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The SRL Pathogenicity Island of *Shigella flexneri* 2a YSH6000

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To the people who believe in me the most:

Warren, Mum, Dad and Natalie

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Summary

Shigella spp. are the causative agent of diseases ranging from a mild diarrhoea to severe dysentery, that result in more than 1.1 million deaths each year. There is increasing difficulty in treating shigellosis as resistance to the antibiotics used in treatment increases. A better understanding of how chromosomal antibiotic resistance determinants and other virulence factors may be spread between bacteria prompted a close study of an element carrying resistance determinants for streptomycin, ampicillin, chloramphenicol and tetracycline. The multi-antibiotic resistance determinant element (MRDE) was identified when *S. flexneri* 2a strain YSH6000 was found to spontaneously lose resistance to the four antibiotics. The resistance determinants were revealed to be carried on a 99 kb deletable element, the MRDE. Further investigation has revealed that the streptomycin, ampicillin, chloramphenicol and tetracycline determinants are carried on the *Shigella* resistance locus (SRL), which is carried on a pathogenicity island (SRL PAI), part of the MRDE. Each of these elements, the SRL, SRL PAI and MRDE, is able to undergo site-specific deletion from the *S. flexneri* chromosome.

The SRL PAI is a 66 257 bp element that is flanked by 14 bp direct repeats. At its right flank, the direct repeat forms the 3'-terminus of the *serX* tRNA gene. The SRL PAI deletes precisely from the chromosome leaving a single copy of the 14 bp direct repeat, as part of an intact *serX*. The deletion of the SRL PAI is dependent on a P4-like integrase, encoded by the PAI. The integrase is most closely related to integrases on enterohaemorrhagic *E. coli* O157:H7 O-islands #43 and #48, and the integrase from the CP4-57 cryptic phage of *E. coli* K-12. Excision of the SRL PAI is enhanced by the overexpression of Rox, a transcriptional regulator related to AlpA from CP4-57. This increased excision of the SRL PAI is dependent on the *int* gene, because excision does not occur in an *int* mutant, even when Rox is over expressed. In a SRL PAI negative background Rox represses integrase transcription, but SRL PAI excision is enhanced in the presence of the PAI. These findings are suggestive of the use of a co-factor, potentially encoded on the SRL PAI, by Rox in the control of *int* transcription.

The SRL PAI consists of approximately 24% prophage related open reading frames. The organisation and conservation of these phage-related ORFs in several PAIs and O-islands has led to the proposal that they form a conserved backbone which has been added to, and deleted from, depending on specific bacterium's requirements for survival. This is similar to the modular theory proposed for the *Staphylococcus aureus* SaPI3. These phage-related ORFs include the integrase, two AlpA homologues, an insertion sequence, an autotransporter, two potential ATPases and a potential member of the RadC family.

Other noteworthy features of the SRL PAI include a homologue of Flu, an autotransporter involved in biofilm formation in enteroaggregative and diffusely adhering *E. coli*, an intact ferric-dicitrate iron transport system, a LysR-like transcriptional regulator and potentially its regulated gene, a member of the Hha/YmoA family of transcriptional regulators, a number of ORFs with no similarity to anything in the database, and a large number of insertion sequences. The genes and regions encoding these features have widely varying G+C% content, suggesting that many were acquired by horizontal transfer.

The ferric-dicitrate iron transport system encoded by the SRL PAI is 99% identical at nucleotide level to the *fec* locus encoded by *E. coli* K-12. This was the first report of a ferric-dicitrate system in *Shigella* spp. The *S. flexneri fec* locus was shown to be functional by the complementation of an *E. coli fec* mutant, fully restoring the ability of the mutant to grow in iron-limited conditions in the presence of citrate. The gene encoding the outer membrane receptor, *fecA*, is transcribed in *S. flexneri* 2a YSH6000, further supporting the functionality of this locus. A mutant was constructed in *fecI* the regulator of transcription of the *fec* structural genes *fecABCDE*. This mutant was used to try to determine a role for *fec* in survival of *S.flexneri* 2a YSH6000 in iron-limited conditions. However, it was revealed that growth of YSH6000 was not significantly different in iron-limited conditions in the presence or absence of the *fec* system. PCR analysis enabled the identification of a second iron transport system in this *S. flexneri* strain, an aerobactin system common to serotype 2a strains. Recent sequencing of a *S. flexneri* 2a strain also identified an additional iron transport system not previously found in *S. flexneri*. Therefore, it is possible that in the conditions tested, the loss of the *fec* locus was compensated for by other iron-transport systems.

Future studies could endeavour to identify additional iron transport systems in this *S. flexneri* strain, and attempt to discover their role in survival. This may include mutagenesis of the various systems to see if the presence of any single system is sufficient to support growth in iron-limited conditions. It may also involve transcriptional analysis of the various systems under specific conditions or in particular environments, to try and discover which system is required for which niche. A better understanding of the regulation of the various genes encoded on the PAI may provide information about the ancestry of this element and thus information about its potential ability to mobilise between species. Work by others is attempting to demonstrate the mobility of the SRL PAI and has so far revealed its excision as a circular form and a role for Int in the integration of the PAI. This would be enhanced by a better understanding of Rox and its effect on *int*.

I, Shelley Narelle Luck, declare that this thesis contains no material which has been accepted for the award of any degree or diploma in any other university and to the best of my knowledge and belief, contains no material which has been previously published or written by another person, except where due reference is made in the text of the thesis.

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~ CHAPTER ONE ~

Introduction

SHIGELLA

THE ORGANISM

Dysentery is caused primarily by two organisms, *Entamoeba histolytica* and *Shigella* spp. In 1875, Losch isolated the causative agent of amoebic dysentery, *Entamoeba histolytica*, and proved its role in infection (Keusch & Bennish, 1991). In 1898, Dr Kioshi Shiga isolated the major causative agent of bacillary dysentery, *Shigella dysenteriae* (Shiga's bacillus), from stool samples obtained during a Japanese outbreak of severe epidemic dysentery (Flexner, 1900). The organism had previously been described by Chantemesse and Widal in 1888, but Shiga provided the epidemiological proof of the bacterium's role in disease. Kruse and Flexner identified serological diversity in disease-causing *Shigella* strains, ultimately leading to the classification of four *Shigella* species within the Enterobactericeae family (Keusch & Bennish, 1991).

TAXONOMY

Shigella are small (2-3 μm x 0.4-0.6 μm), non-motile, non-capsulated, Gram-negative rods. *Shigella* spp. are members of the family *Enterobacteriaceae*, tribe *Escherichiae*. Members of the *Enterobactericeae* family are Gram negative, non-spore forming, usually facultative anaerobic bacteria. This family includes important enteric human pathogens such as *Yersinia*, *Salmonella*, and pathogenic and non-pathogenic *Escherichia coli*. The separation of *E. coli* and *Shigella* spp. into different genera is highly debated due to their similarity at morphological, biochemical and genetic levels. For example, most *Shigella* strains do not produce gas following fermentation and are lactose negative, while most *E. coli* strains produce gas and ferment lactose (Brenner, 1984). However, there are a few serotypes of the various *Shigella* species that do produce gas following fermentation and there are some *E. coli* that are lactose negative. *Shigella* is generally considered non-motile, while *E. coli* is motile, although this is not always the case. While *Shigella* is generally defined as more pathogenic than *E. coli* based on its ability to cause dysentery there are some strains of *E. coli*, such as enteroinvasive *E. coli* (EIEC) that cause dysentery, and EIEC also cross reacts with antisera to some *Shigella* O-antigens. Like *Shigella*, EIEC also possesses an invasion plasmid and an enterotoxin (Levine & Edelman, 1984). There is some debate as to whether the four *Shigella* species and *E. coli* should be reclassified as one species with five subgroups or five species within one genus due to the high level of genetic conservation. One study found that based on multi-locus enzyme electrophoresis (MLEE), most *Shigella* spp. lay within a single phylogenetic cluster amongst reference *E. coli* strains. Only a few *S. flexneri* serotypes fell outside this cluster (Pupo *et al.*, 1997). The clustering of *Shigella* spp. with *E. coli* subtypes by MLEE suggests that there is no phylogenetic distinction between these

genera. More recently, the sequencing of 36 housekeeping genes of *E. coli* K-12 and *S. flexneri* 2a suggested an average distance between the two of 1.12%, less than that between *E. coli* K-12 and O157:H7 (Lan & Reeves, 2002). This is supported by comparison of the genome sequences of *E. coli* K-12, enterohaemorrhagic *E. coli* (EHEC) O157:H7 and *S. flexneri* 2a (Jin *et al.*, 2002).

Currently, the *Shigella* genus consists of four species distinguished by their biochemical and serological characteristics. These species are *S. dysenteriae*, *S. flexneri*, *S. sonnei* and *S. boydii*. *S. flexneri* contains eight serotypes with nine subtypes while *S. dysenteriae* and *S. boydii* contain ten and 15 serotypes respectively. In contrast, *S. sonnei* contains only one serotype.

SHIGELLOSIS

THE DISEASE

Bacillary dysentery is an acute enteric infection. Symptoms usually appear 1-2 days after ingestion of as few as 10 organisms (DuPont *et al.*, 1989). Classic dysentery, following infection with *S. flexneri* type 2a, presents with fever and abdominal cramps in the first 48 hours, with diarrhoea developing after approximately 72 hours. Between 120 and 144 hours after ingestion of *Shigella* the patient's symptoms progress to bloody stools (DuPont *et al.*, 1969). The entire colon may be involved during an infection, but it is the rectum and sigmoid colon that are usually the most affected areas (Speelman *et al.*, 1984). The clinical symptoms of shigellosis, including frequent, small volume, bloody stools, are associated with multiplication of the organisms within the colon and subsequent destruction or ulceration of the colonic mucosa. These bloody stools are seen more often during *S. dysenteriae* type 1 infection than during infection with the other *Shigella* spp. *Shigella* strains carrying a 140-MDa virulence plasmid have the ability to invade the colonic epithelium and this coupled with the toxins produced by *S. dysenteriae* and *S. flexneri* are the causes of classic dysentery (DuPont, 1990).

The disease severity varies depending on the species of *Shigella* involved. *S. dysenteriae* 1 and some serotypes of *S. flexneri* produce toxins that can lead to the most severe form of the disease, causing considerable tissue damage. The disease is progressively less severe with other *S. flexneri*, *S. sonnei* and *S. boydii* serotypes (Rowe & Gross, 1981). Shigellosis in well-nourished patients is usually self-limiting and persists for a much shorter time (up to 7 days) than in malnourished patients. Malnourished patients can suffer from chronic relapses of shigellosis over several months (Mata *et al.*, 1966). As well as nutritional state, immunocompetency, age and the level of exposure in endemic areas (e.g. new travellers, young children) influence the severity of illness following *Shigella* infection.

Differentiation between amoebic and bacillary dysentery is based on the presence/absence of fever, leukocyte numbers in faeces, duration of illness and age. That is, an older person suffering from prolonged illness without fever and lower faecal leukocytes is most likely to be suffering from amoebic dysentery (Speelman *et al.*, 1987).

DIAGNOSIS AND TREATMENT

Diagnosis of shigellosis is aided by patient history, but is dependent on culture and isolation from faecal specimens. Selective bacteriological media are required to highlight the biochemical properties that distinguish *Shigella* from other *Enterobacteriaceae*. Species identification follows initial biochemical analysis and is determined by serological agglutination with group specific anti-O-(somatic) antigen sera (Keusch & Bennish, 1991).

The media used to culture *Shigella* must differentiate it from the normal flora found in human faeces, such as *E. coli*. Media developed for this purpose include MacConkey's bile salts, xylose-lactose-deoxycholate (XLD), Hektoen enteric (HE) and tergitol-7-triphenyl tetrazolium chloride (TTC) agars. These media allow *Shigella* to grow but have inhibitory effects on most normal flora, or distinguish *Shigella* from commensal bacteria by colour reactions based on their inability to ferment lactose. *Shigella* spp. are also negative in tests for urease, phenylalanine deaminase, lysine decarboxylase, arginine decarboxylase, growth in KCN medium, utilisation of citrate or malonate, oxidation of gluconate or gelatin liquefaction (Watanabe & Okamura, 1992). *Shigella* is distinguished from another common enteric pathogen, *Salmonella* by testing for H_2S formation, which is seen with *Salmonella* but not *Shigella* spp. or *E. coli*.

More recently, polymerase chain reaction (PCR) diagnostic systems have been developed for diagnosis of *Shigella* and EIEC infections. One of these PCR based systems detects the *ipaH* gene, found in multiple copies on the virulence plasmid of *Shigella* and EIEC. This system increased the detection of *Shigella* species and EIEC from 58% to 78% among dysenteric patients and from 6% to 27% in family contacts in a study in Thailand (Gaudio *et al.*, 1997). Thus, the PCR based detection system is far more sensitive than culture based detection.

Treatment for shigellosis involves a combination of symptomatic and chemotherapeutic therapy. Dehydration is a major problem and thus fluid and electrolyte levels need to be maintained. Antibiotics have proven to be very effective in controlling symptoms and limiting the clinical course of the illness (Benenson, 1995). Problems with the treatment of shigellosis have arisen as the prevalence of antibiotic resistance has increased. The incidence of ampicillin, tetracycline and chloramphenicol resistance is as high as 81.8%, 96.9% and 72.7% respectively for *Shigella* infections

in Tanzania (Navia *et al.*, 1999). In Spain, a survey found multiple antibiotic resistance in 72% and 63% of *S. sonnei* and *S. flexneri* isolates respectively (Vila *et al.*, 1994). *S. sonnei* strains were most commonly resistant to STX and tetracycline, while for *S. flexneri* strains resistance was most often observed for ampicillin, chloramphenicol and tetracycline. The drugs of choice had been ampicillin and sulfamethoxazole-trimethoprim (STX), but with widespread resistance to these drugs, treatment is now dependent on alternatives such as nalidixic acid, amdinocillin pivoxil or newer quinolones (Salam & Bennish, 1991).

EPIDEMIOLOGY

Cases of dysentery have been reported throughout history, as far back as Hippocrates. *Shigella* endemics appear to have a cyclic pattern each lasting 20 – 30 years (DuPont, 1990). This cyclic pattern reflects shifts in the dominant species causing disease (e.g. *S. dysenteriae* 1 to *S. flexneri* 2a). An example of this occurred in Dhaka, Bangladesh. In 1969 the predominant species was *S. flexneri*, causing 74% of *Shigella* cases, by 1973 *S. dysenteriae* caused 56% of cases and in 1981, *S. flexneri* was predominant again causing 75% of cases (Khan *et al.*, 1985). There is a difference between the species present in various geographic locations, but as a population in one region becomes immune to one species, another flourishes. In developed countries, *S. sonnei* is most prevalent while *S. flexneri* is the predominant endemic species in developing countries (Kotloff *et al.*, 1999). Consequently, the frequency, severity of disease, and the causative species will vary between geographic locations.

Bacillary dysentery is most commonly spread from person-to-person via the faecal-oral route. Water-borne infection is uncommon and is usually associated with faecal contamination. Foodborne transmission is also rare compared to direct contact transmission, and is associated with infected food preparers (Trevejo *et al.*, 1999) and with flies passing the disease between faecal matter and food (DuPont, 1990). A recent outbreak of shigellosis occurred in the United States associated with the contamination of a commercially sold dip (CDC, 2000). The dip had been contaminated with *S. sonnei* prior to packaging.

In developed countries, outbreaks of shigellosis are commonly associated with daycare centres, mental hospitals, jails and institutions with children (Benenson, 1995). In developing countries, they are associated with high-density living and poor sanitation. Approximately two out of three cases of shigellosis and most *Shigella* related deaths occur in children under 10 years old (Benenson, 1995). This coupled with the fact that in some developing countries children have a 50% chance of dying due to diarrhoea by the time they are seven years old (Rowe & Gross, 1981), shows the severe effect that shigellosis can have on a given population.

SHIGELLA AND THE IMMUNE SYSTEM

There has been only limited investigation into the immune response to shigellosis. Most investigation has centred on vaccine development, although some basic information about immunity to *Shigella* infection has been obtained. An early T-cell response begins within six to eight days of infection, followed by a phase of increased CD4+ T-cell (T-helper cells) and T-memory cell numbers (Islam *et al.*, 1995). The T-helper (Th) cells aid in the humoral response which is important during an early response to the disease, as a correlation has been demonstrated between the severity of disease and the level of humoral immunity (Islam *et al.*, 1996). This is supported by findings that Th_1, Th_2 and local B-lymphocyte activity may contribute to the survival of immune mice after *Shigella* challenge (van de Verg *et al.*, 1995). However, there is a decrease in total serum IgG, IgM and total protein as the disease becomes more severe. This contrasts with the increased levels of *Shigella*-antigen-specific serum antibodies associated with severe disease. The high anti-LPS IgA in stools at an early stage of infections suggests that there is a rapid mucosal humoral response to infection, but this early response does not appear sufficient to stem the disease process.

Oberhelman (1991) found that the predominant serum antibody response to *S. flexneri* infections was to IpaC (invasion plasmid antigen) and IpaD in adults, but variable in children. This was supported by findings showing significantly higher serum IgG levels to virulent *S. flexneri* than to avirulent strains, as Ipas are only expressed by virulent species. Generally, mucosal antibody responses were to non-Ipa molecules such as LPS. Serum antibody titres to homologous LPS serotypes were prolonged, in contrast with the short-lived mucosal IgA antibody to homologous LPS serotypes. It is unlikely that antibodies to Ipa proteins are protective as Ipa proteins remain mostly cytoplasmic and are secreted only following epithelial cell contact thus having minimal exposure to the immune system (Watarai *et al.*, 1995a). This is supported by the serotype specific nature of immunity to *Shigella* infection. The protectiveness of anti-LPS antibodies is discussed in 'Vaccine Development'.

The importance of antibodies in overcoming *Shigella* infection is highlighted by the role of Peyer's patches in shigellosis. In a rabbit ileal loop model it was found that loops that contained a Peyer's patch exhibited a stronger early IgA antibody response than loops lacking a Peyer's patch (Keren *et al.*, 1978). However, over time the level of IgA in both types of loop was comparable. As Peyer's patches contain the precursors of IgA plasma cells (Roux *et al.*, 1981), the importance of both Peyer's patches and antibodies in an early immune response is implied. The importance of IgA was demonstrated when mucosal anti-LPS secretory IgA was shown to be protective in mice. This protection is both serotype-specific and local concentration dose-dependent (Phalipon *et al.*, 1995).

M CELLS, MACROPHAGES AND SHIGELLA

During a *Shigella* infection, it is critical for the bacteria to reach the basolateral surface of the epithelial cells. The shigellae initially move out of the gut lumen via uptake by M cells. M cells overlie solitary colonic lymph nodules and are continuously sampling macromolecules and microorganisms from the intestinal lumen to deliver antigens to underlying lymphoid tissue. It has been shown that virulent *Shigella* multiply within M cells after uptake and can lead to ulceration over the Peyer's patches (Wassef *et al.*, 1989). This initial uptake provides *Shigella* with access to the epithelial layers where they are taken up by macrophages.

Macrophages normally destroy internalised bacteria and present bacterial antigens on their cell surface in order to activate the immune response. However, *Shigella* spp. have developed a mechanism to escape macrophages. It has been shown both *in vitro* (Zychlinsky *et al.*, 1992) and *in vivo* (Zychlinsky *et al.*, 1996) that *Shigella* are capable of inducing macrophages to undergo apoptosis once they are within the macrophage's cytoplasm. Macrophage apoptosis has also been demonstrated in *Yersinia* spp. (Monack *et al.*, 1997) and *Salmonella* spp. (Monack *et al.*, 1996). For *Shigella*, this process involves the invasion plasmid antigen, IpaB, binding to Casp-1 (Interleukin-1β converting enzyme, ICE) which results in a bypassing of signal transduction events and caspases upstream of Casp-1 (Chen *et al.*, 1996; Hilbi *et al.*, 1998). Interestingly, *Salmonella* induced apoptosis is due to SipB, an IpaB homologue (Hersh *et al.*, 1999). ICE is homologous to *ced*-3, the cell death gene of *Caenohabiditis elegans*, which is known to promote programmed cell death of mammalian cells (Miura *et al.*, 1993; Yuan *et al.*, 1993). When *Shigella* activates ICE and causes the macrophages to apoptose, IL-1β is released in its activated form causing the inflammatory response that is associated with tissue damage during shigellosis. Increased IL-1 levels are seen only during infection with invasive strains of *S. flexneri*, probably because of the IpaB induced apoptosis and ICE activation requires the 140-MDa plasmid. It has previously been shown that IL-1β plays a role in the pathogenesis of shigellosis through its role as a proinflammatory cytokine, produced mostly by macrophages (Sansonetti *et al.*, 1995). This was demonstrated by protection against invasion and intestinal tissue destruction when animals were treated with an IL-1 receptor antagonist. The use of ICE inhibitors also prevents apoptosis of macrophages (Chen et al., 1996; Hilbi *et al.*, 1997). Recent data suggest that the rapid, uncontrolled inflammation, characteristic of shigellosis, is due mostly to the low IL-1 antagonist to IL-1 ratio that occurs within four hours of infection by *Shigella* in the rabbit-ileal loop model (Arondel *et al.*, 1999).

It should be noted that during *Shigella* infection in the rabbit ileal loop model, T- and B-cells also become apoptotic. However, whether this is directly or indirectly due to *Shigella* invasion is still to be established (Zychlinsky et al., 1996). This study also found a significant difference in apoptosis

caused by virulent versus avirulent *S. flexneri* in the rabbit ileal loop model. It is believed that this is because of the requirement of IpaB for macrophage apoptosis, which is lacking in avirulent strains (Zychlinsky *et al.*, 1994).

POLYMORPHONUCLEAR LEUKOCYTES AND SHIGELLA

Once some of the bacteria have passed through the epithelium via the M cells and are in contact with the epithelial cells they are able to elicit a marked neutrophil (polymorphonuclear leukocytes - PMN) transepithelial migration in the basolateral-to-apical direction. This migration of leukocytes through the epithelial barrier is the primary cause of epithelium destruction (Perdomo *et al.*, 1994). In cell monolayers, McCormick *et al.* (1998) demonstrated that the presence of the bacteria on the basolateral side increased PMN migration ten-fold. Apical exposure of *Shigella* failed to elicit this response, showing the importance of bacterial translocation through the epithelium via the M cells. This process is dependent on genes found on the virulence plasmid and more specifically genes involved in type-III secretion. Virulent *S. flexneri* M90T show stronger adherence and activation of PMNs than a non-invasive strain BS176, lacking the virulence plasmid. Further analysis with M90T also showed that *ipa* mutants (Δ*ipaABCD*, Δ*ipaB*, Δ*ipaC*) produced the same results as the non-invasive strain, suggesting an importance for these genes in PMN activation (Renesto *et al.*, 1996).

The necessity for the PMN migration during *Shigella* infection may be due to the opening of the paracellular pathway and subsequent tissue damage that allows easier bacterial entry into the colonocytes. This notion is emphasised by the finding that bacterial invasion is six times greater in the presence of PMNs in confluent monolayers than in their absence (Perdomo *et al.*, 1994). In conclusion, PMN activation is induced by *Shigella* invasion at the basolateral surface of epithelial cells and is probably mediated by the Ipa proteins, which are released upon contact with the epithelial cells (Watarai et al., 1995a).

GAMMA INTERFERON

Way *et al.* (1998) established that Natural Killer (NK) cell production of IFN-γ is essential for resistance following primary *Shigella* infection. For *S. dysenteriae* 1 infections, up-regulation of IFN-γ production and expression of the IFN-γ receptor can be correlated to immunity to shigellosis (Raqib *et al.*, 1996). This is based on the finding that in the convalescent stage of shigellosis there is a progressive entrapment and binding of IFN-γ to its specific receptor at the local infection site. It has been shown that activating various cell lines with IFN-γ *in vitro* allows the cells to eradicate intracellular *Shigella*. In contrast, non-activated macrophages infected with *Shigella* allow

intracellular replication and intercellular spread. After exposure to *S. dysenteriae* antigens, IFN-γ and IL-10 production increased in peripheral blood mononuclear cells from 95% and 87% of human volunteers respectively, even at the lowest inoculum (Samandari *et al.*, 2000). In contrast, there was no response by antibody-secreting cells at the same inoculum. This supports the notion that a type 1 immune response plays an important role in the human response to *Shigella* infection.

VACCINE DEVELOPMENT

In 1996 the development of a *Shigella* vaccine was made a top priority by the World Health Organisation's (WHO) Steering Committee on Diarrhoeal Disease Vaccines. It aimed to accelerate the development of such a vaccine by bringing together the manufacturers, researchers, drug regulatory agencies and other necessary groups (WHO, 1997).

During *Shigella* infection, natural immunity is directed primarily against the O-specific polysaccharide (O-SP) found in the bacterial LPS. Therefore, much of the effort to develop a vaccine has focused on candidate vaccines using these antigens. Early work showed that heat-killed vaccines were ineffective (Formal *et al.*, 1967). However, (Chakrabarti *et al.*, 1999) showed that a heat-killed *S. flexneri* 2a strain could elicit an immune response. Its main limitation was that it required five doses of vaccine and the antibody response was very specific. The protective efficacy of such vaccines may be improved by the use of mutant forms of cholera-toxin and heat-labile enterotoxin as adjuvants. These adjuvants in conjunction with live attenuated and heat-killed *Shigella* vaccines have been shown to enhance protective efficacy against guinea pig keratoconjunctivitis (Hartman *et al.*, 1999). Because of the lack of success with heat-killed *Shigella* vaccines, most recent work has focused on live, conjugated or acellular vaccines.

LIVE ATTENUATED VACCINES

A considerable amount of research has been performed into the development of a live attenuated vaccine. In 1972, DuPont *et al.* (1972) found that a live vaccine provided protection that was as effective as a primary infection. Since then, some promising live vaccine candidates have been developed.

Viret *et al.*, (1993) developed a *Vibrio cholerae* strain that had defined mutations in the *rfb* locus as a potential live attenuated carrier strain. The *V. cholerae* strain CVD103-HgR was attenuated by deletion of 94% of the gene encoding the toxic, enzymatically active subunit A of the cholera toxin. This carrier strain elicited strong mucosal immunity, as would be required for enteric pathogens such

as *V. cholerae* and *Shigella* spp. The strain was also safe in adults and children. Favre *et al.* (1996) used this *V. cholerae* attenuated carrier strain for the development of a live attenuated *Shigella* vaccine by integrating the *S. sonnei rfb* and *rfc* loci into its chromosome. Immunisation with this hybrid strain resulted in very good antibody response to *S. sonnei* LPS in mice. This potential vaccine candidate is now proceeding to large-scale production and human trials.

An attenuated *S. flexneri* Y strain SFL124 has been developed by Karnell *et al.*, (1992) following work on the mildly reactogenic strain SFL114 (Karnell *et al.*, 1991; Lindberg *et al.*, 1990). *S. flexneri* strain SFL124 was attenuated by deleting the *aroD* gene, making it auxotrophic for aromatic metabolites. This strain binds Congo red, a phenotype associated with the invasion plasmid, and is invasive, but is limited in its ability to multiply intracellularly. Challenge in monkeys resulted in complete protection against the wi d-type parent strain SFL1. In human trials this vaccine elicited an immune response, although not as great as one elicited by natural infection (Li *et al.*, 1993). Immunisation with strain SFL124 resulted in only mild, self-limiting reactogenicity in 9.5% of volunteers (Li *et al.*, 1992). This study also found that three doses resulted in a greater anti-LPS and anti-Ipa (invasion plasmid antigen) antibody response than a single dose. Mucosal immunity with this vaccine strain has been shown to last for at least one year (Li et al., 1993; Li et al., 1992). However, when given to children who have antibodies to *Shigella* LPS and Ipa proteins before immunisation, the vaccine strain elicited an increase in antibody response in only a few children (Li *et al.*, 1994), thus showing no booster effect. It has been shown that it is possible to convert strain SFL124 from a *S. flexneri* serotype Y to a serotype X strain by site-specific integration of the X-specific O-antigen (Guan & Verma, 1998). *S. flexneri* Y strain SFL124 is being used by several groups as a host strain for the development of live attenuated *Shigella* vaccines.

A *S. flexneri* 2a Δ*aroA* Δ*virG* strain was developed by Noriega *et al.* (1994) and designated CVD1203. The strain elicited a high level anti-LPS IgA response and was protective in homologous guinea pig conjunctival challenges. When human volunteers were immunised with this strain some reactogenicity was observed, presenting as fever, diarrhoea and dysentery (Kotloff *et al.*, 1996). The immune response elicited was specific, although dose-dependent. It was decided that further attenuation of this strain was required before production and immunisation trials were scaled up. In 1996, Noriega *et al.* (1996) used a *S. flexneri* 2a Δ*guaB-A* Δ*virG* mutant for investigation as a more attenuated vaccine candidate strain, CVD1205. This new strain elicited strong mucosal IgG and IgA responses to LPS after a single dose, with a marked boost of mucosal IgA levels after a second dose at day 14. Serum IgG and IgA levels were also significantly higher when compared to the controls following the boost at day 14. When tested in guinea pigs, there was protection against keratoconjuctivis after challenge. Immunisation with this strain also resulted in a mild, self-limiting

inflammatory response but in significantly fewer animals than those immunised with CVD1204 (Δ*guaB-A* only).

Further work by this group lead to the development of *S. flexneri* 2a strain CVD1207, a Δ*guaB-A* Δ*virG* Δ*set1* Δ*sen* strain. This strain has the genes encoding enterotoxin 1 (*set1*) (Noriega *et al.*, 1995) and enterotoxin 2 (*sen*) (Nataro *et al.*, 1995), the virulence gene *virG* and the *guaB-A* locus deleted. Strain CVD1207 was used in conjunction with CVD1211 (*S. flexneri* 3a Δ*guaB-A* Δ*virG* Δ*sen*) to elicit cross protection to *S. flexneri* Y, 1b, 2a, 2b, 3a, 3b and 5b serotypes in guinea pigs (Noriega *et al.*, 1999). This type of vaccine allows for a simplified vaccination strategy. As different *Shigella* spp. are endemic in different geographic areas it would be possible to design specific vaccines for different regions. It may also be possible to include *S. dysenteriae* 1 and *S. sonnei* O-SP in such a vaccine, giving a broad protection range. Strain CVD1207 has recently been used in phase 1 clinical trials and has been found to be highly attenuated in humans with doses up to 10^8 CFU (Kotloff *et al.*, 2000). CVD1207 also induced a dose-dependent increase in IgA secreting cells specific for *S. flexneri* 2a O-specific LPS. This vaccine is therefore ready for further clinical trials.

These attenuated strains are promising vaccine candidates, but at this time there has been limited data showing the duration of protection with any of these potential vaccine strains. Before any of them are considered as suitable vaccines, their ability to provide long term protection also needs to be investigated.

ACELLULAR VACCINES

Proteosomes are outer membrane complex vesicles derived from *Neisseria menigitidis*. Peptides alone are usually not immunogenic. However, coupling them to proteosomes results in high levels of specific IgG in mice, without the use of adjuvants. Therefore, proteosomes may provide a mode for peptide-based vaccination (Lowell *et al.*, 1988). The use of protesome-based LPS vaccines against *S. flexneri* 2a and *S. sonnei* resulted in good immune response following intranasal and oral immunisation in mice (Orr *et al.*, 1993). Homologous challenge in guinea pigs showed protection when compared to controls, suggesting that it may be possible to develop safe acellular vaccines against shigellosis. More recently, the intra-nasal administration of *S. flexneri* LPS complexed to meningococcal proteosomes was found to be well-tolerated in human volunteers (Fries *et al.*, 2001). This vaccine also elicited an IgA, IgG and IgM antibody secreting cell LPS-specific response similar to that seen with live vaccine candidates tested in human vaccine trials.

CONJUGATE VACCINES

In order to stimulate a greater specific antibody response following immunisation, several groups have attempted to make conjugate vaccines using O-SP from various *Shigella* spp. and other known immunogens. In 1991, Chu *et al.* (1991) used the *S. dysenteriae* 1 O-SP conjugated to tetanus toxoid as a potential vaccine candidate. The conjugate elicited greater serum IgM and IgG levels in mice than immunisation with O-SP alone. Use of an adjuvant such as alum also enhanced immunogenicity. Pozsgay *et al.* (1999) went a step further and used human serum albumin (HSA) conjugates of synthetic *S. dysenteriae* 1-like saccharides for immunisation. The synthetic saccharides elicited higher levels of serum IgG anti-LPS in mice than conjugates prepared with purified *S. dysenteriae* LPS.

Work on recombinant exoprotein A of *Pseudomonas aeroginosa* (*r*EPA) – *S. sonnei* conjugate vaccines has shown that while initial immunisations significantly raised serum IgG, IgM and IgA levels in adult volunteers, there was no boost effect seen with subsequent doses (Cohen *et al.*, 1996). IgA antibody levels, but not IgG or IgM antibody levels, to the conjugate vaccine dropped to preimmunisation levels within 56 days of immunisation, but the retention of high IgG levels should be sufficient to provide protection. A natural challenge of vaccine trial volunteers in the Israel Defence Force following an outbreak of *S. sonnei* showed a 74% protection efficacy against infection compared to unvaccinated controls (Cohen *et al.*, 1997). It was found that recipients of the Hepatitis B vaccine did not develop a significant antibody response to the *Shigella* vaccine (Cohen et al., 1996). This may be a problem, as people in endemic areas or troops going into such areas should be vaccinated against hepatitis B and shigellosis. However, it should be noted that the levels of antibody recorded post immunisation were comparable to those following natural infection in the Israel Defence Forces with *S. flexneri* 2a, *S. dysenteriae* and *S. sonnei* (Cohen et al., 1997).

Once a *Shigella* vaccine is developed and is ready for use, large-scale immunisation campaigns will be required in developing countries in order to attempt to stem the rate of infection and death, especially in young children.

SHIGELLA AND VIRULENCE

PASSAGE THROUGH THE GUT

Infection occurs following ingestion of virulent bacteria and their passage through the low pH of the stomach. *Shigella* has a higher tolerance for low pH than other closely related enteric pathogens such as *E. coli* and *Salmonella* (Gorden & Small, 1990), explaining why the infectious dose of *Shigella* is so small. As few as 10 bacteria are required to cause disease, while less than 500 bacteria are needed for a 50% infectious dose (DuPont et al., 1989; Levine *et al.*, 1973). The ability of *Shigella* to survive at low pH is due to the stationary phase-specific sigma factor, σ^s (or σ^{38}), a component of RNA polymerase. This factor increases the expression of stationary phase proteins such as catalase HPII and exonuclease III (Lange & Hengge-Aronis, 1991). The stationary phase proteins aid survival of bacteria in harsh environments characterised by high temperature, low pH and in the presence of hydrogen peroxide. The expression of at least two genes, *gadC* and *hdeA*, that are dependent on σ^s, is required for survival by *Shigella* at low pH (Waterman & Small, 1996).

Once the bacteria reach the small intestine they multiply, reaching concentrations as high as 10^7-10^9 bacteria per ml of lumenal fluid (DuPont, 1990). The bacteria then move into the large intestine, where they gain access to the colonic epithelial cells.

M CELLS AND MACROPHAGES

Shigellae gain access to the basolateral surface of epithelial cells via a sequence of events including uptake by M cells, phagocytosis by macrophages, apoptosis of macrophages and polymorphonuclear leukocyte (PMN) migration to the apical surface of the epithelial cells. The intestinal M cells provide a route for the bacteria into the colonic epithelium. The bacteria are then taken up by macrophages, which they are able to kill by apoptosis (Zychlinsky *et al.*, 1992). This killing promotes an inflammatory response by the release of cytokines from infected macrophages. This ultimately leads to more bacteria gaining access to the basolateral surface of the epithelial cells. Access to the basolateral surface of cells is important because it has been shown that *in vitro* shigellae primarily enter epithelial cells through their basolateral pole (Mounier *et al.*, 1992).

INVASION INTO THE EPITHELIAL CELLS

In 1964, LaBrec *et al.* (1964) demonstrated that invasion of epithelial cells by virulent *S. flexneri* 2a plays a critical role in the pathogenesis of bacillary dysentery. This finding is supported by the fact

that avirulent *Shigella* are unable to invade epithelial cells. The ability to invade is dependent on a 140 MDa plasmid that is carried by all virulent strains of *Shigella* (and is absent in avirulent strains) and enteroinvasive *E. coli* (Watanabe & Nakamura, 1986). The large virulence plasmid of a *S. flexneri* 5 strain has recently been sequenced by two groups (Buchrieser *et al.*, 2000; Venkatesan *et al.*, 2001). One of the most interesting findings was that 53% of the ORFs on the plasmid appeared to be IS-element related. This suggests that many of the features of the plasmid have been acquired by IS-mediated events. The virulence plasmid encodes the genes for the invasion plasmid antigens (Ipa), the Mxi-Spa type-III secretion apparatus and other virulence factors, including an enterotoxin (Figure 1a).

Upon contact between epithelial cells and the shigellae, there is a vigorous ruffling movement of the epithelial cell membrane which preceeds the directed-phagocytosis of the shigellae into the epithelial cell (Clerc & Sansonetti, 1987; Ogawa *et al.*, 1968) (Figure 1b). This phagocytosis occurs via the formation of pseudopodia which engulf the bacterium (Hale *et al.*, 1979), in a manner similar to that of professional phagocytes (Clerc *et al.*, 1988; Sansonetti, 2001). The shigellae entry sites are on the epithelial cell membrane at locations with an abundance of $\alpha5\beta1$ integrin molecules and actin polymerisation (Watarai *et al.*, 1996). At this adhesion site a focal adhesion complex that includes F-actin, vinculin and talin is formed. As the bacteria enter the cell they are surrounded by actin filament bundles (pseudopodia).

CONTACT TRIGGERS IPA RELEASE

Contact between the bacteria and the epithelial cell triggers the release of Ipa proteins into the extracellular medium (Watarai *et al.*, 1995a). The release of Ipas is more efficient on the basolateral surface of the epithelial cells than the apical surface. Ipa release is also more efficient during the exponential phase of growth or during cell division (Mounier *et al.*, 1997). This may be because during cell division the Mxi-Spa type-III secretion apparatus can be more easily incorporated into the bacterial membrane allowing greater secretion of the Ipa proteins. Secreted IpaB and IpaC promote the internalisation of the bacteria into the mammalian cells by inducing actin polymerisation (Menard *et al.*, 1996). This process is dependent on Rho, a ras-related, small GTP-binding protein as part of the signalling pathway for invasion (Watarai *et al.*, 1997).

Once the bacteria have gained entry to the cells they lyse the vacuole formed by their uptake and readily multiply within the cytosol. Sansonetti *et al.* (1986) have shown that within four to five hours of entry into epithelial cells bacterial cell numbers may have increased 100-fold.

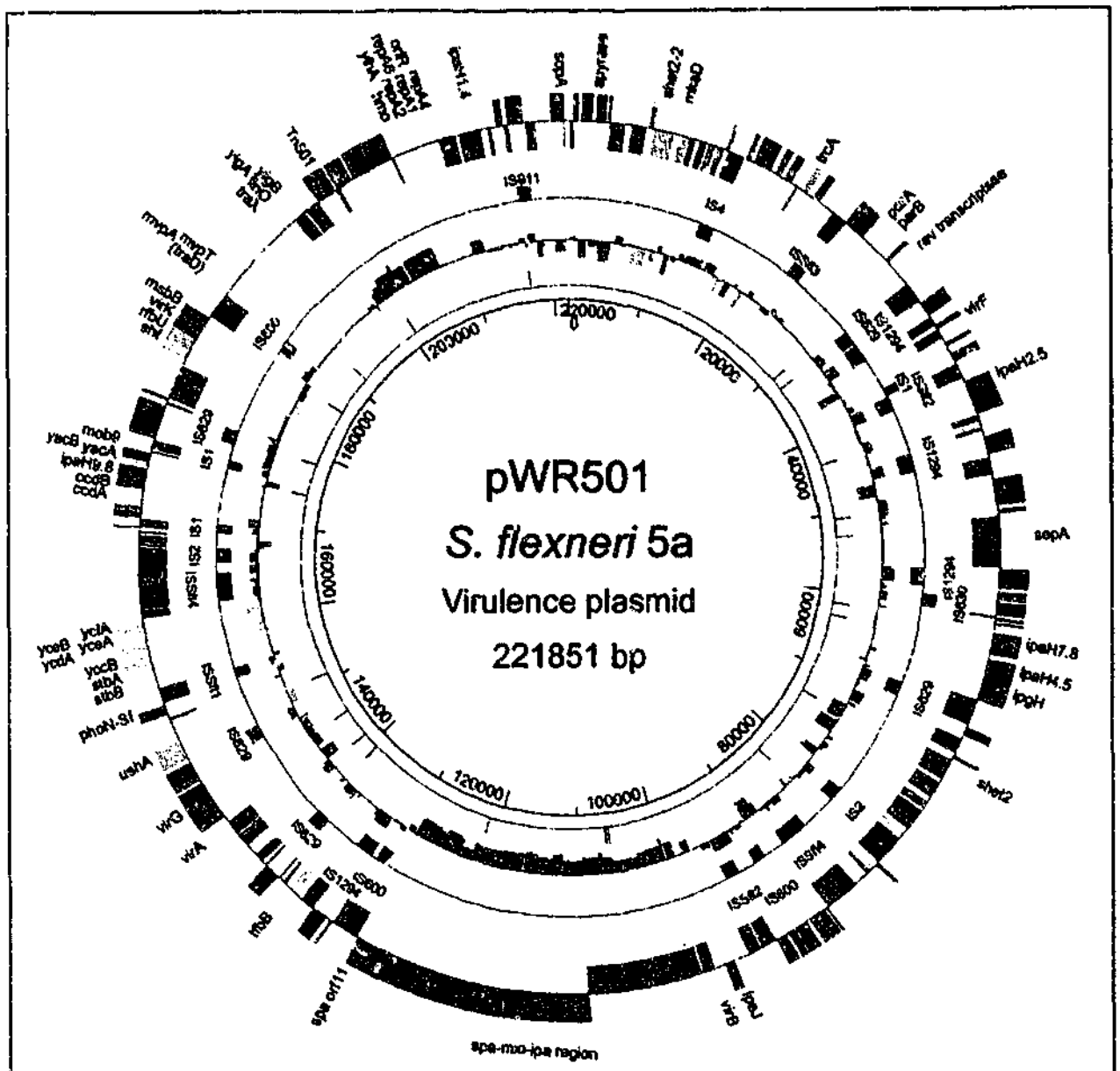


Figure 1a. Schematic representation of the *S. flexneri* 5 virulence plasmid. "Outer ring depicts ORFs and their orientation, colour coded according to functional category: 1, identical or essentially identical to known virulence-associated proteins (red); 2, homologous to known pathogenesis-associated proteins (pink); 3, highly cohomologous to IS elements or transposases (blue); 4, weakly homologus to IS elements and transposases (light blue); 5, homologous to proteins involved in replication, plamsid maintenance or other DNA metabolic functions ( ); 6, no significant similarity to any protein or ORF in the database (brown); 7, homologus or identical to conserved hypothetical ORFs, i.e. proteins of unknown function (orange); and 8, TN*501* insertion-associated genes (green). The second ring graphs G+C content, calculated for each ORF and plotted around the mean value for all ORFs, with each value colour coded for the corresponding ORF. Scale is in base pairs." (Venkatesan *et al.* 2001)

SHIGELLA *MOVEMENT ~ INTRA- AND INTERCELLULAR SPREAD*

Shigella spp. are described as non-motile bacteria. However, they have been found to move within epithelial cells (Ogawa *et al.*, 1968) and flagella have recently been identified on the surface of many *Shigella* strains (Giron, 1995). The presence of flagella appears to be regulated by strict genetic and environmental factors, and their role in intra- and intercellular movement remains undetermined.

Once within the cell's cytosol, the *Shigella* are coated by F-actin which polymerises at one pole of the bacterium to form a 'tail' (Bernardini *et al.*, 1989). This process involves VirG binding to and activating N-WASP, which then interacts with F-actin and mediates the attachment of the actin tail to the bacterium (Egile *et al.*, 1999) (Figure 1c). The F-actin tail is anchored to the plasma membrane and as it elongates the bacteria are propelled to the distal end of the cell (Sanger *et al.*, 1992). This mechanism of intracellular motility appears to be similar to that of *Listeria monocytogenes*. *virG* (*icsA*), is encoded on the *Shigella* virulence plasmid and is essential for intra- and intercellular movement because of its role in F-actin accumulation and polymerisation (Bernardini *et al.*, 1989; Makino *et al.*, 1986). The surface exposed VirG is under the control of VirF, a transcriptional regulator also encoded on the virulence plasmid (Lett *et al.*, 1989).

Figure 1b. Engulfment of *Shigella* by an epithelial cell. "Scanning electron micrograph of *Shigella flexneri* inducing membrane ruffles on the surface of an epithelial cell prior to its uptake". (Philpott *et al.*, 2000)

An additional type of intracellular movement, organelle-like movement (olm) has also been demonstrated by *S. flexneri* (Vasselon *et al.*, 1991). This involves binding and tracking along the stress fibres of the host cell. Although the significance of this movement is unknown, the movement seems to result in microcolony formation near the infected cell's nucleus. It is likely that the movement of bacteria along actin filaments is a combination of olm and VirG-mediated movement because as one promotes cellular colonisation along filaments, the other promotes intercellular spread (Vasselon *et al.*, 1992).

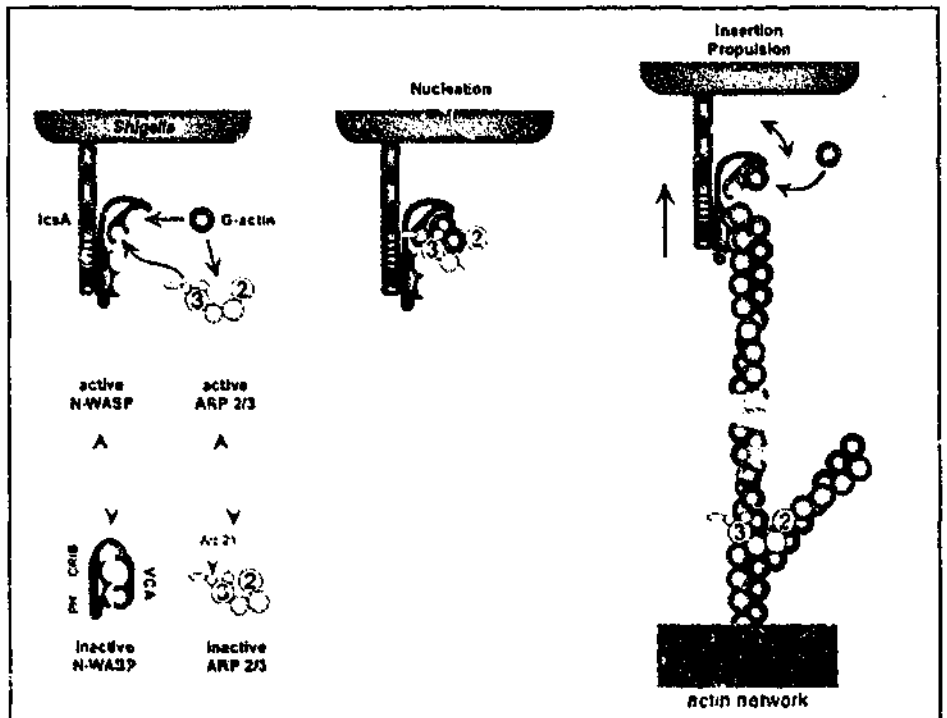


Figure 1c. Working model for actin-based movement of *Shigella*. "Left, Binding of N-WASP to IcsA at Shigella surface activates the connector, exposing the Arp2/3 complex, G-actin, and F-actin binding sites. Center, Nucleation: interaction of VCA with G-actin and Arp2/3 complex in a ternary complex in which the G-actin is positioned at the barbed end. Right, Barbed end growth and movement: the VCA domain of N-WASP shuttles G-actin subunits to the growing barbed end. The filament is maintained in close vicinity to the bacterium surface by binding the NH2-terminal domain of N-WASP." (Egile *et al.*, 1999)

When the bacteria reach the plasma membrane of the cell, a protrusion carrying bacteria, extends up to 18 μm into neighbouring cells (Kadurugamuwa *et al.*, 1991). This intercellular movement is similar to the initial passage from the extracellular environment into the cell. These protrusions are made from cytoskeletal proteins. Following cellular invasion by *Shigella*, actin accumulates at one pole of the bacterium, while the normally peripherally distributed vinculin becomes clustered around the intracellular bacteria in the cell's cytoplasm and intercellular protrusions. This suggests a possible role for vinculin in intercellular spread of shigellae during infection.

