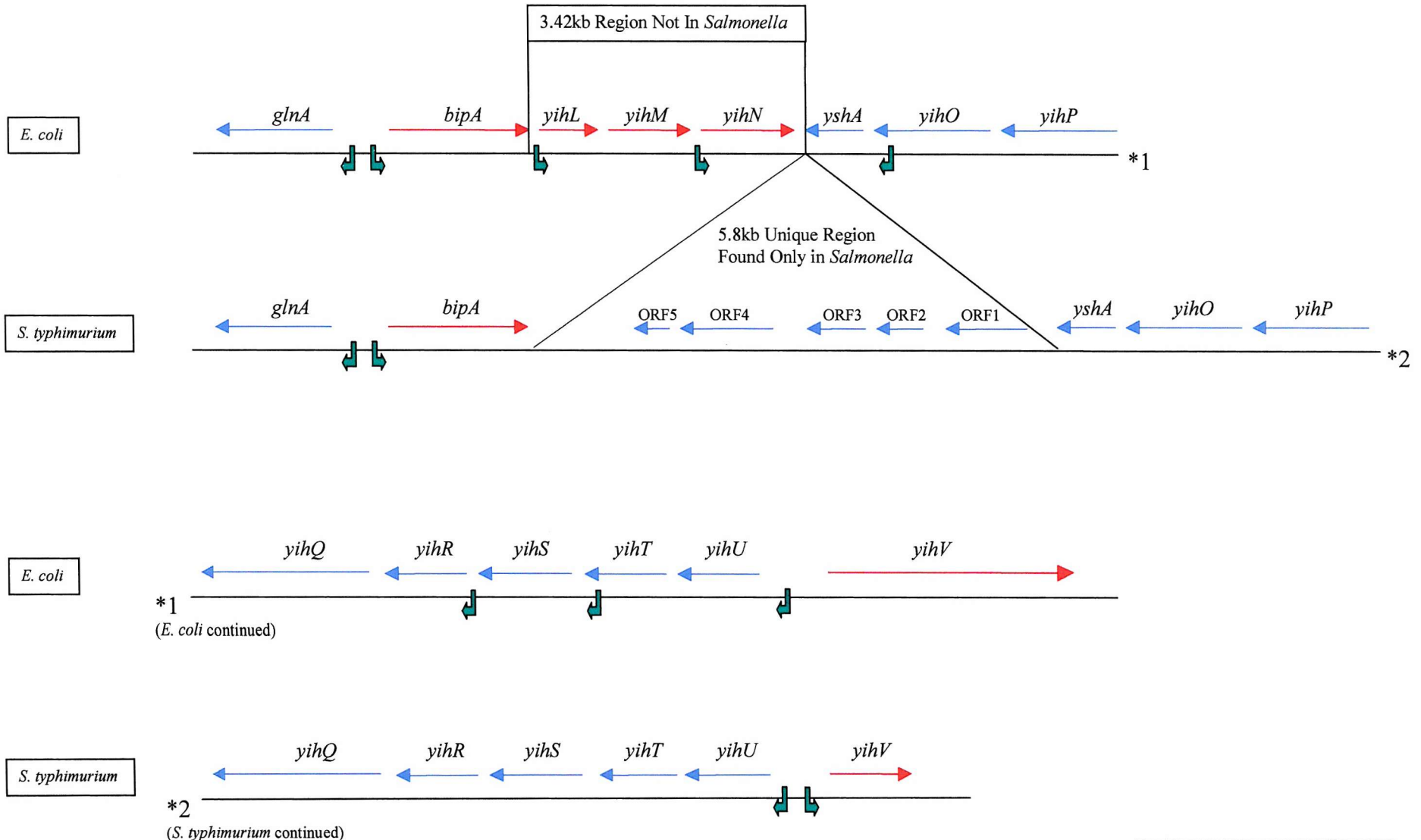


Figure 7-1 Organisation of genes flanking *E. coli* and *S. typhimurium bipA*

Using the full-length *S. enteritidis bipA* sequence BLAST searches were carried out in the NCBI database

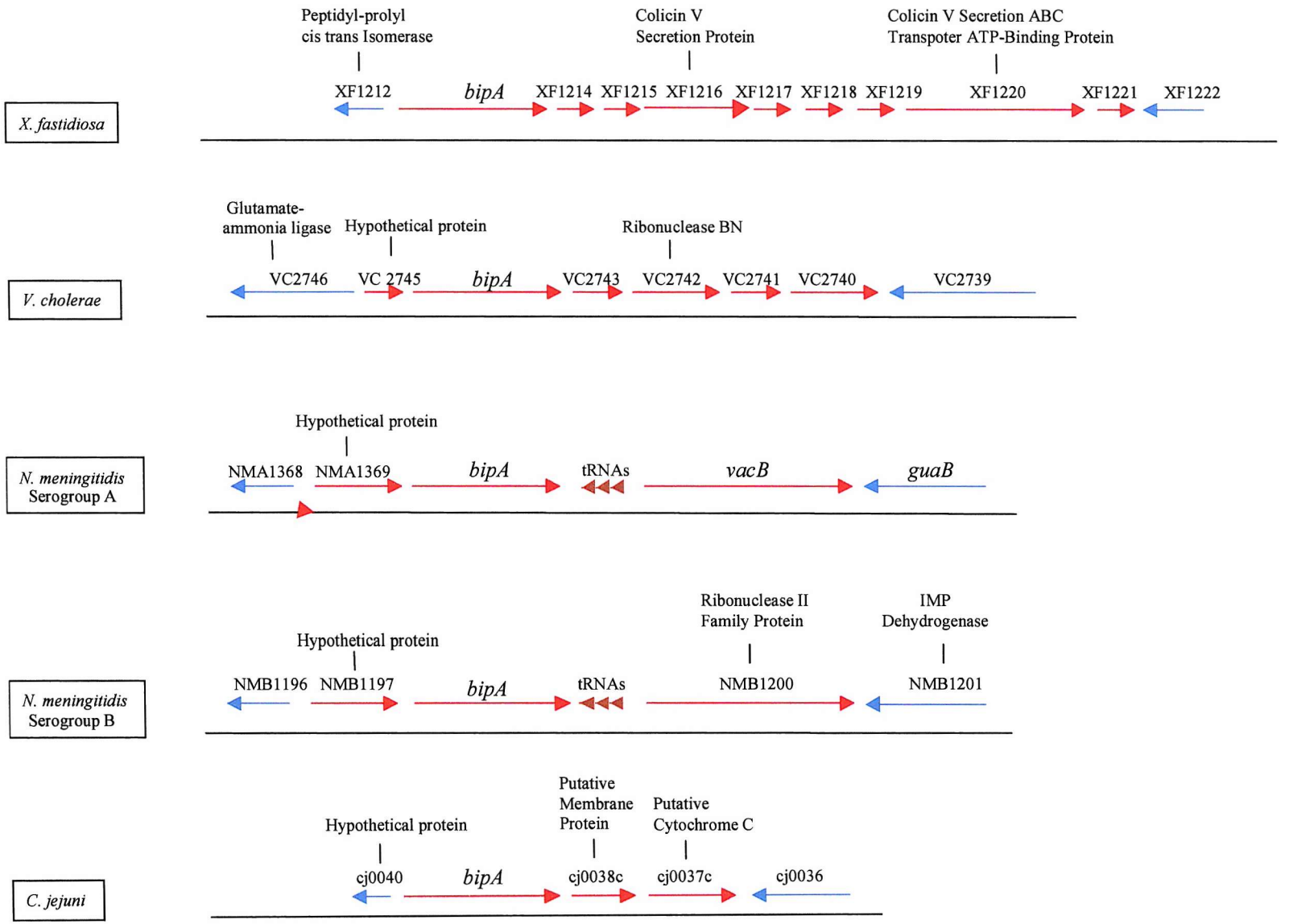


Figure 7-2 Organisation of genes flanking *bipA* in several different eubacteria

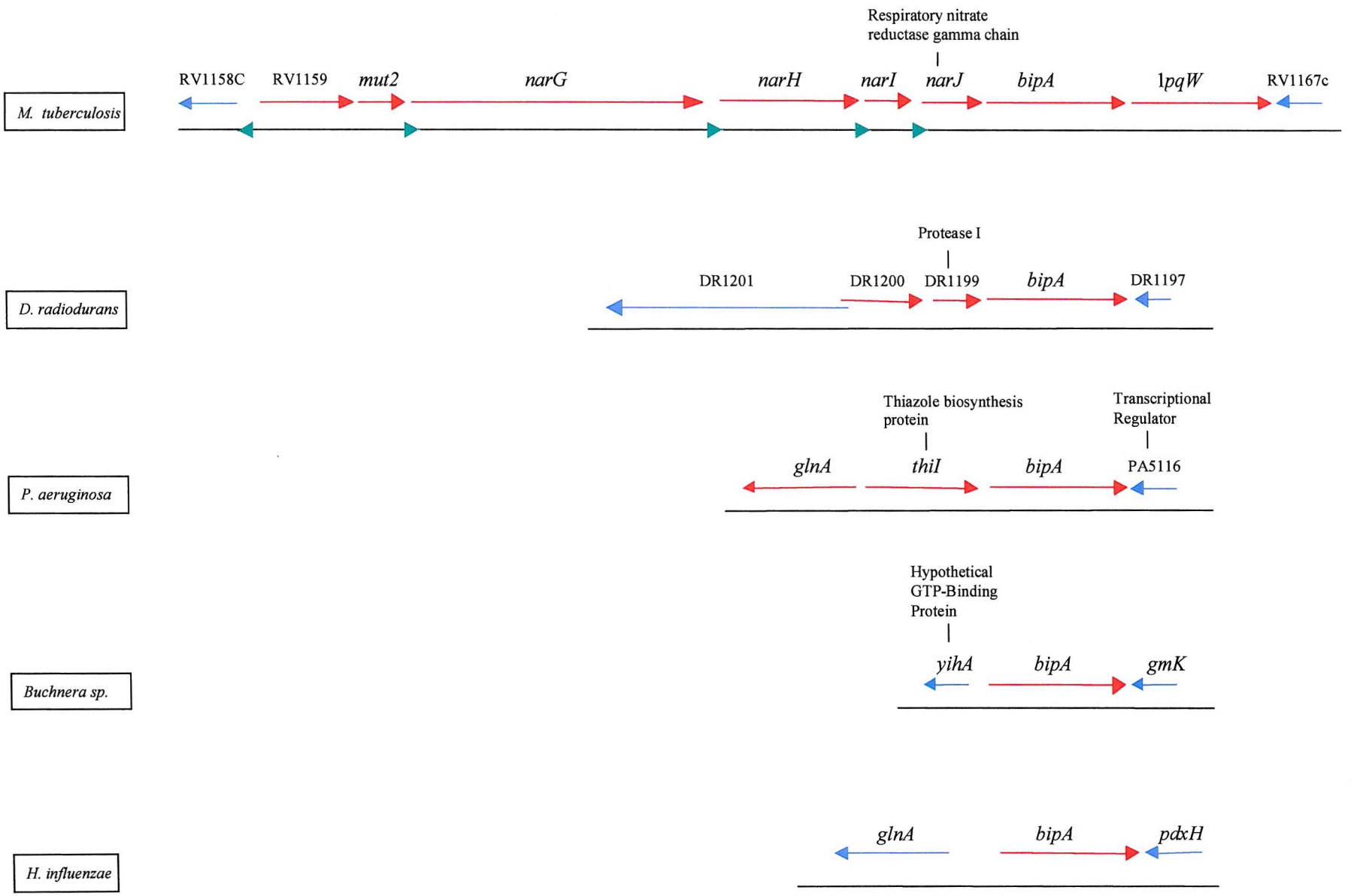


Figure 7-2 (continued) **Organisation of genes flanking *bipA* in several different eubacteria**

