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# Genetics of the SRL

# Pathogenicity Island of *Shigella*

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# SUMMARY

*Shigella* spp. are the causative agents of bacillary dysentery which is responsible for more than 1.1 million deaths every year. Antibiotic therapy remains one of the most effective treatments known for bacillary dysentery, although rising levels of multiple antibiotic resistance make treatment complicated. Multiple antibiotic resistance determinants were originally identified on mobile resistance plasmids, but although chromosomally borne resistance determinants have been reported in *Shigella*, the basis for this resistance has not been widely investigated.

Previous work with *Shigella flexneri* 2a strain YSH6000 identified a chromosomal multi-antibiotic resistance locus, which encodes resistance to streptomycin (Sm), ampicillin (Ap), chloramphenicol (Cm) and tetracycline (Tc). This locus, the *Shigella* resistance locus (SRL), was originally reported to reside on a 99 kb deletable element which was thought to delete from the chromosome via flanking IS elements. This element has since been designated the multiple-antibiotic resistance deletable element (MRDE). However, subsequent sequencing data indicated that the MRDE contained a distinct element of 66 kb in length inserted into the *serX* tRNA gene, designated the SRL pathogenicity island (PAI). This work has revealed the presence of a set of three nested, deletable elements on the YSH6000 chromosome: 1) the SRL, which resides on the SRL PAI; 2) the SRL PAI itself; and 3) the larger MRDE, which contains entirely the other two elements.

The present work determined the boundaries of the MRDE in *S. flexneri* 2a strain YSH6000 which was found to be flanked by two identical IS*91* elements. Sequence data from YSH6000-derived MRDE deletants revealed that the deletion occurred between the two flanking IS*91* elements. Selection for the loss of antibiotic resistance form YSH6000 revealed that the SRL, which is flanked by two IS*1* elements, was also able to undergo independent deletion, leaving a single IS*1* element on the chromosome. Additionally, the SRL PAI was also shown to delete through the recombination of flanking 14 bp direct repeats.

Many PAIs are flanked by direct repeats, insert adjacent to tRNA genes, and contain phage-related integrase genes. Therefore, it has been suggested that PAIs may be mobilised by mechanisms resembling the site-specific recombination of lambdoid phages. Such recombination events are integrase-mediated and lead to the formation of circular intermediates. Mutation of a P4-like integrase gene (*int*) at the left end of the SRL PAI revealed that *int* mediates precise deletion of the PAI through site-specific recombination of the two flanking 14 bp direct repeats. Following this, a stabilised extra-chromosomal circular form of the deleted SRL PAI was detected. Amplification of the junction (*attP* attachment site) of the SRL PAI circular intermediate was carried out, and the *attP* was then shown to be capable of RecA-independent, site-specific *int*-mediated integration into the bacterial tRNA *attB* sites *serX* and *serW*. Thus both deletion and integration of the SRL PAI are similar to site-specific recombination of lambdoid phages, suggesting that the SRL PAI may be mobile.

Distribution of the SRL was therefore investigated and, although absent from other enteric pathogens, the SRL and SRL-related loci were present in a number of clinical *Shigella* isolates. SRL PAI markers were also present in the majority of strains carrying the SRL and SRL-related loci. PCR linkage studies on representative strains demonstrated that the SRL is carried on elements similar in structure and organisation to the YSH6000 SRL PAI, consistent with the hypothesis that the SRL PAI is involved in the spread of multiple-antibiotic resistance in *Shigella*.

# STATEMENT

I, Sally Turner, 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.

(Sally Turner)

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# PUBLICATIONS

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Luck, S. N. **Turner, S. A.**, Rajakumar, K., Sakellaris, H., & Adler, B. (2001). Ferric Dicitrate Transport System (Fec) of *Shigella flexneri* 2a YSH6000 Is Encoded on a Novel Pathogenicity Island Carrying Multiple Antibiotic Resistance Genes. *Infection and Immunity* 69, 6012 – 6021.

**Turner, S. A.**, Luck, S. N., Sakellaris, H., Rajakumar, K. & Adler, B. (2003). Molecular Epidemiology of the SRL Pathogenicity Island. *Antimicrobial Agents and Chemotherapy* 47, 727 – 734.

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# CONFERENCE PROCEEDINGS

Luck, S., **Turner, S.**, Rajakumar, K. & Adler, B. (1999). A 99 kb Deletable Element of *S. flexneri* 2a. *5th Australian Conference on Molecular Analysis of Bacterial Pathogenesis, Victor Harbour, South Australia, Australia.*

**Turner, S.** & Adler, B. (1999). Deletion Events of the MRDE of *Shigella flexneri* 2a YSH6000. *Bacterial Pathogenesis Conference, Department of Microbiology, Monash University, Victoria, Australia.*

**Turner, S. A.**, Luck, S. N., Rajakumar, K., Sakeliaris, H. & Adler, B. (2000). Deletion Events of a Novel *Shigella* Pathogenicity Island. *3rd Louis Pasteur Conference on Evolution of Pathogens and Their Hosts, Institut Pasteur, Paris, France.*

**Turner, S. A.**, Luck, S. N., Rajakumar, K., Sakellaris, H. & Adler, B. (2000). Deletion Events of a Novel *Shigella* Pathogenicity Island. *International Conference on Bacterial and Viral Virulence Factors, Smolenice, Slovakia.*

Luck, S. N., **Turner, S. A.**, Rajakumar, K., Sakellaris, H. & Adler, B. (2000). A Novel Pathogenicity Island of *Shigella flexneri* 2a. *3rd Louis Pasteur Conference on Evolution of Pathogens and Their Hosts, Institut Pasteur, Paris, France.*

Luck, S. N., **Turner, S. A.**, Rajakumar, K., Sakellaris, H. & Adler, B. (2000). A Novel Pathogenicity Island of *Shigella flexneri* 2a. *International Conference on Bacterial and Viral Virulence Factors, Smolenice, Slovakia.*

**Turner, S. A.**, Luck, S. N., Sakellaris, H., Rajakumar, K. & Adler, B. (2001). Deletion Events of a Novel *Shigella* Pathogenicity Island. *6th Australian Conference on Molecular Analysis of Bacterial Pathogenesis, Marysville, Victoria, Australia.*

Luck, S. N., **Turner, S. A.**, Sakellaris, H., Rajakumar, K. & Adler, B. (2001). The SRL Pathogenicity Island of *Shigella flexneri* 2a. *6th Australian Conference on Molecular Analysis of Bacterial Pathogenesis, Marysville, Victoria, Australia.*

Sakellaris, H., Al-Hasani, K., Luck, S., **Turner, S.** & Adler, B. (2001). Mobility of Adhesin and Virulence Genes Carried on the *she* Pathogenicity Island of *Shigella flexneri* 2a. *Gordon Research Conference on Molecular Mechanisms of Microbial Adhesion, Rhode Island, USA.*

**Turner. S. A.**, Luck, S. N., Rajakumar, K., Sakellaris, H., & Adler, B. (2002). Molecular Epidemiology of the SRL Pathogenicity Island in *Shigella*. *Bacterial Pathogenesis Conference, Department of Microbiology, Monash University, Victoria, Australia.*

**Turner, S. A.**, Luck, S. N., Sakellaris, H., & Adler, B. (2003). Integrative module of the SRL Pathogenicity Island. *7th Australian Conference on Molecular Analysis of Bacterial Pathogenesis, Jamberoo, New South Wales, Australia.*

# CHAPTER ONE

## Introduction

# *SHIGELLA*

## Historical Background

Dysentery has long been recognised as one of the most pervasive and important diseases throughout human history. Early reports of dysentery date as far back as the Ebers papyrus of Egypt (1500BC), and a typical dysentery epidemic of the Persian army is described by Herodotus as early as 480 BC (Ackerknecht, 1965). Perhaps one of the greatest historical considerations is the influence that bacillary dysentery has had on military campaigns. From Thucydides in the fifth century to the more recent battles of World War II (Keusch & Bennish, 1991), the mention of dysentery accompanies the descriptions of nearly every war, and it is not uncommon for deaths ascribed to dysentery and other fluxes to out number deaths in action for any given battle (McGrew, 1985).

Dysentery may be divided into two categories on the basis of causative organism: amoebic and bacillary. While the difference can easily be determined with a microscope, it was not until the late 1800s that a distinction between the two was made. In 1859, Lambl first described the amoeba of amoebic dysentery (Ackerknecht, 1965), and work by Losch (1875), Councilman and Lafleur (1891) and Kruse and Pasquale (1893) later described experimental infection and pathogenesis of *Entamoeba histolytica*, proving it to be the causative agent of amoebic dysentery (Bensted, 1956). In 1888, Chantemesse and Widal described an organism that they claimed to be the causative agent of non-amoebic dysentery, but their study lacked convincing description. In 1898 Dr Kiyoshi Shiga, while investigating an epidemic of dysentery in Japan, isolated a bacterium from a patient's stools and demonstrated conclusively that it was responsible for non-amoebic dysentery. Shiga named the organism *Bacillus dysenteriae* (Bensted, 1956). Comprehensive studies carried out by Flexner, Kruse and other investigators not only confirmed Shiga's original findings, but lead to the identification of several other similar, but serologically distinct species (Keusch & Bennish, 1991). In 1950, following a recommendation by Ewing (1949) and in honour of Kiyoshi Shiga, the Shigella Commission of the Congress of the

International Association of Microbiologists proposed the adoption of the generic name *Shigella* for these species (Bensted, 1956).

## Taxonomy

Shigellae are members of the family *Enterobacteriaceae*, tribe *Escherichiae* (DuPont, 1990). The *Enterobacteriaciae* are Gram negative straight rods that are non-sporing. They are motile by peritrichous flagella or non-motile, grow both aerobically and anaerobically, and ferment glucose often with the production of gas. Almost all reduce nitrate to nitrite and have a 38 to 60% guanine-plus-cytosine (G + C) content of DNA (Brenner, 1984). The two genera within the tribe Escherichiae, *Escherichia* and *Shigella*, share more than 80 % nucleotide similarity (Brenner *et al.*, 1969; Brenner *et al.*, 1972) and exhibit many biochemical and phenotypic similarities: they do not produce $H_2S$ in triple sugar iron agar; they do not produce urease; they do not utilise citrate in Simmon's medium; they do not hydrolyse gelatin; and they do not deaminate phenylalanine (Farmer III, 1995).

Members of the genus *Shigella* are further divided into four sub-groups on the basis of biochemical and O-antigen differences. *S. dysenteriae* (group A), consisting of serovars 1 - 13, does not ferment lactose or mannitol. This group contains the original *Bacillus dysenteriae* described by Shiga, which is now referred to as *S. dysenteriae* 1 (Bensted, 1956; Noriega *et al.*, 1999). *S. flexneri* (group B) consisting of 15 serovars (1a-b, 2a-b, 3a-c, 4a-c, 5a-b, 6, X, ') and *S. boydii* (group C) consisting of serovars 1 - 18 do not ferment lactose, and with the exception of *S. flexneri* 6, are able to ferment mannitol. The *S. boydii* serovars each have a qualitatively distinct antigen. However, all *S. flexneri* serovars are antigenically related and this is attributed to possession of the group antigens (or a modification of the group antigens) 3,4. *S. flexneri* 6 is the only *S. flexneri* serovar that does not contain the shared group antigens, and it therefore more closely resembles *S. boydii* immunochemically. In addition to the group antigens, each *S. flexneri* serovar with the exception of X and Y, also has a specific 'type' antigen (types 1 - 6) which correlates to the serovar number (Noriega, *et al.*, 1999; Rowe & Gross, 1984). Although containing a single serovar, *S. sonnei* (group D) exists in two "phases" (I and II), and each phase has a distinctive antigen. *S. sonnei* is able to ferment

mannitol, but unlike other *Shigella* species, it is positive for ornithine decarboxylase and will ferment lactose after prolonged incubation (Rowe & Gross, 1984).

The current scheme for classifying *Shigella* largely follows the methods put forward in the early 20th century and relies very strongly on antigenic similarities (Bensted, 1956). However, with the development of molecular techniques, investigation into the evolutionary relationships within *Shigella*, and in addition with *E. coli*, suggests that the traditionally recognised subgroups are not indicative of the true clonal origins of the species (Pupo *et al.*, 2000). Recent sequencing of two *S. flexneri* 2a strains suggests that *Shigella* is phylogenetically indistinguishable from *E. coli* (Jin *et al.*, 2002; Wei *et al.*, 2003). Indeed, studies comparing evolutionary relationships among *E. coli* and *Shigella* spp. by sequence and multilocus enzyme electrophoresis demonstrate that although most *Shigella* strains are closely related, they sit clearly within the *E. coli* cluster (Fukushima *et al.*, 2002; Pupo *et al.*, 1997). The classification of *Shigella* and *Escherichia* as two separate genera has long been controversial, and there is rising support for the proposal that they be re-classified under a single genus (Lan & Reeves, 2001). Historically, separate classification of these organisms was due to the pathogenic nature of *Shigella* spp. for humans, whereas *E. coli* was known as a commensal organism. However, this classification system was complicated when pathogenic *E. coli* strains were isolated (Pupo, *et al.*, 2000). Enteroinvasive *E. coli* (EIEC) strains, for example, resemble *Shigella* in their pathogenic mechanisms, the clinical illness they produce, and like *Shigella* are non-motile, lactose-negative, and possess a large virulence plasmid. They also cross-react with certain *Shigella* O-antigens (Falkow & Mekalanos, 1990). Nevertheless, despite complications of the traditional classification scheme, *Shigella* and *Escherichia* remain distinct genera. They are differentiated clinically on the basis that *Shigella* isolates are always non-motile, lysine negative, do not rapidly ferment lactose, and with the exception of *S. flexneri* 6, *S. dysenteriae* 3 and *S. boydii* 13 and 14, do not produce gas during carbohydrate fermentation (Find, 1992).

# SHIGELLOSIS

## The Disease

Bacillary dysentery, commonly known as shigellosis, is an acute enteric infection of humans. Symptoms usually appear 1 - 3 days after ingestion of the bacteria (Benenson, 1995) and as few as 10 organisms are sufficient to produce illness in some people (DuPont *et al.*, 1989). Intensity and course of disease is variable and symptoms may range from mild diarrhoea to severe dysentery (Find, 1992) depending both on host and bacterial factors. Typically *S. dysenteriae* causes the most severe disease, with *S. flexneri*, *S. boydii* and *S. sonnei* causing progressively less severe disease (Qadri *et al.*, 1988; Rowe & Gross, 1981). After virulent shigellae are swallowed, the bacteria pass through the stomach and multiply in the small intestine to concentrations approaching $10^9$ viable cells/ml of lumenal contents. This initial manifestation of the disease usually presents with symptoms typical of other enteric disease, including fever, malaise, abdominal pain and watery diarrhoea (Falkow & Mekalanos, 1990). After 1 to 3 days, the organism moves from the small intestine to the colon, invading the colonic epithelial cells thereby leading to inflammation, ulceration and impaired colonic fluid absorption (Find, 1992). At this stage the patient develops symptoms of classic dysentery including frequent passing of low volume stools often streaked with blood and mucus. This may be accompanied by a lowering of temperature, increased pain and tenderness in the lower abdomen, faecal urgency and tenesmus. Shigellosis is typically self-limiting and in previously healthy adults clinical illness lasts approximately 7 days. However, in young, elderly or malnourished individuals, disease may be longer lasting and more severe, with a mortality rate of up to 20 % reported in some epidemics (DuPont, 1990).

## Complications

There is a range of intestinal and systemic complications associated with shigellosis, including the life-threatening complications dehydration, haemolytic-uraemic syndrome (HUS) and Reiter's syndrome (Keusch & Bennish, 1991). Dehydration during shigellosis is not as common as is seen in other diarrhoeal diseases such as cholera, but

is thought to arise because of watery diarrhoea during early shigellosis, increased water loss due to fever and decreased water uptake because of anorexia and inflammation of the intestine (Bennish, 1991). HUS occurs predominantly in patients with *S. dysenteriae* 1 infection, and is described as the combination of haemolytic anaemia, thombocytopenia and acute renal failure. Death usually occurs as a consequence of renal failure or severe anaemia (Bennish, 1991). It is thought HUS is caused in part by circulating Shiga toxin (DuPont, 1990) and thus HUS may also develop after infection with Shiga toxin producing *E. coli* (STEC) (Paton *et al.*, 1999). Reiter's syndrome, which is a form of reactive arthritis, may develop 1 to 3 weeks after infection with *Shigella*. This complication appears to show a genetic predilection for subjects carrying the major histocompatability antigen HLA-B27 gene, although it is believed other host factors may also be involved in its development (Finch *et al.*, 1986; Keusch & Bennish, 1991).

Other life-threatening complications associated with shigellosis include febrile seizures which are usually a result of metabolic abnormalities, malnutrition and hypoglycaemia due to reduced food intake and nutrient uptake, and septicaemia and/or pneumonia due to coliform organisms (and less commonly the infecting *Shigella* strain). Also developing after some cases of shigellosis is conjunctivitis which arises presumably as a result of autoinnoculation, intestinal perforation leading to peritonitis caused by intestinal flora and sometimes the infecting *Shigella* strain, and toxic megacolon which is thought to arise as a result of severe colonic inflammation. Frequency studies based on hospital data reveal that septicaemia and hypoglycaemia are among the most common fatal complications associated with shigellosis (Bennish, 1991; DuPont, 1990).

## Diagnosis and Treatment

Shigellosis should be suspected when a febrile, bloody mucoid diarrhoea occurs. However, definitive diagnosis depends on isolation of the organism from a stool sample (Keusch & Bennish, 1991). In the first few days the stool usually contains large numbers of bacteria, although lower counts are experienced during the latter stages making isolation more difficult (Falkow & Mekalanos, 1990). The samples of choice, in order, are rectal swab, stool and anal swab. However, streaks and collections of

blood, mucus and pus in stool specimens are generally productive and should also be cultured (Gray, 1995). Specimens are usually cultured using both selective media such as deoxycholate citrate agar (DCA) or *Shigella-Salmonella* (SS) agar, and also relatively non-inhibitory media such as MacConkey or eosin methylene blue (EMB) agar (Rowe & Gross, 1984). Following this, suspect colonies are subjected to numerous biochemical tests, and isolates identified as *Shigella* may then be further serotyped using appropriate antisera (Falkow & Mekalanos, 1990). Alternatively, another promising means of rapid diagnosis involves the use of polymerase chain reaction (PCR), which amplifies a gene specific to *Shigella* and EIEC strains. However, PCR diagnosis, although more sensitive than standard culture procedures (Gaudio *et al.*, 1997), is not yet universal in diagnostic laboratories. Colony blot immuno-assays are also being investigated as a means for rapid sample screening, and are especially useful for laboratories not equipped with molecular techniques (Szakal *et al.*, 2003).

In developing countries, where the majority of bacillary dysentery cases occur, there are often no laboratory facilities for isolation of the organism. Thus diagnosis is often dependent on clinical evaluations and may also be aided by direct microscopic examination of a stained faecal smear. This will reveal the presence of polymorphonuclear leukocytes, which is indicative of colitis, and distinguishes it from some other forms of diarrhoea (DuPont, 1990). Microscopic analysis is also an important tool in differentiating between amoebic and bacillary dysentery, as amoebic dysentery does not cause a purulent stool (Keusch & Bennish, 1991).

Treatment for shigellosis usually includes both supportive therapy, such as fluid and electrolyte replacement, when watery diarrhoea or dehydration is evident, and antimicrobial therapy (Benenson, 1995). Shigellosis is one of the few enteric infections for which antimicrobial therapy is clearly effective. Although no controlled studies have shown that antimicrobial therapy will reduce mortality (Salam & Bennish, 1991), treatment with antibiotics reduces both the duration/severity of illness and duration of pathogen excretion (Haltalin *et al.*, 1967). This reduction in pathogen excretion may also be useful for reducing the spread of disease to other individuals, which is an important consideration for areas of poor hygiene (DuPont, 1990). Tetracycline, ampicillin, or its analogue amoxicillin are the drugs of choice for sensitive isolates while trimethoprim-sulfamethoxazole (TMP-SMZ) is the drug of choice when the

sensitivity of the infecting organism is unknown. Adult patients may also be treated with either norfloxacin or ciprofloxacin (Falkow & Mekalanos, 1990; Find, 1992; Therapeutic.Guidelines.Limited, 2000). However, over the past several decades, *Shigella* has become increasingly resistant to most of the widely used antibiotics including ampicillin, tetracycline and TMP-SMZ (Khan, 1985; Salam & Bennish, 1991) and there is a clear need for the development of alternative antimicrobial agents, and especially treatments that are safe for children (Sack *et al.*, 1997).

# EPIDEMIOLOGY

A high incidence of dysentery is often an index of social distress, and throughout history, dysentery has plagued nearly every continent. However, from the beginning of the twentieth century as revolutions in sanitation and public health occurred, the incidence of dysentery in the western world has decreased considerably. The improvements in sanitation, hygiene and public health administration all require funding, and therefore most of the world where dysentery now prevails, is poor (McGrew, 1985).

*Shigella* is responsible for the deaths of more than 1.1 million people annually. There are an estimated 164.7 million episodes every year, with 163.2 million occurring in developing countries, and 1.5 million in industrialised countries (Kotloff *et al.*, 1999). Endemic shigellosis is predominantly a childhood disease and although breastfed children under six months of age are highly resistant to shigellosis (DuPont, 1990), in developing countries the combined effects of infection and nutritional inadequacy of the diet after weaning result in enhanced susceptibility to the disease (Keusch & Bennish, 1991). It is estimated that 69 % of all episodes and 61 % of all deaths attributed to *Shigella* involve children under the age of 5 (Kotloff, *et al.*, 1999).

The etiological distribution of *Shigella* species differs world-wide, and geographical distribution of *Shigella* is recognised to be cyclic, changing every 20 - 30 years. It is thought that this shift in the predominant infecting strain for a given population reflects the time required for the acquisition of herd-immunity before one species is replaced by

another (DuPont, 1990). Currently, *S. flexneri* (predominantly serotype 2a) is the most common cause of dysentery in developing countries, accounting for 60 % of all *Shigella* cases. *S. sonnei* (15 %), *S. boydii* (6 %) and *S. dysenteriae* (6 %) follow this. However, in developed countries, *S. sonnei* (77 %) is clearly the most prevalent infecting species, followed by *S. flexneri* (16 %), *S. boydii* (2 %) and *S. dysenteriae* (1 %)(Kotloff, *et al.*, 1999).

*Shigella* is most commonly spread by person to person contact via the faecal-oral route, and the efficiency of this form of transmission is largely a consequence of the low infectious dose (Keusch & Bennish, 1991). Indirect faecal-oral transmission of *Shigella* via water contaminated with faecal matter, food contaminated by flies which transmit disease between faecal matter and food (DuPont, 1990), and food contaminated by infected food handlers (Trevejo *et al.*, 1999) has also been reported. However, food- and water-borne infection is probably more common in developing countries where inadequate disposal of faecal matter allows a higher incidence of contamination (Faruque *et al.*, 2002; Rowe & Gross, 1981).

Factors that predispose populations to endemic and epidemic shigellosis include over-crowding, poor sanitation, malnutrition and poor personal hygiene (Keusch & Bennish, 1991). In developing countries, outbreaks are associated with areas of high-density living and poor sanitation. In developed countries, outbreaks are associated with daycare centres, mental institutions, refugee camps, jails and any other places where close personal contact and sub-optimal hygiene practices are common (Benenson, 1995). Other groups at high-risk of contracting shigellosis include native populations such as the Aboriginals of Central Australia (Albert *et al.*, 1990) and the Indians of Central America, family members of infected children, some male homosexual populations, and travellers to, and migrants from, developing countries (Keusch & Bennish, 1991).

# IMMUNITY

## The Adaptive Immune Response

Most epidemiological data suggest that immunity to *Shigella* infection is serotype-specific. Endemic shigellosis is primarily a disease of children aged 1 - 4, whereas introduction of a strain not previously prevalent in a population, such as *S. dysenteriae* 1, often results in an epidemic spread of infection involving all age groups (WHO, 1997). This common pattern of infection indicates that the prevalence of certain strains in endemic areas results in immunity which prevents reinfection, but that this immunity is not cross protective (Keusch & Bennish, 1991). Serotype-specific immunity has also been shown to occur experimentally in rhesus monkeys where previous infection with *S. flexneri* 2a protects against re-infection with a homologous strain, but not against infection with *S. sonnei* (Formal *et al.*, 1991).

### *Humoral Immunity*

Much of the research on immunity to *Shigella* infection focuses on the humoral response and it seems likely that this is the major component of protective immunity (Sansonetti & Phalipon, 1996). Indeed, the level of humoral immune response correlates with the disease severity of shigellosis in human infection (Islam *et al.*, 1996) and after natural infection, both mucosal and serum antibodies directed against LPS are detectable (Oberheiman *et al.*, 1991). There is evidence that the presence of serum anti-LPS antibodies in humans is a strong marker of acquired immunity (Cohen *et al.*, 1991). The serotype-specific nature of *Shigella* immunity implicates LPS as the primary target antigen and in agreement with this, a recent study of *S. flexneri* infected patients reported that the anti-LPS antibody response following infection is directed mainly against serotype-specific determinants, with little cross-reactivity to heterologous *S. flexneri* strains (Rasolofo-Razanamparany *et al.*, 2001). The importance of anti-LPS mucosal immunity has been demonstrated using a murine pulmonary infection model in which Phalipon *et al.* (1995) showed that secretory IgA directed against a single O-side chain epitope of *S. flexneri* 5 LPS was sufficient to confer dose-dependent protection against homologous challenge.

Aside from LPS, the most notable antigens that elicit an immune response during human mucosal infection are the invasion plasmid antigens (Ipas) which are required for the entero-invasive phenotype of virulent shigellae. The Ipa proteins are also potent immunogens, but unlike LPS, are serologically related in all *Shigella* spp. and thus induce a cross-reactive immune response (Oberhelman, *et al.*, 1991). Although high titres of secretory IgA directed against the Ipa proteins have been correlated with a decrease in disease duration, none of the anti-IpaA or anti-IpaB antibodies tested in the murine pulmonary model have been protective against infection. These results, in conjunction with the fact that the Ipa proteins are mainly cytoplasmic and are secreted only upon contact with the intestinal target cells, suggest that the role of anti-Ipa antibodies in protective immunity may not be significant (Sansonetti & Phalipon, 1996).

It has long been assumed that mucosal rather than systemic immunity is of major protective importance, as during disease, shigellae remain localised at the colonic mucosa and only rarely disseminate systemically. Indeed, an increase in *Shigella*-specific secretory IgA, which is associated with a mucosal immune response (Islam *et al.*, 1995), and LPS-specific IgA antibody-secreting cells (Rasolofo-Razanamparany, *et al.*, 2001) is observed early in human infection. An early mucosal IgA response, involving the Peyer's patches, also occurs in isolated rabbit ileal loop studies (Keren *et al.*, 1978). However, it has recently been shown that IgA is not required for protective adaptive immunity in the murine pulmonary infection model. Way *et al.* (1999a) showed that IgA deficient mice ($IgA^{-/-}$) and cogenic mice ($IgA^{+/+}$) vaccinated with an attenuated *S. flexneri* 2a strain were equally protected against homologous challenge. This finding may add weight to the hypothesis that serum anti-LPS IgG antibodies contribute to protective immunity (Robbins *et al.*, 1992). Additionally, parenterally injected conjugate vaccines which are expected to elicit a systemic rather than mucosal response, appear to provide some protection against *Shigella* infection (Cohen *et al.*, 1997). Thus it is a distinct possibility that systemic humoral immunity may confer protection in human disease.

### *Cell-Mediated Immunity*

During much of the disease course, shigellae reside within the cytoplasm of the intestinal epithelial cells and are thus protected from the humoral response. Little is known about the role of cell-mediated immunity during *Shigella* infection. However,

cytotoxic T-cells are known to play an important role in protection from other intracellular pathogens such as *Listeria*, *Mycobacterium* and *Leishmania* and it has been suggested they may play a similar role during *Shigella* infection (Sansonetti & Phalipon, 1996). It has also been reported that T-helper cells are activated during *Shigella* infection (van de Verg *et al.*, 1995). However, recent studies in the murine pulmonary infection model show that adaptive immunity to *S. flexneri* 2a is an antibody-mediated, B-lymphocyte dependent process that does not require the presence of either T-helper cells or cytotoxic T-cells (Way *et al.*, 1999b). Thus the role of T-cells during *Shigella* infection remains unclear.

## The Innate Immune Response

Numerous studies support an active role for the innate immune response both in first line defence and in clearing of *Shigella*. Raqib *et al.* (2000) demonstrated that the levels of an array of mediators of the innate immune system are significantly elevated in humans with acute shigellosis, thus suggesting that the innate immune system, including the activities of macrophages and polymorphonuclear lymphocytes (PMNs), is active early in disease. However, macrophages themselves are unable to effectively contain shigellae once phagocytosed (discussed further in *Macrophage Apoptosis*).

PMNs, unlike macrophages and epithelial cells from which *Shigella* is able to escape into the cytoplasm, are able to effectively trap and kill shigellae. Although migration of PMNs through the epithelium is known to contribute to mucosal inflammation during shigellosis, it is proposed that PMNs also play an important role in the clearance and resolution of infection (Mandic-Mulec *et al.*, 1997). In the neutrophil-depleted SCID mouse model, PMNs have also been shown to decrease the number of bacteria progressing to the intracellular environment, thus underlying the importance of their role in innate immunity (Zhang *et al.*, 2001).

Natural Killer (NK) cells have also been shown to be an important component in innate immunity against *Shigella* infection. *In vitro*, NK cells have been shown to lyse epithelial cells infected with *Shigella* (Limpel *et al.*, 1986), and are also thought to contribute to the production of gamma interferon (IFN-γ) during human infection

(Samandari *et al.*, 2000). Immunity to shigellosis in humans has been linked to production of IFN-γ (Raqib *et al.*, 1996), and Way *et al* (1998) proposed that NK cell-mediated IFN-γ is essential for innate resistance to *Shigella* infection, thus indicating that NK cells play a key role in innate protection. However, data from the same laboratory indicated that IFN-γ is not required for development of adaptive immunity in the murine pulmonary infection model, as IFN-γ deficient mice may also acquire immunity after vaccination (Way, *et al.*, 1999b).

# VACCINES

The World Health Organisation (WHO) considers the development of a vaccine against *Shigella* of prime importance, and in 1996 the WHO Steering Committee on Diarrhoeal Disease Vaccines placed *Shigella* vaccine development first in its line of priorities (WHO, 1997). Despite many years of research, a suitable *Shigella* vaccine still remains to be developed. As natural immunity is serotype specific and thought to be directed against LPS, most vaccine approaches have attempted to elicit antibody responses to the O polysaccharide (O-PS) component of the LPS. However, the presence of at least 47 serotypes of *Shigella* complicates the development of effective vaccines. As a result, the three most important serotypes, *S. dysenteriae* 1, *S. flexneri* 2a and *S. sonnei*, are commonly targeted for vaccine research (Noriega, *et al.*, 1999; WHO, 1997).

## Killed Vaccines

Early attempts at vaccine development involved the administration of acetone- or heat-killed shigellae injected subcutaneously. However, these vaccines failed to protect against homologous challenge (Hale & Keren, 1992). Similarly, subcutaneous injection of killed or live *S. flexneri* 2a failed to elicit protective immunity in monkeys (Formal *et al.*, 1967). Initial efforts involving killed oral vaccines were also unsuccessful (Hale & Keren, 1992), and it was not until recently that a potentially successful killed oral vaccine has been reported. Chakrabarti *et al.* (1999) demonstrated that oral administration of a heat killed *S. flexneri* 2a vaccine provided rabbits with 100 %

protection against dysentery and diarrhoea during homologous challenge. Although these results are promising, little more has been reported on this potential vaccine.

## Live Attenuated Strains

With a growing understanding of the genetic basis of the pathogenic mechanisms of *Shigella*, vaccine research has become increasingly concentrated on the development of live attenuated strains. Of particular interest are strains with specific mutations, which will elicit protective immunity without inducing the symptoms of shigellosis. One such promising vaccine is *S. flexneri* 2a strain SC602 which carries deletions of the virulence gene *icsA* (mediating intra-and intercellular spread) and the *iuc* locus (encoding an iron uptake system which aids survival in tissues) (Barzu *et al.*, 1998; Barzu *et al.*, 1996). Like wildtype shigellae, strain SC602 colonises the intestine and is thus thought to be more likely to mimic the natural immune responses observed after natural infection (Teska *et al.*, 1999). Clinical trials showed that at low doses ($10^4$ CFU) SC602 induces only mild diarrhoea or transient fever in 13 % of volunteers, and after homologous challenge none of the vaccinees had fever, dysentery or severe symptoms. This is the first attenuated vaccine to provide protection against shigellosis in a stringent, human challenge model, and at last report is part of ongoing phase 2 trials (Coster *et al.*, 1999). WRSd1, a *S. dysenteriae* 1 vaccine candidate which carries deletions of the *virG* (*icsA*) gene and a 20 kb chromosomal region encompassing the Shiga toxin genes (*stxAB*), has been shown to be safe and immunogenic in rhesus monkeys (Venkatesan *et al.*, 2002). When used in conjunction with SC602, WRSd1 has been shown to protect against homologous challenge, and is thus being readied for phase 1 trials (Venkatesan, *et al.*, 2002).

*S. flexneri* 2a vaccine strain CVD 1203 which carries deletions in *aroA* (a component of the aromatic amino acid biosynthesis pathway which is required during growth in mammalian tissues) and *virG* (*icsA*) has also undergone preliminary human testing, but with less promising results. CVD 1203 was found capable of invading intestinal cells, but was unable to replicate intracellularly or spread, thus mimicking natural *Shigella* infection without causing widespread inflammation. Ocular inoculation of guinea pigs with CVD 1203 elicited production of α-LPS secretory IgA and provided protection

against subsequent homologous challenge (Noriega *et al.*, 1994). However, although some attenuation was observed human clinical trials, CVD 1203 was still found to cause fever, diarrhoea or dysentery in some volunteers, thus rendering it unacceptable for further human trials (Kotloff *et al.*, 1996).

However, another aromatic mutant derived from *S. flexneri* Y strain SFL1 appears less reactogenic when fed to human volunteers. SFL114, which carries a Tn*10* insertional inactivation of *aroD*, was well tolerated when fed to monkeys, eliciting intestinal IgA and serum IgA, IgM and IgG responses and giving 100 % protection against diarrhoea and intestinal ulceration upon challenge (Lindberg *et al.*, 1988). As this strain was prone to reversion, SFL124, a transposon-generated deletion mutation at *aroD* derived from SFL114 was used in subsequent work. Like SFL114, SFL124 was found to elicit a protective immune response in the monkey model (Karnell *et al.*, 1992). When trialled in children, this vaccine strain was found to be safe, with few side-effects reported. Both secretory IgA and serum antibodies to LPS and Ipa proteins were elicited in a dose dependent manner (Li *et al.*, 1994) and although the protective capacity of SFL124 was not determined in this trial, it remains a promising vaccine candidate. SFL124 has also recently been used as a base strain to develop a system for expressing other *S. flexneri* O-antigens, making it a potentially useful tool in eliciting cross-protective responses (Guan & Verma, 1998).

The *S. flexneri* 2a strain CVD 1204 which carries a deletion in the *guaBA* operon (responsible for guanine nucleotide biosynthesis) and its derivative CVD 1205 which carries an additional deletion in *virG* are also attractive live attenuated vaccine candidates. CVD 1204 (Anderson *et al.*, 2000) and CVD 1205 (Noriega *et al.*, 1996) have been shown to induce an anti-LPS immune response upon inoculation in guinea pigs, and also to protect against homologous ocular challenge in 100 % of immunised animals. Interestingly, CVD 1204 has also been used in an attempt to develop a multivalent *Shigella*/ETEC vaccine (Altboum *et al.*, 2001; Koprowski II *et al.*, 2000). Like CVD 1205, *S. flexneri* 2a strain CVD 1207 carries specific deletions in *virG* and *guaBA* but carries additional deletions in the *Shigella* enterotoxin 1 and 2 genes *sen* and *set1*. CVD 1207 has recently been used in phase 1 clinical trials and was found to be highly attenuated in humans, with few cases of diarrhoea occurring in volunteers, even at high dosage. Inoculation of human volunteers with CVD 1207 stimulated an increase

in anti-LPS IgA antibody secreting cells, and a moderate serum immunoglobin response, but its protective efficacy in humans was not investigated (Kotloff *et al.*, 2000). CVD 1207 together with *S. flexneri* 3a strain CVD 1211 (*ΔguaBA ΔvirG Δsen*) bear all of the major antigenic group factors of Group B *Shigella* (excepting *S. flexneri* 6). Serum from guinea pigs inoculated with CVD1207 and 1211 cross-reacted with the heterologous *S. flexneri* serotypes 1a, 1b, 2b, 4b, 5b, Y and the vaccine conferred protection against challenge with serotypes 1b, 2b, 5b and Y (Noriega, *et al.*, 1999). Such results are certainly encouraging, and offer a logical solution to the problem of cross-protection across a wide variety of serotypes.

## Hybrid Vaccines

In an attempt to elicit an immune response without inducing symptoms associated with dysentery, one approach undertaken has been to express *Shigella* antigens in a non-pathogenic or attenuated host such as *E. coli* or *Salmonella* (Lindberg & Pal, 1993). EcSf2a-2, an *E. coli* K-12 strain which carries the *S. flexneri* 5a invasion plasmid (thus conferring the invasion phenotype and Ipa expression), the *S. flexneri* 2a O-antigen genes and a deletion in the *aroD* gene, has been shown to confer some protection against *S. flexneri* 2a in a monkey model. However, human trials revealed that in doses high enough to elicit antibody responses and low-level protection, EcSf2a-2 caused dysentery, fever and diarrhoea in some volunteers (Kotloff *et al.*, 1992). In an attempt to reduce reactogenicity, the derivative strain EcSf2a-3 was developed with an additional deletion in the *virG* gene. However, although well-tolerated when administered at low-level doses, EcSf2a-3 was less immunogenic than EcSf2a-2 in the guinea pig model (Alexander *et al.*, 1996). Recently, the use of mutant forms of cholera toxin and heat-labile enterotoxin as adjuvants to increase immunogenicity of this vaccine has had some success in the guinea pig model, and may provide incentive for progression to clinical trials (Hartman *et al.*, 1999).

Other attempts at manufacturing hybrid vaccines have included a *Salmonella typhi* strain Ty21a expressing *S. sonnei* LPS. Immunisation with this hybrid, although eliciting the production of antibody secreting cells specific for *S. sonnei* LPS in all

human volunteers, failed to protect against re-challenge with wildtype *S. sonnei* (Herrington *et al* , 1990).

## Subunit/Acellular Vaccines

Acellular vaccines, like hybrid vaccines, are designed to elicit immune responses to specific antigens without inducing disease symptoms. However, unlike hybrid vaccines, subunit vaccines do not use a host to deliver these antigens, but rather supply antigens directly to the immune system.

One of the widely reported forms of acellular vaccine is the conjugate vaccine whereby potent immunogens are conjugated to specific antigens in an attempt to stimulate a protective immune response. Tetanus toxoid-O-specific polysaccharide (O-SP) conjugates provided encouraging results in the attempt to develop a conjugate *Shigella* vaccine (Chu *et al.*, 1991) and conjugation of recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA) with O-SP from *S. sonnei* and *S. flexneri* 2a has resulted in the production of two vaccines that are safe in adult humans (Taylor *et al.*, 1993). When trialed in a double blind randomised human study, the *S. sonnei*-rEPA vaccine provided a protective efficacy of 74% (Cohen, *et al.*, 1997), and the post-vaccination increase in specific serum IgG was found to persist for at least 2 years for both the *S. flexneri* 2a-rEPA and *S. sonnei*-rEPA conjugates (Robin *et al.*, 1999). It has recently been reported that treatment of CRM9-(an inactivated diptheria toxin protein) or rEPA-bound *S. flexneri* 2a O-SP with succinic anhydride (SA) increases immunogenicity of the conjugates in mice (Pavliakova *et al.*, 1999). Human trials with SA-treated and untreated conjugates showed they were well tolerated and were more immunogenic than the previous untreated rEPA conjugates. *S. sonnei*-CRM9 and *S. flexneri* 2a-rEPA(SA) have been chosen for future evaluation in children (Passwell *et al.*, 2001). Using a similar approach, these investigators have demonstrated that conjugation of *S. dysenteriae* 1 O-SP to human serum albumin is immunogenic in the mouse model, and clinical evaluation of this conjugate is also planned (Pozsgay *et al.*, 1999).

Other acellular approaches have included proteosome and ribosome vaccines. The proteosome vaccine, which is composed of *Shigella* LPS complexed with *Neisseria meningitidis* outer membrane protein proteosomes, conferred protection

against homologous challenge in the guinea pig model (Orr *et al.*, 1993). The *Shigella* ribosomal vaccine (SRV), which is a preparation from the *S. sonnei* ribosome, serves as a delivery system for polysaccharide O-antigen. It has been shown to be non-toxic in mice, guinea pigs and monkeys and the protective efficiency of SRV against homologous challenge was found to be an encouraging 89 % in rhesus monkeys, thus warranting further investigation in human trials (Levenson *et al.*, 1991). Recent investigation into the invasion mechanism of *Shigella* has revealed the presence of a naturally formed complex that contains all the major virulence antigens, including IpaB, IpaC, IpaD and LPS. Immunisation with this complex conferred significant protection against challenge in the mouse and guinea pig models, providing yet another avenue for the development of an effective acellular *Shigella* vaccine (Turbyfill *et al.*, 2000).

# PATHOGENESIS

The disease process caused by *Shigella* is the result of extraordinarily sophisticated mechanisms of pathogenesis and it was not until recent years that the identification of these processes had truly begun. In the last two decades, the molecular basis of shigellosis has been studied in increasing detail, and although a great deal of the genetic basis of virulence is now known, there is still much to be elucidated. It has been established that an essential element of *Shigella* pathogenesis is an ability to invade epithelial cells. There are several steps involved in this process, including: (i) traversal of the epithelial barrier involving bacterially directed macrophage apoptosis; (ii) bacterial-induced uptake into the epithelial cells; (iii) subsequent lysing of the endocytic vacuole, intracellular multiplication and spread (via actin-based motility) into adjacent epithelial cells, thus resulting in the tissue destruction and mass inflammation associated with shigellosis.

## Organisation of Virulence Loci in *Shigella*

All virulent isolates of *Shigella* carry a ~220 kb plasmid, termed the large virulence plasmid, which encodes the invasive phenotype of this species (Sansonetti *et al.*, 1983; Sansonetti *et al.*, 1982). Sequence analysis of selected genes in a number of species

...dicates that *Shigella* and EIEC carry one of two distinct, though closely related, virulence plasmids. Interestingly, virulence plasmid type appears not to correspond to the current taxonomic scheme, but closely follows the recently reported sequence-based phylogenetic relationships of the strains (as discussed in *Taxonomy*) (Lan *et al.*, 2001).