ULCERATION AND INFLAMMATION

The final step in *Shigella* pathogenesis is the destruction of the invaded enterocytes. In macrophage cell lines, it has been shown that killing can be completed within 4 hours (Sansonetti & Mounier, 1987). This killing of macrophages is not caused by toxins but by the induced apoptosis following ICE activation by IpaB (see "Immunity - Macrophages and polymorphonuclear leukocytes). While much is known about the ability of *Shigella* to cause apoptosis in macrophages, there has been little investigation into the mechanisms for enterocyte destruction. It may be that the level of enterocyte destruction caused by *Shigella* is minimal and that much of the ulceration and inflammation seen during shigellosis is due to macrophage death, which results in inflammatory cytokine release. PMN infiltration into the infected tissue and through the epithelial layers (as discussed in "Immunity") can cause considerable tissue damage and aid further *Shigella* invasion by allowing the bacteria access to the basolateral side of the epithelial cells. Therefore, tissue damage may be mainly due to the immune response to *Shigella* invasion. However, there is evidence that toxins produced by *Shigella* spp. may also be involved in colonic tissue damage.

SHIGELLA TOXINS

The Shiga toxin (stx) is responsible for the bloody, mucoid stools associated with infection with *S. dysenteriae* type 1. This toxin is a holotoxin composed of a single 32 kDa A-subunit that is associated with a pentamer of 7.7 kDa B-subunits. This structure was first reported by Yutsudo *et al.* (1986) who determined the subunit nature of the toxin and the approximate sizes of the two toxin subunits. Shiga toxin is part of a large family of proteins known as RIPs, ribosome-inactivating proteins. Shiga toxin has been found to bind to a well-conserved aminoacyl-tRNA-accepting loop of rRNA (Skinner & Jackson, 1997). Further work has shown that this binding allows Shiga toxin to act as an inhibitor of prokaryotic protein synthesis (Skinner & Jackson, 1998). The importance of this in *S. dysenteriae* pathogenesis has not yet been addressed. A two gene operon, *stxAB*, encodes the toxin. This operon is present as a single copy in the chromosome of *S. dysenteriae* 1 and a single *S. sonnei*

strain CB7888 (Strauch *et al.*, 2001; Strockbine *et al.*, 1988). The *stxAB* genes share greater than 99% sequence identity with the Shiga-like toxin genes (SLT-I) of enterhaemorrhagic *E. coli* (EHEC), the two proteins differing by only a single amino acid. The SLT-I genes show some weak homology to *S. flexneri* 2a DNA but to no other *Shigella* strain tested.

Unlike the EHEC SLTs, the *S. dysenteriae* 1 toxin genes are not encoded by an intact bacteriphage. However, they have been found to amplify and delete as the phage-derived toxins do. A tandem amplification of the *stx* operon has been found in some *S. dysenteriae* 1 chromosomes and deletions of the *stx* genes are induced in anaerobic conditions on chlorate-containing medium (McDonough & Butterton, 1999). IS*600* elements flanking the region containing *stxAB* are believed to be responsible for the amplification and deletion of the genes. The deletion is believed to be aided by *fur*, a global anaerobic regulator gene in the same region. It was postulated that the ability of the *S. dysenteriae* 1 Shiga toxin encoding fragment to amplify and delete may be due to a lambdoid origin as seen with the EHEC SLTs. However, over time a variety of IS-element insertions and chromosomal rearrangements has resulted in the loss of phage function in *S. dysenteriae* 1. Genetic analysis of the *stx* locus of Shiga toxin-producing *E. coli* (STEC) and *S. dysenteriae* 1 strains suggests that the region in both species is phage-borne, although not all loci are encoded by intact, functional phages (Unkmeir & Schmidt, 2000). Interestingly, unlike the *stx* genes from *S. dysenteriae* 1, the *stx* genes of *S. sonnei* are naturally transferable and are carried on an intact phage, 7888 (Strauch *et al.*, 2001).

The importance of Shiga toxin in pathogenicity was demonstrated using macaque monkeys as a model for shigellosis (Fontaine *et al.*, 1988). It was found that toxin production was associated with "blood within stools, a sharp drop in blood polymorphonuclear cells, and histopathological alterations, such as the destruction of capillary vessels within the connective tissue of the colonic mucosa, severe inflammatory vasculitis of the peritoneal mesothelium, and major efflux of inflammatory cells to the intestinal lumen." This damage is believed to be due to colonic vascular destruction caused by the toxin. The same study also found that Shiga toxin did not affect rates of intracellular bacterial multiplication or killing of host cells, suggesting its role was in the inflammation associated with bacillary dysentery. The ability of the SLT-1 in EHEC to cause a superinduction of IL-8 in human colonic epithelial cell lines suggests that it may be important for the initiation of the host inflammatory response to EHEC and *S. dysenteriae* infections (Thorpe *et al.*, 1999).

S. flexneri 2a strains also produce a toxin, ShET1 (*Shigella* enterotoxin 1). This iron-regulated toxin is encoded by two chromosomal open reading frames, *set1AB* (Fasano *et al.*, 1995) carried on a pathogenicity island (PAI), the *she* PAI (Rajakumar *et al.*, 1997). Fluid accumulation in rabbit ileal loops perfused with ShET1 showed that the toxin is responsible for the watery diarrhoea phase of disease associated with *S. flexneri* 2a (Fasano *et al.*, 1997). ShET1 is present in all *S. flexneri* 2a

clinical isolates tested but only in 3.3% of other *Shigella* isolates and in no EIEC strains (Noriega *et al.*, 1995). As it is found almost exclusively in *S. flexneri* 2a strains, the production of ShET1 toxin may explain the predominance of this serotype in the developing world.

A third toxin encoded by the virulence plasmid of EIEC was identified by Nataro *et al.*, (1995) using Ussing chamber toxin assays, subcloning and sequencing. This toxin, designated ShET2, is also encoded by the *S. flexneri* 2a virulence plasmid (Fasano *et al.*, 1995). The gene, *sen* (*Shigella* enterotoxin) was found in 75% and 83% of EIEC and *Shigella* strains tested respectively.

VIRULENCE LOCI OF S. FLEXNERI

S. flexneri virulence requires both chromosomal and plasmid-borne factors. The importance of the 140 MDa plasmid was first described by Sansonetti *et al.* (1982) who discovered that loss and return of the plasmid resulted in avirulence and virulence respectively. The virulence plasmid is conserved across the *Shigella* species and is necessary for virulence in all *Shigella* strains (Watanabe & Nakamura, 1986). Figure 1a shows a schematic representation of the virulence plasmid as sequenced by Venkatesan *et al.* (2001). The plasmid also requires some chromosomal genes for regulation of the genes required for epithelial cell invasion. The next few sections discuss the genes and loci that are important for virulence of *S. flexneri* and their regulatory mechanisms.

THE INVASION PLASMID ANTIGENS (IPA)

The *Shigella* virulence (invasion) plasmid encodes most of the proteins required for cellular invasion. The invasion plasmid antigens (Ipa) are critical for invasion. Ipa were first identified in *S. flexneri* by Baudry *et al.* (1987) as four proteins encoded on a 20 kb region of the 140 MDa plasmid. This region showed striking restriction mapping similarity to a region in *S. sonnei* that was also required for invasion. Early mapping of *ipaBCD* on the virulence plasmid was carried out by Buysse *et al.* (1987). The genes were part of an operon that includes *ipgC* (Figure 1a). Nucleotide sequencing of *ipaB*, *ipaC* and *ipaD* by Venkatesan *et al.* (1991a) predicted proteins of 62 kDa, 42 kDa and 37 kDa respectively. These proteins were shown to be temperature-regulated at the transcriptional level.

Ipa produced by *Shigella* accumulate within the cytoplasm, with only a few membrane-associated molecules of IpaB and IpaC exposed. Upon contact with the basolateral surface of the host epithelial cell there is a rapid release of IpaB, IpaC and IpaD into the extracellular matrix. This release is most efficient at the basolateral rather than apical epithelial cell surface because of the extracellular matrix

proteins, such as fibronectin and collagen type IV, that act as triggers for Ipa release (Watarai *et al.*, 1995a).

The steps of *Shigella* invasion are: adhesion to the cell surface, entry into the cell and lysis of the vacuole to gain access to the epithelial cell cytoplasm. These processes involve IpaA, IpaC and IpaB respectively. The role of IpaB in invasion has been shown by demonstrating that *ipaB* mutants are non-invasive, have decreased actin polymerisation and are unable to lyse the membranes of vacuoles containing internalised bacteria (High *et al.*, 1992). IpaB also mediates contact haemolysis *in vitro* (Blocker *et al.*, 1999; High *et al.*, 1992) and is necessary for *Shigella*-induced apoptosis and escape from macrophages (Zychlinsky *et al.*, 1994).

IpaA is composed of 633 amino acids and has a predicted molecular mass of 70 kDa (Venkatesan *et al.*, 1991b). *ipaA* mutants have a ten-fold reduction in invasion but unlike other *ipa* mutants this reduction is not attributable to impaired bacterial internalisation. The specific role of IpaA has recently been proposed to be in facilitating access of the bacterium to the host cell surface. IpaA is secreted upon contact with the host cell and rapidly associates with vinculin, an actin-binding protein (Tran Van Nhieu *et al.*, 1997). When complexed to vinculin, IpaA is able to induce actin depolymerisation (Bourdet-Sicard *et al.*, 1999). It is postulated that this actin depolymerisation aids in bacterial access to the host cell by making the host cell surface more hospitable for the bacterial cell. The association of IpaA and IpgD in the extracellular milieu suggests that IpgD may also have a role in the association of the bacterium and host cell. This is supported by the fact that IpgD is secreted through the Mxi-Spa type III secretion system in similar amounts to IpaABCD and its ability to modulate the host cell response following contact by the bacterium (Niebuhr *et al.*, 2000).

IpaB, IpaC and IpaD accumulate within the bacterial cytoplasm before cell contact. In the cytoplasm, IpgC individually binds to IpaB and IpaC in order to inhibit the formation of IpaB-IpaC complexes and prevent premature oligomerisation of these proteins (Menard *et al.*, 1994a). That is, IpgC stabilises IpaB and prevents IpaC degradation in the cytoplasm. Upon secretion into the extracellular environment IpaB and IpaC form a complex. The role of the IpaBC complex is implied by findings that latex beads coated with IpaBC complex are taken up by HeLa cells in a phagocytosis similar to that seen during *Shigella* entry into cells (Menard *et al.*, 1996). There is a close association between the secretion of the IpaBC complex and an early recruitment of cytoskeletal-associated proteins. More specifically, the co-localisation of IpaC with actin and cortactin shows the importance of this secreted protein in cytoskeletal rearrangements and the complex formation required for *Shigella* entry (Mounier *et al.*, 1997; Tran Van Nhieu *et al.*, 1999). IpaC is capable of actin polymerisation, unlike IpaA or IpaD, making it critical for entry into the epithelial cells. IpaC is also necessary for the activation of Cdc42, which leads to Rac activation. These GTPases are also required for *Shigella*

entry. IpaC is also required for escape from phagosomes (Barzu *et al.*, 1997), an interaction modulated by pH (De Geyter *et al.*, 1997).

IpaD is required for the modulation of IpaB and IpaC transport (Menard *et al.*, 1994b). IpaD complexes with IpaB and probably Spa32 (see 'Type III Secretion in *S. flexneri*') in the bacterial membrane. Mutation of *ipaD* prevents formation of the IpaBD complex. Consequently, IpaB and IpaC are secreted into the extracellular milieu without the requirement for any environmental signal to trigger their release. *ipaD* mutants are not invasive, possibly because IpaB and IpaC are released too early and the concentration required for epithelial cell entry would not be available upon cell contact. The role of IpaD in protein transport is supported by findings that show the protein to have surface exposed domains (Turbyfill *et al.*, 1998). It has also been shown that insertional mutagenesis of *ipaB* and *ipaD* leads to greater secretion of other *Shigella* proteins via the Mxi-Spa translocon without the necessity of environmental triggers (Parsot *et al.*, 1995).

The transport and release of the Ipa proteins is dependent on proteins encoded by two other operons on the virulence plasmid - the *mxi* and *spa* operons.

TYPE III SECRETION IN S. FLEXNERI

Type III secretion systems are required for the secretion of proteins without signal sequences. These systems are independent of the *sec* pathway, unlike secretion pathways II and IV. The other *sec*-independent pathway, type I, consist of three proteins compared to the approximately 20 proteins of the type III pathway. Type III protein secretion is often regulated by contact with eukaryotic cells. In several Gram-negative bacteria these type III secretion systems are critical for virulence and several are carried on pathogenicity islands. *Salmonella typhi* Typhimurium carries the *spa* genes on SPI-1, as well as a second type III system on SPI-2, which are essential for its ability to invade due to their role in the secretion of the Inv proteins (Gálan, 1996; Shea *et al.*, 1996). The *E. coli* LEE PAI also encodes a type III secretion system that is necessary for the virulence of enteropathogenic *E. coli* (EPEC) (Donnenberg *et al.*, 1997).

The *S. flexneri* type III secretion system is encoded by two operons, consisting of fifteen and eight genes respectively, located on the *Shigella* virulence plasmid. The protein products of these operons form the Mxi-Spa translocon. The *spa* genes are required for the presentation of IpaB and IpaC on the cell surface (Venkatesan *et al.*, 1992). In 1993, Sasakawa *et al.* (1993) confirmed this finding using random mutagenesis of the virulence plasmid and showed that the genes were essential for epithelial cell invasion. Eight of the nine *spa* genes are encoded in a single operon and each gene is critical for invasion. Like the *ipa*, the *spa* genes are regulated by VirB (Sasakawa *et al.*, 1993). The

S. Typhimurium *spa* genes are homologous to the *S. flexneri spa* genes and *S.* Typhimurium *spa* mutants can be complemented with *S. flexneri spa* genes (Groisman & Ochman, 1993). The *yscN/U* gene cluster of *Y. pseudotuberculosis* is also very similar in sequence and genetic organisation to the *spa* operons of *S. flexneri* and *S.* Typhimurium. These type III secretion systems also secrete related invasins, the Ipa, Inv and Yop proteins. The conservation of the type III secretions genes and necessity for invasion in all three species suggests they have a common ancestor.

The contact-triggered release of IpaBC coincides with a decrease in Ipa concentration on the bacterial cell surface. This release of Ipa depends on Spa32, a surface-located protein that forms part of the Mxi-Spa type III secretion system (Watarai *et al.*, 1995a). It has also been found that *spa32* mutants are unable to elicit actin polymerisation at the site of epithelial cell contact, probably due to the lack of Ipa release. A chromosomally located gene, *dsbA* encodes a disulfide oxidoreductase protein that is required for the correct folding of Spa32. Incorrect folding of Spa32 leads to a lack of Ipa transport across the bacterial membrane, preventing Ipa release, making DsbA critical for *Shigella* invasion as well (Watarai *et al.*, 1995b).

The role of the *mxi* operon was proposed following the suggestion that IpaB and IpaC were "truly secreted proteins" whose secretion was facilitated by MxiA (Andrews *et al.*, 1991). Further work on *mxiA* showed it was a homologue of *lcrD* of *Y. pestis*, from the low-calcium response locus. LcrD is an inner membrane regulatory protein with an N-terminal anchor and a role in facilitating the export of proteins to the outer membrane (Andrews & Maurelli, 1992). This suggested the potential role of MxiA in secretion in *S. flexneri*.

Investigation of other Mxi proteins has shown that at least MxiG, MxiM and MxiD are critical for Ipa secretion and therefore cellular invasion. MxiG is a 42 kDa protein associated with the inner and outer membrane of the *S. flexneri* cell. Mutational analysis of *mxiG* has shown that the Mxi-Spa translocon is required for cell entry and intercellular spread (Allaoui *et al.*, 1995). This is most likely due to the requirement for Ipa secretion. MxiD is an outer membrane protein that shows homology to YscC of *Y. enterocolitica*, which is part of the *Yersinia* spp. type III secretion system and is required for extracellular secretion (Allaoui *et al.*, 1993).

MxiM is a lipoprotein that associates with the cell envelope. While it is secreted independently of the type III pathway, it is required for type III protein secretion. It has a role in either the assembly or function (or both) of the Mxi-Spa translocon in the outer, but not inner, membrane (Schuch & Maurelli, 1999). MxiM has no homologues in the databases suggesting that it may have a *Shigella* specific function.

The structure of the *Shigella* type III secretion system has recently been shown to be a needle-like structure (Figure 1d). The structure is composed of a needle and basal components. The major components of this structure are MxiD, MxiG, MxiJ and MxiH (Tamano *et al.*, 2000). MxiH is the needle component and essential for the secretion of effector proteins. Interestingly, the level of MxiH production effects the length of the needle. *spa47* is required for the transport of MxiH. Work by Blocker *et al.* (2001) showed that MxiG and MxiJ appear to form the base of the needle in the bacterial inner membrane and MxiD forms the outer ring in the outer membrane.

As discussed in 'Invasion Plasmid Antigens', site-specific mutation of *ipaB* and *ipaD* results in enhanced secretion of IpaC thought the Mxi-Spa translocon (Menard *et al.*, 1994b). IpaB secretion is also enhanced through the translocon following mutation of *ipaD*. It is possible that IpaB and IpaD could form part of the Mxi-Spa translocon in the outer membrane as they are able to associate with the bacterial membrane. The increased secretion following their mutation suggests that they act as a lock for the pathway. IpaB and IpaD mutations not only results in increased IpaB and IpaC secretion through the Mxi-Spa translocon, but also increased secretion of 15 other proteins, including IpaA. These proteins range in size from 12-35 kDa, excluding the Ipa proteins (Parsot *et al.*, 1995). The larger of these proteins are barely detectable in wild-type strains, but can be readily detected in IpaB and IpaD mutants. All of these proteins require the Mxi-Spa translocon for secretion because mutation of *mxiD* disrupts the translocon and prevents the secretion of these 15 proteins, even in a *mxiD/ipaD* double mutant.

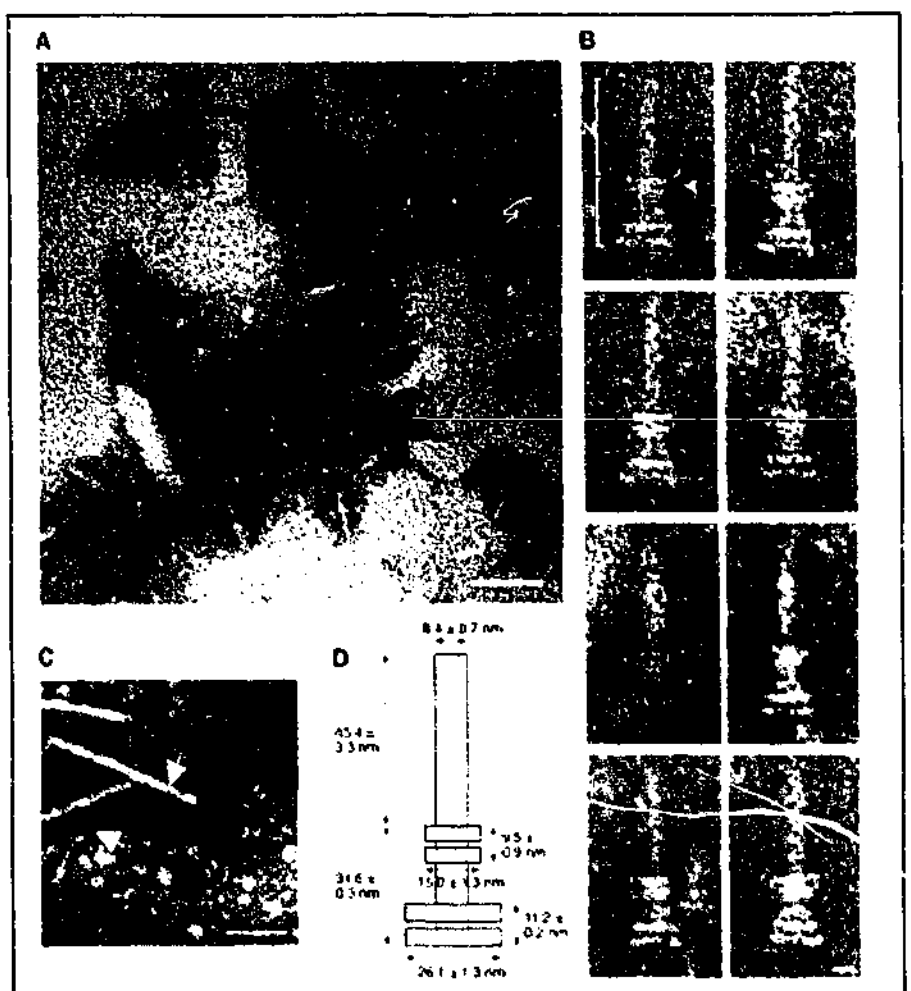


Figure 1d. The *S. flexneri* Needle-like Complex. "(A) Purified type III secretion complexes at a low magnification. Scale bar, 100 nm. (B) Type III secretion complexes at a high magnification. N, indicates the needle, while B indicates the basal part of the complex. The white arrowhead denotes upper (or outer) rings, while the black arrowhead denotes the lower (or inner) rings. Scale bar, 10 nm. (C) Measurement of the size of type III secretion complexes. The purified type III secretion complexes under TEM with TMV (300 nm long, 20 nm wide (yellow arrowheads). Scale bar, 100 nm). (D) Proposed size of the type III secretion complex of *S. flexneri*." (Tamano *et al.*, 2000)

REGULATION OF VIRULENCE IN S. FLEXNERI

Regulation of the *Shigella* invasion system is under the tight control of the VirF/VirB pathway. In 1990, Watanabe *et al.* (1990) identified a protein, InvE, in *S. sonnei* that showed significant sequence similarity to DNA binding proteins, suggesting a regulatory role for the protein. A homologue of *invE* was identified in *S. flexneri* and named *virB*. Adler *et al.* (1989) demonstrated that VirB is a positive regulator of *ipaB*, *ipaC* and *ipaD* transcription at 37°C. This group also proposed that VirF was required for *virB* activation and *virG* regulation.

Tobe *et al.* (1991) found that *virB* was transcribed from its own promoter at 37°C but not at 30°C. However, if *virB* was placed under the control of a *tac* promoter, invasion was possible at both 30°C and 37°C. This showed that temperature-dependent regulation occurred at the *virB* level rather than at the level of the invasion operons (*ipa*, *mxi-spa*). The same group also showed that *virF* is not as tightly controlled by temperature as *virB*. This tight thermoregulation is probably due to the changes in DNA superhelicity of the *virB* regulatory region caused by temperature changes. By inducing negative supercoiling, even at 30°C, it is possible to express *virB*, although it is still dependent on VirF (Tobe *et al.*, 1995).

The AraC-like regulator, VirF, is encoded on the *S. flexneri* virulence plasmid and is conserved amongst *Shigella* and EIEC strains (Sakai *et al.*, 1988). Unlike *virB* it does not act directly on the *ipa* operon; instead it activates *virB* which results in *ipa* and *mxi-spa* expression (Adler *et al.*, 1989). VirF also positively regulates *virG* expression (Sakai *et al.*, 1988). Therefore, VirF has an important role in invasion, intra- and intercellular spread. As well as temperature and pH, oxygen tension also affects *virF* expression. *virF* expression is 30 to 38-fold greater in aerobic than anaerobic conditions (Dorman & Porter, 1998). Interestingly, VirF also has some role in repression of the *virF* gene (Porter & Dorman, 1997a). Environmental and genetic controls of *virF* and *virB* are summarised in Figure 1e.

The importance of H-NS for virulence of *S. flexneri* lies in its control of *virB* and *virF* transcription. Both VirF and H-NS bind to the promoter region of *virB* (Tobe *et al.*, 1993). While VirF activates transcription of *virB*, H-NS blocks transcription. *virB* regulation has been shown to be dependent on DNA supercoiling, and as H-NS blocks *virB* transcription it is possible that H-NS repression of *virB* is due to inhibition of negative supercoiling of the region upstream of *virB*. Prosseda *et al.* (1998) found that *virF* has, around its promoter, an intrinsically curved region for which H-NS has a high-affinity. H-NS repression of *virF* and *virB* is temperature dependent, with transcripton of both genes repressed

at 30°C (Porter & Dorman, 1997a). It was found that H-NS represses *virF* at both low temperature and low pH, ensuring that the invasion genes are switched on in the correct location for invasion.

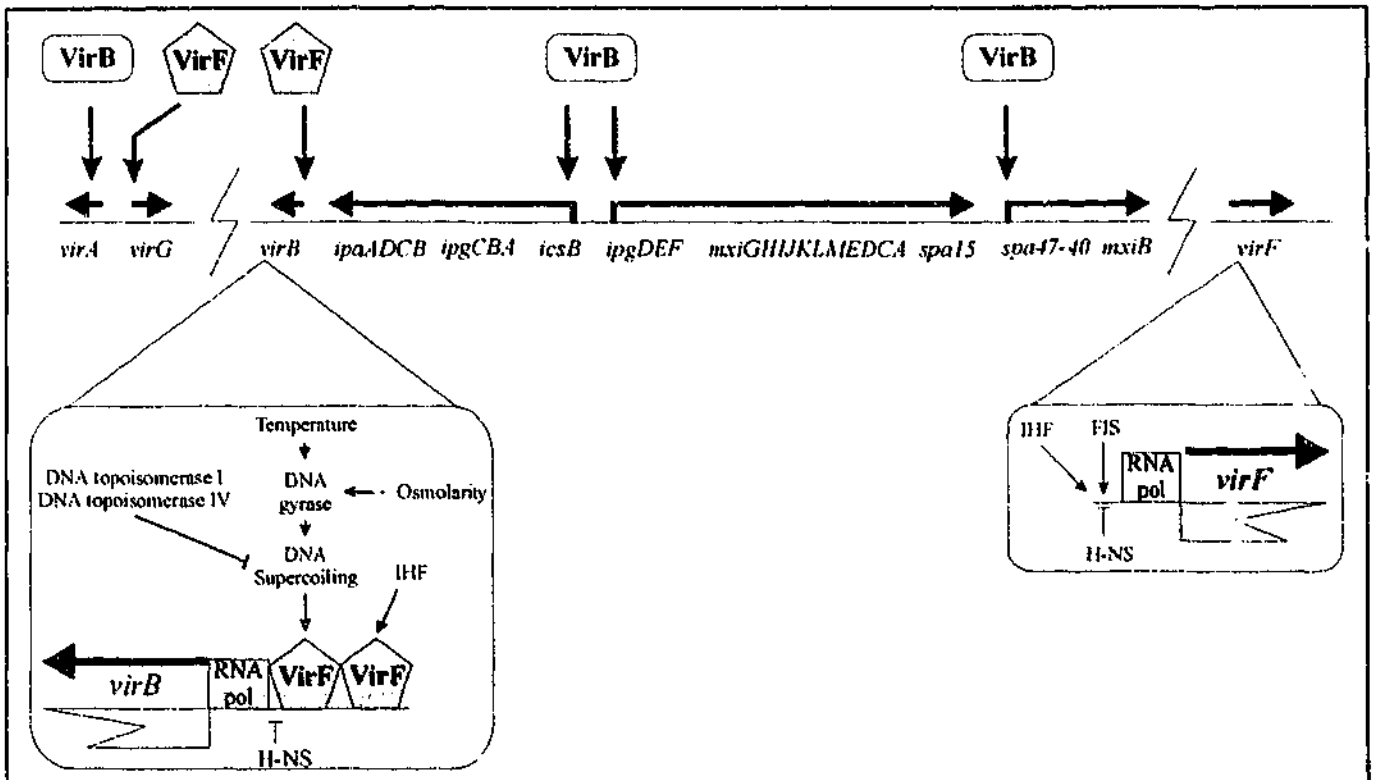


Figure 1e. Regulation of the *Shigella* virulence cascade. (Adapted from Dorman & Porter, 1998). The upper section of the figure summarises the genetic structure of a 31 kb virulence region of the 230 kb plasmid and the regulatory inputs of VirF and VirB. Regulation of *virF* and *virB* expression is summarised in the lower section of the figure. Positive regulation is represented by arrows (➔) while negative regulation is represented by truncated lines (⊥). Not to scale.

In contrast to H-NS, FIS (factor for inversion stimulation) has been found to positively regulate *virF* at 37°C (Falconi *et al.*, 2001). At transition temperatures (32°C) FIS appears to partially counteract H-NS repression of *virF* possibly because two of the four FIS binding sites upstream of *virF* overlap the H-NS binding sites. This would result in a rapid increase in VirF at higher temperatures. The expression of VirF is controlled by several factors including tRNA modification, temperature and DNA superhelicity (Durand *et al.*, 2000). As VirF concentration is the critical factor in the regulation of *Shigella* virulence (Durand *et al.*, 2000) its tight control by positive and negative regulators is essential.

Integration host factor (IHF) is a nucleoid-associated protein that, unlike H-NS, enhances *virB* expression in stationary phase and stimulates *virF* expression in logarithmic and early-stationary phase (Porter & Dorman, 1997b). The primary role of IHF is probably to overcome H-NS repression, as it plays a direct role at the level of *virF* and *virB* transcription. IHF has also been found to bind to the *virG* promoter.

Mills *et al.* (1992) found an insertion of an IS*1*-like element into the *virF* open-reading frame resulted in avirulence of *S. flexneri* strain 2457O. The insertion has been found to recur in the same location and orientation of the ORF approximately once in every 10^4 cell divisions. These variants were

identified because of a different colony morphology on MEA medium, originally noted by Formal and colleagues (DuPont *et al.*, 1972). The attenuated strain, 2457O, was used in clinical trials but was found to revert to the virulence in the patient. Schuch *et al.* (1997) have found that *virF* and *virB* are often deleted or inactivated under laboratory conditions. This group proposed that an avirulent strain might have a selective advantage in the environment because energy is conserved and not spent on unrequired virulence genes. This would mean that strains that are capable of reversion could reactivate their virulence genes when they enter a host and invade giving them an advantage over strains with a deletion of virulence associated regions or loss of the virulence plasmid. The insertion of the IS*1*-like element offers an interesting regulatory mechanism and may imply a role for other IS-elements in regulation.

Table 1 shows other virulence genes from *Shigella* spp. that were not discussed in detail in the text.

Table 1. *Shigella* virulence-related genes

a) PLASMID-BORNE

Gene/ Locus	Role in Virulence	Other Information	Reference
virK	• Required for intra- & intercelluar spreading	• Role in *virG* expression at post-translational level • Essential for virulence	Nakata *et al.*, (1992)
virA	• Required for intercellular spread	• Located near *virG* • Secreted through Mxi-Spa system • Slight affect on *virG* expression • Regulated by *virB* • May have *cis*-acting role on *virG* promoter	Demers *et al.*, (1998) Uchiya *et al.*, (1995)
ipgDEF	• Associated with IpaA	• Part of *mxi* operon • Mutants do not effect bacteria's ability to cause plaques or keratoconjuctivitis in guinea pigs.	Allaoui *et al.*, (1993b)
impB	• Enhanced UV radiation resistance	• Probable role in error-prone DNA repair • May give some strains a selective advantage	Runyen-Janecky *et al.* (1999)
icsB	• Role in intercellular spread	• Mutation effects plaque size but not ability to invade.	Allaoui *et al*, (1992)
apy	• Unknown	• On virulence plasmid of *S. flexneri* & EIEC • apyrase (ATP-diphosphohydrolase) • Expression controlled by temperature, H-NS & *virF*/*virB* cascade	Berlutti *et al.*, (1998)

Table 1 *continued*

b) CHROMOSOME-BORNE

Gene/ Locus	Role in Virulence	Other Information	Reference
vacB	• Regulation of invasion and intra- & intercellular	• Disruption leads to decreased invasive capacity in *Shigella* & EIEC. • Acts on IpaBCD and VirG at translational level	Tobe *et al.* (1992)
virR	• Repression of virulence genes at 30°C	• Encodes H-NS	Maurelli & Sansonetti (1988)
ompC	• Required for intercellular spread and cell killing	• Regulated by OmpR-EnvZ • Expressed at all osmolarities in *S. flexneri* • Possible role intracellular multiplication	Bernardini *et al.* (1990) Bernardini *et al.* (1993)
topA	• Role in supercoiling DNA	• Encodes DNA topoisomerase I • Mutation leads to increase in negatively supercoiled DNA and hypersensitivity to media osmolarity • Mutation results in slower growth rate	Bhriain & Dorman (1993)
sodB	• Resistance to phagocytosis	• Encodes iron-containing superoxide dismutase • Mutation results in avirulence in rabbit ileal loops	Franzon *et al.* (1990)
pic	• Mucinase activity, serum resistance, haemaglutination	• Located on *she* PAI • Secreted protease • SHeT1 enterotoxin also encoded within *pic* gene	Henderson *et al.* (1999)
ispA	• Effects intercellular spread	• Required for septum formation and thus critical for cell division. • Essential for virulence	Hong *et al.* (1998) Mac Siomoin *et al.* (1996)
rpoS	• Regulates acid & base resistance	• Encodes a growth-phase dependent sigma factor σ^{38} • Regulation is dependent on growth conditions (i.e. pH and aerobicity	Small *et al.* (1994)
cydC	• Required for intracellular survival	• Encodes cytochrome *bd*	Way *et al.* (1999)

PATHOGENICITY ISLANDS

A relatively recent but expanding area of investigation into bacterial pathogenesis is the study of 'pathogenicity islands' (PAIs). The term PAI refers to a region of virulence genes that forms a distinct molecular and functional unit on a bacterial chromosome (Lee, 1996). In 1997, Hacker *et al.* (1997) described the defining characteristics of pathogenicity islands:

"i. carriage of one (often many) virulence gene.

ii. Presence in pathogenic strains, and absence or sporadic distribution in less-pathogenic strains of one species or a related species.

iii. Different G+C content in comparison to DNA of host bacteria.

iv. Occupation of large chromosomal regions (often >30 kb).

v. Represent compact, distinct genetic units, often flanked by direct repeats.

vi. Association with tRNA genes and/or insertion sequence (IS) elements at their boundaries.

vii. Presence of (often cryptic) 'mobility' genes (IS elements, integrases, transposases).

viii. Instability."

Based on these criteria, a role for PAIs in microbial evolution has been suggested. Stable transfer and optimal gene expression of PAIs allow for the rapid evolution of bacteria through the simultaneous acquisition (or loss) of a large number of genes (Häcker *et al.*, 1997). For example, acquisition of the LEE PAI encoding enterocyte attachment and effacement by *E. coli* would probably be sufficient to make the strain pathogenic (Groisman & Ochman, 1996).

PAIs have been found in a variety of species and locations within Gram negative and some Gram positive bacterial genomes. At least five unique PAIs have been identified in various strains of *E. coli* and five different PAIs in *Salmonella* strains. Table 2 shows PAIs identified in a variety of Gram negative bacteria and some of their features.

An element related to pathogenicity islands has recently been described by (Hochhut & Waldor, 1999). A CONSTIN is a conjugative self-transmissible, integrating element. The sole CONSTIN identified is in a *Vibrio cholerae* O39 strain that causes epidemic cholera. This strain is important because it is characteristically resistant to the antibiotics sulphamethoxazole, trimethoprim, chloramphenicol and streptomycin. These resistances are encoded on a 62 kb CONSTIN. The element (SXT element) inserts into the *prfC* gene and the 5' end of the element reconstitutes the *prfC* gene upon integration. Unlike the SXT element, the PAIs so far identified have not been shown to be conjugative. It is possible that more CONSTINs will be identified in a range of species, and some current PAIs may prove to be CONSTINs.

The genome of virulent *V. cholerae* has another interesting feature. In order to cause cholera the bacteria must by lysogenised by the CTX-phage that encodes the cholera toxin. For this phage to lysogenise the bacteria, it must bind to the TCP (toxin-coregulated pilus) receptor on the cell surface. This receptor is not encoded by all *V. cholerae* strains. The TCP receptor, a type IV pilus, is encoded on a PAI, the *Vibrio* pathogenicity island (VPI). It has recently been found that this PAI is actually part of a prophage (VPIϕ) (Karaolis *et al.*, 1999). This is the first report of a pathogenicity island being part of a prophage; however, other PAIs have some characterisitics of phages. The LEE PAI of enterohaemorrhagic *E. coli* (EHEC) possesses a string of open reading frames from a proposed prophage, 933L (Perna *et al.*, 1998). Remnants and some intact copies of these prophage genes have also been found on the *she* PAI of *S. flexneri* 2a (Al-Hasani *et al.*, 2001). P4-bacteriophage-like integrases have also been associated with a number of PAIs including *she*, VPI (Karaolis *et al.*, 1998), *D. nodosus* PAI (Cheetham & Katz, 1995) and *Mesorhizobium loti* (Sullivan & Ronson, 1998). This association with phages may explain some of the instability of PAIs as well as their preference for tRNA insertion sites.

Table 2. Gram negative bacterial pathogenicity and genomic islands

Microorganism	PAI	Size (kb)	Role in Virulence	Flanking regions	tRNA*	References
Vibrio cholerae	VPI	39.5	• Cholera toxin phage receptor • ToxT - regulates virulence genes • Essential colonisation factor	att sites *ssrA* - 10sRNA gene	None	Karaolis *et al.* (1998)
Enterotoxigenic *Bacteroides fragilis*	Fragilysin pathogenicity islet	6.3	• Fragilysin (extracellular metalloprotease toxin)	12 bp near perfect repeat	None	Moncrief *et al.* (1998)
Mesorhizobium loti	Symbiosis Island	>500	• Restores prototrophy to strains auxotrophic for biotin, thiamin and nicotinate	left - *phe* right - direct repeats	*phe*	Sullivan & Ronson (1998)
Yersinia spp.[a]	High Pathogenicity Island (HPI)	45[b] 35[c]	• *fyuA-irp* - siderphore Yersiniabactin	IS*100* - *Y. pestis*, *Y.pseudotuberculosis* IS*1400* - *Y. enterocolitica* 17 bp direct repeats - all	*asn*	Buchrieser *et al.*, (1998b) Buchrieser *et al.*, (1998a), Carniel *et al.* (1996), Karch *et al.* (1999), Schubert *et al.* (1998)
Helicobacter pylori type I	cag	37	• *cagA* - virulence associated • Possible toxin gene	IS*605* 31 bp direct repeats	None	Akopyants *et al.* (1998), Censini *et al.* (1996) Covacci *et al.* (1997)
S. flexneri	*she*	46.6	• Pic - exported protease • ShMu - mucinase • SigA - cytotoxic protease • Shet1 - enterotoxin	*pheV*	*pheV*	Rajakumar *et al.* (1997) Al-Hasani *et al.* (2001)
S. flexneri	SHI-2	23.7	• *iuc* cluster - aerobactin • ColV immunity	IS*2* - strain M90T, not SA100 *selC*	*selC*	Moss *et al.* (1999), Vokes *et al.* (1999)
E. coli (EHEC EPEC)	LEE	43.3[b] 35.4[c]	• Type III secretion system • Esp - secreted protein • Intimin adhesion • Attachment & effacement	left - *yicJ* right - *yicI*. (*yicK* lost upon PAI insertion)	*selC*	Elliot *et al.*, (1998), McDaniel & Kaper (1997), Perna *et al.*(1998)
E. coli uropathogenic strain J96	PAI-IV PAI-V	>170 110	• PAI IV: OmpR, cytotoxic necrotising factor (CNF), • P-fimbriae • PAI V: *hly* - haemolysin, • *pap* - P-fimbriae	unknown 135 bp direct repeats	*pheV* *pheR*	Häcker *et al.* (1990) Swenson *et al.* (1996)
E. coli uropathogenic strain CFT073	Unnamed	50	• *hlyCABD* • *pap* operon	*dadX* *metV*	*metV*	Kao *et al.* (1997)
E. coli uropathogenic strain 536	PAI-I PAI-II	70 90	• *hly*, *prf* (fimbriae) • *prf* (fimbriae)		*selC* *leuX*	Blum *et al.* (1994), Häcker *et al.* (1990)
*S.*Typhimurium	SPI-I	40	• Type III secretion (*inv/spa*) • Secreted invasins • Iron transport system (Sit) • Macrophage apoptosis	*fhlA*, *mutS*	None	Chen *et al.* (1996), Gálan (1996), Groisman & Ochman (1997), Mills *et al.* (1995)
*S.*Typhimurium	SPI-II	40	• Type III secretion • *ssrA/B* - two component regulatory system • Role in intracellular survival & growth	*ydhE*, *pykC*	*valV*	Cirillo *et al.*, (1998), Hensel *et al.* (1997), Shea *et al.* (1996), Uchiya *et al.* (1999)
*S.*Typhimurium *S. enterica*	SPI-III	17	• *mgtC* - intracellular macrophage survival & growth in low Mg+	*selC*, *orf307*	*selC*	Blanc-Potard & Groisman (1997), Blanc-Potard *et al.* (1999)
S. enterica	SPI-IV	27	• Type I secretion	*ssb* (tRNA-like structure) *yjcB*	None	Wong *et al.* (1998)
S. dublin	SPI-V	N/D	• Role in murine enteric salmonellosis	19 bp direct repeats	*serT*	Wood *et al.*, (1998)

* tRNA genes are either the insertion/deletion site of of EHEC LEE PAI. (a) Refers to *Yersinia pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. (b) Size of EHEC LEE PAI. (c) Size of EPEC LEE PAI.

In contrast to the enhancement of virulence by the acquisition of new DNA, another theory involves the loss of DNA and virulence enhancement. There are at least two instances of increased virulence of *Shigella* and EIEC strains following loss of DNA present in non-pathogenic *E. coli*. While *E. coli* and *Shigella* share >85% DNA-DNA homology, there are major differences in virulence that appear to arise from the acquisition or loss of regions of DNA. The region encoding lysine decarboxylase (LDC) is present in 90% of *E. coli* but is absent in *Shigella* and EIEC strains. LDC is encoded by *cadA*, which is near minute 93 on the *E. coli* K-12 chromosome. In all *Shigella* spp. and EIEC strains tested the *cadA* region/gene has either been lost or disrupted, although the means by which this has occurred varies between species (Day Jr. *et al.*, 2001). CadA inhibits enterotoxin activity, therefore the loss of this region enhances *Shigella* virulence (Maurelli *et al.*, 1998). As none of the genomic rearrangements disrupting *cadA* have been observed in commensal *E. coli* it has been proposed that this loss of *cadA* has led to the convergent, pathoadaptive evolution of the various *Shigella* species. A second event involving the deletion of 21 kb from the minute 12 region of *E. coli* K-12 also enhances virulence by the deletion of OmpT, a surface protease (Nakata *et al.*, 1993). It is believed that OmpT cleaves VirG, a protein essential for intercellular spreading of *Shigella*, thus affecting the virulence of the bacteria if it is present. Transformation of OmpT into *Shigella* results in a loss of ability to spread to adjacent epithelial cells.

Several possible mechanisms by which microorganisms can enhance their virulence have been discussed here: acquisition of genes on PAIs, loss of genes, conjugative transfer of chromosomal regions (CONSTINs) and, phage transfer of virulence genes. As bacteria acquire different attributes that aid virulence they become more pathogenic and are better able to survive in their particular hosts. The various changes to bacterial genomes that are discussed here are evidence of the very rapid evolution that is possible by bacteria.

IRON TRANSPORT

BACTERIA AND IRON

Iron is a component of cytochrome and iron-sulfur proteins involved in electron transport during respiration and is essential for bacterial survival (Brock *et al.*, 1994). In the environment free iron is readily available, but in the human host, iron is either stored in tissues such as the liver or it is chelated to extracellular proteins such as haemoglobin or transferrin (Moffett *et al.*, 1993). Intracellular organisms such as *Shigella* spp. can scavenge iron from the cells' internal compartments, but they need to be able to obtain iron in the extracellular environment of the host to survive. To obtain iron in the extracellular environment these pathogens need to produce high affinity iron

chelators. In some instances these chelators may act by binding to iron in already chelated forms such as haem, transferrin and lactoferrin.

SIDEROPHORES AND IRON TRANSPORT SYSTEMS

Siderophores are low molecular weight, iron-complexing compounds. Siderophore systems require the periplasmic TonB protein for passage of iron through the outer membrane. TonB is assisted by ExbB and ExbD in transport of iron into cells. *E. coli* strains have up to five different iron (III) transport systems, including several siderophore systems. Enterobactin (Ent) is a catecholate siderophore that is produced by all *E. coli* and *Salmonella* strains, while the dihydroxamate siderophore, aerobactin, is produced by only some strains (Earhart, 1996). Aerobactin has been shown to be superior to Ent in providing iron to microbes in a host. Other forms of iron transport systems in *E. coli* include the ferric-citrate (Fec), ferrichrome (Fhu) and ferrioxamine (Fox) iron uptake systems.

IRON TRANSPORT IN SHIGELLA *SPP.*

All clinical isolates of *Shigella* possess at least one siderophore-mediated iron transport system (Payne, 1989), suggesting a strong requirement for iron uptake. While all *E. coli* strains produce Ent, not all *Shigella* spp. produce this siderophore. Although *S. flexneri* strain 2457 does not produce enterobactin, it is able to utilise Ent via an enterobactin receptor on its cell surface (Payne, 1980). This suggests that *Shigella* scavenges the iron chelators of other bacteria. The ability of *Shigella* to utilise enterobactin is consistent with the fact that *E. coli* and *Shigella* share the intestinal environment. *Salmonella* has also been found to scavenge the iron chelators of other bacteria (Rabsch *et al.*, 1999).