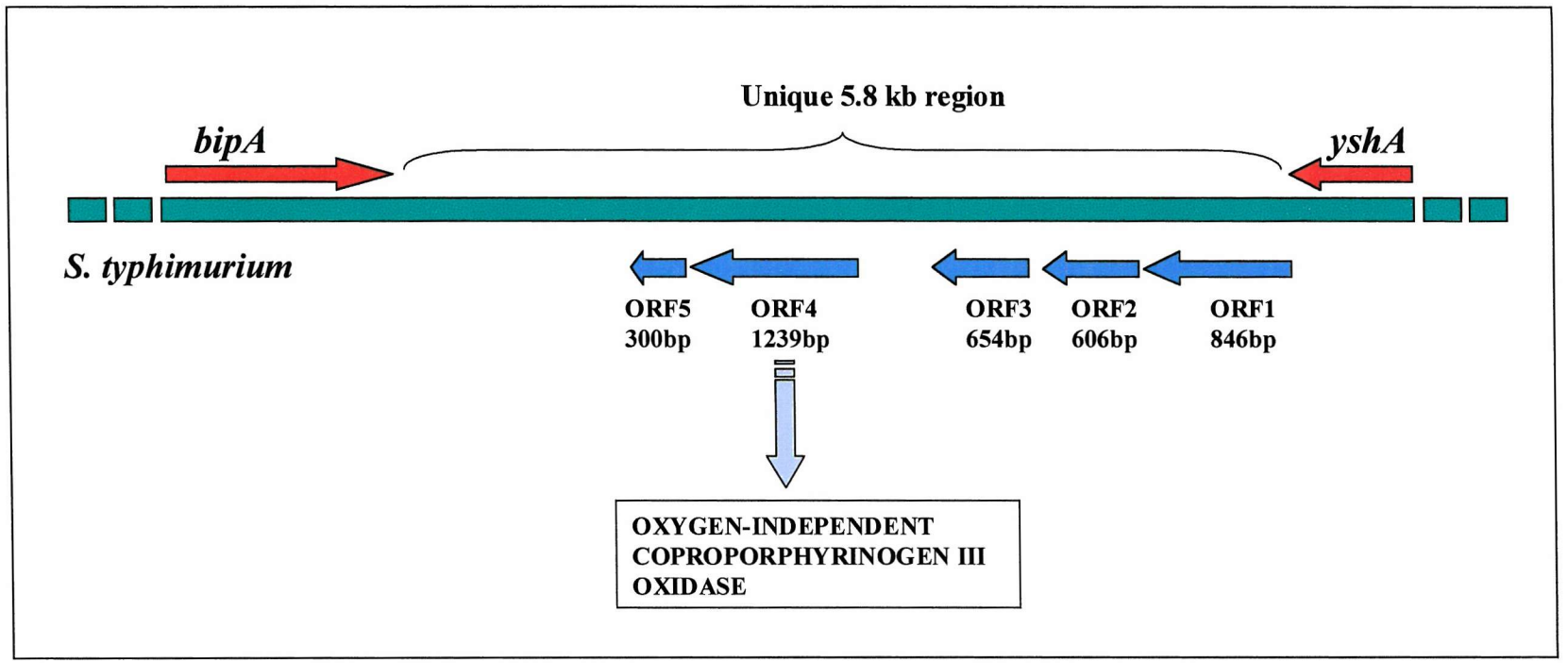


Figure 7-3 Unique ORFs found only in *Salmonella*

The *S. typhimurium* sequence was obtained from Washington University database. The sequence was analysed for putative ORFs using the Omiga 1.1.3 software package. Only ORFs coding for proteins ≥ 100 amino acids are shown

7.4 *In vitro* studies

7.4.1 Cloning and sequencing of flanking regions of *S. enteritidis bipA*

An unusual technique was used to clone the flanking regions of *S. enteritidis bipA*. Plasmid pAW4, used in the previous conjugation experiment (section 3.2.3), was mobilised into S1400/94 to enable homologous recombination to occur between the chromosomal *bipA* gene and the insertionally inactivated form present on the plasmid. Single crossover recombination events were selected for by obtaining kanamycin-resistant, chloramphenicol-resistant colonies. The kanamycin cassette is within *bipA* whereas the chloramphenicol cassette is within the vector. The chromosomal DNA from one such strain was digested with *Mlu1*, recircularised with T4 DNA ligase and transformed into S17(λPir). Since *Mlu1* does not have a site within the vector, this procedure results in the cloning of flanking DNA. Therefore the resulting plasmid, pAW5, contained the flanking regions of *bipA*, which could then be sequenced (Figure 7-4).

Figure 7-5 shows the comparison of the putative ORFs within the unique regions of *S. typhimrium* and *S. typhi* with the sequenced portion of *S. enteritidis*. The *S. enteritidis* and *S. typhi* sequences within this region are very similar and only differ slightly in ORF3 and ORF4. The *S. typhi* ORF3 is only 60bp larger than the *S. enteritidis* ORF3 whereas the *S. typhi* ORF4 is 399bp smaller than the *S. enteritidis* ORF4. In contrast, the *S. typhi* ORF4 is very different in size relative to the *S. typhimurium* ORF4, as there is a difference of 687bp. However, both ORFs share the same stop codon. The *S. typhimurium* 3' sequence of the 5.8kb region is slightly different to that of *S. enteritidis* and *S. typhi* as *S. typhimurium* has no ORF6 and ORF5 is 576bp smaller. Overall however it is encouraging that all three different serovars contain the unique region and that the ORFs show similar trends in all three serovars.

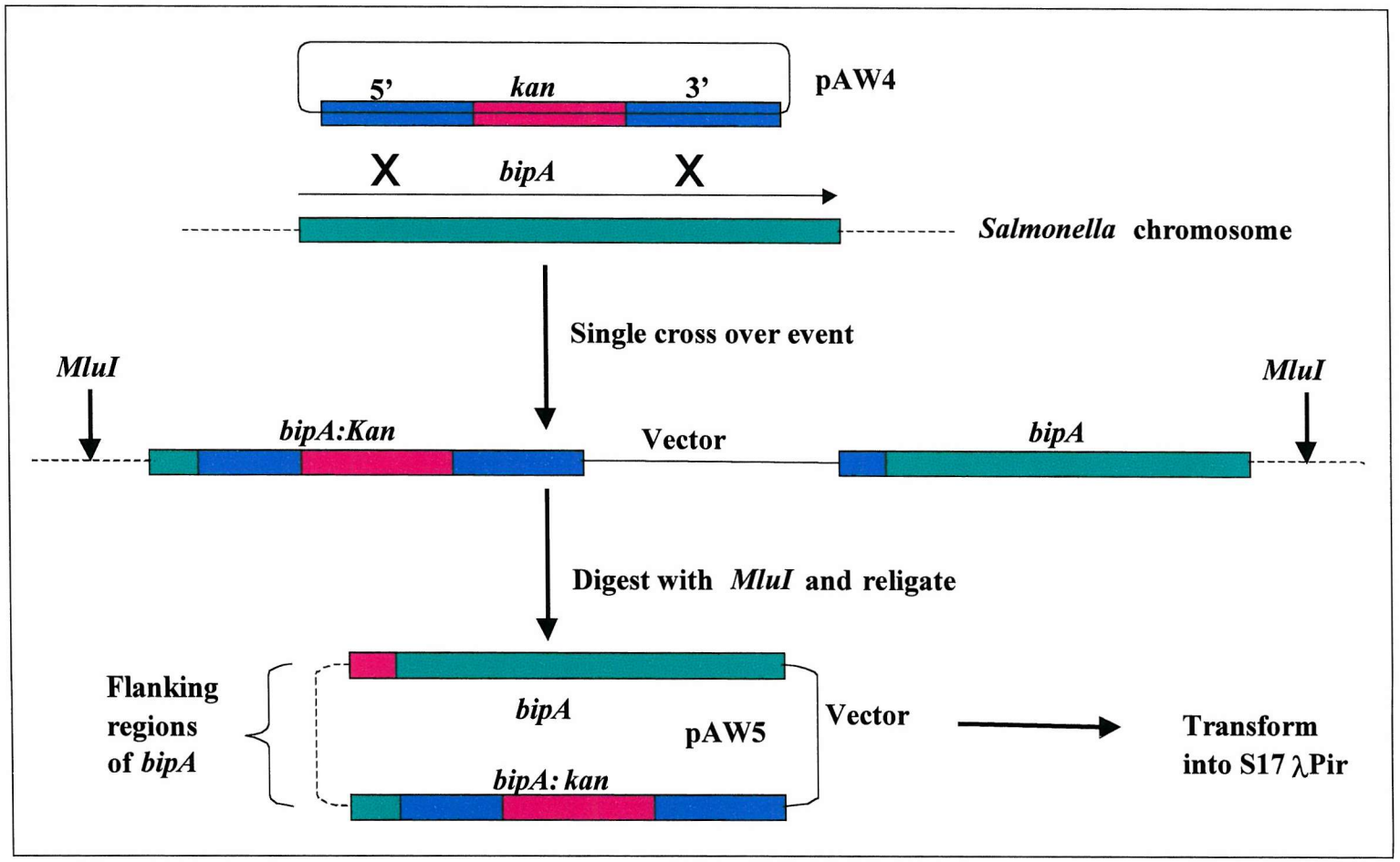


Figure 7-4 Scheme for cloning the flanking regions of *bipA* in *S. enteritidis*

pAW4 was mobilised into S1400 to allow homologous recombination. A single crossover event was selected for and the chromosomal DNA from such strains was digested with *Mlu1*. The digested DNA was religated and transformed into S17(λPir)