Initial mutagenesis studies identified a 31 kb region of the virulence plasmid that was necessary, and sufficient for the invasive phenotype (Sasakawa *et al.*, 1988; Sasakawa *et al.*, 1986). Subsequent sequencing of this region revealed genes organised in two oppositely transcribed clusters (Fig. 1.1) (Buchrieser *et al.*, 2000). This 31 kb region consists primarily of the *mxi-spa* locus, which encodes a type III secretion system. However, it also encodes virulence determinants such as IpaA-D, IpgA-F and IcsB, that are involved in entry, cell to cell spreading and macrophage apoptosis, and VirB the transcriptional regulator of the *ipa*, *mxi* and *spa* operons (Fig. 1.1) (Sansonetti, 2001). Although the entire 31 kb region has been sequenced in relatively few strains, available data suggest that both organisation and sequence of this region is highly conserved across the four *Shigella* species (Parsot & Sansonetti, 1999; Sasakawa, *et al.*, 1988). The *ipa/mxi-spa* locus is itself flanked by IS elements (Venkatesan *et al.*, 2001), and features such as a low G + C content compared with the chromosome suggest that this region of the virulence plasmid has been acquired through horiztonal transfer, and is considered to be a pathogenicity island (PAI) (Parsot & Sansonetti, 1999).

In addition to the *ipa/mxi-spa* PAI, one of the most important virulence factors residing on the virulence plasmid, is *icsA* (also called *virG*) which is responsible for the actin-based motility of *Shigella*, thus permitting spread from one cell to another (Bernardini *et al.*, 1989). SopA (also called IcsP) and VirK are also involved in actin-based motility of *Shigella* (Fig. 1.1) (Egile *et al.*, 1997; Nakata *et al.*, 1992). VirF, the major regulator of the *Shigella* virulence phenotype, also resides on the large virulence plasmid (Fig. 1.1). Other virulence plasmid-encoded proteins such as VirA, which has been shown to be involved in invasion and intercellular spreading (Sasakawa & Yoshida, 2003; Uchiya *et al.*, 1995), and the IpaH family are secreted via type III secretion, yet their precise function remains unknown. It is thought likely that such proteins secreted by the type III secretion system may later be found to be involved in pathogenesis (Buchrieser, *et al.*, 2000; Sansonetti, 2001). The large virulence plasmid-encoded serine protease SepA, and the product of the *ap*v gene are also considered to be potential

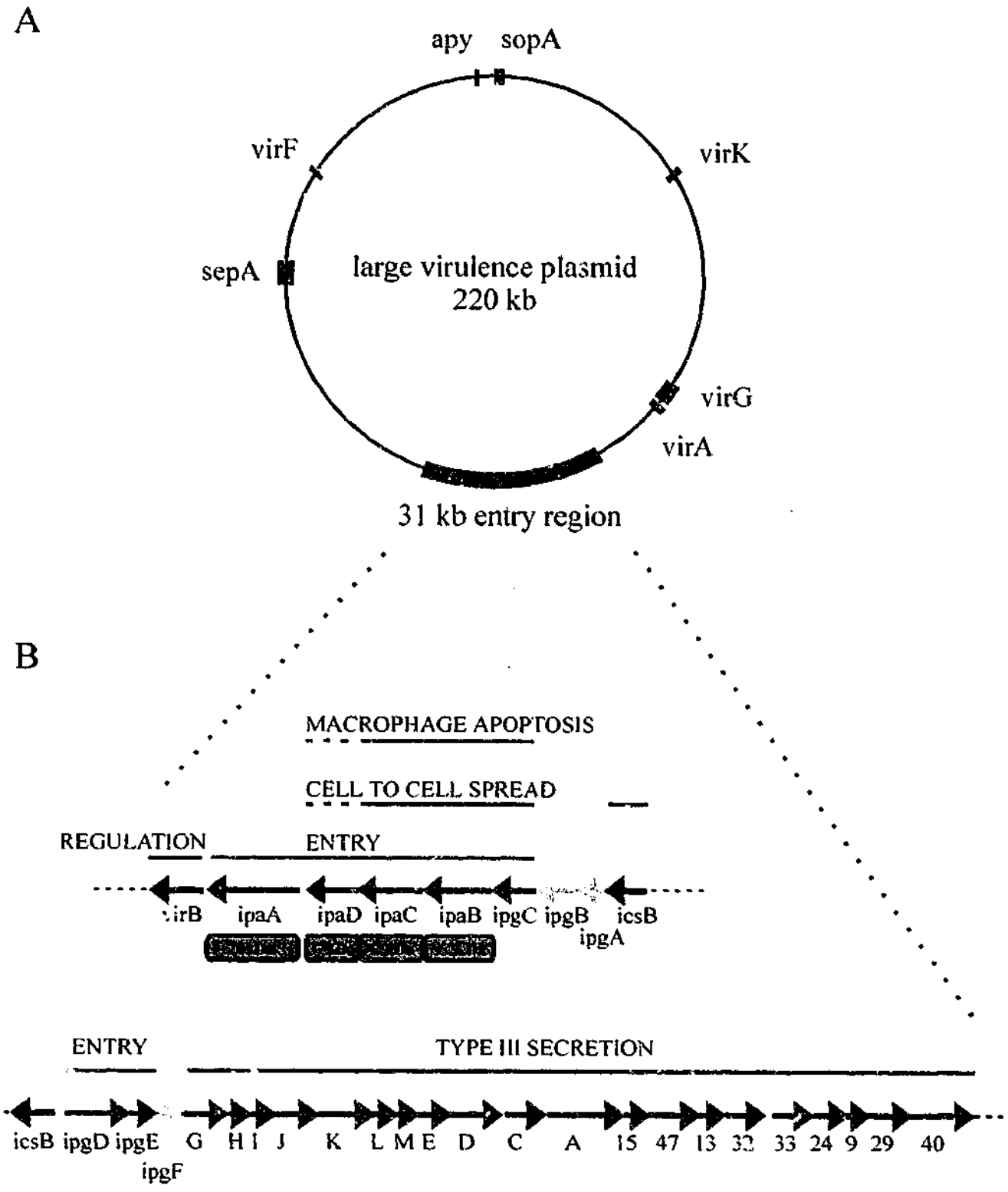



**Figure 1.1** The large virulence plasmid of *S. flexneri* (Picture to scale, modified from Venkatesan *et al.*, 2001; Sansonetti *et al.*, 2001). (A) Circular map of the large virulence plasmid of *S. flexneri* 5a. (B) Organisation of the *S. flexneri* plasmid located 31 kb entry region.

virulence factors (Fig. 1.1) (Benjelloun-Touimi *et al.*, 1998; Berlutti *et al.*, 1998).

In addition to the virulence plasmid genes which direct the bacterial-epithelial cell interactions, there is also a number of chromosomal genes which play a role in the virulence of *Shigella*. These genes may be classified in two categories: (i) genes that regulate the expression of the virulence plasmid genes, such as the H-NS-like *virR* (Maurelli & Sansonetti, 1988); (ii) genes that are important for bacterial survival in the host such as those encoding the synthesis of LPS (Okada *et al.*, 1991; Rajakumar *et al.*, 1994) and iron uptake systems, many of which are encoded on PAIs (Hacker & Kaper, 2000). The *S. dysenteriae* 1 genes for Shiga toxin are also included in this category (Sansonetti, 2001). Interestingly, the absence of certain genes on the chromosome also confers virulence in *Shigella* spp. The deletion of the region, or formation of a "black hole", around the *cadA* gene (the structural gene for lysine decarboxylase) has been shown to increase the virulence of *Shigella* spp. as compared with non-pathogenic *E. coli* strains (Maurelli *et al.*, 1998).

## Gastro-Intestinal Passage of Shigellae

In order to reach the colon, bacteria must first survive passage through the highly acidic conditions of the stomach. Unlike other enteric pathogens such as *Salmonella*, *Shigella* is able to withstand a pH as low as 2.0, thus explaining the low dose required to produce disease (Gorden & Small, 1990; Gorden & Small, 1993). Acid resistance in *Shigella* and *E. coli* is a function of the stationary-phase-specific sigma factor $\sigma^S$, a component of RNA polymerase encoded by *rpoS* (Lange & Hengge-Aronis, 1991; Small *et al.*, 1994) and recent studies suggest that *rpoS*-dependent acid resistance plays an important role in bacterial survival in the gastro-intestinal tract (Price *et al.*, 2000). The $\sigma^S$ factor directs RNA polymerase to otherwise poorly recognised promoters, and is thus a major regulator for late log phase and stationary phase growth. Around 30 late log phase proteins have been found to be under the control of $\sigma^S$, including *hdeAB* which encodes periplasmic proteins, and *gadC* which encodes an inner membrane amino acid antiporter (Sasakawa, 1997; Waterman & Small, 1996).

Following passage through the stomach, shigellae continue through to the small intestine where they multiply to concentrations approaching $10^7$ - $10^9$ bacteria $ml^{-1}$ of lumenal fluid (DuPont, 1990).

## Invasion of Colonic Epithelial Cells

### *M Cells: A Gateway of Entry*

From the lumen of the intestine, shigellae must traverse the epithelial layer, as they are capable of invading epithelial cells efficiently only from the basolateral surface (Mounier *et al.*, 1992). Wassef *et al.* (1989) demonstrated that the initial site of entry in the rabbit ileal loop was M cells, a component of the follicle-associated epithelium (FAE); this finding was later confirmed by Sansonetti *et al.* (1996). M cells function as non-specific transporters, and as their role in mucosal immunity is to sample the intestinal contents by uptaking and transporting antigens from the lumen through to the underlying lymphoid tissue (Jepson & Clark, 1998), some nonspecific uptake of shigellae would be expected. However, it has also been demonstrated that expression of an adhesive or invasive phenotype is required for shigellae to effectively colonise the FAE. Although no specific bacterial or M cell receptor has been identified, there remains a distinct possibility that some form of adhesin is involved in this process (Philpott *et al.*, 2000a). Interestingly, *Shigella* is not the only bacterial species to exploit M cells as a gateway into the host, as enteric pathogens *Salmonella* and *Yersinia* also employ similar tactics (Sansonetti & Phalipon, 1999).

### *Macrophage Apoptosis*

After translocation through the M cell, shigellae are delivered to the underlying lymphoid tissue where they are engulfed by resident phagocytes for processing and presentation to the immune system. However, *in vitro* studies have demonstrated that once in the cytoplasm, shigellae are a powerful cytotoxic force, able to trigger apoptosis (programmed cell death) of infected murine macrophages within 3 hours (Zychlinsky *et al* , 1992). After initial uptake, shigellae lyse the phagocytic vacuole and escape to the cytoplasmic compartment of macrophages. Although IpaB is the main effector implicated in membrane lysis (High *et al.*, 1992), it has also been suggested that IpaC (Barzu *et al.*, 1997) and the virulence plasmid-encoded $IpaH_{7.8}$ (Fernandez-Prada *et al.*,

2000) play a functional role in escape from the phagocytic vacuole. Using a genetic approach, Zychlinsky *et al.* (1994) reported that *Shigella*-induced macrophage apoptosis required IpaB. This finding was later confirmed when microinjection of purifed IpaB into the cytoplasm of murine macrophages was sufficient to cause apoptosis (Chen *et al.*, 1996). It was also found that IpaB binds directly to interleukin-1β converting enzyme (ICE) and that this enzyme, which is activated shortly after *S. flexneri* infection, is absolutely required for *Shigella*-induced apoptosis (Chen, *et al.*, 1996; Hilbi *et al.*, 1998). However, recent studies suggest that binding alone is insufficient to activate ICE (also called Casp-1), and that the association between IpaB and Casp-1 is simply one step in the activation of macrophage apoptosis (Guichon *et al.*, 2001). Work is currently underway to determine other components the apoptotic pathway induced by *Shigella* infection (Hilbi *et al.*, 2000).

Interestingly, the majority of work outlining the apoptotic effects of *Shigella* has been carried out using murine macrophages. Fernandez-Prada *et al.* (1997) demonstrated that although *Shigella*-induced cell death occurs in a human macrophages, it is not due to apoptosis. Rather, they describe an "accidental cell death" termed oncosis, a process which is distinct from apoptosis. Indeed, it appears that human-derived monoblasts are capable of undergoing cell death by either apoptosis or oncosis, and that the type of cell death may be dependent on the stimulus that induces differentiation within the monoblast cell line (Nonaka *et al.*, 1999).

### *Bacterial-Host Interactions During Invasion*

The association between pathogenesis and invasion of epithelial cells was made relatively early in *Shigella* research when LaBrec *et al.* (1964) established that invasion may be equated with the virulence phenotype. In 1979, *in vitro* studies showed that virulent shigellae induce the ordinarily non-phagocytic epithelial cells to engulf bacteria in a process resembling endocytosis (Hale *et al.*, 1979). Indeed, modern microscopic techniques reveal that *Shigella* is able to induce massive host cell membrane ruffling at the site of bacterial contact, with cytoskeletal-mediated membrane protrusions extending out from the surface of the cell and eventually engulfing the bacteria (Fig. 1.2) (Philpott, *et al.*, 2000a). Hale and Bonventre (1979) suggested that *Shigella* surface antigens alone are not sufficient to induce this process, and it was later found

that IpaB, IpaC and IpaD which are secreted into the culture supernatant, were absolutely required for entry into epithelial cells (Menard *et al.*, 1993).

The Ipas are secreted via the type III secretion system encoded by the *mxi-spa* locus on the large virulence plasmid (Buchrieser, *et al.*, 2000). Their release is triggered upon contact of *Shigella* with host cells or components of the extracellular matrix such as fibronectin and collagen type IV (Watarai *et al.*, 1995a); secretion is found to be most efficient during the exponential growth phase (Mounier *et al.*, 1997). Prior to secretion, IpaB and IpaC individually bind the chaperone protein IpgC to prevent proteolysis and instability whilst in the cytoplasm. However, once secreted, IpaB and IpaC associate, probably via the IpaC N terminus (Harrington *et al.*, 2003), and form a soluble complex in the extracellular medium (Menard *et al.*, 1994). Latex beads coated with the Ipa complex are readily internalised by epithelial cells and like wildtype *S. flexneri*, induce membrane projections and actin polymerisation at the site of entry, thus establishing the importance of IpaB and IpaC in the entry process (Menard *et al.*, 1996). Interestingly, microinjected IpaC is capable of inducing formation of membrane extensions in epithelial cells and the C-terminal region is found to be necessary for *Shigella*-induced actin polymerisation observed at the foci of entry (Picking *et al.*, 2001; Tran *et al.*, 2000; Tran Van Nhieu & Sansonetti, 1999). IpaC is also known to interact with lipid vesicles (de Geyter *et al.*, 1997), and indeed the IpaB-IpaC complex is thought to be inserted into the host membrane during entry, forming a pore for injection of other effectors of entry into the host cell cytoplasm (Blocker *et al.*, 1999).

**Figure 1.2** Scanning electron micrograph of *S. flexneri* inducing membrane ruffles on the surface of an epithelial cell during uptake (Philpott *et al.*, 2000).

IpaD also plays an important role in *Shigella* pathogenesis, as *ipaD* mutants are unable to invade epithelial cells (Menard, *et al.*, 1993). This protein is known to have surface-exposed domains (Turbyfill *et al.*, 1998) and is thought to form a complex with IpaB,

controlling the flux of proteins through the type III secretion system (Menard, *et al.*, 1996; Sansonetti, 2001). Although IpaA is not absolutely required for epithelial cell entry, mutation of *ipaA* reduces entry efficiency ten-fold (Tran V'an Nhieu *et al.*, 1997). It is thought that IpaA plays a role in the maturation of the entry foci, inducing both actin depolymerisation and bundling that allows close contact between the bacterium and host cell. This process is due to the binding of IpaA to the head of vinculin, a cytoskeleton-associated protein, which in turn interacts with F-actin (Bourdet-Sicard *et al.*, 1999; Tran Van Nhieu, *et al.*, 1997). IpaA has also been found to bind to IpgD which is encoded on the large virulence plasmid upstream of the *ipa* operon (Fig. 1.1) (Niebuhr *et al.*, 2000). IpgD is stored in the cytoplasm of *Shigella* prior to secretion, where it is bound by the chaperone protein IpgE, presumably to prevent premature interaction with IpaA. However, upon secretion, IpgD is thought to bind and act in concert with IpaA, modulating the host cell responses through possible phosphatase activity (Allaoui *et al.*, 1993a; Niebuhr, *et al.*, 2000).

### *The Role of Rho GTPases and Adhesion Receptors*

Many of the mechanisms employed by *Shigella* to control the cellular signalling of epithelial cells remain unknown, but it is evident that the Ipas and other effectors of invasion are able to influence pathways involved in cytoskeletal re-arrangement. The Rho family of small GTPases is known to play an essential role in organisation and regulation of cytoskeletal structures, and it has been shown that actin polymerisation at the site of *Shigella* entry, and entry itself, is dependent on the family of small Rho GTPases (Menard, *et al.*, 1996). Accumulation of F-actin, vinculin, talin and activation of protein kinase C during uptake of *Shigella* is dependent on Rho (Watarai *et al.*, 1997) and further research implicates the Rho GTPases Cdc42, Rac and Rho in formation of *Shigella* entry foci (Mounier *et al.*, 1999; Tran Van Nhieu *et al.*, 1999). Thus it appears probable that influencing activity of the Rho GTPases is at least one mechanism employed by *Shigella* to manipulate the cellular pathways of epithelial cells.

A number of cell adhesion receptors has also been implicated in *Shigella* entry into epithelial cells. Invasins IpaB, IpaC and IpaD are able to associate directly with $\alpha_5\beta_1$ integrins, which are present on the epithelial cell surface and interact with the cytoskeleton. Interestingly, Watarai *et al.* (1996) demonstrated that an increase in

invasive efficiency of *Shigella* for Chinese hamster ovary (CHO) cells correlated with an increase in the level of $\alpha_5\beta_1$ integrin expression. Consistent with their possible involvement in epithelial cell invasion, $\alpha_5\beta_1$ integrins also have a basolateral distribution, thus correlating with basolateral invasion by *Shigella*. The IpaB-IpaC complex has also been shown to associate with the cell surface receptor CD44 during bacterial entry into HeLa cells, and anti-CD44 blocking antibodies reduced the efficiency of *Shigella* uptake (Tran Van Nhieu & Sansonetti, 1999). CD44 binds ezrin (a membrane-cytoskeleton linker protein) which is highly concentrated in the cellular protrusions induced by the entry process (Skoudy *et al.*, 1999). Together these data suggest a functional role for CD44 in *Shigella* uptake.

***Mucosal Inflammation: A Balance Between Protection and Destruction***

Colonic epithelial cells are known to express an array of pro-inflammatory molecules in response to bacterial invasion (Jung *et al.*, 1995). It has also been shown that *Shigella* induces the transcription of the chemokine IL-8 from such cells (Philpott *et al.*, 2000b). Additionally, due to activation of ICE (as discussed under *Macrophage Apoptosis*), infected macrophages also release increased amounts of IL-1 (Zychlinsky, *et al.*, 1994). The net result of the interactions between these and other immune factors usually results in massive PMN influx and is observed as the acute inflammation typically associated with shigellosis (Philpott, *et al.*, 2000a). Indeed, blocking neutrophil influx using anti-β1-integrin antibodies, IL-1 receptor antagonists, or anti-IL-8 antibodies all result in a decrease of tissue damage, which suggests that the host response, not *Shigella*, is the direct cause of mucosal destruction (Perdomo *et al.*, 1994b; Sansonetti *et al.*, 1995; Sansonetti *et al.*, 1999a).

In addition to causing tissue damage, PMN transmigration has been implicated in disruption of the otherwise impermeable epithelial cell junctions. PMN transmigration has been shown to promote bacterial entry into epithelial cells, as disruption of epithelial cell junctions would increase bacterial entry through the paracellular pathway, thus allowing access to the basolateral surface of epithelial cells where shigellae are known to invade (Mounier, *et al.*, 1992; Perdomo *et al.*, 1994a). Interestingly, PMN transmigration has also been associated with an increase in Shiga toxin translocation across the intestinal epithelial cells (Hurley *et al.*, 2001). Nevertheless, PMN activity

plays an important role in defence against *Shigella* and there is some research to support the theory that tissue damage may not be due to transmigration of PMNs (Zhang, *et al.*, 2001). Interestingly, IL-8 mediated PMN activity also plays a major role in confining *Shigella* invasion to the epithelium (Sansonetti, *et al.*, 1999a), which would effectively limit inflammation.

Overall, these data suggest a model whereby *Shigella* manipulates the host immune response to cause epithelial cell damage, which ultimately favours bacterial invasion, while simultaneously, the host immune response also acts to limit the effects of *Shigella* invasion. Thus the host response to *Shigella* appears to be a paradoxical balance between causing and preventing mucosal damage.

## Intra- and Intercellular Dissemination

After inducing their own uptake into epithelial cells, shigellae escape the endocytic vacuole in a process that was originally attributed to the actions of IpaB (High, *et al.*, 1992). However, recently IpaC, which has been shown to lyse membranes in a pH dependent manner (Barzu, *et al.*, 1997), has also been implicated in vacuole escape (Osiecki *et al.*, 2001). Following release into the cytosol, shigellae multiply rapidly (Sansonetti *et al.*, 1986) and although non-motile in culture media, are seen to move randomly through the host cell cytosol (Sasakawa, 1997). This movement is accomplished through the nucleation of actin at one pole of the bacterial cell surface, thus forming a distinctive F-actin comet tail and propelling the cell forward (Fig. 1.3) (Bernardini, *et al.*, 1989; Sansonetti *et al.*, 1999b). The actin-based motility observed in *Shigella* is mediated by the outer membrane protein IcsA (VirG) which is encoded on the large virulence-plasmid (Fig. 1.1) (Bernardini, *et al.*, 1989; Lett *et al.*, 1989). IcsA does not interact directly with actin, but binds neutral Wiskott-Aldrich syndrome

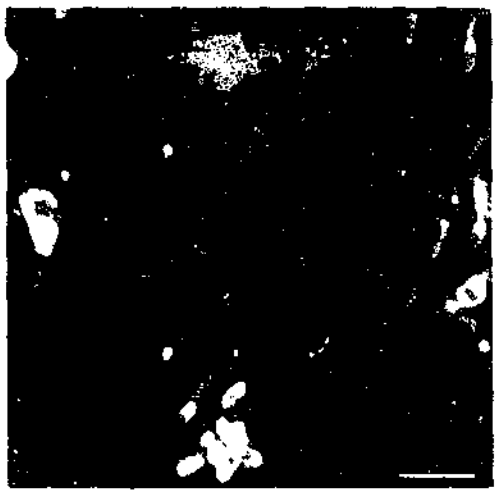


**Figure 1.3** F-actin comet tails labelled with fluorescent phallacidin; bacteria are labelled with antibodies to lipopolysaccharide and then rhodamine (bar = 10 μm) (Sansonetti *et al.*, 1999).

protein (N-WASP) which is then activated to bind the Arp2/3 complex. Actin nucleation occurs as a function of this ternary complex and also involves the recruitment of other actin-related proteins (Egile *et al.*, 1999; Mimuro *et al.*, 2000).

IcsA has been classified as an autotransporter (Henderson *et al.*, 1998; Suzuki *et al.*, 1995) which, after synthesis, is targeted directly to the old pole of the bacterium. However, once present in the outer-membrane, the protein diffuses laterally out from the pole, thus creating a gradient across the bacterial cell surface (Robbins *et al.*, 2001; Steinhauer *et al.*, 1999). Polar distribution of IcsA is absolutely required for actin-based motility, and maintenance of this distribution requires the action of the *Shigella* outer-membrane protease SopA (IcsP), the gene for which is also located on the large virulence plasmid (Fig. 1.1) (Egile, *et al.*, 1997). SopA, which has uniform activity across the bacterial surface, acts to cleave IcsA from the outer-membrane. As there is an increased concentration of IcsA at one pole of the bacterium, SopA restores the exclusive polar localisation of IcsA and is thus an essential component of actin-based motility (Egile, *et al.*, 1997; Robbins, *et al.*, 2001; Steinhauer, *et al.*, 1999).

When contact occurs between the motile bacteria and the inner face of the host cell membrane, a protrusion containing the bacteria is formed and eventually phagocytosed into the neighbouring cell. The protrusions occur predominantly at the epithelial cell adherence junctions and require the expression of cadherins (Sansonetti *et al.*, 1994; Vasselon *et al.*, 1992). Myosin light chain kinase is also involved in this process, indicating that a mechanism resembling phagocytosis may take place (Rathman *et al.*, 2000). In the adjacent cell, shigellae escape the now double membrane vacuole in a process requiring the expression of IpaB, IpaC, and their chaperon protein IpgC (Page *et al.*, 1999). Once free in the cytosol, bacteria multiply and proceed to the next cell, repeating this cycle to create a focus of infection.

In a process distinct from actin-based motility, *Shigella* is also able to move along stress fibres that radiate from adhesion plaques. This movement resembles that of cell organelles and has thus been termed organelle like movement (olm) (Vasselon *et al.*, 1991). The Olm phenotype of *Shigella* has been observed in epithelial cell monolayers, although the importance of this movement *in vivo* has not been investigated (Sansonetti, *et al.*, 1999b).

# The Type III Secretion System of *Shigella*

Type III secretion systems are an essential component of virulence in many Gram negative bacteria, and serve to translocate proteins directly from the bacterial cytoplasm to the eukaryotic cell surface or intracellular environment. This form of secretion is *sec*-independent and occurs via a supramolecular structure spanning the innner and outer membranes. In *Shigella* spp., the type III secretion system is encoded by the *mxi* and *spa* loci (membrane expression of Ipa antigens and secretion of protein antigens) on the large virulence-plasmid (Fig. 1.1) (Allaoui *et al.*, 1993b; Andrews & Maurelli, 1992; Niebuhr, *et al.*, 2000; Venkatesan *et al.*, 1992). Approximately 25 proteins are thought to utilise the *S. flexneri* type III secretion system. Among those known to be secreted are IpaA, IpaB, IpaC, IpaD, and IpgD, and as these effectors are required for uptake into host epithelial cells, the Mxi/Spa apparatus must be considered a prerequisite for *Shigella* virulence (Buchrieser, *et al.*, 2000).

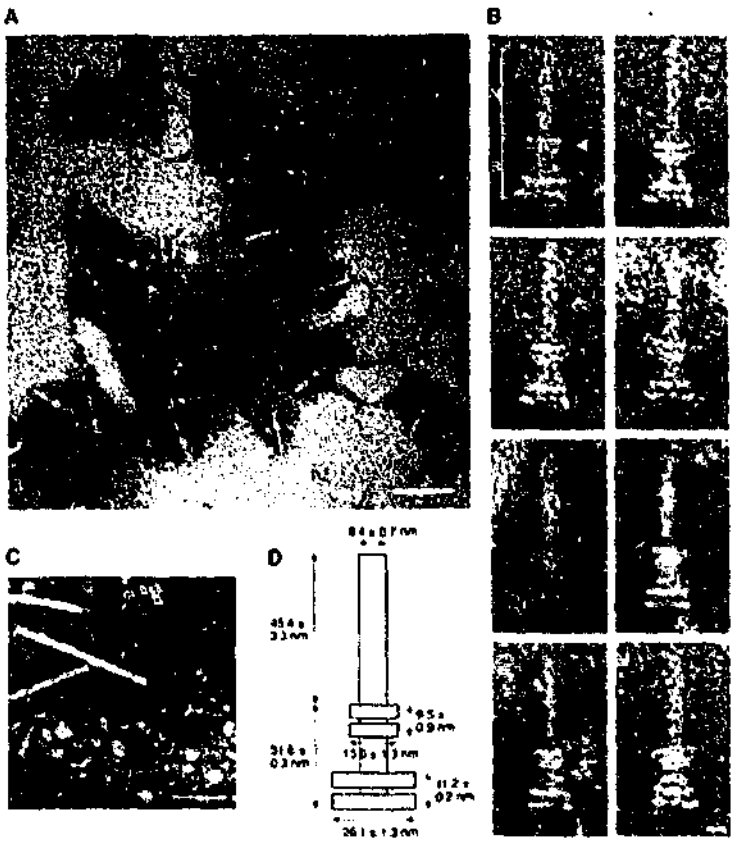



**Figure 1.4** Electron micrograph of type III secretion complexes. **(A)** Purified type III secretion complexes at low magnification. **(B)** Purified type III secretion complexes at high magnification. **(C)** Measurement of size of type III secretion complexes (scale bar 100 nm). **(D)** Proposed size of the type III secretion complexes. (Tamano *et al.*, 2000)

The *mxi/spa* locus comprises approximately 21 kb of DNA and encodes 20 individual proteins all arranged in the same transcriptional orientation (Sansonetti 2001). This locus bears striking sequence similarity to the type III systems of enteric pathogens *Yersinia* and *Salmonella*, suggesting they may have a common ancestor. Interestingly, like *Shigella*, the type III secretion systems of *Salmonella* and EPEC are also carried on PAIs and are required for full expression of

the virulence phenotype (Donnenberg *et al.*, 1997; Hensel *et al.*, 1998).

Recent elucidation of the physical structure of the *Shigella* Mxi/Spa secreton has revealed that it is a hollow structure consisting of two major components: the needle complex; and the basal body (Fig. 1.4) (Blocker *et al.*, 2001; Tamano *et al.*, 2000). The needle complex is required for delivery of secreted proteins and consists primarily of MxiH, and possibly MxiI to a lesser extent. Spa47 has also been implicated in formation of the needle complex (Blocker, *et al.*, 2001; Tamano, *et al.*, 2000). The basal body, which shares considerable similarity with flagellar export machinery, is known to consist at least of MxiD, MxiG, and MxiJ (Blocker, *et al.*, 2001; Tamano, *et al.*, 2000). MxiE has recently been shown to regulate the expression of a number of proteins exported through the type III secretion system, including *virA* and $ipaH_{9.8}$ (Kane *et al.*, 2002; Mavris *et al.*, 2002). The functions of the other proteins in the *mxi/spa* locus are largely unknown, although studies involving MxiM, MxiD, MxiA and many of the Spa proteins indicate these proteins are also required for assembly and/or function of the Type III secretion pathway (Allaoui, *et al.*, 1993b; Andrews & Maurelli, 1992; Schuch & Maurelli, 1999; Schuch & Maurelli, 2001; Venkatesan, *et al.*, 1992; Watarai, *et al.*, 1995a; Watarai *et al.*, 1995b).

## Regulation of Virulence in *Shigella*

The *Shigella* virulence phenotype appears to be regulated by a complex combination of systems, involving multiple inputs from both chromosomal and virulence plasmid encoded genes. The majority of the virulence associated sequence is thought to have arisen following horizontal transfer, as have some of the specific virulence regulators (Buchrieser, *et al.*, 2000). However, *Shigella* is also capable of exploiting more general regulatory features of the host cell, such as those influencing DNA topology (Tobe *et al.*, 1995). Although much of the work on regulatory pathways has been carried out at the genetic level, thus making integration of different systems difficult to comprehend, three major influences on regulation have been elucidated: temperature, osmotic stress and pH. Virulence gene expression is subject to strict environmental conditions including a temperature of 37 °C, an osmolarity similar to that of physiological saline,

and a pH of 7.4 and it is suggested that these controls prevent the inappropriate expression of the virulence phenotype outside the host (Dorman & Porter, 1998).

Two of the major virulence regulators are VirF and VirB, both of which are encoded on the large virulence plasmid (Fig. 1.1). VirF and VirB act at the transcriptional level in a cascade-like regulation system that is ultimately controlled by environmental signals (Porter & Dorman, 1997). VirF is an AraC-like regulator that activates transcription of VirB and IcsA, probably through DNA binding (Adler *et al.*, 1989; Porter & Dorman, 2002). VirB, in turn, activates a number of virulence effectors including IpaA, IpaB, IpaC, IpaD and the *mxi/spa* locus (Adler, *et al.*, 1989; Dorman & Porter, 1998). At 37 °C VirF activates *virB* through binding to an upstream promotor region, but at 30 °C it is thought that a change in DNA supercoiling prevents this interaction (Tobe *et al.*, 1991; Tobe, *et al.*, 1995). Thus, at 30 °C, the invasive phenotype is not expressed.

Interestingly, it appears that quorum-sensing may also play a role in *S. flexneri* regulation, and a *Shigella* quorum-sensing molecule that is active in late log-phase appears to modulate *virB* expression. However, as this molecule is not essential for virulence, the importance of quorum-sensing in regulation of the *Shigella* virulence phenotype is unclear and awaits further investigation (Day Jr & Maurelli, 2001). H-NS (which was originally designated VirR in *Shigella* (Maurelli & Sansonetti, 1988)) has been shown to bind the *virB* promotor, suggesting that it may negatively regulate the gene through exclusion of the RNA polymerase (Tobe *et al.*, 1993). H-NS also exhibits some activity via *virF*, although the major *virF* regulation appears to be under pH control (Porter & Dorman, 1997).

It has been shown that in *S. sonnei*, pH control requires the chromosomally encoded *cpxRA* genes. CpxR and CpxA show homology to the sensor and response regulator family of two-component regulators (Nakayama & Watanabe, 1995). However, as direct binding of CpxR to the *virF* promotor has not been shown, it is not clear whether the effect of the two-component system is direct or indirect. Expression of virulence genes in *Shigella* is also regulated by the osmolarity of the medium, and there is some evidence that another two-component regulatory system participates in this regulation (Bernardini *et al.*, 1990). The OmpR/EnvZ system is encoded by the chromosomal *ompB* locus and loss of this locus is known to downregulate the expression of virulence-

plasmid genes and the porin OmpC. However, unlike OmpC, the virulence region has not been shown to be under direct control of OmpR and it is therefore unclear whether OmpR/EnvZ regulation of virulence gene expression is direct or indirect (Bernardini *et al.*, 1993).

## Toxins

Shiga toxin (Stx) is elaborated only by *S. dysenteriae* 1, although Shiga-like toxins ($Stx_1$, $Stx_2$ and $Stx_2$ variatants) are produced by various Shiga-toxigenic *E. coli* (STEC) strains (O'Brien & Holmes, 1987; Unkmeir & Schmidt, 2000). The chromosomal *stx* locus, comprising the *stxA* and *stxB* genes, encodes the A and B subunits of the toxin. The holotoxin, which has a molecular weight of ~60,000, is formed by the association of a single A subunit with five B subunits (Yutsudo *et al.*, 1986). Stx is not essential for the progression of disease during shigellosis; rather it is associated with destruction of capillary vessels and the resultant bloody stools (Fontaine *et al.*, 1988). Nevertheless, Stx has been attributed neurotoxic and cytotoxic activities (Fujii *et al.*, 2001; Yutsudo, *et al.*, 1986) and further studies indicate that it also stimulates mucosal inflammation (Fontaine, *et al.*, 1988; Thorpe *et al.*, 1999; Thorpe *et al.*, 2001). In conjunction with LPS, Stx is thought to be the major contributor towards the development of HUS following *Shigella* or STEC infection (Louise & Obrig, 1992). Interestingly, recent studies indicate that the actions of Stx may be effectively neutralised by treatment with anti-Stx antibodies or mimics of the Stx receptor (Fujii, *et al.*, 2001; Paton *et al.*, 2001).

The genetic similarity between the Stx of *S. dysenteriae* 1 and Shiga-like toxins of STEC is quite high, and genes for the STEC toxins are frequently encoded in the genome of temperate bacteriophages (Muniesa *et al.*, 2000; Plunkett III *et al.*, 1999; Strockbine *et al.*, 1988; Unkmeir & Schmidt, 2000). The *S. dysenteriae* 1 *stx* locus, although similarly flanked by phage-related sequence (Unkmeir & Schmidt, 2000), was not found to be inducible as a phage (Strockbine, *et al.*, 1988), but interestingly was found to amplify and delete from the chromosome via flanking IS elements (McDonough & Butterton, 1999). This locus was also recently found to be present on temperate bacteriophage 7888 in the *S. sonnei* genome, and the phage was found to be naturally transferable between a wide range of *Shigella* strains (Strauch *et al.*, 2001).

These data suggest that the *stx* genes of *E. coli* and *Shigella* are closely related and were probably acquired through phage-borne horizontal transfer.

The other major toxins elaborated by *Shigella* are the *Shigella* enterotoxins: ShET1 and ShET2. ShET1 is encoded by the *sen* locus on the large virulence plasmid, and is present in approximately 83 % of *Shigella* strains and 75 % of EIEC strains (Nataro *et al.*, 1995). ShET2 is chromosomally-encoded by the *set1* genes, but is detected only in *S. flexneri*, and is found almost exclusively in serotype 2a strains (Noriega *et al.*, 1995; Vargas *et al.*, 1999). This toxin is thought to contribute to watery diarrhoea (Fasano *et al.*, 1997; Fasano *et al.*, 1995) and as *S. flexneri* 2a shows high prevalence in the developing world, ShET2 may potentially play a role in communicability (Noriega, *et al.*, 1995). Notably, the *set1* genes were recently found to be encoded on the 47 kb *she* PAI, suggesting they too were acquired through horizontal transfer (Al-Hasani *et al.*, 2001; Rajakumar *et al.*, 1997b).

# PATHOGENICITY ISLANDS

*Shigella* and *E. coli* are known to share a high degree of nucleotide similarity, although there exist some obvious differences, both genetically and pathogenically. Many of these differences have been attributable to either the loss of genetic material, termed 'Black Holes' (Maurelli, *et al.*, 1998), or the addition of genetic material. Indeed, the *S. flexneri* 2a genome sequence indicates that 64 *Shigella*-islands are present, and are probably acquired from a variety of sources (Jin, *et al.*, 2002; Wei, *et al.*, 2003). Interestingly, many of the important *Shigella* virulence loci such as those encoding the Ipa-Mxi/Spa entry region, Shiga toxin and ShET2, appear to have been acquired via horizontal transfer. Even the large virulence plasmid itself is believed to be a composite of several PAIs (Parsot & Sansonetti, 1999). Thus, it must be considered that PAIs have played an important role in the development of virulence in *Shigella*. A number of other PAI-like elements carrying potential virulence loci, including SHI-2, SHI-3 and the *she* PAI have also been identified in *Shigella* spp. The *she* PAI, which carries the ShET2 genes, has also been found to carry other potential virulence factors such as the autotransporters Pic and SigA (Al-Hasani *et al.*, 2000; Henderson *et al.*, 1999). SHI-2

and SHI-3, which both encode the aerobactin iron acquisition siderophore system, exhibit many PAI-like qualities, though they may be considered to be iron transport islands rather than pathogenicity islands (Moss *et al.*, 1999; Purdy & Payne, 2001; Vokes *et al.*, 1999).

The term "pathogenicity island" was first used to describe large unstable DNA regions in uropathogenic *E. coli* (UPEC) (Blum *et al.*, 1994). However, since its inception, the term PAI has been expanded to refer to unstable chromosomal regions in a number of Gram positive and Gram negative species (Hacker *et al.*, 1997). Although the exact definition of what may be considered a PAI appears to be still evolving, some common features are apparent. These include carriage of virulence genes (such as haemolysins, iron transport systems, type III secretion systems, adhesins), occupation of often large chromosomal regions (10 - 200 kb), association with tRNAs, instability, carriage of mobility genes (such as IS elements and integrases), and differing G + C % compared with the host (Table 1.1) (Hacker, *et al.*, 1997; Hacker & Kaper, 2000).

Many PAI characteristics, such as association with tRNAs, instability, presence of integrases and differing G + C content show remarkable similarity to those of bacteriophages, thus leading to the suggestion that PAIs are acquired from different species via mechanisms resembling phage-mediated horizontal transfer (Hacker & Kaper, 1999). Reminiscent of prophages, PAIs are commonly arranged with short flanking direct repeats (DR) of 9 to 20 bp which are often identical to the 3' sequence of the target tRNA gene (Dozois & Curtiss III, 1999). Additionally, there is a growing number of PAIs that appear to delete precisely through the site-specific recombination of these DRs, including PAI $I_{536}$ and $II_{536}$ of UPEC (Blum, *et al.*, 1994), the *she* PAI of *S. flexneri* (Al-Hasani, *et al.*, 2001), and the HPI of *Y. pseudotuberculosis* (Buchrieser *et al.*, 1998a), thus suggesting a degree of relatedness between phages and PAIs. However, despite the wide distribution of PAIs and demonstration of deletion in some cases, PAI movement has only been demonstrated for the *V. cholerae* VPI, which is believed to be the genome of a prophage (VPIϕ) (Karaolis & Kaper, 1999; Karaolis *et al.*, 1999), the *Salmonella* SaPI, which is mobilised by a phage (Ruzin *et al.*, 2001), and the *V. cholerae* SXT-constin, which resembles a PAI in its integrated form, but is mobile as a conjugative self-transmissible circular element.

**Table 1.1** Pathogenicity and genomic islands of various pathogenic bacterial species.

| Organism | Island name | Genes harboured | Size (kb) | G + C % island (strain) | Deletion frequency | Target site (junction) | Reference |
|---|---|---|---|---|---|---|---|
| *E. coli* 536 UPEC | PAI $I_{536}$ | haemolysin | 70 | | | *selC* (16 bp DR) | (Blum, *et al.*, 1994) |
| *E. coli* 536 UPEC | PAI $II_{536}$ | haemolysin, P-fimbriae | 190 | | | *leuX* (18 bp DR | (Blum, *et al.*, 1994) |
| *E. coli* J96 UPEC | PAI $I_{J96}$ | haemolysin, P-fimbriae | 170 | | | *pheV* | (Swenson *et al.*, 1996) |
| *E. coli* J96 UPEC | PAI $II_{J96}$ | haemolysin, P-fimbriae, cytotoxic necrotising factor 1 (CTNF1)[a] | 110 | | | *pheR* (135 bp DR) | (Swenson, *et al.*, 1996) |
| *E. coli* CFT073 UPEC | PAI $I_{CFT073}$ | haemolysin, P-fimbriae | 58 | 42 (51) | | *metV* (9 bp DR) | (Guyer *et al.*, 1998) |
| *E. coli* E2348/69 EPEC | LEE PAI | type III secretion system, invasion | 35 | 38 (51) | | *selC* | (Elliott *et al.*, 1998; McDaniel & Kaper, 1997) |
| *E. coli* O157:H7 EHEC | LEE PAI | type III secretion system, invasion | 43 | 41 (51) | | *selC* | (Perna *et al.*, 1998) |
| *Shigella* spp. | Entry region | type III secretion system, invasion | 37 | 34 (51) | | plasmids | (Parsot & Sansonetti, 1999) |
| *S. flexneri* 2a | *she* PAI | ShET toxins, proteases | 47 | 49 (51) | $10^{-5} - 10^{-6}$ | *pheV* (22 bp DR) | (Al-Hasani, *et al.*, 2001; Rajakumar, *et al.*, 1997b) |
| *S. flexneri* 2a | SHI II | aerobactin, colicin V immunity | 24 | 49 (51) | | *selC* (no DR) | (Moss, *et al.*, 1999; Vokes, *et al.*, 1999) |
| *S. boydii* | SHI III | aerobactin | 21 | 51 (51) | ?stable | *pheU* | (Purdy & Payne, 2001) |

| Organism | Island name | Genes harboured | Size (kb) | G + C % island (strain) | Deletion frequency | Target site (junction) | Reference |
|---|---|---|---|---|---|---|---|
| *Y. enterocolitica* | HPI | yersiniabactin synthesis, transport | 43 | 51[b] (48) | $5 \times 10^{-7}$ (imprecise) | *asnT* (17 bp DR) | (Bach *et al.*, 1999; Buchrieser *et al.*, 1998b; Rakin *et al.*, 1999) |
| *Y. pseudotuberculosis* | HPI | yersiniabactin synthesis, transport | 36 | | $10^{-4}$ | *asnT, U, V* (17 bp DR) | (Buchrieser, *et al.*, 1998a; Rakin, *et al.*, 1999) |
| *Y. pestis* | HPI | yersiniabactin synthesis, transport, haemin storage | 102 | 46-56 (48) | $10^{-5}$ | *asnT* (IS*100*/17 bp DR) | (Buchrieser, *et al.*, 1998b; Fetherston *et al.*, 1992) |
| *S. enterica* | SPI-2 | type III secretion system, invasion into monocytes | 40 | 41-50[b] (52) | | *valV* | (Cirillo *et al.*, 1998; Hensel *et al.*, 1997; Hensel, *et al.*, 1998) |
| *S. enterica* | SPI-3 | invasion[a], survival in monocytes | 17 | 48 (52) | | *selC* | (Blanc-Potard *et al.*, 1999) |
| *S. enterica* | SPI-4 | invasion[a], survival in monocytes | 25 | 37-54 (52) | | ? tRNA | (Wong *et al.*, 1998) |
| *S. enterica* | SG1 | multi-drug resistance | 43 | 49 (52) | | *thdF* (18 bp DR) | (Boyd *et al.*, 2001; Boyd *et al.*, 2000) |
| *V. cholerae* | VPI | TCP-adhesin, regulator of virulence | 40 | 35 (48) | | *ssrA* (30 bp DR[a]) | (Karaolis & Kaper, 1999; Kovach *et al.*, 1996) |
| *H. pylori* | Cag PAI | type IV secretion, cag-antigen | 40 | 35 (41) | | *glr* (31 bp DR) | (Censini *et al.*, 1996; Covacci *et al.*, 1997) |

[a]information taken from (Hacker & Kaper, 2000)

[b]G + C % determined from partial sequence

# MULTIPLE ANTIBIOTIC RESISTANCE

## Historical Perspective

Antibiotic resistance in *Shigella* was first reported in Japan during the late 1940s, shortly after the introduction of sulfonamide (Su) derivatives as antibiotic therapy for dysentery. Subsequently, newer antibiotics such as streptomycin (Sm), chloramphenicol (Cm) and tetracycline (Tc) were employed as treatment for Su-resistant shigellae, but within four years, resistance to these antibiotics was also emerging. Strains resistant to either Sm or Tc were isolated as early as 1953, but multiple antibiotic resistance was not found until 1955 (Table 1.2) (Watanabe, 1963). This first example of a multiply resistant *Shigella* strain was isolated from a dysentery patient who had just returned from Hong Kong. Shortly after this time, strains multiply resistant to Sm, Cm and Tc were isolated with increasing frequency. Interestingly, multiple resistance to the antibiotics Sm, Cm and Tc arose quickly and became more common than other combinations of resistance, or even singly resistant shigellae (Table 1.2) (Watanabe, 1963).