A number of *Shigella* species encode the hydroxymate aerobactin system. The aerobactin iron transport system is carried on most of the colicin-V plasmids, which are able to confer virulence upon their host strains (Ambrozic *et al.*, 1998; Martinez *et al.*, 1994). The ability of this plasmid family to confer virulence upon its host strain has been shown to be due to the aerobactin system it carries, rather than the colicin toxin also encoded by the plasmid (Williams, 1979). The importance of aerobactin in cytotoxicity and thus virulence was shown by Harjai *et al.* (1994) when they demonstrated that transformation of an incomplete set of aerobactin genes into *E. coli* HB101 resulted in cytotoxic effects not seen with the avirulent parent strain. In a mouse model for ascending pyelonephritis, the transformant also established itself in the renal tissue better than the parent strain, suggesting that the areobactin aided survival of this strain.

The synthesis of aerobactin by *S. flexneri* was discovered in 1980 by Payne (1980). It is associated with colicin V immunity genes located on the chromosome of some *Shigella* strains just as on the colicin-V plasmids (Moss *et al.*, 1999; Vokes *et al.*, 1999). Tn*10* mutagenesis of the *iuc* genes of *S. flexneri* has shown that aerobactin does not affect invasion and killing of cells by the bacterium. Nassif *et al.* (1987) suggested that aerobactin may be important for the growth of *Shigella* in extracellular compartments, as it does not appear to be important for intracellular growth (Headley *et al.*, 1997). It is this extracellular survival in an iron-limited environment that is critical for *Shigella* before it gains access to macrophages or the epithelial cells. Therefore, aerobactin genes may aid in establishment of *Shigella* infection in the colon, the preferred invasion site.

S. dysenteriae encodes a haem receptor, ShuA, on its chromosome. Although ShuA binds to haem, it is not essential for invasion or intracellular multiplication, the major virulence functions of *Shigella* (Wyckoff *et al.*, 1998). In addition, the *shu* locus is not present in all *Shigella* strains, suggesting that it may not have an important role in virulence. Payne (1989) suggested that haemin-binding may be important in gaining access to the interior of epithelial cells by binding to their haem receptors on the cell surface. However, the overall importance of this method of internalisation may not be great because of the high efficiency of access to the interior of the cells provided by the invasion plasmid antigens (Ipa). The importance of haem-binding in virulence is thus unknown.

SOME GRAM NEGATIVE BACTERIA AND THEIR IRON TRANSPORT SYSTEMS

For *Yersinia* spp. the ability to use iron is essential for virulence. The *irp* genes of *Yersinia* spp. encode the siderophore, yersiniabactin, while the *psn* gene encodes the yersiniabactin (pesticin) receptor. Mutations in either the *psn* or *irp* genes render *Y. pestis* avirulent in mice (Bearden *et al.*, 1997). The importance of this iron transport system in *Yersinia* infection is supported by mutational analysis of the regulators of the yersiniabactin system, *ybtF* and *ybtQ*. Mutations in these genes impair growth at 37°C under iron-deficient conditions. *ybtP* mutants are also avirulent in mice (Fetherston *et al.*, 1999).

The marine fish pathogen *Vibrio anguillarum* encodes a siderophore iron transport system on a plasmid associated with high-virulence (Crosa 1980). Pathogenic strains also carry a chromosomally encoded iron-uptake system that is not related to the plasmid-encoded anguibactin system or the enterobactin system of *E. coli* (Lemos *et al.*, 1988). This association of iron transport with high virulence again shows the importance of iron in bacterial pathogenicity.

Other pathogenic bacteria also carry siderophores or other iron transport systems. Some of these include: *Acinetobacter baumannii* (acinetobactin) (Yamamoto *et al.*, 1994), pathogenic *Vibrio* spp.

(haem transport) (Occhino *et al.*, 1998; O'Malley *et al.*, 1999), *Plesiomonas shigelloides* (haem) (Daskaleros *et al.*, 1991), EHEC O157:H7 (haem) (Torres & Payne, 1997), *Gardnerella vaginalis* (Jarosik *et al.*, 1998), pathogenic *Neisseria* (enterobactin and transferrin receptors, haem) (Schryvers & Stojiljkovic, 1999) as well as the *Shigella* spp. and other pathogenic *E. coli*.

IRON TRANSPORT AND PATHOGENICITY ISLANDS

Iron transport systems have been identified on several pathogenicity islands (PAIs). The 58 kb PAI in uropathogenic *E. coli* CFT073 contains a putative iron transport system which includes a homologue of a ferric siderophore receptor (Guyer *et al.*, 1998). The yersiniabactin-encoding locus (*irp*) is located on the high-pathogenicity island of *Y. pestis* (Buchrieser *et al.*, 1998a) and *Y. enterocolitica* (Carniel *et al.*, 1996) as well as a number of pathogenic and commensal *E. coli* (Karch *et al.*, 1999; Schubert *et al.*, 1998). The *Yersinia* PAI is essential for virulence because of its iron-acquistion locus. However, its presence in bacteria that have additional iron transport systems suggests the importance of a variety of iron transport systems for survival within the host, whether in the intra- or extra-cellular environment. Recently, the *iuc* genes encoding aerobactin have been located to a PAI (SHI-2) in at least four *S. flexneri* serotypes (Moss *et al.*, 1999; Vokes *et al.*, 1999) and also the SHI-3 PAI in *S. boydii* (Purdy & Payne, 2001)

The possession of iron transport systems by such a range of pathogenic bacteria, as well as their presence on several pathogenicity islands, shows a strong requirement for iron-uptake systems by bacteria. The role of iron transport systems in the virulence of *Shigella* is still unknown.

THE 99 KB DELETABLE ELEMENT

S. flexneri 2a strain YSH6000 is resistant to streptomycin, ampicillin, chloramphenicol and tetracycline. In 1996, Rajakumar *et al.* (1996) identified a 99 kb deletion from the *Not*I D chromosomal fragment of two independent YSH6000 derivatives. Strain S2430 was a Tn*5*-generated mutant with an insertion in the *shf1/shf2* region of the virulence plasmid (Rajakumar *et al.*, 1996), as well as the deletion of the 99 kb from the chromosome. Strain YSH6000T was derived by repeated passaging of YSH6000 in order to obtain an antibiotic sensitive strain (C. Sasakawa, personal communication). In virulence-related *in vitro* assays, these strains were at least partially attenuated. Both strains showed an approximately 50% decrease in contact haemolysis and S2430 also displayed delayed plaque formation on LLC-MK2 monolayers. These findings, as well as the multi-antibiotic resistance, suggested a role in virulence for the 99 kb element. However, the delayed plaque

formation has since been found to be due to neither the 99 kb deletion nor the Tn*5* insertion into *shf1/shf2* (Rajakumar, unpublished data).

The 99 kb element was found to lie between *ompA* and *pyrC* homologues on the *S. flexneri* 2a chromosome, but its exact boundaries were not defined. Recent work has identified the boundaries of the 99 kb deletion, termed MRDE (multi-antibiotic resistant deletable element), to be identical IS*91*-like elements (Turner *et al.*, 2001). This work found that the MRDE consists of 27 kb of chromosomal DNA flanking a ~72 kb PAI-like element. So far this PAI has been found to encode the *Shigella* resistance locus (SRL); a putative ferric-dicitrate transport system (*fec*); an intact integrase; several IS-elements; and a putative novel mobile element (*shf*), homologous to the *shf* genes on the *Shigella* virulence plasmid (Rajakumar *et al.*, 1996). Insertion of the PAI is potentially in the *serX* tRNA gene.

The simultaneous deletion of a PAI and its flanking chromosomal region does not appear to be unique to *S. flexneri*. Recently, the High-Pathogenicity Island of *Y. enterocolitica* Ye8081 was found to delete approximately 140 kb of chromosomal DNA, including the 35 kb PAI (Bach *et al.*, 1999). This deletion event occurs at a lower frequency than the deletion of the PAI alone. The group postulated that the HPI is therefore situated on an unstable chromosomal fragment. This may also be true for the *S. flexneri* 2a YSH6000 PAI. The chromosomal regions deleted from *Y. enterocolitica* with the PAI have not yet been examined.

Early analysis of the multi-antibiotic resistance of strain YSH6000 by marker rescue identified a locus that encodes the genes for resistance to streptomycin, ampicillin, chloramphenicol and tetracycline (Rajakumar *et al.*, 1997). This locus has since been termed SRL, for *Shigella* resistance locus. This early analysis suggested an NR-1 related origin for the SRL due to similarities in genetic structure and resistance profile. NR-1 is the archetypal conjugative antibiotic-resistance plasmid of *Shigella* (Womble & Rownd, 1988). However, recent work suggests that the SRL may have evolved from a transposon (S. Turner, personal communication).

PROJECT AIMS

The primary aim of this project was the sequencing and characterisation of the SRL pathogenicity island found on the MRDE of *Shigella flexneri* 2a strain YSH6000. This involved subcloning and PCR-based strategies to obtain the sequence, followed by detailed sequence analysis using a range of web-based tools. This process then led to more detailed analysis of specific features of the PAI.

Prior to this study an intact ferric-dictrate iron transport system, Fec, had been identified on the SRL PAI. Following completion of the *fec* locus sequence using subclones obtained in earlier work, the potential importance of this locus in survival of this *S. flexneri* strain under iron-limiting conditions was investigated. The study also involved some transcriptional analysis of this locus and the identification of an alternate iron transport system.

Finally, analysis of a potential transcriptional regulator, Rox, was undertaken. The aim of this was the elucidation of the role of Rox in SRL PAI excision and/or integrase transcription. This process involved analysis of Rox/*int* interaction using gel-mobility assays, site-directed mutagenesis of the *int* promoter region and Real-time RT-PCR to analyse a specific effect on *int* transcription by Rox expression.

~ *CHAPTER TWO* ~

Materials and Methods

STRAINS, PLASMIDS AND GROWTH CONDITIONS

Bacterial Strains and Plasmids

The *Escherichia coli* and *Shigella* strains used in this study are listed in Table 2a. Plasmids used in this study are listed in Table 2b.

Media and Culture Conditions

Bacterial strains were routinely grown in Luria-Bertani (LB) (Ausubel *et al.*, 1995) or 2YT medium (Miller, 1972) at 37°C with aeration. Strains carrying the pCACTUS plasmid were routinely grown at 30°C. Solidified medium was prepared as per LB or 2YT broths with the addition of 1.0% bacterial agar. When antibiotic selection was required the growth medium was supplemented with ampicillin (100 μg^{ml-1}), chloramphenicol (40 μg^{ml-1}), kanamycin (50 μg^{ml-1}), streptomycin (25 μg^{ml-1}) or tetracycline (10 μg^{ml-1}).

RECOMBINANT DNA TECHNIQUES

Preparation of Plasmids from E. coli

Plasmids were routinely isolated by a modified alkaline lysis technique as previously described by LeGouill (1994). 2.5 ml overnight cultures were centrifuged at 11 600 x *g* for 1 min and the cells resuspended in 100 µl of Solution 1. RNase was added to a final concentration of 200 μg^{ml-1}. Lysis was performed by incubation at RT for 2 min in the presence of 200 µl each of Solution 2 and 200 µl chloroform. Following the addition of 150 µl Solution 3, the preparation was centrifuged at 11 600 x *g* for 10 min at 4°C. The aqueous phase was transferred to a fresh microfuge tube and DNA was precipitated with 850 µl of ice-cold 100% ethanol and centrifuged for an additional 11 min. The aqueous phase was then discarded and the pellet washed with ice-cold 70% ethanol, spinning for 5 min at 11 600 x *g*. The pellet was then vacuum dried in a Savant Speed Vac Concentrator for 3 min and the pellet resuspended in 20 µl dH_2O. The DNA was stored at -20°C until required.

Table 2a Bacterial strains used in this study

Strain	Parent	Details/Description	Source/Ref
E. coli			
DH5α		F-ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169*endA1 recA*1hsdR17deoR*thi1supE*441-*gyrA96relA1*	BRL
AA93	Z1418	F- *araD139 ΔlacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi aroB Δfec*	Ochs *et al.* (1995)
SBA264	DH5α	Carrying pGEX-2T	This lab
SBA596	DH5α	Carrying pSBA361	K. Rajakumar
SBA597	DH5α	Carrying pSBA362	K. Rajakumar
SBA740	DH5α	Carrying pSBA484	This study
SBA742	DH5α	Carrying pSBA486	This study
SBA743	DH5α	Carrying pSBA487	This study
SBA744	DH5α	Carrying pSBA488	This study
SBA745	DH5α	Carrying pSBA489	This study
SBA746	DH5α	Carrying pSBA490	This study
SBA747	DH5α	Carrying pSBA491	This study
SBA780	DH5α	Carrying pSBA509	This study
SBA781	DH5α	Carrying pSBA510	This study
SBA783	DH5α	Carrying pSBA512	This study
SBA822	DH5α	Carrying pSBA531	This study
SBA823	DH5α	Carrying pSBA532	This study
SBA824	DH5α	Carrying pSBA533	This study
SBA827	DH5α	Carrying pSBA536	This study
SBA828	DH5α	Carrying pSBA537	This study
SBA831	DH5α	Carrying pSBA540	This study
SBA832	DH5α	Carrying pSBA541	This study
SBA834	DH5α	Carrying pSBA543	This study
SBA844	AA93	Carrying pSBA491	This study
SBA845	AA93	Carrying pWSK129	This study
SBA846	DH5α	Carrying SKpSV662	This study
SBA873	DH5α	Carrying pSBA564	This study
SBA874	DH5α	Carrying pSBA565	This study
SBA882	DH5α	Carrying pSBA573	This study
AL19	DH5 α	Carrying pAL18.	This study
AL36	AA93	Carrying pAL18. Complemented Δ *fec* strain	This study
AL56	DH5 α	Carrying pAL31	This study
AL59	DH5 α	Carrying pAL34	This study
AL60	DH5 α	Carrying pAL35	This study
AL61	DH5 α	Carrying pAL36	This study
AL62	DH5 α	Carrying pAL37	This study
AL63	DH5 α	Carrying pAL38	This study
AL158	DH5 α	Carrying pAL85	This study
AL170	DH5 α	Carrying pAL95	This study
AL171	DH5 α	Carrying pAL96	This study
AL172	DH5 α	Carrying pAL97	This study
AL191	BL21 (DE3)	E. coli B F- *dcm ompT hsdS(rB- mB-) gal* (DE3 prophage)(Stratagene). Carrying pGEX-2T.	This study
AL195	BL21 (DE3)	E. coli B F- *dcm ompT hsdS(rB- mB-) gal* (DE3 prophage). Carrying pAL95	This study
AL211	SG22094	Δ *lon clp*P1::*cat rcsA*166::Km.	S. Gottesman
AL212	SG22094	Δ *lon clp*P1::*cat rscA*166::Km. Carrying pAL95	S. Gottesman
AL301	DH5α	Carrying pAL171	This study
AL349	DH5α	Carrying pAL204	This study
AL370	DH5α	Carrying pAL216	This study
AL371	DH5α	Carrying pAL217	This study
AL372	DH5α	Carrying pAL218	This study

Strain	Parent	Details/Description	Source/Ref
S. flexneri			
YSH6000		Serotype 2a, wild-type Japanese isolate. StrR, ApR, CmR, TetR.	Sasakawa *et al.* (1986)
YSH6000T	YSH6000	MRDE deletant. StrS, ApS, CmS, TetS.	Nakata *et al.* (1992)
SBA1366	YSH6000	SRL deletant. StrS, ApS, CmS, TetS.	Turner *et al.* (2001)
SBA1415	SBA1366	*fecI::kan* allelic exchange.	This study
AL11	YSH6000	Insertion *int* mutant. StrR, ApR, CmR, TetR, KnR.	Turner *et al.* (2001)
AL66	YSH6000	Carrying pAL34	This study
AL67	YSH6000	Carrying pAL31	This study
AL75	YSH6000	Carrying pPBA1100	This study
AL173	SBA1366	Carrying pAL95	This study
AL174	SBA1367	Carrying pAL96	This study
AL175	SBA1368	Carrying pAL97	This study
AL178	SBA1366	Carrying pGEX-2T	This study
AL295	AL11	Carrying pUC19_Tp	This study
AL296	AL11	Carrying pAL85	This study
AL376	YSH6000T	Carrying pAL217	This study
AL377	YSH6000T	Carrying pAL218	This study
AL378	YSH6000T	Carrying pAL217 and pBAD30	This study
AL379	YSH6000T	Carrying pAL218 and pBAD30	This study
AL380	YSH6000T	Carrying pAL217 and pAL216	This study
AL381	YSH6000T	Carrying pAL218 and pAL216	This study

Table 2.2 Plasmids used in this study

Plasmid	Details/Description	Isolator/Ref
pWSK29	pSC101-based low-copy number vector. ApR Δ*lacZ*	Wang
pWSK129	pSC101-based low-copy number vector. KnR Δ*lacZ*	Wang
pPBA1100	*E. coli / P. multocida* shuttle vector.	This Lab
pUC19_Tp	Trimethoprim resistance gene from pR388 inserted into *Ssp*I site of pUC19	This Lab
pGEX-2T	*E. coli* expression vector	Pharmacia Biotech
pCACTUS	pSC101-based-low-copy number, temperature sensitive, suicide vector with *sacB*, CmR	Van den Bosch *et al.* (1997)
pSU2719	pACYC184-derived *E. coli* cloning vector with pUC19 MCS and lacZα	Martinez & de la Cruz (1988)
pUC4-KIXX	Source of KnR from Tn*5*, ApR, KnR	Barany, (1985)
pBAD30	pACYC184 *ori*. Tightly controlled arabinose-inducible promoter. ApR	Guzman *et al.*, (1995)
pSKpSV662	*E. coli fecIRABCDE*	Ochs *et al.*, (1995)
pSBA361	*Bam*HI fragment from YSH6000, within SRL PAI. Contains insert of 27.6 kb in pWSK29. ApR, CmR, TetR.	K. Rajakumar
pSBA362	Same insert as pSBA361 cloned in reverse orientation into pWSK29. ApR, CmR, TetR.	K. Rajakumar
pSBA484	*Pst*I subclone of pSBA361. Contains insert of 600 bp in pWSK129	This study
pSBA486	*Pst*I subclone of pSBA361. Contains insert of 2.9 kb in pWSK129	This study
pSBA487	*Pst*I subclone of pSBA361. Contains inserts of 5.9 kb and 5.3 kb in pWSK129	This study
pSBA488	*Pst*I subclone of pSBA361. Contains insert of 4.8 kb in pWSK129	This study
pSBA489	*Sal*I dropout from pSBA361. Remaining insert was 19.3 kb	This study
pSBA490	*Sal*I dropout from pSBA362. Remaining insert was 8.3 kb	This study
pSBA491	*Hin*dIII subclone of pSBA361. Contains insert of 8.9 kb in pWSK129	This study
pSBA509	*Bam*HI subclone generated by Ap marker resuce from YSH6000 in pWSK129. Approximately 24 kb insert	This study
pSBA510	*Eco*RI subclone generated by Ap marker resuce from YSH6000 in pWSK129	This study
pSBA512	*Sal*I subclone generated by Ap marker resuce from YSH6000 in pWSK129. 23.6 kb insert.	This study
pSBA531	*Eco*RI subclone of pSBA509. Contains inserts of 4.8 kb in pWSK129	This study
pSBA532	*Eco*RI subclone of pSBA509. Contains inserts of 1.5 kb and 7.3 kb in pWSK129	This study
pSBA533	*Eco*RI subclone of pSBA509. Contains inserts of 7.3 kb in pWSK129, overlapping SRL PAI left junction.	This study
pSBA536	*Eco*RI subclone of pSBA530. Contains insert of 8.8 kb in pWSK29	This study
pSBA537	*Eco*RI subclone of pSBA530. Contains insert of 2.3 kb in pWSK29	This study
pSBA540	*Sal*I subclone of pSBA536. Contains insert of 1.4 kb in pWSK129	This study
pSBA541	*Sal*I subclone of pSBA536. Contains insert of 2.8 kb in pWSK129	This study
pSBA543	*Sal*I subclone of pSBA536. Contains insert of 1.8 kb in pWSK129	This study
pSBA564	*Hin*dIII fragment from pSBA491 (*fecIRABCDE*) subcloned into pSU2719.	This study
pSBA565	1.6 kb *kan/ble* from pUC4-KIXX cloned into *Xho*I site of pSBA564	This study
pSBA573	PCR derived 5.5 kb*Bam*HI fragment cloned into pCACTUS. *fecIΩkan*	This study
pAL31	800 bp PCR derived *orf41* gene directionally cloned into *Bam*HI and *Eco*RI sites of pPBA1100	This study
pAL34	800 bp PCR derived *rox* gene directionally cloned into *Bam*HI and *Eco*RI sites of pPBA1100	This study
pAL35	*Pst*I subclone of Long PCR product from SRL PAI. Contains inserts of 2 kb and 300 kb in pWSK129	This study
pAL36	*Pst*I subclone of Long PCR product from SRL PAI. Contains insert of 300 bp kb in pWSK129	This study
pAL37	*Pst*I subclone of Long PCR product from SRL PAI. Contains inserts of 2.2 kb and 2.3 kb in pWSK129	This study
pAL38	*Pst*I subclone of Long PCR product from SRL PAI. Contains insert of 1.8kb in pWSK129	This study
pAL85	*Bam*HI/*Eco*RI insert from pAL34 cloned into *Bam*HI/*Eco*RI site of pUC19-Tp. Encodes *rox*	This study
pAL95	PCR derived *rox* gene and downstream region (~600 bp) cloned in-frame into *Bam*HI/*Eco*RI sites of pGEX-2T - clone #13	This study
pAL96	PCR derived *rox* gene and downstream region (~600 bp) cloned in-frame into *Bam*HI/*Eco*RI sites of pGEX-2T - clone #17	This study

Plasmid	Details/Description	Isolator/Ref
pAL97	PCR derived *rox* gene and downstream region (~600 bp) cloned in-frame into *Bam*HI/*Eco*RI sites of pGEX-2T - clone #20	This study
pAL171	PCR derived fragment of bp 411 to 1145 of SRL PAI for site-directed mutagenesis of *int* promoter region	This study
pAL204	Site-direct mutant of pAL171 (TCCG to GAAT)	This study
pAL216	Insert from pAL34 cloned into *Eco*RI and *Hin*dIII sites of pBAD30	This study
pAL217	Insert cloned from pAL171	This study
pAL218	Insert cloned from pAL204	This study

Preparation of Plasmids from S. flexneri

Plasmids were extracted from *S. flexneri* using QIAprep Spin miniprep Kit columns as described by the manufacturer (Qiagen).

Preparation of Genomic DNA from E. coli *and* S. flexneri

Genomic DNA was extracted from *E. coli* and *S. flexneri* using a modification of the method previously described by Ausubel (1995). Overnight bacterial cultures were transferred to 1.5 ml microfuge tubes and centrifuged at 11 600 x *g* for 1 min at RT. The supernatant was discarded and the bacterial pellet was resuspended in 567 µl TE buffer, 30 µl 10% SDS and 3 µl 20mg $^{ml-1}$ Proteinase K. The mixture was incubated at 37°C for 1 hour. Following incubation, 100 µl 5M NaCl was added and the solution vigorously shaken. 80 µl of CTAB/NaCl was then added and the mixture again vigorously shaken before incubation at 65°C for 10 min. The DNA was then extracted by sequentially using equal volumes of chloroform-isoamyl (24:1), phenol/chloroform-isoamyl (25:24:1) and chloroform-isoamyl. Following the addition of each solution the mixture was inverted at least 10 times and then centrifuged at 11 600 x *g* for 5 min at RT, before the aqueous phase was transferred to a fresh microfuge tube. Following the final chloroform extraction the DNA solution was transferred to a fresh microfuge tube, mixed with 0.6 volumes of isopropanol, and carefully inverted several times. This mixture was centrifuged at 11 600 x *g* for 20 min at 4°C. The aqueous phase was then discarded and the pellet washed with 1 ml of ice-cold 70% ethanol by centrifugation at 11 600 x *g* for 5 min at 4°C. The pellet was then vacuum dried and resuspended in 50-100 µl dH_2O.

Restriction Endonuclease Digestion

DNA was routinely digested with restriction endonucleases provided by New England Biolabs Inc. (NEB) or Roche Molecular Biochemicals. Digestion of plasmid and genomic DNA was performed in 20 µl volumes containing 5-10 U of enzyme with the appropriate reaction buffer supplied by the manufacturer and dH_2O for 2-18 hours at 37°C. Enzymes were inactivated by heating the reaction mix to 75-80°C for 20 min, or by the addition of 1/5 volumes of stop mix.

Preparation of DNA for Sequencing and Ligation

Polyethylene glycol (PEG) precipitation was performed on plasmid DNA to remove unwanted salts before sequence reactions were carried out. Plasmid, 4M NaCl and 13% (v/v) PEG were mixed in a ratio of 4:1:5. The mixture was mixed and incubated on ice for 20 min, followed by 20 min centrifugation at 11 600 x *g* at 4°C. The supernatant was discarded and the pellet washed in 1 ml ice-cold 70% ethanol for 5 min at 11 600 x *g* at 4°C, followed by vacuum drying and resuspension in an appropriate volume of dH_2O and stored at -20°C until required. PCR-derived fragments were prepared for sequencing and ligation by use of the QIAQUICK columns as per the manufacturers (Qiagen) instructions. Unwanted salt and enzymes were removed from restriction endonuclease digests by phenol/chloroform extraction and ethanol precipitation. The digestion mixture was extracted with an equal volume of phenol/chloroform-isoamyl (25:24:1), followed by an equal volume of chloroform-isoamyl (24:1). Each extraction was mixed well and centrifuged at 11 600 x *g* for 5 min at RT. The resulting aqueous phase was transferred to a fresh microfuge tube and mixed with 0.1 volumes of 3M sodium acetate (pH 4.8) and 2.5 volumes of 100% ethanol. The mixture was incubated on ice for 10-20 min and then centrifuged at 11 600 x *g* for 20 min at 4°C. The pellet was then washed with ice-cold 70% ethanol, vacuum dried and resuspended in an appropriate volume of dH_2O.

Agarose Gel Electrophoresis

DNA was electrophoresed in agarose gels consisting of 0.8 – 1% (w/v) agarose dissolved in 1 x TAE. Ethidium bromide was added to the solution before solidification to a final concentration of 0.1 μg ml^{-1}. Stop mix was added to DNA samples prior to loading on the gel. Electrophoresis was performed at 100 V for small fragments and 70 V for large fragments for 1–2 hours in a mini-gel electrophoresis apparatus buffered by 1 x TAE. *Hin*dIII digested Lambda DNA was used as molecular markers for size and concentration of DNA samples. DNA samples were observed on a UV transilluminator and photographed using a video imager, when required.

Ligation of DNA

Ligations were carried out using a 1:10 vector to insert ratio, for most reactions (see sspPCR for alternative ligation method). A 20 μl ligation reaction consisted of 14.5 μl of digested insert DNA, 2 μl of digested vector DNA, 2 μl of T4-ligase buffer (Roche Molecular Biochemicals) and 0.5-1 μl T4 DNA Ligase (0.5-1 Unit). Ligations were incubated overnight at 15°C. Following incubation samples were either vacuum dried, washed twice in 70% ethanol and vacuum dried again or ethanol

precipitated. Following cleaning of ligation mix, samples were resuspended in 5µl of dH_2O. DNA was transformed into *E. coli* cells by either chemical transformation or electroporation. DNA was transformed into *S. flexneri* by electroporation only.

Preparation of Electrocompetent Bacterial Cells

Electrocompetent bacterial cells were prepared by overnight growth of the desired bacteria in 2.5 ml 2YT at 37°C with aeration, followed by a 1 ml inoculation of 250 ml of 2YT and growth at 37°C with aeration to an absorbance at 550 nm of 0.8. Cultures were then centrifuged at 5 000 x *g* for 10 min at 4°C. The resulting pellet was then resuspended in 167 ml of sterile, ice-cold 10% (v/v) glycerol and centrifuged at 5 000 x *g* at 4°C for 10 min. The glycerol wash is then repeated and excess glycerol removed. The pellet is resuspended in 1 ml glycerol and aliquoted into 40 µl aliquots. Aliquots are snap-frozen in a dry ice/ethanol bath and stored at -70°C until required

Electroporation

Electroporation was performed as previously described (Smith & Jesse, 1990). Electroporation was carried out using a BTX Electrocell Manipulator with conditions for *E. coli* and *S. flexneri* of 1.5 kV, 125 Ω and 25µF. Prior to electroporation, electrocompetent bacterial cells were thawed on ice and BTX 1 mM electroporation cuvettes were chilled on ice. The electroporation mixture consisted of 3-5 µl of very clean DNA and 40 µl of electrocompetent bacterial cells. Following the electric pulse the mixture was immediately resuspended in 2 ml of SOC broth and incubated at 37°C, with aeration, for 1-2 hours recovery. Ten-fold dilutions were prepared and 100 µl aliqouts plated onto 2YT or LB plates containing the appropriate antibiotic.

Preparation of Chemically Competent E. coli *DH5α*

Chemically competent cells were prepared by inoculating 100 ml of SOB, in a 2 L flask, with 10 colonies from a fresh, overnight SOB or LB plate. The flask was incubated at 37°C with aeration until the cell density was 4-7 x 10^7 viable cells ml^{-1} (approximate absorbance of 0.3 at 600 nm). The culture was then poured into 50 ml sterile centrifuge tubes and chilled on ice for 10-15 min. The cells were pelleted by centrifugation at 1000 x *g* for 15 min at 4°C. The supernatant was then discarded and the pellet resuspended in 33 ml RF1 (0.48 g RbCl, 0.40g $MnCl_2$, 1.2 ml Potassium acetate [1M, pH 7.5], 0.06g $CaCl_2.2H_2O$, 6 ml glycerol, dH_2O, pH to 5.8 with 0.1 M acetic acid, final volume 40 ml). Bacterial cells were incubated on ice for 1 hour, and then pelleted by centrifugation at 1000 x *g* for 15 min at 4°C. The supernatant was discarded and the cells resuspended in 7 ml of RF2 (0.02g RbCl, 0.4 ml MOPS, 0.22$CaCl_2.2H_2O$, 3 ml glycerol, dH_2O, pH to 6.8 with 0.1 M NaOH, final

volume 20 ml) and incubated on ice for 15 min. The bacterial cells were then aliquoted into microfuge tubes and snap frozen in solid CO_2/ethanol and stored at -70°C until required.

Oligonucleotide Synthesis

The oligonucleotides used for sequencing, PCR amplification and gel mobility assays were synthesised on an Applied Biosystems model 392 DNA/RNA oligonucleotide synthesiser. Khim Hoe, Monash University Department of Microbiology, eluted and purified oligonucleotides from the column according to the manufacturer's instructions. Oligonucleotides were then deprotected by incubation at 55°C for 20 hours, aliquoted and vacuum dried. For use in PCR and sequencing reactions, oligonucleotides were resuspended in 200 µl dH_2O and a 1/10 aliquot used per 25 µl or 15 µl PCR or sequencing reaction, respectively. Oligonucleotides used in this study are listed in Appendix 3.

DNA Sequencing Techniques and Analysis

Automated DNA Sequencing

Nucleotide sequencing was carried out using templates of genomic clones, inverse PCR products, sspPCR products and a long range PCR product. Sequence reactions were conducted using the BigDye™ system (PE Biosystems Inc.). Standard reaction and cycling conditions are described in Appendix 4. Following cycling, samples were prepared for electrophoresis by ethanol precipitation with 50 µl RT 100% ethanol and 2 µl 3 M sodium acetate. The precipitations were incubated at RT for 15 min, followed by centrifugation at 11 600 x *g* at RT for 30 min. Samples were then washed once with 250 µl RT 70% ethanol and spun at 11 600 x *g* at RT for 5 min. The supernatant was removed and the pellet dried under vacuum for 5 min. Reaction products were analyzed on an Applied Biosystems model 373A DNA sequencing system.

Nucleotide Sequence Analysis

Sequence editing was carried out using Sequencher 3.0 for Macintosh. Sequence analysis and database comparisons were performed using BlastN and BlastX (Altschul *et al.*, 1997).

Deduced Amino Acid Sequence Analysis

Analysis of proteins was carried out using previously described web-based analysis tools (Hofmann *et al.*, 1999; Hofmann & Stoffel, 1993; Nakai & Horton, 1999; Nielsen *et al.*, 1997; Schultz *et al.*,

1998). ANGIS (www.angis.org.au) was used for comparative analysis of deduced protein sequences. MacVector software, with a window size of 100 bp, was used for base composition analysis of the SRL PAI.

SOUTHERN HYBRIDISATION

Preparation of DNA Probe

DNA fragments were labelled with digoxigenin labelled d-UTP as described by Roche 'DIG System User's Guide for Filter Hybridisation'. A standard *Taq* PCR reaction (Appendix 4) was used to label the required fragment, with the exception of 1 µl of dNTPs and 0.5 µl of DIG-labelling mix (Roche) being included in the reaction. The resulting PCR product was then electrophoresed through an 0.8% TAE agarose gel at 100 V. The band was run into a small area of low-melting temperature agarose which was then excised from the gel. This gel fragment was then dissolved in 8 ml of standard hybridisation solution by boiling for 10 min. The probe was then either used immediately or stored at -20°C until required.

Southern Blotting

Equal concentrations of DNA samples were routinely digested overnight at 37°C with the appropriate restriction endonuclease in 25 µl reactions as described above. Before loading onto 0.8% TAE agarose gels, 5 µl of Stop mix was added to each reaction. Samples were electrophoresed at 15 V overnight. DNA was then routinely transferred onto positively charged Nylon membrane (Roche) using a TE 80 Transvac Vacuum Blotter. The transfer stack was begun by placing 2 pieces of pre-wet filter paper on to the metal support of the blotter. A pre-wet piece of membrane, the same size as the gel, was then placed on the stack. The pre-cut mask was then placed over the membrane and the gel placed directly over the mask onto the membrane. The vacuum was then started and remained in operation throughout the transfer. The gel was covered in depurination solution and left for 10-20 min. The depurination solution was then removed and the denaturation solution added and left for 10-20 min. Finally, the denaturation solution was replaced with neutralisation solution and left for 10 min before removal. The transfer solution (20 x SSC) was then poured onto the gel and the transfer proceeded for one and a half hours, ensuring the gel was covered with transfer solution at all times. The transfer solution was then removed, the vacuum released and the gel carefully removed.

The nylon membrane, with the transferred DNA, was dried briefly at 37°C. The membrane was placed between two sheets of filter paper and wrapped in foil. This package was then baked in a National Appliance Company oven Model 5831 at 80°C for one and a half hours to fix the DNA to

the membrane. Following fixation, the membrane was either stored at RT until required, or used immediately for hybridisation. Alternatively, the membrane was fixed by using a Stratagene UV Stratalinker® 1800 autocross link procedure (120 000 microjoules).

DNA-DNA Hybridisation

Prior to hybridisation the membrane was pre-hybridised with standard hybridisation solution (Appendix 2) for two hours to decrease non-specific binding of the DNA probe. Pre-hybridisation and hybridisation were routinely performed in a Robbins Scientific Model 1000 hybridisation oven at 65-68°C in Amersham hybridisation bottles. Prior to hybridisation the probe was denatured by boiling for 10 min and was then placed on ice for 3 min. The pre-hybridisation solution was then discarded and the probe poured into the hybridisation bottle. The hybridisation then proceeded overnight.

Detection of Southern Blot

Following hybridisation the probe was poured into a suitable container and stored at -20°C for reuse. Stringency washes were performed to remove probe that had bound non-specifically to the membrane. Two 5 min low stringency washes (2 x SSC/0.1% SDS) were performed at room temperature, followed by two 15 min high stringency washes (0.1 x SSC/0.1% SDS) washes at 65°C. The membrane was then equilibrated in Buffer 1 for 1 min. In a fresh dish, the membrane was blocked with Buffer 2 (5 g skim milk powder in 100 ml of Buffer 1) with gentle agitation for 45 min at RT. Anti-DIG alkaline phosphatase, Fab fragments (Roche) was diluted 1:10 000 (3 µl in 30 ml) in Buffer 2. The blocking solution was then replaced with the antibody solution, which was incubated at RT with gentle agitation for 30 min. The antibody solution was then discarded and in a fresh dish the membrane was washed twice for 15 min in washing buffer (Buffer 1 with 0.3% Tween 20) at RT. The membrane was then equilibrated for 2 min in Buffer 3. In a fresh dish, the membrane was incubated with the substrate (CDP-Star [Roche] diluted 100 µl in 10 ml buffer 3) in the dark at RT for 5 min. The membrane was then sealed in a hybridisation bag and exposed to X-ray film (Fuji) for an appropriate time.

POLYMERASE CHAIN REACTION (PCR)

Standard Taq *Reactions*

PCR mixtures routinely contained 20-100 ng of template DNA, PCR buffer (Roche), 1.25 mM each of dATP, dTTP, dCTP and dGTP, approximately 150 ng of each primer and 0.5 U of *Taq* DNA polymerase (Roche), in a total of 25 µl. PCR amplifications were carried out in 0.2 ml thin-walled tubes using an Applied Biosystems GeneAmp PCR 2400 thermocycler. Thermocycler conditions are shown in Appendix 4. PCR products were analysed by agarose gel electrophoresis.

Expand™ High Fidelity PCR

The Expand™ high fidelity PCR system was used as described by the manufacturer (Roche). Reaction mixes and PCR cycles are outlined in Appendix 4.

Expand™ Long Range PCR

Long range PCR reactions were performed as described by the manufacturer (Roche). The reaction mixes and cycle used to amplify the 12 kb product used to complete the sequence of the SRL PAI is described in Appendix 4. Samples were analysed by electrophoresis in a TAE gel for purity and size.

Inverse PCR

Inverse PCR was performed as described by Ochman (1988). The digestion reaction consisted of 20 µl of *S. flexneri* YSH6000 genomic DNA, 10 µl of Buffer B (NEB), 2 µl of *Eco*RV (NEB) and made up to 100 µl with dH_2O incubated at 37°C for 4 hours. The digestion was then cleaned by phenol/chloroform extraction and ethanol precipitation as described earlier. Following drying of the pellet under vacuum it was resuspended in 40 µl of dH_2O. The digested products were then ligated in a 100 µl reaction containing 20 µl of the digested DNA, 20 µl of 5 x T4 DNA ligation buffer (Roche), 0.2 µl (1 U) T4 ligase (high concentration) and dH_2O. Ligations were incubated at 15°C overnight. Ligations were dried down as much as possible (complete drying not possible due to presence of PEG in buffer), then resuspended by vortexing in 1 ml of 70% ethanol and incubating on ice for 5 min. The mixture was then spun at 11 600 x *g* for 15 min at 4°C, the supernatant was then removed and followed by a second 70% ethanol wash. The mixture was then dried under vacuum and resuspended in 20 µl of dH_2O. The cycles and conditions used to amplify the inverse PCR product are described in Appendix 4.

Single Specific Primer PCR (sspPCR)

As previously described by Novak & Novak (1997) sspPCR is used to identify new sequences beyond points of known sequence. In this study, 30 µl of *S. flexneri* YSH6000 genomic DNA was digested by 2 µl (20-40 U) of a variety of restriction endonucleases (*Cla*I, *Eco*RV, *Hin*cII, *Hin*dIII, *Pst*I and *Sal*I, buffered according to manufacturer's recommendations and the volume of the reaction made up to 120 µl with dH_2O. 2 µl of pWSK129 was digested with 0.5 µl (5-10 U) of the same set of enzymes buffered according to the manufacturer's instructions in a total volume of 20 µl. Digests were incubated at 37°C for 3 hours and then inactivated by heating at 80°C for 20 min. Samples were then ethanol precipitated and both the vector and genomic DNA resuspended in 10 µl. The digestions were then ligated in reactions consisting of 10 µl of digested genomic DNA, 3 µl digested pWSK129, 2 µl 10 mM ATP, 4 µl of 5 x ligation buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 25% w/v polyethylene glycol 8000, 5 mM DTT, 1 mM ATP) and 1 µl (1 U) T4 ligase (Roche). Ligations were incubated overnight at 15°C. The following day ligations were dried down under vacuum, washed twice in 1 ml 70% ethanol, dried again under vacuum and resuspended in 30 µl. Reaction and cycles used for sspPCR reactions are described in Appendix 4. A PCR was performed with the ligation mix for each enzyme and the one with the largest specific band was upscaled to a PCR reaction with a larger volume and this product cleaned and sequenced as described above.

RNA Techniques

Extraction of RNA from Bacterial Cells

Inocula were prepared by growing bacteria overnight at 37°C with aeration in LB broth supplemented with antibiotics where necessary. A 50 ml LB broth was inoculated with a 1/500 dilution of the overnight culture and incubated, with aeration, at 37 °C. Cultures for extraction of *fec* transcripts were grown in Fec media. The bacterial cells were harvested at early exponential phase (2-4 hours). Bacterial cells were pelleted by centrifugation at 1,300 x *g* for 10 min at RT and the supernatant discarded. RNA was extracted using Trizol™ as previously described (Simms *et al.*, 1993). The pellet was homogenised with 1 ml of Trizol™ and transferred to a fresh microfuge tube. The mixture was incubated at 65°C for 15 min, followed by the addition of 0.2 ml of chloroform. This mixture was shaken vigorously for 15 sec, incubated at RT for 15 min and then centrifuged at 11 600 x *g* for 15 min at 4°C. The resulting aqueous phase was transferred to a tube containing an equal volume of phenol/chloroform. This mixture was mixed and spun at 11 600 x *g* for 5 min at 4°C. An additional extraction in an equal volume of chloroform-isoamyl alcohol (24:1) was performed by centrifugation at 11 600 x *g* at 4°C for 5 min. This aqueous solution was then transferred into a fresh tube

containing 1 µl of RNAsin (400 U, Promega). The RNA was precipitated by the addition of 300 µl of isopropanol and incubated at RT for 10 min. The RNA was then pelleted by centrifugation at 4°C for 10 min at 1' 600 x *g*. The supernatant was discarded and the pellet washed in 70% EtOH, by vortexing and centrifugation at 11 600 x *g* at 4°C for 5 min. The pellet was then air dried and resuspended in 100 µl DEPC H_2O by pipetting at RT. The RNA was then precipitated by incubation at -70°C for 30 min in a 1/10 volume of 5 M sodium acetate, pH 5.2 and three volumes of 99% ethanol, followed by centrifugation for 20 min at 11 600 x *g* at 4°C. The supernatant was then removed and the pellet washed in 70% ethanol by spinning at 4°C for 5 min at 11 600 x *g*. Following air drying the RNA was resuspended in DEPC H_2O. Concentration was determined by absorbance at A_{260}. The RNA was then diluted to the desired concentration and stored in aliquots at -70°C until required. Quality of RNA was determined by agarose gel electrophoresis. Before use purified RNA was treated with DNase (15 U for 45 min at 37°C) and the RNA finally purified on RNeasy minicolumns (Qiagen). RNA concentration was then determined by A_{260}.

RNA Dot Blots using a DNA Probe

RNA dot blots were performed as described by the Roche manual for 'DIG System User's Guide for Filter Hybridisation' Samples were diluted into ten-fold serial dilutions in RNA dilution buffer (DEPC dH_2O:20 x SSC:Formaldehyde [5:3:2]). 1 µl aliquots of the ten-fold serial dilutions of each RNA sample were then spotted onto a nylon membrane. Spots were allowed to air dry before fixation using a Stratagene UV Stratalinker® 1800. DNA probes were prepared as described for 'Southern Blotting', with the exception of the probe being dissolved in High-SDS concentration hybridisation buffer. The dot blots were then treated as described by Roche. Pre-hybridisation involved 2 hours incubation at 65°C in high-SDS concentration hybridisation buffer, prior to overnight hybridisation with the DNA probe. RNA dot blots were developed as per 'Southern Hybridisation Development' however, all solutions were made using DEPC treated dH_2O to avoid RNase contamination.

PROTEIN ANALYSIS METHODS

GST Protein Purification

Rox and GST proteins were extracted as described by the manufacturer of the pGEX-2T expression system (Amersham Pharmacia Biotech). Inocula were grown overnight at 37°C with aeration in 50 ml of 2YT supplemented with 0.1% glucose and ampicillin (100 µg/ml). Inocula were diluted 1/10 into 500 ml of fresh 2YT/Ap/0.1% glucose and grown at 37°C with aeration for 1 hour. Cultures where induced with a final concentration of 0.1 mM IPTG. Cultures were then incubated at 37°C with

aeration for a further 3 hours. Cultures where then centrifuged at 5 000 x *g* for 10 min at RT to pellet cells. The supernatant was discarded and the cells resuspended in 10 - 20 ml of ice cold PBS. Bacterial cells were then passed through a Portapower Black Hawk French press Ram Model RC-159 to disrupt cell membranes and release the protein. Alternatively, cultures were sonicated using a Branson Sonic Power Company Sonifier B-12. After disruption, 10% Triton-X100 was added to a final concentration of 1%, mixed and the cellular debris pelleted by centrifugation at 10 000 x *g* at 4°C. The resulting supernatant was pooled and added to 1 ml of glutathione sepharose 4B (Amersham Pharmacia Biotech). This mixture was gently mixed at RT for 2 min, before the addition of 50 ml of ice cold PBS to wash the beads. The beads were pelleted by centrifugation of 500 x *g* for 10 sec at RT. The PBS wash was repeated twice. Finally, 1.5 ml of PBS/glutathione sepharose was transferred to a microfuge tube and spun at 500 x *g* to pellet the beads and the supernatant was carefully removed. The fusion protein and the GST only were eluted by applying 0.5 - 1 ml of elution solution (50 mM Tris-HCl pH 8.0, 5 mM reduced glutathione) to the beads. The beads and elutant were gently mixed at RT for 2 min and then centrifuged for 10 sec at 500 x *g*. The supernatant was collected. Two additional elutions were performed to maximise the protein recovered. Recovered protein was analysed by SDS-PAGE.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were routinely electrophoresed on 12.5% SDS-PAGE gels. Gels were cast between two glass plates separateted by 1.5 mM spacers. The 12.5% resolving gel (Appendix 2) was poured and overlaid with 50% (v/v) tertiary butanol to ensure a flat surface. After the resolving gel had set the butantol was poured off and the remaining gel was washed thoroughly with dH_2O. The 4% stacking gel (Appendix 2) was poured and an appropriate comb inserted. To calculate concentration of recovered proteins they were electrophoresed along side known BSA standards. Prior to loading, samples were mixed with sample buffer in a 1:1 ratio and boiled for 5 min. Samples were then centrifuged briefly and loaded onto the gel, along with low molecular weight markers (Amersham Pharmacia Biotech). Electrophoresis was performed in a Bio-Rad Protean II mini-gel apparatus buffered with PAGE running buffer. Gels were electrophoresed at 140 V until the dye front was through the stacking gel and 200 V for the remainder of electrophoresis.

Coomassie Blue Staining

To visualise proteins SDS-PAGE gels were stained with 0.2% Coomassie Brillant Blue (BDH Chemicals Pty. Ltd.) for 1 hour at room temperature with gentle shaking. Gels were destained by several rinses in a solution of 7% (v/v) acetic acid and 10% (v/v) methanol over several hours or

overnight. Gels were preserved by washing in a glycerol/methanol solution (45% v/v methanol, 2% v/v glycerol) and drying in cellulose paper.