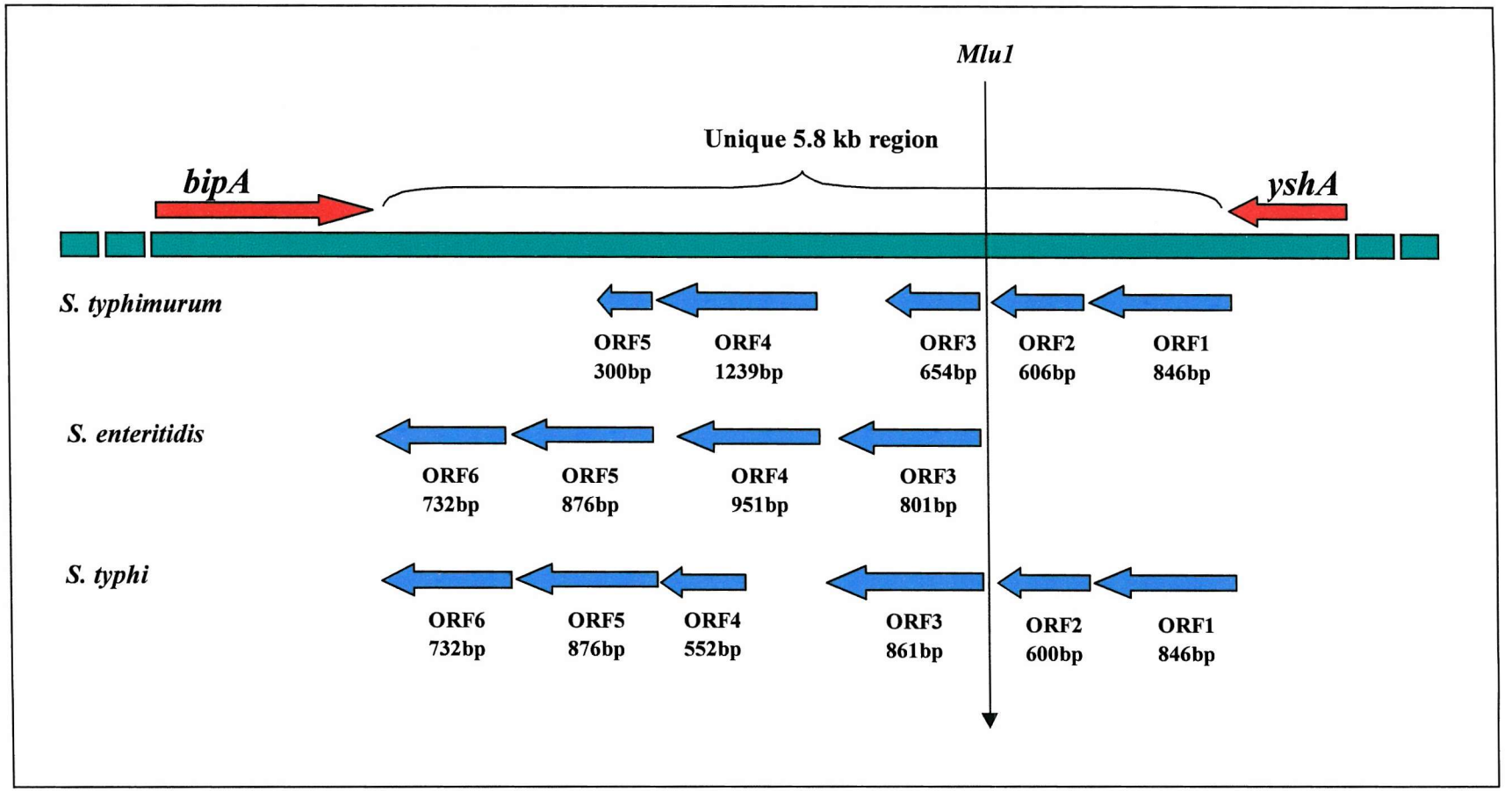


Figure 7-5 Comparing unique ORFs of *S. typhimurium*, *S. enteritidis* and *S. typhi*

S. typhimurium and *S. typhi* sequences were obtained from Washington University database and the Sanger Center database respectively. The *S. enteritidis* sequence was obtained from sequencing pAW5. All sequences were analysed for putative ORFs using the Omiga 1.1.3 software package.

7.5 Southern blotting profiles

7.5.1 Distribution of *bipA* in other *Salmonella* serovars

Previous chapters have shown that *S. enteritidis bipA* regulates several different processes that are thought to be associated with virulence. For example, *bipA* regulates the expression of flagella and certain fimbriae (Chapter 4) and is also associated with the oxidative stress response (Chapter 5). However, very little work has been carried out on the distribution of *bipA* in other *Salmonella* serotypes. If *bipA* is only present in specific serotypes it could be hypothesised that that *bipA* may be associated with host specificity. Accordingly, the distribution of *bipA* in a range of *Salmonella* serotypes was investigated. To ensure that the results obtained were not artefacts due to spontaneous mutation to create or destroy specific sites, five *S. typhimurium* and *S. enteritidis* serotypes of the same phage type were used. In the case of the other serotypes only two of each strain was used.

Southern hybridisation experiments were carried out to confirm the presence of *bipA* in different *Salmonella* serotypes. Total genomic DNA was extracted from each strain and the DNA digested to completion using *MluI*. The DNA was resolved by agarose gel electrophoresis, transferred to nylon membranes and hybridised with a probe consisting of the *bipA* gene. Analysis of the resulting autoradiograms showed that all of the *Salmonella* strains contained *bipA* (Figure 7-6). An approximately 9.50 kb *MluI* fragment hybridised with the *bipA* probe in *S. enteritidis*, *S. typhimurium*, *S. dublin*, *S. derby*, *S. pullorum*, *S. binza S. agona*, and *S. kedougou*. In contrast, an approximately 12.4 kb fragment hybridised with the *bipA* probe in *S. montevideo*, which was the same as the positive control *E. coli* K12. In the case of *S. heidelberg* and *S. hadar* a 6.5 kb fragment hybridised with the *bipA* probe. In *S. arizona* two fragments of approximately 6.5 kb and 4.4 kb hybridised with the *bipA* probe. These results suggest that BipA is present in all of the *Salmonella* serovars examined.

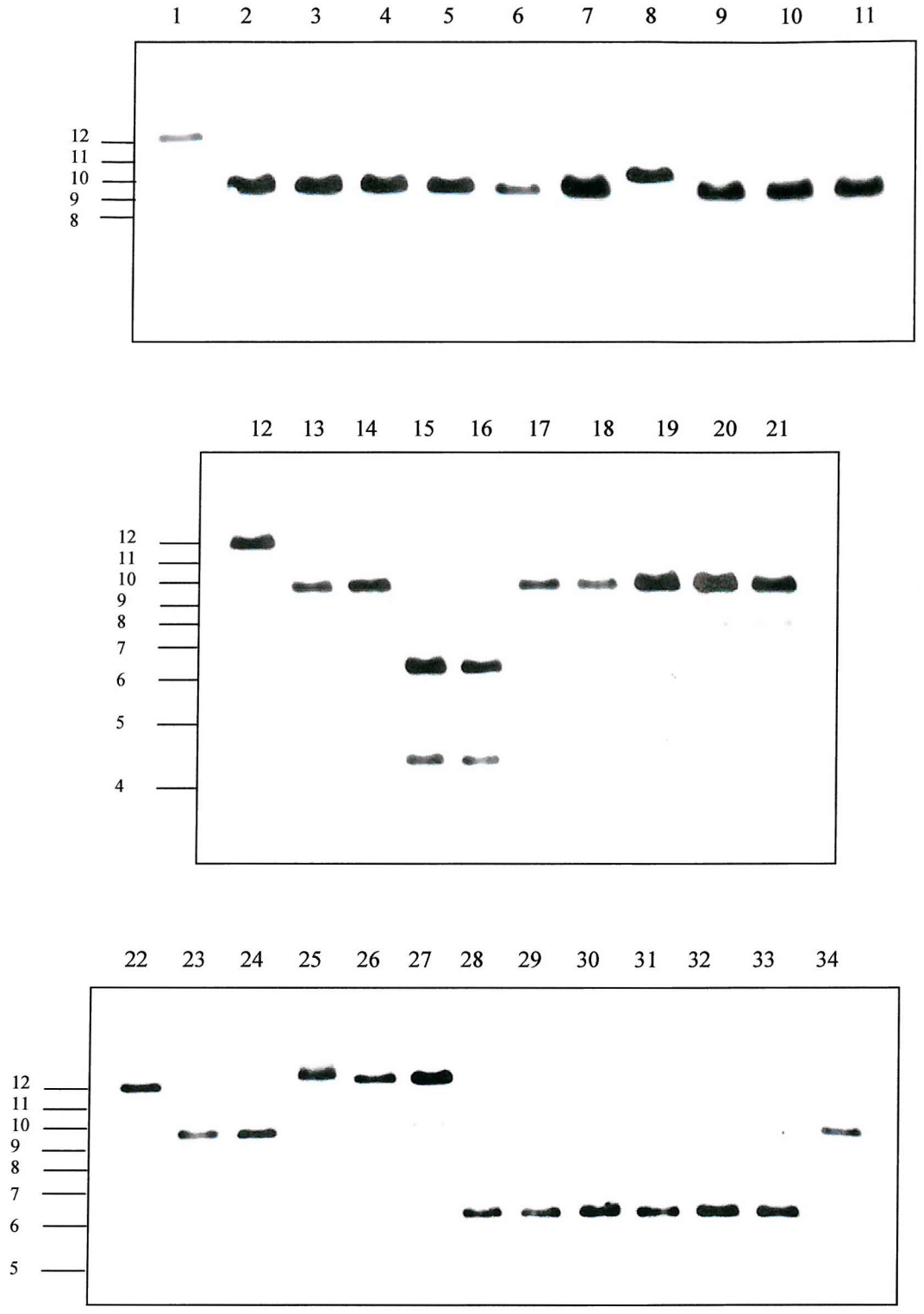


Figure 7-6 Distribution of BipA in *Salmonella* serovars

Comparison of *Mlu1*-digested chromosomal DNA from several *Salmonella* serotypes by Southern hybridisation using *bipA* as a probe. Lane 1: Positive control *E. coli* K12, lanes 2-6: *S. enteritidis*, lane 7: *S. typhimurium*, lane 8: *S. typhimurium* (strain 3148), lanes 9-11: *S. typhimurium*, lane 12: positive control, *E. coli* K12. Lane 13: *S. agona*, lane 14: *S. pullorum*, lanes 15-16: *S. arizona*, lanes 17-18: *S. dublin*, lanes 19-20: *S. kedougou*, lane 21: *S. derby*, lane 22: positive control, *E. coli* K12. Lanes 23-24: *S. binza*, lanes 25-27: *S. montevideo*, lanes 28-30: *S. heidelberg*, lanes 31-33: *S. hadar*, lane 34: *S. enteritidis*. Molecular markers are given in kb.

7.6 Further analysis of unique ORFs

Many virulence genes are located on pathogenicity islands that have been acquired by horizontal gene transfer. Previous bioinformatic studies looking at the regions that flanked *bipA* showed a unique 5.8kb region downstream of *Salmonella bipA* (section 7.3). The unique region in *Salmonella* was found to have a lower GC content which suggested that it had been obtained from gene transfer. It was thus of interest to investigate if this region was *Salmonella* specific and if so to investigate the DNA-DNA hybridisation profiles of the flanking regions of *bipA*.

7.6.1 Distribution of unique ORFs in several different eubacteria.

Several different eubacteria, including all strains from the *E. coli* reference collection (details given in Materials and Methods in Table 2-2 and Table 2-3), were screened for the unique ORFs by colony dot blots. The different strains were grown overnight on a membrane overlaid on a LB plate. The cells were then lysed *in situ* and the DNA probed. As the 5.8kb region was too large to use as a single probe two different probes had to be used to confirm the presence of the whole of the region. One probe consisted of the 5' region of the ORFs whereas the other consisted of the 3' region. No hybridisation was seen with either the 3' or 5' probe in any of the different strains suggesting that the region is unique to *Salmonella* (results not shown as all negative).