**Table 1.2** Statistics of antibiotic-resistant *Shigella* in Japan (Watanabe *et al.*, 1963).

| Year | No. of strains tested | No. of strains resistant to: | | | |
|---|---|---|---|---|---|
| | | Sm | Tc | Cm | Sm, Cm & Tc |
| 1953 | 4,900 | 5 | 2 | 0 | 0 |
| 1954 | 4,876 | 11 | 0 | 0 | 0 |
| 1955 | 5,327 | 4 | 0 | 0 | 1 |
| 1956 | 4,399 | 8 | 4 | 0 | 0 |
| 1957 | 4,873 | 13 | 45 | 0 | 37 |
| 1958 | 6,563 | 18 | 20 | 0 | 193 |
| 1959 | 4,071 | 16 | 32 | 0 | 74 |
| 1960 | 3,396 | 29 | 36 | 0 | 308 |

Studies by Japanese investigators Akiba *et al.* (1960) and Ochiai (Pfeifer *et al.*, 1999; Watanabe, 1963) lead to the discovery that these resistances could be transmitted *in vitro* between *Shigella* and *E. coli* strains by a process requiring cell to cell contact. These findings were extended when it was demonstrated that multiple antibiotic resistance could be trasferred to almost every genus of the family *Enterobacteriaceae* by a process of cell to cell contact thought to be conjugation (Harada *et al.*, 1960; Nakaya *et al.*, 1960). These and other findings lead to the proposal that the resistance determinants were carried by some form of transmissible factor (Nakaya, *et al.*, 1960; Watanabe & Fukasawa, 1960).

## Resistance Plasmids

Resistance plasmids (R plasmids) confer resistance to antibiotics and various other inhibitors of growth, and are recognised in a wide range of Gram negative and Gram positive bacteria. They belong to a diverse family consisting of many incompatability groups (Brock *et al.*, 1994). The R plasmid NR1 (also called R100), is a 94.5 kb self-transmissible, multiple antibiotic resistance plasmid. It belongs to the incompatability grouping IncFII, and is the original R factor referred to by Nakaya *et al.* (1960) (Womble & Rownd, 1988). Consistent with the structure of IncFII R plasmids, NR1 is composed of two distinct genetic and functional domains: (i) the resistance transfer factor (RTF) which encodes the genes for self-transmissibility (*tra*) and autonomous replication (*rep*); and (ii) a resistance determinant (r-det) which is 23 kb and harbours the majority of the resistance genes (Fig. 1.5). The NR1 r-det is bounded by direct repeats of IS*1* and resembles a number of transposons, being transferable itself as Tn*2670* (Hanni *et al.*, 1982; Iida *et al.*, 1981). The NR1 r-det appears to be a composite of an intact copy of Tn*21* (which encodes mercury ion resistance (*mer*), spectimycin and streptomycin resistance (*aadA1*) and sulphonimde resistance (*sul1*) and a Tn*9*-like transposon which encodes chloramphenicol resitance (*catA1*) gene (Fig. 1.5) (Liebert *et al.*, 1999; Womble & Rownd, 1988). Interestingly, located in the RTF region of NR1 is the transposon Tn*10*, which encodes resistance to tetracycline (Fig. 1.5) (Womble & Rownd, 1988).

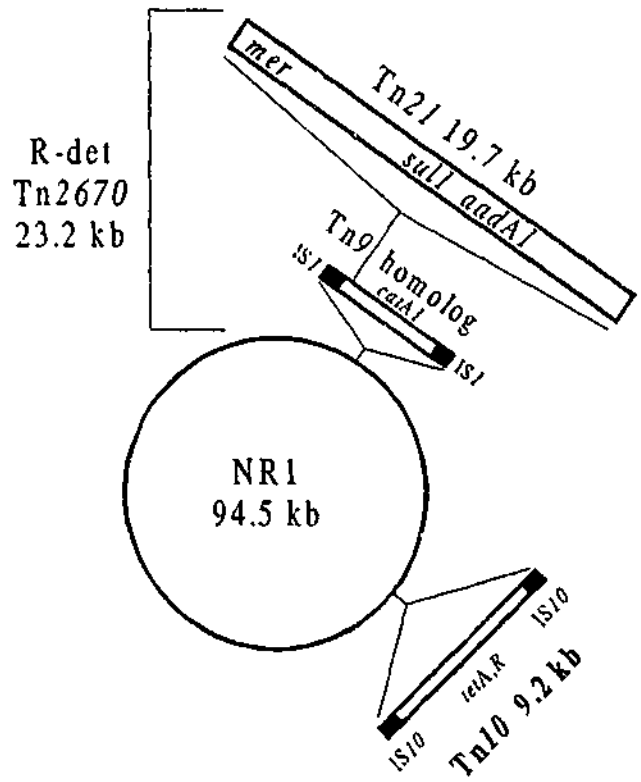



**Figure 1.5** Circular map of NR1. Inserts indicate mobile genetic elements (Liebert *et al.*, 1999).

## Multiple Antibiotic Resitance in *Shigella*

As discussed previously, antimicrobial therapy is among the most effective treatments known for dysentery; thus, multiple antibiotic resistance of *Shigella* poses a substantial threat. Since the first report of multiple antibiotic resistance in *Shigella* during the 1950s, levels of multiple antibiotic resistance have been on the increase. The patterns of antimicrobial resistance in *Shigella* in Bangladesh are representative of those in many developing countries (Sack, *et al.*, 1997), and Khan *et al.* (1985) demonstrated a clear increase in resistance to antibiotics such as Tc, Cm and Sm over a 14 year period. Indeed, a clinical study in Brazil during 1988 to 1993 reported that most *Shigella* isolates were resistant to four or more antimicrobial agents (Lima *et al.*, 1995). Similarly, characterisation of *S. flexneri* strains in Somalia during 1983, 1984, 1988, and 1989 revealed that all but three of 112 strains were resistant at least to spectinomycin (Sp), ampicillin (Ap), Cm and Tc (Casalino *et al.*, 1994).

In many clinical investigations, multiple antibiotic resistance is found to be transferrable to antibiotic sensitive strains by conjugation, and is thus believed to be due to the presence of R plasmids. However, the presence of resistance determinants that are neither transferable nor mobilisable has also been identified in *Shigella* (Casalino, *et al.*, 1994; Gebre-Yohannes & Drasar, 1990; Ling *et al.*, 1993; Navia *et al.*, 1999). Indeed, Ling *et al.* (1993) reported that one of the most common *Shigella* resistance profiles, resistance to Sm, Ap, Cm and Tc, is almost always non-transferable. These non-transferable resistances are generally considered to be chromosomally encoded, and may have arisen following transposon integration. However, despite the prevalence of non-transferable resistance, the genetic basis for chromosomal resistance in *Shigella* has not been widely investigated.

# THE 99 KB DELETABLE ELEMENT

In 1997, Rajakumar *et al.* reported the identification of a chromosomal multi-antibiotic resistance locus in *S. flexneri* 2a strain YSH6000. This locus, which has since been designated the *Shigella* resistance locus (SRL), was found to encode resistance to Sm,

Ap, Cm and Tc, and exhibited both organisational and sequence similarity to corresponding regions of NR1. However, the SRL exhibited several differences from NR1, including the insertion of a $\beta$-lactamase encoding *oxa1* cassette, the absence of the sulfonamide and mercury resistance determinants, and a substantial deletion between the Cm and Tc resistance determinants (Rajakumar *et al.*, 1997a).

The SRL was known to be carried on the *S. flexneri* 2a YSH6000 chromosome in a spontaneously deletable 99 kb region. Both the YSH6000 derivatives S2430 and YSH600T exhibited a 99 kb deletion in the chromosome which coincided with a loss of antibiotic resistance to Sm, Ap, Cm and Tc. An SRL-specific probe indicated that this deletion was responsible for the loss of resistance (Rajakumar *et al.*, 1996). The 99 kb region, which has since been designated the multiple-antibiotic resistance deletable element (MRDE), was mapped to *Not*I fragment D, bounded by the *S. flexneri* homologues of *ompA* and *pyrC* (Rajakumar, *et al.*, 1996). Further sequence analysis indicated that deletion of the MRDE in YSH6000 and S2430 occurred at the *S. flexneri* homologous of *putA* and *mdoA*, possibly through flanking IS elements (Turner, 1998). Sample sequencing of the MRDE revealed that in addition to the SRL, it contained an intact ferric dicitrate transport system (*fec*), and appeared to consist both of chromosomal DNA, and a foreign element deemed the SRL PAI inserted probably at the *serX* tRNA gene (Luck, 1998; Turner, 1998).

# OUTLINE OF THESIS

The research presented in **Chapter 2** initially focuses on characterising, and demonstrating the reproducibility of the deletion of the MRDE from the YSH6000 chromosome. Subsequently, selection for $Tc^S$ derivatives of YSH6000 was used to explore the independent deletion of the SRL PAI and the SRL. The role of the SRL PAI P4-like integrase gene (*int*) in deletion of the SRL PAI was also investigated. **Chapter 3** presents data on the mechanism of SRL PAI integration. The role of the SRL PAI *attP* and *int* in the integration of a small circular form of the PAI into the target sequences *serX* and *serW* was also examined. Data presented in **Chapter 4** include a survey of 71 *Shigella* isolates and 28 other enteric pathogens. PCR and Southern hybridisation were used to examine clinical strains for the presence of the SRL and SRL PAI markers, and PCR linkage studies were used to determine the relatedness between these elements and the SRL PAI.

# REFERENCES


**Ackerknecht, E. H. (1965).** *History and geography of the most important diseases.* New York: Hafner Publishing Company, Inc.

**Adler, B., Sasakawa, C., Tobe, T., Makino, S., Komatsu, K. & Yoshikawa, M. (1989).** A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. *Molecular Microbiology* **3**, 627 - 635.

**Akiba, T., Koyama, K., Ishiki, Y., Kimura, S. & Fukushima, T. (1960).** On the mechanisms of the development of nultiple-drug-resistant clones of *Shigella*. *Japanese Journal of Microbiology* **4**, 219 - 227.

**Albert, M. J., Singh, K. V., Murray, B. E. & Erlich, J. (1990).** Molecular epidemiology of shigella infection in Central Australia. *Epidemiology and Infection.* **105**, 51-57.

**Alexander, W. A., Hartman, A. B., Oaks, E. V. & Venkatesan, M. M. (1996).** Construction and characterization of *virG* (*icsA*)-deleted *Escherichia coli* K12-*Shigella flexneri* hybrid vaccine strains. *Vaccine* **14**, 1053 - 1061.

**Al-Hasani, K., Henderson, I. R., Sakellaris, H., Rajakumar, K., Grant, T., Nataro, J. P., Robins-Browne, R. & Adler, B. (2000).** The sigA gene which Is borne on the *she* pathogenicity island of *Shigella flexneri* 2a encodes an exported cytopathic protease involved in intestinal fluid accumulation. *Infection and Immunity* **68**, 2457 - 2463.

**Al-Hasani, K., Rajakumar, K., Bulach, D., Robins-Browne, R., Adler, B. & Sakellaris, H. (2001).** Genetic organization of the *she* pathogenicity island in *Shigella flexneri* 2a. *Microbial Pathogenesis* **30**, 1 - 8.

**Allaoui, A., Menard, R., Sansonetti, P. J. & Parsot, C. (1993a).** Characterization of the *Shigella* flexneri *ipgD* and *ipgF* genes, which are located in the proximal part of the mxi locus. *Infection and Immunity* **61**, 1707 - 1714.

**Allaoui, A., Sansonetti, P. J. & Parsot, C. (1993b).** MxiD, an outer membrane protein necessary for the secretion of the *Shigella flexneri* Ipa invasins. *Molecular Microbiology* 7, 59 - 68.

**Altboum, Z., Barry, E. M., Losonsky, G., Galen, J. E. & Levine, M. M. (2001).** Attenuated *Shigella flexneri* 2a *ΔguaBA* strain CVD 1204 expressing enterotoxigenic *Escherichia coli* (ETEC) CS2 and CS3 fimbriae as a live mucosal vaccine against *Shigella* and ETEC infection. *Infection and Immunity* **69**, 3150 - 3158.

**Anderson, R. J., Pasetti, M. F., Sztein, M. B., Levine, M. M. & Noriega, F. R. (2000).** Δ*guaBA* attenuated *Shigella flexneri* 2a strain CVD 1204 as a *Shigella* vaccine and as a live mucosal delivery system for fragment C of tetanus toxin. *Vaccine* **18**, 2193 - 2202.

**Andrews, G. P. & Maurelli, A. T. (1992).** MxiA of *Shigella flexneri* 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low-calcium-response protein, LcrD, of *Yersinia pestis*. *Infection and Immunity* **60**, 3287-95.

**Bach, S., Buchrieser, C., Prentice, M., Guiyoule, A., Msadek, T. & Carniel, E. (1999).** The high-pathogenicity island of *Yersinia enterocolitica* Ye8081 undergoes low-frequency deletion but not precise excision, suggesting recent stabilization in the genome. *Infection and Immunity* **67**, 5091 - 5099.

**Barzu, S., Arondel, J., Guillot, S., Sansonetti, P. J. & Phalipon, A. (1998).** Immunogenicity of IpaC-hybrid proteins expressed in the *Shigella flexneri* 2a vaccine candidate SC602. *Infection and Immunity* **66**, 77 - 82.

**Barzu, S., Benjelloun-Touimi, Z., Phalipon, A., Sansonetti, P. & Parsot, C. (1997).** Functional analysis of the *Shigella flexneri* IpaC invasin by insertional mutagenesis. *Infection and Immunity* **65**, 1599 - 1605.

**Barzu, S., Fontaine, A., Sansonetti, P. & Phalipon, A. (1996).** Induction of a local anti-IpaC antibody response in mice by use of a *Shigella flexneri* 2a vaccine candidate: implications for use of IpaC as a protein carrier. *Infection and Immunity* **64**, 1190 - 1196.

**Benenson, A. S. (1995).** Shigellosis. In *Contol of Communicable Disease Manual*, pp. 421 - 425. Edited by A. S. Beneson. Washington D.C.: American Public Health Authority.

**Benjelloun-Touimi, Z., Si Tahar, M., Montecucco, M., Sansonetti, P. J. & Parsot, C. (1998).** SepA, the 110 kDa protein secreted by *Shigella flexneri*: two-domain structure and proteolytic activity. *Microbiology* **144**, 1815 - 1822.

**Bennish, M. L. (1991).** Potentially lethal complications of shigellosis. *Reviews of Infectious Diseases* **13 Suppl 4**, S319 - S324.

**Bensted, H. J. (1956).** Dysentery bacilli - *Shigella*. A brief historical review. *Canadian Journal of Microbiology* **2**, 163 - 174.

**Berlutti, F., Casalino, M., Zagaglia, C., Fradiani, P. A., Visca, P. & Nicoletti, M. (1998).** Expression of the virulence plasmid-carried apyrase gene (*apy*) of enteroinvasive *Escherichia coli* and *Shigella flexneri* Is under the control of H-NS and the VirF and VirB regulatory cascade. *Infection and Immunity* **66**, 4957 - 4964.

**Bernardini, M. L., Fontaine, A. & Sansonetti, P. J. (1990).** The two-component regulatory system ompR-envZ controls the virulence of *Shigella flexneri*. *Journal of Bacteriology* **172**, 6274 - 6281.

**Bernardini, M. L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M. & Sansonetti, P. J. (1989).** Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 3867 - 3871.

**Bernardini, M. L., Sanna, M. G., Fontaine, A. & Sansonetti, P. J. (1993).** OmpC is involved in invasion of epithelial cells by *Shigella flexneri*. *Infection and Immunity* **61**, 3625 - 3635.

**Blanc-Potard, A.-B., Solomon, F., Kayser, J. & Groisman, E. A. (1999).** The SPI-3 pathogenicity island of *Salmonella enterica*. *Journal of Bacteriology* **181**, 998 - 1004.

**Blocker, A., Gounon, P., Larquet, E., Niebuhr, K., Cabiaux, V., Parsot, C. & Sansonetti, P. (1999).** The tripartite type III secretion of *Shigella flexneri* inserts IpaB and IpaC into host membranes. *The Journal of Cell Biology* **147**, 683 - 693.

**Blocker, A., Jouihri, N., Larquet, E., Gounon, P., Ebel, F., Parsot, C., Sansonetti, P. & Allaoui, A. (2001).** Structure and composition of the *Shigella flexneri* "needle complex", a part of its type III secretion. *Molecular Microbiology* **39**, 652 - 663.

**Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H. & Hacker, J. (1994).** Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. *Infection and Immunity* **62**, 606 - 614.

**Bourdet-Sicard, R., Rudiger, M., Sansonetti, P. J. & Tran Van Nhieu, G. (1999).** Vinculin is unfolded by the *Shigella* protein IpaA, and the complex promotes F-actin depolymerization. *EMBO Journal* **18**, 5853 - 5862.

**Boyd, D., Peters, G. A., Cloeckaert, A., Boumedine, K. S., Chaslus-Dancla, E., Imberechts, H. & Mulvey, M. R. (2001).** Complete nucleotide sequence of a 43-Kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *Journal of Bacteriology* **183**, 5725 - 5732.

**Boyd, D. A., Peters, G. A., Ng, L.-K. & Mulvey, M. R. (2000).** Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhymurium DT104. *FEMS Microbiology Letters* **189**, 285 - 291.

**Brenner, D. J. (1984).** Enterobacteriaceae. In *Bergey's Manual of Systematic Bacteriology*, pp. 408-481. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

**Brenner, D. J., Fanning, G. R., Johnson, K. E., Citarella, R. V. & Falkow, S. (1969).** Polynucleotide sequence relationships among members of *Enterobacteriaceae*. *Journal of Bacteriology* **98**, 637 - 650.

**Brenner, D. J., Fanning, G. R., Skerman, F. J. & Falkow, S. (1972).** Polynucleotide sequence divergence among strains of *Escherichia coli* and slosely related organisms. *Journal of Bacteriology* **109**, 953 - 965.

**Brock, T. D., Madigan, M. T., Martinko, J. M. & Parker, J. (1994).** *Biology of microorganisms*, 7th edn. Englewood Cliffs: Prentice-Hall.

**Buchrieser, C., Brosch, R., Bach, S., Guiyoule, A. & Carniel, E. (1998a).** The high-pathogenicity island of *Yersinia pseudotuberculosis* can be inserted into any of the three chromosomal *asn* tRNA genes. *Molecular Microbiology* **30**, 965 - 978.

**Buchrieser, C., Glaser, P., Rusniok, C., Nedjari, H., d'Hauteville, H., Kunst, F., Sansonetti, P. & Parsot, C. (2000).** The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Molecular Microbiology* **38**, 760 - 771.

**Buchrieser, C., Prentice, M. & Carniel, E. (1998b).** The 102-Kilobase unstable region of *Yersinia pestis* comprises a High-Pathogenicity island linked to a pigmentation segment which undergoes internal rearrangement. *Journal of Bacteriology* **180**, 2321 - 2329.

**Casalino, M., Nicoletti, M., Salvia, A., Colonna, B., Pazzani, C., Calconi, A., Mohamud, K. A. & Maimone, F. (1994).** Characterization of endemic *Shigella flexneri* strains in Somalia: antimicrobial resistance, plasmid profiles, and serotype correlation. *Journal of Clinical Microbiology* **32**, 1179 - 1183.

**Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. & Covacci, A. (1996).** *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 14648 - 14653.

**Chakrabarti, M., Battacharya, J., Battacharya, M., Nair, G., Battacharya, S. & Mahalanabis, D. (1999).** Killed oral *Shigella* vaccine made from *Shigella flexneri* 2a protects against challenge in the rabbit model of shigellosis. *Acta Paediatrica* **88**, 161-165.

**Chen, Y., Smith, M. R., Thirumalai, K. & Zychlinsky, A. (1996).** A bacterial invasin induces macrophage apoptosis by binding directly to ICE. *EMBO Journal* **15**, 3853 - 3860.

**Chu, C. Y., Liu, B. K., Watson, D., Szu, S. S., Bryla, D., Shiloach, J., Schneerson, R. & Robbins, J. B. (1991).** Preparation, characterization, and immunogenicity of conjugates composed of the O-specific polysaccharide of *Shigella dysenteriae* type 1 (Shiga's bacillus) bound to tetanus toxoid. *Infection and Immunity* **59**, 4450 - 4458.

**Cirillo, D. M., Valdivia, R. H., Monack, D. M. & Falkow, S. (1998).** Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Molecular Microbiology* **30**, 175 - 188.

**Cohen, D., Ashkenazi, S., Green, M. S., Gdalevich, M., Robin, G., Slepon, R., Yavzori, M., Orr, N., Block, C., Ashkenazi, I., Shemer, J., Taylor, D. N., Hale, T. L., Sadoff, J. C., Pavliakova, D., Schneerson, R. & Robbins, J. B. (1997).** Double-blind vaccine-controlled randomised efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults. *Lancet* **349**, 155 - 159.

**Cohen, D., Green, M. S., Block, C., Slepon, R. & Ofek, I. (1991).** Prospective study of the association between serum antibodies to lipopolysaccharide O Antigen and the attack rate of Shigellosis. *Journal of Clinical Microbiology* **29**, 386 - 389.

**Coster, T. S., Hoge, C. W., van de Verg, L. L., Hartman, A. B., Oaks, E. V., Venkatesan, M. M., Cohen, D., Robin, G., Fontaine-Thompson, A., Sansonetti, P. J. & Hale, T. L. (1999).** Vaccination against Shigellosis with attenuated *Shigella flexneri* 2a strain SC602. *Infection and Immunity* **67**, 3437 - 3443.

**Covacci, A., Falkow, S., Berg, D. E. & Rappuoli, R. (1997).** Did the inheritance of a pathogenicity island modify the virulence of *Helicobacter pylori*? *Trends in Microbiology* **5**, 205 - 208.

**Day Jr, W. A. & Maurelli, A. T. (2001).** *Shigella flexneri* LuxS quorum-sensing system modulates *virB* expression but is not essential for virulence. *Infection and Immunity* **69**, 15 - 23.

**de Geyter, C., Vogt, B., Benjelloun-Touimi, Z., Sansonetti, P. J., Ruysschaert, J.-M., Parsot, C. & Cabiaux, V. (1997).** Purification of IpaC, a protein involved in entry of *Shigella flexneri* into epithelial cells and characterization of its interaction with lipid membranes. *FEBS Letters* **400**, 149 - 154.

**Donnenberg, M. S., Kaper, J. B. & Finlay, B. B. (1997).** Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *Trends in Microbiology* **5**, 109 - 114.

**Dorman, C. J. & Porter, M. E. (1998).** The Shigella virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. *Molecular Microbiology* **29**, 677 - 684.

**Dozois, C. M. & Curtiss III, R. (1999).** Pathogenic diversity of *Escherichia coli* and the emergence of 'exotic' islands in the gene stream. *Veterinary Research* **30**, 157 - 179.

**DuPont, H. L. (1990).** *Shigella* species (bacillary dysentery). In *Principles and practice of infectious diseases*, pp. 1716-1722. Edited by G. L. Mandell, G. D. Jr. & J. E. Bennett. New York: Churchill Livingstone Inc.

**DuPont, H. L., Levine, M. M., Hornick, R. B. & Formal, S. B. (1989).** Inoculum size in shigellosis and implications for expected mode of transmission. *Journal of Infectious Diseases* **159**, 1126-1128.

**Egile, C., d'Hauteville, H., Parsot, C. & Sansonetti, P. J. (1997).** SopA, the outer membrane protease responsible for polar localization of IcsA in *Shigella flexneri*. *Molecular Microbiology* **23**, 1063 - 1073.

**Egile, C., Loisel, T. P., Laurent, V., Li, R., Pantaloni, D., Sansonetti, P. J. & Carlier, M.-F. (1999).** Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *The Journal of Cell Biology* **146**, 1319 - 1332.

**Elliott, S. J., Wainwright, L. A., McDaniel, T. K., Lai, L., McNamara, B. P., Donnenberg, M. S. & Kaper, J. B. (1998).** The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escerichia coli* E2348/69. *Molecular Microbiology* **28**, 1 - 4.

**Ewing, W. H. (1949).** Shigella nomenclature. *Journal of Bacteriology* **57**, 633 - 638.

**Falkow, S. & Mekalanos, J. (1990).** The enteric bacilli and vibrios. In *Microbiology*, pp. 561 - 587. Edited by B. D. Davis, R. Dulecco, H. N. Eisen & H. S. Ginsberg. Philadelphia: J. B. Lippincott Company.

**Farmer III, J. J. (1995).** *Enterobactericaceae*: introduction and identification. In *Manual of Clinical Microbiology*, pp. 438 - 449. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Yolken. Washington, D. C.: ASM Press.

**Faruque, S. M., Khan, R., Kamruzzaman, M., Yamasaki, S., Ahmad, Q. S., Azim, T., Nair, G. B., Takeda, Y. & Sack, D. A. (2002).** Isolation of *Shigella dysenteriae* type 1 and *S. flexneri* strains from surface waters in Bangladesh: comparative molecular analysis of environmental *Shigella* isolates versus clinical strains. *Applied and Environmental Microbiology* **68**, 3908 - 3913.

**Fasano, A., Noriega, F. R., Liao, F. M., Wang, W. & Levine, M. M. (1997).** Effect of *Shigella* enterotoxin 1 (ShET1) on rabbit intestine in vitro and in vivo. *Gut* **40**, 505 - 511.

**Fasano, A., Noriega, F. R., Maneval, D. R., Jr., Chanasongcram, S., Russell, R., Guandalini, S. & Levine, M. M. (1995).** *Shigella* enterotoxin 1: an enterotoxin of *Shigella flexneri* 2a active in rabbit small intestine in vivo and in vitro. *Journal of Clinical Investigation* **95**, 2853 - 2861.

**Fernandez-Prada, C., Hoover, D. L., Tall, B. D. & Venkatesan, M. M. (1997).** Human monocyte-derived macrophages infected with virulent *Shigella flexneri* in vitro undergo a rapid cytolytic event similar to oncosis but not apoptosis. *Infection and Immunity* **65**, 1486 - 1496.

**Fernandez-Prada, C. M., Hoover, K. L., Tall, B. D., Hartman, A. B., Kopelowitz, J. & Venkatesan, M. M. (2000).** *Shigella flexneri* IpaH7.8 facilitates escape of virulent bacteria from the endocytic vacuoles of mouse and human macrophages. *Infection and Immunity* **68**, 3608 - 3619.

**Fetherston, J. D., Schuetze, P. & Perry, R. D. (1992).** Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. *Molecular Microbiology* **6**, 2693 - 2704.

**Finch, M., Rodey, G., Lawrence, D. & Blake, P. (1986).** Epidemic reiter's syndrome following an outbreak of Shigellosis. *European Journal of Epidemiology* **2**, 26 - 30.

**Find (1992).** Enterobactericacea: *Salmonella* and *Shigella*, intestinal pathogens. In *Zinsser microbiology*, pp. 556 - 565. Edited by W. K. Joklik, H. P. Willett, d. B. Amons & C. M. Wilfert. Connecticut: Appleton & Lange.

**Fontaine, A., Arondel, J. & Sansonetti, P. J. (1988).** Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a Tox- mutant of *Shigella dysenteriae* 1. *Infection and Immunity* **56**, 3099 - 3109.

**Formal, S. B., Maenza, R. M., Austin, S. & LaBrec, E. H. (1967).** Failure of parenteral vaccines to protect monkeys against experimental shigellosis. *Proceedings of the Society for Experimental Biology and Medicine* **125**, 347 - 349.

**Formal, S. B., Oaks, E. V., Olsen, R. E., Wingfield-Eggleston, M., Snoy, P. J. & Cogan, J. P. (1991).** Effect of prior infection with virulent *Shigella flexneri* 2a on the resistance of monkeys to subsequent infection with *Shigella sonnei*. *Journal of Infectious Diseases* **164**, 533 - 537.

**Fujii, J., Kinoshita, Y., Yutsudo, T., Taniguchi, H., Obrig, T. & Yoshida, S.-I. (2001).** Toxicity of Shiga toxin 1 in the central nervous system of rabbits. *Infection and Immunity* **69**, 6545 - 6548.

**Fukushima, M., Kakinuma, K. & Kawaguchi, R. (2002).** Phylogenetic analysis of *Salmonella*, *Shigella*, and *Escherichia coli* strains on the basis of the *gyrB* gene sequence. *Journal of Clinical Microbiology* **40**, 2779 - 2785.

**Gaudio, P. A., Sethabutr, O., Echeverria, P. & Hoge, C. W. (1997).** Utility of a polymerase chain reaction diagnsotic system in a study of the epidemiology of shigellosis among dysentery patients, family contacts, and well controls living in a shigellosis-endemic area. *Journal of Infectious Diseases* **176**, 1013 - 1018.

**Gebre-Yohannes, A. & Drasar, B. S. (1990).** Plasmid profiles of antibiotic-resistance Shigella dysenteriae types 2, 3, 4, 6 and 7 isolated in Ethiopia during 1976 - 85. *Epidemiology and Infection.* **105**, 65 - 72.

**Gorden, J. & Small, P. (1990).** Quantitative analysis of the effects of variable pH on the growth of *Shigella flexneri*, *Salmonella typhimurium*, and entero-invasive *Escherichia coli*. *Abstracts of the 90th Annual Meeting of the American Society of Microbiology*, 64.

**Gorden, J. & Small, P. L. C. (1993).** Acid resistance in enteric bacteria. *Infection and Immunity* **61**, 364 - 367.

**Gray, L. D. (1995).** *Escherichia, Salmonella, Shigella,* and *Yersinia.* In *Manual of Clinical Microbiology*, pp. 450 - 456. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Yolken. Washington, D. C.: ASM Press.

**Guan, S. & Verma, N. K. (1998).** Serotype conversion of a *Shigella flexneri* candidate vaccine strain via a novel site-specific chromosome-integration system. *FEMS Microbiology Letters* **166**, 79 - 87.

**Guichon, A., Hersh, D., Smith, M. R. & Zychlinsky, A. (2001).** Structure-function analysis of the *Shigella* virulence factor IpaB. *Journal of Bacteriology* **183**, 1269 - 1276.

**Guyer, D. M., Kao, J. S. & Mobley, H. L. (1998).** Genomic analysis of a pathogenicity island in uropathogenic Escherichia coli CFT073: distribution of homologous sequences among isolates from patients with pyelonephritis, cystitis, and Catheter-associated bacteriuria and from fecal samples. *Infection and Immunity* **66**, 4411 - 4417.

**Hacker, J., Blum-Oehler, G., Muhldorfer, I. & Tschape, H. (1997).** Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Molecular Microbiology* **23**, 1089 - 1097.

**Hacker, J. & Kaper, J. B. (1999).** The concept of pathogenicity islands. In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 1 - 11. Edited by J. B. Kaper & J. Hacker. Washington: American Society for Microbiology.

**Hacker, J. & Kaper, J. B. (2000).** Pathogenicity islands and the evolution of microbes. *Annual Review of Microbiology* **54**, 641 - 679.

**Hale, T. L. & Bonventre, P. F. (1979).** *Shigella* infection of Henle intestinal epithelial cells: Role of the bacterium. *Infection and Immunity* **24**, 879 - 886.

**Hale, T. L. & Keren, D. F. (1992).** Pathogenesis and immunology in shigellosis: applications for vaccine development. *Current Topics in Microbiology and Immunology* **180**, 117 - 137.

**Hale, T. L., Morris, R. E. & Bonventre, P. F. (1979).** *Shigella* infection of henle intestinal epithelial cells: role of the host cell. *Infection and Immunity* **24**, 887 - 894.

**Haltalin, K. C., Nelson, J. D., Ring, R. D., Sladoje, M. & Hinton, L. V. (1967).** Double-blind treatment study of shigellosis comparing ampicillin, sulfadiazine, and placebo. *Journal of Pediatrics* **70**, 970 - 981.

**Hanni, C. M., J., Iida, S. & Arber, W. (1982).** Occurrence and properties of composite transposon Tn*2672*: evolution of multiple drug resistance transposons. *Journal of Bacteriology* **150**, 1266 - 1273.

**Harada, K., Suzuki, M., Kameda, M. & Mitsuhashi, S. (1960).** On the drug-resistance of enteric bacteria: 2) transmission of the drug-resistance among Enterobacteriaceae. *Japanese Journal of Microbiology* **30**, 289 - 299.

**Harrington, A. T., Hearn, P. D., Picking, W. L., Barker, J. R., Wessel, A. & Picking, W. D. (2003).** Structural characterization of the N terminus of IpaC from *Shigella flexneri*. *Infection and Immunity* **71**, 1255 - 1264.

**Hartman, A. B., Van de Verg, L. L. & Venkatesan, M. M. (1999).** Native and mutant forms of cholera toxin and heat-labile enterotoxin effectively enhnace protective efficacy of live Attenuated and heat-killed *Shigella* vaccines. *Infection and Immunity* **67**, 5841 - 5847.

**Henderson, I. R., Czeczulin, J., Eslava, C., Noriega, F. & Nataro, J. P. (1999).** Characterization of Pic, a secreted protease of *Shigella flexneri* and enteroaggregative *Escherichia coli*. *Infection and Immunity* **67**, 5587 - 5596.

**Henderson, I. R., Navarro-Garcia, F. & Nataro, J. P. (1998).** The great escape: structure and unction of the autotransporter proteins. *Trends in Microbiology* **6**, 370 - 378.

**Hensel, M., Shea, J. E., Baumler, A. J., Gleeson, C., Blattner, F. & Holden, D. W. (1997).** Analysis of the boundaries of *Salmoella* pathogenicity island 2 and the corresponding chromosomal region of *Escherichia coli* K-12. *Journal of Bacteriology* **179**, 1105 - 1111.

**Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vazquez-Torres, A., Gleeson, C., Fang, F. C. & Holden, D. W. (1998).** Genes encoding putative effector proteinsof the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Molecular Microbiology* **30**, 163 - 174.

**Herrington, D. A., Van De Verg, L., Formal, S. B., Hale, T. L., Tall, B. D., Cryz, S. J., Tramont, E. C. & Levine, M. M. (1990).** Studies in volunteers to evaluate candidate *Shigella* vaccines: further experience with a bivalent *Salmonella typi-Shigella sonnei* vaccine and protection conferred by previous *Shigella sonnei* disease. *Vaccine* **8**, 353 - 357.

**High, N., Mounier, J., Prevost, M. C. & Sansonetti, P. J. (1992).** IpaB of *Shigella flexneri* causes entry into epithelial cells and escape from the phagocytic vacuole. *EMBO Journal* **11**, 1991 - 1999.

**Hilbi, H., Moss, J. E., Hersh, D., Chen, Y., Arondel, J., Banerjee, S., Flavell, R. A., Yuan, J., Sansonetti, P. J. & Zychlinsky, A. (1998).** Shigella-induced apoptosis is dependent on Caspase-1 which binds to IpaB. *The Journal of Biological Chemistry* **273**, 32895 - 32900.

**Hilbi, H., Puro, R. J. & Zychlinksy, A. (2000).** Tripeptidyl peptidase II promotes maturation of caspase-1 in *Shigella flexneri*-induced macrophage apoptosis. *Infection and Immunity* **68**, 5502 - 5508.

**Hurley, B. P., Thorpe, C. M. & Acheson, D. W. K. (2001).** Shiga toxin translocation across intestinal epithelial cells Is enhanced by neutrophil transmigration. *Infection and Immunity* **69**, 6148 - 6155.

**Iida, S., Hanni, C., Echarti, C. & Arber, W. (1981).** Is the IS1-flanked r-determinant of the R plasmid NR1 a transposon. *Journal of General Microbiology* **126**, 413 - 425.

**Islam, D., Bardhan, P. K., Lindberg, A. A. & Christensson, B. (1995).** *Shigella* infection induces cellular activation of T and B cells and distinct species-related changes in peripheral blood lymphocyte subsets during the course of the disease. *Infection and Immunity* **63**, 2941 - 2949.

**Islam, D., Wretlind, B., Hammarstrom, L., Christensson, B. & Lindberg, A. A. (1996).** Semiquantitative estimation of *Shigella* antigen-specific antibodies: correlation with disease severity during shigellosis. *APMIS* **104**, 563 - 574.

**Jepson, M. A. & Clark, M. A. (1998).** Studying M cells and their role in infection. *Trends in Microbiology* **6**, 359 - 365.

**Jin, Q., Yuan, Z., Xu, J., Wang, Y., Shen, Y., Lu, W., Wang, J. Y., Liu, H., Yang, J., Yang, F., Zhang, X., Zhang, J., Yang, G., Wu, H., Qu, D., Dong, J., Sun, L., Xue, Y., Zhao, A., Gao, Y., Zhu, J., Kan, B., Ding, K., Chen, S., Cheng, H., Yao, Z., He, B., Chen, R., Ma, D., Qiang, B., Wen, Y., Hou, Y. & Yu, J. (2002).** Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. *Nucleic Acids Research* **30**, 4432 - 4441.

**Jung, H. C., Eckmann, L., Yang, S.-K., Pamja, A., Fierer, J., Morzycka-Wroblewska, E. & Kagnoff, M. F. (1995).** A dinstinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *The Journal of Clinical Investigation* **95**, 55 - 65.

**Kane, C. D., Schuch, R., Day Jr, W. A. & Maurelli, A. T. (2002).** MxiE regulates intracellular expression of factors secreted by the *Shigella flenxeri* 2a type III secretion system. *Journal of Bacteriology* **184**, 4409 - 4419.

**Karaolis, D. K. R. & Kaper, J. B. (1999).** Pathogenicity islands and other mobile virulence elements of *Vibrio cholerae*. In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 167 - 187. Edited by J. B. Kaper & J. Hacker. Washington: American Society for Microbiology.

**Karaolis, D. K. R., Somara, S., Maneval Jr, D. R., Johnson, J. A. & Kaper, J. B. (1999).** A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature* **399**, 375 - 379.

**Karnell, A., Stocker, B. A. D., Katakura, S., Reinholt, F. P. & Lindberg, A. A. (1992).** Live oral auxotrophic *Shigella flexneri* SFL124 vaccine with a deleted *aroD* gene: Characterization and monkey protection studies. *Vaccine* **10**, 398 - 394.

**Keren, D. F., Holt, P. S., Collins, H. H., Gemski, P. & Formal, S. B. (1978).** The role of Peyer's patches in the local immune response of rabbit ileum to live bacteria. *Journal of Immunology* **120**, 1892 - 1896.

**Keusch, G. T. & Bennish, M. L. (1991).** Shigellosis. In *Bacterial Infections of Humans - Epidemiology and Control*, pp. 593 - 620. Edited by A. S. Evans & P. S. Brachman. New York: Plenum Medical Book Company.

**Khan, M. U. (1985).** Fourteen years of shigellosis in Dhaka: an epidemiological analysis. *International Journal of Epidemiology* **14**, 607 - 613.

**Koprowski II, H., Levine, M. M., Anderson, R. J., Losonsky, G., Pizza, M. & Barry, E. M. (2000).** Attenuated *Shigella flexneri* 2a vaccine strain CVD 1204 expressing colonization factor antigen I and mutant heat-labile enterotoxin of Enterotoxigenic *Escherichia coli*. *Infection and Immunity* **68**, 4884 - 4892.

**Kotloff, D. L., Herrington, D. A., Hale, T. L., Newland, J. W., van de Verg, L., Cogan, J. P., Snoy, P. J., Sadoff, J. C., Formal, S. B. & Levine, M. M. (1992).** Safety, immunogenicity, and efficacy in monkeys and humans of invasive *Escherichia*

*coli* K-12 hybrid vaccine candidates expressing *Shigella flexneri* 2a somatic antigen. *Infection and Immunity* **68**, 2218 - 2224.

**Kotloff, K. L., Noriega, F., Losonsky, G. A., Sztein, M. B., Wasserman, S. S., Nataro, J. P. & Levine, M. M. (1996).** Safety, immunogenicity, and transmissibility in humans of CVD 1203, a live oral *Shigella flexneri* 2a vaccine candidate attenuated by deletions in aroA and virG. *Infection and Immunity* **64**, 4542 - 4548.

**Kotloff, K. L., Noriega, F. R., Samandari, T., Sztein, M. B., Losonsky, G. A., Nataro, J. P., Picking, W. D., Barry, E. M. & Levine, M. M. (2000).** *Shigella flexneri* 2a strain CVD 1207, with specific deletions in *virG, sen, set*, and *guaBA*, Is highly attenuated in humans. *Infection and Immunity* **68**, 1304 - 1039.

**Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Swerdlow, D. L., Sansonetti, P. J., Adak, G. K. & Levine, M. M. (1999).** Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bulletin of the World Health Organization* **77**, 651 - 665.

**Kovach, M. E., Shaffer, M. D. & Peterson, K. M. (1996).** A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. *Microbiology* **142**, 2165 - 2174.

**LaBrec, E. H., Schneider, H., Magnani, T. J. & Formal, S. B. (1964).** Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. *Journal of Bacteriology* **88**, 1503 - 1518.

**Lan, R., Lumb, B., Ryan, D. & Reeves, P. R. (2001).** Molecular evolution of large virulence plasmid in *Shigella* clones and enteroinvasive *Escherichia coli*. *Infection and Immunity* **69**, 6303 - 6309.

**Lan, R. & Reeves, P. R. (2001).** When does a clone deserve a name? A perspective on bacterial species based on population genetics. *Trends in Microbiology* **9**, 419 - 424.