Western Blotting

Following the running of a 12.5% SDS-PAGE gel, including See-Blue Plus II markers (Invitrogen) for determining molecular mass, proteins were transferred onto nitrocellulose membranes using a Bio-Rad transblot apparatus. The gel was layered in a stack of sponge, filter paper, gel, nitrocellulose, filter paper and sponge. The transfer was carried out at 4°C for 1 hour at 100 V. The transfer buffer consisted of 100 mM glycine, 12.5 mM Tris and 10% methanol. Following transfer, the membrane was blocked by shaking for 30 min at RT in 5% skim milk powder dissolved in TBS-T. The blocking solution was removed and the primary antibody diluted in TBS-T was applied and incubated at RT, with shaking, for 1 hour. The primary antibody was then removed and the membrane was washed four times in TBS-T for 5 min at RT, with shaking. The secondary antibody diluted in TBS-T was then applied and incubated at RT, with shaking, for 1 hour. The secondary antibody was then removed and the membrane was again washed four times in TBS-T for 5 min at RT, with shaking, with an additional TBS only wash. The chloronapthol substrate solution (15 mg 4-chloronapthol in 5 ml methanol mixed with 15 µl of hydrogen peroxide in 25 ml of TBS) was then applied to the membrane and the colour allowed to develop for as long as required. The reaction was stopped by washing the membrane in dH_2O.

GEL MOBILITY ASSAY

DIG-DNA Labelling (3'-end labelling)

Following manufacturer's instructions (DIG-DNA Gel Shift Kit, Roche) double-stranded DNA fragments ranging from 35 bp to 297 bp were 3'-end labelled with a single digoxigenin molecule. The labelling reaction was as follows:

5 x labelling buffer (Roche)	4 µl
$CoCl_2$ Solution (Roche)	4 µl
Double-stranded DNA	490 ng
DIG-11-ddUTP (Roche)	1 µl
Terminal transferase (Roche)	1 µl
dH_2O	Make final volume 20 µl

The mixture was incubated at 37°C for 15 min, then placed on ice. The labelled DNA was precipitated with 2 µl 4 M LiCl and 60 µl chilled 100% ethanol, and incubated at -70°C for 30 min. The mixture was then centrifuged at 11 600 x *g* for 15 min at 4°C. The resulting pellet was washed three times by addition of 500 µl chilled 70% ethanol and centrifugation at 11 600 x *g* for 5 min at 4°C. The washed pellet was dried under vacuum and dissolved in 10 µl of TEN buffer.

Labelling efficiency was determined by perfoming a dot blot analysis of 10-fold serial dilutions of the labelled DNA and comparing to standards provided with the Roche 'DIG-DNA Gel Shift Kit', and detected as described below.

DIG-DNA/Protein Binding Reaction

The binding reaction between the digoxigenin labelled DNA fragments and Rox protein were performed as described by Roche (DIG-DNA Gel Shift Kit) with several modifications. The binding buffer supplied by Roche (100 mM Hepes, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, Tween®-20 1% w/v, 150 mM KCl [x5 buffer]) was replaced by the buffer previously described by Polo (1996) (100 mM KCl, 5% glycerol, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1mM DTT, 50 µg/ml bovine serum albumin). $ZnCl_2$ was used in a range of concentrations, and found to be required for binding. A standard reaction is presented below:

5 x Binding buffer	5 µl	
poly [d(I-C)] (1µg/µl)	1 µl	
poly L-lysine (0.1µg/µl)	1 µl	
$ZnCl_2$ (250 mM)	0.5 µl	
DIG-labelled DNA (15 fmol)	1-2 µl	
Rox Protein	12 µl	(amount of protein ranged from 0.78 ng to 400 ng)
dH_2O	make to 25 µl	

Reactions were mixed very gently and incubated at room temperature for 15-30 min. Tubes were then placed on ice and 5 µl of loading buffer (0.25 x TBE:Glycerol in a 6:4 ratio) was added. Samples were then immediately loaded onto an acrylamide gel (described below).

Non-denaturing PAGE

Gel mobility assays were routinely performed in 4% non-denaturing polyacrylamide gels. The gels were made by the addition of 5 ml 40% acrylamide:bis solution (29:1 ratio), 1.25 ml 10 x TBE, 2.5 ml

glycerol and 41.25 ml ultra-pure dH_2O. This solution was then passed through a 0.2 μM filter and degassed for less than 5 min. In order to polymerise the gel 350 μl of 10% ammonium persulfate was added. 1 ml of the solution was removed and 15 μl of TEMED (Sigma) was added before this mixture was applied to the internal edges of the gel apparatus to quick-seal against leakage. This was allowed to set for 5 min before the addition of 40 μl of TEMED to the remaining 49 ml gel mixture. The mixture was then poured into the large BioRad Protean II apparatus and allowed to set overnight at 4°C.

The gels were run in a BioRad Protean II apparatus with 0.5% TBE in the upper tank and 0.25% TBE in the lower tank. The gels were prerun at 4°C for 2 hours at constant amperage of 13-15 mA before the binding reactions were applied. A lane was also loaded with 30 μl loading buffer with bromophenol blue to allow the progress of the samples to be followed. Binding reactions were electrophoresed for 4 hours at 13-15 mA at 4°C, until the dye front had migrated half to two-thirds through the gel.

After electrophoresis, the samples were transferred to nylon membrane by capillary transfer as described by Roche in the 'Dig DNA Gel Shift Kit'. The transfer took place overnight using 0.25% TBE as the carrier.

Chemiluminscent Detection

Following transfer to nylon membrane, the DNA was fixed by a UV Stratalinka (as described previously). The membrane was then equilibrated in Buffer 1 for 5 min. The membrane was blocked for 30 min in 1% Blocking Solution (Roche), followed by 30 min incubation with anti-DIG-alkaline phosphatase, Fab fragments (Roche) diluted 1/10 000 in Blocking Solution (Roche). The membrane was washed twice for 15 min in washing buffer (Buffer1 + 0.3% Tween-20) and then equilibrated for 3 min in Buffer 3. The membrane was incubated in a sealed bag with a 1/100 dilution of CSPD (Roche) in the dark at RT with shaking for 10 min, then placed in a fresh, sealed bag and incubated in the dark at 37°C for a further 10 min. The membrane was then exposed to X-ray film (Fuji) for as long as required.

IRON-LIMITED GROWTH ASSAY

Modified Fec medium (Ochs *et al.*, 1995) for growth in iron-limited conditions consisted of LB containing 0.4 mM 2',2-dipyridyl, 1 mM citrate, and kanamycin when required. Inocula were prepared by growing strains overnight in 2.5 ml of LB or LB containing kanamycin for the maintenance of plasmids. To remove exogenous iron, bacteria were centrifuged at 11 600 x *g* for

one min and resuspended in modified Fec medium. Following a second wash in modified Fec medium bacterial suspensions were standardized by absorbance at 600 nm. Fifty ml of the modified Fec medium were inoculated with 0.1 ml of the standardized bacterial suspension. Aerated cultures were incubated at 37°C. Two ml samples were taken over a 24 hour period (2 h, 4 h, 6 h, 8 h, 12 h and 24 h) and the absorbance at 600 nm was measured. Four cultures of each strain were grown simultaneously. Viable counts were also performed at 0 h and 24 h to compare with absorbance readings.

PATHOGENICITY ISLAND EXCISION ASSAY

Bacterial genome extractions were performed as described above. Genomic preparations were standardised by absorbance at 260 nm prior to the PCR assay. The excision of the SRL PAI was determined by PCR using inward facing primers designed on either side of the SRL PAI, BAP679 and BAP1157 (Appendix 3). As a control for the standardisation of the various genomic preparations, a PCR for the presence of *recA* (primers BAP1643 and BAP1644) was performed simultaneously. Serial 10-fold dilutions of the various genomic preparations were performed and PCRs carried out at each dilution. The PCR cycle was the standard *Taq* PCR with a 1 min extension time and 59°C annealing temperature. DNA positive and negative controls were also included. PCR products were analysed by electrophoresis on 0.8% TAE agarose.

SITE-DIRECTED MUTAGENESIS

Site-directed mutagenesis of four base pairs on a plasmid insert was performed utilising a PCR-based method. Overlapping oligonucleotides were designed with the mutated bases located centrally (as described by the QuikChange Site-Directed Mutagenesis Kit [Stratagene]). The mutation was introduced by amplification of the plasmid using KOD polymerase (Novagen) as described by the manufacturer (Appendix 4). For mutagenesis 250 µl of PCR reaction was used, and 16 PCR cycles. The PCR product mix was treated with *Dpn*I (1 µl of 20 U/µl per 100µl of reaction mix) by incubation at 37°C overnight to digest any template DNA. The reaction was cleaned using a QIAQUICK column (Qiagen). The resulting solution was then concentrated in a Savant Speed Vac Concentrator and resuspended in a final volume of 6 µl. This solution was then electroporated into the strain of interest (described above). Transformation mix was plated onto appropriate selective LB plates and grown overnight at 37°C. Plasmid DNA was extracted from any resulting colonies and sequenced to confirm mutation.

REAL-TIME REVERSE TRANSCRIPTION PCR

Real-Time was performed as described by PE Biosystems (SYBR® Green PCR Master Mix & RT-PCR Protocol). RNA utilised for Real-Time PCR was extracted as described previously. Primers were designed using Primer Express software (ABI) (Appendix 3). Oligonucleotide concentration was determined as described by PE Biosystems, and stored at a final concentration of 625 nM. Reverse transcription reactions consisted of: 20 µg of total RNA; 30 µg of random hexamers; 10 U Superscript II (Gibco/BRL) reverse transcriptase; 500 µM concentrations of dATP, dCTP, dGTP and dTTP. The reverse transcription reaction was performed by incubation at 42°C for 2.5 hr, followed by 15 min at 70°C. cDNA was diluted 320-fold prior to Real-Time PCR. A typical Real-Time PCR reaction consisted of:

SYBR Green PCR Master Mix (x2) (ABI)	10 µl
625 nM OligoI	1.6 µl
625 nM OligoII	1.6 µl
dH_2O	4.4 µl

The reaction mix was made and dispensed into a 96-well tray compatible with the ABI Prism 7700 Sequence Detection System. 2.4 µl of template cDNA was then added to the wells. Real-Time PCR data was standardised and cDNA concentrations determined by comparison with a gene-specific standard curve from known concentrations of *S. flexneri* genomic DNA. Amplification of a single product for each RT-PCR was confirmed by melting curve analysis.

~ CHAPTER THREE ~

THE SRL PATHOGENICITY ISLAND

INTRODUCTION

Bacterial genomes show remarkable plasticity because of their ability to both acquire and lose regions of their chromosomes. This plasticity provides bacteria with the potential to evolve rapidly, and thus enhances their chance of survival. The ability to sequence entire genomes has provided us with examples of bacterial species whose genomes clearly show plasticity, i.e. variation is apparent between very closely related species. Consequently, the genome sequence of one bacterial strain is not representative of the entire species. There are many differences between bacterial genomes of the same genus, species and serotype. For instance, *Escherichia coli* K-12 has several regions that vary when compared to other *E. coli* strains. An example is the ferric dicitrate transport system found at minute 97 of the *E. coli* K-12 chromosome, but which is absent in many subsequently examined *E. coli* strains (Ochman, 2000). Genome variations are particularly obvious when comparing pathogenic to non-pathogenic members of the same species. The differences between *E. coli* K-12 and recently sequenced enterohaemorrhagic *E. coli* (EHEC) O157:H7 strains are significant. Approximately 25% of the EHEC O157:H7 genome, from two independently isolated strains, is not present in *E. coli* K-12 (Hayashi *et al.*, 2001; Perna *et al.*, 2001; Strockbine *et al.*, 1988). In addition, 567 *orfs* (~0.53 Mb) present in *E. coli* K-12 are absent in the EHEC O157:H7 strains, including the *fec* locus and restriction and modification genes. In both sequenced EHEC strains, the regions differing from *E. coli* K-12 (S-loops in O157 Sakai strain and O-islands in EDL933 strain) had significantly different G+C contents from the *E. coli* backbone, suggesting that they were acquired by horizontal transfer. The genome of EHEC O157:H7 strain EDL933 was found to contain 177 O-islands greater than 50 bp in length. There is even diversity between the two O157:H7 strains sequenced. For example, EDL933 has duplicate O-islands inserted at *serX* and *serW*. In contrast, the Sakai strain only has an homologous S-loop, SpLE1, inserted into *serX*, and there are variations within the O-islands and the S-loop as well (Hayashi *et al.*, 2001; Perna *et al.*, 2001). As more genomes are sequenced, comparative genomics will provide more evidence for the plasticity of bacterial genomes, and as such, will allow a better understanding of the way in which bacteria share and exchange their genetic material.

There are many mechanisms for the acquisition of foreign DNA; some of these are listed in Table 3a, along with examples of how they may enhance bacterial virulence. Of interest in this study is the area of pathogenicity islands (PAI) and genomic islands (GEI). These are large regions of the chromosome that appear to be genetically and functionally distinct units (Häcker *et al.*, 1997; Lee, 1996). These elements are usually incorporated into bacterial chromosomes by site-specific integration into or adjacent to tRNA genes and some are capable of excision. These elements often have a G+C content that varies from that of the host bacterium, suggesting they were acquired by horizontal transfer. The difference between a PAI and a GEI is the presence of pathogenesis

determinants. For example, the LEE PAI of EPEC and EHEC is termed a pathogenicity island because it has a role in virulence due to the carriage of a type III secretion system and the effector genes required for causing A/E lesions associated with disease (Elliot *et al.*, 1998). An example of a genomic island is the 500 kb island in *Mesorhizobium loti*, which encodes the genes for nodulation and nitogen fixation (Sullivan & Ronson, 1998).

Table 3a Mechanisms for acquisition of bacterial virulence factors (adapted from Dobrint & Hacker 2001)

Genetic element or mechanism	Example of altered pathogenesis	Reference
IS Elements/Composite Transposons/Integrons	Spread of antibiotic resistance determinants	Hall & Collis, (1995), Stokes & Hall (1989)
Plasmids	Virulence plasmid of *Shigella*	Sasakawa *et al.* (1992)
Bacteriophages	*Vibrio cholerae* CTX phage, EHEC STX phage	Boyd & Brüssow, (2002), Waldor & Mekalanos (1996)
Pathogenicity /Genomic Islands	VPI of *V. cholerae*	Karaolis *et al.* (1998)

This study was initiated following the discovery of a 99 kb element that was capable of deleting from the chromosome of *S. flexneri* 2a YSH6000 (Rajakumar *et al.*, 1996). This element encoded resistance determinants for streptomycin, ampicillin, chloramphenicol and tetracycline, in an organisation similar to that seen in the archetypal antibiotic resistance plasmid, NR-1 (Rajakumar *et al.*, 1997). Further work on this element found that it consisted of a series of nested elements within the chromosome, consisting of the 99 kb element (multi-antibiotic resistant deletable element – MRDE), the *Shigella* resistance locus (SRL) and a pathogenicity island (SRL PAI) (Figure 3a) (Turner *et al.*, 2001).

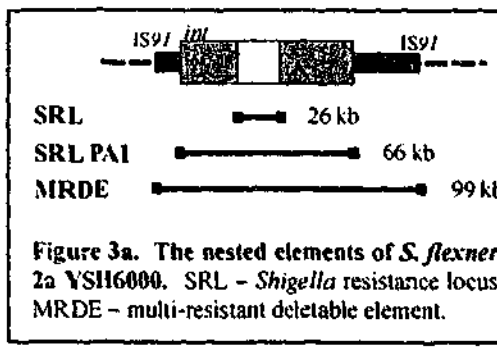


Figure 3a. The nested elements of *S. flexneri* 2a YSH6000. SRL – *Shigella* resistance locus; MRDE – multi-resistant deletable element.

This project has focused on the island within the MRDE. The SRL PAI is 66 257 bp in length, has integrated into a *serX* tRNA gene and carries at least 22 prophage-related ORFs, including a P4-like integrase. The island has been sequenced in its entirety and this chapter focuses on the overall features of the island and analysis of the sequence.

RESULTS AND DISCUSSION

SEQUENCING OF THE SRL PATHOGENICITY ISLAND

The antibiotic resistance determinants of strain *S. flexneri* 2a YSH6000 delete spontaneously from the chromosome at a frequency of 10^{-5}-10^{-6} (Turner *et al.*, 2001) suggesting that the SRL may be carried on a mobile element. To gain a better understanding of the element, the regions flanking the SRL were sequenced using plasmid clones obtained by marker rescue of the resistance determinants encoded by the SRL. *Bam*HI fragments of 27.6 kb inserted in pSBA361 and 24 kb, inserted within pSBA509, were cloned by selection for the tetracycline and ampicillin resistance markers on the PAI,

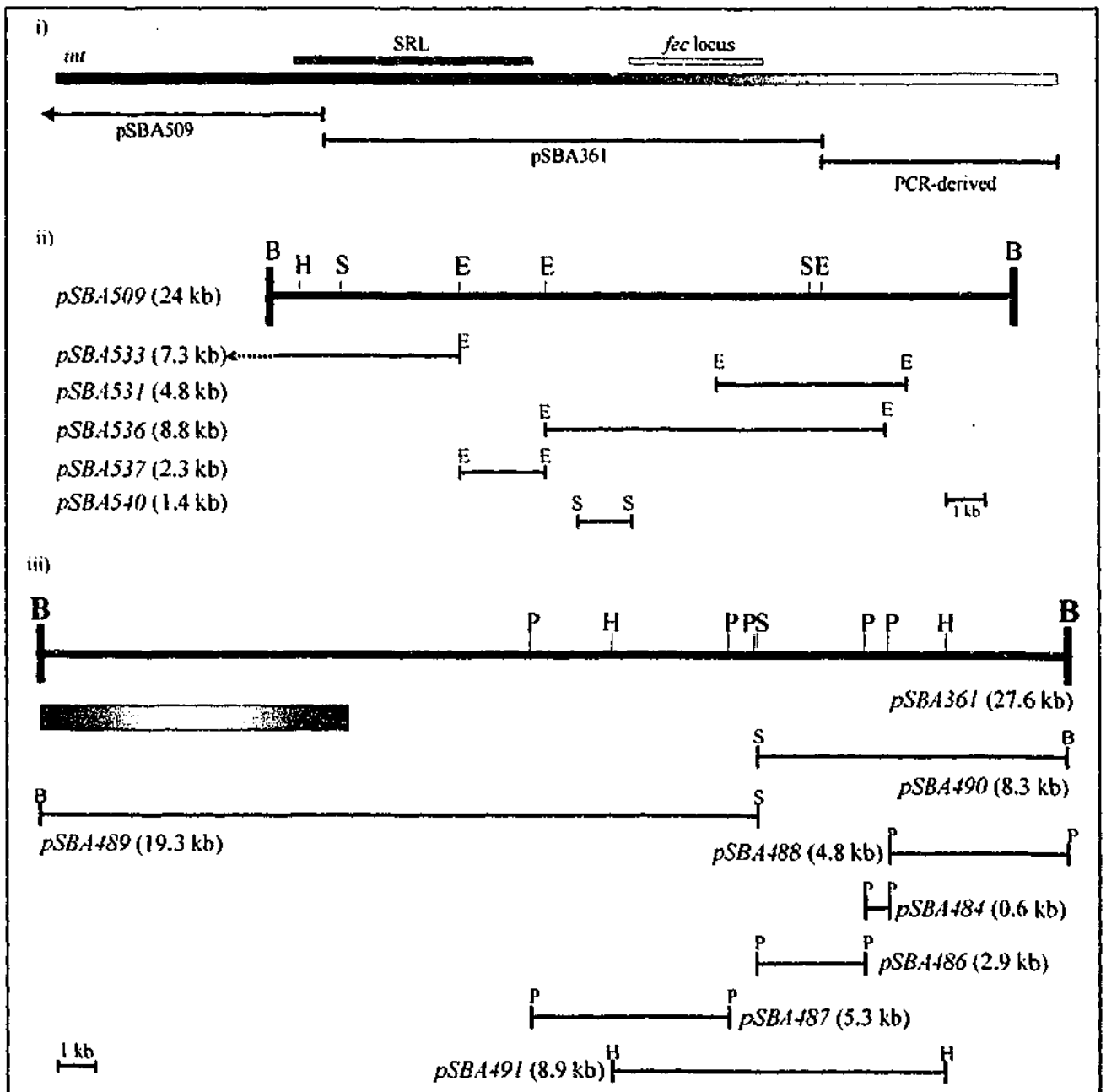


Figure 3b. i) Overview of SRL PAI and major clones. ii) Subclones of pSBA509. iii) Subclones of pSBA361. The size of the various inserts are in brackets. B, *Bam*HI; E, *Eco*RI; H, *HindIII*; P, *PstI*; S, *SalI*. The broken line represents vector region. The shaded box in figure ii) represents the area previously sequenced by Rajakumar *et al.* (1996).

respectively. These *Bam*HI clones were subcloned with a range of restriction endonucleases and the various subclones sequenced using both vector based primers and primers designed from known sequence (Figure 3b) (Appendix 5). The *Bam*HI clones and subclones were generated and shotgun sequenced as part of previous work. Completion of sequencing and annotation was performed as part of this study.

In order to sequence beyond the regions obtained by marker rescue, PCR-based strategies were employed (Figure 3c). The right end of the island was sequenced by designing primers upstream of the *E. coli serX* gene and "walking" into the island by sspPCR. This approach was taken following the sequencing of a PCR product across the deletion point of the island. Turner *et al.* (2001) identified the deletion point by PCR analysis of a strain that had spontaneously lost the SRL PAI. Sequence beyond the known left end of the island was obtained by inverse PCR, sspPCR and the ends of the island were linked by long range PCR.

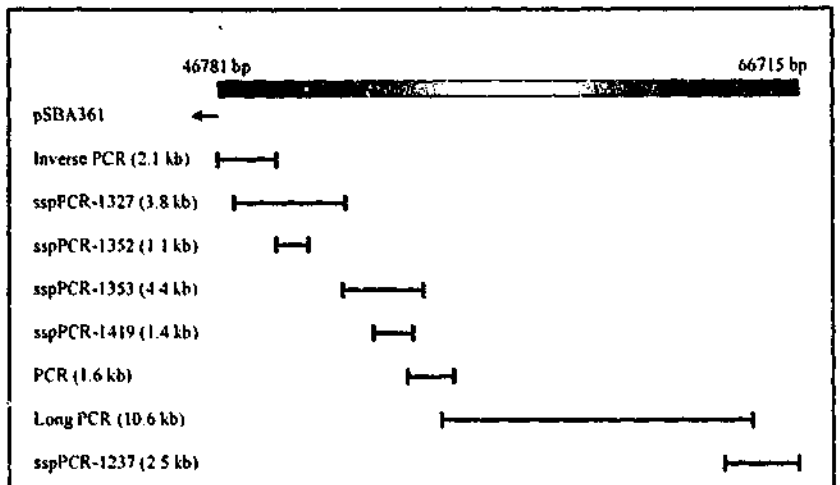


Figure 3c. PCR derived products for sequencing the SRL PAI. The numbers above the shaded area represent the nucleotides of the Genbank submitted sequence (Acc. No. AF326777). Numbers in brackets denote the size of the PCR product.

OVERVIEW OF THE SRL ISLAND

The SRL PAI is 66 257 bp in length (Fig. 3d), beginning 161 bp upstream of the *int* gene and ending 14 bp upstream of the 3' terminus of the intact *serX* gene. It contains 59 open reading frames (Table 3b, Fig. 3d), excluding the ORFs associated with insertion sequences, and has an average mol % G+C content of 49.8%. Although the overall G+C content is not significantly different from that of the *S. flexneri* chromosome (51%), significant deviations occur in the regions homologous to Tn*2603*, Tn*10* and the *fec* locus, which have G+C contents of 57%, 39% and 58%, respectively. This is consistent with the fact that Tn*2603* and Tn*10* are laterally acquired elements and suggests that the *fec* locus may also have been laterally acquired by the PAI. Other ORFs with G+C that are significantly different from that of *S. flexneri* are shown in Table 3c, as well as the average G+C across the island. These regions of variation, with higher and lower G+C than *S. flexneri* suggest that the SRL PAI may have accumulated genetic information from a variety of sources. This is made more apparent as the structure and sequence of the island are characterised in more detail.

Table 3b. Open reading frames contained within the SRL PAI

ORF No.	Gene	Position (bp)	Related Protein*	%Sim#	Protein Accession no./ Reference
1	*int*	619-1836	Integrase CP4-57 (SlpA)	49	P32053 (U36840)
2	*orf2*	3535-2006	YfjI	45	P52124
3	*orf3(rox)*	3812-3507	AlpA (CP4-57)	66	P33997
4	*orf4*	3868-5730	No similarity	-	-
5	*orf5*	7104-7697	No similarity	-	-
6	*orf6*	8449-8745	YfjJ (frameshift)	73	P52125
7	*orf7*	11918-10980	LysR-like transcriptional regulator	79	P39376
8	*orf8*	12300-12989	Hypothetical protein in LysR-AraE	63	P03813
9	*orf9*	13055-14353	DcuA (anaerobic dicarboxylate transporter)	70	P04539
10	*aadA1*	16963-16172	AadA1 (Streptomycin resistance)	95	P04826
11	*oxa-1*	17906-17076	Oxa-1 (Ampicillin resistance)	99	P13661
12	*intI1*	18116-19129	IntI1	99	P09999
13	*tnpM*	19098-19682	TnpM	100	P04162
14	*tnpR*	19808-20368	TnpR	98	P04130
15	*tnpA*	20371-23337	TnpA	98	BAA78805.1
16	*cat*	24641-23982	Cat (Chloramphenicol Resistance)	98	P00483
17	*orf17*	25759-26256	YdjB	79	BAA78832.1
18	*orf18*	26365-26949	JemC	79	AF162223
19	*tetR*	27553-26927	TetR	98	P04483
20	*tetA(B)*	27632-28837	TetA(B) (Tetracycline resistance)	99	P02980
21	*tetC*	29543-28950	TetC	99	BAA78836
22	*tetD*	29631-30047	TetD	100	BAA78837
23	*orf23*	32594-31983	CapU (Hexosyltransferase homolog)	99/73	AAD34405, AB011549
24	*orf24*	33389-32622	Shf	93/92.3	AF134403, U61977
25	*orf25*	34749-35096	Hypothetical ORF o137	98	S56511
26	*fecE*	36488-35721	FecE	100	P15031
27	*fecD*	37445-36492	FecD	100	P15029
28	*fecC*	38419-37442	FecC	100	P15030
29	*fecB*	39339-38437	FecB	98	P15028
30	*orf30*	40424-39384	FecA precursor	100	P13036
31	*fecA*	41707-40421	FecA	100	M63115
32	*fecR*	42747-41794	FecR	100	P23485
33	*fecI*	43265-42744	FecI	100	P23484
34	*orf34*	45173-46531	Putative periplasmic protein (*C. jejuni*)	52	AL139076
35	*orf35*	48155-46770	L0015	98	AAC31494
36	*orf36*	48552-48205	L0014	98	AAC31493
37	*orf37*	48950-48549	L0013	99	AAC31492
38	*orf38*	49805-49284	No similarity	-	-
39	*orf39*	50934-50365	YfjJ	55	P52125
40	*orf40*	51855-51679	Hha/YmoA (*Y. enterocolitica*)	75/75	P23870, P27720
41	*orf41*	52491-52769	Vis (P4)	53	Polo *et al.*, 1996
42	*orf42*	52858-53466	No similarity	-	-
43	*orf43*	53637-53888	No similarity	-	-
44	*orf44*	54534-54319	No similarity	-	-
45	*orf45*	54667-55584	No similarity	-	-
46	*orf46*	55669-56541	YfjP, YeeP	65/97	P52131, P76359
47	*orf47*	56924-59761	Ag43 (Flu)	78	P39180
48	*orf48*	60145-60705	YfjQ	88	P52132
49	*orf49*	61390-61530	YfjX, KlcA	81/51	P52139, P52603
50	*orf50*	61782-62273	YfjY, YeeS	80/99	p52140, P76362
51	*orf51*	62336-62557	YeeT	98	P76363
52	*orf52*	62720-63094	YeeU, YfjZ	91/75	P76364, P52141
53	*orf53*	63141-63515	L0007, YeeV, YpjJ	93/87/74	AAC31485.1, P76365, O46953
54	*orf54*	63512-64003	L0008, YeeW	96/77	AAC31487.1, P76366
55	*orf55*	64015-64212	L0009	88	AAC31488.1
56	*orf56*	64297-64860	L0010	85	AAC31489.1
57	*orf57*	64878-64651	L0011	80	AAC31490.1
58	*orf58*	64929-65933	IS*1328* transposase (*Y. enterocolitica*)	83	O56897
59	*orf59*	66204-66452	L0012	90	AAC31491.1

* Related proteins are from *E. coli* unless otherwise stated. # % similarity at amino acid level

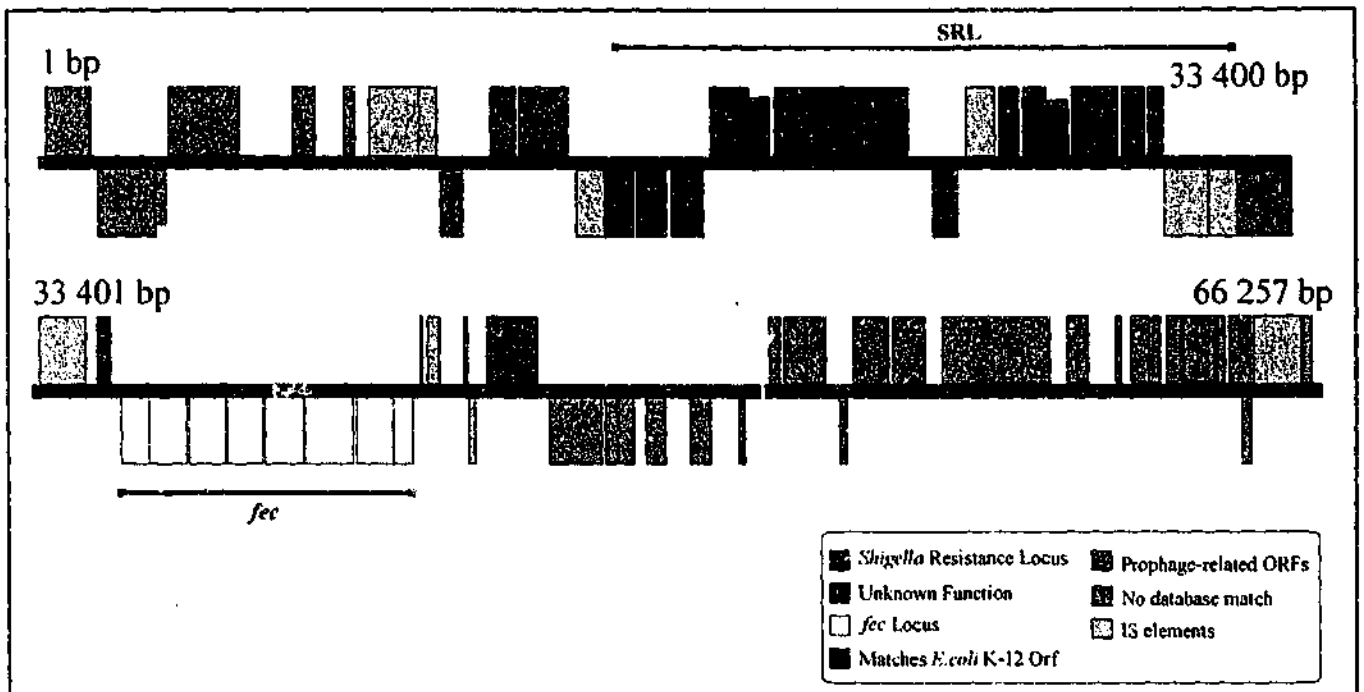


Figure 3d. The genetic organisation of the SRL island. The open reading frames (*orfs*) are represented by boxes above the line (forward orientation) or below the line (reverse orientation). The positions of the SRL and the *fec* loci are denoted by horizontal lines. Overlapping *orfs* are denoted by a variation in size.

Table 3c. G+C% mol comparison of regions within the SRL PAI

Region (bp)	G+C mol (%)	Phage related gene/region	G+C mol (%)	Unknown *orfs*/regions	G+C mol (%)
1 – 10 000	42.6	*orf2-orf3* (619 – 3812)	44.9	*orf4-orf5* (3868-7697)	37.5
10 001 – 20 000	49.6	*orf6* (8449-8745)	45.8	*orf7-orf8* (10980-12989)	44.4
20 001 – 30 000	48.8	*orf35-orf37* (46770-48950)	56.1	*orf23-orf24* (31983-33389)	40.4
30 001 – 40 000	51.4	*orf39* (50404-50940)	57.5	*orf 38* (49284-49805)	53.1
40 001 – 50 000	52.8	*orf41* (52491-52769)	56	*orf40* (51855-41679)	52.6
50 001 – 60 000	51.9	*orf46-orf52* (55669-63094)	55.5	*orf42-45* (52858-55584)	45.5
60 001 – 66 715*	51.3	*orf53-orf57* (63141-64878)	50.9		
		orf59 (66204—66452)	47.8		

*G+C based on sequence submitted as accession number AF326777, which includes 458 bp upstream of the SRL PAI

INTEGRATION OF THE SRL PAI INTO THE CHROMOSOME

Analysis of the DNA sequence showed that a large genetic element has inserted into the 3' terminal region of the *serX* tRNA gene in YSH6000 (Fig. 3c). The element is bounded on the *serX*-distal side by a 14 bp direct repeat (DR) of the 3'-terminal 14 bp sequence of *serX* (Fig. 3e). The DNA sequences upstream of *serX* and downstream of the *serX*-distal DR are almost identical to sequences that are contiguous with the *serX* gene in *E. coli*, a species closely related to *S. flexneri*. Notably, the 3' termini of tRNA genes commonly serve as integration sites for PAIs and prophages (Häcker, *et al.*,

1997). In addition, the 3' terminus of *serX* has sequence similarity to a P4-phage *att* site (Figure 3e), implying that it may act as an integration site for prophage-like or PAI-like elements. The sequence of the genetic element revealed the presence of a P4 bacteriophage-like integrase gene 161 bp upstream of the 14 bp *serX*-distal DR. The integration of the element into the 3' end of a tRNA gene, the presence of an integrase gene near one boundary of the element and the recent finding that the element undergoes integrase-mediated precise excision to restore the *serX* gene as it is organised in *E. coli* (Turner *et al.*, 2001; Van den Bosch *et al.*, 1997) led us to conclude that the element is a PAI, which we have termed the SRL PAI.

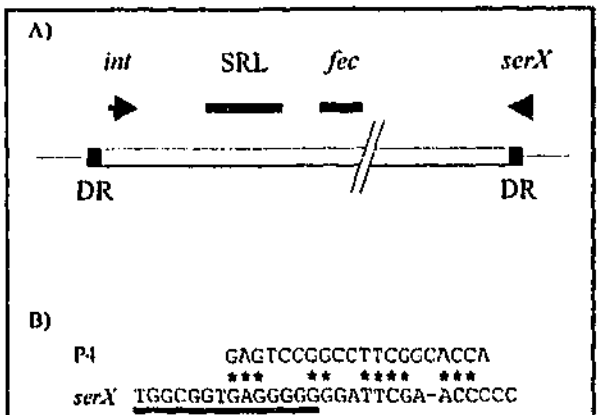


Figure 3e. Structure of the SRL PAI and the flanking direct repeats (DR). (A) The SRL PAI is represented by the open box. The shaded boxes represent the 14 bp DR. The right-hand DR is the last 14 bp of *serX*. (B) The14 bp DR and the potential *att* site. The lower sequence shows the 3' end of the *S. flexneri* YSH6000 *serX* gene. The region representing the 14 bp DR is underlined. The upper sequence represents the P4 core *att* site. *, conserved nucleotide.

Interestingly, there was a significant discrepancy in the lengths of the 66.2 kb SRL PAI and the previously described 99 kb deletable element carrying multiple antibiotic resistance in YSH6000 (Rajakumar *et al.*, 1997). This discrepancy has been explained by the recent finding that the SRL PAI is entirely contained within the larger distinct genetic element, termed the MRDE (Multi-antibiotic Resistant Deletable Element), which is flanked by IS*91*-like elements and is also capable of precise excision from the chromosome (Rajakumar *et al.*, 1997; Turner *et al.*, 2001) (Fig. 3a). This is similar to the *Y. pestis* 6/69 High-PAI which is contained within a larger, deletable 102 kb chromosomal region, that also carries genes for hemin utilisation. The boundaries of the 102 kb region are defined by IS*100* elements which mediate the spontaneous deletion of the High-PAI and flanking chromosomal DNA in this strain (Buchrieser *et al.*, 1998a).

SEQUENCE ANALYSIS OF THE SRL PAI

Integrase

The deduced product of the *int* gene, located near the left boundary of the SRL PAI, has significant sequence similarity at the amino acid level to several integrase proteins from the P4 bacteriophage Int family, including those from the CP4-57 cryptic prophage of *E. coli* (49% similar) and the *V. cholerae* pathogenicity island (52% similar). Integrases from the other *S. flexneri* PAIs, *she* PAI and SHI-2, showed 47% and 45% similarity to the SRL PAI Int respectively. The putative Int protein encoded on the SRL PAI possesses a conserved motif (R, HXXR, Y) necessary for the function of P4-like

integrases (Abremski & Hoess, 1992; Argos *et al.*, 1986), suggesting that the integrase may be functional. The function of the integrase is discussed in more detail in Chapter 4.

Prophage Related Open Reading Frames

Twelve ORFs on the SRL PAI had significant amino acid sequence similarity, ranging from 45-88%, to ORFs encoded by the CP4-57 prophage (Figure 3f). Although the physical spacing of the SRL PAI *orfs* differed from that of their homologues in CP4-57, their order and orientation were conserved, with the exception of *orf2* and *orf3* which were inverted when compared to their CP4-57 homologues *yfjI* and *alpA*. There is also a second, truncated copy of *yfjI*, *orf6* which is in the same orientation as the other CP4-57 homologues. *orf39* is in the opposite orientation with respect to its CP4-57 encoded homologue, *yfjJ*. Orf6, Orf39 and Orf48 are truncated in comparison to their CP4-57 homologues (Orf6 and Orf39 have N-terminal truncations and Orf48 has a C-terminal truncation). Of the 12 SRL PAI ORFs homologous to CP4-57 ORFs, seven have homologues in CP4-44, another cryptic prophage in *E. coli* K-12 (Blattner *et al.*, 1997). Similarity between the SRL PAI ORFs and ORFs on CP4-44 ranged from 87% to 99%. The SRL PAI also carries homologues of ORFs L0007 - L0015 from a third prophage-related element, CP-933L, situated on the EHEC LEE PAI. Two of these ORFs, Orf53 and Orf54 are also common to CP4-44. Thus, a total of 22 SRL PAI ORFs, comprising approximately 24% of the PAI sequence, appear to have a prophage origin.

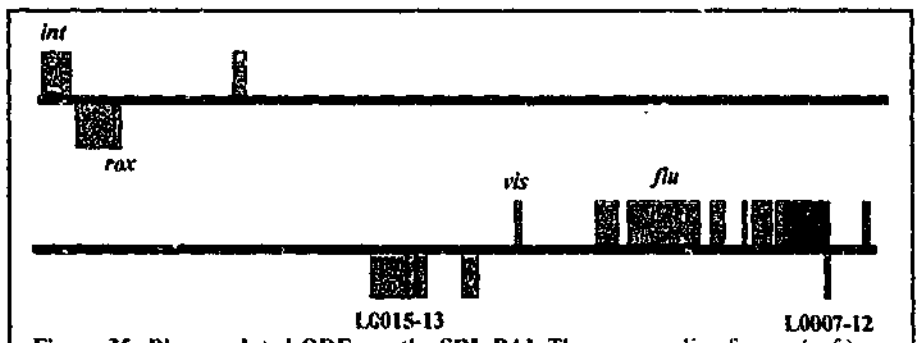


Figure 3f. Phage related ORFs on the SRL PAI. The open reading frames (*orfs*) are represented by boxes above the line (forward orientation) or below the line (reverse orientation). The pink ORFs denote CP-4 homologues and purple ORFs denote homologues of the EHEC 933L prophage. ORFs with known homologues are labelled.

Recently, a PAI-like element has been identified in uropathogenic *E. coli* CFT073 that appears to carry two partial copies of a CP4-like prophage. The PAI II_{CFT073} is lacking an integrase gene. However, like the *S. flexneri* 2a YSH6000 SRL and *she* PAIs it carries homologues of both the CP4-like prophages and the EHEC 933L prophage. Figure 3g compares the organisation of the known CP4-like prophages.

The recent sequencing of the EHEC O157:H7 EDL933 genome has revealed two identical elements encoding tellurite resistance and urease genes with a conserved backbone remarkably similar to the SRL PAI. These elements insert into *serX* and *serW* on the EHEC O157:H7 EDL933 chromosome, and have been designated O-islands #43 and #48, respectively (Perna *et al.*, 2001). A similar element,

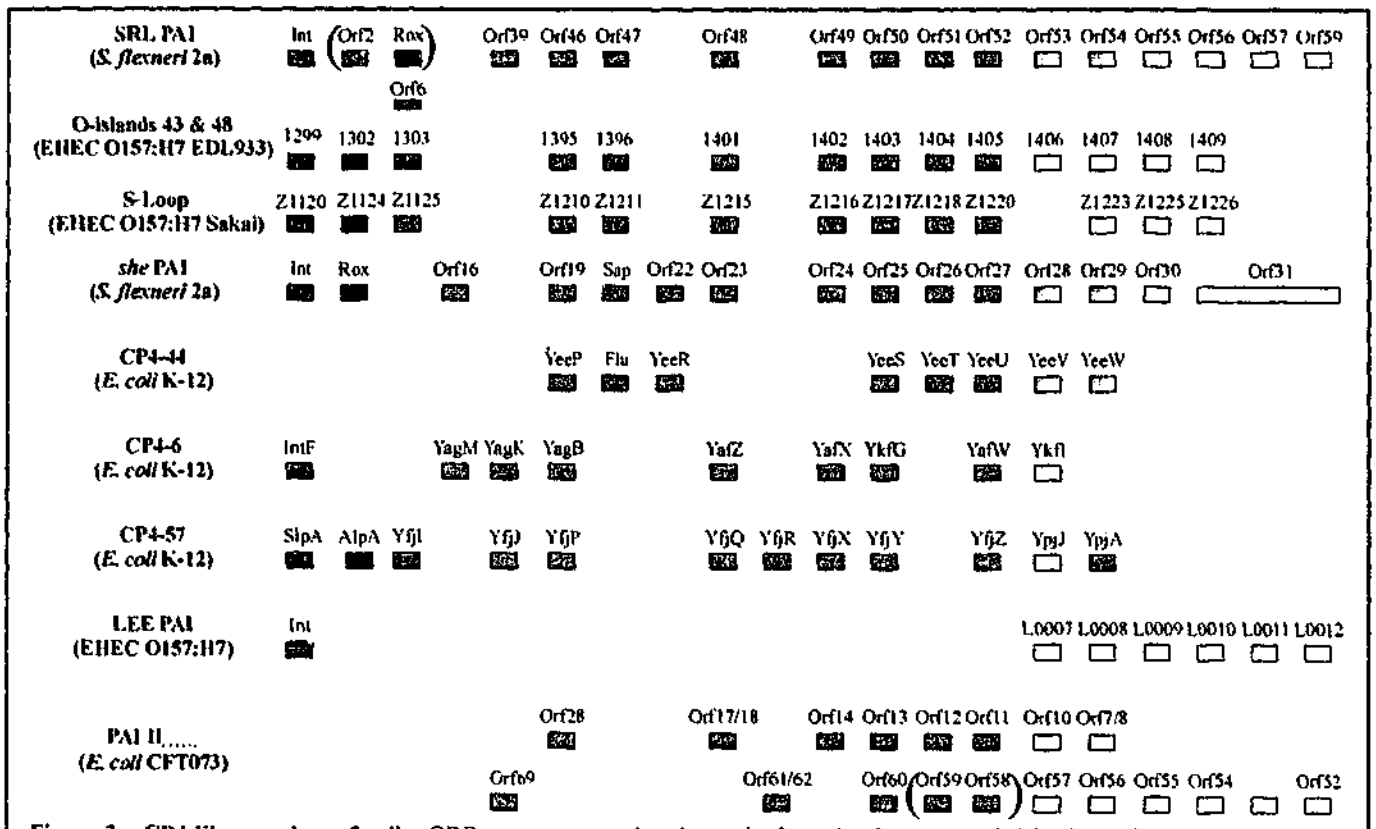


Figure 3g. CP4-like prophage family. ORFs are represented as boxes in the order they are carried in the various elements. ORFs represented by pink boxes have homologues on the CP4-like prophages of *E. coli* K-12, while yellow boxes denote ORFs encoded on the EHEC 933L prophage. ORFs with pink and yellow shading are present in both parent prophages. Int homologues are represented by blue boxes. Rox homologues are denoted by purple boxes. Green boxes show homologues of Ag43 (Flu). Homologues of Yfjl are denoted in orange. ORFs in the reverse orientation are indicated by parentheses.

also present in the EHEC O157:H7 Sakai strain inserted at *serX*, but not at *serW*, is designated SpLE1 (Hayashi *et al.*, 2001). Genes within the P4 integrase family show very little or no identity at nucleotide level and similarity at amino acid level is usually confined to conserved motifs (Abremski and Hoess, 1992;Argos *et al.*, 1986). However, the *int* genes on the two EHEC elements (O-island #43 and #48 in EDL933) show 99% identity at nucleotide level to the SRL *int*, before a divergence for the last 29 nucleotides. The sequences upstream of the O-island #43 and SRL PAI *int* genes before the 14 bp direct repeat are also identical. Of particular importance is the presence of AlpA homologues on each of the EHEC elements. Remarkably, the AlpA homologue on the SRL PAI, Orf3 (Rox), is also very similar (79% amino acid similarity) to the EHEC homologues. As discussed in Chapter 4, Orf3 is believed to regulate *int* transcription. O-islands #43 and #48 also have other prophage homologues that are present on the SRL PAI (Figure 3g). Notably, there are homologues of L0007-L0011 at the right end of the element suggesting that the SRL PAI and EHEC islands shared a common ancestor and then over time each evolved specific features that were beneficial to the different organisms. In EHEC strains there may have been a selection for the maintenance of tellurite resistance and urease genes carried on the O-islands, while *Shigella* species may have benefited from multiple antibiotic resistance and an additional iron-transport system. The conservation of the *int* genes suggests that this divergence happened relatively recently.