7.6.2 Southern Hybridisation profiles of region downstream of *bipA*

Southern hybridisation experiments were carried out to confirm the presence of the unique region found downstream of *bipA* in different *Salmonella* serotypes. Total genomic DNA was extracted from each strain and the DNA digested to completion using an enzyme that cut within the region being probed. The DNA was resolved by agarose gel electrophoresis and transferred to nylon membranes. The presence of the ORFs was confirmed by probing with a radiolabelled 3' or 5' fragment of the unique region. The *Mlu1*-, *EcoRI*- (Figure -7-7, Figure 7-8 and Figure 7-9), *Nde1*- and *PvuI*-digested blots (Figure 7-11, Figure 7-12 and Figure

7-13) show that, within the different serovars, there are differences in the genetic makeup downstream of *bipA* (summarised in Figure 7-10 and Figure 7-14). Approximately 9.5kb and 8.9kb *MluI* fragments hybridised with the 5' probe in *S. typhimurium*, *S. dublin*, *S. derby* compared to 9.5kb and 6.5kb fragments in *S. enteritidis* and *S. pullorum* and 9.5kb and 3.4kb fragments in *S. binza S. agona* and *S. kedougou*. In contrast *S. heidelberg*, *S. hadar* and *S. montevideo* only gave one *MluI* fragment that hybridised to the probe. A 12.4kb fragment was seen in *S. montevideo,* compared to a 6.5kb fragment in *S. heidelberg* and *S. hadar*. In the *EcoRI*-digested blots fragments of approximately 6.7kb and 4.1kb hybridised with the 5' probe in *S. typhimurium*, *S. dublin*, *S. derby*, *S. enteritidis, S. pullorum*, *S. binza, S. agona* and *S. kedougou* compared to a 5.7kb fragment in *S. heidelberg*, *S. hadar* and *S. montevideo.* Interestingly, no hybridisation was seen with *S. arizona* in either the *MluI*- or *EcoRI*-digested blots.

Hybridisation with the 3' probe also varied with different serovars. Two *NdeI*-digested fragments of 12kb hybridised with the 3'probe in *S. typhimurium*, *S. dublin*, *S. derby* compared to 12kb and 10kb fragments in *S. enteritidis* and *S. pullorum*. In *S. binza* and *S. kedougou* fragments of 12kb and 3.8kb hybridised compared to fragments of 9kb and 3.8kb in *S. agona*. In the *PvuI*-digested blots fragments of approximately 3kb and 1.6kb hybridised with the probe in *S. typhimurium*, *S. dublin*, *S. derby*, *S. enteritidis, S. pullorum*, *S. binza* and *S. kedougou*. Interestingly, no hybridisation was seen with the probe in *S. arizona, S. heidelberg*, *S. hadar* or *S. montevideo* in either the *NdeI*- or *PvuI*-digested blots.

The *MluI*- and *NdeI*-digested blots suggested that in the serovars *S. typhimurium*, *S. dublin*, *S. derby*, *S. enteritidis, S. pullorum*, *S. binza* and *S. kedougou* there is genetic polymorphism downstream of the unique region. However, the *EcoRI*- and *PvuI*-digested and blots suggested that the genetic makeup within the unique region may be similar as the *EcoRI* and *PvuI* sites are the same in all of the serovars. *EcoRI* cuts within *bipA* and just outside the unique region, whereas *PvuI* cuts within *bipA* and at the end of the unique region. However, in the serovars *S. heidelberg*, *S. hadar* and *S. montevideo* there seems to be polymorphism

downstream and within the unique region as these serovars did not hybridise with the 3' region of the ORFs and the *MluI* sites are different.

The Southern blots carried out showed that several of the serovars have identical restriction sites which meant that these serovars could be divided into groups (groups 1-4). Unfortunately, when comparing these groups with the Kauffmann-White scheme there is no relationship between either the group that the serovar belongs or with the somatic antigens that each serovar exhibits (Table 7-1).

Serotype	**Group**	**Somatic antigen**	
S. typhimurium	B	4,5,12	Group 1
S. dublin	D1	9,12	
S. derby	B	1,4,12	
S. enteritidis	D1	9,12	Group 2
S. pullorum	D1	1,9,12	
S. binza	E2	3,15	Group 3
S. kedougou	G2	13,23	
S. heidelberg	B	4,5,12	Group 4
S. hadar	C2	6,8	

Table 7-1: Relationship of Southern profiles with the Kauffmann-White scheme

Interestingly, the *S. typhimurium* strain 3148 showed identical DNA-DNA hybridisation profiles as *S. montevideo* suggesting that this serovar may have been wrongly typed when it was obtained. Further work would however need to be carried out to confirm this.

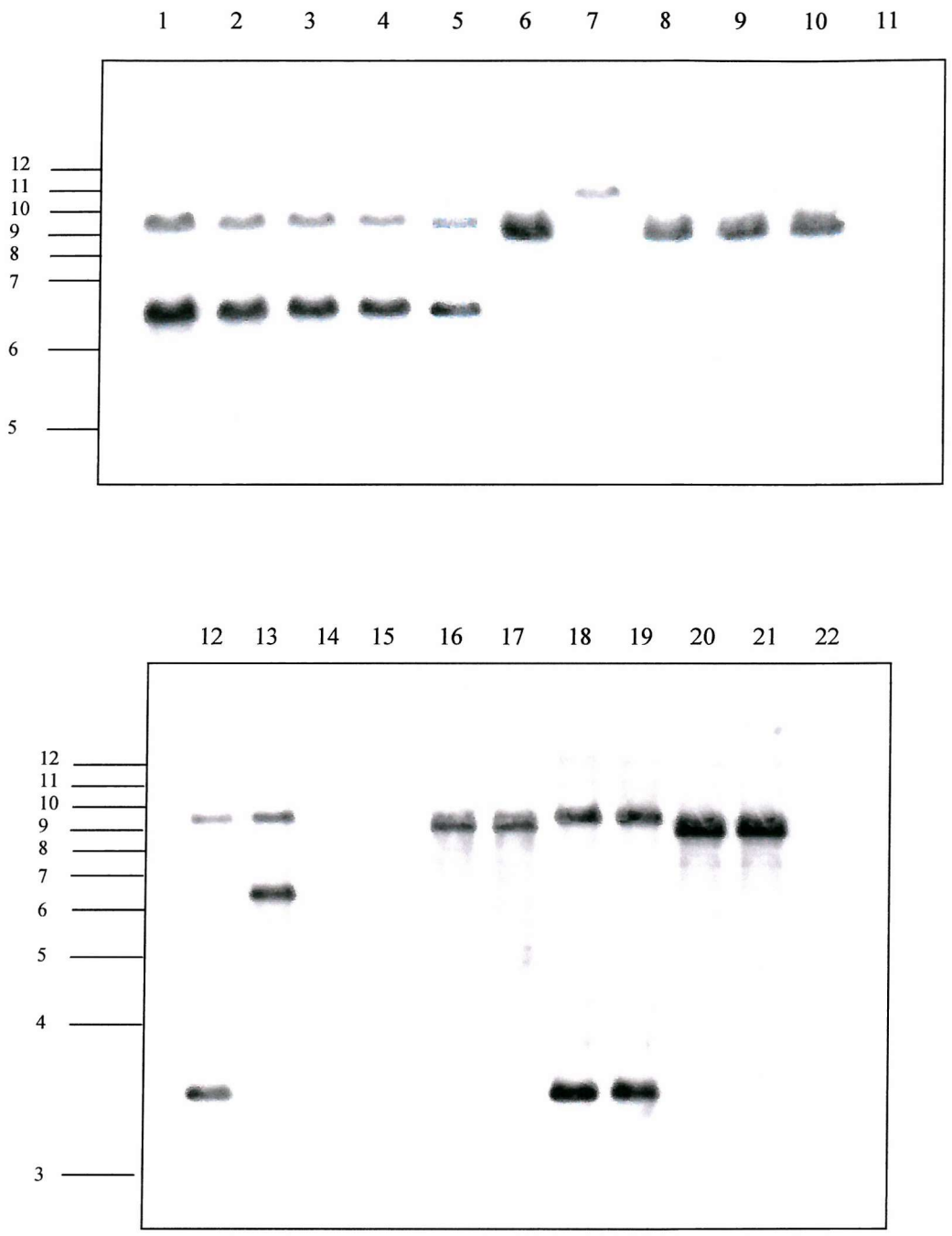


Figure -7-7 *Mlu1* profiles of several different *Salmonella* serovars when probed with 5' region of unique ORFs

Comparison of *Mlu1*-digested chromosomal DNA from several *Salmonella* serotypes by Southern hybridisation using 5' region of ORFs as a probe. Lanes 1-5: *S. enteritidis*, lanes 6: *S. typhimurium*, lane 7: *S. typhimurium* (strain 3148), lanes 8-10: *S. typhimurium*, lane 11: negative control *E. coli* K12, lane 12: *S. agona*, lane 13: *S. pullorum*, lanes 14-15: *S. arizona*, lanes 16-17: *S. dublin*, lanes 18-19: *S. kedougou*, lanes 20-21: *S. derby*. Molecular markers are given in kb.

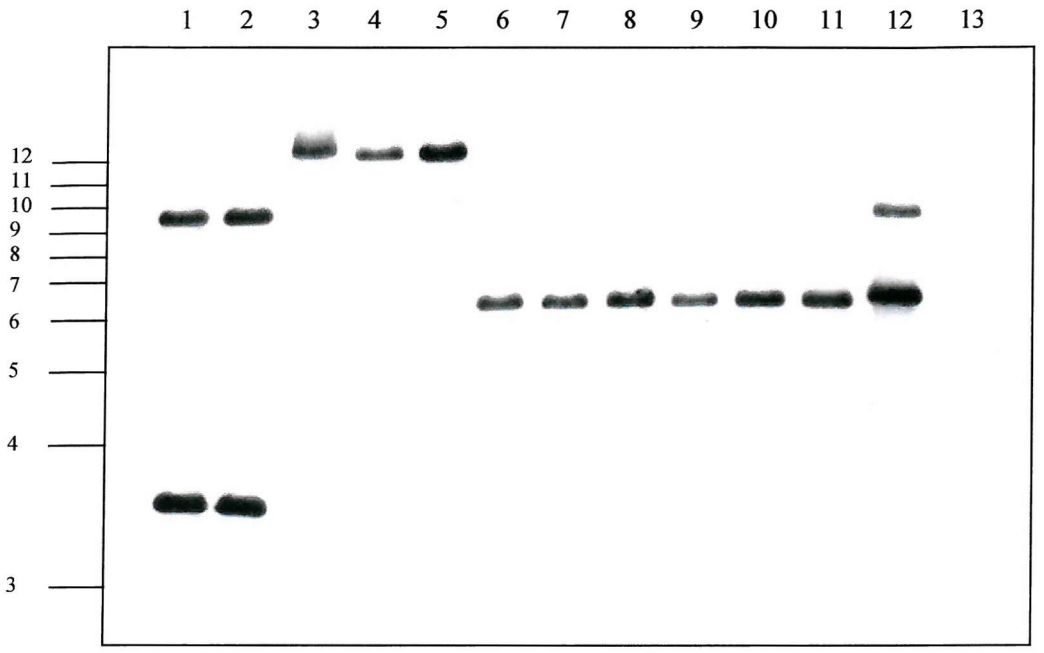


Figure 7-8 ***Mlu1*** **profiles of several different** ***Salmonella*** **serovars when probed with 5' region of unique ORFs**

Comparison of *Mlu1*-digested chromosomal DNA from several *Salmonella* serotypes by Southern hybridisation using 5' region of ORFs as a probe. Lane 1-2: *S. binza*, lanes 3-5: *S. montevideo*, lanes 6-8: *S. heidelberg*, lanes 9-11: *S. hadar*, lane 12: *S. enteritidis*, lane 13: negative control *E. coli* K12. Molecular markers are given in kb.