**Lange, R. & Hengge-Aronis, R. (1991).** Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Molecular Microbiology* **5**, 49 - 59.

**Lett, M. C., Sasakawa, C., Okada, N., Sakai, T., Makino, S., Yamada, M., Komatsu, K. & Yoshikawa, M. (1989).** *virG*, a plasmid-coded virulence gene of *Shigella flexneri*: identification of the VirG protein and determination of the complete coding sequence. *Journal of Bacteriology* **171**, 353 - 359.

**Levenson, V. I., Egorova, T. P., Belkin, Z. P., Fedosova, V. G., Subbotina, J. L., Rukhadze, E. Z., Dzhikidze, E. K. & Stassilevich, Z. K. (1991).** Protective ribosomal preparation from *Shigella sonnei* as a parenteral candidate vaccine. *Infection and Immunity* **59**, 3619 - 3618.

**Li, A., Cam, P. D., Islam, D., Minh, N. B., Huan, P. T., Rong, Z. C., Karlsson, K., Lindberg, G. & Lindberg, A. A. (1994).** Immune responses in Vietnamese children after a single dose of the auxotrophic, live *Shigella flexneri* Y vaccine strain SFL124. *Journal of Infection* **28**, 11 - 23.

**Liebert, C. A., Hall, R. M. & Summers, A. O. (1999).** Transposon Tn*21*, flagship of the floating genome. *Microbiology and Molecular Biology Reviews* **63**, 507 - 522.

**Lima, A. A. M., Lima, N. L., Pinho, M. C. N., Barros Jnr, E. A., Teixeira, M. J., Martins, M. C. V. & Guerrant, R. L. (1995).** High frequency of strains multiply resistant to ampicillin, trimethoprim-sulfamethoxazole, streptomycin, chloramphenicol, and tetracycline isolated from patients with Shigellosis in Northeastern Brazil during the period 1988 to 1993. *Antimicrobial Agents and Chemotherapy* **39**, 256 - 259.

**Limpel, G. R., Niesel, D. W. & Klimpel, K. D. (1986).** Natural cytotoxic effector cell activity against *Shigella flexneri*-infected HeLa cells. *Journal of Immunology* **136**, 1081 - 1086.

**Lindberg, A. A., Karnell, A., Stocker, B. A., Katakura, S., Sweiha, H. & Reinholt, F. P. (1988).** Development of an auxotrophic oral live *Shigella flexneri* vaccine. *Vaccine* **6**, 146 - 150.

**Lindberg, A. A. & Pal, T. (1993).** Strategies for development of potential candidate *Shigella* vaccines. *Vaccine* **11**, 168 - 179.

**Ling, J. M., Shaw, P. C., Kam, K. M., Cheng, A. F. & French, G. L. (1993).** Molecular studies of plasmids of multiply-resistant *Shigella* spp. in Hong Kong. *Epidemiology and Infection* **110**, 437 - 446.

**Louise, C. B. & Obrig, T. G. (1992).** Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells in vitro. *Infection and Immunity* **60**, 1536 - 1543.

**Luck, S. N. (1998).** Characterisation of a 99 kb chromomsal element in *Shigella flexneri* 2a. *B. Sc. (Hons) Thesis*. Monash University: Melbourne.

**Mandic-Mulec, I., Weiss, J. & Zychlinsky, A. (1997).** *Shigella flexneri* Is trapped in polymorphonuclear leukocyte vacuoles and efficiently killed. *Infection and Immunity* **65**, 110 - 115.

**Maurelli, A. T., Fernadez, R. E., Bloch, C. A., Rode, C. K. & Fasano, A. (1998).** "Black holes" and bacterial pathogenicity: A large genomic deletion that enhances the virulence of *Shigella* spp. and enteroinvasive *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 3949 - 3948.

**Maurelli, A. T. & Sansonetti, P. J. (1988).** Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 2820 - 2824.

**Mavris, M., Page, A.-L., Tournebize, R., Demers, B., Sansonetti, P. & Parsot, C. (2002).** Regulation of transcription by the activity of the *Shigella* flexneri type III secretion apparatus. *Molecular Microbiology* **43**, 1543 - 1553.

**McDaniel, T. K. & Kaper, J. B. (1997).** A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Molecular Microbiology* **23**, 399 - 407.

**McDonough, M. A. & Butterton, J. R. (1999).** Spontaneous tandem amplification and deletion of the Shiga toxin operon in *Shigella dysenteriae* 1. *Molecular Microbiology* **34**, 1058 - 1069.

**McGrew, R. E. (1985).** *Encyclopedia of medical history.* New York: McGraw-Hill Book Company.

**Menard, R., Prevost, M. C., Gounon, P., Sansonetti, P. & Dehio, C. (1996).** The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 1254 - 1258.

**Menard, R., Sansonetti, P., Parsot, C. & Vasselon, T. (1994).** Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*. *Cell* **79**, 515 - 525.

**Menard, R., Sansonetti, P. J. & Parsot, C. (1993).** Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *Journal of Bacteriology* **175**, 5899 - 5906.

**Mimuro, H., Suzuki, T., Suetsugu, S., Miki, H., Takenawa, T. & Sasakawa, C. (2000).** Prolifin is Required for Sustaining Efficient Intra- and Intercellular Spreading of *Shigella flexneri*. *The Journal of Biological Chemistry* **275**, 28893 - 28901.

**Moss, J. E., Cardozo, T. J., Zychlinksy, A. & Groisman, E. A. (1999).** The *selC*-associated SHI-2 pathogenicity island of *Shigella flexneri*. *Molecular Microbiology* **33**, 74 - 83.

**Mounier, J., Bahrani, F. K. & Sansonetti, P. J. (1997).** Secretion of *Shigella flexneri* Ipa invasins on contact with epithelial cells and subsequent entry of the bacterium into cells are growth stage dependent. *Infection and Immunity* **65**, 774 - 782.

**Mounier, J., Laurent, V., Hall, A., Fort, P., Carlier, M.-F., Sansonetti, P. J. & Egile, C. (1999).** Rho family GTPases control entry of *Shigella flexneri* into epithelial cells but not intracellular motility. *Journal of Cell Science* **112**, 2069 - 2080.

**Mounier, J., Vasselon, T., Hellio, R., Lesourd, M. & Sansonetti, P. (1992).** *Shigella flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole. *Infection and Immunity* **60**, 237 - 248.

**Muniesa, M., Recktenwald, J., Bielaszewska, M., Karch, H. & Schmidt, H. (2000).** Characterization of a Shiga toxin 2e-converting bacteriophage from an *Escherichia coli* strain of human origin. *Infection and Immunity* **68**, 4850 - 4855.

**Nakata, N., Sasakawa, C., Okada, N., Tobe, T., Fukuda, I., Suzuki, T., Komatsu, K. & Yoshikawa, M. (1992).** Identification and characterization of *virK*, a virulence-associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. *Molecular Microbiology* **6**, 2387 - 2395.

**Nakaya, R., Nakamura, A. & Murata, Y. (1960).** Resistance transfer agents in *Shigella*. *Biochemical and Biophysical Research Communications* **3**, 654 - 659.

**Nakayama, S.-I. & Watanabe, H. (1995).** Involvement of *cpxA*, a sensor of a two-component regulatory system in the pH-dependent regulation of expression of *Shigella sonnei virF* gene. *Journal of Bacteriology* **177**, 5062 - 5069.

**Nataro, J. P., Seriwatana, J., Fasano, A., Maneval, D. R., Guers, L. D., Noriega, F. R., Duovsky, F., Levine, M. M. & Morris Jr, J. G. (1995).** Identification and cloning

of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. *Infection and Immunity* **63**, 4721 - 4728.

**Navia, M. M., Capitano, L., Ruiz, J., Vargas, M., Urassa, H., Schellemberg, D., Gascon, J. & Vila, J. (1999).** Typing and characterization of mechanisms of resistance of *Shigella* spp. isolated from feces of children under 5 years of age from Ifakar, Tanzania. *Journal of Clinical Microbiology* **37**, 3113 - 3117.

**Niebuhr, K., Jouihri, N., Allaoui, A., Gounon, P., Sansonetti, P. J. & Parsot, C. (2000).** IpgD, a protein secreted by the type III secretion machinery of *Shigella flexneri*, is chaperoned by IpgE and implicated in entry focus formation. *Molecular Microbiology* **38**, 8 - 19.

**Nonaka, T., Kuwae, A., Sasakawa, C. & Imajoh-Ohmi, S. (1999).** *Shigella flexneri* YSH6000 induces two types of cell death, apoptosis and oncosis, in the differentiated human monoblastic cell line U937. *FEMS Microbiology Letters* **174**, 89 - 95.

**Noriega, F. R., Liao, F. M., Formal, S. B., Fasano, A. & Levine, M. M. (1995).** Prevalence of *Shigella* enterotoxin 1 among *Shigella* clinical isolates of diverse serotypes. *Journal of Infectious Diseases* **172**, 1408 - 1410.

**Noriega, F. R., Liao, F. M., Maneval, D. R., Ren, S., Formal, S. B. & Levine, M. M. (1999).** Strategy for cross-protection among *Shigella flexneri* serotypes. *Infection and Immunity* **67**, 782 - 788.

**Noriega, F. R., Losonsky, G., Lauderbaugh, C., Liao, F. M., Wang, J. Y. & Levine, M. M. (1996).** Engineered Δ*guaB-A* Δ*virG Shigella flexneri* 2a strain CVD 1205: Construction, safety, immunogenicity and potential efficacy as a mucosal vaccine. *Infection and Immunity* **64**, 3055 - 3061.

**Noriega, F. R., Wang, J. Y., Losonsky, G., Maneval, D. R., Hone, D. M. & Levine, M. M. (1994).** Construction and characterization of attenuated Δ*aroA* Δ*virG Shigella*

*flexneri* 2a strain CVD 1203, a prototype live oral vaccine. *Infection and Immunity* **62**, 5168 - 5172.

**Oberhelman, R. A., Kopecko, D. J., Salazar-Lindo, E., Gotuzzo, E., Buysse, J. M., Venkatesan, M. M., Yi, A., Fernandez-Prada, C., Guzman, M. & Leon-Barua, R. (1991).** Prospective study of systemic and mucosal immune responses in dysenteric patients to specific *Shigella* invasion plasmid antigens and lipopolysaccharides. *Infection and Immunity* **59**, 2341 - 2350.

**O'Brien, A. D. & Holmes, R. K. (1987).** Shiga and Shiga-like toxins. *Microbiological Reviews* **51**, 206 - 220.

**Okada, N., Sasakawa, C., Tobe, T., Yamada, M., Nagai, S., Talukder, K. A., Komatsu, K., Kanegasaki, S. & Yoshikawa, M. (1991).** Virulence-associated chromosomal loci of Shigella flexneri identified by random Tn5 insertion mutagenesis. *Molecular Microbiology* **5**, 187 - 195.

**Orr, N., Robin, G., Cohen, D., Arnon, R. & Lowell, G. H. (1993).** Immunogenicity and efficacy of oral or intranasal *Shigella flexneri* 2a and *Shigella sonnei* proteosome-lipopolysaccharide vaccines in animals models. *Infection and Immunity* **61**, 2390 - 2395.

**Osiecki, J. C., Barder, J., Picking, W. L., Barnoski Serfis, A., Berring, E., Shah, S., Harrington, A. & Picking, W. D. (2001).** IpaC from *Shigella* and SipC from *Salmonella* possess similar biochemical properties but are funcitonally distinct. *Molecular Microbiology* **42**, 469 - 481.

**Page, A.-L., Ohayon, H., Sansonetti, P. J. & Parsot, C. (1999).** The secreted IpaB and IpaC invasins and their cytoplasmic chaperone IpgC are required for intercellular dissemination of *Shigella flexneri*. *Cellular Microbiology* **1**, 183 - 193.

**Parsot, C. & Sansonetti, P. J. (1999).** The virulence plasmid of Shigellae: an archipelago of Pathogenicity islands? In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 151 - 165. Edited by J. B. Kaper & J. Hacker. Washington: American Society for Microbiology.

**Passwell, J. H., Harley, E., Ashkenazi, S., Chuc, C., Miron, D., Ramon, R., Farzan, N., Shiloach, J., Bryla, D. A., Majadly, F., Roberson, R., Robbins, J. B. & Schneerson, R. (2001).** Safety and immunogenicity of improved *Shigella* O-specific polysaccharide-protein conjugate vaccines in adults in Israel. *Infection and Immunity* **69**, 1351 - 1357.

**Paton, A. W., Morona, R. & Paton, J. C. (2001).** Neutralization of Shiga toxins Stx1, Stx2c, and Stx2e by recombinant bacteria expressing mimics of Globotriose and Globotetraose. *Infection and Immunity* **69**, 1967 - 1970.

**Paton, A. W., Woodrow, M. C., Doyle, R. M., A., L. & Paton, J. C. (1999).** Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. *Journal of Clinical Microbiology* **37**, 3357 - 3361.

**Pavliakova, D., Chu, C., Bystricky, S., Tolson, N., Shiloach, J., Kaufman, J. B., Bryla, D. A., Robbins, J. B. & Schneerson, R. (1999).** Treatment with succinic anhydride improves the immunogenicity of *Shigella flexneri* type 2a O-specific polysaccharide-protein conjugates in mice. *Infection and Immunity* **67**, 5526 - 5529.

**Perdomo, J. J., Gounon, P. & Sansonetti, P. J. (1994a).** Polymorphonuclear leukocyte transmigration promotes invasion of colonic epithelial monolayer by *Shigella flexneri*. *Journal of Clinical Investigation* **93**, 633 - 643.

**Perdomo, O. J. J., Cavailoon, J. M., Huerre, M., Ohayon, H., Gounon, P. & Sansonetti, P. J. (1994b).** Acute inflammation causes epithelial invasion and mucosal destruction in experimental Shigellosis. *Journal of Experimental Medicine* **180**, 1307 - 1319.

**Perna, N. T., Mayhew, F., Posfal, G., Elliott, S., Donnenberg, M. S., Kaper, J. B. & Blattner, F. R. (1998).** Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity* **66**, 3810 - 3817.

**Pfeifer, C. G., Marcus, S. L., Steele-Mortimer, O., Knodler, L. A. & Finlay, B. B. (1999).** *Salmonella typhimurium* virulence genes are induced upon bacterial invasion into phagocytic and onphagocytic cells. *Infection and Immunity* **67**, 5690 - 5698.

**Phalipon, A., Kaufmann, M., Michetti, P., Cavaillon, J. M., Huerre, M., Sansonetti, P. & Kraehenbuhl, J. P. (1995).** Monoclonal immunoglobulin A antibody directed against serotype-specific epitope of *Shigella flexneri* lipopolysaccharide protects against murine experimental shigellosis. *Journal of Experimental Medicine* **182**, 769 - 778.

**Philpott, D. J., Edgeworth, J. D. & Sansonetti, P. J. (2000a).** The pathogenesis of *Shigella flexneri* infection: lessons from *in vitro* and *in vivo* studies. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* **355**, 575 - 586.

**Philpott, D. J., Yamaoka, S., Israel, A. & Sansonetti, P. J. (2000b).** Invasive *Shigella flexneri* actiates NF-kB through a lipopolysaccharide-dependent innate intracellular response and leads to IL-8 expression in epithelial cells. *The Journal of Immunology* **165**, 903 -914.

**Picking, W. L., Coye, L., Osiecki, J. C., Serfis, A. B., Schaper, E. & Picking, W. D. (2001).** Identification of functional regions within invasion plasmid antigen C (IpaC) of *Shigella flexneri*. *Molecular Microbiology* **39**, 100 - 111.

**Plunkett III, G., Rose, D. J., Durfee, T. J. & Blattner, F. R. (1999).** Sequence of Shiga toxin 2 phage 933W from *Escherichia coli* O157:H7: Shiga toxin as a phage late-gene product. *Journal of Bacteriology* **181**, 1767 - 1778.

**Porter, M. E. & Dorman, C. J. (1997).** Differential regulation of the plasmid-encoded genes in the *Shigella flexneri* virulence regulon. *Molecular and General Genetics* **256**, 93 - 103.

**Porter, M. E. & Dorman, C. J. (2002).** In Vivo DNA binding and oligomerization properties of the *Shigella flexneri* AraC-like transcriptional regulator VirF as identified by random and site-specific mutagenesis. *Journal of Bacteriology* **184**, 531 - 539.

**Pozsgay, V., Chu, C., Pannell, L., Wolfe, J., Robbins, J. B. & Schneerson, R. (1999).** Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from *Shigella dysenteriae* type 1. *Proceedings of the National Acadamy of Sciences of the United States of America* **96**, 5194 - 5197.

**Price, S. B., Cheng, C.-M., Kaspar, C. W., Wright, J. C., DeGraves, F. J., Penfounds, T. A., Castanie-Cornet, M.-P. & Foster, J. W. (2000).** Role of *rpoS* in acid resistance and fecal shedding of *Escherichia coli* O157:H7. *Applied and Environmental Microbiology* **66**, 632 - 637.

**Pupo, G. M., Karaolis, D. K., Lan, R. & Reeves, P. R. (1997).** Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and mdh sequence studies. *Infection and Immunity* **65**, 2685 - 2692.

**Pupo, G. M., Lan, R. & Reeves, P. R. (2000).** Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characterstics. *Proceedings of the National Academy of Sciences, USA* **97**, 10567 - 10572.

**Purdy, G. E. & Payne, S. M. (2001).** The SHI-3 iron transport island of *Shigella boydii* O-1392 carries the genes for aerobactin synthesis and transport. *Journal of Bacteriology* **183**, 4176 - 4182.

**Qadri, F., Hossain, S. A., Ciznar, I., Haider, K., Ljungh, A., Wadstrom, T. & Sack, D. A. (1988).** Congo red binding and salt aggregation as indicators of virulence in Shigella species. *Journal of Clinical Microbiology* **26**, 1343 - 1348.

**Rajakumar, K., Bulach, D., Davies, J., Ambrose, L., Sasakawa, C. & Adler, B. (1997a).** Identification of a chromosomal *Shigella flexneri* multi-antibiotic resistance locus which shares sequence and organizational similarity with the resistance region of the plasmid NR1. *Plasmid* **37**, 159 - 168.

**Rajakumar, K., Jost, B. H., Sasakawa, C., Okada, N., Yoshikawa, M. & Adler, B. (1994).** Nucleotide sequence of the rhamnose biosynthetic operon of *Shigella flexneri* 2a and role of lipopolysaccharide in virulence. *Journal of Bacteriology* **176**, 2362 - 2373.

**Rajakumar, K., Sasakawa, C. & Adler, B. (1996).** A spontaneous 99-kb chromosomal deletion results in multi-antibiotic susceptibility and an attenuation of contact haemolysis in *Shigella flexneri* 2a. *Journal of Medical Microbiology* **45**, 64 - 75.

**Rajakumar, K., Sasakawa, C. & Adler, B. (1997b).** Use of a novel approach, termed island probing, identifies the *Shigella flexneri she* pathogenicity island which encodes a homolog of the immunoglobulin A protease-like family of proteins. *Infection and Immunity* **65**, 4606 - 4614.

**Rakin, A., Noelting, C., Schubert, S. & Heesemann, J. (1999).** Common and specific characteristics of the high-pathogenicity island of *Yersinia enterolytica*. *Infection and Immunity* **67**, 5265 - 5274.

**Raqib, R., Ljungdahl, A., Lindberg, A. A., Andersson, U. & Andersson, J. (1996).** Local entrapment of interferon gamma in the recovery from *Shigella dysenteriae* type 1 infection. *Gut* **38**, 328 - 336.

**Raqib, R., Mia, S. M. S., Qadri, F., Alam, T. I., Alam, N. H., Chowdhury, A. K., Mathan, M. M. & Andersson, J. (2000).** Innate immune responses in children and adults with Shigellosis. *Infection and Immunity* **68**, 3620 - 3629.

**Rasolofo-Razanamparany, V., Cassel-Beraud, A.-M., Roux, J., Sansonetti, P. J. & Phalipon, A. (2001).** Predominance of serotype-specific mucosal antibody response in *Shigella flexneri*-infected human living in an area of endemicity. *Infection and Immunity* **69**, 5230 - 5234.

**Rathman, M., de Lanerolle, P., Ohayon, H., Gounon, P. & Sansonetti, P. (2000).** Myson light chain kinase plays an essential role in *S. flexneri* dissemination. *Journal of Cell Science* **113**, 3375 - 3386.

**Robbins, J. B., Chu, C. & Schneerson, R. (1992).** Hypothesis for vaccine development: protective immunity to enteric diseases caused by nontyphoidal Salmonellae and Shigellae may be conferred by serum IgG antibodies to the O-specific polysaccharide of their lipopolysaccharides. *Clinical Infectious Diseases* **15**, 346 - 361.

**Robbins, J. R., Monack, D., McCallum, S. J., Vegas, A., Pham, E., Goldberg, M. B. & Theriot, J. A. (2001).** The making of a gradient: IcsA (VirG) polarity in *Shigella flexneri*. *Molecular Microbiology* **41**, 861 - 872.

**Robin, G., Keisari, Y., Slepon, R., Ashkenazi, S. & Cohen, D. (1999).** Quantitative analysis of IgG class and subclass and IgA serum response to *Shigella sonnei* and *Shigella flexneri* 2a polysaccharides following vaccination with *Shigella* conjugate vaccines. *Vaccine* **17**, 3109 - 3115.

**Rowe, B. & Gross, R. J. (1981).** The Genus *Shigella*. In *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria*, pp. 1248 - 1259. Edited by M. P. Starr, H. Stolp, H. G. Truper, A. Balows & H. G. Schlegel. Heidelberg: Springer-Verlag.

**Rowe, B. & Gross, R. J. (1984).** *Shigella*. In *Bergey's Manual of Systematic Bacteriology*, pp. 423 - 427. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

**Ruzin, A., Lindsay, J. & Novick, R. P. (2001).** Molecular genetics of SaPI1 - a mobile pathogenicity island in *Staphylococcus aureus*. *Molecular Microbiology* **41**, 365 - 377.

Sack, **R. B., Rahman, M., Yunus, M. & Khan, E. H. (1997).** Antimicrobial resistance in organisms causing diarrheal disease. *Clinical Infectious Diseases* **24(Suppl 1)**, S102 - S105.

**Salam, M. A. & Bennish, M. L. (1991).** Antimicrobial therapy for shigellosis. *Reviews of Infectious Diseases* **13**, S332 - S341.

**Samandari, T., Kotloff, K. L., Losonsky, G. A., Picking, W. D., Sansonetti, P. J., Levine, M. M. & Sztein, M. B. (2000).** Production of IFN-g and IL-10 to *Shigella* invasins by mononuclear cells from volunteers orally inoculated with a shiga toxin-deleted *Shigella dysenteriae* type 1 strain. *The Journal of Immunology* **164**, 2221 - 2232.

**Sansonetti, P. & Phalipon, A. (1996).** Shigellosis: from molecular pathogenesis of infection to protective immunity and vaccine devleopment. *Research in Immunology* **147**, 595 - 602.

**Sansonetti, P. J. (2001).** Rupture, invasion and inflammatorydestruction of the intestinal barrier by *Shigella*, making sense of prokaryote-eukaryote cross-talks. *FEMS Microbiology Reviews* **25**, 3 - 14.

**Sansonetti, P. J., Arondel, J., Cantey, J. R., Prevost, M.-C. & Huerre, M. (1996).** Infection of rabbit Peyer's patches by *Shigella flexneri*: Effect of adhesive or invasive bacterial phenotypes on follicle-associated epithelium. *Infection and Immunity* **64**, 2752 - 2764.

**Sansonetti, P. J., Arondel, J., Cavaillon, J. M. & Huerre, M. (1995).** Role of interleukin-1 in the pathogenesis of experimental shigellosis. *The Journal of Clinical Investigation* **96**, 884 - 892.

**Sansonetti, P. J., Arondel, J., Huerre, M., Harada, A. & Matsushima, K. (1999a).** Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis. *Infection and Immunity* **67**, 1471 - 1480.

**Sansonetti, P. J., Hale, T. L., Dammin, G. J., Kapfer, C., Colins, H. & Formal, S. B. (1983).** Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. *Infection and Immunity* **39**, 1392 - 1402.

**Sansonetti, P. J., Kopecko, D. J. & Formal, S. B. (1982).** Involvementof a large plasmid in the invasive abiliy of Shigella flexneri. *Infection and Immunity* **35**, 852 - 860.

**Sansonetti, P. J., Mounier, J., Prevost, M. C. & Mege, R. M. (1994).** Cadherin expression is required for the spread of *Shigella flexneri* between epithelial cells. *Cell* **76**, 829 - 839.

**Sansonetti, P. J. & Phalipon, A. (1999).** M cells as ports of entry for enteroinvasive pathogens: Mechanisms of interaction, consequences for the disease process. *Seminars in Immunology* **11**, 193 - 203.

**Sansonetti, P. J., Ryter, A., Clerc, P., Maurelli, A. T. & Mounier, J. (1986).** Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infection and Immunity* **51**, 461 - 469.

**Sansonetti, P. J., Tran Van Nhieu, G. & Egile, C. (1999b).** Rupture of the intestinal epithelial barrier and mucosal invasion by *Shigella flexneri*. *Clinical Infectious Diseases* **28**, 466 - 475.

**Sasakawa, C. (1997).** Early stages of *Shigella* interaction with host cells. *Journal of Infectious Chemotherapy* **3**, 63 - 72.

**Sasakawa, C., Kamata, K., Sakai, T., Makino, S., Yamada, M., Okada, N. & Yoshikawa, M. (1988).** Virulence-associated genetic regions comprising 31 kilobases of the 230-kilobase plasmid in *Shigella flexneri* 2a. *Journal of Bacteriology* **170**, 2480 - 2484.

**Sasakawa, C., Makino, S., Kamata, K. & Yoshikawa, M. (1986).** Isolation, characterization, and mapping of Tn5 insertions into the 140-megadalton invasion plasmid defective in the mouse Sereny test in *Shigella flexneri* 2a. *Infection and Immunity* **54**, 32 - 36.

**Sasakawa, C. & Yoshida, S. (2003).** Exploiting host microtubule dynamics: a new aspect of bacterial invasion. *Trends in Microbiology* **11**, 139 - 143.

**Schuch, R. & Maurelli, A. T. (1999).** The Mxi-Spa Type III secretory pathway of *Shigella flexneri* requires an outer membrane lipoprotein, MxiM, for invasin translocation. *Infection and Immunity* **67**, 1982 - 1991.

**Schuch, R. & Maurelli, A. T. (2001).** MxiM and MxiJ, base elements of the Mxi-Spa Type III secretion system of *Shigella*, interact with and stabilize the MxiD secretin in the cell envelope. *Journal of Bacteriology* **183**, 6991 - 6998.

**Skoudy, A., Tran Van Nhieu, G., Mantis, N., Arpin, M., Mounier, J., Gounon, P. & Sansonetti, P. (1999).** A functional role for ezrin during *Shigella flexneri* entry into epithelial cells. *Journal of Cell Science* **112**, 2059 - 2068.

**Small, P., Blankenhorn, D., Welty, D., Zinser, E. & Slonczewski, J. L. (1994).** Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. *Journal of Bacteriology* **176**, 1729 - 1737.

**Steinhauer, J., Agha, R., Pham, T., Varga, A. W. & Goldberg, M. B. (1999).** The unipolar *Shigella* surface protein IcsA is targeted directly to the bacterial old pole: IcsP cleavage of IcsA occurs over the entire bacterial surface. *Molecular Microbiology* **32**, 367 - 377.

**Strauch, E., Lurz, R. & Beutin, L. (2001).** Characterization of a Shiga toxin-encoding temperate bacteriophage of *Shigella sonnei*. *Infection and Immunity* **69**, 7588 - 7595.

**Strockbine, N. A., Jackson, M. P., Sung, L. M., Holmes, R. D. & O'Brien, A. D. (1988).** Cloning and sequencing of the genes for Shiga toxin from *Shigella dysenteriae* type 1. *Journal of Bacteriology* **170**, 1116 - 1122.

**Suzuki, T., Lett, M. C. & Sasakawa, C. (1995).** Extracellular transport of VirG protein in *Shigella*. *Journal of Biological Chemistry* **270**, 30874 - 30880.

**Swenson, D. L., Bukanov, N. O., Berg, D. E. & Welch, R. A. (1996).** Two pathogenicity islands in uropathogenic *Escherichia coli* J96: cosmid cloning and sample sequencing. *Infection and Immunity* **64**, 3736 - 3743.

**Szakal, D. D., Schneider, G. & Pal, T. (2003).** A colony blot immune assay to identify enteroinvasive *Escherichia coli* and *Shigella* in stool samples. *Diagnostic Microbiology and Infectious Disease* **45**, 165 - 171.

**Tamano, K., Aizawa, S., Katayama, E., Nonaka, T., Imajoh-Ohmi, S., Kuwae, A., Nagai, S. & Sasakawa, C. (2000).** Supramolecular structure of the *Shigella* type III secretion machinery: the needle part is changeable in length and essential for delivery of effectors. *EMBO Journal* **19**, 3876 - 3887.

**Taylor, D. N., Trofa, A. C., Sadoff, J., Chu, C., Bryla, D., Shiloach, J., Cohen, D., Ashkenazi, S., Lerman, Y. & Egan, W. (1993).** Synthesis, characterization, and clinical evaluation of conjugate vaccines composed of the O-specific polysaccharides of *Shigella dysenteriae* type 1, *Shigella flexneri* type 2a, and *Shigella sonnei* (Plesiomonas shigelloides) bound to bacterial toxoids. *Infection and Immunity* **61**, 3678 - 3687.

**Teska, J. D., Coster, T., Byrne, W. R., Colbert, J. R., Taylor, D., Venkatesan, M. & Hale, T. L. (1999).** Novel self-sampling culture method to monitor excretion of live, oral *Shigella flexneri* 2a vaccine SC602 during a community-based phase 1 trial. *Journal of Laboratory and Clinical Medicine.* **134**, 141 - 146.

**Therapeutic.Guidelines.Limited (2000).** *Therapeutic Guidelines: Antibiotic.* North Melbourne: Therapeutic Guidelines Limited.

**Thorpe, C. M., Hurley, B. P., Lincicome, L. L., Jacewicz, M. S., Keusch, G. T. & Acheson, D. W. K. (1999).** Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. *Infection and Immunity* **67**, 5985 - 5993.

**Thorpe, C. M., Smith, W. E., Hurley, B. P. & Acheson, D. W. K. (2001).** Shiga toxins induce, superinduce, and stabilize a variety of C-X-C chemokine mRNAs in intestinal epithelial cells, resulting in increased chemokine expression. *Infection and Immunity* **69**, 6140 - 6147.

**Tobe, T., Nagai, S., Okada, N., Adler, B., Yoshikawa, M. & Sasakawa, C. (1991).** Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the *virB* gene on the large plasmid. *Molecular Microbiology* **5**, 887 - 893.

**Tobe, T., Yoshikawa, M., Mizuno, T. & Sasakawa, C. (1993).** Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by VirF and repression by H-NS. *Journal of Bacteriology* **175**, 6142 - 6149.

**Tobe, T., Yoshikawa, M. & Sasakawa, C. (1995).** Thermoregulation of *virB* transcription in *Shigella flexneri* by sensing of changes in local DNA superhelicity. *Journal of Bacteriology* **177**, 1094 - 1097.

**Tran, N., Serfis, A. B., Osiecki, J. C., Picking, W. L., Coye, L., Davis, R. & Picking, W. D. (2000).** Interaction of *Shigella flexneri* IpaC with model membranes correlates with effects on cultured cells. *Infection and Immunity* **68**, 3710 - 3715.

**Tran Van Nhieu, G., Ben-Ze'ev, A. & Sansonetti, P. J. (1997).** Modulation of bacterial entry into epithelial cells by association between vinculin and the *Shigella* IpaA invasin. *EMBO Journal* **16**, 2717 - 2729.

**Tran Van Nhieu, G., Caron, E., Hall, A. & Sansonetti, P. J. (1999).** IpaC induces actin polymerization and filopodia formation during *Shigella* entry into epithelial cells. *EMBO Journal* **18**, 3249 - 3262.

**Tran Van Nhieu, G. & Sansonetti, P. J. (1999).** Mechanism of *Shigella* entry into epithelial cells. *Current Opinion in Microbiology* **2**, 51 - 55.

**Trevejo, R. T., Abbott, S. L., Wolfe, M. I., Meshulam, J., Yong, D. & Flores, G. R. (1999).** An untypeable *Shigella flexneri* strain associated with an outbreak in California. *Journal of Clinical Microbiology* **37**, 2352 - 2353.

**Turbyfill, K. R., Hartman, A. B. & Oaks, E. (2000).** Isolation and characterization of a *Shigella flexneri* invasin complex subunit vaccine. *Infection and Immunity* **68**, 6624 - 6632.

**Turbyfill, K. R., Mertz, J. A., Mallett, C. P. & Oaks, E. (1998).** Identifcation of epitope and surface-exposed domains of *Shigella flexneri* invasion plasmid antigen D (IpaD). *Infection and Immunity* **66**, 1999 - 2006.

**Turner, S. A. (1998).** Characterisation of the right flanking region and deletion endpoints of a 99 kb chromosomal element in *Shigella flexneri* 2a. *B. Sc. (Hons) Thesis.* Monash University: Melbourne.

**Uchiya, K., Tobe, T., Komatsu, K., Suzuki, T.. Watarai, M., Fukuda, I., Yoshikawa, M. & Sasakawa, C. (1995).** Identification of a novel virulence gene, *virA*, on the large plasmid of *Shigella*, involved in invasion and intercellular spreading. *Molecular Microbiology* **17**, 241 - 250.

**Unkmeir, A. & Schmidt, H. (2000).** Structural analysis of phage-borne *stx* genes and their flanking sequences in Shiga toxin-producing *Escherichia coli* and *Shigella dysenteriae* type 1 strains. *Infection and immunity* **68**, 4856 - 4864.

**van de Verg, L. L., Mallett, C. P., Collins, H. H., Larsen, T., Hammack, C. & Hale, T. L. (1995).** Antibody and cytokine responses in a mouse pulmonary model of *Shigella flexneri* serotype 2a infection. *Infection and Immunity* **63**, 1947 - 1954.

**Vargas, M., Gascon, J., De Anta, M. T. J. & Vila, J. (1999).** Prevalence of *Shigella* enterotoxins 1 and 2 among *Shigella* strains isolated from patients with traveler's diarrhea. *Journal of Clinical Microbiology* **37**, 3608 - 3611.

**Vasselon, T., Mounier, J., Hellio, R. & Sansonetti, P. J. (1992).** Movement along actin filaments of the perijunctional area and De Novo polymerization of cellular actin are required for *Shigella flexneri* colonization of epithelial Caco-2 cell monolayers. *Infection and Immunity* **60**, 1031 - 1040.

**Vasselon, T., Mounier, J., Prevost, M.-C., Hellio, R. & Sansonetti, P. J. (1991).** Stress fiber-based movement of *Shigella flexneri* within cells. *Infection and Immunity* **59**, 1723 - 1732.

**Venkatesan, M. M., Buysse, J. M. & Oaks, E. V. (1992).** Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the *spa* locus. *Journal of Bacteriology* **174**, 1990 - 2001.

**Venkatesan, M. M., Goldberg, M. B., Rose, D. J., Grotbeck, E. J., Burland, V. & Blattner, F. R. (2001).** Complete DNA sequence and analysis of the large virulence plasmid of *Shigella flexneri*. *Infection and Immunity* **69**, 3271 - 3285.

**Venkatesan, M. M., Hartman, A. B., Newland, J. W., Ivanova, V. S., Hale, T. L., McDonough, M. & Butterton, J. (2002).** Construction, characterization, an animal testing of WRSd1, a *Shigella dysenteriae* 1 vaccine. *Infection and Immunity* **70**, 2950 - 2958.

**Vokes, S. A., Reeves, S. A., Torres, A. G. & Payne, S. M. (1999).** The aerobactin iron transport systen genes in *Shigella flexneri* are present within a pathogenicity island. *Molecular Microbiology* **33**, 63 - 73.

**Wassef, J. S., Keren, D. F. & Mailloux, J. L. (1989).** Role of M cells in initial antigen uprtake and in ulcer formation in the rabbit intestinal loop model of Shigellosis. *Infection and Immunity* **57**, 858 - 863.

**Watanabe, T. (1963).** Infective heredity of multiple drug resitance in bacteria. *Bacteriological Reviews* **27**, 87 - 115.

**Watanabe, T. & Fukasawa, T. (1960).** "Resistance transfer factor" an episome in Enterobacteriaceae. *Biochemical and Biophysical Research Communications* **3**, 660 - 665.

**Watarai, M., Funato, S. & Sasakawa, C. (1996).** Interaction of Ipa proteins of *Shigella flexneri* with $\alpha_5\beta_1$ integrin promotes entry of the bacteria into mammalian cells. *Journal of Experimental Medicine* **183**, 991 - 999.

**Watarai, M., Kamata, Y., Kozaki, S. & Sasakawa, C. (1997).** rho, a small GTP-binding protein, is essential for *Shigella* invasion of epithelial cells. *Journal of Experimental Medicine* **185**, 281 - 292.

**Watarai, M., Tobe, T., Yoshikawa, M. & Sasakawa, C. (1995a).** Contact of *Shigella* with host cells triggers release of Ipa invasins and is an essential function of invasiveness. *EMBO Journal* **14**, 2461 - 2470.

**Watarai, M., Tobe, T., Yoshikawa, M. & Sasakawa, C. (1995b).** Disulfide oxidoreductase activity of *Shigella flexneri* is required for release of Ipa proteins and invasion of epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 4927 - 4931.

**Waterman, S. R. & Small, P. L. C. (1996).** Identification of sigma s-dependent genes associated with the stationary-phase acid-resistance phenotype of *Shigella flexneri*. *Molecular Microbiology* **21**, 925 - 940.

**Way, S., Borczuk, A. C., Dominitz, R. & Goldberg, M. B. (1998).** An essential role for gamma interferon in innate resistance to *Shigella flexneri* infection. *Infection and Immunity* **66**, 1342 - 1348.

**Way, S. S., Borczuk, A. C. & Goldberg, M. B. (1999a).** Adaptive immune response to *Shigella flexneri* 2a *cydC* in immunocompetent mice and mice lacking immunoglobulin A. *Infection and Immunity* **67**, 2001 - 2004.

**Way, S. S., Borczuk, A. C. & Goldberg, M. B. (1999b).** Thymic Independence of adaptive immunity to the intracellular pathogen *Shigella flexneri* serotype 2a. *Infection and Immunity* **67**, 3970 - 3979.

**Wei, J., Goldberg, M. B., Burland, V., Venkatesan, M. M., Deng, W., Fournier, G., Mayhew, G. F., Plunkett III, G., Rose, D. J., Darling, A., Mau, B., Perna, N. T., Payne, S. M., Runyen-Janciky, L. J., Zhou, S., Chwartz, D. C. & Blattner, F. (2003).** Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. *Infection and Immunity* **71**, 2775 - 2786.

**WHO (1997).** Vaccine research and development. New strategies for accelerating *Shigella* vaccine development. *Weekly Epidemiological Record* **72**, 73 - 79.

**Womble, D. D. & Rownd, R. H. (1988).** Genetic and physical map of plasmid NR1: Comparison with other IncFII antibiotic resistance plasmids. *Microbiological Reviews* **52**, 433 - 451.

**Wong, K.-K., McCelland, M., Stillwell, L. C., Sisk, E. C., Thurston, S. J. & Saffer, J. D. (1998).** Identification and sequence analysis of a 27-kilobase chromosomal fragment containing a *Salmonella* pathogenicity island located at 92 minutes on the chromosome map of *Salmonella enterica* serovar Typhimurium LT2. *Infection and Immunity* **66**, 3365 - 3371.

**Yutsudo, T., Honda, T., Takeda, Y. & Miwatani, T. (1986).** Physico-chemical and biological properties of purified Shiga toxin from *Shigella dysenteriae*. In *Advances in Research on Cholera and Related Diarrheas*, pp. 153 - 160. Edited by S. Kuwahara & N. F. Pierce. Tokyo: KTK Scientific Publishers.

**Zhang, Z., Jin, L., Champion, G., Seydel, K. B. & Stanley Jr, S. L. (2001).** Shigella infection in a SCID mouse-human intestinal xonegraft model: role for neutrophils in containing bacterial dissemination in human intestine. *Infection and Immunity* **69**, 3240 - 3247.

**Zychlinsky, A., Kenny, B., Menard, R., Prevost, M.-C., Holland, I. B. & Sansonetti, P. J. (1994).** IpaB mediates macrophage apoptosis induced by *Shigella flexneri*. *Molecular Microbiology* **11**, 619 - 627.

**Zychlinsky, A., Prevost, M. C. & Sansonetti, P. J. (1992).** *Shigella flexneri* induces apoptosis in infected macrophages. *Nature* **358**, 167 - 169.

2

# CHAPTER TWO

## Nested Deletion Events of the SRL Pathogenicity Island of *Shigella flexneri* 2a

*Based on:*

**Turner, S. A., Luck, S. N., Sakellaris, H., Rajakumar, K. & Adler, B. (2001).** Nested Deletion Events of the SRL Pathogenicity Island of *Shigella flexneri* 2a. *Journal of Bacteriology* **183**, 5535 – 5543.

# INTRODUCTION

*Shigella* spp., the causative agents of bacillary dysentery, are responsible for the deaths of more than 1.1 million people every year (Kotloff *et al.*, 1999). Infections are transmitted via the fecal-oral route either as a result of person-to-person contact or through ingestion of contaminated food or water, and result in watery diarrhoea which may progress to the bloody mucoid stools typical of bacillary dysentery (DuPont *et al.*, 1989). In developing countries individuals affected by acute diarrhoea are commonly treated using oral rehydration and antimicrobial therapy. However, rehydration therapy alone provides little benefit to patients with dysentery caused by invasive enteropathogens such as *Shigella*, and the global importance of dysentery in developing countries has increased as a result of ineffective treatment (Khan, 1985). In addition, resistance to antibiotics such as tetracycline (Tc) and ampicillin (Ap), that were once highly efficacious in treatment of shigellosis, has grown considerably in the last few decades (Sack *et al.*, 1997). In many cases, resistance genes are found to reside on easily transferable R-plasmids. However, chromosomally-borne resistance genes have recently been identified in a number of studies (Casalino *et al.*, 1994; Gebre-Yohannes & Drasar, 1990; Ling *et al.*, 1993; Rajakumar *et al.*, 1997a; Rajakumar *et al.*, 1996), although the basis of chromosomal resistance has not been widely investigated.

Rajakumar *et al.* (1996) described a spontaneous 99 kb chromosomal deletion that resulted in multi-antibiotic susceptibility in *Shigella flexneri* 2a YSH6000. The resistance locus carried on the 99 kb element was found to encode resistance determinants to streptomycin (Sm), chloramphenicol (Cm), Ap and Tc. These determinants have since been collectively designated the SRL, for Shigella resistance locus, and the SRL exhibits similarity in sequence and organisation to those of the antibiotic resistance loci of NR-1, an R-plasmid commonly found in *Shigella*, and the transposon Tn*2603* (Rajakumar, *et al.*, 1997a). Although the nature and exact location of the 99 kb multiple-antibiotic resistance deletable element (MRDE) harbouring the SRL was not determined, it was mapped to a region of the chromosome on *Not*I fragment D bounded by the *S. flexneri* homologues of the

*Escherichia coli* genes *ompA* and *pyrC* (Rajakumar, *et al.*, 1996). The instability of the MRDE was reminiscent of the behaviour of other large chromosomal regions in *E. coli* and *Yersinia pestis*, referred to as pathogenicity islands (PAIs) (Blum *et al.*, 1994), suggesting that the MRDE may also be a PAI-like element.