The presence of prophage related *orfs* in conserved organisation lends weight to the suggestion that many PAIs may be cryptic prophages that have lost their mobility. It does not appear that the *orfs* on the SRL PAI encode a packaging system for a phage and so its ability to move horizontally may have

been lost. However, as this PAI has a P4 *int* gene and the prophage-related ORFs show homology to cryptic P4 bacteriophages, it is possible that this island utilises a helper phage, just as P4 does. Without bacteriophage P2, P4 propagates as a high copy number plasmid or remains withing the host chromosome, but is unable to enter the lytic cycle and thus move into new host cells (Lindqvist *et al.*, 1993). It is possible that the SRL PAI uses a similar mechanism. Ohnishi *et al.*, (2001) suggests that some of the EHEC O157:H7 SpLEs elements, which are very similar to the SRL PAI, may use a P2/P4-like system for transfer. The use of accessory phages for mobilisation has been described for two PAIs. Firstly, the SaPI1 in *Staphylococcus aureus* utilises the temperate staphylococcal phage 80α in order to excise, undergo encapsidation and transfer to a new recipient (Ruzin *et al.*, 2001). Secondly, the 39.5 kb VPI of *V. cholerae* is able to be transferred between O1 serotype strains by a generalised transducing phage, CP-T1 (O'Shea & Boyd, 2002). The VPI then integrates in a site-specific manner to the same chromosomal locus in all strains. In order to determine if there is a bacteriophage capable of aiding SRL PAI movement a large pool of bacteriophages known to infect *Shigella* needs to be tested with *S. flexneri* YSH6000.

Iron Transport System

Sequencing of the regions surrounding the SRL using marker rescued clones (described previously) revealed a locus that was homologous to the ferric-dicitrate (*fec*) transport genes located at minute 97.3 of the *E. coli* K-12 chromsome (Veitinger & Braun, 1992). The *Shigella* locus has an identical organisation to the *E. coli* K-12 *fec* locus, consisting of the regulatory genes, *fecI* and *fecR*, and the downstream structural genes, *fecABCDE*. The *S. flexneri fec* genes showed greater than 99% nucleotide identity with the *E. coli* K-12 genes, but there were differences in the regions flanking the locus. The *E. coli* K-12 locus is flanked upstream by IS*1* and downstream by an IS*911* element that is insertionally disrupted by an IS*30* and a truncated IS*2*. In contrast, the *S. flexneri fec* locus was flanked downstream by an intact IS*911*. The sequence directly downstrea of IS*911* is identical in *E. coli* and *S. flexneri*. Upstream of the *S. flexneri fec* locus were the first 61 bp of IS*1* followed by remnants of IS*3*, IS*629* and IS*903*-like elements (Figure 3h). Prior to this report the entire *fec* locus had been identified only in *E. coli* K-12 and B strains (Lin *et al.*, 1999; Pressler *et al.*, 1988; Wagegg & Braun, 1981), although *fecA*, *fecD* and *fecE* homologues had been reported in *Helicobacter pylori* (Velayudhan *et al.*, 2000) and *fecBCD* homologues in *Pasteurella multocida* PM70 (May *et al.*, 2001). This is the first report of an intact ferric dicitrate (*fec*) transport system in a *Shigella* spp. Further characterisation of this iron transport system is reported in Chapter 5.

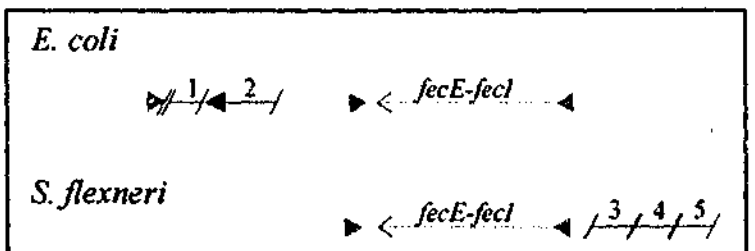


Figure 3h. Comparison of *S. flexneri* and *E. coli fec* loci flanking regions. The blue arrows denote IS*911*, the pink arrow denotes IS*1F*. The numbers 1, 2, 3, 4, and 5 represent remnants of IS elements IS*2*, IS*30*, IS*3*, IS*629* and IS*903*-like respectively.

Shig ·la *Resistance Locus (SRL)*

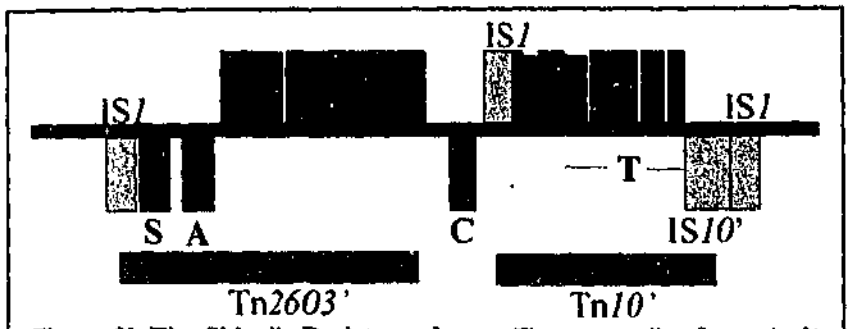


Figure 3i. The *Shigella* Resistance Locus. The open reading frames (*orfs*) are represented by boxes above the line (forward orientation) or below the line (reverse orientation). Grey boxes denote insertion sequences. Genes or operons encoding resistance to streptomycin, ampicillin, chloramphenicol and tetracycline are labelled with S, A, C and T respectively. Regions showing homology to Tn*2603* and Tn*10* are denoted by pale blue boxes.

Previously described by Rajakumar *et al.* (1997), the *Shigella* resistance locus (SRL) spans 15 898 bp in the centre of the SRL PAI. In addition to the locus as described by Rajakumar *et al.* (1997), the boundaries of the SRL were defined as part of this study, and several gaps in the sequence were completed. The complete organisation of the SRL is shown in Figure 3i. The SRL is flanked by directly repeated IS*1* elements. This, coupled with its ability to excise precisely (Turner *et al.*, 2001), suggest that it may be a transposon. Rajakumar *et al.* (1997) reported the similarity in organisation between the SRL and NR-1, the archetypal resistance plasmid. This similarity is mostly due to the carriage of the streptomycin, ampicillin, chloramphenicol and tetracycline resistance determinants on transposons. NR1 is a self-transmissible R-plasmid in the FII incompatilibility group. Its R-determinants consist of a Tn*2671* (Iida *et al.*, 1981) and a Tn*10* encoding tetracycline resistance (Womble & Rownd, 1988). While transposons, integrons and plasmids are well known to be sources for the dissemination and stability of antibiotic resistance (Hall & Collis, 1995; Salyers & Amábile-Cuevas, 1997), this is one of only four genomic islands that encode such resistance determinants. A mobile element termed a CONSTIN, carried in some *Vibrio cholerae* strains encodes resistance to sulfamethoxazole, trimethoprim, chloramphenicol and streptomycin (Hochhut *et al.*, 2000). Methicillin resistance in *Staphylococcus aureus* N315 has been shown to be carried on a genomic island, although the island lacks an integrase (Ito *et al.*, 1999). There is also an island in *S. enterica* sv Typhimurium DT104 that encodes resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Boyd *et al.*, 2000; Boyd *et al.*, 2001). The multiple drug resistance determinants on this island are mostly carried on integrons, although the integrons and genes encoding these resistances are different from those on the SRL PAI. Interestingly, the *S. enterica* sv Typhimurium genomic island also has a CP4-like integrase.

Turner *et al.* (2003) have examined 71 *Shigella* strains for the presence of the SRL PAI. Of the 71, 31 had the same resistance pattern as *S. flexneri* 2a YSH6000. With the exception of two strains whose resistances were plasmid-borne, all of the *Shigella* strains with multiple antibiotic resistance also possessed a SRL *int* homologue. The resistance determinants in the majority of the strains were shown by PCR to be linked, indicating an organisation identical to that of the SRL. This provides evidence for the role of PAIs in the dissemination of antibiotic resistance determinants. Given the

phage ancestry of this island, perhaps bacteriophages may also be involved in the spread of antibiotic resistance. Further work will show whether this element is capable of horizontal transfer to new *Shigella* or *E. coli* strains and thus act as a vector for the spread of multiple antibiotic resistance determinants.

Insertion Sequences

The SRL PAI carries six intact insertion sequences and seven IS remnants that have undergone deletions (Table 3d). Together they constitute 14% of the SRL PAI sequence. IS elements and their remnants, including IS*1*, IS*200*, IS*600* IS*629* and IS*1328* which are present on the SRL PAI, are commonly found in other PAIs (Blanc-Potard *et al.*, 1999; Elliot *et al.*, 1998; Mellies *et al.*, 2001; Vokes *et al.*, 1999). Notably, the recently sequenced *S. flexerni* 2a strain 301 does not contain any remnants of IS*200* or IS*1328* (Jin *et al.*, 2002). Of interest is the presence of an IS*1328* transposase at the right end of the PAI. IS*1328* is associated with the *fyuA/irp2* gene cluster of *Y. enterocolitica* (Rakin & Heeseman, 1995) in the High-PAI (Carniel *et al.*, 1996), which is essential for *Yersinia* virulence. On the SRL PAI, sequences either side of the transposase matched part of the *Y. enterocolitica* IS*1328* at nucleotide level; conversely the transposase was only similar (83%) at amino acid level. The distribution of IS*1328* is relatively limited, with related transposases being identified only on an *Enterobacter aerogenes* plasmid R751 and near an AraC-like regulatory gene in *Pseudomonas aeruginosa*.

Orf35, Orf36 and Orf37 which show significant similarity to L0015, L0014 and L0013 of the EHEC 933L prophage, may represent an IS element. Analysis of members of the IS*66* family of IS elements has led to the designation of L0013, L0014 and L0015 as an IS element. Database searching by Schneiker *et al.* (1999) showed that the 933L prophage ORFs are related to IS*Rm14* of *Sinorhizobium meliloti*, and therefore they were designated as part of an IS element, IS*Ec8*. Schneiker *et al.* (1999) proposed that IS*Ec8* has the structural characteristics of elements within the IS*66* family. Members of this family have features including size of approximately 2.7 kb and three consecutive ORFs oriented in the same direction, with an ATGA or GTGA lying between the first two ORFs (Schneiker *et al.*, 1999). Like other members of the family, IS*Ec8* and the SRL PAI homologue consist of three consecutive ORFs oriented in the same direction; the first and second ORFs (*l0013/l0014* and *orf37/orf36*) do overlap and share a common ATGA sequence. The inverted terminal repeats identifed by Han *et al.* (2001) for IS*Ec8* are also present in the SRL PAI flanking *orf35* and *orf37*. The IS element homologue on the SRL PAI is 2 288 bp in length and corresponds to base pairs 49021-46733. It is unlikely that either IS*Ec8* or the SRL PAI homologue are functional, because both contain a number of deletions in all three ORFs when compared to IS*Rm14* homologues. The SRL PAI homologue also has an additional 154 bp deletion in *orf35* compared to L0015 of IS*Ec8*. The

truncation of IS*Ec8*-like ORFs homologues is relatively common among *Shigella* and *E. coli*. In the EHEC O157:H7 Sakai genome there are 11 copies of IS*Ec8*, four with truncations or deletions (http://genome.gen-info.osaka-u.ac.jp/bacteria/o157/sptable1.html). Sequencing of *S. flexneri* 5 virulence plasmid pWR501 identified 3 intact IS*Sfl4* (equivalent of IS*Ec8*) and 6 partial copies. Of the three full length IS*Sfl4* elements, only one had an intact L0015, one had an internal deletion and another a frameshift (Venkatesan *et al.*, 2001).

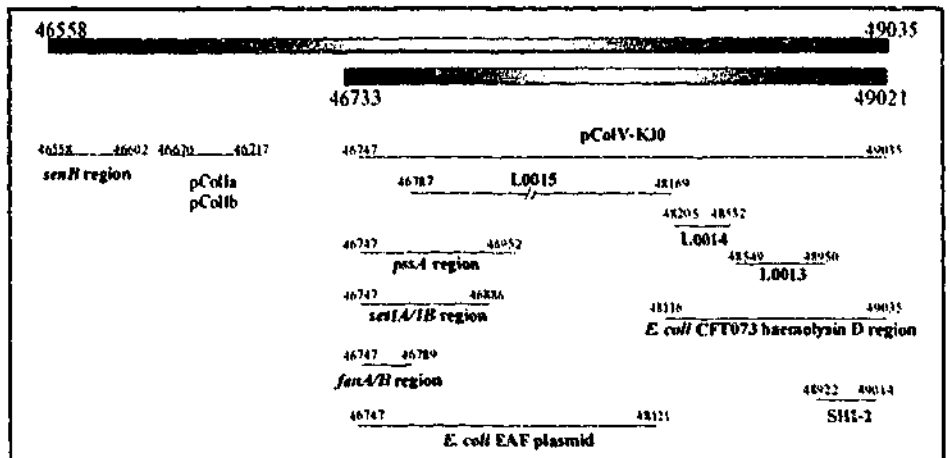


Figure 3j. IS*Ec8* and virulence associated loci. The green box represents a region of the SRL PAI. The purple box represents IS*Ec8*. The numbers on each line represent the corresponding nucleotides on the SRL PAI.

The IS*Ec8* homologue, 175 bp upstream and 15 bp downstream are associated with a number of virulence loci. Figure 3j displays this 2477 bp region and the virulence loci associated with it. These virulence loci are known to encode two toxins (*senB* and *set1AB*), three members of the IgA-protease family (*sigA*, *pic* and *pssA*), a regulator in the synthesis of the K99 fimbrial subunit component, FanC (*fanC*), the genes required for colicin immunity on two colicin I plasmids (pColIa and pColIb), and the colicin V plamsid, pColV-K30. The region between *orf34* and *orf35* on the SRL PAI has no open reading frames but is associated with several virulence loci. This association was identified previously but no function was assigned (Djafari *et al.*, 1997).

The association of this IS element, IS*Ec8*, and its homologues with so many known virulence loci is noteworthy. Has the IS element aided in the dissemination of virulence factors between *E. coli* and *Shigella* spp.? Has it had a role in converting commensal *E. coli* to pathogenic *E. coli*? There are no copies of this IS element present in *E. coli* K-12, but it is present in many pathogenic strains. IS elements are known to have a role in the movement of virulence determinants. For example, the aerobactin locus in *S. flexneri* has been shown to be able to moved on a transposon flanked by IS*1* elements (de Lorenzo *et al.*, 1988).

Putative Transcriptional Regulators

Hha

Orf40 encodes a homologue of the *E. coli* haemolysin expression modulating protein, Hha, with the closest homologue being RmoA, a member of the Hha/YmoA Protein family, showing 90% amino acid similarity across the central region of the protein. The central region of this protein is highly conserved amongst the members of the family. Orf40 appears to be truncated at the amino-terminus as it is between nine and fourteen residues shorter than other family members, and conservation of amino acid residues is not observed until approximately the 25th residue of Orf40 (Figure 3k). Orf40 does possess a potential ribosome binding site and –10 box upstream of the proposed GUG start codon, although conservation of these sites are weak. GUG has been shown to act as an initiation codon in some instances (Kovak, 1983). Therefore, transcriptional analysis of this *orf* is required before any conclusions about its ability to function can be drawn.

```
Hha      MSEKPLTKTD Y MRLR CQT ID  RVI  NKYELSDN L AVFYS  DH  LAE TMNK Y DK PSSVWKF IR
YmoA     -----MTKTD Y MRLRKCTT ID  RVI  NKYELSDD L ELFYS  DH  LAE TMNK Y DK PPTVWQH VK
RmoA     ---MAKTKQE W YQLR CSS AN  KIIR NRDSLLNS R ESFNS  DH  LAE ITGK Y DR PKEIWKY VR
pO157    ---MEKTKQE W YQLR CSS WN  KIIH NRDSLSTS R ESFNS  DH  LAE ITGK Y DR PKEIWKY VR
Orf40    ---------- ----VAARNT SGDAGKVNSH TR KLTLA L EAFNS VDN  LAE TMNK Y DRAPASVWKY VT
                       .  .    .. . ..  *  *   * ** *.* ****  .*** *. * . *.  .
```

Figure 3k. Comparison of Orf40 and Hha/YmoA class proteins. Residues highly conserved amongst Hha and H-NS class proteins are highlighted in ▮. Asterisks (*) denote identical residues; Dots (.) represent conservative amino acid changes. Accession numbers for the sequences shown are: Hha – P23870; YmoA – P2720; RmoA – CAA74168; pO157 – T42104; Orf40 – AF326777 40.

YmoA and Hha are members of a class of proteins that modulate bacterial gene expression as a response to environmental stimuli (de la Cruz *et al.*, 1992). Hha has been shown to modulate α-haemolysin synthesis at the transcriptional level (Carmona *et al.*, 1993; Nieto *et al.*, 1991). Hha also has a role in modulating expression of an *E. coli* outer membrane protein, OmpA, and IIA^{Glc}, of the phosphotransfer system, and the adhesin encoded by the Vir plasmid in bovine diarrheagenic *E. coli* (Balsalobre *et al.*, 1999; Mouriño *et al.*, 1996). YmoA modulates the expression of the *yop* genes, *yadA* and *yst* enterotoxin all of which have a role in *Yersinia enterocolitica* virulence (Cornelis *et al.*, 1991). Interestingly, *hha* mutants resulted in both the repression and derepression of various proteins. For example, at low temperatures the α-haemolysin is usually repressed, but it was derepressed in a *hha* mutant. Conversely, the expression of OmpA and IIA^{Glc} was markedly decreased (59% and 81% respectively) in a *hha* mutant (Balsalobre *et al.*, 1999). Similar results were seen with *ymoA* mutants (Cornelis *et al.*, 1991). It has been demonstrated that Hha can complement the *ymoA* mutation (Mikulskis & Cornelis, 1994) and vice versa (Balsalobre *et al.*, 1996) demonstrating the functional similarity of these two genes.

Hha and YmoA are both small proteins that show some resemblance to bacterial histones. Both proteins also have an abundance of charged amino acid residues. While Hha does not have a typical

helix-turn-helix motif, it has been shown that this class of expression modulators is capable of affecting DNA topology (Carmona *et al.*, 1993). Other members of this family are RmoA which is carried on the R100 plasmid and has a role in modulating R100 conjugation in response to environmental signals such as osmolarity (Nieto & Juarez, 1996; Nieto *et al.*, 1998) and a hypothetical protein encoded by the pO157 virulence plasmid from EHEC O157:H7. This family of proteins is highly conserved, especially at the carboxy-terminus where it is hypothesised that they interact with H-NS. There are several residues that are conserved between the members of the Hha and H-NS families (Figure 3k). There is also evidence that Hha and H-NS are capable of interacting directly (Nieto *et al.*, 2002). Therefore, it has been proposed that Hha is probably part of a regulatory cascade that responds to environmental stimuli to modulate the expression of multi-gene systems (Balsalobre *et al.*, 1999).

Of interest to this study is the finding that Hha stimulates transposition of the *E. coli* insertion sequence elements IS*1*, IS*5* and IS*10* (Mikulskis & Cornelis, 1994). The same study also found that the *hha* gene itself may be a hotspot for IS element insertion. In a later study an increase in IS transposition was observed when *hha* was supplied in a medium copy vector and proposed that Hha may have a role in IS stability in *E. coli* (Balsalobre *et al.*, 1996). A single copy of the *hha* gene has been found in a number of enterobacteria including *S. flexneri*, *S. dysenteriae*, *Citrobacter diversus*, *Klebsiella pneumoniae* and *S.* Typhimurium. This may be important because of the number of IS elements on the SRL PAI and also the movement of the SRL, possibly be IS*1*-mediated events.

LysR-family

Orf7 is potentially a LysR-like transcriptional regulator. LysR transcriptional regulators are the second most common family of transcriptional regulators, after two-component systems (Schell, 1993). Interestingly, many are found on self-transmissible plasmids, which themselves are often associated with virulence genes. The main characteristics of LysR-like regulators include a size between 276 and 324 residues and divergent transcription from a promoter very near or overlapping the regulated target gene promoter. Homology of Orf7 to most members of the LysR family is very low, however, there is little conservation between any members of this family (approximately 14 residues out of 279 between LysR, CysB, IrgB, IlvY and YeeY). In contrast, Orf7 shows significant similarity to two putative LysR-like transcriptional regulators (Figure 3l), YjiE and a putative regulator in *S. enterica* sv Typhi (NP_458936.1), the functions of which are unknown. It is worth noting that some members of the LysR family, LysR, IrgB and IlvY have helix-turn-helix (HTH) motifs as defined by Dodd and Egan (1990), while others, CysB and YeeY, do not. Orf7, NP458936 and YjiE all possess a potential HTH. The function of various LysR-like transcriptional regulators was reviewed by Schell (1993), and included regulators of β-lactamases (AmpR, BlaA), amino acid biosynthesis (CysB, IlvY, LysR, MetR), iron-regulated virulence factors (IrgB) and oxidation stress

```
Orf7      -------MKNIETKWLYDFLTLEACR[redacted]LEAAIGVVLFDRTTTPLQLTEEGKLFHSQTRSL
NP458936 MDVTGAGLHNIETKWLYDFLTLEKCR[redacted]LEHAVGVELFNRQVSPLQLSEQGKIFHSQVRHL
YjiE      MDDCGAILHNIETKWLYDFLTLEKCR[redacted]LEQAIGVELFNRQVTPLQLSEQGKIFHSQIRHL
                ..************** **.*****  **.*********.*** *.** ** *  .****.*.**.**** * *

Orf7      LQQLECNLGELNGQSLLGVPNIKIAAAHSLSLSVLPKLVHSLTAYGGEFVYHVEAIDVVQAVNTLREGKSDFIISFRDEDL
NP458936 LQQLESNLTELRGGSDYTLRKIKIAAAHSLSLGLLPTIVKQMPT---QFTYAVEAIDVDQAVDMLREGQSDFIFSYHDENL
YjiE      LQQLESNLAELRGGSDYAQRKIKIAAAHSLSLGLLPSIISQMPP---LFTWAIEAIDVDEAVDKLREGQSDCIFSFHDEDL
          *****.** ** * *      ********** .** ..  .      *   .***** .**  ****.** * *..** *

Orf7      MQSPFCCLKLFESELYPVCAADAQGF VFDITQPQVPLLNYTATSYMGRLVNRHLAEVGGITGRTIFISSMSELLKNMALN
NP458936 QQAPFDNIRLFESRLFPVCANNGRGEPRYTLEQPHFPLLNYSQNSYMGRLINRTLTRHAELSFSTFFVSSMSELLKQVAMD
YjiE      LEAPFDHIRLFESQLFPVCASDEHGEALFNLAQPHFPLLNYSRNSYMGRLINRTLTRHSELSFSTFFVSSMSELLKQVALD
           ..**  ..**** *.****   .*   . . **. *****. .******.** *.    ..  * *.********..*.

Orf7      GYGIAWLPIWSIVDELQTKRLICLDAAKLTVPIQAYIYRMNTRLNRTAENLWRILQEHMPDDLIQQISMEEPARRN
NP458936 GCGIAWLPEYAIRQEITDGRLIVLDADELVIPIQAYAYRMNTRMSQVAETFWRDLRGLQAAL--------------
YjiE      GCGIAWLPEYAIQQEIRSGKLVVLNRDELVIPIQAYAYRMNTRMNPVAERFWRELRELEIVLS-------------
          * ******  .*  *.   .*. *    * .***** ******.   **  ** *.
```

Figure 3l. Alignment of ORf7 LysR-like Homologues. Identical residues are denoted with an asterisks (*) and conserved residues with a dot (.). The accession numbers of proteins aligned are: Orf7 – AF326777_7; NP_458936; YjiE – P39376. The proposed helix-turn-helices are denoted by [redacted] highlighting.

response, aggregation and phage restriction (OxyR). A common feature of the LysR family is that they regulate a gene that is divertly transcribed directly upstream. This feature coupled with its homology to NP_458936 proposes a possible function for Orf7. Both Orf7 and NP_458936 are transcribed divergently to putative aspartate racemase genes, which themselves share significant homology (63%) (Figure 3m), suggesting that they may act as transcriptional regulators of an aspartate racemase. Further experimental work is required to determine if Orf7 does have a role in the regulation of Orf8.


```
ii)
NP_458935       MKHTIGILGGMGPAATADMLEKFVELRHVSCDQQHIPLIVSSIPDIPDRTACLLSGGPSPYRYLERYLHMLED
Orf8            ----------MGPAATVDLFNKFVTFTAAQCDQEHIPLIISSIPDIPDRTDALMHHGRSPLPVMRDYMHKLED
                          ****** *.  ***      ***.*****.********** .*.  * **   .  *.* ***

NP_458935       AGAECIVIPCNTAHYWFDDLQNVAKARMISILDATLGDIPPSAR-HVGLLATNATLATGLYQKKALARGLTLI
Orf8            AGAECIVIPCNTAHYWFKELKDACHIDILSIVETTINEVRACGKTRIGLLATNATLYMGLYQKGIESLGFTCV
                ***************** .*.  ..  ..**...*....  . . ..********* *****   . * * .

NP_458935       QPEDAGQALVMQAIYTLKRGDKTAAQALLLPQIDSLIARGAQAIIMGCTEIPLIVAGHERAIACPMIDSTASL
Orf8            SPDADGQKKVMESIYSLKAGNIAHAQKLMNEQAEILFSRGAEILVLGCTEIPVILAQAVKEQPSRYIDSTASL
                 *.  **  **..**.** *  . ** *.  * . * .***. ...******.*.*   .   .  *******

NP_458935       VRAAIRWYESWPDTRASLTGEQRLTA
Orf8            VRAGIKWYENRVGKHYLLTQ------
                *** ' .***      .  **
```

Figure 3m. i) Organisation of LysR-like regulators and their regulated gene. ii) Alignment of Orf8 with a homologue from *Salmonella enterica* sv Typhi. In part i) different regulons are denoted by different colours. Black, blue, pink and peach denote regulons in *S. flexneri*, *S.* Typhimurium, *E. coli* and *V. cholerae* respectively. In part ii) identical residues are denoted by as asterisks (*) and conservative substitutions with a dot (.).

Vis

Orf41 shows significant similarity to Vis, a transcriptional regulator carried on the P4 bacteriophage, which has been shown to regulate transcription from late promoters. Vis negatively and positively regulates transcription from the P_{LL} and P_{sid} promoters, respectively (Polo *et al.*, 1996). As yet, a role for Vis in the regulation of P4 *int* has not been demonstrated. Interestingly, Vis shows significant similarity to AlpA, a transcriptional regulator encoded by the CP4-57 cryptic prophage, which also

encodes a homologue to the SRL Int protein. The potential role of this ORF in SRL PAI excision is discussed in Chapter 4.

ALPA

The SRL PAI encodes a second homologue of Vis, Orf3. Orf3 is more closely related to AlpA, encoded by the CP4-57 prophage (66%), than to Vis (53%). AlpA has been shown to regulate the excision of CP4-57 from the *E. coli* K-12 chromsome by increasing transcription of *slpA*, the integrase gene (Kirby *et al.*, 1994; Trempy *et al.*, 1994). The potential function of Orf3 is examined in Chapter 4.

Ag43 (Flu)

orf47 is homologous to the *flu* gene (90% identity) found on the CP4-44 cryptic prophage, *sap* (92%) on the *she* PAI and a hypothetical *orf* in EHEC O157:H7 (95%). Orf47 is also similar to YpjA (37%) encoded by the cryptic prophage CP4-57. Flu, originally designated Ag43 (Owen *et al.*, 1987), is an autotransported protein that mediates autoaggregation in *E. coli* (Henderson *et al.*, 1997). Recently, a role for Flu has been found in cell-to-cell and cell-to-surface interaction in biofilms in minimal media (Danese *et al.*, 2000). The Flu protein is also homologous to YpjA encoded by CP4-57, although the similarity of YpjA to Orf47 is only 38% compared to 75% similarity between Orf47 and Flu and 87% similarity between Sap (*she* PAI) and Flu (Al-Hasani *et al.*, 2001). Most of the divergence between Orf47 and Flu is confined to the central regions, consistent with the role of the N-terminal signal sequence and C-terminal β-barrel domain in autotransport (Henderson *et al.*, 1998). Differences in the central region may confer different binding specificities for Flu and Orf47 that could be important in the colonisation of different niches within the host and the environment.

Shf Locus

Rajakumar *et al.* (1996) identified two overlapping genes, *shf1* and *shf2* on the virulence plasmid of *S. flexneri* S2430, a derivative of YSH6000, which showed characteristics common to some mobile elements such as retroviral integrases and IS transposases. Recent work revealed that *shf* is a single ORF on the *S. flexneri* 5 M90T virulence plasmid (Buchrieser *et al.*, 2000). Because of these conflicting data and the finding of a single *shf* gene on the chromosome, the *shf* region on the plasmid of *S. flexneri* 2a YSH6000T, which lacks a chromosomal *shf* gene but carries the same virulence plasmid as S2430, was sequenced from a PCR product. Sequencing of the resulting amplicon revealed the thymine reported at position 773 by Rajakumar *et al.* (1996) was absent in the YSH6000T plasmid *shf* locus. Correction of this sequencing error revealed that there is a single *shf* orf on the virulence plasmid in YSH6000T. This *orf* shows 97% sequence identity with *orf24* on the

SRL PAI. As reported by Buchrieser *et al.* (2000), this gene has close homologues (>83% similarity at the amino acid level) on pAA2 of enteroaggregative and diffusely adhering *E. coli*, pO157 of EHEC (Burland *et al.*, 1998) and a chromosomal locus bearing two overlapping genes, *sat1* and *sat2*, in *S. enterica* sv Typhimurium. In *Shigella* and *E. coli*, *shf* is part of a larger locus that includes a hexosyltransferase homologue, *capU*, an *msbB* homologue and an IS*911* remnant. Figure 3n shows a schematic representation of the organisation of these genes in the various *Shigella* and *E. coli* loci. Although the specific functions of the genes in the *shf* loci are unknown, its conservation on several virulence-related plasmids and a PAI imply a potential role in virulence. Venkatesan *et al.* (2001) have proposed that the *shf* locus has a role in carbohydrate transport and metabolism based on COG analysis and the other proteins encoded by the operon. COG analysis places *shf* in the xylanases/chitin deacetylase family. Of the other ORFs, a CapU homologue, RfbU, has been described as a sugar hydrolase in *V. cholerae* and MsbB is an acyltransferase involved in O-antigen fatty acyl modification. VirK post transcriptionally regulates VirG expression (Nakata *et al.*, 1993), but its presence in this locus suggests it may also have a role in O-antigen biosynthesis.

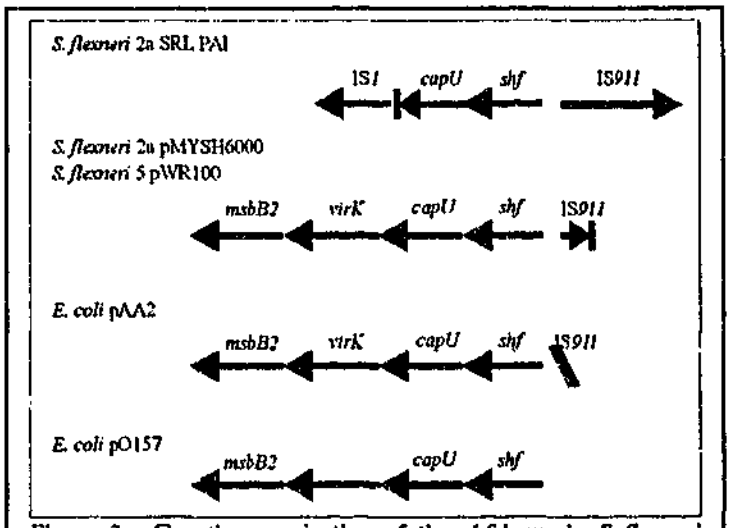


Figure 3n. Genetic organisation of the *shf* locus in *S. flexneri* and *E. coli*. This figure shows the extent of the conserved *shf* locus in several bacterial strains. pMYSH6000 is carried by YSH6000, the same strain that encodes the SRL PAI. pWR100 is borne by an *S. flexneri* 5 strain M90T (Venkatesan *et al.*, 2001). pAA2 is from an enteroaggregative *E. coli* strain O42 (Czeczulin *et al.*, 1999) and pO157 is carried by enterohemorrhagic *E. coli* O157:H7 (Burland *et al.*, 1998). The *shf* and *capU* genes are conserved in all loci, although truncated in the *S. flexneri* chromosome due to an IS*1* insertion. An IS*911* element is found upstream of three of the loci, although it is intact only on the *S. flexneri* SRL PAI chromosomal locus and is oriented in the reverse direction on pAA2 in enteroaggregative *E. coli*. In addition, the plasmid-borne loci exhibit a conserved organisation with regards to *shf*, *capU*, *virK* and *msbB2*, with pO157 having an alternative gene in place of *virK*.

DcuA

Orf9 shows similarity (70%) to the anaerobic dicarboxylate carrier, DcuA, from *E. coli* K-12. Interestingly, in *E. coli dcuA* lies directly downstream of the aspartase gene, *aspA*, while Orf9 lies directly downstream of an aspartate racemase. Genes encoding enzymes with a role in C_4-metabolism have been found adjacent to other *dcuA/B* homolgoues. These include an aspartase (*aspA*) next to *dcuA* in *Serratia marcescens* and genes encoding asparaginases (*asnA* and *asnB*) in *Haemophilus influenzae*, *Wolinella succinogenes* and *Helicobacter pylori* (Golby *et al.*, 1998). *E. coli* encodes three homologues of C_4-dicarboxylate carriers, *dcuA*, *dcuB* and *dcuC* and mutation of all 3 genes is required for the loss of the ability to grow under anaerobic conditions using fumurate, malate or

aspartate (Six *et al.*, 1994; Zientz *et al.*, 1996). It has been proposed that DcuA, DcuB and DcuC are integral membrane proteins due to their high proportion of hydrophobic amino acid residues. Six *et al.* (1994) proposed several models for the transmembrane structure of DcuA and DcuB, with the most probable possessing 12 transmembrane domains. Interestingly, Orf9 also appears to have 12 transmembrane domains (TMPred - (Hofmann & Stoffel, 1993)) (Figure 3o). Predictive analysis of Orf9 using Psort (Nakai & Horton, 1999) also suggests that, like DcuA, Orf9 is most likely an integral inner membrane protein.

It is worth noting that *dcuA* is constitutively expressed in *E. coli*, while *dcuB* is only highly expressed under anaerobic conditions (Golby *et al.*, 1998). This varied expression can be explained by the finding that FNR, the major anaerobic regulator, was shown to affect *dcuB* but not *dcuA* expression. Work needs to be carried out to determine if Orf9 functions in a manner similar to DcuA. This could be achieved in the *dcuA/dcuB/dcuC* negative background, to see if Orf9 could restore growth using fumurate as a carbon source. It should also be determined if the loss of the SRL PAI, and specifically *orf9*, affects the ability of *S. flexneri* to use fumurate as a carbon source under various growth conditions. It should be noted that homologues of *dcuA*, *dcuB* and *dcuC* (SF4292, SF4100 and SF0659 respectively), with 100% nucleotide identity to the *E. coli* K-12 *orfs* (P04539, P14409, Q47134), are present in the recently sequenced *S. flexneri* 2a strain 301. The presence of these genes may make the presence of *orf9* on the SRL PAI redundant. However, it is also possible that Orf9 is involved in the transport of different C_4-metabolites and so it may still

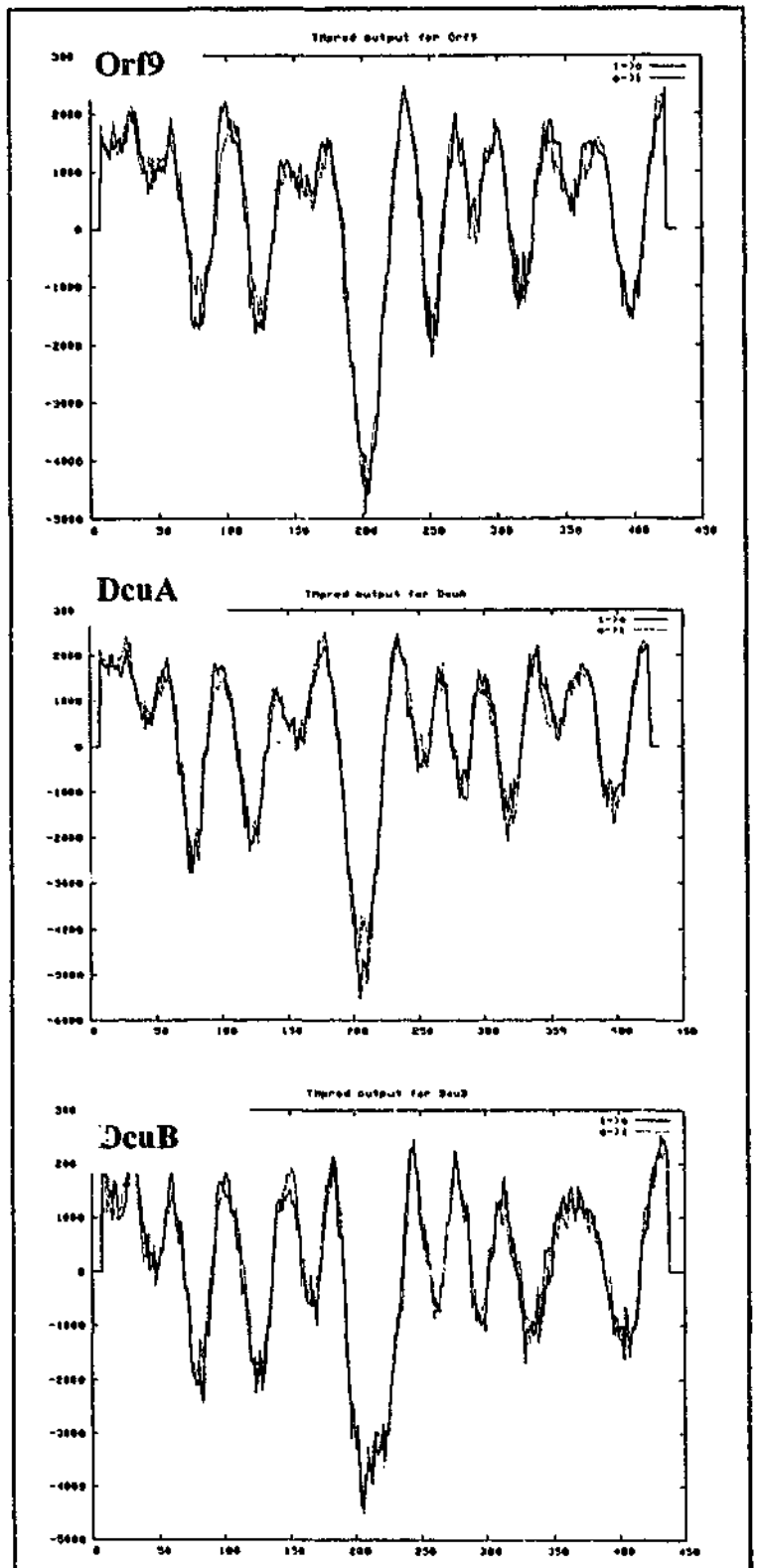


Figure 3o. Hydropathy plots of Orf9, DcuA and DcuB. The program used to determine the indicated transmembrane domains was TMPred (Hofmann, 1993)

be of some importance.

ORFs with No Homologues

There are several ORFs on the PAI that show no homology to any ORFs of known function (Figure 3p); this includes most of the prophage-related ORFs. Table 3d summarises the characterisitics of these ORFs.

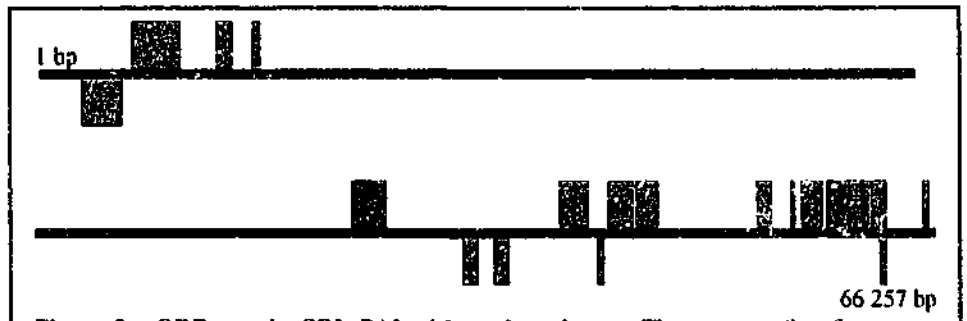


Figure 3p. ORFs on the SRL PAI with no homologues. The open reading frames are represented by boxes above the line (forward orientation) or below the line (reverse orientation). The pink ORFs denote prophage-related ORFs. The peach ORFs indicated ORFs with no database matches. The blue ORF is the homologue of *Campylobacter jejuni* periplasmic protein with no known function.

The Pfam motifs detected may give some indication of the roles of some of the proteins. Orf45 had a helix-turn-helix (HTH) motif (E-value 3.9e-3) similar to those seen in the CopG family. CopG is a small transcriptional regulator which regulates the replication of a streptococcal plasmid which encodes it (del Solar *et al.*, 1998). Orf50 has a motif found in members of the RadC family. These proteins have a role in *recA*-dependent DNA repair following exposure to UV radiation (Felzenszwalb *et al.*, 1984). The presence of the signal peptide for Orf34 was predicted by both SMART and SignalP with the cleavage site being located between amino acids 20 and 21 (ALA-AQ). The presence of potential coiled coil domains was predicted by the COILS program (Lupas *et al.*, 1991). The probability of a coiled coil being present in Orf4 and Orf42 was greater than 0.8. Coiled-coil domains are often found within functionally important domains of proteins (Lupas *et al.*, 1991). Potential functions have been proposed for homologues of Orf46 and Orf52 present on O-islands #43 and #48 in EHEC O157:H7. It has been proposed that the YeeP homologue, Orf46, is a putative histone and the Orf52 homologue is described as a putative structural protein.

Table 3d. ORFs on the SRL PAI with no homologues

ORF	Mol Mass (kDa)	Pfam Sequence Motifs[a]	SMART Sequence Motifs[b]	Predicted Sub-cellular localisation[c]	Predicted Transmembrane Domains[d]
Orf2*	57.9	-	AAA (66-206) Low Complex (138-144) Low Complex (173-185)	cytoplasm	1
Orf4	73.3		COIL (207-235) Low Complex (211-228)	inner membrane	0
Orf5	23.4	-	-	cytoplasm	0
Orf6*	11.4	-	-	cytoplasm	0
Orf34	49.1	-	SIGNAL (aa1-20)	periplasmic space/ outer membrane	2
Orf38	19.8	-	-	cytoplasm	0
Orf39*	21.8	-	-	cytoplasm	
Orf42	24.0	-	COIL (157-184)	cytoplasm	1
Orf43	9.5	-	-	cytoplasm	0
Orf44	7.9	-	-	cytoplasm	1
Orf45	36.2	HTH_4 (copG family)	-	cytoplasm	1 potential
Orf46*	32.4	-	AAA (35-239)	cytoplasm	0
Orf48*	21.8	-	-	cytoplasm	0
Orf49*	4.7	-	Low Complex (4-15)	cytoplasm	2
Orf50*	18.1	RadC family	Low Complex (100-113)	cytoplasm	0
Orf51*	8.4	-	-	cytoplasm	0
Orf52*	13.8	-	-	cytoplasm	0
Orf53*	14.1	-	-	cytoplasm	0
Orf54*	18.2	-	-	cytoplasm	0
Orf 55*	7.4	-	-	cytoplasm	0
Orf56*	20.6	-	-	cytoplasm	0
Orf57*	8.1	-	-	cytoplasm	0
Orf59*	9.4	-	-	cytoplasm	0

* prophage related ORF; [a]Pfam (Bateman *et al.*, 2002); [b]SMART (Schultz *et al.*, 1998); [c]P-SORT (Nakai & Horton, 1999); [d]TM-PRED (Hofmann & Stoffel, 1993); AAA-ATPase family (ATPases associated with diverse cellular activities (Neuwald *et al.*, 1999); Coil – Coiled Coil Domain (Lupas *et al.*, 1991); Signal – signal peptide (Nielsen *et al.*, 1997); Low Complex – Region of low complexity (Wootton & Fedheren, 1996).

CONCLUDING REMARKS

The SRL PAI appears to be a hot spot for the insertion of DNA "modules". The concept of a modular island was proposed by Yarwood *et al.* (2002) for the SaPI3 of *Staphylococcus aureus*. It was proposed that various modules were added, deleted or changed by recombination to form the island. This theory could be applied to the SRL PAI and EHEC O157:H7 O-islands #43 and #48. These islands may have begun with the CP4-like prophage backbone mobilising into both EHEC O157:H7 and *Shigella* at some point in time. Subsequently, the various islands acquired "modules" that were beneficial to each particular host (Figure 3q). In *Shigella*, this could have begun with the SRL, most likely as a transposon. Other SRL PAI may modules include the *fec* locus, *orf7*/*orf8*/*orf9*, *shf* and the various IS elements. A modular island could also see the loss of unwanted modules over time. This hypothesis is supported by two key points. Firstly, the conservation of the *int* genes and the various prophage homologues on the SRL PAI and the EHEC islands suggests that they share a common backbone. Secondly, findings by Turner *et al.* (2003) have identified variants of the island that are

missing the region of the PAI ranging from the *fec* locus to Orf47. These variants may represent a more ancestral form of the SRL PAI yet to acquire the *fec*-Orf47 region or a more recent form that has lost this region. The modular theory is further supported by findings of other islands with similar backbones in uropathogenic *E. coli* strain CFT073, a second island in *S. flexneri* 2a and in *E. coli* K-12.

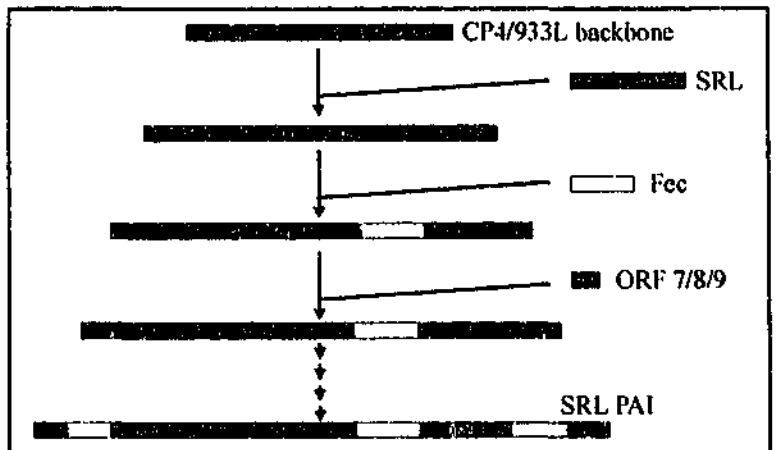


Figure 3q. Modular Theory of SRL PAI Evolution

The role of bacteriophages in horizontal transfer of virulence determinants is largely understudied. However, genomic sequencing is changing our understanding of the role of the phage. Boyd and Brüssow (2002) discuss the "mutualistic role of bacteriophages in the evolution of important bacterial pathogens," including the acquisition of toxin genes and O-antigen modification systems by *E. coli* and *Shigella* spp. This new role of the bacteriophage in bacterial evolution has now been demonstrated by the transfer of large chromsomal regions including PAIs or cryptic prophages as seen in *S. aureus* and *V. cholerae*. In time, it may become apparent that many PAIs are actually cryptic prophages and as such will be able to be mobilised by bacteriophages.