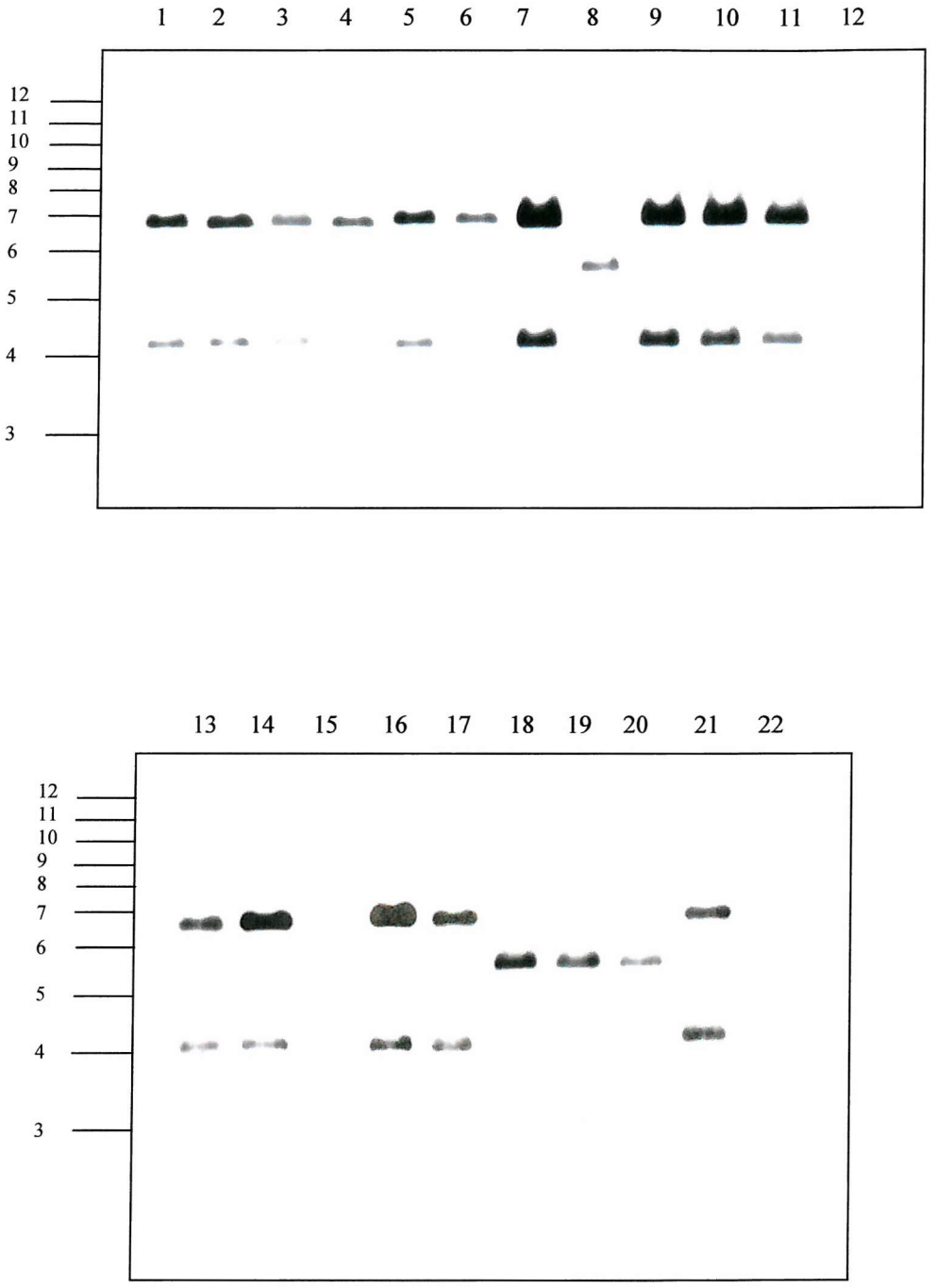
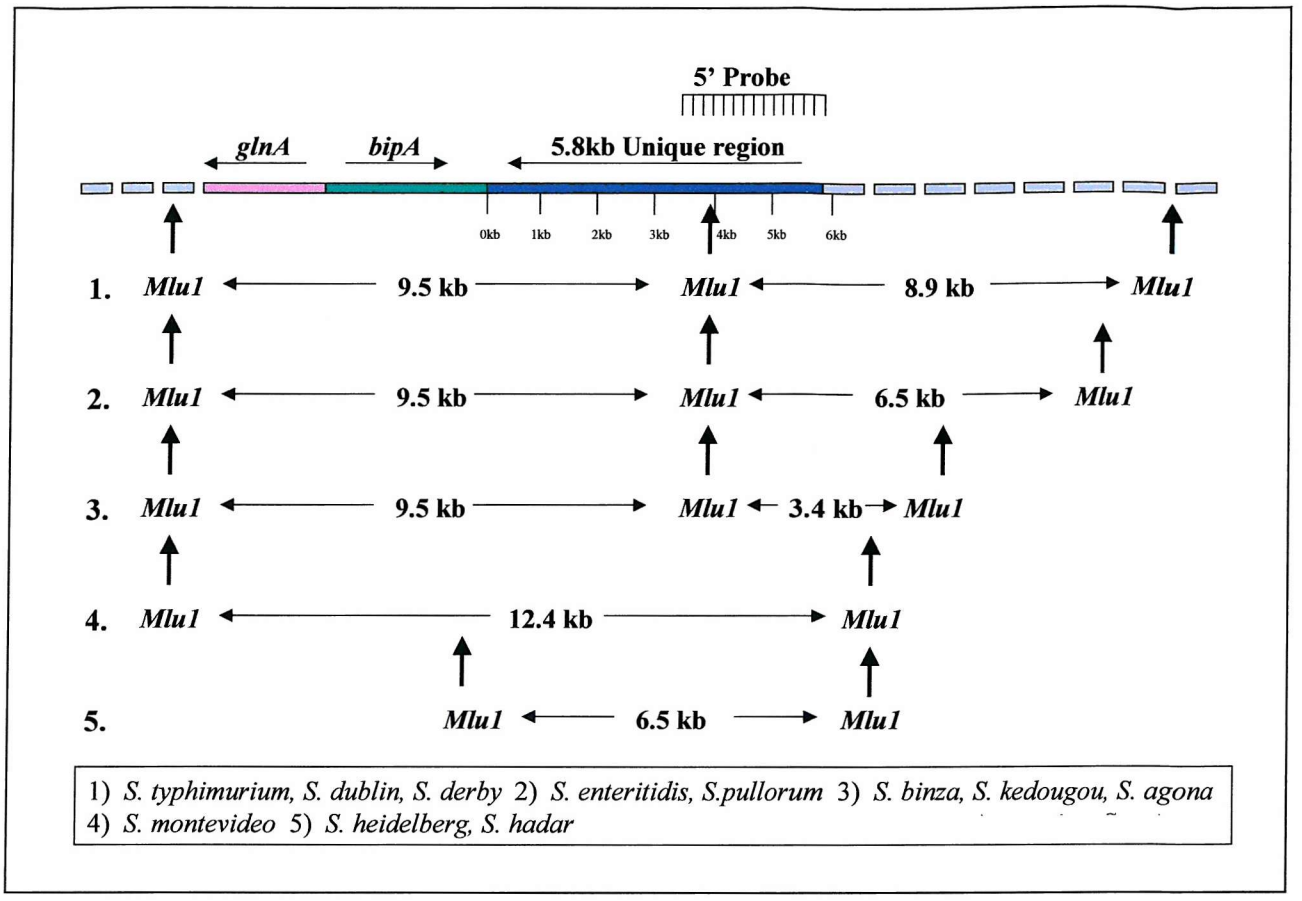


Figure 7-9 ***EcoRI* profiles of several different *Salmonella* serovars when probed with 5' region of unique ORFs**

Comparison of *EcoRI*-digested chromosomal DNA from several *Salmonella* serotypes by Southern hybridisation using 5' region of ORFs as a probe. Lanes 1-3: *S. dublin*, lanes 4 - 6: *S. derby*, lane 7: *S. typhimurium*, lane 8: *S. typhimurium* (strain 3148), lanes 9-11: *S. typhimurium*, lane 12: negative control *E. coli* K12. Lane 13: *S. agona*, lane 14: *S. pullorum*, lane 15: *S. arizona*, lane 16: *S. kedougou*, lane 17: *S. binza*, lane 18: *S. montevideo*, lane 19: *S. heidelberg*, lane 20: *S. hadar*, lane 21: *S. enteritidis,* lane 22*:* negative control *E. coli* K12. Molecular markers given in kb.



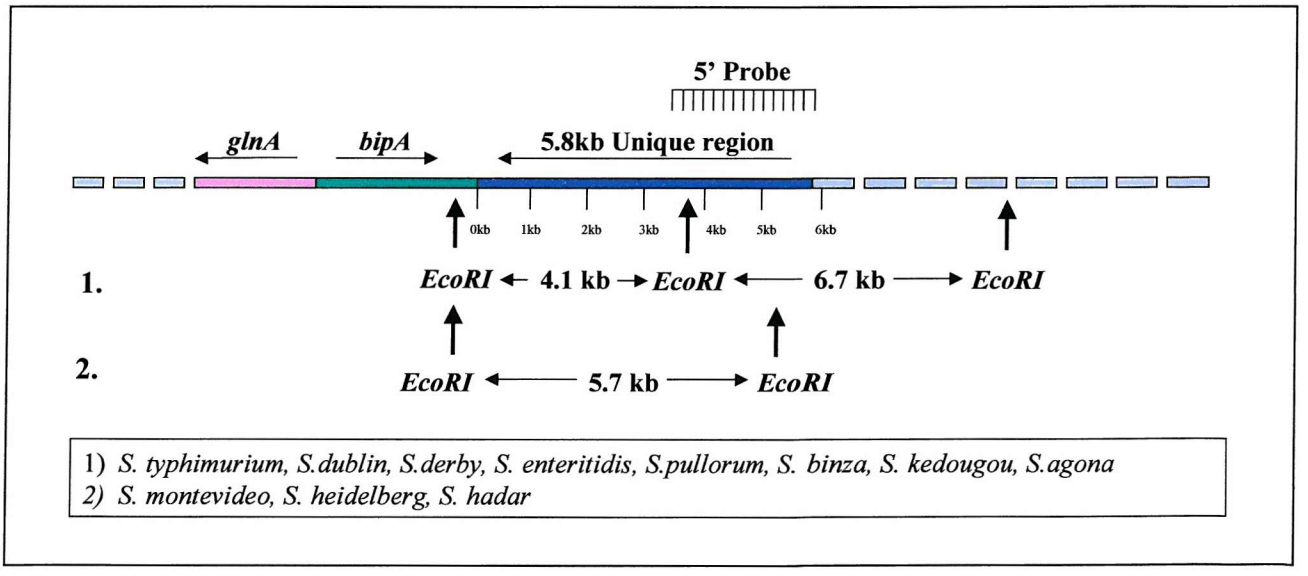


Figure 7-10 Possible profiles of different *Salmonella* serovars

Diagrams have been produced from the information obtained from the *MluI*– and *EcoRI*–digested Southern blots.