The term pathogenicity island (PAI) was first used to describe large unstable DNA regions in uropathogenic *E. coli* (UPEC) (Blum, *et al.*, 1994). However, the term is now used more generally to refer to sections of chromosome throughout a number of species that are often unstable and which frequently carry virulence genes (Hacker *et al.*, 1997). Since the introduction of the term PAI, islands have been identified in many species including *E. coli*, *Yersinia* spp., *Helicobacter pylori*, *Salmonella* spp. and *S. flexneri* (Hacker & Kaper, 2000). In addition to virulence genes, PAIs also encode mobility elements such as integrases and IS elements, and commonly integrate into, or adjacent to, tRNA genes. These characteristics, which show remarkable similarity to those of bacteriophages, in conjunction with G + C content that often differs from that of the host chromosome, have lead to the suggestion that PAIs are acquired from different species via phage-mediated horizontal transfer (Hacker & Kaper, 2000). Indeed, it has recently been reported that the *Vibrio cholerae* PAI (VPI), which plays a role in the emergence of epidemic and pandemic cholera, is the genome of a prophage, and the VPIΦ has been shown to transfer to one VPIΦ-negative *V. cholerae* strain (Karaolis & Kaper, 1999; Karaolis *et al.*, 1999). Additionally, the staphylococal pathogenicity island (SaPI) is known to be packaged and mobilised by a helper phage (Ruzin *et al.*, 2001).

In most cases, the instability of PAIs is due to their precise excision from the chromosome via recombination between identical sequences situated on either side of the element. PAIs are commonly arranged with short flanking direct repeats (DRs) of 9-20 bp which are analogous to phage *att* sites. These repeats are often identical to the 3' sequence of the target tRNA gene, and upon PAI deletion only one copy of the DR remains on the chromosome (Dozois & Curtiss III, 1999). IS elements have also been found in the flanking region of some PAIs (Hacker & Kaper, 2000) and recombination between two

flanking IS*100* elements has been shown to occur upon deletion of the HPI from *Y. pestis* (Bach *et al.*, 1999; Fetherston *et al.*, 1992).

In this study, we investigated the various types of deletion events leading to loss of multiple antibiotic resistance in *S. flexneri* 2a strain YSH6000. We report here that the antibiotic resistance genes of the SRL are lost following at least 3 distinct events including the precise deletion of the MRDE and the independent deletion of the SRL and a PAI-like element termed the SRL PAI, both of which are contained entirely within the larger MRDE.

# MATERIALS AND METHODS

## Bacterial Strains, Media and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1. Strains were grown routinely at 37 °C in Luria Bertani medium (Ausubel *et al.*, 1993) with the addition of ampicillin (100 μg/ml), kanamycin (50 μg/ml), trimethoprim (50 μg/ml), chloramphenicol (40 μg/ml) or tetracycline (10 μg/ml) when necessary.

## Molecular Biological Techniques

Genomic DNA was isolated by the small-scale preparation as described previously (Ausubel, *et al.*, 1993). Plasmid DNA was isolated by a modification of the alkaline lysis method (Morelle, 1989). Standard cloning procedures using the vector pWSK29, pJP5603 or pBRTp$^r\Delta$, were employed. *E. coli* DH5α was transformed using the rubidium chloride method (Glover, 1985). DNA from both plasmids and PCR products was prepared for sequence analysis using the PRISM™ Ready Reaction Dye Deoxy™ Terminator Cycle and chromatograms were produced on an Applied Biosystems model 373A DNA sequencing system.

**Table 2.1** Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| *S. flexneri* | | |
| YSH6000 | Wild type *S. flexneri* 2a Japanese isolate, $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ | (Sasakawa *et al.*, 1986) |
| YSH6000T | MRDE deletant of YSH6000, $Sm^s$ $Ap^s$ $Cm^s$ $Tc^s$ | (Nakata *et al.*, 1992) |
| S2430 | MRDE deletant of YSH6000, $Sm^s$ $Ap^s$ $Cm^s$ $Tc^s$ | (Okada *et al.*, 1991) |
| SBA1304 | Wild-type *S. dysenteriae* 3 Japanese isolate | This study |
| SBA1363 | Spontaneous SRL PAI deletant of YSH6000, $Sm^s$ $Ap^s$ $Cm^s$ $Tc^s$ | This study |
| SBA1365 | Spontaneous MRDE deletant of YSH6000, $Sm^s$ $Ap^s$ $Cm^s$ $Tc^s$ | This study |
| SBA1366 | Spontaneous SRL deletant of YSH6000, $Sm^s$ $Ap^s$ $Cm^s$ $Tc^s$ | This study |
| SBA1367 | Spontaneous SRL PAI deletant of YSH6000, $Sm^s$ $Ap^s$ $Cm^s$ $Tc^s$ | This study |
| SBA1368 | Spontaneous SRL deletant of YSH6000, $Sm^s$ $Ap^s$ $Cm^s$ $Tc^s$ | This study |
| SBA1369 | Spontaneous MRDE deletant of YSH6000, $Sm^s$ $Ap^s$ $Cm^s$ $Tc^s$ | This study |
| AL11 | YSH6000 insertional *int* mutant, $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ $Kn^r$ | This study |
| AL108 | YSH6000 harbouring pBRTp$^r\Delta$, $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ $Tp^r$ | This study |
| AL109 | AL11 harbouring pBRTp$^r\Delta$, $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ $Kn^r$ $Tp^r$ | This study |
| AL110 | AL11 harbouring pAL66 $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ $Kn^r$ $Tp^r$ | This study |
| *E. coli* | | |
| DH5α | F-ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169*endA1 recA*1hsdR17deo$^R$*thi1supE*441$^-$*gyrA96rel*$^{A1}$ | Bethesda Research Laboratories |
| S17-1 (λ*pir*) | RP4-2($Tc^s$::Mu)($Kn^s$::Tn7) *recA* λ*pir*, $Sm^r$ $Tp^r$ | (Miller & Mekalanos, 1988) |
| JM109 (λ*pir*) | *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ (*lac-proAB*) λ*pir* | (Miller & Mekalanos, 1988) |
| Plasmids | | |
| pWSK29 | pSC101-based low-copy-number vector Δ*lacZ*, $Ap^r$, 5.4 kb | (Wang & Kushner, 1991) |
| pWSK129 | pSC101-based low-copy-number vector Δ*lacZ*, $Kn^r$, 6.7 kb | (Wang & Kushner, 1991) |
| pBRTp$^r\Delta$ | pBR322 based cloning vector. $Tp^r$ $Tc^r$, 4.8 kb | C. Sasakawa |
| pJP5603 | R6K based suicide vector, $Kn^r$, 3.1 kb | (Penfold & Pemberton, 1992) |
| pSBA480 | 0.5 kb fragment *solA* fragment of YSH6000 (*Cla*I dropout of pSBA358) in pWSK29 | (Turner, 1998) |
| pSBA509 | pWSK129 harbouring part of SRL PAI and flanking regions | S. N. Luck |
| pSBA533 | pWSK129 harbouring 14 kb *Eco*RI subclone from pSBA509 | S. N. Luck |
| pSBA574 | pWSK29 harbouring SBA1366 SRL deletion region, $Ap^r$ | This study |
| pSBA575 | pWSK29 harbouring SBA1368 SRL deletion region, $Ap^r$ | This study |
| pAL11 | pJP5603 harbouring 666 bp internal SRL PAI *integrase* fragment, 3.7 kb | This study |
| pAL64 | pWSK29 harbouring 1.3 kb PCR fragment containing *int* | This study |
| pAL66 | pBRTp$^r\Delta$ harbouring 1.3 kb *int* fragment | This study |

## Selection of Tetracycline-Sensitive Derivatives of YSH6000

Tetracycline sensitive derivatives of YSH6000 were selected by plating dilutions of YSH6000 (grown in LB broth to a density of approximately $10^9$ CFU $ml^{-1}$) onto LB agar supplemented with fusaric acid (12 μg/ml), chlortetracycline (50 μg/ml) and 0.1 mM $ZnCl_2$ (Maloy & Nunn, 1981). Plates were incubated for 24 – 40 h at 37 °C.

## Preparation of High Molecular Weight (HMW) Genomic DNA and Pulsed Field Gel Electrophoresis (PFGE)

HMW DNA was prepared as described by Smith & Cantor (1987) with modifications described previously (Rajakumar, *et al.*, 1996). Low melting temperature agarose plugs (100 μl) containing around 2 μg of DNA were pre-equilibrated with digestion buffer before incubation for 16 h at 37 °C with 10 U *Not*I in a 250 μl reaction mixture (50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 100 mM NaCl; 1 mM dithioerythritol, DTE; bovine serum albumin, BSA 100 mg/L). After digestion, the agarose plugs were incubated in ES (0.5 M EDTA, pH 9.5; lauroyl sarcosine 1 %) at 50 °C for 2 h and equilibrated in TE (10 mM Tris, pH 8.0; 1 mM EDTA) at room temperature before PFGE in a CHEF BioRad system in 1 % agarose gels and 0.5 x TBE running buffer (44.5 mM Tris; 44.5 mM boric acid; 1 mM EDTA). Gels were electrophoresed at 180 V for 24 h with pulse times of 20 – 25 s, at 14 °C.

## Southern Hybridisation

After electrophoresis, DNA was transferred to a charged nylon membrane (Roche) using a vacuum blotting apparatus (TE80 Transvac, Hoefer) or by capillary transfer in 20 X SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0). Overnight hybridisation and subsequent washings were performed under high stringency conditions at 68 °C as recommended in the Roche digoxigenin labelling and detection kit. Probes were labelled by PCR amplification with digoxigenin as specified by Boehringer Mannheim. The 500 bp *solA* probe was amplified using primers T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-

GTAATACGACTCACTATAGGGC-3') from a pSBA480 template. The 0.5 kb *pholI* probe was amplified using primers BAP507 (5'-AATAAACCCTTCCCGCTTCC-3') and T3 from a pSBA509 template. The 2.0 kb *csg* probe was amplified using primers *csgA* forward (5'-AAAGAATTCGCTCTGGCAGGTGTTGTTCC-3') and *csgA* reverse (5'-AAAAAGTCGACTTAACCAAAGCCAACCTGAGTCACG-3') from an SBA1304 template. The 1.0 kb *fec* probe was amplified using primers BAP914 (5'-GCTCCCATTTCGCTCGGC-3') and BAP935 (5'-GTTGTCGTCATAAGAGCGG-3') using a YSH6000 template. The 1.0 kb *int* probe was amplified using BAP1005 (5'-GCTGGATTGGGAACTTACC-3') and T7 from a pSBA533 template.

## PCR Amplification of Deletion Point Junctions

Amplifications were performed on chromosomal DNA with the following oligonucleotide primers. Mapping the MRDE deletion endpoint by inverse PCR, BAP499 (5'-CGGGAAGAATACCTGTTGATG-3') and BAP531 (5'-TATTTGATGCTATGAAGAAGGGGG-3'); MRDE deletion region, BAP649 (5'-AGCGGCAGCGGTATTCAC-3') and BAP530 (5'-TTAATCTCTTCTTCACTTCGCCCC-3') or BAP470 (5'-TCCAGCCACCTTTAGCGG-3'); SRL deletion region, BAP1249 (5'-TATCCCGCTTGCCGTCGC-3') and BAP694 (5'-AGCGGCAGCGGTATTCAC-3'); PAI deletion region, BAP679 (5'-GTGCTGCTTTCGGTGTGC-3') and BAP1157 (5'-GCCAGCATTTCAACAGGAGG-3').

## Mutant Construction

Primers BAP1354 (5'-GCGGATTCCCCTGGCTTCGC-3') and BAP1355 (5'-TTGGATTCAGGGGCGGGGGAAATGGG-3') were used to amplify a 666 bp internal fragment of the integrase gene (bp 714 – 1380, GenBank AF326777) which was then ligated into the T-tailed *Hinc*II site of pJP5603. The recombinant plasmid carrying the *int* fragment, pAL11, isolated in JM109(λ*pir*), was transferred by conjugation into YSH6000 using the mobilising strain S17-1(λ*pir*). Exconjugants carrying a single crossover mutation

of *int* were obtained by selection on Kn/Ap LB plates and confirmed by PCR and Southern hybridisation.

## Mutant Complementation

A 1344 bp product (bp 586 – 1930 GenBank AF326777) containing the entire *int* ORF was amplified by PCR with primers BAP1636 (5'-TGGATGGGATCCCAGAGTGACGGGAATTAGC-3') and BAP1637 (5'-ATGCCAGGATCCCATTACGAACTGGCATTG-3'). Both primers included *Bam*HI sites at the 5' ends and this product was cloned into the T-tailed *Eco*RV site of pWSK29, designated pAL64, and sequenced to confirm that no errors were incorporated. The 1.3 kb *Bam*HI fragment from pAL64 containing the *int* fragment was then cloned into the *Bam*HI site of pBRTp$^r\Delta$, and clones selected by sensitivity to Tc. Orientation of the *int* fragment was confirmed by restriction enzyme digest to be in the same direction as the interrupted Tc$^r$ gene.

## Computer Analysis

Sequencing chromatograms were analysed using the Sequencher program (GeneCodes Corporation, Ann Arbor, MI, USA). Nucleotide sequence similarity searches with sequences in the databases were performed using the BlastN or BlastX program (Altschul *et al.*, 1997). Protein sequence alignments were performed in eclustalw available on the Australian National Genomic Information Server (ANGIS [http://www.angis.org.au]).

# RESULTS

## Background

The MRDE of *S. flexneri* 2a YSH6000 had previously been mapped to chromosomal *NotI* fragment D (Rajakumar, *et al.*, 1996). Through comparisons with the spontaneous MRDE deletants YSH6000T and S2430 the right endpoint was mapped to a 2.4 kb *Eco*RI - *Eco*RV fragment adjacent to *mdoA* (Fig. 2.1). As the position of the left endpoint of the MRDE was unknown, inverse PCR with primers situated within this 2.4 kb fragment was employed to amplify a product which traversed the deletion site in strain YSH6000T. A 6 kb PCR product was amplified from *Sal*I digested and religated YSH6000T genomic DNA using primers BAP499 and BAP531 (Fig. 2.1b). This inverse PCR product extended across the MRDE deletion point in YSH6000T, and 4 kb into the left flanking region. Sequence analysis revealed that this fragment contained the right end of an IS*91* element followed by a sequence exhibiting high level identity to the 3' region of the *E. coli putA* gene, which is situated approximately 31 kb upstream of *mdoA* in *E. coli*. These data showed that a single copy of the IS*91* element spanned the deletion region in YSH6000T and suggested two possible explanations. There may have been a single IS*91* element adjacent to the *mdoA* locus that, after the deletion event, spanned the region between this locus and the *putA* sequence. Alternatively, an IS*91* element had been present at each locus in YSH6000

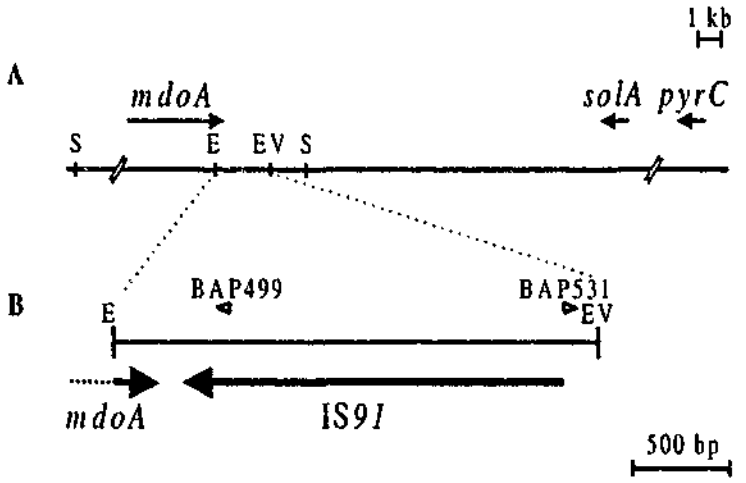



**Figure 2.1** Map of the right flank of the YSH6000 Multiple-antibiotic Resistance Deletable Element (MRDE). (A) Schematic representation of the YSH6000 *mdoA – pyrC* region. (B) The 2.4 kb *Eco*RI/*Eco*RV fragment bearing the right deletion endpoint of the MRDE. Chromosomal DNA is represented as a thin black line and genes are represented as arrows. Arrow heads indicate the positions of the primers BAP499 and BAP531 used to amplify across the MRDE deletion region in YSH6000T using inverse PCR of *Sal*I (S) digested and religated genomic DNA. Abbreviations for restriction sites: E, *Eco*RI; EV, *Eco*RV; S, *Sal*I.

and after the deletion event, only one copy of the element remained on the chromosome (Turner, 1998).

## Mapping the MRDE Deletion Point

To resolve these two alternatives, direct PCR and sequence analysis of both the left flanking region of the MRDE in YSH6000 and the deletion region of YSH6000T and S2430 was carried out. Identical intact IS*91* elements were found at both flanks of the MRDE in YSH6000, confirming that two distinct IS*91* elements were present, one downstream of the *mdoA* locus and the other interrupting the *putA* sequence. The data also showed that an identical intact IS*91* was present at the deletion points in both S2430 and YSH6000T. The organisational similarity between *E. coli* and *S. flexneri* together with the known sequence of the pathogenicity island (the SRL PAI) located within this region (Luck *et al.*, 2001) allowed us to propose a structure for the MRDE in YSH6000, and the corresponding region bearing the deletion point in YSH6000T and S2430 (Fig. 2.2a). This organisation suggests that the MRDE was not acquired or evolved as a unit, but rather, is composed of a PAI that is situated within a distinct deletable region of the chromosome defined by two IS*91* elements.

Although the *S. flexneri* IS*91* showed considerable similarity to the *E. coli* IS*91*, some differences were noted. IS*91* is known to show absolute insertion specificity for the tetramers GAAC or CAAG, always inserting such that the right inverted repeat ($IR_R$) is adjacent to either of these target sequences (Mendiola & de la Cruz, 1989). The left flanking IS*91* in YSH6000 also exhibits this specificity, but the right flanking IS*91* is inserted adjacent to the sequence CGAG (Fig. 2.2a), implying that this element may have a different insertion specificity from that of the *E. coli* IS*91*. Additionally, the sequences showing similarity to the two major ORFs of IS*91*, ORF121 which is implicated in insertion specificity (Bernales *et al.*, 1999) and TnpA, the transposase ORF (Mendiola *et al.*, 1992) are shortened by 12 and 31 amino acids respectively at the carboxy termini when compared with their *E. coli* homologues.

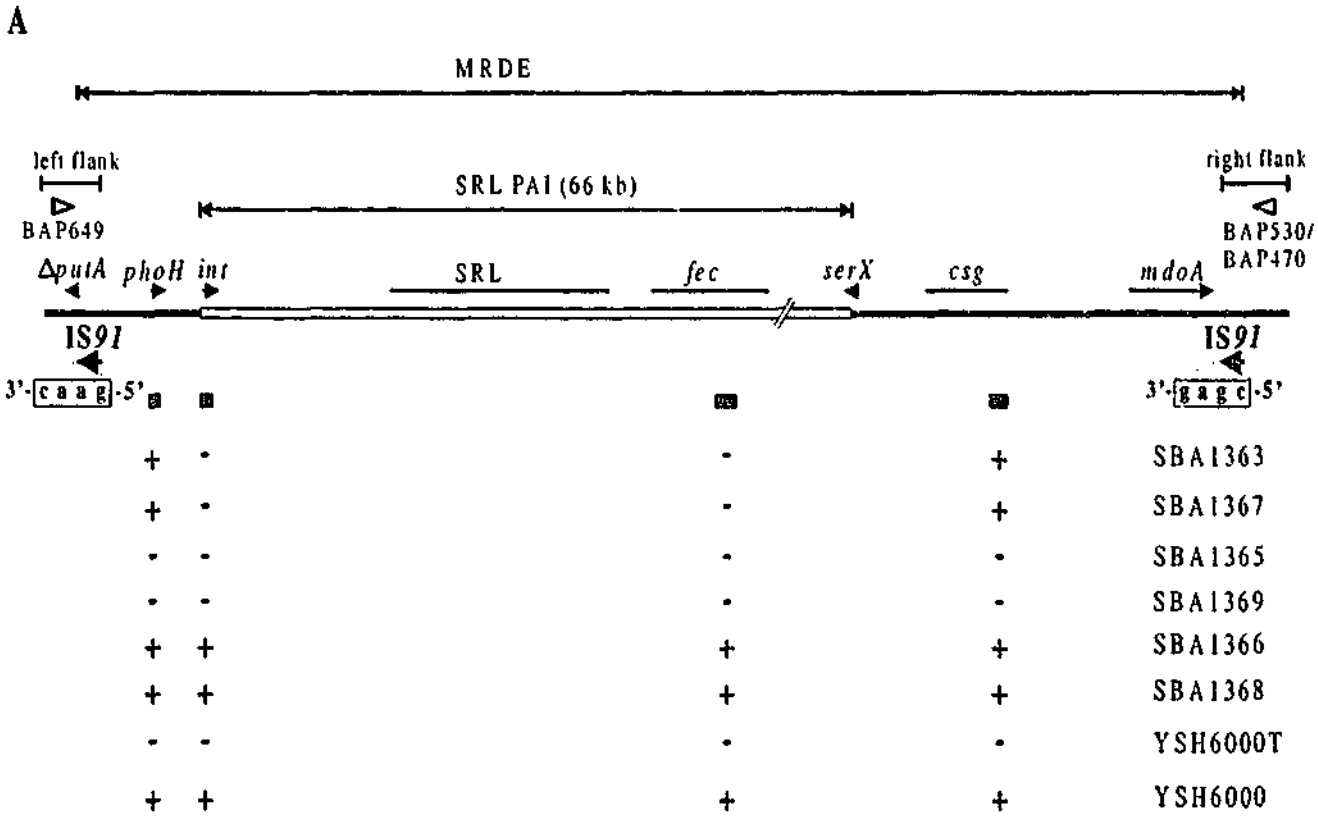



**Figure 2.2** Deletion of the YSH6000 Multiple-antibiotic Resistance Deletable Element (MRDE). (A) Schematic representation of the genetic organisation of the wild type YSH6000 MRDE. (B) Schematic representation of the resultant structure across the deletion point following loss of the MRDE element in YSH6000T, S2430, SBA1365 and SBA1369. The boundaries of the MRDE are defined by the two IS*91* elements indicated by arrows. The SRL PAI is indicated as a white box. Genes and IS elements are represented as arrows, with truncations being indicated by a delta symbol. The *fec*, *csg* and SRL loci are shown as thin black lines. The tetramer immediately downstream of the IS*91* elements at the left and right flank of the MRDE is indicated in the box below each element. Shaded boxes represent the position of probes used to determine the presence (+) or absence (-) of these regions within six streptomycin, ampicillin, chloramphenicol and tetracycline sensitive strains, YSH6000T or YSH6000. The arrow heads represent primers BAP649 and BAP530/BAP470 used to amplify across the deletion endpoint. The physical distance between *putA* and *csg* is based on PCR and/or sequence analysis, while the distance between *csg* and *mdoA* is calculated based on the original sizing of the MRDE at 99 kb (Rajakumar *et al.*, 1996). Regions designated the MRDE left and right flank common to both wild type and MRDE deletants are indicated above the diagram with black bars. (C) Detection of the MRDE deletion point. PCR products amplified using primers BAP649 and BAP470 (Fig. 2.2a and Fig. 2.2b). Lanes: 0, *Hin*dIII-digested λ DNA size markers (kb) *Hin*dIII ladder; 1, YSH6000; 2, YSH6000T; 3, SBA1365; 4, SBA1369.

## Spontaneous Deletion of the MRDE

In order to assess whether the MRDE deletes reproducibly from the same point in the chromosome, we selected for the loss of tetracycline (Tc) resistance by growth of YSH6000 on LB medium supplemented with fusaric acid. Colonies that grew on fusaric acid medium were confirmed for sensitivity to Tc and further tested for susceptibility to the antibiotics streptomycin (Sm), ampicillin (Ap) and chloramphenicol (Cm). Using this method, six $Sm^S$, $Ap^S$, $Cm^S$ and $Tc^S$ strains were identified: SBA1363, and SBA1365 - 1369. PCR using inwardly directed primers BAP649 and BAP470 was employed to confirm MRDE deletions, as the intact deletion region would be amplified only in MRDE$^-$ strains (Fig. 2.2). Products were amplified in strains SBA1365 and SBA1369 (Fig. 2.2c, lanes 3 and 4); sequencing revealed that these strains carried a single IS*91* element at the deletion point, confirming that the MRDE deletions in these strains were identical to those in YSH6000T and S2430 (Fig. 2.2b).

## Novel Deletions Leading to Loss of Antibiotic Resistance

As typical MRDE deletions could be confirmed by PCR in only two of the six multi-antibiotic sensitive strains, the basis for antibiotic sensitivity in the remaining strains was investigated further. Pulsed field gel electrophoresis (PFGE) of *Not*I-digested chromosomal

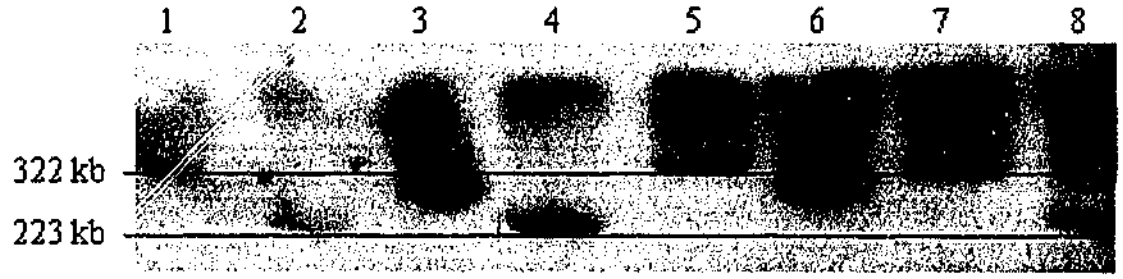



**Figure 2.3.** Southern hybridisation analysis of *Not*I digested DNA resolved by PFGE. Membrane probed with *solA* which resides outside the MRDE endpoint on the *Not*I fragment D of YSH6000 (Fig. 2.1). Lanes: 1, YSH6000; 2, YSH6000T; 3, SBA1363; 4, SBA1365; 5, SBA1366; 6, SBA1367; 7, SBA1368; 8, SBA1369. Numbers on the left indicate the size of *Not*I fragment D in YSH6000 and YSH6000T according to Rajakumar *et al.*, (1996).

DNA showed that in both SBA1365 and SBA1369 the *Not*I fragment D carrying the MRDE suffered a deletion of the same size as those observed in YSH6000T and S2430 (Fig. 2.3, lane 4 and 8). However, in the remaining strains a variety of smaller deletions was observed (Fig. 2.3, lane 3, 5, 6 and 7). To localise these deletions more precisely, strains were analysed by Southern hybridisation with a series of probes corresponding to four regions within the MRDE (Fig. 2.2a). With the exception of SBA1365 and SBA1369, which had undergone deletion events identical to that in YSH6000T, the deletions were confined to the region between *phoH* and *csg* (Fig. 2.2a).

## Deletion of the SRL

Sequence analysis of the region responsible for antibiotic resistance in *S. flexneri* YSH6000 revealed that the MRDE harbours a cluster of resistance genes, now called the SRL (Fig. 2.2a). The SRL shows significant similarity to the resistance region in the *Shigella* R-plasmid NR-1, but also includes an *oxa-1* cassette, encoding β -lactamase (Rajakumar, *et al.*, 1996), therefore conferring resistance to the antibiotics Sm, Ap, Cm and Tc. The SRL is 16.7 kb in length including the two flanking 768 bp IS*1* elements (Fig. 2.4a). Each IS*1* contains an intact *insAB'* and Δ*insA-B'-insB* ORF, both of which have been implicated in transposition (Mahillon & Chandler, 1998; Matsutani, 1994), suggesting that they are still functional. However, the IS*1* elements were not identical, with the left and right IS*1* elements showing 99 % and 97 % identity respectively to the *E. coli* IS*1* nucleotide sequence.

Although the profiles of SBA1366 and SBA1368 indicated that all probed areas were present (Fig. 2.2a), these strains were susceptible to all four antibiotics to which resistance was encoded by the SRL. For this reason it was considered that these two strains might harbour deletions of the resistance locus itself. Inward facing primers, BAP1249 and BAP694, situated on either side of the SRL were used to amplify this region. These primers are separated by 18.5 kb in the wild type and thus do not result in the amplification of a product (Fig. 2.4a), but amplification of a 2.6 kb product occurred in both SBA1366

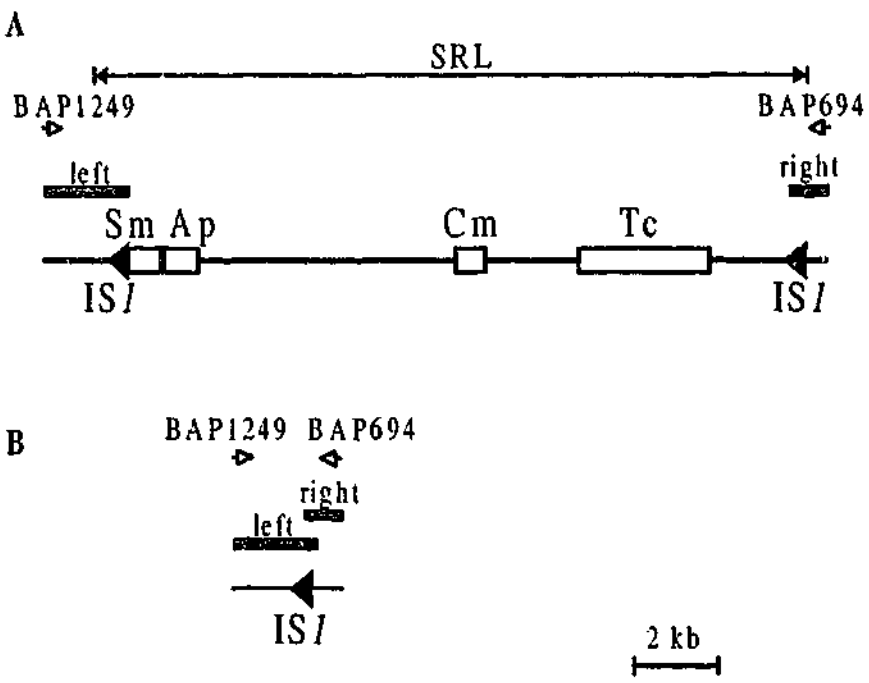



**Figure 2.4** Deletion of the *Shigella* resistance locus (SRL). (A) Diagramatic representation of the SRL in YSH6000. (B) Schematic representation of the resultant structure across the deletion point following loss of the SRL element in SBA1366 and SBA1368. IS elements are represented as arrows and chromosomal DNA by thin black lines. Determinants encoding resistance to Streptomycin (Sm), Ampicillin (Ap), Chloramphenicol (Cm) and Tetracycline (Tc) are represented as white boxes labelled Sm, Ap, Cm and Tc respectively. Left and right flanking regions common to both wild type and SRL deletants are indicated by shaded boxes, with the region corresponding to the identical 487 bps of each IS*1* shaded black.

and SBA1368. The PCR products were cloned into *Eco*RV-digested, T-tailed pWSK29 and designated pSBA574 and pSBA575 respectively. Sequence analysis revealed that these fragments contained sequences identical to that flanking the left end of the SRL, a stretch of sequence identical to the left IS*1* element, followed directly by sequence identical to that flanking the right end of the SRL (Fig. 2.4b). These data indicate that the SRL was able to delete from the chromosome independently of the MRDE, and that upon deletion, a single IS*1* element identical to the left IS*1* of the SRL, spanned the deleted area. However, it is not possible to determine whether the IS*1* element spanning the deletion point has its origin entirely from the left IS*1* of the SRL or if it comprises a composite of the left and right IS*1* elements, which share an identical sequence throughout bp 1 - 487 (Fig. 2.4).

## Deletion of the SRL PAI

The region surrounding the SRL has recently been sequenced, revealing the presence of a 66 kb element which displays characteristics of a PAI. This element, designated the SRL PAI is contained completely within the MRDE (Fig. 2.2a). The SRL PAI encodes an integrase-like gene at the left boundary, numerous IS elements and phage related sequences, and also encodes a ferric dicitrate transport system (*fec*) (Luck, *et al.*, 2001). The genetic organisation in the wild type strain YSH6000 showed the SRL PAI to be flanked by 14 bp direct repeats (DRs) which correspond to the 3' terminus of the tRNA gene *serX* (5'-GGGGGAGTGGCGGT-3'). The right flank of the PAI harboured an intact *serX*, but similarity to *E. coli* sequence ended directly downstream of the 14 bp sequence. At the left end of the SRL PAI, the 14 bp repeat was followed by a stretch of sequence similar to that found downstream of the *serX* tRNA gene in *E. coli*, indicating that the PAI had inserted into the 3' end of the *serX* gene (Fig. 2.5a). Although deletion of this element had not been

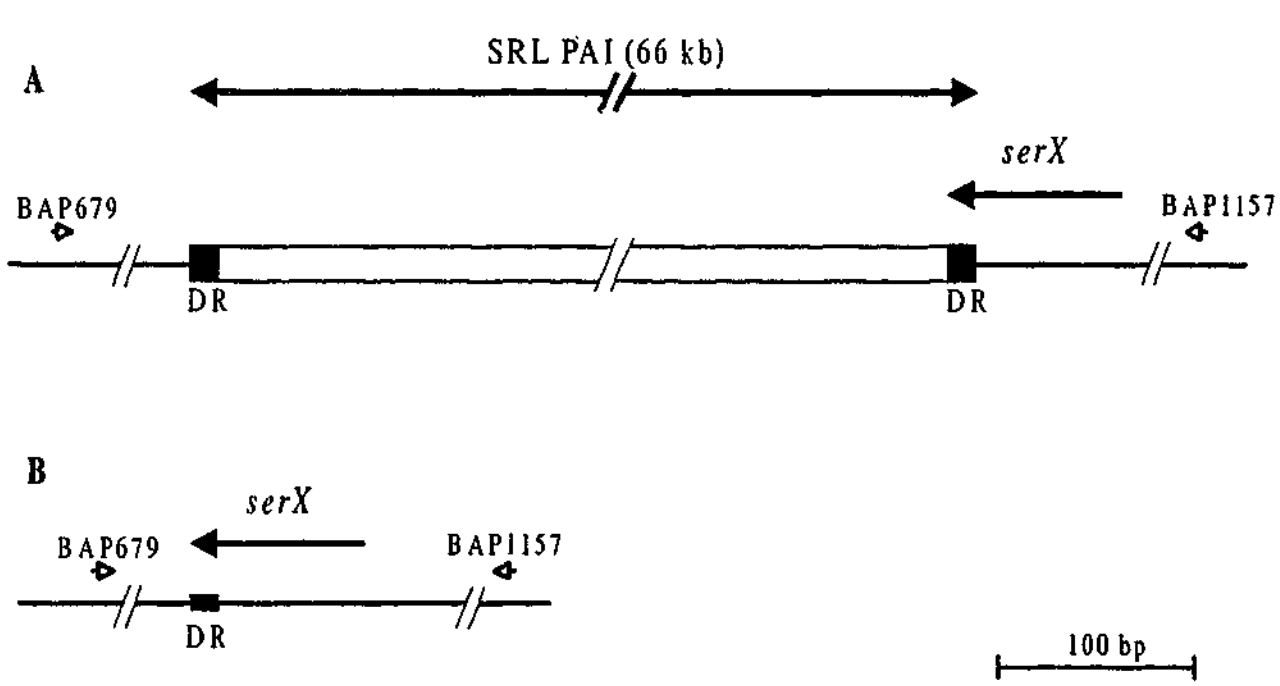



**Figure 2.5** Deletion of the SRL PAI. (A) Diagramatic representation of the genetic organisation of the left and right SRL PAI flanking regions in YSH6000. (B) Schematic representation of the resultant structure across the deletion point following loss of the SRL PAI in SBA1363 and SBA1367. Chromosomal DNA is presented as a thin black line whereas the SRL PAI is indicated as a white box and genes as arrows. The 14 bp direct repeats (DRs) are shown as black boxes. Also shown are the locations of primers BAP1157 and BAP679 used to amplify across the SRL PAI deletion point.

previously reported, the Southern hybridisation profiles of SBA1363 and SBA1367 suggested that the PAI might be capable of excision (Fig. 2.2a). Inward facing primers situated either side of *serX* were used to amplify a product spanning the right and left flanking regions of the PAI. An approximately 1.1 kb product was amplified in both SBA1363 and SBA1367 and sequence analysis of this fragment revealed the presence of an intact *serX* gene, with only a single copy of the 14 bp repeat present. Upstream and downstream regions were identical to the left and right flanking regions of the PAI, demonstrating that the SRL PAI itself is capable of precise excision from the chromosome leaving behind an intact *serX* gene (Fig. 2.5b).

## The Role of Integrase in Excision of the SRL PAI

Hacker *et al.* (1997) suggested that flanking repeats may act as targets for site-specific recombinases, facilitating integration and/or excision of PAIs. It was thought that the 14 bp DR may therefore represent the core sequence of the SRL PAI attachment (*att*) site, acting in a manner similar to the *att* sites of site-specific bacteriophages (Campbell, 1992). The presence of mobility genes encoding determinants, such as integrases, on pathogenicity islands had previously been noted (Hacker, *et al.*, 1997; Hacker & Kaper, 1999), but with the exception of the PAI-like *Vibrio cholerae* SXT element (Hochhut & Waldor, 1999), the role of integrases in the excision of integrated PAIs had not been demonstrated. The SRL PAI encodes an ORF at its left boundary that shows similarity at the amino acid level to several integrase proteins from the P4 prophage Int family (Luck, *et al.*, 2001). This *orf*, designated *int*, encodes a putative protein of 405 amino acids, and contains the highly conserved HxxR, Y motif necessary for integrase function (Argos *et al.*, 1986). As the model of PAI deletion described above implicates the involvement of a site-specific recombinase, the role of the SRL PAI *int* in deletion of the element was investigated.

An insertion mutation in the *int* gene was constructed in *S. flexneri* 2a strain YSH6000, and confirmed by Southern hybridisation (Fig. 2.6). The frequency of spontaneous SRL PAI excision in YSH6000 and the *int* mutant strain, AL11, was compared. Spontaneous SRL

PAI excisants were isolated by taking advantage of the selective properties of fusaric acid against tetracycline resistance encoded by the SRL PAI (see Materials and Methods). Tetracycline sensitive derivative strains were further tested for susceptibility to the antibiotics Sm, Ap and Cm, and PAI deletions were confirmed by PCR using primers to amplify a 1.1 kb product across the intact *serX* gene as performed previously for SBA1363 and SBA1367 (Fig. 2.5). By comparing the number of PAI excisants to the total cell count, the PAI deletion frequency was estimated to be approximately $10^{-5}$ per cell in the wild type strain YSH6000. However, precise excision of the SRL PAI was not detected at all in the mutant strain AL11 (detection limit = 1.1 x $10^{-7}$ per cell). Since deletion of the MRDE occurs at a rate of $10^{-6}$ per cell in both the wild type and AL11, the *int* mutation was responsible for at least a 10-fold decrease in the SRL PAI excision rate, suggesting that the integrase gene is essential for precise excision of the PAI.

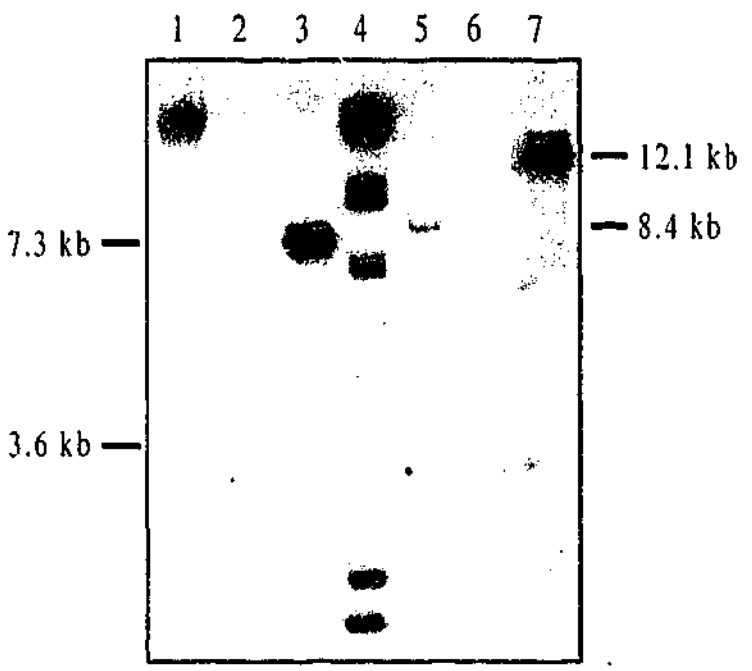



**Figure 2.6** Southern hybridisation analysis with *int* probe. *Cla*I digests: lane 1 – 3. *Eco*RV digests: lane 5 –7.
Lane 1 & 5: YSH6000 (wild type integrase)
Lane 2 & 6: YSH600T (no integrase)
Lane 3 & 7: AL11 (insertional integrase mutant)
Lane 4: DIG-labelled λ *Hind*III markers
Numbers to the left and right indicate a 3.7 kb increase in fragment size due to insertion of pAL11 into the chromosome in AL11

To confirm that the loss of PAI excision in this strain was due to inactivation of the integrase, AL11 was complemented with pBRTp$^r\Delta$ carrying an intact *int* gene (pAL66). The resultant strain, designated AL110, YSH6000/pBRTp$^r\Delta$ (AL108) and AL11/pBRTp$^r\Delta$ (AL109) were tested for spontaneous SRL PAI excision using a PCR assay. Genomic DNA extracted from AL108, AL109 and AL110 was standardised for concentration and assayed for excision of the PAI using inward facing primers, BAP1157 and BAP679, to amplify

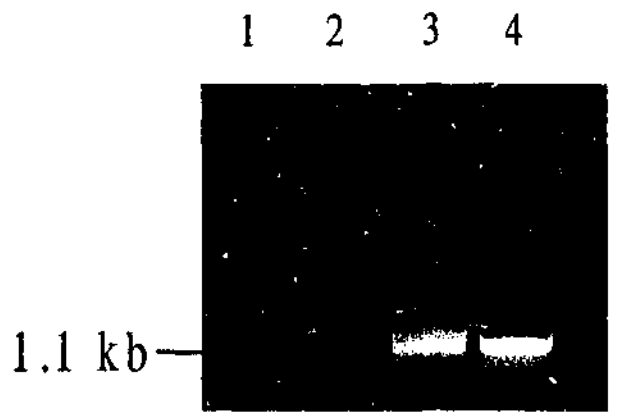



**Figure 2.7** Complementation of the *int* mutation. PCR products spanning the SRL PAI excision site amplified using primers BAP1157 and BAP679 (Fig. 2.5). From 100 µl PCR reactions, all was loaded for lanes 1 and 2, 75 µl for lane 3 and 25 µl for lane 4. Lanes: 1, AL108 (YSH6000/pBRTp$^r\Delta$); 2, AL109 (AL11/pBRTp$^r\Delta$); 3, AL110 (AL11/pAL66); 4, SBA1363 (YSH6000 derived spontaneous PAI deletant).