Future work must include transcriptional and translational analysis of the SRL PAI *orfs* in order to identify which ORFs may still be functional. This analysis would involve RT-PCR using primer pairs from each *orf* to see which are transcribed. Microarray technology and RealTime-PCR could be utilised to examine the affect of overexpression of various transcriptional regulators and determine which *orfs* they may regulate. It could also be used to determine what environmental signals are required for regulation of different genes. Translational analysis would be carried out by the cloning of the various *orfs* into expression vectors and other plasmids with their native promoters in order to determine if they are capable of expression.

~ *CHAPTER FOUR* ~

Rox, a Regulator of SRL PAI Excision in **S. flexneri**

INTRODUCTION

Bacteria utilise many genetic elements for the acquisition of foreign DNA, including insertion sequences, transposons, plasmids, bacteriophages and pathogenicity islands (PAIs). PAIs are large regions of the chromosome that are genetically and functionally distinct units (Häcker *et al.*, 1997). These elements are usually incorporated into bacterial chromosomes by site-specific integration at tRNA genes and some are capable of excision. The importance of genetic acquisition and loss is highlighted by the recent sequencing of the *S. flexneri* 2a genome, which showed that the major differences between *S. flexneri* and *E. coli* have arisen primarily through the loss or acquisition of DNA, including PAIs and phages (Jin *et al.*, 2002).

Very little is known about the movement of PAIs, either into or out of bacterial genomes. Several PAIs have been shown to be capable of deletion, including the SRL and *she* PAIs (Rajakumar *et al.*, 1997; Turner *et al.*, 2001). Like many PAIs, the SRL PAI encodes an integrase that is homologous to members of the P4 family of integrases. Work by Turner *et al* (2001) has shown that disruption of the *int* gene results in stability of the SRL PAI, indicating that *int* is essential for excision. The *int* gene of the *Y. enterocolitica* HIGH-PAI has a role in integration of this element (Rakin *et al.*, 2001). However, until now there has been no information regarding the regulation of PAI integrases.

AlpA is a transcriptional regulator encoded by a cryptic prophage, CP4-57, of *E. coli* K-12. AlpA up-regulates transcription of *slpA*, the CP4-57 integrase gene, which mediates the loss of the element from the chromosome (Kirby *et al.*, 1994; Trempy *et al.*, 1994). Two AlpA homologues were identified on the SRL PAI suggesting that SRL PAI excision may also be controlled by transcriptional activation of its integrase gene.

RESULTS

ALPA HOMOLOGUES

Sequence analysis of the SRL PAI revealed two homologues of the AlpA transcriptional regulator, namely Orf3 (Rox – regulator of excision) and Orf41. AlpA regulates the excision of the CP4-57 element of *E. coli* K-12 by upregulating the transcription of *slpA*, the CP4-57 integrase (Trempy *et al.*, 1994). Rox shows 66% similarity to AlpA, while Orf41 is more closely related (53% similarity) to Vis, another AlpA homologue (Figure 4a). Vis is a transcriptional regulator encoded by the P4 bacteriophage genome, which has been shown to regulate transcription from late promoters, P_{LL} and P_{sid} (Polo *et al.*, 1996). As yet, a role for Vis in the regulation of P4 *int* has not been demonstrated. The recent sequencing of EHEC O157:H7 EDL933 strains has revealed AlpA homologues on duplicate O-islands #43 and #48, which share a near identical integrase with the SRL PAI. Unlike *alpA*, the EHEC homologue shows significant nucleotide identity with *rox* (Figure 4b). Rox, but not Orf41, contains a helix-turn-helix (HTH) motif that is consistent with a DNA binding motif, and a HTH is present in AlpA and Vis.

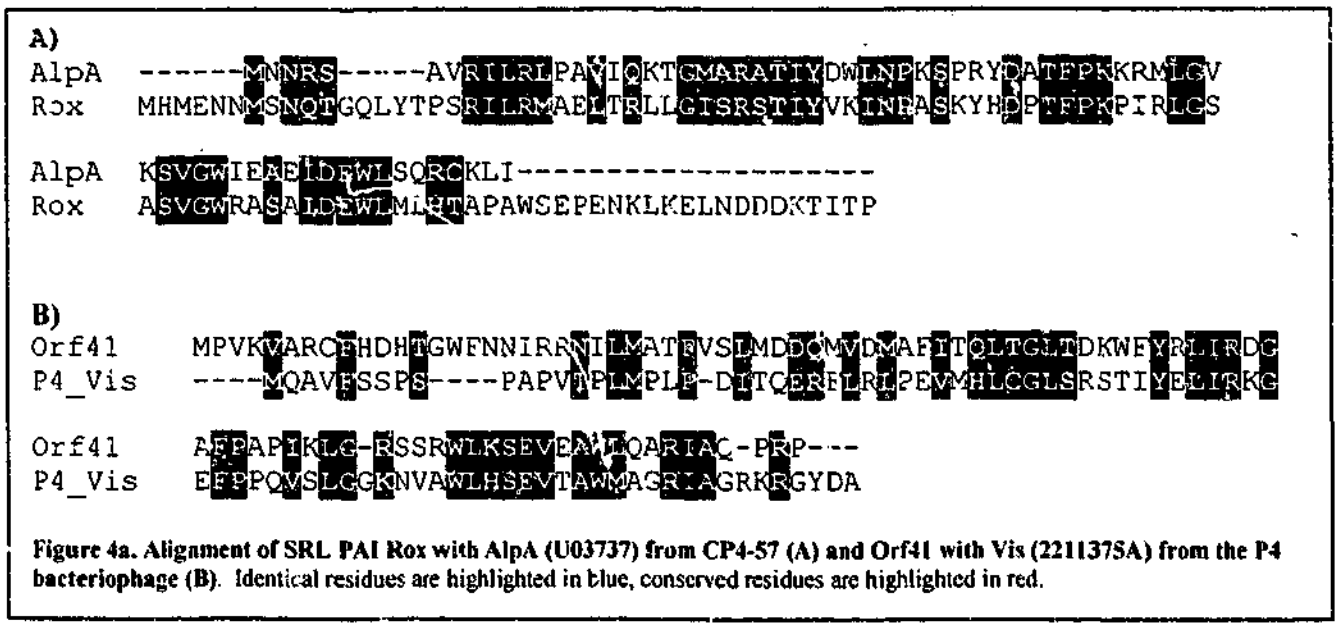


Figure 4a. Alignment of SRL PAI Rox with AlpA (U03737) from CP4-57 (A) and Orf41 with Vis (2211375A) from the P4 bacteriophage (B). Identical residues are highlighted in blue, conserved residues are highlighted in red.

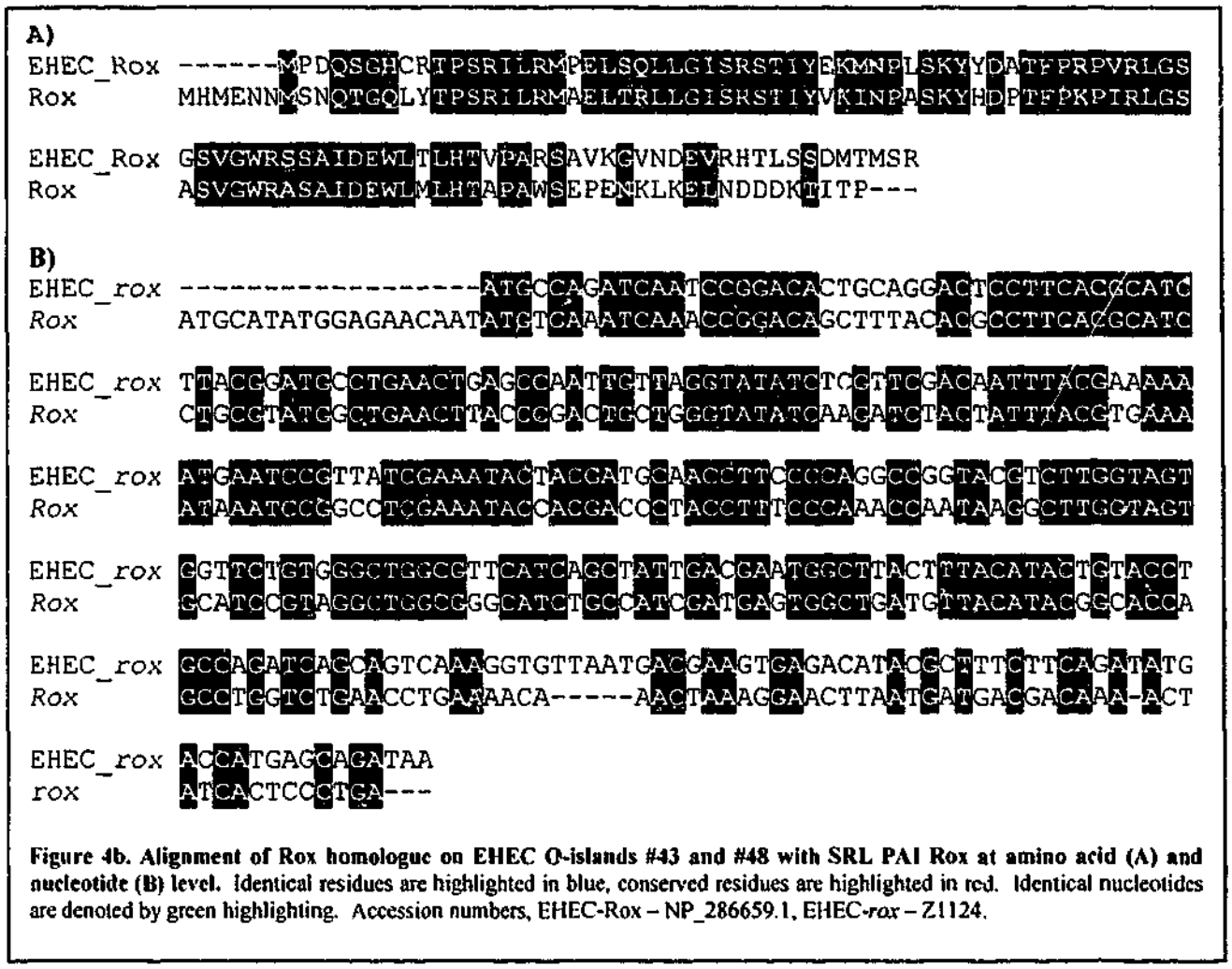


Figure 4b. Alignment of Rox homologue on EHEC O-islands #43 and #48 with SRL PAI Rox at amino acid (A) and nucleotide (B) level. Identical residues are highlighted in blue, conserved residues are highlighted in red. Identical nucleotides are denoted by green highlighting. Accession numbers, EHEC-Rox – NP_286659.1, EHEC-*rox* – Z1124.

EFFECT OF ROX AND ORF41 ON SRL PAI EXCISION

rox and *orf41* were amplified by PCR from *S. flexneri* YSH6000 genomic DNA and directionally cloned into the *Eco*RI and *Bam*HI sites of pPBA1100 giving rise to plasmids pAL34 and pAL31, respectively. Accurate amplification of PCR products was confirmed by sequencing the inserts of the plasmids obtained. These plasmids, along with pPBA1100, were separately transformed into *S. flexneri* 2a strain YSH6000 by electroporation, resulting in the strains AL66 (pAL34), AL67 (pAL31) and AL75 (pPBA1100) (Table 2a). Each strain was grown overnight with aeration in 2.5 ml of 2YT medium supplemented with 50 μg/ml kanamycin and 10mM IPTG. Extraction of genomic DNA was performed as described previously (Ausubel *et al.*, 1995). Total DNA was standardised to 1.4 mg/ml for each strain. Excision of the SRL PAI was assayed by PCR using inward facing primers on either side of the SRL PAI (BAP679/BAP1157 [Appendix 3]) (Figure 4c). PCR reactions for amplification of *recA* (BAP1643/BAP1644) served as controls for template concentration. Results for *recA* controls showed a 10-fold difference in concentration between wild-type and AL66 template. However, there was a 10^4-fold increase in excision of the SRL PAI when Rox was overexpressed compared to the wild-type (Figure 4c). In contrast, Orf41 did not have any effect on SRL PAI excision. Therefore, Rox, but not Orf41, appeared to have a role in SRL PAI excision.

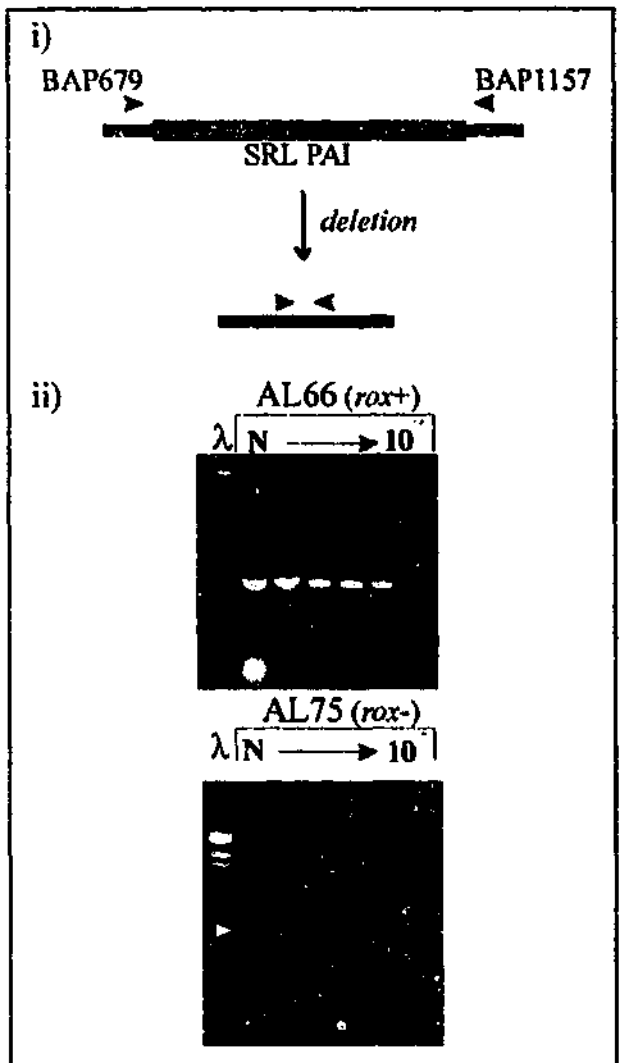


Figure 4c. The effect of Rox on SRL PAI excision. i) Red denotes the SRL PAI and blue denotes YSH6000 backbone. Primers used to determine if the PAI has excised are shown. ii) λ represents lambda *HindIII* markers. Each lane contains 10-fold serial dilutions of genomic DNA from left to right. Wild-type SRL PAI excision can be seen by a PCR product marked by the yellow arrow.

In order to determine if the effect of Rox on PAI excision was dependent on the SRL PAI integrase, the PCR excision assay was performed in an *int* mutant, AL11. AL11 was transformed with pAL85 and pUC19-Tp, giving rise to strains AL295 and AL296, respectively. The excision assay was performed as previously described, using strains AL325 (wild-type *int* with pAL85), YSH6000 (parent) and SBA1363 (SRL PAI$^-$) as a controls. The results showed that PAI excision is dependent on Rox and Int, as excision was not observed in the *int* mutant background, but was clearly visible when wild-type *int* was present (data not shown).

PURIFICATION OF ROX PROTEIN

In order to perform functional assays on Rox, a purifed protein was required. *rox* was amplified by PCR and cloned in-frame into the *Bam*HI and *Eco*RI sites of the expression vector pGEX-2T to produce an N-terminal glutathione S-transferase (GST) fusion protein. The construct was transformed into *E. coli* DH5α. The various clones were screened for inserts by plasmid extraction, restriction digests, PCR and sequencing of clones with inserts. Plasmids with the correct, in-frame sequence, designated pAL95, pAL96 and pAL97, were carried by strains AL170, AL171 and AL172, respectively. Whole cell lysates were examined for induction of the Rox protein. However, the results were unclear, as a protein that appeared to be smaller than the expected 39 kDa GST-Rox fusion was induced (data not shown). To confirm the ability of these plasmids to produce a functional GST-Rox fusion, the three plasmids were introduced into SBA1366, giving rise to strains AL173, AL174 and AL175. These *Shigella* clones were tested using the PCR excision assay described above to ensure that the fusion protein was functional. All clones showed an increase in PAI excision when compared to AL178 (SBA1366, pGEX-2T) (Figure 4d). Therefore, the GST-Rox fusion protein was active.

In order to purify Rox, large-scale protein extractions were performed (Chapter 2). GST has a molecular mass of 27.5 kDa. The predicted size of the GST-Rox fusion protein was 39 kDa. Initial extraction attempts yielded a number of breakdown products and only a small proportion of the

purified protein constituting full-length GST-Rox fusion protein (Figure 4e). Various methods and strategies were undertaken in order to reduce the degradation of GST-Rox. The results of the various expression conditions used are summarised in Table 4a.

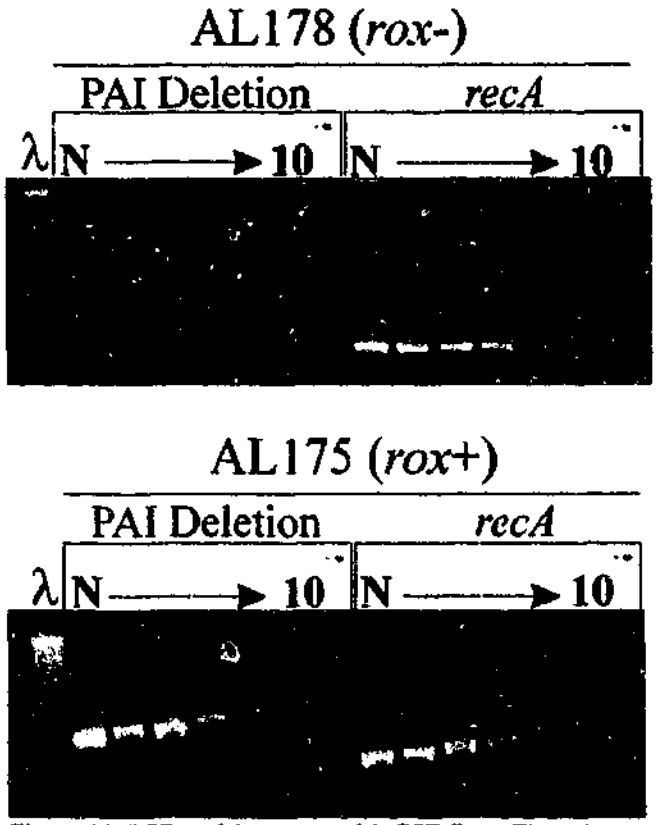


Figure 4d. PCR excision assay with GST-Rox. The red arrow shows the product in the wildtype strain. Each lane is a 10-fold serial dilution of genomic DNA from left to right. λ, represents lambda *HindIII* markers. AL178, pGEX-2T control; AL175, pGEX-2T/Rox

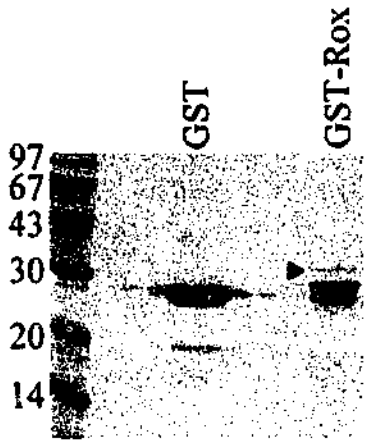


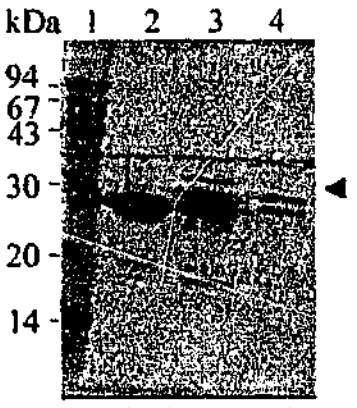
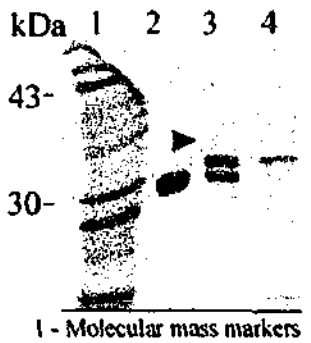
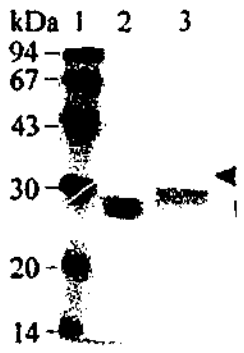
Figure 4e. Coomassie Blue stained SDS-PAGE gel of purified GST-Rox fusion. The molecular masses of standard markers (kDa) are shown on the left. The full length fusion product is indicated by the arrowhead.

Table 4a. Expression conditions used for purification of GST-Rox fusion protein

Condition/Variable	Figure (4f-4i)	Result/Comments
▪ French pressed versus sonicated cells		No difference in degradation was observed
▪ Growth at 30°C ▪ Presence/absence glucose	**Figure 4f** kDa 1 2 3 4 5 94 67 43 30 20 14 1 - Molecular mass markers 2 - GST only 3 - GST-Rox 37C, Glucose, IPTG 4 - GST-Rox 30C, IPTG 5 - GST-Rox 30C, Glucose, IPTG	Breakdown products were observed in all conditions tested. Concentration of full length GST-Rox fusion protein extracted was highest at 37°C with glucose.

► Arrowheads denote expected position of GST-Rox fusion protein

Table 4a continued

Condition/Variable	Figure (4f-4	Result/Comments
• Expression in BL21 (DE3)		GST-Rox was not expressed well in BL21 (DE3). Full length fusion protein was never observed.
• Addition of DTT and Sarkosyl during protein extraction to attempt to reduce protein degradation in DH5α background	Figure 4g  1 - Molecular mass markers 2 - GST only 3 - GST-Rox 37C 4 - GST-Rox 30C	Did not eliminate degradation at either 37°C or 30°C.
• Expression in *E. coli* strain SG22094 to reduce the effect of host strain proteases	Figure 4h.  1 - Molecular mass markers 2 - GST only 3 - GST-Rox DH5α 4 - GST-Rox SG22094	There appeared to be less breakdown products from the GST-Rox fusion protein when it is expressed in SG22094 compared to DH5α.
• Treatment with a protease inhibitor cocktail during protein extraction	Figure 4i.  	Did not eliminate degradation occurring during extraction

▸ **Arrowheads denote expected position of GST-Rox fusion protein**

Therefore, none of these methods resulted in a significant decrease in protein degradation. A Western blot was performed using anti-GST antibody to confirm that the protein being purified contained GST (Figure 4j). This suggested that BL21 (DE3) had the most breakdown products and SG22094 had the least. However, extraction from SG22094 resulted in less full length GST-Rox compared to extraction from a DH5α background. Therefore, future experiments and extractions were performed

using the DH5α strain transformed with pAL95, AL170. AL170 was grown at 37°C in the presence of glucose and induced with IPTG (see Chapter 2 for details).

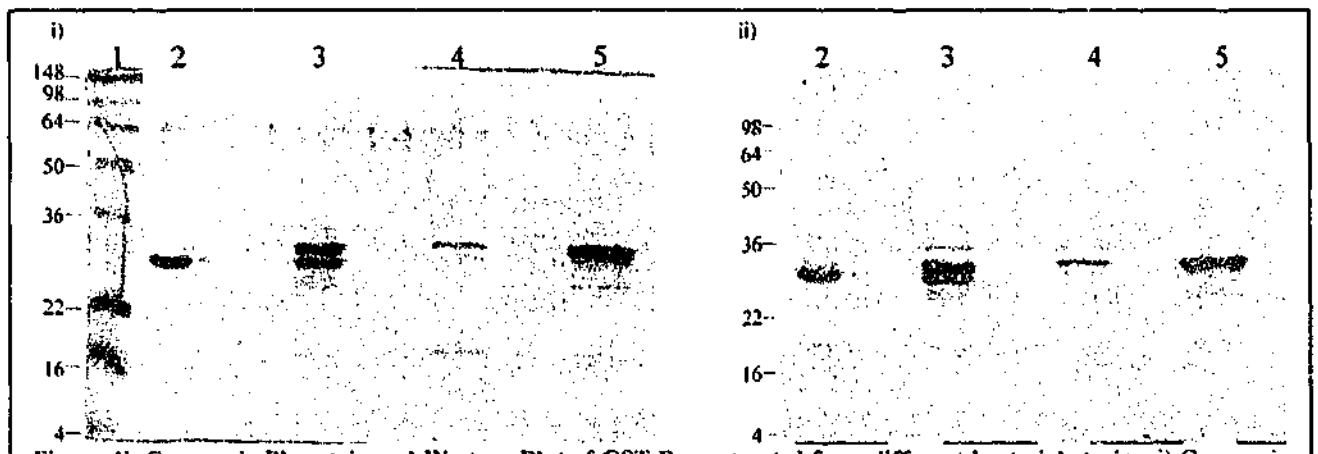


Figure 4j. Coomassie Blue stain and Western Blot of GST-Rox extracted from different bacterial strains. i) Coomassie Blue stained SDS-PAGE gel, loaded and run simultaneously with SDS-PAGE gel transferred for Western Blotting. Equal volumes of purified protein were loaded on both gels. Lane 1, SeeBluell Markers; Lane 2, GST only; Lane 3, GST-Rox DH5α; Lane 4, GST-Rox SG22094; Lane 5, GST-Rox BL21 (DE3). ii) Western Blot probed with rabbit anti-GST antibody (1/5000)

BINDING OF ROX TO int

i)

Consensus	**YYRTCCGR-RY**
SRL PAI *int*	TTGTCCGATGT
P_{sid}	TTGTCCGGTGT
P_{LL}	TTATCCGGTGC
slpA I	TCATCCGATGA
slpA II	AAGTCCGAAGA

ii)

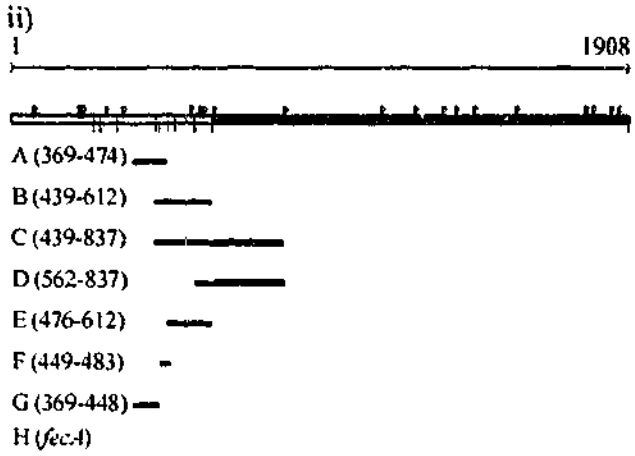


Figure 4k. Vis binding site and fragments used for gel mobility assay. i) Alignment of proposed Vis binding site. Highlighted nucleotides are divergent from the consensus. P_{sid} and P_{LL} are from P4 and *slpA* is from CP4-57. ii) The fragments used for gel mobility assay of GST-Rox binding. The numbers in brackets represent the positions of the fragments on the SRL PAI (Acc. No AF326777). The proposed binding site is denoted in pink, and the *int* ORF in blue. Fragment H represents 116 bp within the *fecA* coding region on the SRL PAI and was used as a negative control. Red lines and green 'p' denote potential stop and start codons, respectively.

In order to determine if Rox acted directly on the integrase gene, gel mobility assays were undertaken. As it was believed that Rox was a transcriptional regulator and therefore acted on the promoter region of *int*, several DNA fragments were designed to identify a potential binding site. A potential binding site has been identifed for Vis from bacteriophage P4 (Polo *et al.*, 1996). This potential binding site is also conserved upstream of *slpA* where AlpA acts (Figure 4k) and is present upstream of the SRL PAI *int*. Thus, fragments were designed to determine if this binding site was utilised by Rox (Figure 4k).

Initial gel mobility shift experiments were performed with the 'DIG Gel Shift Kit' as described by the manufacturer (Roche), utilising the binding buffer and conditions recommended (Chapter 2). However, there was no variation in mobility when the various fragments were incubated with GST-Rox. Therefore, conditions were adjusted to more closely parallel those used by Polo *et al.* (1996) when showing Vis binding. These changes included the addition of glycerol to the 4% acrylamide gel, and a change in binding buffers (Chapter 2). It was also suggested that cations may

be required for DNA binding and therefore $ZnCl_2$ was included in reactions and examined for its effect on binding.

Initial $ZnCl_2$ concentrations of 80 mM were used as recommended by J. Cheung & V. Adams (personal communication). At 80 mM $ZnCl_2$ retardation of the test fragment (B) (containing the potential binding site) was observed. However, when all DNA fragments were tested it was determined that the shift was non-specific at this concentration in the presence of GST-Rox (Preparation A), but not GST alone (Figure 4l). To attempt to eliminate non-specific retardation of the DNA fragments, titrations of a fresh preparation of Rox (Preparation B) were performed to compare retardation of test fragment (B) and the negative control fragment (H) (*fecA*) (Figure 4m). While there appeared to be a decrease in non-specific shifts as GST-Rox concentration was decreased, at 0.78 ng of full length GST-Rox, there was still non-specific shifting of the negative control fragment (H). Therefore, titration of $ZnCl_2$ in the binding reaction was undertaken, using 0.78 ng of GST-Rox. This showed that the non-specific shift seen for the negative control fragment (H) at 80 mM $ZnCl_2$ was lost at 20 mM $ZnCl_2$ (Figure 4n). Therefore, future experiments were performed using 2 ng of protein and 10 mM $ZnCl_2$. 2 ng of GST-Rox fusion protein was chosen to ensure that the efficiency of the binding reactions was not limited by protein concentration.

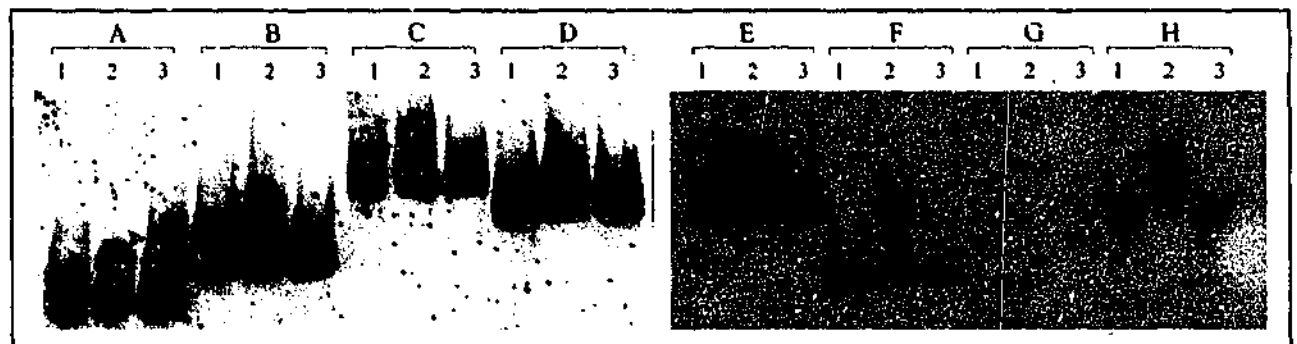


Figure 4l. Gel mobility assay with 80 mM $ZnCl_2$. Each of the various fragments (A-H) was used in binding reactions with 1, no protein; 2, GST-Rox (Preparation A); 3, GST only. The red arrowheads show the position of any retarded fragments. Total GST-Rox was 400 ng.

Experiments performed the following week using the same stock of all solutions showed no shift for any fragment. Fresh solutions were prepared, but the DNA mobility within the gel had changed. This was believed to be due to a problem with the poly-[d(I-C)] which is required for entry of the DNA into the gel. Therefore, the poly-[d(I-C)] was prepared again. The result was the correct entry of fragments into the gel, but again no shift was observed. As breakdown of GST-Rox was observed during purification, it was considered that GST-Rox may be degrading while in storage at -70°C. A fresh batch of GST-Rox was prepared (Preparation C) and run on an SDS-PAGE gel, along with GST-Rox that had been used for the previous experiments (Preparation B). It was noted that the band believed to represent the full length GST-Rox fusion had been lost in the old sample, when compared to a gel run when first extracted (Figure 4o). Both gels were loaded with the equal volumes of protein from the freezer stock. In order to attempt to overcome this problem, GST-Rox and GST alone were

extracted from AL170 and SBA264 and a protease inhibitor cocktail treatment was included in the extraction. These samples were then used in the gel mobility assay. Again no shift was observed.

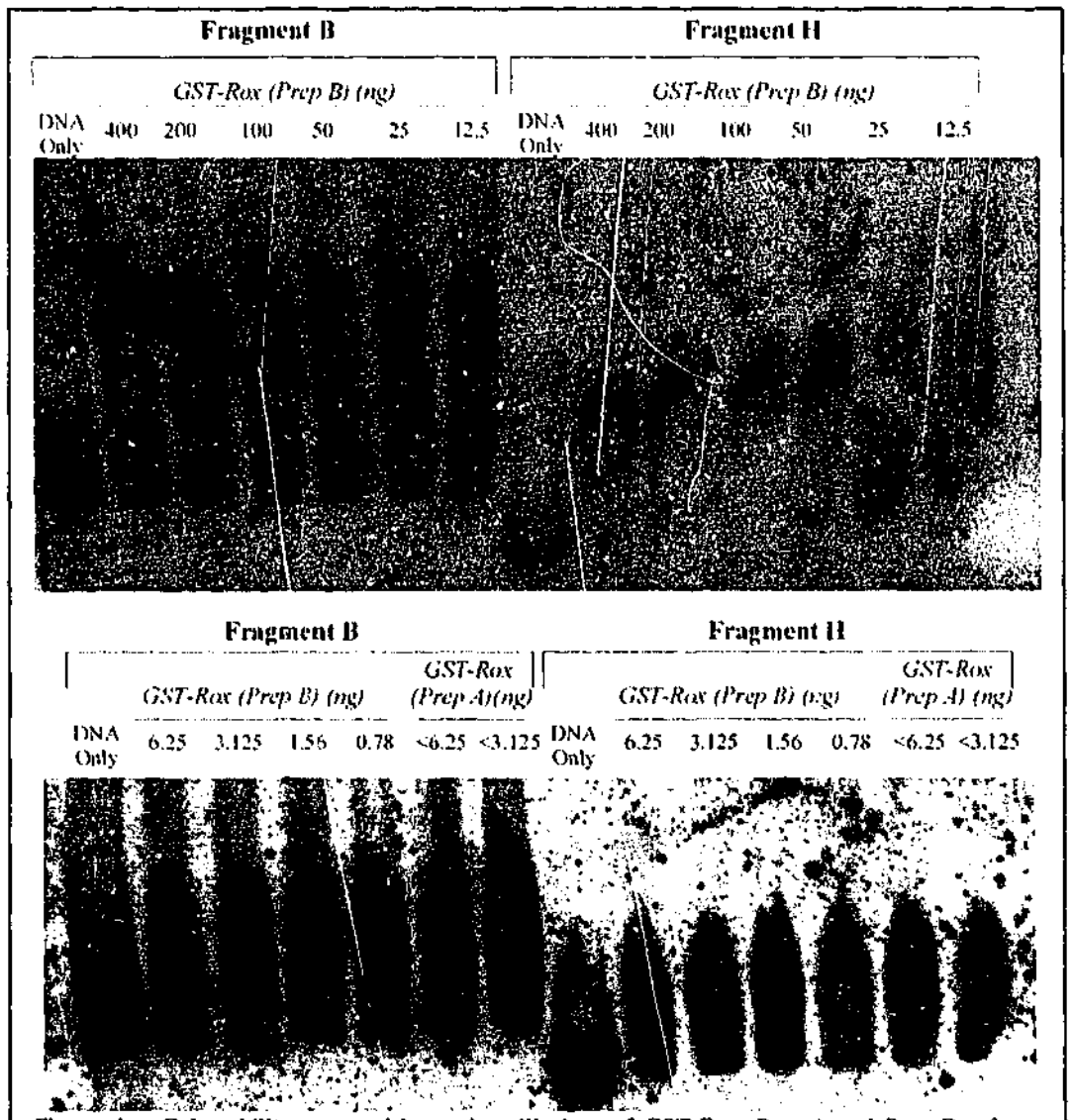


Figure 4m. Gel mobility assay with varying dilutions of GST-Rox. Prep A and Prep B refer to preparations of GST-Rox obtained on different dates. Protein amounts refers to full-length GST-Rox

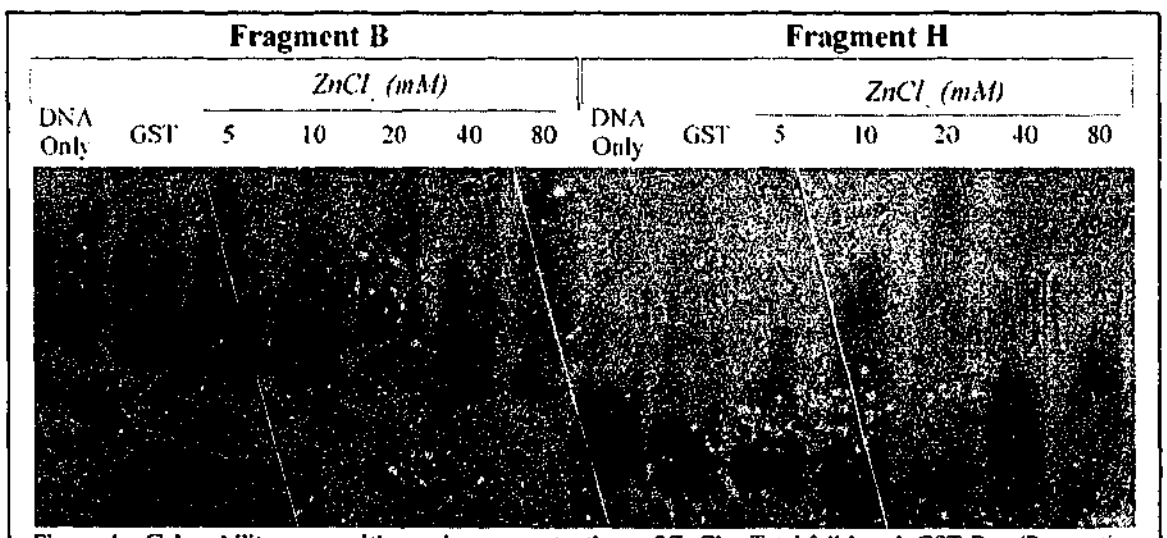


Figure 4n. Gel mobility assay with varying concentrations of $ZnCl_2$. Total full-length GST-Rox (Preparation B) was 0.78 ng. Note the loss of fragment H retardation at 20 mM $ZnCl_2$ and lower.

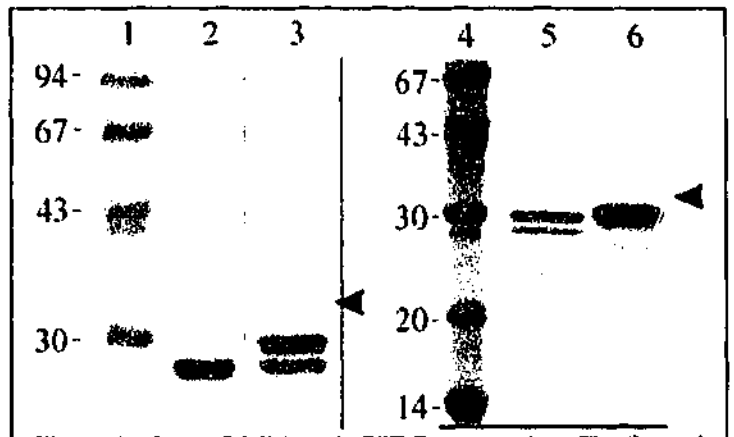


Figure 4o. Loss of full length GST-Rox over time. The first gel was run three weeks before the second gel. Lane 1, Molecular mass markers (kDa); Lane 2, GST; Lane 3, GST-Rox Preparation B. Lane 4, Molecular mass markers (kDa); Lane 5, GST-Rox Preparation C; Lane 6, GST-Rox Preparation B. Arrowhead denotes expected position of full-length fusion protein.

Other variables tested during attempted gel mobility analysis were:

- Variation of the concentration of the non-denaturing polyacrylamide gel between 4-15% acrylamide
- Modification of electrophoresis conditions (variation in voltage and amperage and buffering conditions)
- Addition of $MgCl_2$ to binding reaction

However, none of these conditions resulted in consistent retardation of fragments in the presence of GST-Rox. Due to the inability to reproducibly observe any retardation of DNA fragments in the presence of GST-Rox, other methods were undertaken to show an interaction between Rox and *int*.

DOES ROX AFFECT INT TRANSCRIPTION?

In order to examine the role of the potential binding site upstream of the *int* gene, as well as the role of Rox on *int* transcription, Real-time PCR was employed. To determine if the potential binding site upstream of *int* did have a role in *int* regulation, a mutation was introduced into the potential binding site. Site-directed mutagenesis was undertaken to mutate the four conserved nucleotides within the potential binding site (5'-YYR**TCCG**RNRY-3' to 5'-YYR**GAAT**RNRY-3'). Site-directed mutagenesis was performed as described in Chapter 2. As site-directed mutagenesis results in a plasmid with the desired mutation, a plasmid-based system was employed for Real-time PCR. A PCR derived fragment, consisting of 209 bp upstream of *int* and the first 526 bp of the gene (Figure 4p) was cloned into pWSK29. The resulting plasmid, pAL171, was extracted from AL301 and sequenced to confirm correct sequence prior to mutagenesis. A clone was obtained with the correctly mutated sequence (pAL204). The inserts from pAL171 and pAL204 were subsequently cloned into pWSK129, yielding the plasmids pAL217 and pAL218 respectively. This was done to provide antibiotic resistances compatible with pBAD30 expression vectors utilised for tightly controlled expression of Rox. pAL217 and pAL218 were transformed into *S. flexneri* YSH6000T, giving rise to strains AL376 and AL377 respectively. YSH6000T was chosen because of its antibiotic resistance profile, and lack of background Rox expression or *int* transcription. AL376 and AL377 were each transformed with pAL216 and pBAD30; strains used are summarised in Table

Table 4b. Strains used in Real-Time PCR analysis of *int* transcription.