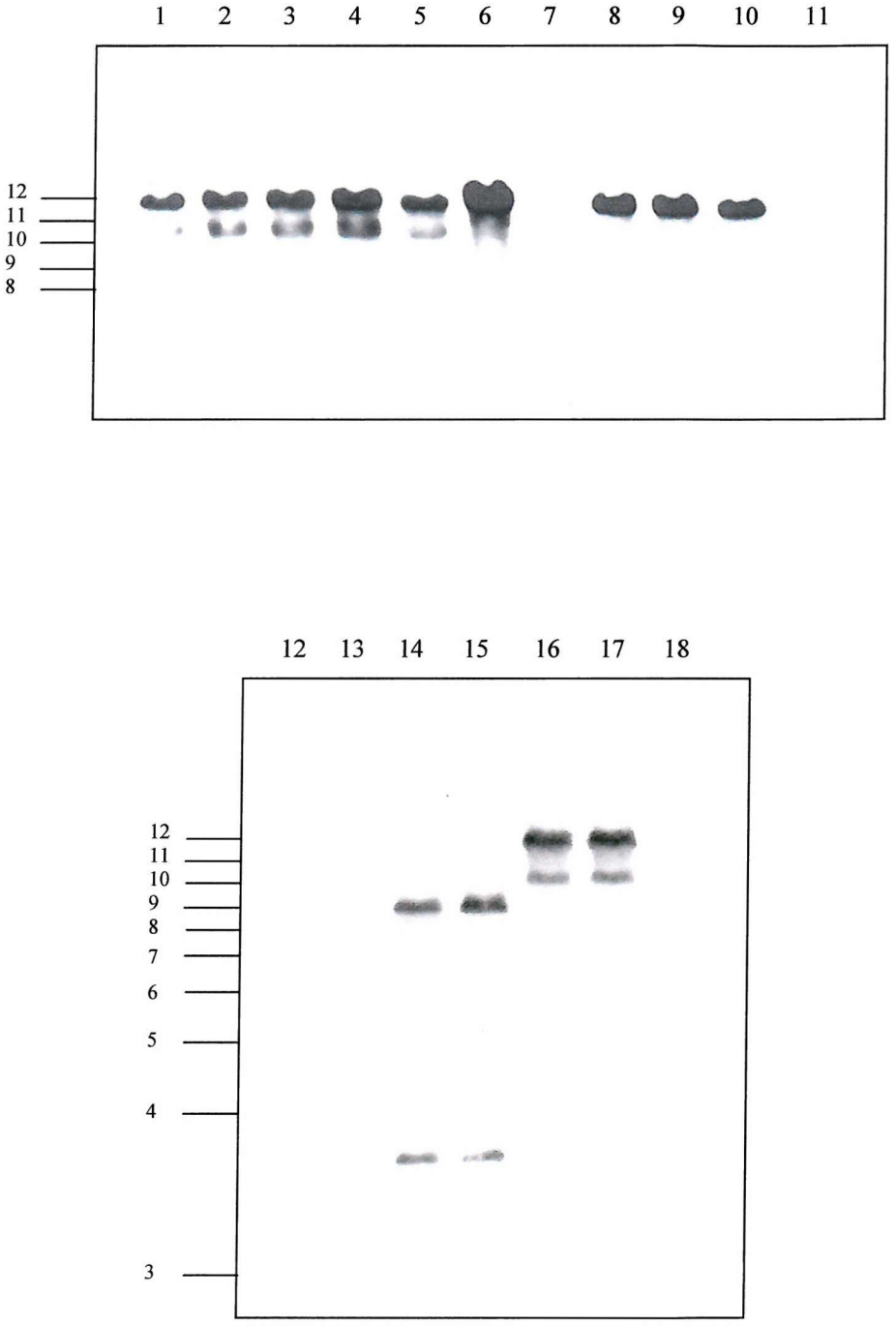


Figure 7-11 ***NdeI*** **profiles of several different** ***Salmonella*** **serovars when probed with 3' region of unique ORFs**

Comparison of *NdeI*-digested chromosomal DNA from several *Salmonella* serotypes by Southern hybridisation using 3' region of ORFs as a probe. Lanes 1-5: *S. enteritidis*, lane 6: *S. typhimurium*, lane 7: *S. typhimurium* (strain 3148), lanes 8-10: *S. typhimurium*, lane 11 negative control *E. coli* K12, lane 12-13: *S. arizona,* lanes 14-15*: S. agona*, lanes 16-17: *S. pullorum*, lane 18: negative control *E. coli* K12.

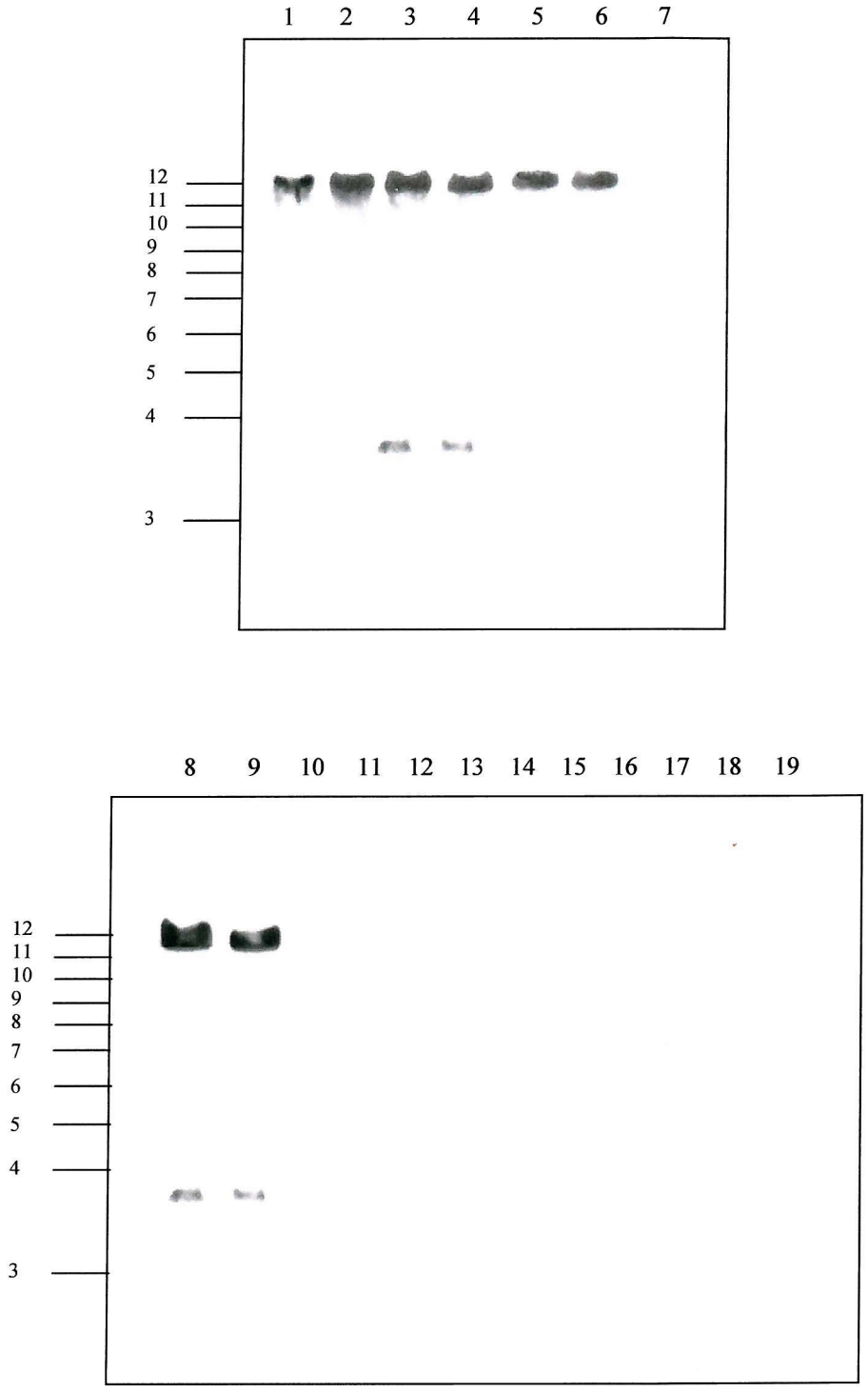


Figure 7-12 ***NdeI*** **profiles of several different** ***Salmonella*** **serovars when probed with 3' region of unique ORFs**

Comparison of *NdeI*-digested chromosomal DNA from several *Salmonella* serotypes by Southern hybridisation using 3' region of ORFs as a probe. Lanes 1-2: *S. dublin*, lanes 3-4: *S. kedougou*, lanes 5-6: *S. derby*, lane 7: negative control *E. coli* K12, lane 8-9: *S. binza,* lane 10-12: *S. montevideo*, lanes 13-15: *S. heidelberg*, lanes 16-18: *S. hadar*, lane 19: negative control *E. coli* K12

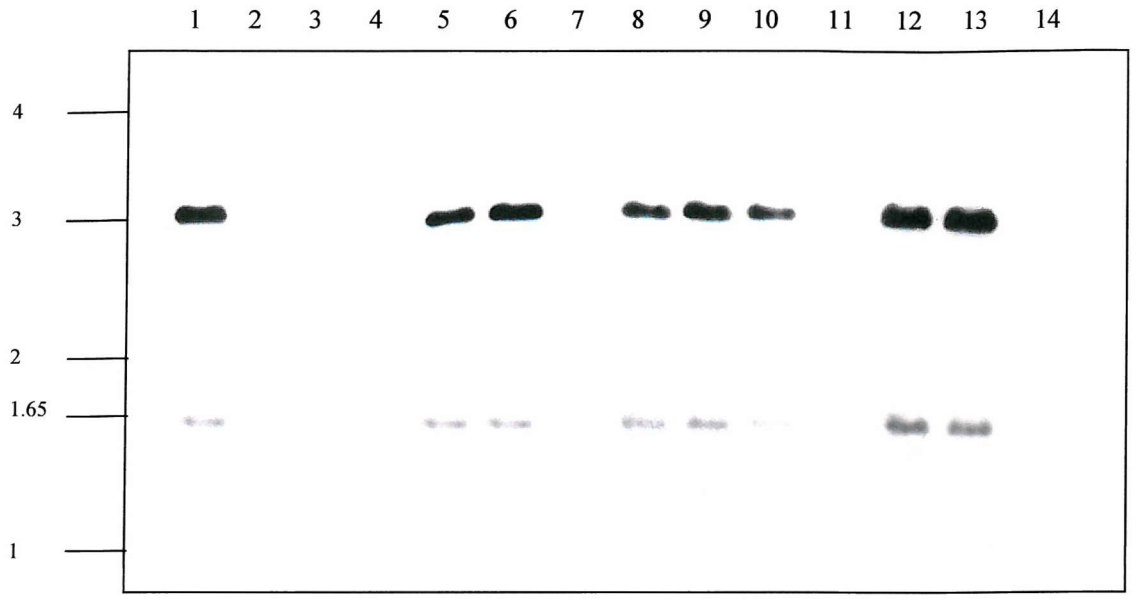
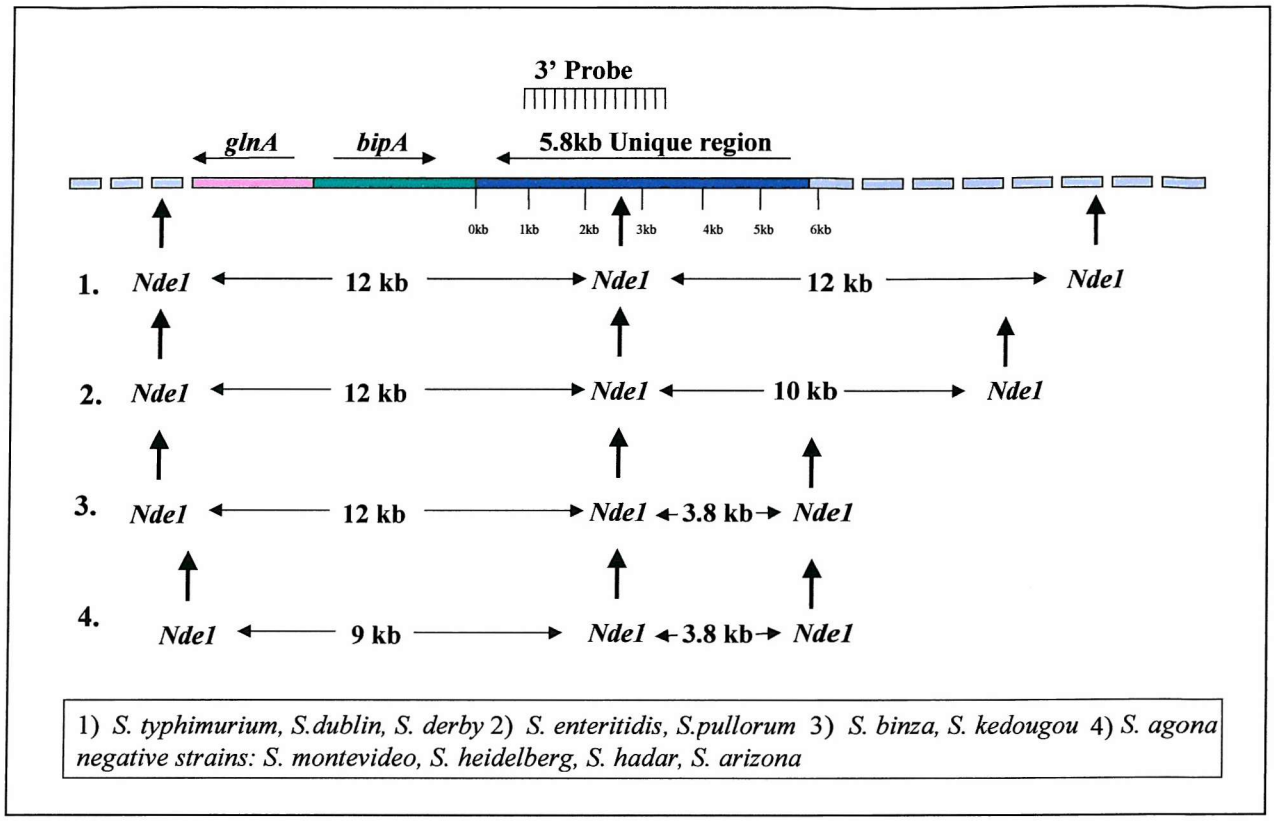