*serX* as described previously. Although PAI excision is an infrequent event, a PCR product could be detected using wild type AL108 DNA (Fig. 2.7, lane 1), but as expected, no PCR product was detected when using AL109 DNA as template (Fig. 2.7, lane 2) confirming the previous findings that strains lacking a functional *int* gene are unable to undergo PAI deletion. An amplification product across the PAI deletion point was again detected using the *int* complemented strain AL110, confirming that *int* was required for SRL PAI excision (Fig. 2.7, lane 3).

# DISCUSSION

In this study we demonstrated three independent mechanisms for the deletion of the resistance locus of *S. flexneri* 2a YSH6000: deletion of the MRDE involving IS*91* elements, deletion of the SRL involving IS*1* elements, and deletion of the SRL PAI occurring via *int*-mediated recombination of 14 bp DRs located at each extremity of the element. In many ways, the different deletion events involving the SRL PAI resemble the variety of deletion events that the HPI undergoes in different species of *Yersinia*. Like the SRL PAI, in *Y. pseudotuberculosis* IP32637, the HPI deletes via recombination between flanking 17 bp DRs (Buchrieser *et al.*, 1998). However, like the MRDE, in *Y. pestis* and *Y. enterocolitica* Ye8081 deletion of the HPI is associated with loss of flanking chromosome by either homologous recombination of flanking IS*100* elements, or by as yet undefined mechanisms, respectively (Bach, *et al.*, 1999; Fetherston, *et al.*, 1992). To the best of our knowledge, the SRL PAI of *S. flexneri* 2a YSH6000 is the first PAI that undergoes both integrase mediated

and non-integrase mediated excision in the same strain. Besides the SXT element of *V. cholerae* (Hochhut & Waldor, 1999), the SRL PAI is only the second such element to be described which carries multi-antibiotic resistance determinants.

The structuring of the three nested elements is itself unique, and although the MRDE was the first element to be described as carrying the SRL (Rajakumar, *et al.*, 1997a; Rajakumar, *et al.*, 1996), it seems unlikely that the entire 99 kb inserted *en bloc* into the *Shigella flexneri* 2a YSH6000 chromosome. Rather, it would appear that the insertion of the IS*91* elements and the SRL PAI were distinct events. This hypothesis is supported by the occurrence of independent deletions involving the PAI and MRDE, and also explains the remarkable sequence and organisational conservation of the regions surrounding the PAI and the corresponding region in the *E. coli* chromosome. Additionally, had the entire MRDE inserted into the *S. flexneri* chromosome this would presumably lead to a duplication of the regions between the *mdoA* locus and *putA*, a phenomenon which was not observed in YSH6000.

We have shown that the *int* gene was not required for MRDE deletion, and the loss of one of the flanking IS*91* elements after deletion suggested that this element itself may be involved in excision of the MRDE. IS*91* is the prototype of a small, unique family of IS elements that are believed to propagate by a rolling-circle replication mechanism (Mahillon & Chandler, 1998). IS*91* was originally isolated from a haemolysin encoding plasmid of *E. coli* and has since been implicated in the spread of haemolysin (*hly*) genes (Zabala *et al.*, 1982; Zabala *et al.*, 1984). *hly* genes have been reported on several PAIs in *E. coli* (Hacker *et al.*, 1999) and although no *hly* genes were discovered on the SRL PAI, it does carry an ORF showing similarity to a haemolysin expression modulating protein of *E. coli* (Hha) (Luck, *et al.*, 2001). It is an intriguing possibility that *hly* determinants may have originally been present on the SRL PAI.

IS-mediated deletion of adjacent DNA has been demonstrated for some IS elements (Galas & Chandler, 1989). However, it has been reported that IS*91* is unable to cause deletions of

adjacent DNA (Bernales, *et al.*, 1999); thus the deletion of the MRDE is probably not mediated by transposition of the elements themselves. It is likely that MRDE deletion is the result of *recA*-mediated homologous recombination between the two flanking elements, which would cause a looping out of the intervening region, resulting in a single residual copy of IS*91* element on the chromosome. Such RecA-dependant "adjacent deletions" have been shown to occur for other IS elements (Kleckner, 1989) and work to determine if the MRDE deletion is the result of RecA dependant homologous recombination may be carried out in the future.

Deletion of the SRL also appears to involve IS elements. Two potentially functional IS*1* elements flank the SRL, although upon deletion, only a single IS*1* element remains on the chromosome. IS*1* is known to mediate deletions of adjacent DNA that end precisely at one boundary of the element (Galas & Chandler, 1989). The loss of the SRL may be an example of such a deletion.

Alternatively, the SRL may delete via homologous recombination between the flanking IS*1* elements. As described previously, the YSH6000 SRL shares many similarities with the resistance determinant (r-det) of NR1, an archetypal resistance plasmid of *Shigella* (Rajakumar, *et al.*, 1997a). The r-det of NR1, which has a size of 23.3 kb and is flanked by direct repeats of IS*1*, is also a transposable unit (Womble & Rownd, 1988). Using the phage P1 as a carrier, the IS*1* flanked r-det (Tn*2671*) was shown to move both to the site of the resident IS*1* in the P1 genome, and to another region of the P1 genome (Iida & Arber, 1980). It was proposed that the former mechanism would involve an intermediate circular form of the r-det carrying a single copy of the IS*1* that excises from NR1 via homologous recombination between the flanking IS*1* elements (Iida & Arber, 1980). The organisation of the YSH6000 SRL deletants suggests that a similar recombination event may have taken place between the two flanking SRL IS*1* elements, thus leaving a single copy of an IS*1* element on the chromosome. Interestingly, after mobilisation to P1, Tn*2671* was subsequently mobilised to *E. coli* recipients, and then to the genome of phage P7, demonstrating the existence of a natural mechanism for spread of antibiotic resistance genes

(Iida *et al.*, 1981). This highlights the potential of the SRL to spread to other genomes, independent of mechanisms involving the MRDE or SRL PAI deletions.

In this study we have demonstrated that the SRL PAI of *S. flexneri* 2a strain YSH6000, which is flanked by 14 bp DRs, excises from the 3' end of the *serX* tRNA gene via mechanisms that resemble the site-specific recombination exhibited by some prophages. The absence of detectable precise deletion of the SRL PAI in the *int* mutant suggests that recombination between the flanking DRs is *int*-mediated site-specific recombination, a mechanism similar to that observed for some phages. Phage transduction has previously been implicated in the mobility of some PAIs (eg *V. cholerae* VPI and the staphylococcal SaPI), and bacteriophage proteins encoded on PAIs are often assumed to have played a role in original mobilisation of the elements (Hacker & Kaper, 2000). Indeed, the excision function of element-encoded integrase genes has been demonstrated with minimal-PAI cassettes for the HPI (Rakin *et al.*, 2001) and SaPIbov2 (Ubeda *et al.*, 2003). However, with the exception of the SXT element of *V. cholerae* (Hochhut & Waldor, 1999), the role of phage-like integrases in the excision of integrated PAIs has not been demonstrated. Here, the *S. flexneri* 2a strain YSH6000 SRL PAI integrase was shown to be required for the precise excision of the element, confirming that the integrase is functional and plays an important role in the deletion of the SRL PAI.

The SRL PAI is one of a growing number of PAIs that appear to delete precisely through site-specific recombination of short flanking DRs. It is probable that like phages these PAIs originally integrated into the 3' termini of tRNA genes, with the DR sequence acting in a manner similar to the core of *attB* sites during phage integration (Campbell, 1992). Other elements in this category may include SaPIbov2 with 18bp DRs (Ubeda, *et al.*, 2003), PAI $I_{536}$ and $II_{536}$ of UPEC with 16 bp and 18 bp DRs respectively (Blum, *et al.*, 1994), the *she* PAI of *S. flexneri* with 22 bp DRs (Al-Hasani *et al.*, 2001b), and the HPI of *Y. pseudotuberculosis* with 17 bp DRs (Buchrieser, *et al.*, 1998). Additionally, these five PAIs also carry sequences near one end of the element exhibiting similarity to the P4 family of phage-like integrases (Al-Hasani, *et al.*, 2001b; Buchrieser, *et al.*, 1998; Hacker, *et al.*,

1999). Indeed, many other PAIs have been found to possess sequences with similarity to integrases and other phage-related ORFs. Interestingly, the insertion site of the HPI was found to contain a region showing partial similarity to the P4 *attB* site (Buchrieser, *et al.*, 1998). The 3' end of *serX* near the SRL PAI integration site also possesses a sequence that matches 12 of the 20 bp of the P4 *attB* (Luck, *et al.*, 2001). The *she* PAI in *S. flexneri* and HPI of *Y. pseudotuberculosis* have been found inserted in at least two different *phe* and *asn* tRNA genes possessing the target DR of each, respectively (Al-Hasani *et al.*, 2001a; Buchrieser, *et al.*, 1998). Similarly, the SRL PAI has been found inserted in both *serX* and a paralogue of this gene, *serW*, in *Shigella* (Chapter 4) indicating that, like the chromosomal integration of phages, there is a high level of insertion specificity for these PAIs.

In this study, the SRL PAI was shown to have a deletion rate of approximately $10^{-5}$, which is consistent with those of PAI $I_{536}$ and $II_{536}$ ($10^{-4}$ – $10^{-5}$) (Hacker, *et al.*, 1997), the HPI ($10^{-4}$) (Buchrieser, *et al.*, 1998) and the *she* PAI ($10^{-5}$ – $10^{-6}$) (Rajakumar *et al.*, 1997b). Other similarities include flanking DRs, the presence of integrases and evidence of site-specific deletion. In light of evidence presented here that the *int* gene is required for precise excision of the SRL PAI, the shared features of these PAIs provide a strong argument that integrase dependent, site-specific recombination is likely to be a common mechanism of excision amongst these elements. Indeed, the P4-like integrase from the *Y. pestis* HPI has been shown to act in a site-specific manner (Rakin, *et al.*, 2001) while in the HPI of *Y. enterocolitica*, interruption of the integrase gene and loss of conservation in the flanking 17 bp DRs are thought to be responsible for the 'stabilisation' of this element in the chromosome. In the HPI of *Y. pseudotuberculosis*, the integrase gene and DRs are intact, probably explaining why this element undergoes precise excision from the chromosome (Bach, *et al.*, 1999). It is interesting to note that although the *int* sequences of both PAI $I_{536}$ and $II_{536}$ of UPEC are thought to be non-functional, these PAIs delete from the chromosome at a rate similar to the SRL PAI (Hacker, *et al.*, 1999; Hacker, *et al.*, 1997), implying that other recombinases may mediate the excision of these elements.

Further evidence for the importance of integrases is found in the *V. cholerae* SXT element, which exhibits many similarities to PAIs including mobility, insertion into a specific chromosomal site and the presence of 17 bp DRs flanking the inserted element. The *int* gene of the SXT element is necessary for excision and/or production of an extrachromosomal circular form of the element, which is required for the transfer of SXT to both *V. cholerae* and *E. coli* recipients (Hochhut & Waldor, 1999). Recently Rakin *et al.* provided evidence that the integrase from *Y. pestis* HPI promotes both excision and integration of a minimal integrative HPI module into the *asn*-tRNA target site (Rakin, *et al.*, 2001). These findings demonstrate that the *int* of the SRL PAI and other PAIs may play an equally important role not only in excision, but also in acquisition and dissemination of the PAI-like elements on which they reside.

Importantly, from a clinical perspective these elements pose a substantial future risk given their ability to alter dramatically both the virulence and the antibiotic susceptibility profile of a pathogen.

# REFERENCES


**Al-Hasani, K., Adler, B., Rajakumar, K. & Sakellaris, H. (2001a).** Distribution and strcutural variation of the *she* pathogencity island in enteric bacterial pathogens. *Journal of Medical Microbiology* **50**, 780 - 786.

**Al-Hasani, K., Rajakumar, K., Bulach, D., Robins-Browne, R., Adler, B. & Sakellaris, H. (2001b).** Genetic organization of the *she* pathogenicity island in *Shigella flexneri* 2a. *Microbial Pathogenesis* **30**, 1 - 8.

**Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997).** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389 - 3402.

**Argos, P., Landy, A., Abremski, K., Egan, J. B., Haggard-Ljungquist, E., Heoss, R. H., Kahn, M. L., Kalionis, B., Narayana, S. V. L., Pierson III, L. S., Sternberg, N. & Leong, J. M. (1986).** The integrase family of site-specific recombinases: regional similarities and global diversity. *EMBO Journal* **5**, 433 - 440.

**Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1993).** *Current protocols in molecular biology*. New York: Greene Publishing and Wiley Interscience.

**Bach, S., Buchrieser, C., Prentice, M., Guiyoule, A., Msadek, T. & Carniel, E. (1999).** The high-pathogenicity island of *Yersinia enterocolitica* Ye8081 undergoes low-frequency deletion but not precise excision, suggesting recent stabilization in the genome. *Infection and Immunity* **67**, 5091 - 5099.

**Bernales, I., Mendiola, M. V. & de la Cruz, F. (1999).** Intramolecular transposition of insertion sequence IS91 results in second-site simple insertions. *Molecular Microbiology* **33**, 223 - 234.

**Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H. & Hacker, J. (1994).** Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. *Infection and Immunity* **62**, 606 - 614.

**Buchrieser, C., Brosch, R., Bach, S., Guiyoule, A. & Carniel, E. (1998).** The high-pathogenicity island of *Yersinia pseudotuberculosis* can be inserted into any of the three chromosomal *asn* tRNA genes. *Molecular Microbiology* **30**, 965 - 978.

**Campbell, A. M. (1992).** Chromosomal insertion sites for phages and plasmids. *Journal of Bacteriology* **174**, 7495 - 7499.

**Casalino, M., Nicoletti, M., Salvia, A., Colonna, B., Pazzani, C., Calconi, A., Mohamud, K. A. & Maimone, F. (1994).** Characterization of endemic *Shigella flexneri* strains in Somalia: antimicrobial resistance, plasmid profiles, and serotype correlation. *Journal of Clinical Microbiology* **32**, 1179 - 1183.

**Dozois, C. M. & Curtiss III, R. (1999).** Pathogenic diversity of *Escherichia coli* and the emergence of 'exotic' islands in the gene stream. *Veterinary Research* **30**, 157 - 179.

**DuPont, H. L., Levine, M. M., Hornick, R. B. & Formal, S. B. (1989).** Inoculum size in shigellosis and implications for expected mode of transmission. *Journal of Infectious Diseases* **159**, 1126-1128.

**Fetherston, J. D., Schuetze, P. & Perry, R. D. (1992).** Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. *Molecular Microbiology* **6**, 2693 - 2704.

**Galas, D. & Chandler, M. (1989).** Bacterial insertion sequences. In *Mobile DNA*, pp. 109 - 162. Edited by D. E. Berg & M. M. Howe. Washington, D.C.: American Society for Microbiology.

**Gebre-Yohannes, A. & Drasar, B. S. (1990).** Plasmid profiles of antibiotic-resistance Shigella dysenteriae types 2, 3, 4, 6 and 7 isolated in Ethiopia during 1976 - 85. *Epidemiology and Infection.* **105**, 65 - 72.

**Glover, D. M. (1985).** *DNA cloning : a practical approach.* Oxford: IRL Press Limited.

**Hacker, J., Blum-Oehler, G., Janke, B., Nagy, G. & Goebel, W. (1999).** Pathogenicity islands of extraintestinal *Escherichia coli.* In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 59 - 76. Edited by J. B. Kaper & J. Hacker. Washington: American Society for Microbiology.

**Hacker, J., Blum-Oehler, G., Muhldorfer, I. & Tschape, H. (1997).** Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Molecular Microbiology* **23**, 1089 - 1097.

**Hacker, J. & Kaper, J. B. (1999).** The concept of pathogenicity islands. In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 1 - 11. Edited by J. B. Kaper & J. Hacker. Washington: American Society for Microbiology.

**Hacker, J. & Kaper, J. B. (2000).** Pathogenicity islands and the evolution of microbes. *Annual Review of Microbiology* **54**, 641 - 679.

**Hochhut, B. & Waldor, M. K. (1999).** Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. *Molecular Microbiology* **32**, 99 - 110.

**Iida, S. & Arber, W. (1980).** On the role of IS*1* in the formation of hybrids between the bacteriophage P1 and the R plasmid NR1. *Molecular and General Genetics* **177**, 261 - 270.

**Iida, S., Hanni, C., Echarti, C. & Arber, W. (1981).** Is the IS1-flanked r-determinant of the R plasmid NR1 a transposon. *Journal of General Microbiology* **126**, 413 - 425.

**Karaolis, D. K. R. & Kaper, J. B. (1999).** Pathogenicity islands and other mobile virulence elements of *Vibrio cholerae*. In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 167 - 187. Edited by J. B. Kaper & J. Hacker. Washington: American Society for Microbiology.

**Karaolis, D. K. R., Somara, S., Maneval Jr, D. R., Johnson, J. A. & Kaper, J. B. (1999).** A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature* **399**, 375 - 379.

**Khan, M. U. (1985).** Fourteen years of shigellosis in Dhaka: an epidemiological analysis. *International Journal of Epidemiology* **14**, 607 - 613.

**Kleckner, N. (1989).** Transposon Tn*10*. In *Mobile DNA*, pp. 227 -268. Edited by D. E. Berg & M. M. Howe. Washington: American Society for Microbiology.

**Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Swerdlow, D. L., Sansonetti, P. J., Adak, G. K. & Levine, M. M. (1999).** Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bulletin of the World Health Organization* **77**, 651 - 665.

**Ling, J. M., Shaw, P. C., Kam, K. M., Cheng, A. F. & French, G. L. (1993).** Molecular studies of plasmids of multiply-resistant *Shigella* spp. in Hong Kong. *Epidemiology and Infection* **110**, 437 - 446.

**Luck, S. N., Turner, S. A., Sakellaris, H., Rajakumar, K. & Adler, B. (2001).** Ferric dicitrate transport system (Fec) of *Shigella flexneri* 2a YSH6000 Is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. *Infection and Immunity* **69**, 6012 - 6021.

**Mahillon, J. & Chandler, M. (1998).** Insertion sequences. *Microbiology and Molecular Biology Reviews* **62**, 725 - 774.

**Maloy, S. R. & Nunn, W. D. (1981).** Selection for loss of tetracycline resistance by *Escherichia coli*. *Journal of Bacteriology* **145**, 1110 - 1112.

**Matsutani, S. (1994).** Genetic evidence for IS1 transposition regulated by InsA and the (delta)InsA-B'-InsB species, which is generated by translation from two alternative internal initiation sites and frameshifting. *Journal of Molecular Biology* **240**, 52 - 65.

**Mendiola, M. V. & de la Cruz, F. (1989).** Specificity of insertion of IS91, and insertion sequence present in alpha-haemolysin plasmids of *Escherichia coli*. *Molecular Microbiology* **3**, 979 - 984.

**Mendiola, M. V., Jubete, Y. & de la Cruz, F. (1992).** DNA sequence of IS*91* and Identification of the transposase gene. *Journal of Bacteriology* **174**, 1345 - 1351.

**Miller, V. C. & Mekalanos, J. J. (1988).** A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* regions *toxR*. *Journal of Bacteriology* **170**, 2575 - 2583.

**Morelle, G. (1989).** A plasmid extraction procedure on a mimiprep scale. *Focus* **11**, 7 - 8.

**Nakata, N., Sasakawa, C., Okada, N., Tobe, T., Fukuda, I., Suzuki, T., Komatsu, K. & Yoshikawa, M. (1992).** Identification and characterization of *virK*, a virulence-associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. *Molecular Microbiology* **6**, 2387 - 2395.

**Okada, N., Sasakawa, C., Tobe, T., Yamada, M., Nagai, S., Talukder, K. A., Komatsu, K., Kanegasaki, S. & Yoshikawa, M. (1991).** Virulence-associated chromosomal loci of Shigella flexneri identified by random Tn5 insertion mutagenesis. *Molecular Microbiology* **5**, 187 - 195.

**Penfold, R. J. & Pemberton, J. M. (1992).** An improved suicide vector for construction of chromosomal insertion mutations in bacteria. *Gene* **118**, 145 - 146.

**Rajakumar, K., Bulach, D., Davies, J., Ambrose, L., Sasakawa, C. & Adler, B. (1997a).** Identification of a chromosomal *Shigella flexneri* multi-antibiotic resistance locus which shares sequence and organizational similarity with the resistance region of the plasmid NR1. *Plasmid* **37**, 159 - 168.

**Rajakumar, K., Sasakawa, C. & Adler, B. (1996).** A spontaneous 99-kb chromosomal deletion results in multi-antibiotic susceptibility and an attenuation of contact haemolysis in *Shigella flexneri* 2a. *Journal of Medical Microbiology* **45**, 64 - 75.

**Rajakumar, K., Sasakawa, C. & Adler, B. (1997b).** Use of a novel approach, termed island probing, identifies the *Shigella flexneri she* pathogenicity island which encodes a homolog of the immunoglobulin A protease-like family of proteins. *Infection and Immunity* **65**, 4606 - 4614.

**Rakin, A., Noelting, C., Schropp, P. & Heesemann, J. (2001).** Integrative module of the high-pathogenicity island of *Yersinia*. *Molecular Microbiology* **39**, 407 - 415.

**Ruzin, A., Lindsay, J. & Novick, R. P. (2001).** Molecular genetics of SaPI1 - a mobile pathogenicity island in *Staphylococcus aureus*. *Molecular Microbiology* **41**, 365 - 377.

**Sack, R. B., Rahman, M., Yunus, M. & Khan, E. H. (1997).** Antimicrobial resistance in organisms causing diarrheal disease. *Clinical Infectious Diseases* **24(Suppl 1)**, S102 - S105.

**Sasakawa, C., Kamata, K., Sakai, T., Murayama, S. Y., Makino, S. & Yoshikawa, M. (1986).** Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. *Infection and Immunity* **51**, 470 - 475.

**Smith, C. L. & Cantor, C. R. (1987).** Purification, specific gragmentation, and separation of large DNA molecules. *Methods in Enzymology* **155**, 449 - 467.

**Turner, S. A. (1998).** Characterisation of the right flanking region and deletion endpoints of a 99 kb chromosomal element in *Shigella flexneri* 2a. *B. Sc. (Hons) Thesis*. Monash University: Melbourne.

**Ubeda, C., Angeles, T., Cucarella, C., Trotonda, P., Foster, T. J., Lasa, I. & Penades, J. R. (2003).** Sip, an integrase protein with excision, circularization and integration activities, defines a new family of mobile *Staphylococcus aureus* pathogenicity isalnds. *Molecular Microbiology* **49**, 193 - 210.

**Wang, R. F. & Kushner, S. R. (1991).** Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**, 195 - 199.

**Womble, D. D. & Rownd, R. H. (1988).** Genetic and physical map of plasmid NR1: Comparison with other IncFII antibiotic resistance plasmids. *Microbiological Reviews* **52**, 433 - 451.

**Zabala, J. C., de la Cruz, F. & Oritz, J. M. (1982).** Several copies of the same insertion sequence are present in alpha-hemolytic plasmids belonging to four different incompitability groups. *Journal of Bacteriology* **151**, 472 - 476.

**Zabala, J. C., Garcia-Lobo, J. M., Diaz-Aroca, E., de la Cruz, F. & Oritz, J. M. (1984).** *Escherichia coli* alpha-haemolysin synthesis and export genes are flanked by a direct repetition of IS91-like elements. *Molecular and General Genetics* **197**, 90 - 97.

3

# CHAPTER THREE

## The Role of the SRL PAI *attP* in Integrase-Mediated Integration

*Based on:*

**Turner, S. A., Luck, S. N., Sakellaris, H., Adler, B. (submitted: Antibmicrobial Agents and Chemotherapy).** The Role of *attP* in Integrase-mediated Integration of the SRL Pathogenicity Island of *Shigella flexneri.*

# INTRODUCTION

*Shigella* spp. are the causative agents of bacillary dysentery, a disease responsible for the deaths of over 1.1 million people annually (Kotloff *et al.*, 1999). The infection, which is spread via the faecal-oral route, is commonly treated with a combination of rehydration and antimicrobial therapy. However, over the past few decades, treatment has become increasingly difficult as resistance has emerged to most of the widely used therapeutic antibiotics (Khan, 1985; Salam & Bennish, 1991).

Multiple antibiotic resistance was first reported in *Shigella* as early as the 1950s (Watanabe, 1963), and since this time multi-resistance gene clusters have been identified on R-plasmids, transposons and integrons. Recently, a cluster of genes known as the *Shigella* resistance locus (SRL) was identified on the chromosome of *S. flexneri* 2a strain YSH6000. In this strain the SRL, which confers resistance to the antibiotics streptomycin (Sm), ampicillin (Ap), chloramphenicol (Cm) and tetracycline (Tc), is present on a pathogenicity island (PAI) designated the SRL PAI (Luck *et al.*, 2001). Data presented in Chapter 4 indicates the SRL PAI is present in numerous *Shigella* strains, and it is thus hypothesised that the SRL PAI may be involved in the spread of multiple-antibiotic resistance in *Shigella* spp.

PAIs, which are believed to be acquired by horizontal gene transfer, frequently carry phage-related integrase genes, are often integrated adjacent to tRNA genes and may be flanked by short direct repeats (DRs) which resemble phage attachment (*att*) sites (Blum *et al.*, 1994). It is thought that the genetic similarities of PAIs and phages may extend to related mechanisms of integration and excision (Hacker & Kaper, 1999). However, genetic instability of PAIs has been observed only in a small number of cases, with only two PAIs shown to be mobilisable, and very little known about mechanisms of PAI transfer in general (Davis & Waldor, 2002). In contrast, the mechanism by which the SRL PAI excises from the *serX* tRNA gene in *S. flexneri* 2a YSH6000 is known to be integrase-mediated and site-specific (Chapter 2). These data, in conjunction with the presence of the SRL PAI in other *Shigella* strains (Chapter 4), suggest that the SRL PAI, may still be mobile. However, nothing is known of the excised form of the SRL PAI, nor its ability to integrate into a new host.

# MATERIALS AND METHODS

## Bacterial Strains, Media and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1. Strains were grown routinely at 37 °C in Luria Bertani medium (Ausubel *et al.*, 1993), and broth cultures were incubated with shaking. Ampicillin (100 μg/ml), kanamycin 50 μg/ml) trimethoprim (50 μg/ml), chloramphenicol (40 μg/ml), tetracycline (10 μg/ml) or naladixic acid (10 μg/ml) were added to plates or broth where appropriate.

## Molecular Biological Techniques

Genomic DNA was isolated by the small-scale preparation as described previously (Ausubel, *et al.*, 1993). Plasmid DNA was isolated by a modification of the alkaline lysis method (Morelle, 1989). Standard cloning procedures using the vector pJP5603 were employed. *E. coli* DH5α was transformed using the transformation storage buffer (TSB) method (Chung & Miller, 1988). DNA from both plasmids and PCR products was prepared for sequence analysis using the PRISM™ Ready Reaction Dye Deoxy™ Terminator Cycle and chromatograms were produced on an Applied Biosystems model 373A DNA sequencing system.

## Separation of Plasmid and Chromosomal DNA

Plasmid and chromosomal DNA were separated using a modification of the Eckhardt in-well lysis method (Priefer, 1984). Approximately $10^7$ – $10^8$ cells were resuspended in lysozyme mixture (7 % Ficoll, 20 % sucrose, 1 U/ml RNase, 1 mg/ml lysozyme in TBE) and loaded into the wells of 0.4 % and 0.8 % agarose gels. Samples were then overlayed with SDS mixture (1 % SDS, 5 % sucrose bromophenol blue in TBE) and electrophoresed for 30 min at 5 mA, followed by 2 h at 100 mA.

**Table 3.1** Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| *S. flexneri* | | |
| YSH6000 | Wild type *S. flexneri* 2a Japanese isolate, $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ | (Sasakawa *et al.*, 1986) |
| SBA1373 | YSH6000 single cross over pCACTUS:*fec*, $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ $Kn^r$ | S. N. Luck |
| AL325 | SBA1373 harbouring pAL85 | This study |
| *E. coli* | | |
| DH5α | F-ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169*endA1* *recA*1hsdR17deo$^R$*thi1supE*441$^-$*gyrA96rel*$^{A1}$ | Bethesda Research Laboratories |
| S17-1(λ*pir*) | RP4-2($Tc^s$::Mu)($Kn^s$::Tn7) *recA* λ*pir*, $Sm^r$ $Tp^r$ | (Miller & Mekalanos, 1988) |
| JM109(λ*pir*) | *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ (*lac-proAB*) λ*pir* | (Miller & Mekalanos, 1988) |
| SBA573 | C600 harbouring R100-1 | C. Sasakawa |
| SBA447 | DH5α harboring pWSK29 | (Wang and Kushner 1991) |
| AL104 | DH5α harboring pAL64 | (Chapter 2) |
| AL403 | S17-1 harbouring pAL226 | This study |
| Plasmids | | |
| pCACTUS | pSC101-based low copy number suicide vector with *sacB*, $Cm^r$ | (Van den Bosch *et al.*, 1997) |
| pJP5603 | R6K based suicide vecor; $Kn^r$ | (Penfold & Pemberton, 1992) |
| R100-1 | *finO* mutant of R100 depressed for transfer | (Yoshioka *et al.*, 1987) |
| pAL64 | pWSK29 harbouring 1.3 kb *int* fragment | (Chapter 2) |
| pAL85 | pUC19Tp harbouring *rox* gene on 750 bp PCR product | S. N. Luck |
| pAL226 | pJP5603 harbouring the 553 bp POP' region | This study |

## Southern Hybridisation

After electrophoresis, DNA was transferred to a charged nylon membrane (Roche) using capillary transfer in 20 X SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0). Overnight hybridisation and subsequent washings were performed under high stringency conditions at 68 °C as recommended in the Roche digoxigenin labelling and detection kit. Probes were labelled by PCR amplification with digoxigenin as specified by Boehringer Mannheim. The *cat* probe was amplified from YSH6000 genomic DNA using primers BAP2025 (5'-TTACGCCCCGCCCTGCCAC-3') and BAP2026 (5'-CGGGAGCTGGTGATATGG-3'). The *serX* and *serW* probes were amplified from DH5α genomic DNA using primer pairs BAP679 (5'-GTGCTGCTTTCGGTGTGC-3') and BAP1157 (5'GCCAGCATTTCAACAGGAGG-3'), and BAP1462 (5'-

ATACTCCACCCGCCCACC-3') and BAP1783 (5'-TGTTTTACCGCCTGATGGG-3') respectively.

## Construction of the *attP* Integrative Plasmid

Plasmid pAL85 harbouring the *rox* gene was transformed into strain SBA1373, which incorporates the temperature sensitive plasmid pCACTUS in the SRL PAI, to construct the strain AL325. Genomic DNA was extracted from AL325 grown at 25 °C, a temperature permissive for replication of pCACTUS, and used as template in amplification of the SRL PAI *attP* with primers BAP2275 (5'-CTTTAAGAATTCCTCCGCGCATATCACG-3') and BAP2276 (5'-GAAAAATCTAGATCCCCCGCCCCTTTACTG-3'). The *XbaI/Eco*RI digested *attP* PCR product was ligated to vector pJP5603, constructing the plasmid pAL226 which was isolated in JM109(λ*pir*). pAL226 was then introduced to the mobilising strain S17-1(λ*pir*) and the resulting strain, AL403, was used in overnight conjugations with *E. coli* strains SBA447 and AL104 to test the ability of the *attP* (pAL226) to recombine with the *E. coli* chromosome.

## PCR Amplification

PCR amplifications were performed on chromosomal DNA using the Taq DNA polymerase system as specified by Roche. Integration of pAL226 into *serX* was detected by PCR using primer pairs BAP2276/BAP679 and BAP2275/BAP1157. Integration of pAL226 into *serW* was detected by PCR using primer pairs BAP2276/BAP1462 and BAP2275/BAP1783. Template for colony PCR was prepared by boiling a single colony in 20 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA) for 10 min, pelleting by centrifugation at 13,000 rpm for 2 min, and adding 2 µl of the supernatant to the PCR mix. An additional 8 min incubation of 94 °C was added to the start of the standard PCR cycle.

### Computer Analysis

Sequencing chromatograms were analysed using the Sequencher program (GeneCodes Corporation, Ann Arbor, MI, USA). Nucleotide sequence similarity searches with sequences in the databases were performed using the BlastN or BlastX program (Altschul *et al.*, 1997). Analysis of *attP* for the presence of repeats was carried out using the MacVector progam (www.accelrys.com).

# RESULTS AND DISCUSSION

## Circular Form of the SRL PAI

The SRL PAI of strain YSH6000 is flanked by 14 bp DRs that correspond to the 3' end of the *serX* tRNA gene. Integrase-mediated excision results in the loss of the PAI and one copy of the DR, while an intact *serX* gene and a single copy of the DR remain on the chromosome. Thus, the SRL PAI excises via mechanisms that resemble the site-specific recombination exhibited by lambdoid phages (Chapter 2). Lambdoid phages are also known to integrate and excise from their chromosomal *attB* sites via a circular extra-chromosomal intermediate (Campbell, 1992); thus, we tested whether the SRL PAI also formed a similar circular intermediate upon deletion.

Deletion was predicted to take place via recombination of the *attL* (*attBOP'*) and *attR* (*attPOB'*) sites, which would result in the reconstitution of the chromosomal *attB* (*attBOB'*) site (demonstrated previously with BAP679 and BAP1157 (Chapter 2) Fig. 3.1), and also the formation of the *attP* (*attPOP'*) site carried on the circular form of the SRL PAI (Fig. 3.1). The *attP* junction on the SRL PAI circular intermediate was not detectable by PCR in YSH6000. As the excised form of the SRL PAI does not replicate, and the frequency of spontaneous PAI excision is low ($10^{-5}$ – $10^{-6}$) (Chapter 2), formation of the circle may be below the level of detection by PCR. For this reason, a YSH6000-derivative strain AL325, which incorporates a temperature-sensitive pCACTUS *ori* as a single crossover in the SRL PAI was constructed. To increase the sensitivity of the assay, hyper-excision of the SRL PAI was induced by the

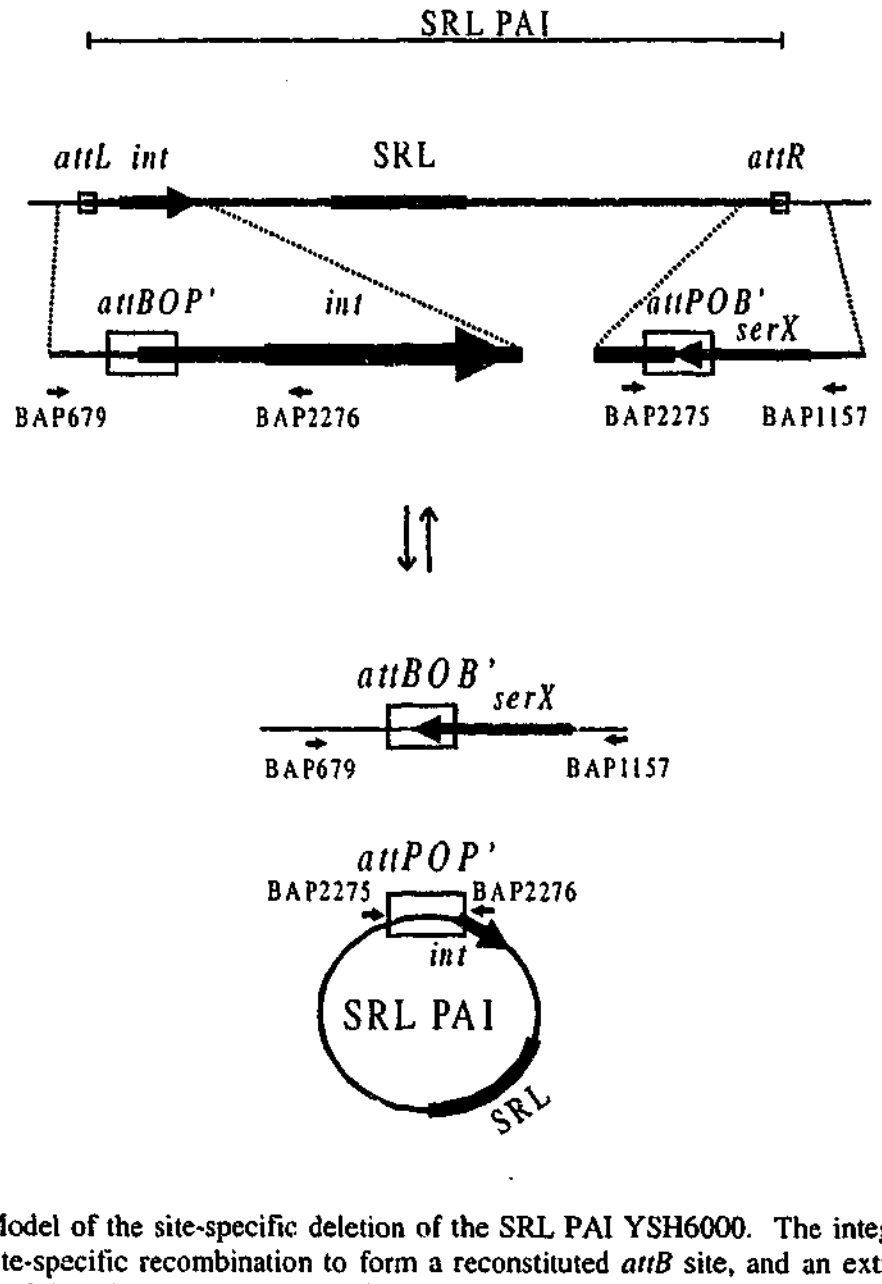



**Figure 3.1** Model of the site-specific deletion of the SRL PAI YSH6000. The integrated SRL PAI deletes via site-specific recombination to form a reconstituted *attB* site, and an extra-chromosomal circular form of the SRL PAI. Thin black lines represent chromosomal DNA, and a thick black line represents the SRL PAI. The SRL is represented by a black box, and *attL* (*attBOP'*), *attR* (*attPOB'*), *attP* (*attPOP'*) and *attB* (*attBOB'*) sites are represented as open boxes. Integrase (*int*) and *serX* genes are represented as large arrows and the position and orientation of primers used in amplifications are indicated with small arrows. Diagram not to scale.

introduction of the *rox* gene carried on pUC19Tp (S. N. Luck, manuscript in preparation). Outward facing primers BAP2275 and BAP2276 were used in a PCR with AL325 chromosomal template to successfully amplify the *attP* junction of an SRL PAI circular molecule formed upon recombination of the left (*attL*) and right (*attR*) PAI boundaries (Fig. 3.1). The resulting 553 bp fragment, designated *attP* due to the prophage-like organisation of the SRL PAI, was cloned into the suicide vector pJP5603 and sequenced. Sequence analysis of the resultant plasmid, pAL226, revealed that the SRL PAI *attP* (POP') consisted of the left end of the SRL PAI (P'), an exact copy of the

14 bp DR (O), followed by the right end of the SRL PAI (P) (Fig. 3.1). These results are thus consistent with the presence of a circular form of the SRL PAI.

To confirm that the SRL PAI was present as an extra-chromosomal molecule, Eckhardt gel electrophoresis, an in-well lysis method which allows plasmid DNA to enter the gel matrix while retarding chromosomal DNA, was employed with strains AL325, YSH6000 and SBA573 (carrying plasmid R100-1) which served as a positive control. Following electrophoresis, DNA was analysed by Southern hybridisation with a probe corresponding to the *cat* gene, which is harboured on R100-1 and the SRL PAI. Hybridisation with the *cat* probe occurred in the lanes corresponding with AL325 and the positive control SBA573, but not with YSH6000 (Fig. 3.2). Hybridisation with DNA from AL325 and the positive control SBA573 (R100-1) indicated that the *cat* gene is present on an extra-chromosomal molecule, thus confirming an extra-chromosomal location of the *cat* gene in both these strains. However, absence of hybridisation with YSH6000 is consistent with the presence of the *cat* gene on the chromosome which would be prevented from entering the gel matrix. These results indicate that the SRL PAI, although present on the chromosome in YSH6000, is present extra-chromosomally in the PAI-stabilised strain AL325 and is consistent with the presence of a stabilised, circular SRL PAI intermediate.

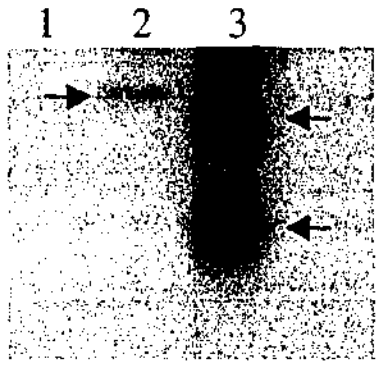



**Figure 3.2** Southern hybridisation analysis of an Eckhardt in-well lysis gel electrophoresis. Membrane probed with the *cat* gene. Arrows indicate *cat*-specific DNA. Lanes: 1, YSH6000; 2, AL325; 3, SBA573 (R100-1). The two bands observed in lane 3 correspond to the open circular and super-coiled forms of the R100-1 plasmid. Similarly, two bands were observed in lane 2 upon longer exposure of the membrane

## Site-Specific Recombination of the SRL PAI *attP*

Sequence analysis of the SRL PAI *attP* site revealed the presence of several DRs (Fig. 3.3). Integration systems often require a combination of accessory elements, and the Lambda *attP* site contains several DRs for the binding of Integrase and the accessory protein IHF (integration host factor) (Nash, 1996). Sequences resembling an IHF binding site WATCAANNNNTTR (W = A or T, R = A or G, N = A, G, C or T)


| | |
|---|---|
| DR1 | GATGGCATAC |
| DR2 | TAAACAAT |
| IHF | WATCAANNNNTTR (*E. coli*) |
| | AAaCAATAACaTG (SRL PAI *attP* -30) |
| | AAaCAATGTATTA (SRL PAI *attP* +59) |

**Figure 3.3** Schematic representation of the SRL PAI *attP* (*POP'*) structure. The 14 bp O core is shown as a black box. Numbers correspond to the positions of the DRs, taking the centre of the core region as position 0. Short horizontal arrows indicate DR1 and DR2, with sequence given below. Sequences showing similarity to IHF binding sites are indicated with open boxes, and alignments with the *E. coli* IHF binding site is shown below with mis-matched bases in lower-case. Figure not to scale.

(Friedman, 1988) are also present in the SRL PAI *attP* (Fig. 3.3). It is interesting to note that the positions of DR1 in the SRL PAI *attP* (-84, -72, +124, +136) (Fig. 3.3) show some similarity to the spacing of integrase binding sites in the Lambda *attP* arm sites (+80, +70, +60, –110, –140) (Campbell, 1992).

Previous analysis indicated that the SRL PAI is found adjacent to the identical paralogous tRNA genes *serX* and *serW* in a number of *Shigella* isolates (Chapter 4). To determine if the integration mechanism of the SRL PAI resembled phage-like site-specific integration, the SRL PAI *attP* was tested for its ability to undergo recombination with *serX* and *serW*, which would represent the chromosomal *attB* sites (Fig. 3.4). pAL226, which carries the SRL *attP* on the suicide plasmid pJP5603, was transferred to the mobilising *E. coli* strain S17-1 λ*pir* to construct AL403 ($Kn^r$). AL403 was then mated with AL104 ($Nal^r$, $Ap^r$), a RecA-deficient *E. coli* DH5α strain harboring the SRL PAI *int* gene on the plasmid pWSK29 (Chapter 2). Transconjugants were selected by plating on Nal+Ap+Kn and tested by PCR for the insertion of pAL226 into *serX* (using primer pairs BAP2276/BAP679 and BAP2275/BAP1157) or *serW* (using primer pairs BAP2276/BAP1462 and BAP2275/BAP1783) (Fig. 3.4). In all transconjugants tested, PCR product size and Southern hybridisation using *serX* or *serW*

as a probe were consistent with the insertion of pAL226 into one or both of these sites. Sequencing of the BAP2276/BAP679 and BAP2276/BAP1462 products confirmed the precise insertion of the SRL PAI POP' which results in an arrangement identical to that of the wildtype YSH6000 *attR* (*attPOB'*) (Fig. 3.1, Fig. 3.4). Thus, in the presence of *int*, the SRL PAI *attP* is sufficient for site-specific recombination with the chromosomal *attB* sites partially contained within *serX* or *serW*.