Strain	Plasmids	Genotype
AL378	pAL217/pBAD	int^{+} rox^{+}
AL379	pAL218/pBAD	int^{mut} rox^{+}
AL380	pAL217/pAL216	int^{+} rox^{-}
AL381	pAL218/pAL216	int^{mut} rox^{-}

```
401  ACGAAAAAAA AGCCCGTACT TTGGTACGCG CTCTTCTTTA AATATGGCGG
     TGCTTTTTTT TCGGGCATGA AAGCATGCGC GAGAAGAAAT TTATACCGCC

451  TGAGGGGGAC CGCCTGAAAT AAATCTGACT TAATCCAGGG GTGTTATATA
     ACTCCCCCTG GCGGACTTTA TTTAGACTGA ATTAGGTCCC CACAATATAT

                                                  GAAT
501  ATTTCTTTAT AAACAATGTA TTACACTTTT TGTTGTCCGA TGTTATCTAA
     TAAAGAAATA TTTGTTACAT AATGTGAAAA ACAACAGGCT ACAATAGATT
                                                  CTTA

551  TGTATTCTTA GTGAAGCGAG GGAGTAATGG CATACTGGAT GGCATACCAG
     ACATAAGAAT CACTTCGCTC CCTCATTACC GTATGACCTA CCGTATGGTC

601  AGTGACGGGA ATTAGCGTAT GGCAGTATTG ACGGATACGA AAGCAAGACA
     TCACTGCCCT TAATCGCATA CCGTCATAAC TGCCTATGCT TTCGTTCTGT

651  TATCAAACCT GATGACAAAC CATTGCCCCA TGGGGGAATT ACCGGACTGA
     ATAGTTTGGA CTACTGTTTG GTAACGGGGT ACCCCCTTAA TGGCCTGACT

701  CCCTTCATCC TTCTTCAGTA AAGGGGCGGG GGAAATGGGT TTTTCGTTAT
     GGGAAGTAGG AAGAAGTCAT TTCCCCGCCC CCTTTACCCA AAAAGCAATA

751  GTAAGTCCGG TGACACAAAA AAGACGTAAT GCTGGATTGG GAACTTACCC
     CATTCAGGCC ACTGTGTTTT TTCTGCATTA CGACCTAACC CTTGAATGGG

801  AGAGGTCAGT ATTGCTGAAG CTGCACGTAC TGCCCGGATA ATGCGAGAGC
     TCTCCAGTCA TAACGACTTC GACGTGCATG ACGGGCCTAT TACGCTCTCG

851  AACTTGCTGC AGGTGATGAT CCTCTGGAGA TTAAAAAGGC TGAATCTGAG
     TTGAACGACG TCCACTACTA GGAGACCTCT AATTTTTCCG ACTTAGACTC

901  AAAGTCGTTA TCCCAACATT TGCCGATGCA GCCAGGCGTG TACATGCAGA
     TTTCAGCAAT AGGGTTGTAA ACGGCTACGT CGGTCCGCAC ATGTACGTCT

951  ACTGTCTCCT GCATGCGAGA ATCCAAAGCA TGTAAGGCAG TGGTTATCGA
     TGACAGAGGA CGTACGCTCT TAGGTTTCGT ACATTCCGTC ACCAATAGCT

1001 CGCTTGAGAA TTACGCGTTT CCTCAACTGG GAGCAAAAAC GCTGGATTCG
     GCGAACTCTT AATGCGCAAA GGAGTTGACC CTCGTTTTTG CGACCTAAGC

1051 ATTACGGCTG CGGACGTGGC AGAAACACTG CGTCCAGTCT GGTTAACCTT
     TAATGCCGAC GCCTGCACCG TCTTTGTGAC GCAGGTCAGA CCAATTGGAA

1101 GTCAGAAACG GCAAGCCGGG TTAAACAGCG CATTCATGTT GTTATGCAGT
     CAGTCTTTGC CGTTCGGCCC AATTTGTCGC GTAAGTACAA CAATACGTCA
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Figure 4p. Sequence details for site-directed mutagenesis of the upstream region of the SRL PAI *int*. Numbers are representative of the position of the sequence from Accession Number AF326777. Primers used for cloning into pWSK29 are highlighted in blue. Primers for site-directed mutagenesis are highlighted in green. The base pairs to be mutated are highlighted in purple. Primers used for Real-Time PCR are highlighted in red. The start codon of *int* is highlighted in pink.

4b. RNA was extracted as previously described, with the following modification; cells were grown for one hour in LB Kn50/Ap100, then induced with 1 mM of arabinose for an additional two hours before RNA extraction.

Assays of *gyrB* transcription served as controls for RNA concentration in Real-time PCR. Transcription of *rox* was also compared in all strains. As expected AL380 and AL381 transcribed high-levels of *rox* in contrast to AL378 and AL379, which did not transcribe *rox*. Real-time PCR analysis showed a significant difference ($p = 0.02$) in *int* transcription in three biological replicates of AL378 and AL380, using a one-tailed unpaired t-test. The data showed that in the presence of Rox, *int* transcription was repressed 1.7-fold. The mutation in the upstream region of *int* also had some effect on *int* transcription, as no significant difference was observed between AL379 and AL381. A statistically significant difference ($p = 0.03$) was observed between AL380 and AL381, suggesting that the mutated bases do have a role in Rox control of *int* transcription, possibly affecting Rox binding.

DISCUSSION

Little is known about the mechanisms of PAI mobility, and more specifically integration and deletion. Turner *et al.* (2001) showed that the SRL PAI *int* is required for excision of the SRL PAI and it has been demonstrated that HPI-*int* and SRL PAI *int* modules, are capable of site-specific integration (Rakin *et al.*, 2001; Turner *et al.*, *manuscript in preparation*). Together, this implies the importance of *int* genes for PAI mobility. However, there is little understanding about how this process is regulated. The HPI *int* is under the control of its own promoter when it is in a circular form, but upon integration this promoter is replaced with the *asn* tRNA promoter, coupling *int* transcription to that of the tRNA gene (Rakin *et al.*, 2001). However, this is not the case for the SRL PAI *int*, as the integrase is at the distal end of the PAI to the intact *serX* gene. This study lead to the identification of a gene, *rox*, that encodes a protein that we believe has a role in the regulation of *int* expression.

A role for Rox in SRL PAI excision was initially established using a PCR assay for SRL PAI excision. It was demonstrated that when Rox was overexpressed, the SRL PAI deleted at a 10^3-fold greater rate than in the absence of Rox overexpression. We were able to show that this process was dependent on the SRL PAI *int* gene, because an *int* mutant, AL11, was no longer capable of excision, even when Rox was overexpressed.

The next step was to test whether Rox interacted directly with a sequence upstream of *int*. The use of gel mobility assays is commonly used to show an interaction between DNA and protein. However, Rox was highly unstable, even upon storage at -70°C and treatment with protease inhibitors, making it difficult to control the amount of GST-Rox fusion protein utilised in the reaction. GST-Rox was also found to bind non-specifically to DNA at high concentrations of $ZnCl_2$. Two distinct bands of different mobility were present in experiments with 80 mM $ZnCl_2$ for the test fragment (B) but, not the negative control fragment (H), as in Figure 4m. Fragments of differing mobility were also observed at 40 and 80 mM $ZnCl_2$ concentrations for fragment B in Figure 4n. Multiple species were also observed for fragment C in Figure 4m. This observation was made on numerous occasions when high concentrations of $ZnCl_2$ were used in binding reactions. Multiple retarded species usually represent multiple binding sites for a protein on a specific DNA target. The presence of two species at higher $ZnCl_2$ concentrations for specific fragments, but not non-specific fragments suggests that one species may represent the specific binding of GST-Rox to DNA and the other non-specific binding at high $ZnCl_2$ concentrations. Therefore, while specific binding may have occurred at high $ZnCl_2$ concentrations with fragments expected to bind Rox, a single specific retardation product was not observed consistently. Other methods, such as South-Western assays, were attempted in order to demonstrate binding of a region upstream of the SRL PAI *int* to GST-Rox. However, results from

these experiments were also inconclusive. Gel mobility assays with Rox should be pursued, firstly by attempting to obtain a more stable form of Rox, perhaps by expressing it as a different fusion protein. His-tagged and maltose-binding protein fusions may be more stable and should be attempted. A closer examination of the multiple species observed at high $ZnCl_2$ concentrations may also be warranted.

The Real-time PCR data suggested that the role of Rox in the regulation of *int* expression is two-fold. It is known that Rox drives Int dependent deletion of the SRL PAI, and thus must up-regulate *int* expression. However, the Real-time PCR data demonstrated that Rox also has a role in the down-regulation of *int* transcription. The reliability of this result is supported by the finding that mutation within the proposed binding site upstream of *int* returned transcription to wild-type levels, even in the presence of Rox. Therefore, the repression of *int* transcription was due to Rox. A plausible explanation for this is that Rox requires a co-factor for the activation of *int*, and this co-factor is encoded on the SRL PAI or MRDE. That is, in the absence of the co-factor Rox represses *int* transcription, possibly by preventing the binding of RNA-polymerase, as seen with other regulators (Schell, 1993). However, in the presence of the co-factor the binding of RNA polymerase to the *int* promoter may be altered and activation is possible (Figure 4q).

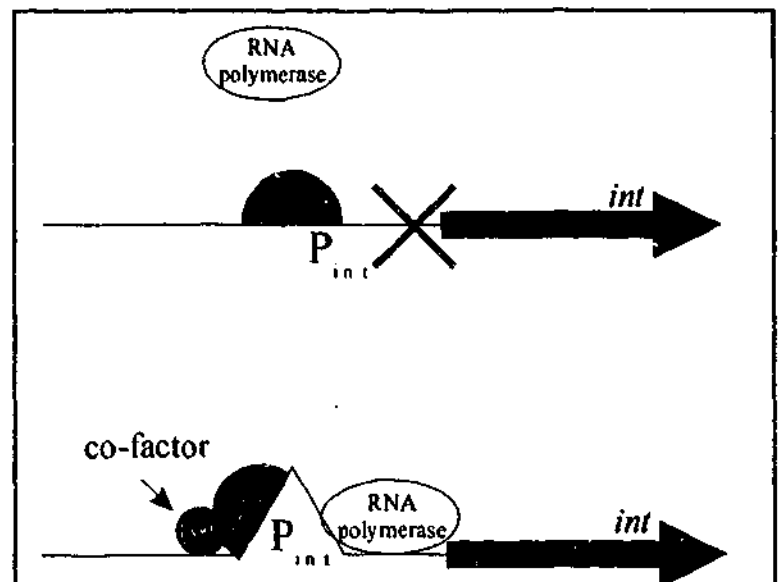

Figure 4q. Model of Rox-co-factor regulation of *int* transcription. P_{int} represents the *int* promoter. The interaction between Rox and the co-factor results in a change in the topology of the DNA allowing access for RNA polymerase and thus transcription.

The requirement for co-inducers by transcriptional regulators is not a new concept. Many members of the LysR-type transcriptional regulators (LTTR) family use co-inducers. LTTRs are often transcribed divergently from their target gene. In the absence of their specific co-inducer they bind to the overlapping promoters and negatively autoregulate their own transcription as well as that of their target genes (Schell, 1993). In some cases, the co-inducer is believed to affect the bending of the DNA and thus allow RNA polymerase to interact with the promoter region. An example of this is CysB, which autoregulates itself, but in the presence of its co-inducer, N-acetyl-L-serine, initiates transcription of *cysJIH* and *cysK* (Ostrowski & Kredich, 1991). Some LTTRs utilise their oxidative state for activation. For example, OxyR negatively autoregulates in its reduced state, but upon oxidation it positively regulates a range of genes around the chromosome (Storz *et al.*, 1990). This is the opposite to what was observed for Rox, because repression was seen in the absence of the SRL

PAI and activation in the presence of the PAI. However, such a co-inducer may be required. Whether it is encoded by the SRL PAI or is a small molecule that is only present at certain times in the cell cycle or under certain conditions is unknown.

The LTTR GcvR interacts with GcvA to repress the *gcvTHP* operon (Ghrist & Stauffer, 1995), but in the absence of GcvA, GcvR is not sufficient to repress the operon. These proteins have been shown to interact directly *in vivo* using a LexA-based system and gel mobility assays (Ghrist *et al.*, 2001). In order to activate the *gcvTHP* operon, glycine must bind to GcvR, allowing GcvA to activate the operon (Heil *et al.*, 2002). Thus, the regulation of this operon requires at least two proteins and a co-inducer. Rox may function in a similar way, in that it may require interaction with a second protein to induce the correct confirmation at the *int* promoter and activate transcription.

An interesting example of a protein that both up and down regulates genes is SoxS. SoxS is part of the superoxide-stress response cascade and therefore up-regulates genes such as *tolC*, *micF* and *marA*. Rob has been shown to repress the same genes up-regulated by SoxS. In order for SoxS to up-regulate these genes it must first down-regulate *rob* transcription (Michán *et al.*, 2002). Superoxide stress leads to SoxS production, controlled by SoxR (Hidalgo *et al.*, 1998), which leads to *rob* repression and thus makes the promoters of the other SoxS targets available. Thus, it is not unreasonable for a protein to be able to both up- and down-regulate gene transcription.

It should be considered that Rox may not be acting directly on *int* but at an earlier point in a cascade that leads to changes in *int* expression. However, several pieces of evidence suggest that this is unlikely. Firstly, Rox repressed *int* transcription in the absence of the SRL PAI. Therefore, any co-factors encoded on the PAI did not influence this interaction. This is supported by the insignificant difference in *int* transcription in the binding-site mutant in the presence and absence of Rox. Secondly, the gel mobility assays suggest that Rox was capable of binding specifically to the *int* promoter region, under the correct conditions. However, it is possible that Rox also acts at other sites on the SRL PAI and the repression of *int* may be a secondary effect. In the presence of the SRL PAI, Rox may activate another gene that encodes the positive *int* regulator. This regulator may have a higher affinity for the *int* promoter than Rox and thereby displaces it.

The bacteriophage P2 utilises a two protein system to control its excision and integration. Excess Int is deleterious to the integrative process, as Int also controls excision of P2; therefore, tight control of *int* expression is critical for the P2 lifecycle. The Cox protein negatively autoregulates the P_e promoter in P2; this promoter is required for Cox and Int transcription (Saha *et al.*, 1987). As Cox negatively regulates Int, it can control integration. Cox can also act *in trans* and activate the P_{LL} promoter of bacteriophage P4 and weakly repress the P_{LE} promoter (Saha *et al.*, 1989). The multiple

functions of Cox have lead to the suggestion that it acts nonspecifically by binding and bending DNA to allow or inhibit the binding of other factors (Yu & Haggard-Ljungquist, 1993). Alternatively, Cox may specifically bind DNA and interact with various proteins to regulate its various targets. Cox and Rox are a very similar size, 101 and 102 amino acids respectively, making Rox 22 residues longer than its AlpA homologue. Therefore, it is conceivable that Rox may function in a similar way to Cox. We have shown that it binds readily to any DNA fragment and thus it may be the protein-protein interactions that are important for regulation.

A homologue of Rox is Vis, encoded by *orf88* of the P4 bacteriophage. Vis regulates transcription from P4 late promoters, up-regulating transcription from the P_{sid} promoter and down-regulating transcription from the P_{LL} promoter. Vis alone is not able to activate transcription from P_{sid} and it has been proposed that it may enhance the activity of the P4 positive regulator δ (Polo *et al.*, 1996). Unpublished results have shown that as well as binding to P_{sid} and P_{LL}, Vis is also able to bind to the P4 *int-att* region (Polo *et al.*, 1996). Pierson III and Kahn (1987) proposed that P2, P4 or *E. coli* proteins may affect P4 *int* synthesis and thus concentration, because their results suggested that *int* may be expressed at a constitutive, low-level when P4 is in the lysogenic state. Given that the concentration of P2 Int affects integration of that bacteriophage, it is reasonable to speculate whether P4 Int concentration also affects the phage's ability to integrate and excise. P4 Int also autoregulates itself, which would control the concentration of Int within the cell. The importance of this in regards to Rox is that the total concentration of Int is important for integration and excision, and so Rox may act similarly to Cox, by controlling Int levels. In the absence of the co-factor, Rox restricts the level of Int in the cell, promoting maintenance of the island. However, in the presence of the co-factor, the level of Int is increased and thus excision of the island is favoured. This is similar to the effect Cox has on P4, where it activates the late promoter, P_{LL} (Saha *et al.*, 1989) and induces the lytic cycle (Saha *et al.*, 1987), possibly by upregulating *int* expression through Vis.

A possible scenario for Rox activity is that it binds to the *int* promoter, but for activation, it requires a co-factor. The most important next step is to determine if there is a co-factor on the SRL PAI required for *int* activation. This would be done by repeating the Real-time PCR experiment using an *S. flexneri* YSH6000 *int* mutant with and without the overexpression of Rox. This experiment has been done for the *she* PAI, which has a similar backbone to the SRL PAI and encodes Rox and P4 Int homologues (Sakellaris *et al.*, *manuscript in preparation*). The experiment found that when Rox was overexpressed transcription of *she int* increased 1.6-fold, a significant difference. The low level of difference seen for *int* transcription in the presence of Rox may be due to autoregulation by Int, as seen in P4. If the SRL PAI Rox system is similar to that of the *she* PAI then there is a co-factor on the SRL PAI required for *int* activation.

In conclusion, Rox has a role in regulation of the transcription of the SRL PAI *int* gene. Rox is able to down-regulate *int* transcription, but increase excision of the SRL PAI. The data presented here suggest that for activation of *int*, Rox requires a co-factor encoded on the SRL PAI.

~ CHAPTER FIVE ~

The Ferric-Dicitrate Iron Transport System of S. flexneri *2a*

INTRODUCTION

Iron is an essential component of the cytochrome and iron-sulfur proteins of the electron-transport system which mediates bacterial respiration (Neilands, 1981). While iron is readily available in the environment, in the human host it is stored in tissues such as the liver or chelated by extracellular proteins such as transferrin and lactoferrin (Aisen & Leibman, 1972; Gray-Owen & Schryvers, 1996; Moffett *et al.*, 1993). Intracellular pathogens such as *Shigella* can scavenge iron from within the cells, but they must also obtain iron from the extracellular environment of the host. To achieve this, many bacteria produce high-affinity, low molecular weight iron chelators, called siderophores, to scavenge extracellular iron (Crosa, 1989; Neilands, 1981).

Several enteric bacteria including *E. coli*, *Shigella* and *Yersinia* use siderophores to obtain iron. *E. coli* strains have been found to encode up to five iron transport systems (Earhart, 1996) and all clinical isolates of *Shigella* spp. carry at least one siderophore based iron-uptake system (Payne, 1989). *E. coli* and some *S. flexneri* and *S. boydii* strains produce the catechol siderophore enterobactin (Payne, 1980; Payne *et al.*, 1983), while other *S. flexneri*, *S. boydii* and *S. sonnei* strains also produce the dihydroxymate siderophore aerobactin (Lawlor & Payne, 1984). The aerobactin locus in *S. flexneri* is carried on two PAIs, SHI-2 and SHI-3 (Moss *et al.*, 1999; Vokes *et al.*, 1999;Purdy & Payne, 2001). In this study we have identified another system, a ferric-dicitrate transport system homologous to the *E. coli* K-12 Fec system, encoded by genes carried on a PAI-like element in *S. flexneri* 2a.

Interestingly, the *fec* locus is absent in many *E. coli* strains (Ochman, 2000) making at least that region of the *E. coli* K-12 chromosome atypical for *E. coli*. The ferric-dicitrate transport system is encoded by two operons located at minute 97.3 of the *E. coli* K-12 genome (Veitinger & Braun, 1992) and is also present in *E. coli* B (Pressler *et al.*, 1988). The *fec* locus of *E. coli* K-12 consists of two regulatory genes, *fecI* and *fecR*, upstream of the structural genes, *fecABCDE* (Figure 5a).

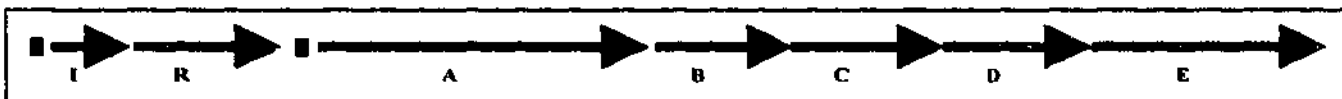

Figure 5a. Schematic representation of the *E. coli* K-12 *fec* locus. The shaded boxes represent promoter regions upstream of *fecI* and *fecA*.

The Fec-transport system transports ferric ions from the extracellular environment into the bacterial cell cytoplasm (Figure 5b). This process is induced by the binding of Fe^{3+} to outer membrane bound FecA, in the presence of at least 0.1 mM citrate (Hussein *et al.*, 1981; Zimmerman *et al.*, 1984). Binding of ferric-dicitrate to FecA leads to complex formation between FecA and TonB which results in a transfer of stored potential energy from TonB to the FecA receptor, promoting transport of ferric-

dicitrate across the outer membrane (Ferguson *et al.*, 2002; Kim *et al.*, 1997). This interaction with TonB is also required for the initiation of transcription of the *fec* operon (Kim *et al.*, 1997). The transfer of signal from FecA to FecI involves interaction between the C-terminus of FecA and the N-terminus of the periplasmic portion of FecR following ferric-dicitrate binding to FecA (Enz *et al.*, 2000). This results in a conformational change in the cytoplasmic portion of FecR, allowing the C-terminus of FecR to interact with region 4 of FecI, where its helix-turn-helix motif is proposed to be (Enz *et al.*, 2000; Mahren *et al.*, 2002). The result is activation of FecI, which ultimately leads to transcription of *fecABCDE*. Figure 5b summarises the mode of iron transport into the cell by the ferric-dicitrate transport system.

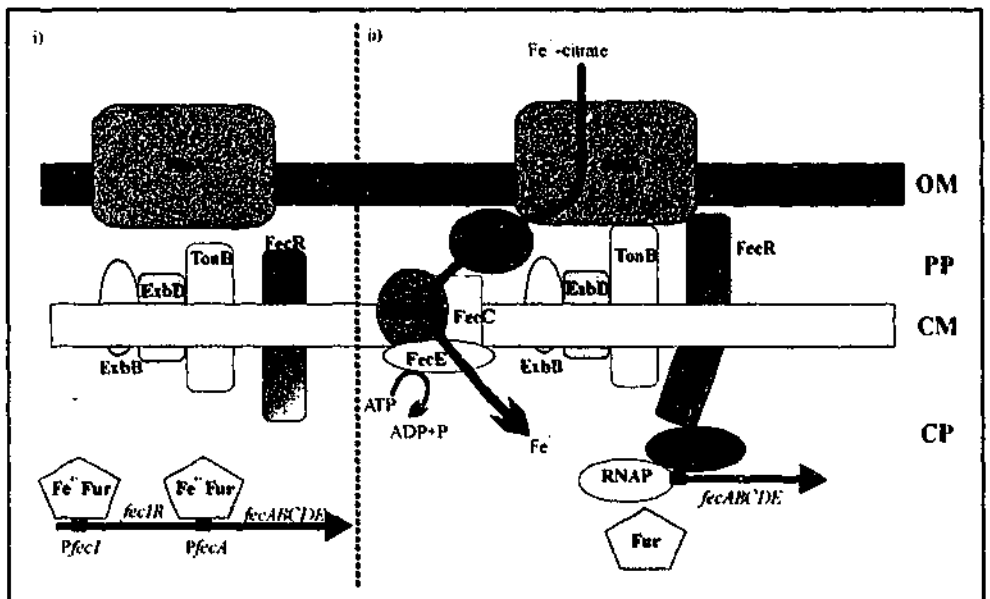

Figure 5b. Transport of Fe^{3+} from the extracellular environment via the ferric dicitrate transport system (adapted from Crosa, 1997]). i) In the presence of intracellular iron. ii) In the absence of intracellular iron. P_{fecI} and P_{fecA} denote the *fecI* and *fecA* promoters respectively. OM, PP, CM and CP denote outer membrane, periplasm, cytoplasmic membrane and cytoplasm resepctively.

FecI is an RNA polymerase sigma factor, and is part of the extracytoplasmic function (ECF) family (Lonetto *et al.*, 1994). This family encodes sigma factors that respond to extracytoplasmic stimuli and regulate extracytoplasmic functions (Enz *et al.*, 1995; Lonetto *et al.*, 1994). FecI possesses a helix-turn-helix motif that is highly conserved amongst the ECF sigma factors (Ochs *et al.*, 1996), providing a means for DNA binding. FecI and FecR are required for the positive regulation of *fec* transporter gene (*fecABCDE*) transcription in the presence of citrate, while iron and Fur act as repressors (Ochs *et al.*, 1996; Ochs *et al.*, 1995). Fur regulation results from the binding of Fe^{2+}-Fur to the *fecI* and *fecA* promoters (Angerer & Braun, 1998). Iron limitation results in the availability of these promoters and so FecI can promote *fecABCDE* transcription from the *fecA* promoter (Angerer *et al.*, 1995). Expression of *fecA* is much greater than *fecBCDE* expression, probably due to the hairpin structure near the 3'-end of *fecA* acting as a terminator (Enz *et al.*, 1995). While the other structural Fec proteins (FecBCDE) are required solely for transport, FecA also has a role in induction of the system (Harle *et al.*, 1995), possibly explaining the difference in transcriptional levels between *fecA* and the other structural genes. FecB is a periplasmic protein that has been shown to bind directly to ferric citrate (Braun, 1997). FecC and FecD are hydrophobic proteins, located in the cytoplasmic membrane that are associated with FecE on the cytoplasmic side of the cytoplasmic membrane. FecE

contains two ATP binding motifs, suggesting that ATP is the energy source for the transport of iron across the cytoplasmic membrane (Schultz-Hauser *et al.*, 1992).

In work prior to the commencement of this thesis, a homologue of the *E. coli* ferric dicitrate system was identified, associated with the SRL of *S. flexneri* 2a YSH6000. 5.8 kb of the *fec* locus had been sequenced. Completion of the *S. flexneri* 2a YSH6000 *fec* locus sequence and functional analysis of this *fec* locus was to be completed as part of the current investigation.

RESULTS

SEQUENCE ANALYSIS OF THE FEC LOCUS AND DISTRIBUTION IN SHIGELLA SPP.

The *fec* locus was associated with the SRL, based on the presence of both loci on pSBA361 (discussed in Chapter 3). Previous subcloning of pSBA361 with *Pst*I and *Hin*dIII resulted in subclones that carried the intact *fec* locus (pSBA491) or incomplete *fec* locus (pSBA484, pSBA486, pSBA487, pSBA488) (Figure 3b). Primer walking along pSBA491 and pSBA488 using primers designed from previously derived sequence (Appendix 3), completed the sequence of the *S. flexneri* 2a YSH6000 *fec* locus. The *fec* locus on the SRL PAI was found to be 7 544 bp in length, from *fecI* to *fecE*, and was homologous to the *fec* system encoded by two operons (*fecIR* and *fecABCDE*) located at minute 97.3 of the *E. coli* K-12 genome (Veitinger & Braun, 1992). The *S. flexneri fec* genes had greater than 99% identity at nucleotide level to the *E. coli* K-12 genes, but differences were observed in the regions flanking the loci (Figure 5c). The *E. coli* K-12 locus is flanked upstream by IS*1*F and downstream by an IS*911* element that is insertionally disrupted by an IS*30* and a truncated IS*2*. The *S. flexneri* SRL PAI *fec* locus is flanked downstream by an intact IS*911*. The sequence directly upstream of IS*911* showed 100% identity at nucleotide level between *E. coli* and *S. flexneri*. Upstream of the *S. flexneri* *fec* locus were the first 61 bp of IS*1*F followed by remnants of several IS elements; copies of IS*3*, an IS*903*-like element and IS*629* were all significantly truncated.

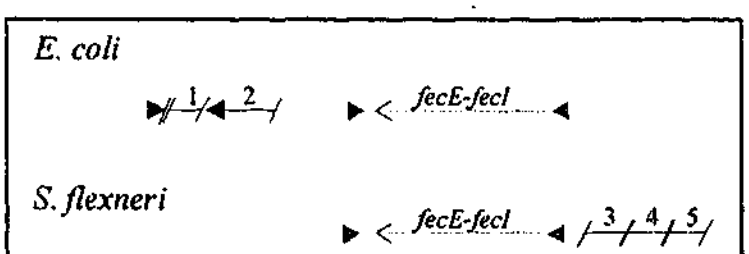

Figure 5c. Comparison of *S. flexneri* and *E. coli fec* loci flanking regions. The blue arrows denote IS*911*, the pink arrow denotes IS*1*F. The numbers 1, 2, 3, 4, and 5 represent remnants of IS elements IS*2*, IS*30*, IS*3*, IS*629* and IS*903*-like respectively.

Table 5a. Distribution of *fec* and selected SRL PAI markers in *Shigella* spp.

Strain	Species	*int*	SACT[a]	*fecA*	*fecE-tetD*
SBA1299[b]	*S. flexneri* 1a	+	+	+	+
SBA1300	*S. flexneri* 2b	+	+	+	+
SBA1302	*S. sonnei* 1	+	+	+	+
SBA1303[b]	*S. sonnei* 1	+	+	+	+
SBA1304[b]	*S. dysenteriae* 3	+	+	-	-
SBA1306	*S. dysenteriae* 3	+	+	+	+
SBA1308[b]	*S. boydii* 4	+	+	+	+
SBA1386[b]	*S. boydii* 1	+	+	-	-
SBA1388	*S. flexneri* 2a	-	+	+	-
SBA1389	*S. flexneri* 3a	+	+	+	-
SBA1391[b]	*S. flexneri* 4	+	+	+	+
SBA1392	*S. flexneri* 6	+	+	+	+

[a] SACT – streptomycin, ampicillin, chloramphenicol and tetracycline resistance determinants. [b] These strains show linkage at other SRL PAI markers (Turner *et al.*, 2003)

As this was the first report of a ferric dicitrate system in *Shigella*, Southern hybridisation was carried out on a variety of *Shigella* strains to determine the distribution of the *fec* locus (performed by S. Turner, 1999). This experiment showed that the *fec* locus is widely distributed amongst *Shigella* spp. and also in at least one EIEC strain (data not shown). Strains from all four *Shigella* species encode *fecA* homologues, although they were not always associated with the presence of a SRL PAI *int* homologue (Turner *et al.*, 2001), and therefore may not always be located on an intact PAI. A long range PCR was performed on a selection of strains carrying a *fecA* homologue and resistance determinants for streptomycin, ampicillin, chloramphenicol and tetracycline in an attempt to link the *fec* and SRL loci. Interestingly, a linkage could be shown only in strains that carried the four antibiotic resistance determinants encoded by the SRL PAI, as well as the SRL PAI *int* gene (Table 5B). Turner *et al.* (2003) also demonstrated linkage between other markers throughout the SRL PAI. Interestingly, in strains SBA1304 and SBA1386, when *fec* was not present, a large region of the PAI consisting of at least *fecE* to *orf48* was also missing. This data suggests that the *fec* region of the SRL PAI has been either acquired or lost by the SRL PAI at a different time to the SRL and several other SRL PAI markers.

FUNCTIONAL ANALYSIS OF THE S. FLEXNERI FEC *LOCUS*

Complementation of an E. coli *Δ*fec *Mutant*

The ferric dicitrate iron-transport system has been well characterised in *E. coli* K-12. It is capable of maintaining the growth of *E. coli* under iron-limited conditions in the absence of other iron-uptake systems (Wagegg & Braun, 1981). Because of the high similarity between the *S. flexneri* and *E. coli* *fec* loci, the function of the SRL PAI *fec* locus was tested in an *E. coli* Δ*fec* strain (AA93) (Table 2a). The AA93 strain had the entire *fec* locus (*fecIRABCDE*) deleted from the chromosome. The entire *S. flexneri fec* locus had been cloned into the *Hin*dIII site of the low copy plasmid pWSK129, giving rise to pSBA491. pSBA491 and an empty pWSK129 vector (plasmid control) were introduced separately into AA93 giving rise to strains SBA844 and SBA845 respectively. Complementation was tested by growth under iron-limited conditions supplemented with citrate, as required for *fec* function

(Chapter 2). An *E. coli* wild-type *fecIRABCDE* bearing plasmid, pSV662, was used as a control for the expression of the *fec* locus in AA93. Complementation of the Δ*fec* strain by the wild-type locus in pSV662 was observed (data not shown), showing the system to be a suitable test for *S. flexneri* Fec function. The growth of AA93 under iron-limited conditions was restored by complementation with the cloned *S. flexneri fec* locus (pSBA491) but not with the cloning vector alone (Figure 5d). These results demonstrated that the *S. flexneri fec* locus was functional.

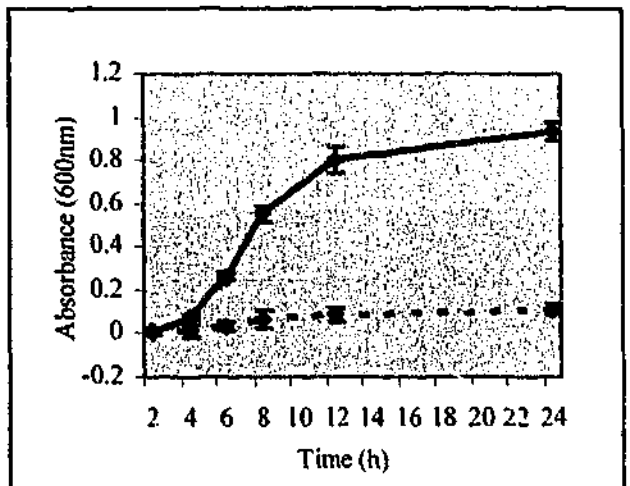

Figure 5d. Growth of *E. coli* AA93 with pWSK29 (Δ*fec*) (dashed line) compared to SBA844 (Δ*fec*, pSBA491) (solid line) under iron-limited conditions. Error bars represent 2 standard deviations.

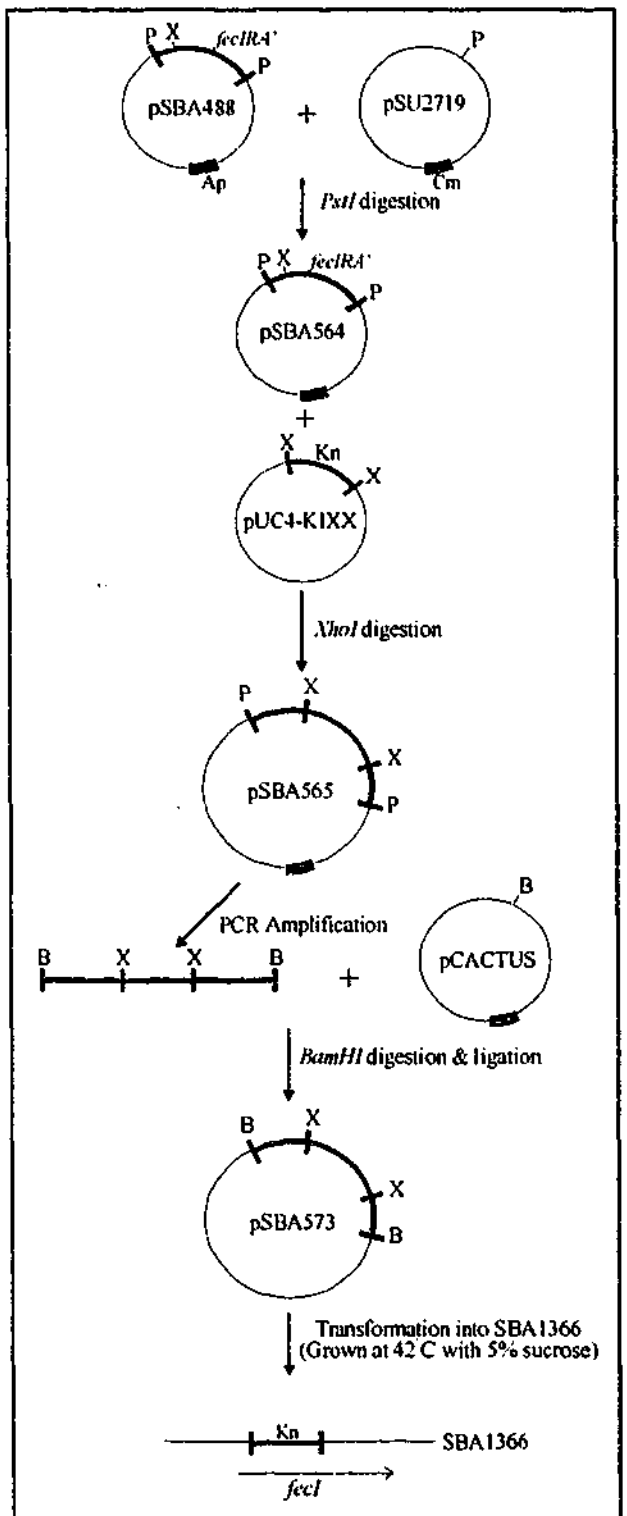

Figure 5e. Construction of a *fecI* insertional mutant. The various plasmids shown are listed in Materials and Methods (Chapter 2). Restriction sites used are denoted by B – *Bam*HI; P – *Pst*I; X – *Xho*I. The antibiotic resistance cassettes for each plasmid are labelled: Ap – ampicillin; Cm – chloramphenicol; Kn - kanamycin

Construction of a S. flexneri fecI *mutant*

Following determination of the functional ability of the *S. flexneri fec* locus, it was necessary to investigate the role of the Fec system in the growth of *S. flexneri* in iron-limited conditions. This was performed by the construction of a specific *fec* mutant. Previous mutation of the *E. coli fecI* gene had shown that it was essential for the transcription of the *fecABCDE* operon and thus function of the locus (Ochs *et al.*, 1996), making it a good candidate for mutation in this study. The 4.8 kb *Pst*I fragment from pSBA488 was inserted into the *Pst*I site of pSU2719 to provide a unique *Xho*I site within *fecI* giving rise to pSBA564 (Figure 5e). The *fecI* gene was inactivated by insertion of the 1.6 kb *kan* cassette from pUC4-KIXX into the unique *Xho*I site in the *fecI* gene on pSBA564. A 5.1 kb PCR product containing *fecI*Ωkan, *fecR* and the 5'-end of *fecA* was cloned into the *Bam*HI site of the pCACTUS suicide vector. The *S. flexneri* strain SBA1366 chosen for mutation was a

derivative of YSH6000 lacking the SRL, to facilitate subsequent complementation of the mutation, if desired. Following introduction of the construct into SBA1366, a double crossover mutant, SBA1415, was selected for by growth at 42°C in the presence of kanamycin and sucrose. The genotype of SBA1415 was confirmed by PCR using primers within and external to the pCACTUS construct that showed the expected increase in size of 1.6 kb (Figure 5f). The wild-type parent was compared to SBA1415 and YSH6000T (MRDE deletant) to confirm the double crossover mutation of *fecI*.

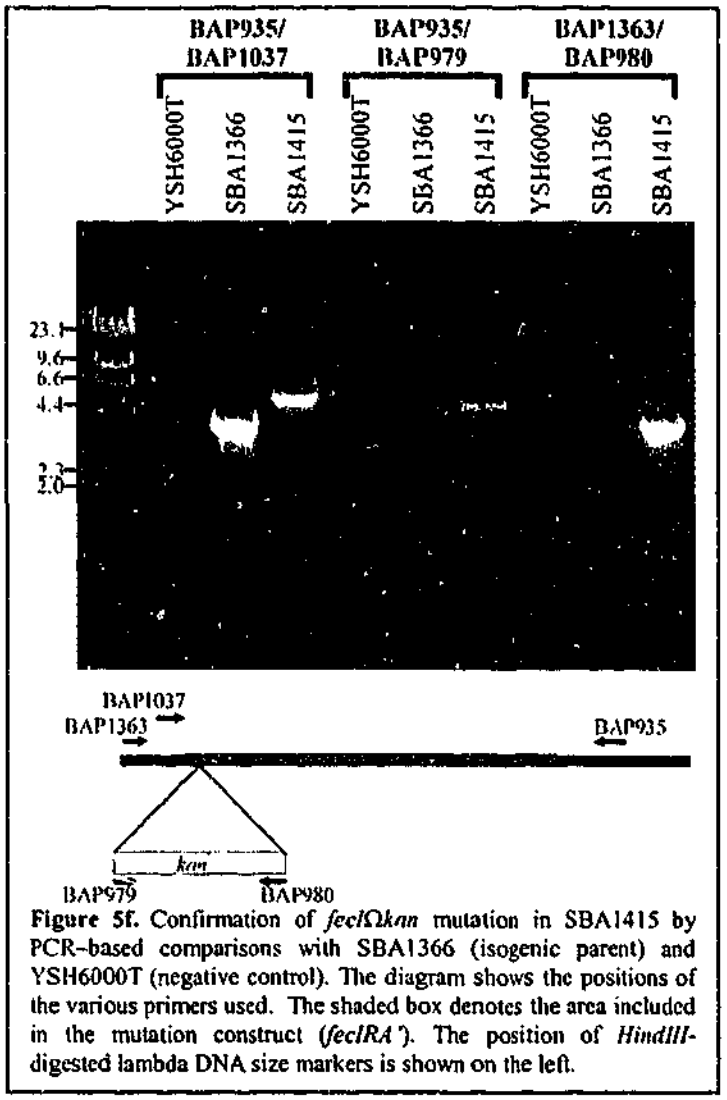

Figure 5f. Confirmation of *fecIΩkan* mutation in SBA1415 by PCR-based comparisons with SBA1366 (isogenic parent) and YSH6000T (negative control). The diagram shows the positions of the various primers used. The shaded box denotes the area included in the mutation construct (*fecIRA'*). The position of *HindIII*-digested lambda DNA size markers is shown on the left.

Analysis of the Growth of S. flexneri in Iron-Limited Conditions

To test whether the *fec* iron-transport system was required for the growth of *S. flexneri* 2a YSH6000 under iron-limited conditions the *fecI*Ωkan mutant was compared to its isogenic parent (SBA1366) and YSH6000T (MRDE deletant). When cultured under iron-limited conditions in medium supplemented with citrate there was no significant difference in growth between any of the strains (Figure 5g); neither mutation of *fecI* nor the loss of the *fec* locus had a significant effect on the growth of *S. flexneri* 2a.

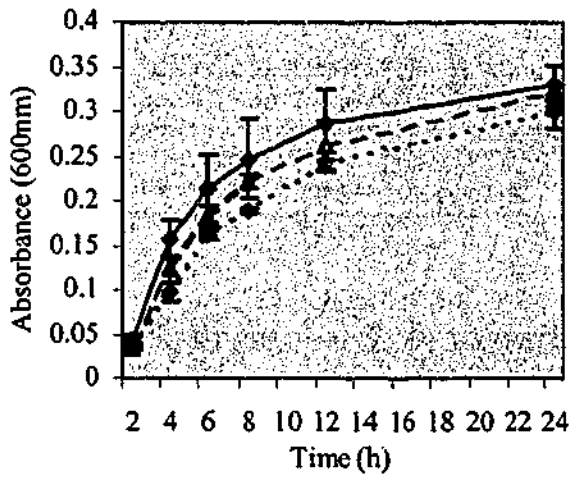

Figure 5g. Growth of SBA1415 (*fecIΩkan*) (dashed line) compared to SBA1366 (isogenic parent) (solid line) and YSH6000T (MRDE deletant) (dotted line) under iron-limited conditions. Error bars represent 2 standard deviations.

There were several possible explanations for the insignificant difference in growth under iron-limited conditions between the wild-type strain and the *fecI* mutant. These explanations included the possibility that the *fec* locus was not transcribed in *S. flexneri* or that the *S. flexneri fecI* mutant may not exhibit reduced transcription of the *fec* structural genes. The latter hypothesis would be possible if transcription of the *fecABCDE* operon in SBA1415 is initiated by an alternative sigma factor that recognises the *fecA* promoter. To confirm that the wild-type strain transcribed *fecA* and the *fecI* mutant did not, an RNA dot blot was performed. RNA was extracted from YSH6000, YSH6000T, AA93, SBA844, SBA1366 and SBA1415 cells grown in iron-limited conditions supplemented with citrate. A *recA* probe confirmed that equal amounts of RNA for each matched pair were loaded on the dot blot (Figure 5h). A *fecA* DNA probe was unable to detect *fecA* mRNA in the *S. flexneri fecI* mutant, SBA1415, but transcripts were readily detectable in the isogenic parent strain, SBA1366 and in the wild-type strain, YSH6000. As expected, *fecA* transcripts were undetectable in the negative control strains YSH6000T and AA93, which do not carry the *fec* locus, but a transcript was detected in an AA93 strain complemented with the *S. flexneri fec* locus (SBA844) (Figure 5h). These results demonstrated that the *fec* locus was expressed in YSH6000 in a *fecI*-dependent manner and therefore suggest that this strain may encode additional iron-uptake systems that compensate for the *fec* mutation when grown in laboratory culture media.

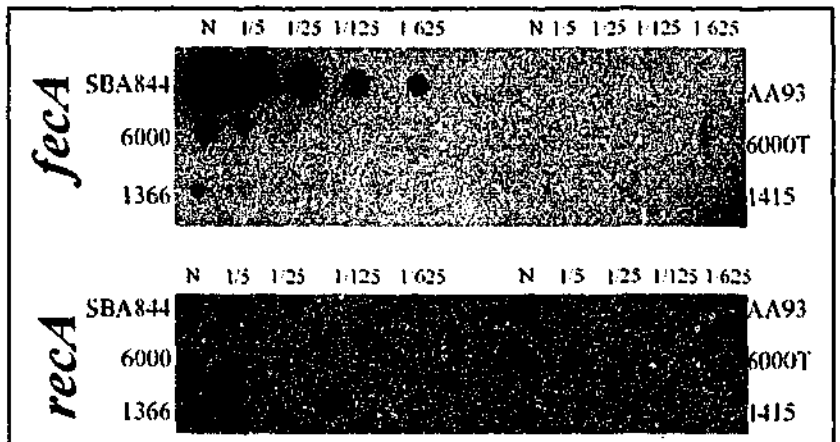

Figure 5h. RNA dot blot using *fecA* and *recA* DNA as probes. RNA from SBA844, AA93, YSH6000, YSH6000T, SBA1366 and SBA1415 spotted. N represents the neat (undiluted sample). Samples were matched for loading in the following pairs: SBA844/AA93, YSH6000/YSH6000T, SBA1366/SBA1415. RNase treated samples not shown.

The Identification of Other Iron-Transport Systems in S. flexneri *2a YSH6000*

It is possible that other iron-transport systems are present in this strain of *S. flexneri*. However, Southern hybridisation has shown that the *fec* locus was present in single copy in YSH6000 and absent in YSH6000T (work conducted prior to the commencement of this thesis). Therefore, if a second iron-uptake system exists in YSH6000, it must belong to another class. Recently, an aerobactin-mediated iron-uptake systems was found on a pathogenicity island, SHI-2, on the

chromosomes of several *S. flexneri* serotypes (Moss *et al.*, 1999; Vokes *et al.*, 1999) and a second PAI, SHI-3, was identified in representatives of all *Shigella* species (Purdy & Payne, 2001). To test whether such a system existed in YSH6000 we examined this strain for the presence of *iucA*, the first gene in the aerobactin synthesis operon. A PCR product was amplified from strains YSH6000 and YSH6000T using primers designed from the SHI-2 PAI *iucA* region (Figure 5i). It is worth noting that the *iucA* genes on SHI-2 and SHI-3 are 99% identical at nucleotide level and it is therefore not possible to determine which locus YSH6000 is carrying from this data alone. The sequence of the PCR product from YSH6000T confirmed that an *iucA* gene identical to that from the SHI-2 PAI was present in YSH6000T, and thus outside the MRDE. Therefore, it appears possible that an aerobactin locus and/or other types of iron-uptake systems may have compensated for the loss of *fec* function in SBA1415.

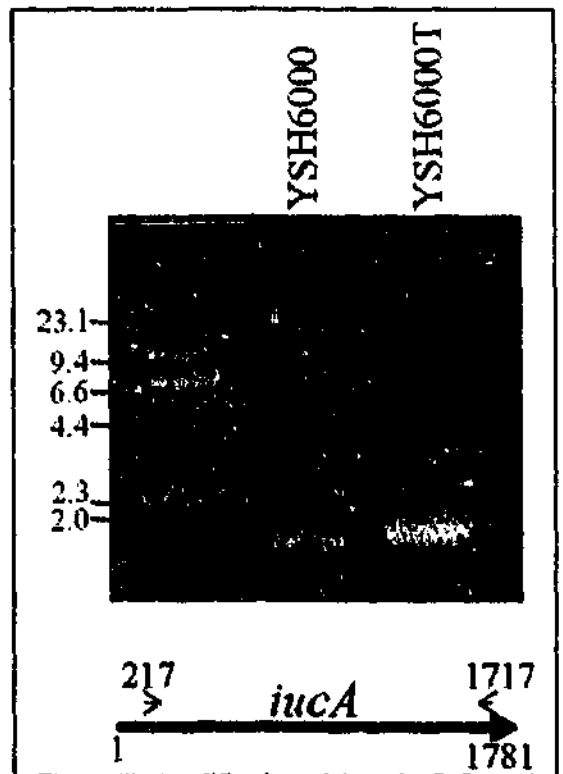

Figure 5i. Amplification of *iucA* in *S. flexneri* 2a. The position of the primers within the SHI-2 *iucA* gene are denoted by the red arrows. The position of *Hin*dIII-digested lambda DNA size markers is shown on the left.

DISCUSSION

The importance of iron to bacterial survival in the host has been well documented. It has been shown that animals on low iron diets have enhanced resistance to infection, and that this resistance is overcome by injection of sufficient iron to restore normal serum iron levels (Puschmann & Ganzoni, 1977). Creating an iron overload in animal models has been shown to increase susceptibility to infection by a variety of bacterial species including *Vibrio cholerae*, *V. vulnificus*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *E. coli* K-12 and *E. coli* 0111 (Bullen *et al.*, 1968; Field *et al.*, 1986; Fletcher & Goldstein, 1970; Ford & Hayhoe, 1976; Robins-Browne & Prpic, 1985; Wright *et al.*, 1981). The growth of many microorganisms in body fluids, cells and tissues is stimulated by excess iron. To prevent this, human hosts use an iron-withholding defence system that results in most of the serum iron being bound tightly to host proteins (Weinberg, 1993). Transferrin or lactoferrin are present in nearly all bodily fluids and act as a first line of defence against microbial invasion (Weinberg, 1993). To combat this, bacteria such as *Neisseriae*, *Haemophilus* and *Pasteurella* species produce transferrin-binding proteins that enable them to scavenge the transferrin-bound iron (Genco & Desai, 1996; Gray-Owen & Schryvers, 1996; Ogunnariwo *et al.*, 1991). To counteract this binding to transferrin the host uses its acute-phase response triggered by microbes and microbial products

such as endotoxin (Litwin & Calderwood, 1993) to induce hypoferremia, which is mediated primarily by cytokines such as IL-1 (Dinarello, 1984) and TNF-α (Beutler & Cerami, 1987). This includes an increase in the release of iron-free lactoferrin. An exchange of iron between ferric-transferrin and iron-free lactoferrin occurs, which results in ferric-lactoferrin that is rapidly cleared by macrophages, removing available iron and iron-bound transferrin, starving the bacteria of iron (Van Snick *et al.*, 1974). There is also a supression of intestinal absorption of iron to reduce the availability of free iron (Weinberg, 1993). This struggle between host and pathogen for iron clearly demonstrates its importance for infection.

IRON TRANSPORT AND MOBILE ELEMENTS

In recent times it has become common to find iron transport systems of Gram negative bacteria located on mobile elements. For example, the aerobactin iron transport system of *E. coli* K-30 is carried on a colicin plasmid (pColV), along with the genes encoding colicin V resistance (Williams, 1979; Williams & George, 1979). Interestingly, the aerobactin locus on pColV-K30 is flanked by IS*1* elements and a transposition-like event occurs that results in movement of the entire aerobactin locus (de Lorenzo *et al.*, 1988). The anguibactin iron transport system, which has been shown to have a role in survival of the marine fish pathogen, *Vibrio anguillarum*, is carried on a plasmid that is required for the high virulence phenotype of this bacteria (Crosa, 1980). The anguibactin system is closely related to the yersiniabactin iron uptake system, which is required for full *Yersinia* virulence (Bearden & Perry, 1999; Heesemann *et al.*, 1993). *Yersinia* spp. were the first found to encode an iron transport system on a pathogenicity island. The yersiniabactin locus (*irp*) is essential for virulence in *Yersinia* and is located on the High-PAI of *Y. pestis* (Buchrieser *et al.*, 1998a), *Y. enterocolitica* (Carniel *et al.*, 1996) and *Y. pseudotuberculosis* (Buchrieser *et al.*, 1998b) as well as on a related element present in a number of pathogenic and commensal *E. coli* strains (Karch *et al.*, 1999; Schubert *et al.*, 1998).