Figure 7-13 *PvuI* profiles of several different *Salmonella* serovars when probed with 3' region of unique ORFs

Comparison of *PvuI*-digested chromosomal DNA from several *Salmonella* serotypes by Southern hybridisation using 3' region of ORFs as a probe. Lane 1: *S. binza*, lane 2: *S. montevideo*, lane 3: *S. heidelberg*, lane 4: *S. hadar* lane 5: *S. agona,* lane 6: *S. pullorum*, lane 7: *S. arizona*, lane 8: *S. dublin*, lane 9: *S. kedougou*, lane 10: *S.* derby, lane 11: *S. typhimurium* (strain 3148), lane 12: *S. typhimurium*, lane 13: S. *enteritidis*, lane 14: negative control *E. coli* K12.



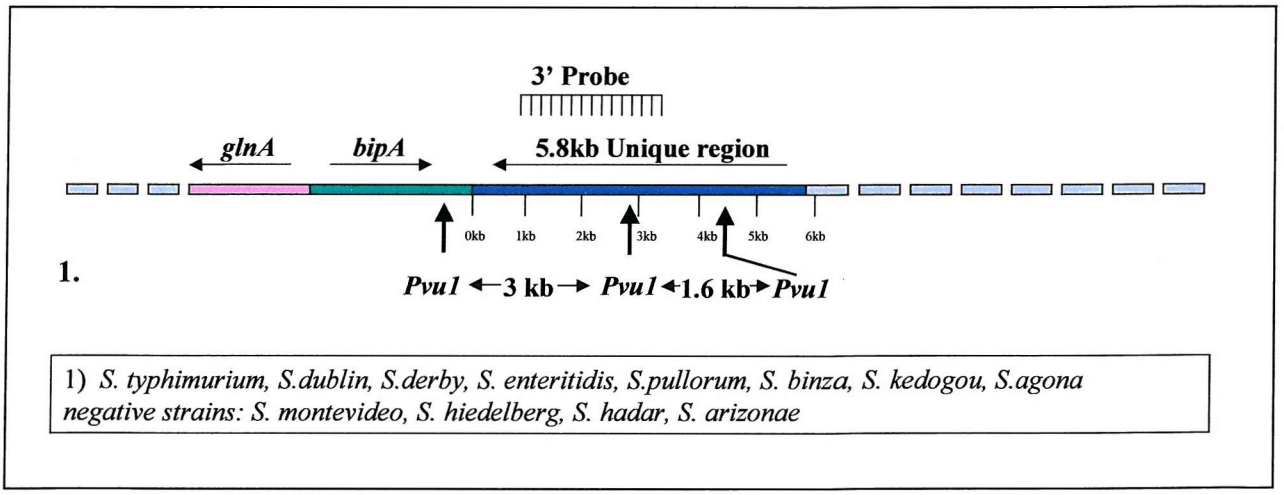


Figure 7-14 Possible profiles of different *Salmonella* serovars

Diagrams have been produced from the information obtained from the *NdeI*– and *PvuI*–digested Southern blots.

7.7 Discussion

Investigations into phylogenetic diversity and host adaptation of *Salmonella* spp. have uncovered various virulence attributes seen only in pathogenic species. Analysis of *Salmonella in vivo*-induced genes located in low GC content areas have uncovered regions that are host specific which may therefore distinguish their host range. These genes may contribute to the degree of host adaptation, host specificity, tissue tropism and disease manifestation (Conner *et al.*, 1998).

The evolution of *Salmonella* from *E. coli* is thought to have occurred in three phases (Baumler, 1997a). Phase 1 involved the acquisition of a pathogenicity island, that was required for the intestinal phase of infection, to give the species *S. bongori* (Mills *et al.*, 1995; Galan, 1996). The second phase led to two distinct lineages of *Salmonella*: *S. enterica* and *S. bongori* (Reeves *et al.*, 1989). This stage involved the acquisition of a second pathogenicity island necessary for the colonisation of deeper tissues and hence the hosts ability to cause a systemic infection (Ochman *et al.*, 1996; Shea *et al.*, 1996). The third phase involved the lineage of *S. enterica* into 6 groups with subgroup I containing most of the pathogenic species for warm-blooded vertebrates, whereas most of the other subgroups were generally isolated from reptiles (Farmer *et al.*, 1984). With the exception of *S. arizona*, which belongs to subgroup III, all of the *Salmonella* serotypes used in this chapter belong to subgroup I.

Investigations into the flanking regions of *bipA* uncovered a unique 5.8kb atypical base composition region that is specific to several *Salmonella* serotypes. These serotypes include *S. typhimurium*, *S. dublin*, *S. derby*, *S. enteritidis*, *S. pullorum*, *S. binza*, *S. kedougou*, *S. heidelberg*, *S. hadar* and *S. montevideo*, but not *S. arizona*. Interestingly *S. arizona* belongs to subgroup III and is mainly isolated from cold-blooded animals, whereas all the other serotypes belong to subgroup I and are therefore mainly isolated from warm-blooded animals. This suggests that the genetic element not found in *S. arizona* and other eubacteria may be involved in *Salmonella* host

range. Future work could involve investigating if this region was present in subgroups II, IV, V, and VI to determine if it is only present in serovars that infect warm-blooded animals.

A lower GC content often initially distinguishes pathogenicity islands (Marcus *et al.,* 2000). The region downstream of *bipA* was found to have a lower GC content of 48.11%, suggesting that this region may be a pathogenicity island. Further analysis of the region indicated at least five putative ORFs and ORF4 shared 40% sequence homology to the oxygen-independent coproporphyrinogen III oxidase. Coproporphyrinogen III oxidase, an enzyme involved in heme biosynthesis, catalyses the oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX. Heme biosynthesis is an integral part of bacterial survival therefore this region may be involved in virulence. Alternatively, there are several factors that suggest this region may not be a pathogenicity island. *Salmonella* pathogenicity islands are highly conserved between the different *Salmonella* serotypes. This is not however the case with this unique region as it is not conserved in the serotypes *S. heidelberg*, *S. hadar* and *S. montevideo.* These serovars did not hybridise with the 3' region of the ORFs which indicted a genetic polymorphism within this area. In contrast, the region is highly conserved in the serotypes .*S. typhimurium*, *S. dublin*, *S. derby*, *S. enteritidis, S. pullorum*, *S. binza* and *S. kedougou.* A knockout mutant would need to be constructed to identify if this region was a new pathogenicity island. Also, coupled *in vitro* transcription/translation of linear DNA templates could be carried out to determine the authenticity of the putative ORFs.

CHAPTER 8

8 General Discussion

8.1 The significance of *S. enteritidis*

The broad-host range species, *S. enteritidis*, is able to cause gastroenteritis in humans and it is one of the most common causes of food poisoning worldwide (Baumler *et al.*, 2000). The interest in the virulence properties of this serovar is therefore extensive. The BipA GTPase has been implicated in several virulence properties in EPEC and *S. typhimurium* (Farris *et al.*, 1998; Barker *et al.*, 2000). BipA interacts with ribosomes, increases antibacterial resistance, is involved in EPEC pseudopod cytoskeletal rearrangements and plays a role in the expression of several effector proteins encoded on the LEE locus (Farris *et al.*, 1998; Barker *et al.*, 2000; R. Owens., unpublished data; A. Grant., unpublished data). It therefore seemed logical to investigate the virulence and stress response attributes of BipA in *S. enteritidis*.

8.1.1 The regulatory targets of BipA

The roles of fimbriae and flagella in *Salmonella* pathogenesis are equivocal. Fimbriae are considered to be virulence factors as they are involved in initial adherence to eukaryotic cell surfaces (Peralta *et al.*, 1994; Thiagarajan *et al.*, 1996; Sukupolvi *et al.*, 1997; Dibb-Fuller *et al.*, 1999; Wilson *et al.*, 2000) and several studies have implicated flagella in the survival within macrophages and to invasion of host cells (Weinstein *et al.*, 1984; Fields *et al.*, 1986). In contrast, other studies have found no such findings (Lockman and Curtiss, 1992; Thorns *et al.*, 1996; Ogunniyi *et al.*, 1997; Allen-Vercoe and Woodward, 1999b; Rajashekara *et al.*, 2000).

BipA positively regulates the expression of SEF14 and flagella and plays a role in macrophage survival, but not in host cell adhesion or invasion. Edwards *et al.* (2000) observed that SEF14 fimbriae contribute to systemic disease as these fimbriae mediate the efficient uptake and

survival of the bacteria in macrophages. Additionally, non-flagellated and non-motile strains were unable to survive as well as their parent strains within mouse macrophages (Weinstein *et al.,* 1984; Fields *et al.,* 1986). These results suggest that *bipA* may regulate the expression of SEF14 and flagella, which in turn, regulate the invasion and survival of *Salmonella* within macrophages (Figure 8-1). However, this hypothesis is disputable as other studies have indicated no role for flagella in macrophage survival or virulence (Lockman and Curtiss, 1992). In addition, further analysis in this study indicated that BipA positively regulates not only flagella but also cell motility in general. This suggests that BipA operates relatively early on the flagella assembly pathway.