## The SRL PAI *attP* x *attB* Recombination Requires *int*

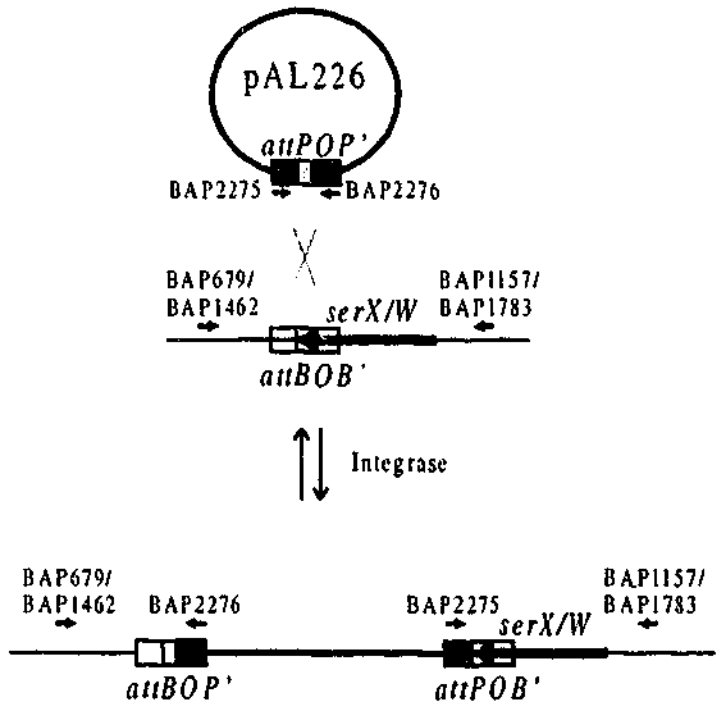



**Figure 3.4** Schematic representation of the integration of the SRL PAI *attP* (carried on plasmid pAL226) into the chromosomal *attB* sites. Chromosomal DNA is represented by thin black lines while the plasmid pAL226 is represented by thick black lines. *att* sites are represented by black or white boxes, with the common O core site represented as a shaded box. Genes are represented as large arrows and the position and orientation of primers used in amplifications are indicated with small black arrows. Diagram not to scale.

To test the ability of the SRL PAI *int* to catalyse the *attP* x *attB* recombination, matings of the donor AL403 (harboring the SRL PAI *attP*) was carried out with both an *int* positive and an *int* negative, RecA-deficient DH5α recipient. Site-specific integration into the *serX* or *serW* tRNA genes was determined by PCR with the primer pairs BAP2275/1157 and BAP2275/1783 (Fig. 3.4). Integrants were obtained with a frequency of $2 \times 10^{-4}$ per donor in the *int* positive background. However, no integration was detected in the *int*-negative background (detection limit $3.4 \times 10^{5}$). Thus, site-specific integration of the SRL PAI *attP* requires the presence of the SRL PAI integrase.

Together with previous findings (Chapter 2), these data indicate that the integration and excision mechanisms of the SRL PAI are closely related to those of lambdoid phages. First, the integrated SRL PAI forms a circular extra-chromosomal intermediate through

recombination of the left and right junctions. Second, integration via site-specific recombination occurs in a 14 bp sequence found both in the circular form of the SRL PAI and at the 3' end of the tRNA genes *serX* and *serW*. Finally, both excision and integration of the SRL PAI require the P4 phage-related SRL PAI *int* gene.

Many PAIs and genomic islands are flanked by short direct repeats, are situated adjacent to tRNA genes, and carry integrase-related sequences. Thus it has been suggested that, although in the majority of cases direct evidence of integration and excision is lacking, site-specific recombination is likely to play a role in the mobility of these elements (Hacker & Kaper, 1999). Certainly an integrative module of the *Yersinia* HPI has been shown to undergo site-specific integration (Rakin *et al.*, 2001), and similarly the *Staphylococcus* SaPIbov2 undergoes site-specific, integrase-mediated integration and excision (Ubeda *et al.*, 2003). Indeed, as the SRL PAI is able to undergo *int*-dependent, site-specific excision (Chapter 2) and integration, it seems credible that site-specific recombination plays a central role in the mobility of the SRL PAI.

To date, site-specific recombination is known to be involved where mobility of genomic islands and PAIs has been demonstrated. The conjugative integrating *V. cholerae* SXT element has been shown to employ integrase-mediated, site-specific recombination to transfer as a circular intermediate (Hochhut & Waldor, 1999), and the *V. cholerae* pathogenicity island (VPI) is thought to undergo recombination of *att*-like sequences and be packaged as a phage (Karaolis *et al.*, 1999). However, unlike these elements, sequence analysis indicates the SRL PAI does not posses similarity to genes encoding conjugative transfer or phage coat proteins (Luck, *et al.*, 2001). It therefore remains unclear how transfer of the SRL PAI occurs. Interestingly, the *Salmonella* SaPI is encapsidated and mobilised by the staphylococcal phage 80α in a relationship that has been likened to that of coliphages P2 and P4 (Chapter 2). It remains a possibility that the SRL PAI may be similarly mobilised, and future work will focus on the role of accessory elements in mobilisation of the PAI.

It is interesting to note that several genomic islands carrying multi-resistance determinants are now thought to employ site-specific recombination as a means for

lateral transfer. Such elements include the SXT element of *V. cholerae* (Hochhut & Waldor, 1999), the *Salmonella* genomic island 1 (SGI1) (Boyd *et al.*, 2001; Boyd *et al.*, 2000) and the SRL PAI. Thus, the studies presented here are not only a useful tool in examining the mechanistic aspects of PAI dissemination, but also provide an interesting insight into the potential mechanisms for spread of multiple antibiotic resistance.

# REFERENCES


**Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997).** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389 - 3402.

**Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1993).** *Current protocols in molecular biology*. New York: Greene Publishing and Wiley Interscience.

**Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H. & Hacker, J. (1994).** Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. *Infection and Immunity* **62**, 606 - 614.

**Boyd, D., Peters, G. A., Clockaert, A., Boumedine, K. S., Chaslus-Dancla, E., Imberechts, H. & Mulvey, M. R. (2001).** Complete nucleotide sequence of a 43-Kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *Journal of Bacteriology* **183**, 5725 - 5732.

**Boyd, D. A., Peters, G. A., Ng, L.-K. & Mulvey, M. R. (2000).** Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhymurium DT104. *FEMS Microbiology Letters* **189**, 285 - 291.

**Campbell, A. M. (1992).** Chromosomal insertion sites for phages and plasmids. *Journal of Bacteriology* **174**, 7495 - 7499.

**Chung, C. T. & Miller, R. H. (1988).** A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Research* **16**, 3580.

**Davis, B. M. & Waldor, M. K. (2002).** Mobile genetic elements and bacterial pathogenesis. In *Mobile DNA II*, pp. 1040 - 1059. Edited by M. L. Craig, R. Craigie, M. Gellert & A. M. Lambowitz. Herndon: ASM Press.

**Friedman, D. I. (1988).** Integration host factor: a protein for all reasons. *Cell* **55**, 545 - 554.

**Hacker, J. & Kaper, J. B. (1999).** The concept of pathogenicity islands. In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 1 - 11. Edited by J. B. Kaper & J. Hacker. Washington: American Society for Microbiology.

**Hochhut, B. & Waldor, M. K. (1999).** Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. *Molecular Microbiology* **32**, 99 - 110.

**Karaolis, D. K. R., Somara, S., Maneval Jr, D. R., Johnson, J. A. & Kaper, J. B. (1999).** A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature* **399**, 375 - 379.

**Khan, M. U. (1985).** Fourteen years of shigellosis in Dhaka: an epidemiological analysis. *International Journal of Epidemiology* **14**, 607 - 613.

**Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Swerdlow, D. L., Sansonetti, P. J., Adak, G. K. & Levine, M. M. (1999).** Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bulletin of the World Health Organization* **77**, 651 - 665.

**Luck, S. N., Turner, S. A., Sakellaris, H., Rajakumar, K. & Adler, B. (2001).** Ferric dicitrate transport system (Fec) of *Shigella flexneri* 2a YSH6000 is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. *Infection and Immunity* **69**, 6012 - 6021.

**Miller, V. C. & Mekalanos, J. J. (1988).** A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and

virulence determinants in *Vibrio cholerae* regions *toxR*. *Journal of Bacteriology* **170**, 2575 - 2583.

**Morelle, G. (1989).** A plasmid extraction procedure on a mimiprep scale. *Focus* **11**, 7 - 8.

**Nash, H. A. (1996).** Site-specific recombination: integration, excision, resolution, and inversion of defined DNA segments. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 2363 - 2376. Edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, C. C. L. Edmund, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger. Washington DC: ASM Press.

**Penfold, R. J. & Pemberton, J. M. (1992).** An improved suicide vector for construction of chromosomal insertion mutations in bacteria. *Gene* **118**, 145 - 146.

**Priefer, U. (1984).** Characterization of plasmid DNA by agarose gel electrophoresis. In *Advanced Molecular Genetics*, pp. 32 - 33. Edited by A. Puhler & K. N. Timmis. New York: Springer-Verlag.

**Rakin, A., Noelting, C., Schropp, P. & Heesemann, J. (2001).** Integrative module of the high-pathogenicity island of *Yersinia*. *Molecular Microbiology* **39**, 407 - 415.

**Salam, M. A. & Bennish, M. L. (1991).** Antimicrobial therapy for shigellosis. *Reviews of Infectious Diseases* **13**, S332 - S341.

**Sasakawa, C., Kamata, K., Sakai, T., Murayama, S. Y., Makino, S. & Yoshikawa, M. (1986).** Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. *Infection and Immunity* **51**, 470 - 475.

**Ubeda, C., Angeles, T., Cucarella, C., Trotonda, P., Foster, T. J., Lasa, I. & Penades, J. R. (2003).** Sip, an integrase protein with excision, circularization and integration activities, defines a new family of mobile *Staphylococcus aureus* pathogenicity isalnds. *Molecular Microbiology* **49**, 193 - 210.

**Van den Bosch, L., Manning, P. & Morona, R. (1997).** Regulation of O-antigen chain length is required for *Shigella flexneri* virulence. *Molecular Microbiology* **23**, 765 - 775.

**Watanabe, T. (1963).** Infective heredity of multiple drug resitance in bacteria. *Bacteriological Reviews* **27**, 87 - 115.

**Yoshioka, Y., Ohtsubo, H. & Ohtsubo, E. (1987).** Repressor gene *finO* in plasmids R100 and F: constitutive transfer of plasmid F is caused by insertion of IS*3* into F *finO*. *Journal of Bacteriology* **169**, 619 - 623.

4

# CHAPTER FOUR

## Molecular Epidemiology of the SRL Pathogenicity Island

*Based on:*

**Turner, S. A., Luck, S. N., Sakellaris, H., Rajakumar, K. & Adler, B. (2003).** Molecular Epidemiology of the SRL Pathogenicity Island. *Antimicrobial Agents and Chemotherapy.* **47**, 727 – 734.

# INTRODUCTION

*Shigella* spp. are a common cause of bacillary dysentery and are responsible for the deaths of more than one million people annually, the majority of which occur in developing countries (Kotloff *et al.*, 1999). Infection is transmitted via the faecal-oral route and is characterised by excretion of stools containing white cells and blood (DuPont *et al.*, 1989). Shigellosis is one of the few enteric infections for which antimicrobial therapy is clearly effective. Although treatment with antibiotics has not been shown to reduce mortality (Salam & Bennish, 1991), it does reduce both the duration and severity of illness and duration of pathogen excretion (Haltalin *et al.*, 1967), which is an important consideration in prevention of further transmission. However, over the past several decades treatment has become increasingly difficult due to emerging resistance to most of the widely used antibiotics (Khan, 1985; Salam & Bennish, 1991).

Multiple resistance in *Shigella* to antibiotics such as sulfonamides (Su), streptomycin (Sm), ampicillin (Ap), chloramphenicol (Cm) and tetracycline (Tc), was first reported in Japan shortly after their introduction as therapeutic agents for the treatment of shigellosis. In the majority of cases, resistance was found to be borne on conjugative R-plasmids and the inherent mobility of R-plasmids was thought to explain the rapid increase in the number of multiply resistant *Shigella* strains observed during the 1950s (Watanabe, 1963). NR1, the archetypal *Shigella* R-plasmid, which encodes resistance to mercury, Su, Ap, Cm and Tc was isolated during this time (Nakaya *et al.*, 1960; Womble & Rownd, 1988). Recently, in addition to plasmid-borne resistance, there have been several reports of chromosomally borne resistance (Casalino *et al.*, 1994; Gebre-Yohannes & Drasar, 1990; Ling *et al.*, 1993). However, the basis of chromosomal resistance in *Shigella* has remained largely unexplored.

In 1997, Rajakumar *et al.* reported the identification of a chromosomal multi-antibiotic resistance locus in *Shigella flexneri* 2a strain YSH6000. This locus, which has since been designated the *Shigella* resistance locus (SRL), encodes resistance to Sm, Ap, Cm and Tc, and exhibits both organisational and sequence similarity to corresponding regions of NR1. However, the SRL also exhibits several differences from NR1,

including the absence of the mercury and Su resistance determinants, a 17.5 kb deletion between the Cm and Tc resistance determinants, and the precise insertion of a β-lactamase-encoding *oxa-1* cassette into the Tn*21*-borne integron In2 (Luck *et al.*, 2001; Rajakumar, *et al.*, 1997). Although insertion of the *oxa-1* cassette into this location of Tn*21* has been demonstrated experimentally (Tanaka *et al.*, 1985) and is observed in the closely related plasmid-borne Tn*2603* (Ouellette *et al.*, 1987), the unique combination and placement of resistance determinants distinguishes the SRL even from these close relatives (Rajakumar, *et al.*, 1997).

The SRL was originally reported to reside on a 99 kb deletable element which has since been designated the multiple-antibiotic resistance deletable element (MRDE) (Rajakumar *et al.*, 1996) (Chapter 2). Recent studies have revealed that the 16.7 kb IS*1*–flanked SRL is borne on a 66 kb pathogenicity island (PAI), designated the SRL PAI (Luck, *et al.*, 2001). The SRL is lost from the *S. flexneri* 2a strain YSH6000 via three distinct but separate mechanisms involving deletion of the SRL itself, integrase-mediated deletion of the PAI and deletion of the MRDE (an IS*91*-flanked element encompassing the SRL PAI and some surrounding chromosomal DNA). All deletions result in the loss of the SRL from the chromosome (Chapter 2). As the IS*1*-flanked r-determinant (r-det) of NR1 and PAIs are mobile in other systems, it was considered there was potential for dissemination of SRL within *Shigella* via one of these mechanisms (Chapter 2). In this study, we present evidence that resistance conferred by the SRL is widespread among clinical *Shigella* isolates, and that dissemination of the SRL may be mediated by the SRL PAI.

# MATERIALS AND METHODS

## Bacterial Strains, Media and Growth Conditions

Bacterial strains used in this study are listed in Table 4.1. Strains were grown routinely at 37 °C in Luria Bertani medium (Ausubel *et al.*, 1993) with the addition of ampicillin (100 μg/ml), streptomycin (25 μg/ml), chloramphenicol (40 μg/ml) or tetracycline (10 μg/ml) when necessary.

**Table 4.1** Bacterial strains (complete listings in Appendix 2)

| Strain | Relevant characteristics (No. of strains in group) | Reference or source* |
|---|---|---|
| *E. coli spp.* | | |
| SBA573 | *E. coli* C600 harbouring the multiple-antibiotic resistant plasmid R100-1 $Sm^r$ $Cm^r$ $Tc^r$ | CS |
| *Shigella spp.* | | |
| YSH6000 | Wild type *S. flexneri* 2a Japanese isolate $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ | (Sasakawa *et al.*, 1986) |
| YSH6000T | MRDE deletant of YSH6000 | (Nakata *et al.*, 1992) |
| SBA 1363 | Spontaneous SRL PAI deletant of YSH6000 | Chapter 2 |
| SBA 1367 | Spontaneous SRL PAI deletant of YSH6000 | Chapter 2 |
| *S. flexneri* | *S. flexneri* 1a (3) | CS |
| | *S. flexneri* 1b (3) | CS, DL |
| | *S. flexneri* 2a (12) | CS, DL |
| | *S. flexneri* 2b (5) | CS, DL |
| | *S. flexneri* 3 (1) | CS |
| | *S. flexneri* 3a (4) | CS, DL |
| | *S. flexneri* 3b (1) | CS |
| | *S. flexneri* 3c (1) | CS |
| | *S. flexneri* 4 (1) | DL |
| | *S. flexneri* 4a (3) | CS, DL |
| | *S. flexneri* 4b (1) | CS |
| | *S. flexneri* 5 (2) | CS |
| | *S. flexneri* 6 (4) | CS, DL |
| | *S. flexneri* X (1) | CS |
| | *S. flexneri* Y (1) | CS |
| *S. sonnei* | *S. sonnei* (11) | CS, DL |
| *S. boydii* | *S. boydii* 1 (1) | CS |
| | *S. boydii* 2 (1) | CS |
| | *S. boydii* 3 (1) | CS |
| | *S. boydii* 4 (2) | CS |
| | *S. boydii* 7 (1) | CS |
| | *S. boydii* 8 (2) | CS |
| *S. dysenteriae* | *S. dysenteriae* 1 (1) | CS |
| | *S. dysenteriae* 3 (4) | CS |
| | *S. dysenteriae* 4 (1) | CS |
| | *S. dysenteriae* 5 (1) | CS |
| | *S. dysenteriae* 6 (1) | CS |
| | *S. dysenteriae* 9 (1) | CS |
| Pathogenic *E. coli* | EIEC (12) | RRB, CS |
| | EHEC (3) | RRB |
| | EPEC (3) | RRB |
| | ETEC (3) | RRB |
| | EAEC (3) | RRB |
| *Salmonella spp.* | *Salmonella enterica* serovar Typhimurium (2) | RRB |
| *Yersinia spp.* | *Yersinia enterocolitica* (2) | RRB |

*CS, C. Sasakawa, University of Tokyo, Tokyo, Japan; DL, D. Lightfoot, University of Melbourne, Parkville, Australia; RRB, R. Robins-Browne, Royal Children's Hospital, Parkville, Australia.

## Molecular Biological Techniques

Genomic DNA was isolated by a small-scale preparation as described previously (Ausubel, *et al.*, 1993). Plasmid DNA was isolated by a modification of the alkaline lysis method (Morelle, 1989). DNA was sequenced as described previously (Chapter 2)

## Selection of Tetracycline-Sensitive Derivatives of SBA1299 and SBA1391

Tetracycline sensitive derivatives were selected by plating dilutions of culture grown in LB broth to a density of approximately $10^9$ CFU $ml^{-1}$ onto LB agar supplemented with fusaric acid (12 μg/ml), heat-inactivated chlortetracycline (50 μg/ml) and 0.1 mM $ZnCl_2$ (Maloy & Nunn, 1981). Agar plates were incubated for 24 – 40 h at 37 °C.

## Southern Hybridisation

After electrophoresis, DNA was transferred to a charged nylon membrane (Roche) using a vacuum blotting apparatus (TE80 Transvac, Hoefer) or by capillary transfer in 20 X SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0). Overnight hybridisation and subsequent washings were performed under high stringency conditions at 68 °C as recommended in the Roche digoxigenin labelling and detection kit. Probes were labelled by PCR amplification with digoxigenin as specified by Roche. The 1.0 kb *int* probe and 1.0 kb *fec* probe were amplified as described previously (Chapter 2). The *cat* probe was amplified with primers BAP2025 and BAP2026 (Table 4.2)

## Separation of Plasmid and Chromosomal DNA

Plasmid and chromosomal DNA were separated using a modification of the Eckhardt in-well lysis method (Priefer, 1984). Approximately $10^7$ -- $10^8$ cells were resuspended in lysozyme mixture (7 % Ficoll, 20 % sucrose, 1 U/ml RNase, 1 mg/ml lysozyme in TBE) and loaded into the wells of 0.4 % and 0.8 % agarose gels. Samples were then overlayed with SDS mixture (1 % SDS, 5 % sucrose bromophenol blue in TBE) and electrophoresed for 30 min at 5 mA, followed by 2 h at 100 mA.

## PCR Amplification and Linkage of SRL PAI Markers

PCR amplifications were performed on chromosomal DNA using the Taq DNA polymerase system as specified by Roche. PCR amplifications of DNA fragments of over 5.0 kb were performed using the Expand Long Template PCR buffer system 3 as specified by Roche. Colony PCRs were performed on single colonies added directly to the PCR mix, with an initial 8 min incubation at 94 °C.

**Table 4.2.** Primers used in this study

| Target DNA | Primer name | Primer sequence (5'–3') | size of PCR product (kb) |
|---|---|---|---|
| *orf58* | BAP2022 | CGCTGTTAAGGTAAATCCC | 0.2 |
| | BAP2023 | AGCGAGCCATAAAGTGTGC | |
| *aadA1 + oxa-1* | BAP2024 | TGACGGGCTGATACTGGG | 1.3 |
| | BAP1553 | AAAACCCCCAAAGGAATGGAG | |
| *oxa-1 + cat* | BAP1559 | GCTACTTTCGAGCCATGC | 7.0 |
| | BAP2026 | CGGTGAGCTGGTGATATGG | |
| *cat + tetA* | BAP2025 | TTACGCCCCGCCCTGCCAC | 4.0 |
| | BAP1622 | CCCCTAACCAACCGAACC | |
| *int + rox* | BAP1012 | TGGGCGGATTATGTGATG | 2.2 |
| | BAP1620 | CCGGAATTCCTCTTCCGCTTGTGTGCC | |
| *rox + orf6* | BAP1621 | GCGCGGATCCCAGCAGCAGCATTTTCCG | 6.0 |
| | BAP1377 | AAGGAGTGGCATCGTTAGC | |
| *orf5 + oxa-1* | BAP1388 | AACACGGTATTATTCTGGGC | 10.0 |
| | BAP1553 | AAAACCCCCAAAGGAATGGAG | |
| *tetC + fecD* | BAP1013 | GCATAAACCAGCCATTGAG | 8.3 |
| | BAP514 | GCGCTGCTGACCCGACTGG | |
| *fecD + orf34* | BAP936 | AGACAAACCACGGCGCAC | 9.4 |
| | BAP563 | GCTCACCAGACGCGTAAAC | |
| *orf34 + orf41* | BAP1440 | CCTGGCGGCACAACCTAC | 7.8 |
| | BAP1619 | GCGCGGATCCTCGGATGACACACGCCCC | |
| *orf41 + orf45* | BAP1618 | CCGGAATTCTAGCTTACTCTGGCAAATCC | 3.6 |
| | BAP1552 | ATGCCAATCACGGGTTCG | |
| *orf45 + orf48* | BAP1531 | TTATGATCCCCCGGAACG | 6.9 |
| | BAP1689 | AGACACGGGGTTCCAGGG | |
| *orf48 + orf58* | BAP1661 | GGCGCTGGCACAGGCGGC | 4.4 |
| | BAP2023 | AGCGAGCCATAAAGTGTGC | |
| *int + ⇓serX*[a] | BAP670 | GTGCTGCTTTCGGTGTGC | 1.1 |
| | BAP689 | CCGGGCAGTACGTGCAGC | |
| *int + ⇓serW*[b] | BAP1462 | ATACTCCACCCGCCCACC | 0.4 |
| | BAP689 | CCGGGCAGTACGTGCAGC | |
| *orf58 + ⇑serX*[c] | BAP2022 | CGCTGTTAAGGTAAATCCC | 2.2 |
| | BAP1157 | GCCAGCATTTCAACAGGAGG | |
| *orf58 + ⇑serW*[d] | BAP2022 | CGCTGTTAAGGTAAATCCC | 2.2 |
| | BAP1783 | TGTTTTACCGCCTGATGGG | |
| *csgA* | csgAUp | AAAGAATTCGCTCTGGCAGGTGTTGTTCC | 1.8 |
| | csgADn | AAAAAGTCGACTTAACCAAAGCCAACCTGA GTCACG | |

[a] ⇓*serX* signifies sequence downstream of *serX*
[b] ⇓ *serW* signifies sequence downstream of *serW*
[c] ⇑ *serX* signifies sequence upstream of *serX*
[d] ⇑ *serW* signifies sequence upstream of *serW*

## Computer Analysis

Nucieotide sequence similarity searches of the databases were preformed using the BlastN or BlastX program (Altschul *et al.*, 1997). Sequence data were analysed using the Sequencher program (GeneCodes Corporation, Ann Arbor, MI.).

# RESULTS

## Distribution of the SRL Among *Shigella* Isolates

To determine whether the combination of resistances encoded by the SRL was present in other *Shigella* species, 71 clinical *Shigella* isolates including representatives from 27 different serotypes were tested for their ability to grow in the presence of Sm, Ap, Cm and Tc. Of these strains, 31 were found to be resistant to all four antibiotics, including strains from each *Shigella* species (strains listed in Table 4.3). To establish whether these resistances were due to the presence of the SRL or other resistance loci, PCRs linking the coding regions of *aadA1* ($Sm^r$) with *oxa-1* ($Ap^r$), *oxa-1* with *catA* ($Cm^r$), and *catA* with *tetA(B)* ($Tc^r$) (Fig. 4.1a) were performed on all isolates exhibiting resistance to the four antibiotics. These linkages enabled a distinction to be made between the SRL and the closely related resistance locus of NR1, as the NR1 locus does not contain the *oxa-1* cassette, and carries an additional ~17.5 kb between the *cat* and *tetA(B)* sequences (Rajakumar, *et al.*, 1997). Physical linkage of all four determinants was demonstrated in the majority (22/31) of strains (Table 4.4), indicating a conservation of the genetic organisation of the YSH6000 SRL in these isolates. With the exception of one strain in which no linkages were demonstrated, all strains exhibited linkage of 2 or 3 resistance determinants (Table 4.4) suggesting the presence of a locus either identical to, or sharing some similarity with, the organisation of the YSH6000 SRL.

**Table 4.3.** Distribution of SRL PAI markers in $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ *Shigella* isolates (for expanded listing see Appendix 2)

| Species (number of strains) | Presence of SRL PAI markers: | | | Linkage of *int* to[a]: | |
|---|---|---|---|---|---|
| | *int* | *fec* | *orf58* | ⇓*serX* | ⇓*serW* |
| *S. flexneri* 3c (1) | - | - | - | ... | ... |
| *S. flexneri* 2a (1) | - | + | - | ... | ... |
| *S. flexneri* 3a (1) | + | + | + | - | - |
| *S. boydii* 8 (1) | + | - | + | -[b] | - |
| *S. boydii* 4 (1) | + | + | + | -[b] | - |
| *S. flexneri* 1b (1) | + | + | + | -[b] | - |
| *S. flexneri* 2a (1) | + | + | + | -[b] | - |
| *S. flexneri* 6 (1) | + | + | + | -[b] | - |
| *S. boydii* 8 (1) | + | - | + | - | + |
| *S. dysenteriae* 3 (2) | + | - | + | - | + |
| *S. sonnei Form* I (1) | + | + | - | - | + |
| *S. dysenteriae* 3 (2) | + | + | + | - | + |
| *S. flexneri* 2a (1) | + | + | + | - | + |
| *S. flexneri* 3b (1) | + | + | + | - | + |
| *S. flexneri* 6 (1) | + | + | + | - | + |
| *S. sonnei* Form I (1) | + | + | + | - | + |
| *S. flexneri* 2a (5) | + | - | + | + | - |
| *S. flexneri* 1a (1) | + | + | + | + | - |
| *S. flexneri* 2a (1) | + | + | + | + | - |
| *S. flexneri* 2b (1) | + | + | + | + | - |
| *S. flexneri* 4 (1) | + | + | + | + | - |
| *S. flexneri* 4a (2) | + | + | + | + | - |
| *S. flexneri* 2b (2) | + | + | + | + | + |

[a] +, PCR linkage was demonstrated; -, PCR linkage was not demonstrated; ⇓*serX* and ⇓*serW* , linkage of *int* to sequences downstream of *serX* and *serW* respectively; ..., not tested

[b] linkage of *int* to ⇓*serX* was negative, but linkage of *orf58* to sequences upstream of *serX* was positive

## Distribution of the SRL PAI Markers Among *Shigella* Isolates

In order to discover whether the SRL-related loci were present as part of the SRL PAI, or as independent loci, strains were tested for the presence of three SRL PAI markers. Probes corresponding to the integrase gene (*int*), which has been shown to be necessary for site-specific deletion of the SRL PAI (Chapter 2) and integration of the SRL PAI *attP* (Chapter 3) (region A, Fig. 4.1a) and *fecA*, a critical component of the *fec* iron transport locus (Wagegg & Braun, 1981) (region B, Fig. 4.1a), were used in a high stringency Southern analysis of the 31 $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ strains (Table 4.3). Additionally, primers amplifying a region internal to *orf58* of the SRL PAI (Luck, *et al.*, 2001), which has no database matches at the nucleotide level, were used to analyse these strains by PCR (region C, Fig. 4.1a) (Table 4.3).

**Table 4.4.** Linkage of resistance determinants in Sm$^r$ Ap$^r$ Cm$^r$ Tc$^r$ *Shigella* isolates (for expanded listing see Appendix 2)

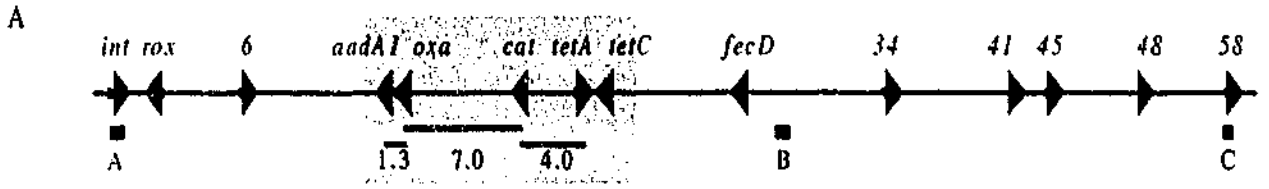
| Linkage[a] | | | No. of strains (n=31) |
|---|---|---|---|
| *aadA1* + *oxa-1* | *oxa-1* + *cat* | *cat* + *tetA* | |
| - | - | - | 1 |
| - | - | + | 1[b] |
| + | - | - | 1[c] |
| + | - | + | 3 |
| + | + | - | 3 |
| + | + | + | 22 |

[a] +, PCR linkage was demonstrated; -, PCR linkage was not demonstrated.
[b] strain SBA1298.
[c] strain SBA1388.

The results indicated that sequences homologous to the SRL PAI *int* were present in all but two of the 31 Sm$^r$ Ap$^r$ Cm$^r$ Tc$^r$ strains, and with one exception, multi-resistant strains that carry the *int* marker also carry the *orf58* marker (Table 4.3). Interestingly, SBA1298 and SBA1388, the only two Sm$^r$ Ap$^r$ Cm$^r$ Tc$^r$ *int* negative strains identified, were among the strains that showed the least similarity to the SRL in SRL-linkage studies (Table 4.4). Thus, in the majority of strains that contain the *int* marker, resistance to Sm Ap Cm and Tc (100 % of strains, 28/29 which are SRL-related) and the *orf58* marker (97 % of strains) are also present, suggesting that the resistance determinants in these strains are borne on elements similar to the SRL PAI. Moreover, the *int* marker was absent in the 40 other strains, indicating a perfect correlation between the presence of *int* and the four antibiotic resistances (Appendix 2). Interestingly, the *orf58* marker was present in 24 % of the 42 strains negative for the *int* marker (Appendix 2). The majority of strains that possessed the *int* marker were also positive for the *fecA* marker (69 %) (Table 4.3). However, 45 % of the *int*-negative strains also possess the *fecA* marker (Appendix 2). These data and the finding that *fec* is a chromosomal locus in closely related *Escherichia coli* K-12 (Hussein *et al.*, 1981), make it difficult to assess whether *fecA* is consistently carried on the SRL PAI in *int*-positive strains.

## The SRL PAI is Widespread Among Sm$^r$ Ap$^r$ Cm$^r$ Tc$^r$ *Shigella* Isolates

In order to examine whether the presence of the SRL PAI markers in the Sm$^r$ Ap$^r$ Cm$^r$ Tc$^r$ isolates indicated the presence of an intact, ordered set of genes representing the SRL PAI, a representative of each *Shigella* species was chosen for further characterisation. Genomic DNA from the strains SBA1299 (*S. flexneri* 1a), SBA1303 (*S. sonnei* Form I), SBA1308 (*S. boydii* 4) and SBA1391 (*S. flexneri* 4a) which were




B

| SBA | 2.2 | 6.0 | 10.0 | 1.3 | 7.0 | 4.0 | 8.3 | 9.4 | 7.8 | 3.6 | 6.9 | 4.4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1299 | 2.2 | 6.0 | 12 | 1.3 | 7.0 | 4.0 | 10 | 9.4 | 7.8 | 3.6 | 9.4 | 4.4 |
| 1303 | 2.2 | 6.0 | 12 | 1.3 | 7.0 | 4.0 | 8.5 | 9.4 | 7.8 | 4.4 | - | 4.4 |
| 1304 | 2.2 | 6.0 | 12 | 1.3 | 7.0 | 4.0 | - | - | - | - | - | 4.4 |
| 1308 | 3.8 | 6.8 | 12 | 1.3 | 7.0 | 4.0 | 8.5 | 10 | 7.8 | 3.6 | 6.9 | 4.4 |
| 1386 | 2.2 | 6.0 | 12 | 1.3 | 7.0 | 4.0 | - | - | - | - | - | 4.4 |
| 1391 | 2.2 | 6.0 | >15 | 1.3 | 7.0 | 4.0 | 8.3 | 8.7 | 7.8 | 3.6 | 9.4 | 4.4 |

**Figure 4.1** Schematic representation of the genetic organisation of the SRL PAI in strain YSH6000. (A) The SRL PAI is represented as a thin black line with the position and direction of relevant genes and *orfs* represented as arrows. The SRL is indicated by the large shaded box. Thick lines represent the regions amplified in linkage analysis of the SRL, with corresponding numbers indicating their size (kb) in the YSH6000 SRL PAI. Small black boxes represent the position of markers that were probed by Southern analysis or PCR amplification: A, *int*; B, *fecA*; and C. *orf58*. (B) Thick black lines represent the regions amplified in linkage analyses of SBA1299 (*S. flexneri* 1a), SBA1303 (*S. sonnei* Form I), SBA1304 (*S. dysenteriae* 3), SBA1308 (*S. boydii* 4), SBA1386 (*S. boydii* 8), SBA1391 (*S. flexneri* 4a) with corresponding numbers indicating their size (kb) in the YSH6000 SRL PAI.

positive for the *int*, *fecA* and *orf58* markers, SBA1304 (*S. dysenteriae* 3) and SBA1386 (*S. boydii* 8), which were positive for the *int* and *orf58* markers, but negative for the *fecA* marker, was analyzed by PCR for physical linkage of 13 loci spanning the YSH6000 SRL PAI (Fig. 4.1). In strains SBA1299, SBA1308 and SBA1391, physical linkage of all 13 loci was demonstrated, revealing the presence of elements that appear to have a similar structure to the YSH6000 SRL PAI (Fig. 4.1). All loci, with the exception of *orf45* and *orf48*, were also linked in strain SBA1303 (Fig. 4.1). As *orf45* and *orf48* sequences bearing the amplifying primer binding sites were present in SBA1303 (as determined by previous PCRs), it was considered likely that the insertion of additional DNA may account for the failure to amplify this region. Although PCR extension times were increased to allow the amplification of 12 - 14 kb products, no products were amplified implying that either the loci are not linked, or these regions exceed 14 kb in length. In SBA1304 and SBA1386, which were negative for the *fecA* marker, all loci were linked except for a large region extending from *tetC* to *orf48* (Fig. 4.1). Thus it appears that absence of the *fecA* marker may correspond to the absence of a larger region in both SBA1304 and SBA1386. Hence, despite minor differences in linkage patterns and sizes, these data demonstrate the presence of

elements in all four *Shigella* species that have similar structures to the *S. flexneri* 2a strain YSH6000 SRL PAI.

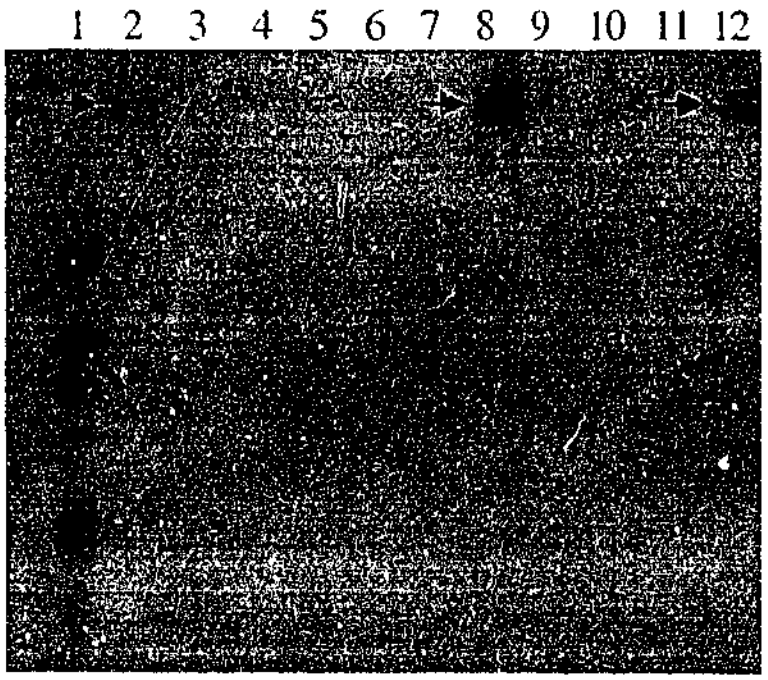



**Figure 4.2** Southern hybridisation analysis of an Eckhardt in-well lysis gel electrophoresis. Membrane probed with the *cat* gene. Lanes: 1, DIG-labelled λ*Hind*III markers; 2, SBA1298; 3, SBA1299; 4, SBA1303; 5, SBA1304; 6, SBA1308; 7, SBA1386; 8, SBA1388; 9, SBA1391; 10, YSH6000 (negative control); 11, SBA1363; 12, SBA573 (R100-1: positive control). Bands in lane 2, 8 and 12 are marked by arrows and indicate that the *cat* gene is present on a plasmid which is able to enter the gel matrix. Membrane was stripped and re-probed with the chromosomal marker *solA* (Chapter 2) to confirm there was no chromosomal spill-over.

To test whether these SRL PAI-like elements were plasmid- or chromosomally-borne, Eckhardt gel electrophoresis was performed on these six strains and DNA was analysed by Southern hybridisation with a *cat* gene probe, using YSH6000 as a plasmid-negative control and the NR1-related R100-1 as a plasmid-positive control (Fig. 4.2). The results confirmed a chromosomal location for the *cat* gene, and therefore the SRL PAI-like elements, in each of the six *Shigella* isolates. In contrast, strains SBA1298 (*S. flexneri* 3c) and SBA1388 (*S. flexneri* 2a), in which the resistances to Sm, Ap, Cm, and Tc were not thought to be related to the presence of the SRL PAI, because of the absence of an *int* gene, possessed a plasmid borne *cat* gene.

## Chromosomal Deletion of the SRL Locus

In *S. flexneri* 2a strain YSH6000, the SRL PAI is known to undergo precise, integrase-mediated deletion from the chromosome. This deletion can be identified by selection of strains on fusaric acid media followed by PCR amplification of the deletion point junction (Chapter 2). To determine whether the elements in SBA1299, SBA1303, SBA1304, SBA1308, SBA1386 and SBA1391 were capable of undergoing similar

deletions, these strains were also grown on fusaric acid. However, only strains SBA1299 (*S. flexneri* 1a) and SBA1391 (*S. flexneri* 4a) were found to be fusaric acid sensitive, and therefore suitable for selecting the loss of the PAI using this method. $Tc^S$ derivates of SBA1299 and SBA1391 obtained by this selection method were subsequently tested for susceptibility to the antibiotics Sm, Ap and Cm. All SBA1299 derivatives were found to be $Sm^r$ and were thought to possess some additional determinant other than the SRL *aadA1* gene conferring this resistance. Thus 10 $Sm^r$ $Ap^S$ $Cm^S$ $Tc^S$ SBA1299 derivatives and 2 SBA1391 $Sm^S$ $Ap^S$ $Cm^S$ $Tc^S$ derivatives were considered to be potential PAI deletants, and therefore selected for PCR characterisation. However, amplification of *serX*, at the SRL PAI deletion point junction, was not successful in any of the SBA1299 and SBA1391 derivatives, suggesting that precise deletion of the element had not occurred.

Each derivative was tested by PCR for the presence of the markers *int*, *fecA* and *orf58*. Nine SBA1299 derivates and both of the SBA1391 derivatives were negative for all of these markers, and were therefore consistent with PAI deletants. The deletion derivatives were further tested by PCR for the presence of the chromosomally-borne *csgA* gene which is approximately 7 kb downstream of *orf58* in YSH6000. This gene was absent from all nine SBA1299 derivatives and both of the SBA1391 derivatives, indicating that DNA flanking the PAI-like element was also deleted with the PAI markers. In YSH6000, imprecise deletion of the SRL PAI, which removes flanking chromosomal DNA including the *csgA* gene, occurs from the *mdoA* and *putA* loci due to the presence of flanking IS*91* elements on the chromosome (Chapter 2). The deletion of this region, known as the MRDE, was tested by PCR amplification across the deletion point junction identified in YSH6000. This amplification was positive in four of the SBA1299 derivatives and both of the SBA1391 derivatives, demonstrating that deletion events similar to MRDE deletion in YSH6000 had occurred in at least six of the derivative strains. In strains in which the MRDE deletion point junction was not amplified, it is likely that loss of the PAI markers occurs by another type of deletion mechanism. As IS elements have been implicated in deletion of part or all of the SRL PAI in strain YSH6000 (Chapter 2), it is possible that additional IS elements present in SBA1299 may be involved in the deletion of the SRL PAI from these strains.

## Distribution of the SRL PAI Among Other Members of the *Enterobacteriaceae*

The dissemination of the SRL PAI throughout many *Shigella* species, in conjunction with the report that the *Yersinia* high-PAI is present in different members of the family *Enterobacteriaceae* (Schubert *et al.*, 2000; Schubert *et al.*, 1998) prompted the investigation of several other enteric pathogens for the presence of the SRL PAI. Twenty eight members of the family *Enterbacteriaceae* including representatives of enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), *Salmonella enterica* serovar Typhimurium, and *Yersinia enterocolitica* were tested for antibiotic resistance. Of the twenty eight strains, only a single *Y. enterocolitica* strain was found to have a $Sm^r$ $Ap^r$ $Cm^r$ $Tc^r$ phenotype. However, PCR linkage of the SRL determinants was not achieved in this strain, suggesting that the resistance profile was not due to the presence of the SRL.