Since then, iron transport systems have been identified on several PAIs. The SitABCD system on SPI-I of *S.* Typhimurium is required for full virulence in a mouse model of disease (Janakiraman & Slauch, 2000). A putative iron transport system has also been identified on a PAI in *Streptococcus pneumoniae* (Brown *et al.*, 2001). Another putative iron transport system is present on PAI_{CFT073} of uropathogenic *E. coli* CFT073 (Guyer *et al.*, 1998). Notably, aside from *E. coli* CFT073, the cluster of genes encoding this putative iron transport system (*prrA*, *modD*, *fepC* and *yc73*) is found exclusively in *E. coli* O157:H7 strains encoding the *stx* gene, although it does not appear to be on the PAI_{CFT073} (Ye & Xu, 2001). The Shiga toxin is known to be iron-regulated, making the strict association of an iron transport system and the toxin very interesting, although a specific link between Shiga toxin and this gene cluster has not yet been shown experimentally. Recently, the genes coding

for aerobactin biosynthesis and its receptor have been identified as part of SHI-2 and SHI-3 in at least four *S. flexneri* serotypes and all four *Shigella* species, respectively (Moss *et al.*, 1999; Purdy & Payne, 2001; Vokes *et al.*, 1999). And now a *fec* locus, homologous to that in *E. coli* K-12 has been identified on a PAI in *S. flexneri* 2a. Interestingly, in *E. coli* K-12 the *fec* locus is in a region of the chromosome that shows similarity to a PAI, including the presence of an integrase, and is just downstream of the P4 insertion site, *leuX*.

The potential role of iron transport systems in virulence is enhanced by studies showing how iron regulates virulence factors in a number of bacterial species (Table 5b). Interestingly, many of the virulence factors regulated by iron are also located on mobile elements such as PAIs and bacteriophages. As iron is available in different forms in different environments, bacteria that have more than one means to take up iron would be placed at an advantage. The presence of iron transport systems on mobile elements is explained in a similar way to the presence of antibiotic resistance determinants. A bacterium's ability to survive in hostile environments, whether that is the presence of antibiotics or low iron levels is a measure of its fitness. Thus, the ability to acquire genes that aid survival of a bacterial population in these harsh environments is beneficial. Their carriage on mobile elements such as PAIs, plasmids and transposons enhances acquisition of new genes by horizontal transfer. Another example of this is the Flu antigen, discussed in Chapter 3, which aids in biofilm formation, which is beneficial to the bacterial population and has homologues on cryptic bacteriophages and PAIs. In the case of the SRL PAI there is an iron transport system, multiple antibiotic resistance determinants and a homologue of Flu encoded on a single element. Is this element therefore a "survival" island rather than a pathogenicity island because it encodes features that will aid survival in hostile environments rather than defined virulence factors?

Table 5b. Iron regulated virulence factors found in pathogenic bacteria

ORGANISM	Iron Regulated Virulence Factors
S. dysenteriae 1	Shiga toxin
Pathogenic *E. coli*	Aerobactin, Shiga-like toxin 1, α-haemolysin
Serratia marcescens	Haemolysin
Vibrio anguillarum	Anguibactin (iron transport system)
Neisseria gonorrohoeae	Transferrin binding proteins 1 & 2
Pseudomonas aeroginosa	Exotoxin A, Elastase, Alkaline Protease
Corynebacterium diphtheriae	Diphtheria toxin

DISTRIBUTION OF THE FEC *LOCUS*

The ability of *E. coli* to use sodium citrate as a growth source in strains unable to use enterobactin was first reported in 1967 by Young *et al.* As most experimental work done in *E. coli* has been performed in *E. coli* K-12 strains, which carry the ferric citrate iron transport system, the apparent rarity of the Fec iron transport system compared to that of enterobactin, has gone mostly unnoticed.

Until now, intact copies of this iron uptake system have been found only in commensal strains *E. coli* B and *E. coli* K-12, while many other *E. coli* strains tested, including pathogenic *E. coli*, do not possess the *fec* genes (Ochman, 2000). This study is the first report of a ferric citrate system in *Shigella*. It is therefore worth noting that the *fec* locus in *S. flexneri* is identical to that in *E. coli* K-12. However, genes related to *fec* genes are not confined to *E. coli* and *S. flexneri*. Sequencing of the *Pasteurella multocida* genome has revealed homologues of *fecBCDE*. This locus is missing the critical regulatory genes and the gene encoding the outer membrane receptor, however, the order and orientation of the four remaining transport genes is conserved. It is therefore possible that these genes may encode proteins that are utilised for the transport of other forms of iron, aside from ferric-citrate. *V. cholerae* strains (classical and El Tor) have a citrate dependent iron transport system, and citrate is known to stimulate vibriobactin production (Sigel *et al.*, 1985). However, there has been no report of a *fec*-related iron transport system in *V. cholerae*. Outer membrane extracts of *Klebsiella pneumoniae* strains isolated from cows with bovine mastitis react strongly with anti-FecA polyclonal serum, suggesting that they may encode a Fec system (Lin *et al.*, 1999). In *Helicobacter pylori* strain NCTC 11637 there are three homologues of the *fecA* gene as well as *orfs* that show similarity to *fecD* and *fecE*. In this strain of *H. pylori* it has been found that *feoB* is required for iron acquisition and is essential for colonisation in the mouse model (Velayudhan *et al.*, 2000). One of the *fecA* homologues in *H. pylori* is adjacent to *feoB*. It has been proposed that *feoB* is essential for the establishment of infection in the mouse stomach and that FeoB is capable of utilising $Fe^{2+}$and Fe^{3+}-citrate. Therefore, it is possible that FecA is the outer membrane receptor for ferric citrate that then interacts with FeoB. Other bacteria that have been shown to utilise an inducible ferric citrate system for iron uptake include *Vibrio anguillarum, Listeria monocytogenes*, the cyanobacterium *Synechocystis* PCC6803 and *Mycobacterium smegmatis* (Mazoy *et al.*, 1997; Adams *et al.*, 1990; Labouré & Briat, 1993; Messenger & Ratledge., 1982). It is interesting that these remnants of the *fec* locus and utilisation of ferric citrate are not confined to the *Enterobactericeae* but are observed in quite distinct bacteria that colonise distinct environments. It poses the question: is Fec an ancient iron transport system that was inherited vertically by distinct bacterial lineages or was it acquired by horizontal transfer? The G+C content of the *fec* locus (58%) when compared to the *E. coli* (50%) and *S. flexneri* (51%) genomes is suggestive of horizontal acquisition of the locus. Lateral transfer is also supported the different sites of insertion in different strains. For example, near *leuX* in *E. coli* K-12 compared to *serX* in *S. flexneri*. The IS elements flanking the *fec* locus are also indicative of horizontal transfer.

MULTIPLE IRON TRANSPORT SYSTEMS

The presence of multiple iron-transport systems on a single chromosome is not uncommon. Pathogenic Neisseriae produce outer membrane receptors that enable them to utilise haemoglobin, transferrin and lactoferrin as sources for iron independently of the TonB system required by most

Gram negative iron uptake systems (Desai *et al.*, 2000). Haemoglobin, transferrin, ferritin and ferric-citrate each up and down regulate different genes in *Pasteurella multocida*, showing that this species can utilise a number of iron sources (Paustian *et al.*, 2002). *E. coli* strains may encode up to five iron (III) transport systems (Earhart, 1996) and all clinical isolates of *Shigella* spp. carry at least one siderophore mediated iron-uptake system (Payne, 1989), highlighting the importance of iron-transport for enterobacterial survival. Siderophores utilised by *Shigella* spp. include enterobactin and aerobactin (Lawlor & Payne, 1984; Payne, 1980) and now ferric-dicitrate. Multiple iron transport systems may be important for the uptake of different forms of iron available in different environments. For example, haem uptake by *S. dysenteriae* and *E. coli* O157:H7 (Mills & Payne, 1995) strains may be important for obtaining iron from the blood during systemic infection, or for utilising iron in the blood that is lost through the colon as part of the bloody stools associated with *S. dysenteriae* or *E. coli* O157:H7 infection. In contrast, aerobactin and ferric-citrate may be important for the establishment of infection in the colon by obtaining iron from the extracellular environment. Given the importance of iron-transport systems for bacterial growth, possession of several different uptake systems may provide a competitive advantage to *Shigella* which has to survive inside and outside the host and thus be able to obtain iron from different sources. The recently sequenced *S. flexneri* 2a strain 301 genome possesses at least two iron transport systems, aerobactin and a homologue of the *S.* Typhimurium SitABCD system, but neither a Fec nor intact enterobactin system (Jin *et al.*, 2002).

The importance of multiple iron transport systems has been demonstrated in other pathogens. *V. cholerae* encodes two major iron transport systems, a vibriobactin system unique to *Vibrio* spp. and also a haem utilisation system. It has been shown that loss of vibriobactin production or transport has no effect on virulence or growth *in vivo* when tested in an animal model of infection (Sigel *et al.*, 1985). The same has been shown following mutation of the haem utilisation system (Henderson & Payne, 1993). Further analysis showed that double mutations of *hutA*, the gene encoding the outer membrane protein required for haem utilisation, and *vib* required for vibriobactin synthesis resulted in impaired survival *in vivo* (Henderson & Payne, 1994; Tashima *et al.*, 1996). In a mouse model of disease no difference was observed between the wild-type strain and single mutants at an inoculum of 10^7 (Henderson & Payne, 1994). However, a strain deficient in both haem utilisation and vibriobactin synthesis had a 230-fold reduction in its ability to colonise the intestine. This effect was dose-dependent because at 10^6 CFU a difference was observed between wild-type *V. cholerae* and single mutants in both fluid accumulation and bacterial recovery from the intestine. Interestingly, the difference was most significant in the vibriobactin and double mutants, rather than the haem utilisation mutant, suggesting that vibriobactin is the more critical of these two iron transport systems. Tashima *et al.* (1996) also showed that double mutation of the haem utilisation system and vibriobactin synthesis in *V. cholerae* resulted in significant impairment of the mutated strain when

compared with the wild-type in *in vivo* growth competition assays in rabbit ileal loops. This has some bearing on the findings of this investigation. We observed no significant difference in growth rate when comparing a wild-type *S. flexneri* 2a strain with strains lacking the ferric dicitrate iron transport system under iron-limited conditions. However, it is possible that additional mutation of the aerobactin locus would result in some impairment of growth. The effect of mutation of both known iron transport systems could then also be tested in the rabbit ileal loop model in a competition assay similar to that carried out for *V. cholerae*. It should be noted that mutation of a gene encoding a protein expressed under iron-stress conditions by *V. cholerae*, *irgA*, resulted in significant differences in the competition assay, without mutation of the haem utilisation or vibriobactin systems (Tashima *et al.*, 1996). This TonB-dependent outer membrane protein may represent a transport system for an undefined iron source and may be the most critical for *V. cholerae* iron transport. This may also be true for *S. flexneri*; hence the only way to determine if other transport systems are playing a role in *S. flexneri* iron uptake will be to mutate aerobactin and any other iron transport system that can be identified. Time constraints precluded these experiments as part of this study.

Iron transport systems in *Yersinia* spp. have been well studied. Early work showed that strains within each *Yersinia* species were capable of utilising haem but not ferric citrate as an iron source (Perry & Brubaker, 1979). The yersiniabactin locus is encoded by the HPI PAI in all highly pathogenic strains of *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* (Buchrieser *et al.*, 1998a; Fetherston *et al.*, 1992). It has been demonstrated that mutation of the yersiniabactin biosynthesis genes (*ybt*) in *Y. pestis* results in avirulence in a mouse model (Bearden *et al.*, 1997). In contrast, a YfuABC inorganic iron transport system, also found in *Y. pestis*, does not appear to be essential for growth, *in vitro* and *in vivo* (Gong *et al.*, 2001). Characterisation showed the locus was expressed under iron-deficient conditions, but mutation had no effect on the virulence of the bacterium in the mouse model. It was proposed that this system might have a role in an alternative rodent host, i.e. rat versus mouse, the flea gut or a different mammalian organ system. It has been demonstrated for *Y. enterocolitica*, that there is differential expression of the yersiniabactin receptor (FyuA) and heme-uptake receptor (HemR) depending on the bacteria's location within the host (Jacobi, 2001). For example, HemR and FyuA were produced at high levels in the peritoneal cavity, but not in the intestinal lumen or liver. Work on *S.* Typhimurium has shown that TonB-mediated iron uptake aided colonisation of the Peyer's patches and mesenteric lymph node, but an alternative, undefined system was required for colonisation of the liver and spleen (Tsolis *et al.*, 1996). A similar scenario may be true for the *fec* system in *S. flexneri* and all other bacteria that carry multiple iron transport systems. Each system may be critical in a given environment and by carrying more than one system the bacteria are better able to obtain iron in a variety of environmental conditions. For example, using the rabbit ileal loop model of shigellosis, the aerobactin locus has been shown to provide a selective advantage for growth within tissues by invasive strains of *S. flexneri* in an inoculum-dependent manner (Nassif *et al.*, 1987).

That is, wild-type strains showed greater fluid accumulation in the model than an *iuc* mutant. However, it was also shown that aerobactin synthesis was not crucial for intracellular growth or movement (Headley *et al.*, 1997). In contrast, a recent study has shown that there are several iron-regulated genes in *S. flexneri* that are specifically induced by the eukaryotic intracellular environment, including genes encoding proteins involved in other siderophore-mediated iron transport (Runyen-Janecky & Payne, 2002). Survival in an iron-limited extracellular environment is essential for *Shigella* bacteria before they gain access to sources of iron within macrophages or epithelial cells. Therefore, having numerous iron transport systems may aid in establishment of *Shigella* infection in the colon, the preferred site for *Shigella* invasion.

THE COW MODEL

The specific importance of a ferric dicitrate iron transport system may not be explained by bacterial behaviour in the human host, but rather in a bovine host. *E. coli* is one of the major causes of bovine mastitis. A study found that all isolates of *E. coli* and *Klebsiella pneumoniae* from 19 separate bovine mastitis infections expressed *fecA* (Lin *et al.*, 1999). The concentration of citrate in bovine and human milk is 7 mM and 3.7 mM, respectively (Jenness, 1974; Peaker & Linzell, 1975), high enough for the induction of the ferric dicitrate system. The citrate concentration of milk also increases up to 46 fold just prior to milk secretion in goats, cows and women (Peaker & Linzell, 1975). Early work into iron transport found that the presence of citrate in media represses iron uptake via the enterobactin pathway, suggesting a switch from Ent to Fec iron uptake (Rosenberg & G., 1974). Enterobactin is the dominant iron transport system during non-lactating periods of cows, however its role diminishes in the mammary gland as the concentration of transferrin decreases and the concentration of citrate increases (Bishop *et al.*, 1976). The growth inhibition observed following the addition of apo-lactoferrin in *in vitro* grown bovine mastitis-causing *E. coli* was reversed by the addition of citrate to the growth medium, suggesting a switch to the Fec system for the uptake of iron (Bishop *et al.*, 1976). Bacteria lacking a ferric-dicitrate transport system would be at a selective disadvantage following this switch. This is a good example of a bacterium's environment controlling which iron transport system it uses. It is possible that at some point in the *S. flexneri* life cycle it is encountering a niche of high citrate where the presence of a ferric-dicitrate iron transport gives it a selective advantage.

The use of antibiotics has not changed the incidence of bovine intramammary infections (Munch-Petersen, 1968). However, a shift away from the *Streptococcus* and *Staphylococcus* spp. and towards *E. coli*, *Klebsiella* spp. and *Aerobacter aerogenes* has been observed (Newbould, 1974). It is possible that the association of the *fec* locus with antibiotic resistance determinants on a PAI may give some indication of the origin of this PAI. *Shigella* spp. are very closely related to *E. coli* and are considered

by some to be *E. coli* subspecies (Pupo *et al.*, 1997). As such, the sharing of genetic material between these organisms should be expected, especially as they would interact in similar environments. The established role of *fec* in the pathogenesis of bovine mastitis and the use of antibiotics to increase milk production and treat bovine infections may be the selective pressure responsible for the physical linkage of the *fec* locus and resistance determinants on the same PAI. The SRL PAI *int* and the SRL cluster have been identified in strains dating back to the 1970s (Turner *et al.*, 2003). Therefore, it is worth noting that in the past streptomycin, ampicillin, chloramphenicol and tetracycline, along with other compounds, were among the most commonly used antibiotics to control mastitis (Bossuyt *et al.*, 1976). The presence and conservation of genes encoding a ferric-dicitrate iron transport system on this PAI is most likely due to the selective pressure to retain the resistance determinants; retention of the *fec* locus in *S. flexneri* may be coincidental. However, as time progresses we may find the number of enteric bacteria encoding ferric-dicitrate transport systems growing as the antibiotic resistance determinants are passed on by horizontal transfer. The retention of an intact, functional iron transport system on the SRL PAI suggests that the *fec* locus, while possibly a by-product of antibiotic resistance spread, still has a role in bacterial survival or virulence.

IN VITRO *VS* IN VIVO *CONDITIONS*

A possible explanation for the insignificant difference in growth between wild-type and *fec* mutant strains could be the assay system itself. The importance of iron-uptake systems is most likely to be apparent *in vivo* rather than *in vitro*. *S.* Typhimurium carries an iron-transport system, SitABCD as part of SPI-1 at centisome 63. SitABCD is capable of restoring growth of SAB11, an *E. coli* HB101 *ent*$^{-}$ strain incapable of growth in the absence of exogenous siderophores. However, when the *S.* Typhimurium *sit* locus was mutated no difference was observed in *in vitro* virulence phenotypes between the wild-type and mutant strains (Zhou *et al.*, 1999). In contrast, in a Balb/C mouse model a *sit* null mutant was significantly attenuated when compared to the wild-type strain (Janakiraman & Slauch, 2000), suggesting that while an importance for iron-uptake systems may not be apparent in *in vitro* assays, the importance of these systems *in vivo* or environmentally is largely undetermined. It is therefore probable that to observe a difference in growth or survival between the wild-type, SBA1366, and *fecI* mutant strain, SBA1415, an *in vivo* assay will be necessary.

The potential role of Fec in iron uptake during infection of the human host should not be overlooked. Serum has a median citrate concentration of 0.1 mM (Tomisek *et al.*, 1975) and gastric juices have a concentration of 2.5 mM (Piper *et al.*, 1967), not including the citrate present in pancreatic juices, food additives, confectionary, drinks and pharmaceuticals. Thus, the level of citrate in serum and the gut is sufficient to induce the Fec system, which is inducible at concentrations of 0.1 mM or greater

(Hussein *et al.*, 1981). This supports the hypothesis that Fec is important in a niche environment, potentially within the host, where ferric-citrate is readily available.

The importance of the potentially mobile SRL PAI and its carriage of multiple antibiotic resistance determinants (SRL) and a functional iron-transport system (Fec) is still not fully understood. However, it is interesting to consider the possibility that the linkage of these two loci confers an advantage to their host in the hostile environments it encounters.

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APPENDIX 1 – Abbreviations

A_{260}	optical density at 260 nm
Ap	ampicillin
ApR	ampicillin resistant
ApS	ampicillin sensitive
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
Cm	chloramphenicol
CmR	chloramphenicol resistant
CmS	chloramphenicol sensitive
°C	degrees Celsius
CTAB	cetyltrimethylammonium bromide (hexadecyltrimethylammonium bromide)
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dH_2O	ultra-pure water
DIG	digoxigenin
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EHEC	enterohaemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
g	grams
x *g*	relative centrifugal force, expressed as units of gravitational force
GST	glutathione S-transferase
h	hour
HTH	helix-turn-helix
IPTG	isopropyl-B-D-Thiogalactopyranoside
IS	insertion sequence(s)
kb	kilobase
kDa	kilodalton
Kn	kanamycin
KnR	kanamycin resistant
KnS	kanamycin sensitive

l	litre(s)
LB	Luria-Bertani
LPS	lipopolysaccharide
M	Molar
mA	milliamps
Mb	megabase pairs
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
mg	milligram(s)
ml	millilitre(s)
min	minute(s)
ml^{-1}	per milliltre
MRDE	multi-antibiotic resistant deletable element
mRNA	messenger RNA
NaCl	sodium chloride
ng	nanogram(s)
nm	nanometers
Orf	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PAI	pathogenicity island
PEG	polyethylene glycol
PCR	polymerase chain reaction
pH	$\log_{10}[H^+]$
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SSC	salt, sodium citrate buffer
sec	seconds
σ	sigma
SRL	*Shigella* resistance locus
sspPCR	strand-specific primer polymerase chain reaction
Str	streptomycin
StrR	streptomycin resistant
StrS	streptomycin sensitive
STX	sulfamethoxazole-trimethoprim

TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramthylethylenediamine
Tet	tetracycline
TetR	tetracycline resistant
TetS	tetracycline sensitive
U	units
UV	ultrviolet
V	volts
v/v	volumer per volume
w/v	weight per volume

APPENDIX 2 – Solutions and Buffers

Media

Luria-Bertani (LB) (Ausubel *et al.*, 1995)

Tryptone (Oxoid)	4 g
Yeast Extract (Oxoid)	2 g
NaCl	2 g
dH_2O	up to 400 ml

- For LB Agar add 4.5 g of Bacteriological Agar (Oxoid)
- Autoclave

2YT (Miller, 1972)

Tryptone (Oxoid)	6.4 g
Yeast Extract (Oxoid)	4 g
NaCl	2 g
dH_2O	up to 400 ml

- For LB Agar add 4.5 g of Bacteriological Agar (Oxoid)
- Autoclave

Fec Media (Ochs *et al.*, 1995)

Tryptone (Oxoid)	4 g
Yeast Extract (Oxoid)	2 g
NaCl	2 g
dH_2O	up to 400 ml

- Autoclave
- Add 800 µl of 100 mM 2,2'-dipyridyl (Sigma) and 800 µl of filter sterilised 0.5 M citrate.

SOB Broth

Tryptone	2 g
Yeast Extract	0.5 g
4 M NaCl	256 µl
1 M KCl	248 µl
dH_2O	make up to 100ml

- Autoclave

SOC Broth

To 2 ml SOB broth add 40 µl sterile 10% glucose and 20 µl sterile 1 M $MgCl_2/MgSO_4$.

Protein Analysis

PBS (x10)

NaCl	80 g
K_2HPO_4	12.1 g
KH_2PO_4	3.4 g

- Make up to 1 litre with dH_2O and adjust pH to 7.2

Resolving Gel Buffer (pH 8.8)

Tris	18.17 g
Na.dodecyl (lauryl) sulfate	0.4g

- Add 70 ml dH_2O, then adjust pH to 8.8 with concentrated HCl, then make up to 100 ml with dH_2O.

12.5% Acrylamide Gel

Resolving Gel	*1 Gel*
Resolving Buffer	1.88 ml
30% Acrylamide	3.13 ml
dH_2O	2.5 ml
10% Ammonium persulfate	25 µl
TEMED (Sigma)	5 µl

Stacking Gel	
Stacking Buffer (0.5M Tris, 0.4% SDS)	0.76 ml
30% Acrylamide	0.4 ml
dH_2O	1.8 ml
10% Ammonium persulfate	15 µl
TEMED (Sigma)	5 µl

Sample buffer (x2)

0.5 M Tris-HCl, pH 6.8 with 2% SDS	4 ml
Glycerol	2 ml
10% SDS	1.7 ml
2-mercaptoethanol	1 ml
0.1% aqueous bromophenol blue (0.1g/ml)	1.3 ml

- Aliquot into 1 ml volumes and store at -20°C

PAGE Running Buffer (x5)

Tris	15 g
Glycine	72 g
SDS	5g

- Make up to 1 litre with dH_2O. Do not pH.

Coomassie Brillant Blue Stain

Coomassie	1 g
Methanol	125 ml
Acetic Acid	35 ml
dH_2O	340 ml

- Weigh out dry stain and dissolve in methanol, then add water and acetic acid.
- Filter after dissolving.

TBS-T

Tris	0.606 g
NaCl	0.877 g

- Make up to 70 ml with dH_2O and pH to 7.0 with concentrated HCl.
- Make up to 100 ml volume.
- Add 50 µl of Tween-20 (Sigma) for a final concentration of 0.05%

Other Solutions

TAE (Tris, Acetate, EDTA) (x50)

Tris	484 g
Glacial Acetic Acid	114.2 ml
Na_2EDTA	74.4 g

- Make up to 2 L with dH_2O. Do not pH.
- Use at a 1/50 dilution

Solution 1

Glucose	0.9 g
Tris-Base	0.33 g
EDTA	0.37 g
dH_2O	make up to 100 ml

- Autoclave

Solution 2

1 M NaOH	1 ml
10% SDS	0.5 ml
dH_2O	3.5 ml

- Mix dH_2O and SDS, then add NaOH. Prepare just before use.

Solution 3

3 M KAc

Autoclave

CTAB/NaCl

CTAB	4.1 g
NaCl	10 g
dH_2O	make up to 100 ml

- Heat to 65°C to dissolve

Stop Mix

Sucrose	50 g
$Na_2EDTA.2H_2O$	3.7 g
Bromophenol Blue	0.05 g

- Dissolve in 50 ml H_2O (may need to be heated)
- pH to 7.0 with NaOH
- Adjust volume to 100 ml
- Autoclave at 108°C for 40 min.
- Store at 4°C or RT

Southern Hybridisation

Depurination Solution (0.25 M HCl)

20.8 ml concentrated HCl
dH_2O up to 1 litre

Denaturation Solution (0.5 M NaOH, 1.5 M NaCl)

87.7 g NaCl
20 g NaOH
dH_2O up to 1 litre

Neutralisation Solution (1.0 M Tris-HCl, pH 7.0, 1.5 M NaCl)

157.6 g Tris-HCl
87.7 g NaCl
dH_2O up to 1 litre

20 x SSC (3 M NaCl, 0.3 M sodium citrate)

174g NaCl
88.2 g sodium citrate
- pH to 7.0 (+20°C), autoclave

Buffer 1 (Maleic Acid)

0.1 M Maleic Acid
0.15 M NaCl
- pH to 7.5 (+20°C) (pH with concentrated NaOH), Autoclave

Buffer 3

100 mM Tris-HCl, pH 9.5 (+20°C)

100 mM NaCl

Standard Hybridisation Buffer

5 x SSC

0.1% N-lauroylsarcosine

0.02% SDS

1% Skim Milk Powder

High-SDS Concentration Hybridisation Buffer

Add in the following order:

100% deionised formamide	250 ml
30 x SSC	83 ml
1 M sodium phosphate, pH 7.0	25 ml
10% Blocking Solutions (Roche)	100 ml
10% N-lauroylsarcosine	5 ml

Pour solution into a large flask and add 35 g of SDS. Heat the solution while stirring to dissolve the SDS, then fill to 500 ml volume with autoclaved dH_2O. Store solution at +20°C and heat to 65°C for use.

RNA

DEPC treated dH_2O

Autoclave dH_2O and in a fumehood with gloves add DEPC (diethylpyrocarbonate) to a final concentration of 0.1% w/v. Shake well. Remove lid and leave open in operating fumehood overnight. Autoclave.

Appendix 3 – Oligonucleotides

BAP	Sequence (5'-3')	Purpose
476	GGCAGCGTCACAATCCAC	To sequence SRL PAI and for inverse PCR
486	GACAGCACCGCCCATACG	To sequence SRL PAI
513	TTTTCTGATTCGTTTGGTGG	To sequence SRL PAI downstream of the *fec* locus
514	GCGCTGCTGACCCGACTGG	To sequence *fecE* on the SRL PAI
526	CCGTTCCTTGGTGCTTATTC	To sequence SRL PAI
527	CATTAATAACAGTGCGCCGG	To sequence SRL PAI
550	CATGGGCGGATTATGTGATG	To sequence SRL PAI
551	CGTAGGCTGGCGGGCATC	To sequence SRL PAI
562	AAAAGGGAGAAGGACGGG	To sequence *fec* locus (*fecE*)
563	GCTCACCAGACGCGTAAAC	To sequence SRL PAI
564	CAGCGCCCTTCTGGTAGC	To sequence SRL PAI
565	GCCCGCAGACCTTCGCCG	To sequence *fec* locus
602	GCTGCATAGCGGTCCGAC	To sequence SRL PAI
603	ACCCTGAAGTTTTGCCCG	To sequence *fec* locus
604	AGCCCCAGTACGCCTTGG	To sequence SRL PAI
605	TGTGGCGTAGTGTTGGCG	To sequence SRL PAI
606	GGTTTGGGTTCTTACGGGC	To sequence *fec* locus
607	GGAAACAGCTATGACCATG	Reverse Primer
608	AATTAACCCTCACTAAAGGG	T3 Primer
609	TCGAGGTCGACGGTATC	KS Primer
610	CGCTCTAGAACTAGTGGATC	SK Primer
611	GTAATACGACTCACTATAGGGC	T7 Primer
612	GTA AAA CGA CGG CCA GT	Universal Primer (M13-20 Primer)
636	CCCTGCTGTGGCTGACCG	To sequence *fec* locus
637	AAATTATACAGCACCAGGGC	To sequence SRL PAI
638	AAGCGGATGGACGATGAG	To sequence *fec* locus
678	TGTGTTGTTCTGAGGTGAGC	To sequence SRL PAI
679	GTGCTGCTTTCGGTGTGC	To sequence SRL PAI
680	CAACATAACGCTTTTCGGG	To sequence SRL PAI
688	TGTATTTCCGTTGGGGGC	To sequence SRL PAI
689	CCGGGCAGTACGTGCAGC	To sequence SRL PAI (*int*)
694	TTTTTCATCGTTGCTGGC	To sequence SRL PAI
914	GCTCCCATTTCGCTCGGC	To sequence *fec* locus
915	AGGCGATGTAAACCCCCG	To sequence *fec* locus
916	CCCATCAACCGCATCAGG	To sequence *fec* locus
917	CCCTGCTGTGGCTGACCG	To sequence *fec* locus
934	CTACACCCAAACCCTGCG	To sequence *fec* locus
935	GTTGTCGTCATAAGAGCGG	To sequence *fec* locus
936	AGACAAACCACGGCGCAC	To sequence *fec* locus
978	CTCGGGTTACTGTTGTGCC	To sequence SRL PAI
1004	AATGGACGAACAGTGGGG	To sequence SRL PAI
1005	GCTGGATTGGGAACTTACC	To sequence SRL PAI (*int*)
1012	TGGGCGGATTATGTGATG	To sequence SRL PAI (*int*)
1013	GCATAAACCAGCCATTGAG	To sequence SRL PAI
1037	GCCGCGTGTCCACTATTG	To sequence SRL PAI
1038	ATCAAGTTTCTCAGCCCG	To sequence SRL PAI
1049	GCCAACCAAAGCGAGAAC	To sequence SRL PAI
1082	CACGGACTCATTGGCACC	To sequence SRL PAI
1083	CGGAAAAGAGGATTGCGG	To sequence MRDE
1102	ACATGGTTACGCTTTGGGG	To sequence SRL PAI
1105	GGAGCGCCTTCACCTGAG	To determine orientation of pKIXX cassette in *fecI*
1173	ATTAACGACAGAAATCCTGCC	To sequence SRL PAI
1174	CGGGGATGTGTTATTCAGC	To sequence SRL PAI and for inverse PCR
1247	AAAAAAGGATCCCCCATTTCCG CCGTCAGC	To clone *fecI::kan* from pSBA565 into pCACTUS *Bam*HI site
1248	AAAAAAGGATCCTTACCGAAAT CCCCATCC	To clone *fecI::kan* from pSBA565 into pCACTUS *Bam*HI site
1290	GCCATGAGGGCGTTACAC	To sequence right flank of PAI
1291	TGGTCACCCCCGTTTTTG	To sequence right flank of PAI
1310	CCGGTCCCATCTCACCAG	To sequence SRL PAI
1311	GGTCACGCCAGATTTCGC	To sequence SRL PAI

BAP	Sequence (5'-3')	Purpose
1327	TACGCCCACAACCGCCCG	To sequence SRL PAI
1328	ATAGCAGCTTTCCCTCGC	For sspPCR on SRL PAI
1338	ATTTGTGCGATACGGCGG	To sequence SRL PAI
1347	CGGGAGTTTGGGGAGTGC	To sequence SRL PAI
1352	ACCATCCGCAATACGAAAC	To sequence SRL PAI
1353	ACGGGTAAGGCTGTGAGG	For sspPCR on SRL PAI
1359	TGGCACACATCACGGCTG	*fecI* (forward primer)
1360	CCAGACGGAACAGCAGGC	*fecI* (reverse primer)
1362	TCGCTGGCGATGGTACAG	To sequence SRL PAI
1363	GGTTGCGAATGTCAGGGC	To sequence SRL PAI
1364	CCTTGCTGTTCTTCTACGGC	To sequence SRL PAI
1365	CGGCAACTGGAAGGCGTC	To sequence SRL PAI
1366	TGGTGCTCGGTTCATTGG	To sequence SRL PAI
1367	GCGAGCACCTGAGCGAGC	To sequence SRL PAI
1368	GCCTCAAGCAGTCCAGCG	To sequence SRL PAI
1369	TCAATGGCTAAGGCGTCG	To sequence SRL PAI
1370	CGATCTTGGCGCTGCTGC	To sequence SRL PAI
1371	CCGCCAGTATGTCAAGTGC	To sequence SRL PAI
1372	AGTGGTTGGTGAGAGAAGGAC	To sequence SRL PAI
1373	CAGCGCCAACAACATCGG	To sequence SRL PAI
1374	ATTCGATCACGGATTGGG	To sequence SRL PAI
1375	TAATCTTTCGCAACCCGC	To sequence SRL PAI
1376	CTGTGTGAGTGGAAAAGCG	To sequence SRL PAI
1377	AAGGAGTGGCATCGTTAGC	To sequence SRL PAI
1378	GAACATGCAACCCATCAATC	To sequence SRL PAI
1379	GGCACACAAGCGGAAGAG	To sequence SRL PAI
1387	TGGTGTGTGGGTATGTTCTG	To sequence SRL PAI
1388	AACACGGTATTATTCTGGGC	To sequence SRL PAI
1389	GTTCGGACATAAGCCCCC	To sequence SRL PAI
1390	ATATTTCGGGTTACGCCG	To sequence SRL PAI
1391	CGTTCTGGCGCGGGTAGC	To sequence SRL PAI
1392	ACGTCATCAAGCAAACCTG	To sequence SRL PAI
1393	CAGCTCCGCAAGGGTAAG	To sequence SRL PAI
1415	GGCGAACAAACGATGCTC	To sequence SRL PAI
1416	CCGGACTCCAAGGACGAC	To sequence SRL PAI
1417	TAGCGTGGCATTGGTGGC	To sequence SRL PAI
1418	AGACGGACATCATTCAGCC	To sequence SRL PAI
1419	TAGCTTACTCTGGCAAATCC	To sequence SRL PAI
1420	CATAGAAGAGGCAGTCAACGC	To sequence SRL PAI
1421	GAATATCTCCTCGGCAGCC	To sequence SRL PAI
1439	GGGGCTTACCGTGGCAGG	To sequence SRL PAI
1440	CCTGGCGGCACAACCTAC	To sequence SRL PAI
1441	TCGTCAGGCATTGGCGGC	To sequence SRL PAI
1444	CTGGCATACCGCACAACG	To sequence SRL PAI
1450	TCCTGTCCGCCGCCGAGC	To sequence SRL PAI
1451	TTGAAGACTGGAAAGAGAACCT GAAACGG	For long-range PCR amplification to join left and right ends of SRL PAI.
1452	TTCATGCCCAAAATCAGAGATA CAGCG	For long-range PCR amplification to join left and right ends of SRL PAI.
1464	TAGGCCGTTGCTTTCAGG	To sequence SRL PAI
1480	TGCATTTGCCTTTTTAGCG	To sequence SRL PAI
1495	CCCGGCACTGTGGAAGAG	To sequence SRL PAI
1505	CCACACCGACAAACGAGG	To sequence SRL PAI
1529	GTATCCAGTTCCCAGCCG	To sequence SRL PAI
1530	CGACCATGCCGCCAGTCC	For sspPCR and sequencing of SRL PAI
1531	TTATGATCCCCCGGAACG	To sequence SRL PAI
1552	ATGCCAATCACGGGTTCG	To sequence SRL PAI
1560	TCCTCCTGTTTCTCTTGCG	To sequence SRL PAI
1591	CCGTTGCGGGAATTTGGC	To sequence SRL PAI
1593	TTCCCCTCTCTCATCCGC	To sequence SRL PAI
1595	ACCAGACGGTACACGGGC	To sequence SRL PAI
1618	CCGGAATTCTAGCTTACTCTGGC AAATCC	To directionally clone *vis* gene from SRL PAI (*Eco*RI)
1619	GCGCGGATCCTCGGATGACACA CGCCCC	To directionally clone *vis* gene from SRL PAI (*Bam*HI)

BAP	Sequence (5'-3')	Purpose
1620	CCGGAATTCCTCTTCCGCTTGTG TGCC	To directionally clone *alpA* gene of SRL PAI (*Eco*RI)
1621	GCGCGGATCCCAGCAGCAGCAT TTTCCG	To directionally clone *alpA* gene of SRL PAI (*Bam*HI)
1622	CCCCTAACCAACCGAACC	To sequence SRL PAI
1623	GCCGGTTACCTTCCCTTCCTCCT GC	For long-range PCR amplification to join left and right ends of SRL PAI.
1624	ATTCCTTTGCCGAGAGCG	To sequence SRL PAI
1639	CGGCTGGTGGGTCTTTGC	To sequence *fec* locus
1643	CTACGCACGTAAACTGGGCG	*S.flexneri recA* gene (forward primer)
1644	ACCGGTAGTGGTTTCCGGG	*S.flexneri recA* gene (reverse primer)
1648	GGAAGGGGGACCAGATGC	To sequence SRL PAI
1655	TGACACACGAAGAACTGATGC	To sequence SRL PAI
1656	CGCTGCTGCCGCTGACGC	To sequence SRL PAI
1657	ATGCCTGCCGGGTTTACC	To sequence SRL PAI
1658	GTTTTTGACAGCCCCAGG	To sequence SRL PAI
1659	ATATCGTTGTGGCTGCCC	To sequence SRL PAI
1660	ATGGGGACAGCCGCCGCC	To sequence SRL PAI
1661	CTTTAACCCGTCACAACCAC	To sequence SRL PAI
1662	CCGGGCAGAAAGGAAAGC	To sequence SRL PAI
1663	AAAACGGATTCAGCAGCAC	To sequence SRL PAI
1664	AGATGGTGACGGGAGACG	To sequence SRL PAI
1678	TCTGGCTTGTGAGGAGGC	To sequence *fec* locus
1688	GGGGGAAGTCTGAGTGGC	To sequence SRL PAI
1689	AGACACGGGGTTCCAGGG	To sequence SRL PAI
1690	GCCGTTGTTATCGTGACCG	To sequence SRL PAI
1691	GGCGCTGGCACAGGCGGC	To sequence SRL PAI
1796	GCGCGGATCCCGACATGCAATG CATATGGAG	To amplify *rox* for in-frame cloning into pGEX-2T (forward primer)
1797	CCGGAATTCCAGCAGCAGCATT TTCCG	To amplify *rox* for in-frame cloning into pGEX-2T (reverse primer)
1833	ATTTCTCCCCAACCCAGC	*S. flexneri iucA* (aerobactin) (forward primer)
1834	CCGAAGGGTCAACAATGG	*S. flexneri iucA* (aerobactin) (reverse primer)
1875	CAGTTACCGCTTTTTGTGGC	To sequence SRL PAI
1940	CCGGGCAGTACGTGCAGC	For Rox gel shift assay (reverse primer) (Fragments C & D)
1941	TTCCCGTCACTCTGGTATGC	For Rox gel shift assay (reverse primer) (Fragments B & E)
1942	ATTTATTTCAGGCGGTCCC	For Rox gel shift assay (reverse primer) (Fragments A)
1943	ACGTGGGCGGGGGCACCC	For Rox gel shift assay (forward primer) (Fragments A & G)
1944	TAAATATGGCGGTGAGGGG	For Rox gel shift assay (forward primer) (Fragments B & C)
1945	TGAAGCGAGGGACTAATGG	For Rox gel shift assay (forward primer) (Fragment D)
2048	TGACTTAATCCAGGGGTG	For Rox gel shift assay (forward primer) (Fragment E)
2049	GGTGAGGGGGACCGCCTGAAAT AAATCTGACTTAA	For Rox gel shift assay (forward primer) (Fragment F)
2050	TTAAGTCAGATTTATTTCAGGCG GTCCCCCTCACC	For Rox gel shift assay (reverse primer) (Fragment F)
2153	CCCGACTGCTGGGTATATCAA	For Real Time PCR *rox* (forward primer)
2154	CAAGCCTTATTGGTTTGGGAAA	For Real Time PCR *rox* (reverse primer)
2191	GATGGGAGAATCCAAAGCA	For Real-time PCR *int* (forward primer)
2192	CCGTAATCGAATCCAGCGTTT	For Real-time PCR *int* (reverse primer)
2193	AGCCCGTACTTTCGTACG	To clone *int* upstream fragment for mutagenesis (forward primer)
2194	ATAACAACATGAATGCGCTG	To clone *int* upstream fragment for mutagenesis (forward primer)
2195	CAATGTATTACACTTTTTGTTGT GAATATGTTATCTAATGTATTCT TAGTGAAGCGAGGGAC	For site-directed mutagenesis of Rox potential binding site (forward primer)
2196	GTCCCTCGCTTCACTAAGAATAC ATTAGATAACATATTCCAACAA AAAGTGTAATACATTG	For site-directed mutagenesis of Rox potential binding site (reverse primer)
2217	GCGTCGGCGGCACATACC	To sequence SRL PAI
2307	TCTCCACCGAAAAAGACGGTAT	Real Time PCR – *S. flexneri gyrB* (forward primer)
2308	CGCTGCGGAATGTTGTTG	Real Time PCR – *S. flexneri gyrB* (reverse primer)

APPENDIX 4 - PCR Cycles and Reactions

Standard* Taq *PCR

DNA	20-100 ng
Oligo I	approx 15 ng
Oligo I	approx 15 ng
2.5 mM dNTPs	0.5 µl
PCR Buffer (Roche)	2.5 µl
Taq DNA polymerase (Roche)	0.1 µl (0.5 U)
dH_2O	up to 25 µl
	25µl

Standard cycle for 0.5 - 1.5 kb DNA fragment

92°C	-	2 min	
92°C	-	44 sec	
60°C	-	40 sec	30 cycles
70°C	-	1 min	
70°C	-	7 min	

Expand™ High Fidelity PCR

Mix 1		*Mix 2*	
10 mM dNTP	4 µl	Expand ™ Buffer (Roche)	5 µl
Template (0.1-0.75 µg)	2 µl	Expand™ Enzyme	0.37 µl
Oligo I (approx 30 ng)	2 µl	dH_2O	19.63 µl
Oligo II(approx 30 ng)	2 µl		25 µl
dH_2O	15 µl		
	25 µl		

Each mix was made separately and then combined immediately prior to PCR cycling. Fragments below 6 kb were amplified as described for 'Standard *Taq* PCR', while fragments larger than 6 kb were amplified using the cycle below (extension time varied depending on fragment length:

PCR Cycle

94°C	-	2 min		
92°C	-	10 sec		
63°C	-	30 sec		10 cycles
68°C	-	3 to 8 min		
92°C	-	10 sec		
63°C	-	30 sec		15 cycles
68°C	-	3 to 8 min	+ 5 sec/cycle	
72°C	-	7 min		

Long Range Expand™ PCR

Mix 1		*Mix 2*	
10 mM dNTP	3 µl	Buffer 3 (Roche)	5 µl
YSH6000 genomic DNA	3 µl	Expand™ Enzyme	0.75 µl
Oligo I (approx 300 ng)	2 µl	dH_2O	19.25 µl
Oligo I (approx 300 ng)	2 µl		25 µl
dH_2O	15 µl		
	25 µl		

Each mix was made separately and then combined immediately prior to PCR cycling.

PCR Cycle

94°C	-	2 min		
92°C	-	10 sec		
63°C	-	30 sec		10 cycles
68°C	-	20 min		
92°C	-	10 sec		
63°C	-	30 sec		15 cycles
68°C	-	20 min	+ 10 sec/cycle	
72°C	-	7 min		

Inverse PCR

Reaction Mix

Clean Ligation Mix	2 µl
Oligo I (approx 50 ng)	3 µl
Oligo I (approx 50 ng)	3 µl
2.5 mM dNTPs	4 µl
PCR Buffer (Roche)	10 µl
Taq DNA polymerase (Roche)	0.4 µl (2U)
dH_2O	27.6 µl
	50 µl

PCR Cycle

94°C	-	2 min	
94°C	-	15 sec	
55°C	-	30 sec	10 cycles
68°C	-	2 min 30 sec	
94°C	-	15 sec	
55°C	-	30 sec	15 cycles
68°C	-	2 min 30 sec + 5 sec/cycle	
72°C	-	7 min	

Single-specific primer PCR (sspPCR)

Reaction Mix

Template	1 µl
Specific oligonucleotide (approx 15 ng)	1 µl
T3 (approx 48 ng)	3.1 µl
2.5 mM dNTPs	0.5 µl
PCR Buffer (Roche)	2.5 µl
Taq DNA polymerase	0.2 µl (1 U)
dH_2O	16.7 µl
	25 µl

PCR cycle was performed as per 'Standard *Taq* PCR', with a 1 min 30 sec extension time.

KOD Polymerase

Reaction Mix

Buffer (Novagen)	5 µl
2mM dNTPs (Novagen)	5 µl
$MgSO_4$ (Novagen)	2 µl
Plasmid DNA	1 µl (0.006-6 ng plasmid/up to 12 ng genomic)
Oligo I	5 pmol/µl, final concentration 0.3 µM
Oligo II	5 pmol/µl, final concentration 0.3 µM
KOD Polymerase	1.25 µl
dH_2O	make up to 50 µl
	50 µl

94°C	-	2 min	
94°C	-	15 sec	
58°C	-	30 sec	30 cycles (16 cycles for mutagenesis)
68°C	-	6 min 30 sec	

Addendum

List of references cited in Tables 1, 2 and 3a not included in the bibliography:

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