The transmission of *Salmonella* on animate and inanimate surfaces may be a vital factor in the spread of disease (Morris *et al.,* 1970a; Morris and Wells, 1970b; Tadesse and Cizek, 1994). Important factors involved in adherence to these surfaces include bacterial surface hydrophobicity, charge, cell density and the production of exopolysaccharides. Several studies have also implicated flagella and fimbriae. This however, is still ambiguous (Baier, 1980; Rogers, 1979). Previous reports suggest a role for type-1 fimbriae and flagella in the non-specific adherence of *S. typhimurium* to mineral particles (albite, biotite, felspar and magnetite) and glass (Stenstrom and Kjelleberg, 1985; Dickson and Koohmaraie, 1989). Also, the reduced adhesion of an isogenic SEF14/SEF21 mutant and SEF17 mutant to Teflon and stainless steel and the reduced biofilm formation in the SEF17 mutant, indicated a role for these surface appendages in adhesion to these inanimate surfaces (Austin *et al.,* 1998). In addition, strains unable to elaborate SEF14, SEF17 or flagella were found to adhere poorly to polystyrene. SEF17 and flagella mutants both showed reduced adherence at 25°C, whereas a SEF14 mutant showed reduced adherence at 37°C. Consequently, these results indicated that these appendages played a role in the bacterial cell aggregation on inanimate surfaces (Woodward *et al.,* 2000). Interestingly, the expression of SEF17 and flagella was reduced in the *bipA* null mutant at 25°C

whereas at 37°C SEF14 expression was reduced. This suggests that BipA may coregulate the expression of these surface appendages and that this coregulation is dependent on the environmental conditions. For example, at ambient temperatures the expression of SEF17 and flagella will be upregulated, whereas at higher temperatures the expression of SEF14 will be upregulated. BipA may either enhance transcription or translation. Alternatively, it might interact with the fimbrial proteins to allow the adherence of this surface appendage (Figure 8-1). In contrast, BipA negatively regulates the expression of SEF21, therefore it may be involved in decreasing the expression of SEF21 when it is no longer needed (Figure 8-1). These results indicate that it would be worthwhile to ascertain the adhesion and biofilm properties of the *bipA* null mutant.

When pathogenic microbes are engulfed by macrophages they must withstand the oxidative killing by means of the oxidative stress response. However, the role of this response in *Salmonella* virulence has recently been reviewed, as functional OxyR or SoxRS regulons are not required for survival in macrophages (Fang *et al.,* 1997; Taylor *et al.,* 1998). Evidence suggests that *Salmonella* interferes with the trafficking of the oxidase-containing vesicles (Vazquez-Torres *et al.,* 2000). In despite of this *Salmonella* are still exposed to reactive oxygen species in the non-host environment (Kappus and Sies, 1981). This resistance may be an important factor in the transmissibility of *Salmonella* and other food-borne pathogens as it enables the bacteria to survive in environments outside the host. BipA positively regulates the hydrogen peroxide oxidative stress response in *S. enteritidis* and *S. typhimurium* (G. Howell., unpublished results). The OxyR regulon enables *Salmonella* to resist the effects of hydrogen peroxide (Farr and Kogoma, 1991). Therefore, *bipA* may act as a transcriptional or translational activator that positively regulates the *oxyR* regulon or BipA may interact with proteins encoded by the regulon (Figure 8-1). Interestingly, Humphrey *et al.* (1995) have shown that *S. enteritidis* isolates that are more tolerant to hydrogen peroxide also survive longer on surfaces. Future work could

incorporate the assessment of not only the adherence but also the survival of the *bipA* null mutant on inanimate surfaces. If survival were reduced, a plasmid containing the full-length *bipA* gene would need to be inserted into the H_2O_2 sensitive strains to see if survival was restored. It would also be beneficial to assess the survival of the flagella, SEF17 and SEF14 mutants used by Woodward *et al.* (1999). Again, if survival were reduced plasmids containing genes that expressed each appendage would need to be inserted into the H_2O_2 sensitive strains and the survival determined. If in both cases survival increased, a link between fimbrial expression and H_2O_2 survival could be made. One hypothesis could be that BipA regulates the H_2O_2 response, which activates the expression of certain fimbriae, which in turn modulates the adherence and survival of the bacteria on inanimate surfaces (Figure 8-2).

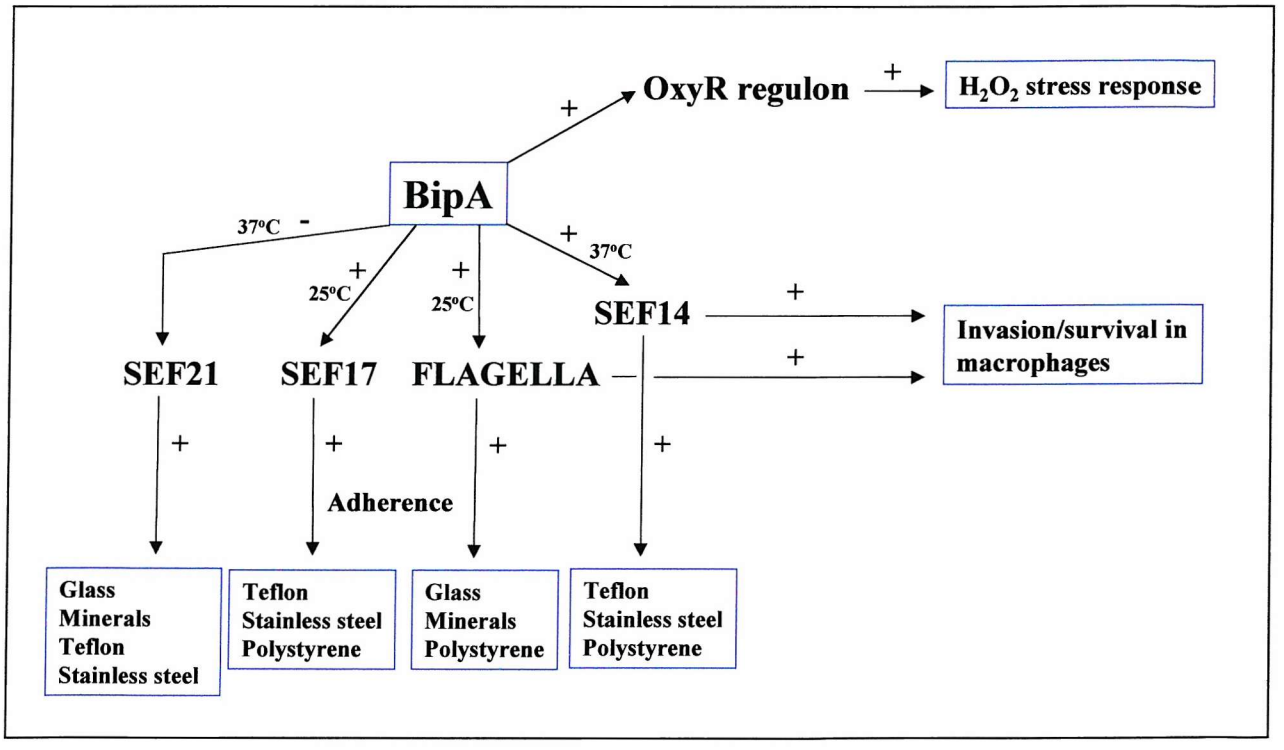


Figure 8-1 Regulatory targets of BipA

Only the temperature at which *bipA* regulates the expression of the fimbriae the most are given. The symbol, +, represents positive regulation, whereas negative regulation is represented by a -.

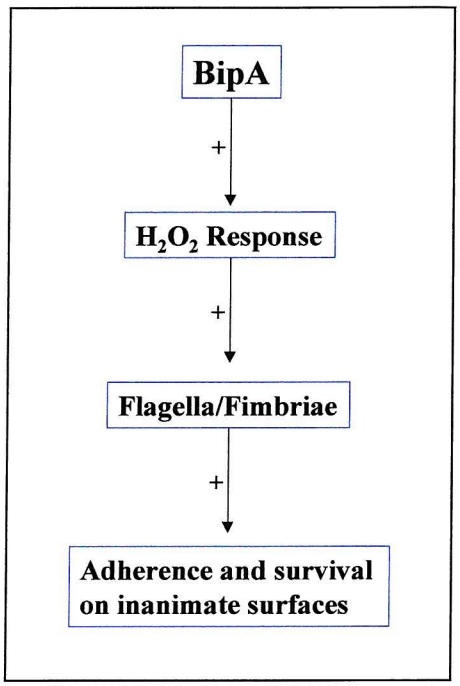


Figure 8-2 A possible pathway for the BipA regulation of surface appendages

The symbol, +, represents positive regulation. BipA regulates the hydrogen peroxide response, which in turn activates the expression of certain surface appendages, which then modulates adherence and survival on inanimate surfaces.

8.1.2 BipA plays a role in survival in the natural environment but not in virulence

Flagella and fimbriae have been implicated in virulence and strains unable to survive in macrophages are avirulent (Fields *et al.,* 1986). Consequently, because BipA affects the expression of several different surface appendages and is involved in the invasion/survival in macrophages (Figure 8-1), it would not be unexpected if the BipA mutant were attenuated *in vivo*. However, studies in the thesis showed that this was not the case. The *bipA* null mutant was as virulent as its parent strain in the BALB/c mouse model and only a slight reduction, compared to the wild type, was seen in the in the initial colonisation and invasion of the caeca and livers of SPF chickens. These results suggest that the effects of *bipA* in the host are not essential to virulence. However, collating these results and the effects of BipA on the H_2O_2 response, it

could be concluded that BipA is more involved in the survival of *S. enteritidis* outside the host. Determining the factors that enable *Salmonella* to survive in the natural environment is as important as determining the virulence attributes of *S. enteritidis* as cross-contamination during catering is known to be important in food poisoning outbreaks caused by *S. enteritidis* (Anonymous, 1993). BipA may enhance survival outside the host, which consequently, will increase *Salmonella* transmission from host to inanimate and animate surfaces.

BipA is unrelated to know virulence or global regulatory proteins but shares substantial sequence similarity to GTPases that interact with ribosomes, notably the Tet(Q)/Tet(M) tetracycline resistant proteins and EF-G. Conserved sequences are found throughout BipA, in particular the sequence conservation extends to domain IV of EF-G. Interestingly, this domain shares structural homology with the anti-codon arm of tRNA in the ternary complex of elongation factor Tu and is thought to interact with the small ribosomal subunit (Nissen *et al.*, 1995; Rodnina *et al.*, 1997). Similarly, studies carried out by Owens *et al.* (unpublished results) have shown that BipA does interact with ribosomes and that BipA GTPase activity is stimulated in the presence of ribosomes. These results and the structural homology to tRNA suggest that BipA may regulate the target proteins, such as flagella and fimbrial proteins and proteins involved in the oxidative stress response, by a novel mechanism operating at the level of the ribosome.

In conclusion, further investigations into the role of BipA in the non-host environment would prove invalid in resolving the unknown aspects of environmental survival.

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