Isolates were also tested for the presence of *int* and *fecA* by Southern hybridisation and *orf58* by PCR. *fecA* was detected in one EIEC, two ETEC and three EAEC strains, while *orf58* was detected in four EIEC and one EHEC strain. Regions hybridising to the *int* probe were found in two EHEC and one EPEC strain (Appendix 2). Recent sequencing of the EHEC O157:H7 EDL 933 genome revealed the presence of a 1203 bp *orf*, present on identical O-islands #43 and #48, which is nearly identical to the SRL PAI *int* gene (Perna *et al.*, 2001). These genes share 97 % nucleotide sequence identity over the first 1189 bp, but diverge abruptly after this point. PCR amplification with primers designed to anneal to this divergent region confirmed that the *int* positive EPEC and EHEC strains identified in this study yielded an amplified product with primers specific for the EHEC integrase gene, but not with primers specific for the SRL PAI *int*.

## Chromosomal Insertion Sites of SRL PAI-Related Elements

The YSH6000 SRL PAI was found to be inserted into the 3' end of the tRNA gene *serX* and the *int* gene was situated adjacent to the 14 bp direct repeat with a sequence

identical to the 3' terminus of the *serX* gene (Luck, *et al.*, 2001). By pairing a primer situated in the *int* gene with one downstream of *serX*, the presence of the SRL PAI in this position may be confirmed by PCR. This method was used to test the 29 *int*-positive *Shigella* strains (Table 4.3), and revealed that in 13 of these strains, *int* was linked to *serX*. *E. coli* possesses a paralogue of *serX*, *serW*, at minute 20. At the nucleotide level, *serW* displays 100 % identity to *serX*, and thus also contains the 14 bp sequence thought to be targeted by the SRL PAI integrase (Luck, *et al.*, 2001) (Chapter 2). As it has been noted that the HPI and *she* PAI are also found inserted into different paralogues of the *asn* and *phe* tRNA genes respectively (Al-Hasani *et al.*, 2001; Buchrieser *et al.*, 1998), the *Shigella* isolates were also tested for linkage of *int* to a sequence downstream of *serW*. Ten strains were found to be positive for this association (Table 4.3). In the six strains where neither of these linkages could be demonstrated, linkage of *orf58* at the right end of the PAI, to sequences upstream of both tRNA genes was tested. Five strains exhibited linkage of *orf58* to sequences upstream of *serX* (Table 4.3). In the remaining one strain, both *int* and *orf58* were found not to be linked to either *serX* or *serW* sequences. Interestingly, in the two *int* positive EHEC strains, *int* was linked to *serX* and *serW*, and in the *int* positive EPEC strain, *int* was linked to *serX* (Appendix 2).

# DISCUSSION

In this study we have demonstrated that loci showing similarity to the SRL, which mediates resistance to Sm, Ap, Cm and Tc, are widespread among *Shigella* isolates of all four species. Additionally, the *S. flexneri* 2a strain YSH6000 SRL PAI *int* and *orf58* markers were found correlated with these loci, suggesting that the SRL PAI may be linked to the dissemination of the SRL throughout *Shigella* species. PCR linkage analysis revealed that the SRL PAI is essentially conserved both in organisation in these strains, although some variation was observed. Interestingly the *fec* locus, which is present on the YSH6000 SRL PAI, is not always present in strains that carry the SRL-related loci and other SRL PAI markers. PCR linkage analysis of two such strains revealed that although the majority of the PAI organisation and structure was conserved, the absence of the same region which spans the entirety of the *fec* locus and some

surrounding sequences, was observed in both isolates. Thus, it appears that the *fec* locus may have been lost from these strains, or these elements may represent a *fec* negative predecessor of the SRL PAI. The absence of the same *fec* carrying regions from these strains, the fact that the locus is flanked with phage and IS-related sequence (Luck, *et al.*, 2001), together with the presence of *fec* markers in PAI-negative *Shigella* isolates and its sporadic distribution throughout *E. coli* (this study) (Hayashi *et al.*, 2001; Hussein, *et al.*, 1981; Pressler *et al.*, 1998) may suggest that the *fec* locus is independently mobile. Interestingly *orf58*, which was found to be present in 97 % of *int*-positive *Shigella* strains, was also present in 11 % of *int*-negative strains. As *orf58* exhibits some similarity to the IS*1328* transposase of *Yersinia enterocolitica* (Luck, *et al.*, 2001), it is possible that the presence of this marker in *int*-negative strains may be due to its independent mobility.

Structural variation is not unique to the SRL PAI, and is also found at one end of the *Yersinia* HPI (Rakin *et al.*, 2001) and the *Shigella she* PAI (Al-Hasani, *et al.*, 2001). Interestingly, unlike the *she* PAI, which has a great diversity in structure, there appear to be few structural SRL PAI variants in *Shigella*, based on the markers that were tested. The extraordinarily high correlation between the presence of the *int* and SRL-related loci is dissimilar to the *V. cholerae* PAI-like SXT element, in which *int* sequences of the element were found to be associated with a variety of different multi-resistance gene clusters (Hochhut *et al.*, 2001). Interestingly, previous studies have demonstrated that the IS*1*-flanked YSH6000 SRL is capable of independent excision from the SRL PAI, a phenomenon which might allow for its independent spread (Chapter 2). However, thus far there have been no SRL positive isolates that do not carry the SRL PAI *int* marker, suggesting that the SRL PAI acquired the SRL before widespread dissemination, or that there is a strong selective pressure to maintain the SRL as part of the SRL PAI.

In this study, using primers specific for the EHEC O-island #43 and #48 *int* gene, which is remarkably similar to the SRL PAI *int*, sequences were amplified in two $Sm^S$ $Ap^S$ $Cm^S$ $Tc^S$ EHEC isolates and one $Sm^S$ $Ap^S$ $Cm^S$ $Tc^S$ EPEC isolate. The identical O-islands #43 and #48 do not encode antibiotic resistance, but show the same insertion specificity, and like the SRL PAI in YSH6000, are also flanked by the same 14 bp direct repeat which corresponds to the 3' of the *serX* and *serW* tRNA genes (Perna, *et al.*, 2001). As we have determined that the SRL PAI may also occupy both of these sites in the *Shigella*

chromosome, the considerable similarity between these integrases appears to be sufficient to confer the insertion site specificity of both elements. Excluding this *int* sequence similarity, the EHEC O-islands show no nucleotide sequence similarity to the SRL PAI. However, analysis of ORFs revealed that the EHEC O-islands and the SRL PAI share a common backbone, and based on this similarity it appears likely that both the SRL and *fec* regions were acquired as distinct elements by the SRL PAI. It is interesting to note that although O-island #43 and #48 exist in some *E. coli*, the SRL-encoded multiple antibiotic resistance was never correlated with these markers in the *E. coli* strains investigated. The SRL-PAI markers *orf58* and *fecA* were also present in some *E. coli* isolates in this study. However, as discussed previously, *orf58* shows some similarity to an IS element transposase and the *fec* locus is a common chromosomal marker in *E. coli*; therefore their presence in these strains was not unexpected. Hence, although several enteric pathogens possess markers of the SRL PAI, no one strain contained more than a single marker (Appendix 2), suggesting that the SRL PAI is not present in the strains tested in this study. Thus, although elements bearing resemblance to the YSH6000 SRL PAI are relatively common in *Shigella*, it appears that similar elements are not present in the other enteric pathogens. Such genus specific distribution is in contrast to the HPI which is found widely distributed throughout the family *Enterobacteriaceae* (Clermont *et al.*, 2001; Karch *et al.*, 1999; Schubert, *et al.*, 2000; Schubert, *et al.*, 1998; Xu *et al.*, 2000). It is possible that early and extensive use of antibiotics to treat *Shigella* infections may have influenced the distribution of the SRL PAI. It would be interesting to determine whether SRL PAI-related elements were present in enterobacterial strains isolated before the introduction of antibiotic therapy, as it has been noted that conjugative plasmids which presumably gave rise to R plasmids, were quite common in enterobacteria of the pre-antibiotic era (Hughes & Datta, 1983).

It is generally believed that PAIs have been transferred horizontally (Hacker *et al.*, 1997; Hacker & Kaper, 1999; Lee, 1996). However, such transfer has only been demonstrated for the PAI-like *V. cholerae* self-transmissible conjugative SXT element (Hochhut & Waldor, 1999), the *V. cholerae* VPI and the staphylococcal SaPI (Karaolis *et al.*, 1999; Ruzin *et al.*, 2001). Although horizontal transfer of the SRL PAI has not been demonstrated, previous studies have shown that it undergoes both site-specific, integrase-dependent deletion and integration (Chapter 2; Chapter 3). Additionally, this

element appears to be widespread throughout *Shigella*, absent from some *Shigella* and *E. coli* strains, and may occupy at least two distinct chromosomal loci. Together, these data support the hypothesis that the SRL PAI is, or was at some point, mobile. If this hypothesis is correct, PAIs may be a thus far unexplored mechanism of antibiotic resistance spread.

It is interesting to note that the SRL itself incorporates components of plasmid, transposon and integron encoded resistance determinants, being composed of loci that resemble the NR1 plasmid r-det, various transposons (including Tn*2670*, Tn*21* and Tn*10*), and the Tn*21*-borne integron In2 bearing an additional *oxa-1* gene cassette (Luck, *et al.*, 2001; Rajakumar, *et al.*, 1997). Indeed, a clinical study of *Enterobacteriaceae* isolates noted that in addition to integron-borne antibiotic resistance, an association existed between the presence of integrons and non integron-borne resistance genes. This genetic linkage of integrons with non integron-borne resistance factors to older antibiotics such as Cm and Tc (White *et al.*, 2001), is exemplified by the SRL and suggests that similar loci may be more common throughout the *Enterobacteriaceae*.

Multi-antibiotic resistance was originally reported to be plasmid-borne (Watanabe, 1963), and this is still considered to be the primary form of resistance in *Shigella* isolates. However, we have demonstrated linkage of the SRL-PAI *int* to the chromosomal markers *serX* and *serW* in the majority of strains that carry the SRL-related loci, suggesting that these resistance determinants are not plasmid borne, but reside on the chromosome. Further characterisation of six such strains illustrated that the PAI-borne SRL was indeed situated on the chromosome. Additionally, there have been increasing reports of chromosomal multi-antibiotic resistance, especially to the antibiotics Sm, Cm, Tc and Ap (Casalino, *et al.*, 1994; Gebre-Yohannes & Drasar, 1990; Ling, *et al.*, 1993). A study in Somalia reported that all but three of 112 *S. flexneri* strains isolated between 1983 and 1989 carry chromosomally linked resistance determinants to the antibiotics Ap. Cm, Tc and spectinomycin (Casalino, *et al.*, 1994). Both spectinomycin and Sm resistance are conferred by the *aadA1* cassette (Rajakumar, *et al.*, 1997). Similarly, a study from Tanzania reported that the common combination of resistance to Ap, Cm and Tc was not directly related to plasmid profiles, and that $Ap^r$ was usually due to the presence of *oxa-1* (Navia *et al.*, 1999). Previously,

*oxa-1* mediated $Ap^r$ was less common in Gram negative bacteria than resistance mediated by TEM β-lactamases (Richmond *et al.*, 1980). Importantly, these data indicate that chromosomal loci bearing some resemblance to the PAI-borne SRL are not only present in the Japanese and Australian isolates investigated in this study, but may also be prevalent in a wider range of *Shigella* strains distributed globally.

The SRL PAI is the first PAI to be linked with antibiotic resistance in a wide variety of bacterial strains. However, resistance to the antibiotics sulfamethoxazole, trimethroprim and streptomycin are encoded on the PAI-like SXT element (Waldor *et al.*, 1996), while the *Salmonella* genomic island 1 which has been detected in a few *Salmonella* serovars, also encodes multi-drug resistance (Boyd *et al.*, 2001; Boyd *et al.*, 2000). Thus, dissemination of antibiotic resistance genes on PAIs and PAI-like elements may be an important mechanism of horizontal transfer not only among *Shigella* isolates, but also within other bacterial populations. The clinical implications of such transfer are potentially serious, as selection for these elements poses the danger of not only increasing the prevalence of multi-antibiotic resistance, but also of changing the virulence profile of such strains.

# REFERENCES


**Al-Hasani, K., Adler, B., Rajakumar, K. & Sakellaris, H. (2001).** Distribution and strcutural variation of the *she* pathogencity island in enteric bacterial pathogens. *Journal of Medical Microbiology* **50**, 780 - 786.

**Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997).** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389 - 3402.

**Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1993).** *Current protocols in molecular biology.* New York: Greene Publishing and Wiley Interscience.

**Boyd, D., Peters, G. A., Clockaert, A., Boumedine, K. S., Chaslus-Dancla, E., Imberechts, H. & Mulvey, M. R. (2001).** Complete nucleotide sequence of a 43-Kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *Journal of Bacteriology* **183**, 5725 - 5732.

**Boyd, D. A., Peters, G. A., Ng, L.-K. & Mulvey, M. R. (2000).** Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhymurium DT104. *FEMS Microbiology Letters* **189**, 285 - 291.

**Buchrieser, C., Brosch, R., Bach, S., Guiyoule, A. & Carniel, E. (1998).** The high-pathogenicity island of *Yersinia pseudotuberculosis* can be inserted into any of the three chromosomal *asn* tRNA genes. *Molecular Microbiology* **30**, 965 - 978.

**Casalino, M., Nicoletti, M., Salvia, A., Colonna, B., Pazzani, C., Calconi, A., Mohamud, K. A. & Maimone, F. (1994).** Characterization of endemic *Shigella flexneri* strains in Somalia: antimicrobial resistance, plasmid profiles, and serotype correlation. *Journal of Clinical Microbiology* **32**, 1179 - 1183.

**Clermont, O., Bonacorsi, S. & Bingen, E. (2001).** The *Yersinia* high-pathogenicity island is highly predominant in virulence-associated phylgoenetic groups of *Escherichia coli*. *FEMS Microbiology Letters* **196**, 153 - 157.

**DuPont, H. L., Levine, M. M., Hornick, R. B. & Formal, S. B. (1989).** Inoculum size in shigellosis and implications for expected mode of transmission. *Journal of Infectious Diseases* **159**, 1126-1128.

**Gebre-Yohannes, A. & Drasar, B. S. (1990).** Plasmid profiles of antibiotic-resistance Shigella dysenteriae types 2, 3, 4, 6 and 7 isolated in Ethiopia during 1976 - 85. *Epidemiology and Infection.* **105**, 65 - 72.

**Hacker, J., Blum-Oehler, G., Muhldorfer, I. & Tschape, H. (1997).** Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Molecular Microbiology* **23**, 1089 - 1097.

**Hacker, J. & Kaper, J. B. (1999).** The concept of pathogenicity islands. In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 1 - 11. Edited by J. B. Kaper & J. Hacker. Washington: American Society for Microbiology.

**Haltalin, K. C., Nelson, J. D., Ring, R. D., Sladoje, M. & Hinton, L. V. (1967).** Double-blind treatment study of shigellosis comparing ampicillin, sulfadiazine, and placebo. *Journal of Pediatrics* **70**, 970 - 981.

**Hayashi, T., Makino, K., Osnishi, M., Kurokawa, K., Ishii, D., Yokoyama, K., Han, C.-G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M. & Shinagawa, H. (2001).** Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Research* **8**, 11 - 22.

**Hochhut, B., Lotfi, Y., mazel, D., Faruque, S. M., Woodgate, R. & Waldor, M. K. (2001).** Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrobial Agents and Chemotherapy* **45**, 2991 - 3000.

**Hochhut, B. & Waldor, M. K. (1999).** Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. *Molecular Microbiology* **32**, 99 - 110.

**Hughes, V. M. & Datta, N. (1983).** Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature* **302**, 725 - 726.

**Hussein, S., Hantke, K. & Braun, V. (1981).** Citrate-dependent iron transport system in *Escherichia coli* K-12. *European Journal of Biochemistry* **117**, 431-437.

**Karaolis, D. K. R., Somara, S., Maneval Jr, D. R., Johnson, J. A. & Kaper, J. B. (1999).** A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature* **399**, 375 - 379.

**Karch, H., Schubert, S., Zhang, D., Zhang, W., Schmidt, H., Olschlager, T. & Hacker, J. (1999).** A genomic island, termed high-pathogenicity island, is present in certain Non-O157 Shiga toxin-producing *Escherichia coli* clonal lineages. *Infection and Immunity* **67**, 5994 - 6001.

**Khan, M. U. (1985).** Fourteen years of shigellosis in Dhaka: an epidemiological analysis. *International Journal of Epidemiology* **14**, 607 - 613.

**Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Swerdlow, D. L., Sansonetti, P. J., Adak, G. K. & Levine, M. M. (1999).** Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bulletin of the World Health Organization* **77**, 651 - 665.

**Lee, C. A. (1996).** Pathogenicity islands and the evolution of bacterial pathogens. *Infectious Agents and Disease* **5**, 1 - 7.

**Ling, J. M., Shaw, P. C., Kam, K. M., Cheng, A. F. & French, G. L. (1993).** Molecular studies of plasmids of multiply-resistant *Shigella* spp. in Hong Kong. *Epidemiology and Infection* **110**, 437 - 446.

**Luck, S. N., Turner, S. A., Sakellaris, H., Rajakumar, K. & Adler, B. (2001).** Ferric dicitrate transport system (Fec) of *Shigella flexneri* 2a YSH6000 Is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. *Infection and Immunity* **69**, 6012 - 6021.

**Maloy, S. R. & Nunn, W. D. (1981).** Selection for loss of tetracycline resistance by *Escherichia coli*. *Journal of Bacteriology* **145**, 1110 - 1112.

**Morelle, G. (1989).** A plasmid extraction procedure on a mimiprep scale. *Focus* **11**, 7 - 8.

**Nakata, N., Sasakawa, C., Okada, N., Tobe, T., Fukuda, I., Suzuki, T., Komatsu, K. & Yoshikawa, M. (1992).** Identification and characterization of *virK*, a virulence-associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. *Molecular Microbiology* **6**, 2387 - 2395.

**Nakaya, R., Nakamura, A. & Murata, Y. (1960).** Resistance transfer agents in *Shigella*. *Biochemical and Biophysical Research Communications* **3**, 654 - 659.

**Navia, M. M., Capitano, L., Ruiz, J., Vargas, M., Urassa, H., Schellemberg, D., Gascon, J. & Vila, J. (1999).** Typing and characterization of mechanisms of resistance of *Shigella* spp. isolated from feces of children under 5 years of age from Ifakar, Tanzania. *Journal of Clinical Microbiology* **37**, 3113 - 3117.

**Ouellette, M., Bissonnette, L. & Roy, P. H. (1987).** Precise insertion of antibiotic resistance determinants into Tn*21*-like transposons: Nucleotide sequence of the OXA-1 B-lactamase gene. *Proceedings of the National Acadamy of Sciences of the United States of America* **84**, 7378 - 7382.

**Perna, N. T., Plunkett III, G., Burland, V., Mau, B., Glasner, J. M., Rose, D. J., Mayhew, G. F., Evans, P. S., Gregor, J., Kirkpatrick, H. A., Posfal, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Lim, A., Dimalanta, E. T., Potamousis, K. D., Apodaca, J., Anantharaman, T. X., Lin, J.,**

**Yen, G., Schwartz, D. C., Welch, R. A. & Blattner, F. R. (2001).** Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* **409**, 529 - 533.

**Pressler, U., Staudenmaier, H., Zimmermann, L. & Braun, V. (1998).** Genetics of the iron dicitrate transport system of *Escherichia coli*. *Journal of Bacteriology* **170**, 2716 - 2724.

**Priefer, U. (1984).** Characterization of plasmid DNA by agarose gel electrophoresis. In *Advanced Molecular Genetics*, pp. 32 - 33. Edited by A. Puhler & K. N. Timmis. New York: Springer-Verlag.

**Rajakumar, K., Bulach, D., Davies, J., Ambrose, L., Sasakawa, C. & Adler, B. (1997).** Identification of a chromosomal *Shigella flexneri* multi-antibiotic resistance locus which shares sequence and organizational similarity with the resistance region of the plasmid NR1. *Plasmid* **37**, 159 - 168.

**Rajakumar, K., Sasakawa, C. & Adler, B. (1996).** A spontaneous 99-kb chromosomal deletion results in multi-antibiotic susceptibility and an attenuation of contact haemolysis in *Shigella flexneri* 2a. *Journal of Medical Microbiology* **45**, 64 - 75.

**Rakin, A., Noelting, C., Schropp, P. & Heesemann, J. (2001).** Integrative module of the high-pathogenicity island of *Yersinia*. *Molecular Microbiology* **39**, 407 - 415.

**Richmond, M. H., Bennett, P. M., Choi, C.-L., Brown, N., Brunton, J., Grinsted, J. & Wallace, L. (1980).** The genetic basis of the spread of beta-lactamase synthesis among plasmid-carrying bacteria. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* **289**, 349 - 359.

**Ruzin, A., Lindsay, J. & Novick, R. P. (2001).** Molecular genetics of SaPI1 - a mobile pathogenicity island in *Staphylococcus aureus*. *Molecular Microbiology* **41**, 365 - 377.

**Salam, M. A. & Bennish, M. L. (1991).** Antimicrobial therapy for shigellosis. *Reviews of Infectious Diseases* **13**, S332 - S341.

**Sasakawa, C., Kamata, K., Sakai, T., Murayama, S. Y., Makino, S. & Yoshikawa, M. (1986).** Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. *Infection and Immunity* **51**, 470 - 475.

**Schubert, S., Cuenca, S., Fischer, D. & Heesemann, J. (2000).** High-pathogenicity island of *Yersinia pestis* in Enterobacteriaceae isolated from blood cultures and urine samples: prevalence and functional expression. *Journal of Infectious Diseases* **182**, 1268 - 1271.

**Schubert, S., Rakin, A., Karch, H., Carniel, E. & Heesemann, J. (1998).** Prevalence of the "high-pathogenicity island" of *Yersinia* species among *Escherichia coli* strains that are pathogenic to humans. *Infection and Immunity* **66**, 480 - 485.

**Tanaka, M., Matsushita, K. & Yamamoto, T. (1985).** Genesis of a complex transposon encoding the OXA-1 (Type II) b-lactamase gene. *Antimicrobial Agents and Chemotherapy* **28**, 227 - 234.

**Wagegg, W. & Braun, V. (1981).** Ferric citrate transport in *Escherichia coli* requires outer membrane recepor protein FecA. *Journal of Bacteriology* **145**, 156 - 163.

**Waldor, M. K., Tschape, H. & Mekalanos, J. J. (1996).** A new type of conjugative transpopson encodes resistance to sulfamethoxazole, trimpethoprim, and strepotmycin in *Vibrio cholerae* O139. *Journal of Bacteriology* **178**, 4157 - 4165.

**Watanabe, T. (1963).** Infective heredity of multiple drug resitance in bacteria. *Bacteriological Reviews* **27**, 87 - 115.

**White, P. A., McIver, C. J. & Rawlinson, W. D. (2001).** Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy* **45**, 2658 - 2661.

**Womble, D. D. & Rownd, R. H. (1988).** Genetic and physical map of plasmid NR1: Comparison with other IncFII antibiotic resistance plasmids. *Microbiological Reviews* **52**, 433 - 451.

**Xu, J.-G., Cheng, B., Wen, X., Cui, S. & Ye, C. (2000).** High-pathogenicity island of *Yersinia* spp. in *Escherichia coli* strains isolated from diarrhea patients in China. *Journal of Clinical Microbiology* **38**, 4672 - 4675.

A

# APPENDICES

# APPENDIX 1

## List of Abbreviations

| | |
|---|---|
| aa | amino acids |
| Ap | ampicillin |
| $Ap^r$ | ampicillin resistant |
| $Ap^s$ | ampicillin sensitive |
| bp | base pair(s) |
| Cm | chloramphenicol |
| $Cm^r$ | chloramphenicol resistant |
| $Cm^s$ | chloramphenicol sensitive |
| CFU | colony forming unit(s) |
| °C | degrees Celsius |
| DNA | deoxyribonucleic acid |
| $dH_2O$ | distilled water |
| DAEC | diffusely adherent *E. coli* |
| DIG | digoxigenin |
| DR | direct repeat |
| EAEC | enteroaggregative *E. coli* |
| EHEC | enterohaemorrhagic *E. coli* |
| EIEC | enteroinvasive *E. coli* |
| EPEC | enteropathogenic *E. coli* |
| ETEC | enterotoxigenic *E. coli* |
| EDTA | ethylene diamine tetra acetic acid |
| Fig. | Figure |
| g | grams |
| h | hour(s) |
| HMW | high molecular weight |
| HPI | high-pathogenicity island |
| Ig | immunoglobulin |
| IgA | immunoglobulin class A |
| IgG | immunoglobulin class G |
| IgM | immunoglobulin class M |
| IS | insertion sequence |

| | |
|---|---|
| Kn | kanamycin |
| $Kn^r$ | kanamycin resistant |
| kb | kilobases |
| kDa | kilodaltons |
| λ | lambda |
| LB | Luria Bertani broth |
| LMT | low melting temperature |
| LPS | lipopolysaccharide |
| M | molar |
| μg | micrograms |
| μl | microlitres |
| min | minutes |
| mA | milliamps |
| ml | millilitres |
| mM | millimolar |
| MRDE | multiple antibiotic-resistance deletable element |
| ng | nanograms |
| NK | natural killer |
| No. | number |
| ORF(s) | open reading frame |
| PAI | pathogenicity island |
| % | percent |
| PCR | polymerase chain reaction |
| PMN | polymorphonucleocyte |
| r-det | resistance determinant |
| R plasmid | resistance plasmid |
| RP | reverse primer |
| RNA | ribonucleic acid |
| SSC | salt, sodium citrate buffer |
| STEC | Shigella-toxigenic *E. coli* |
| SRL | *Shigella* resistance locus |
| SDS | sodium dodecyl sulphate |
| Sm | streptomycin |
| $Sm^r$ | streptomycin resistant |

| | |
|---|---|
| $Sm^s$ | streptomycin sensitive |
| Su | sulfonamide |
| Tc | tetracycline |
| $Tc^r$ | tetracycline resistant |
| $Tc^s$ | tetracycline sensitive |
| tRNA | transfer RNA |
| Tn | transposon |
| Tp | trimethoprim |
| $Tp^r$ | trimethoprim resistant |
| TAE | tris-acetate EDTA |
| TBE | tris-borate EDTA |
| T3 | T3 primer |
| T7 | T7 primer |
| U | units |
| UP | universal primer |
| UPEC | uropathogenic *E. coli* |
| V | volts |

# APPENDIX 2

## Complete List of Strains and Results (Chapter 4)

a) Complete List of *Shigella* Strains and Results

| Strain | Species | Linkage of *int* to[a]: | | Presence of SRL PAI marker: | | | | linkage of resistance determinants[a]: | | | Source[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ⇓*serX* | ⇓*serW* | *int* | $Sm^r/Ap^r/Cm^r/Tc^r$ | *fec* | *orf58* | *aadA1 + oxa-1* | *oxa-1 + cat* | *cat + tetA* | |
| SBA1381 | *S. boydii* 1 | | | - | ---- | + | + | | | | CS |
| SBA1382 | *S. boydii* 2 | | | - | ---- | + | + | | | | CS |
| SBA1383 | *S. boydii* 3 | | | - | ---- | - | - | | | | CS |
| SBA1384 | *S. boydii* 4 | | | - | ---- | + | + | | | | CS |
| SBA1385 | *S. boydii* 7 | | | - | ---- | - | + | | | | CS |
| SBA1397 | *S. dysenteriae* 4 | | | - | ---- | - | + | | | | CS |
| SBA1398 | *S. dysenteriae* 5 | | | - | ---- | - | - | | | | CS |
| SBA1396 | *S. dysenteriae* 6 | | | - | ---- | - | + | | | | CS |
| AL127 | *S. flexneri* 1a | | | - | ---- | - | - | | | | CS |
| AL128 | *S. flexneri* 1a | | | - | ---- | - | - | | | | CS |
| SBA1173 | *S. flexneri* 1b | | | - | ---- | - | - | | | | DL |
| AL129 | *S. flexneri* 1b | | | - | ---- | - | - | | | | CS |
| SBA1321 | *S. flexneri* 2a | | | - | ---- | - | - | | | | DL |
| AL130 | *S. flexneri* 2a | | | - | ---- | - | - | | | | CS |
| AL131 | *S. flexneri* 2b | | | - | ---- | - | - | | | | CS |
| AL134 | *S. flexneri* 3 | | | - | ---- | - | - | | | | CS |
| AL135 | *S. flexneri* 4a | | | - | ---- | - | - | | | | CS |
| AL136 | *S. flexneri* 4b | | | - | ---- | - | - | | | | CS |
| SBA1387 | *S. flexneri* 5 | | | - | ---- | + | + | | | | CS |
| AL137 | *S. flexneri* 5 | | | - | ---- | - | + | | | | CS |
| AL132 | *S. flexneri* X | | | - | ---- | - | - | | | | CS |
| AL133 | *S. flexneri* Y | | | - | ---- | - | - | | | | CS |
| SBA1375 | *S. sonnei* | | | - | ---- | + | - | | | | CS |
| SBA1376 | *S. sonnei* | | | - | ---- | + | - | | | | CS |
| SBA1377 | *S. sonnei* | | | - | ---- | + | - | | | | CS |
| SBA1378 | *S. sonnei* | | | - | ---- | + | - | | | | CS |
| SBA1379 | *S. sonnei* | | | - | ---- | + | - | | | | CS |
| SBA1380 | *S. sonnei* | | | - | ---- | + | - | | | | CS |
| SBA1395 | *S. dysenteriae* 9 | | | - | +--- | + | + | | | | CS |
| SBA1393 | *S. dysenteriae* 1 | | | - | -++ | + | - | | | | CS |
| SBA1407 | *S. flexneri* 3a | | | - | +-+ | + | - | | | | DL |
| AL122 | *S. flexneri* 3a | | | - | +-+ | - | - | | | | CS |
| AL125 | *S. flexneri* 3a | | | - | +-+ | - | - | | | | CS |
| SBA1405 | *S. flexneri* 6 | | | - | +-+ | - | + | | | | DL |

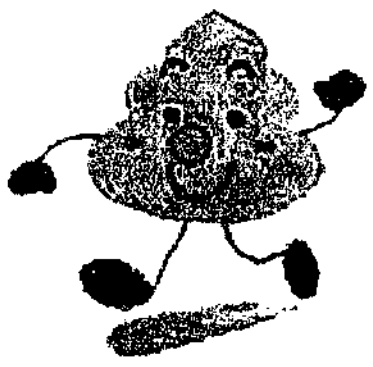
| Strain | Species | Linkage of *int* to[a]: | | Presence of SRL PAI marker: | | | | linkage of resistance determinants[a]: | | | Source[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ⇓*serX* | ⇓*serW* | *int* | Sm$^r$/Ap$^r$/ Cm$^r$/Tc$^r$ | *fec* | *orf58* | *aadA1* + *oxa-1* | *oxa-1* + *cat* | *cat* + *tetA* | |
| SBA1406 | *S. flexneri* 6 | | | - | +-+ | - | - | | | | DL |
| SBA1399 | *S. sonnei* bio g | | | - | +-+ | + | - | | | | DL |
| SBA1401 | *S. flexneri* 2b | | | - | ++- | + | - | | | | DL |
| SBA1400 | *S. sonnei* bio a | | | - | ++- | + | - | | | | DL |
| SBA1301 | *S. sonnei* Form I | | | - | +-++ | + | - | | | | CS |
| AL123 | *S. flexneri* 2a | | | - | ++-+ | - | - | | | | CS |
| SBA1388 | *S. flexneri* 2a | | | - | ++++ | + | - | + | - | - | CS |
| SBA1298 | *S. flexneri* 3c | | | - | ++++ | - | - | - | - | + | CS |
| SBA1308 | *S. boydii* 4 | - | - | + | ++++ | + | + | + | + | + | CS |
| SBA1307 | *S. boydii* 8 | - | - | + | ++++ | - | + | + | + | + | CS |
| SBA1386 | *S. boydii* 8 | - | + | + | ++++ | - | + | + | + | + | CS |
| SBA1304 | *S. dysenteriae* 3 | - | + | + | ++++ | - | + | + | + | + | CS |
| SBA1305 | *S. dysenteriae* 3 | - | + | + | ++++ | - | + | + | + | + | CS |
| SBA1306 | *S. dysenteriae* 3 | - | + | + | ++++ | + | + | + | - | + | CS |
| SBA1394 | *S. dysenteriae* 3 | - | + | + | ++++ | + | + | + | + | + | CS |
| SBA1299 | *S. flexneri* 1a | + | - | + | ++++ | + | + | + | + | + | CS |
| AL126 | *S. flexneri* 1b | - | - | + | ++++ | + | + | + | - | + | CS |
| SBA1317 | *S. flexneri* 2a | + | - | + | ++++ | - | + | + | + | - | DL |
| SBA1318 | *S. flexneri* 2a | + | - | + | ++++ | - | + | + | + | + | DL |
| SBA1319 | *S. flexneri* 2a | + | - | + | ++++ | - | + | + | + | + | DL |
| SBA1320 | *S. flexneri* 2a | - | + | + | ++++ | + | + | + | + | + | DL |
| SBA1322 | *S. flexneri* 2a | + | - | + | ++++ | + | + | + | + | + | DL |
| SBA1323 | *S. flexneri* 2a | + | - | + | ++++ | - | + | + | + | + | DL |
| SBA1402 | *S. flexneri* 2a | + | - | + | ++++ | - | + | + | + | - | DL |
| SBA1404 | *S. flexneri* 2a | - | - | + | ++++ | + | + | + | + | - | DL |
| SBA1300 | *S. flexneri* 2b | + | - | + | ++++ | + | + | + | + | + | CS |
| SBA1316 | *S. flexneri* 2b | + | + | + | ++++ | + | + | + | + | + | DL |
| SBA1390 | *S. flexneri* 2b | + | + | + | ++++ | + | + | + | + | + | CS |
| SBA1389 | *S. flexneri* 3a | - | - | + | ++++ | + | + | - | - | - | CS |
| SBA1297 | *S. flexneri* 3b | - | + | + | ++++ | + | + | + | - | + | CS |
| SBA1403 | *S. flexneri* 4 | + | - | + | ++++ | + | + | + | + | + | DL |
| SBA1391 | *S. flexneri* 4a | + | - | + | ++++ | + | + | + | + | + | CS |
| SBA1408 | *S. flexneri* 4a | + | - | + | ++++ | + | + | + | + | + | DL |
| SBA1392 | *S. flexneri* 6 | - | + | + | ++++ | + | + | + | + | + | CS |
| SBA1302 | *S. sonnei* Form I | - | + | + | ++++ | + | - | + | + | + | CS |
| SBA1303 | *S. sonnei* Form I | - | + | + | ++++ | + | + | + | + | + | CS |
| AL124 | *S. flexneri* 6 | - | - | + | ++++ | + | + | + | + | + | CS |

b) Complete List of Strains and Results of Other Members of the *Enterobacteriaceae*

| Strain | Species | Linkage of *int* to[a]: | | Presence of SRL PAI marker: | | | | linkage of resistance determinants[a]: | | | Source[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ⇓*serX* | ⇓*serW* | *int* | $Sm^r/Ap^r/Cm^r/Tc^r$ | *fec* | *orf58* | *aadA1 + oxa-1* | *oxa-1 + cat* | *cat + tetA* | |
| SBA885 | EIEC 28ac | | | - | ---- | - | + | | | | RRB |
| SBA886 | EIEC 167 | | | - | +--+ | - | + | | | | RRB |
| SBA887 | EIEC 112ac | | | - | ---- | - | - | | | | RRB |
| SBA888 | EIEC 124 | | | - | ---- | - | - | | | | RRB |
| SBA889 | EIEC O28 | | | - | +--+ | - | + | | | | CS |
| SBA890 | EIEC O29 | | | - | ---- | - | - | | | | CS |
| SBA891 | EIEC O124 | | | - | +--+ | - | - | | | | CS |
| SBA892 | EIEC O136 | | | - | ---+ | - | - | | | | CS |
| SBA893 | EIEC O143 | | | - | ---+ | + | - | | | | CS |
| SBA894 | EIEC O144 | | | - | +--+ | - | - | | | | CS |
| SBA895 | EIEC O152 | | | - | -+++ | - | - | | | | CS |
| SBA896 | EIEC O164 | | | - | ---- | - | + | | | | CS |
| AL21 | *S. typhimurium* | | | - | ---- | - | - | | | | RRB |
| AL22 | *S. typhimurium* | | | - | ---- | - | - | | | | RRB |
| AL24 | EHEC | + | + | + | ---- | - | - | | | | RRB |
| AL25 | EHEC | + | + | + | ---- | - | - | | | | RRB |
| AL26 | EHEC | | | - | ---- | - | + | | | | RRB |
| AL27 | *Y. enterocolytica* | | | - | ++++ | - | - | - | - | - | RRB |
| AL28 | *Y. enterocolytica* | | | - | ++-- | - | - | | | | RRB |
| AL30 | EPEC | | | - | ---- | - | - | | | | RRB |
| AL31 | EPEC | | | - | ---- | - | - | | | | RRB |
| AL32 | EPEC | + | - | + | ---- | - | - | | | | RRB |
| AL96 | ETEC | | | - | ---- | + | - | | | | RRB |
| AL97 | ETEC | | | - | +-++ | + | - | | | | RRB |
| AL98 | ETEC | | | - | ---- | - | - | | | | RRB |
| AL99 | EAEC | | | - | +-++ | + | - | | | | RRB |
| AL100 | EAEC | | | - | ++-+ | + | - | | | | RRB |
| AL101 | EAEC | | | - | +--- | + | - | | | | RRB |

[a] +, PCR linkage was demonstrated; -, PCR linkage was not demonstrated; , PCR linkage was not performed.

[b] CS, C. Sasakawa, University of Tokyo, Japan; DL, D. Lightfoot, University of Melbourne, Parkville, Australia; RRB, R. Robins-Browne, Royal Children's Hospital, Parkville, Australia.



## AMENDMENTS

| | Replace: | With: |
|---|---|---|
| p3 para 2, line 2 | *Enterobacteriaciae* | *Enterobacteriaceae* |
| p3 para 2, line 6 | Escherichiae | *Escherichiae* |
| p6 para 1, line 5 | components | components of |
| p23 para 1, line 9 | and Casp-1 | and ICE |
| p24, figure legend, line 4 | 2000 | 2000a |
| p28, para 3, line 8 | chaperon | chaperone |
| p33, para 3, line 2 | pathogenically | phenotypically |
| p35, line 3 of table | (18 bp DR | (18 bp DR) |
| p37, para 2, line 1 | Ochiai (Pfeifer | Ochiai (in Pfeifer |
| p38, line 12 | (1960) | in 1960 |
| p38, line 23 | spectimycin | spectinomycin |
| p39, para 1, line 6 | Khan *et al.* | Khan |
| p40, para 2, line 10 | homologous | homologues |
| p42, para 3 | nultiple | multiple |
| p42, para 8 | mxi | *mxi* |
| p43, para 1 | lpa | Ipa |
| p44, para 2 | *Contol* | *Control* |
| p44, para 6; p55, para 1; p57, para 3 | Is | is |
| p45, para 7; p46, para 7; p122, para 4 | Kilobase | kilobase |
| p46, para 3 | slosely | closely |
| p49, para 4 | *Escerichia* | *Escherichia* |
| p50, para 7 | Enterobactericacea | Enterobacteriaceae |
| p51 para 6; p104, para 3; p145, para 3 | Shigella dysenteriae | *Shigella dysenteriae* |
| p51 para 6; p104, para 3; p145, para3 | *Infection.* | *Infection* |
| p52, para 4 | Escherichia coli | *Escherichia coli* |
| p53, para 2 | henle | Henle |
| p54, para 1 | unction | function |
| p54, para 2 | *Salmoella* | *Salmonella* |
| p54, para 3 | proteinsof | proteins of |
| p55, para 7 | dinstinct | distinct |
| p59, para 6; p147, para 1 | Is | is |
| p60, para 7 | ipa | *ipa* |
| p60, para 8 | Prolifin | Profilin |
| p63, para 4; p107, para 3 | Shigella flexneri | *Shigella flexneri* |
| p63, para 8 | Shigellae | *Shigellae* |
| p66, para 3 | mdh | *mdh* |
| p67, para 5; p157, table | *enterocolytica* | *enterocolitica* |
| p67, para 8 | Myson | Myosin |
| p68, para 1 | Salmonellae | *Salmonellae* |
| p68, para 1 | Shigellae | *Shigellae* |
| p69, para 1 | IFN-g | IFN-γ |
| p69, para 3 | inflammatorydestruction | inflammatory destruction |
| p69, para 8 | Involvementof | Involvement of |
| p69, para 8 | abiliy | ability |
| p75, para 4 | an | and |
| p75, para 5 | systen | system |
| p75, para 6 | uprtake | uptake |
| p76, para 2 | rho | Rho |
| p84, para 2, line 1 | HMW | High molecular weight (HMW) |
| p86, para 3 | eclustalw | Clustal W |
| p88, para 3, line 2 | *IS91* | The *E. coli* IS*91* |
| p102, para 1; p144, para 1 | strcutural | structural |
| p107, para 1 | mimiprep | miniprep |
| p108, para 5 | gragmentation | fragmention |
| p109, para 2 | incompitability | incompatability |
| p125, para 2 | resitance | resistance |
| p145, para 1 | phylogoenetic | phylogenetic |
| p147, para 7 | B-lactamase | β-lactamase |
| p149, para 2 | Enterobacteriaceae | *Enterobacteriaceae* |
| p149, para 4 | b-lactamase | β-lactamase |
| p149, para 5 | recepor | receptor |

Chapter 1

p2 para 2: delete "work by Losch (1875), Councilman and Lafleur (1891) and Kruse and Pasquale (1893)" and read "work by Losch (in 1875), Councilman and Lafleur (in 1891) and Kruse and Pasquale (in 1893)"

p3 para 2: delete "and exhibit many " and read ", share 3.9 Mb of common backbone sequence (Jin et al., 2002), and exhibit many "

p4 para 2: delete "when pathogenic *E. coli* strains were isolated (Pupo, et al., 2000)." and read "when pathogenic *E. coli* strains were isolated."

p5 para 2: delete "dehydration, haemolytic-uraemic syndrome (HUS) and Reiter's syndrome" and read "dehydration and haemolytic-uraemic syndrome (HUS)"

p6 para 1: delete "may develop 1 to 3 weeks after" and read "may develop after"

p6 para 2: delete "Also developing after some cases of shigellosis is conjunctivitis" and read "Other conditions also developing after some cases of shigellosis are conjunctivitis"

p19 para 3: delete "SopA (also called IcsP)" and read "SopA" . Delete "serine protease SepA, and the product of the *apy* gene" and read "serine protease and autotransporter protein SepA (which is thought to play a role in tissue invasion), and the product of the *apy* gene (which is a proposed cytotoxin)"

p20. Delete figure legend and insert: "**Figure 1.1** Schematic representation of the large virulence plasmid of *S. flexneri* (Picture to scale, modified from Venkatesan *et al.*, 2001; Sansonetti, 2001). (A) Circular map of the large virulence plasmid of *S. flexneri* 5a indicating major genes and loci involved in pathogenesis. (B) Detailed genetic organisation of the *S. flexneri* plasmid-borne 31 kb region which is required for entry of *Shigella* into epithelial cells. Arrows represent size and orientation of individual genes"

p27 figure legend: after "**Figure 1.3**" insert "Fluorescence microscopy showing individual motile *Shigella* cells (in red) and the propelling F-actin comet tails associated with the cells (in green)."

p28: add at the end of para 2: "Interestingly, recent data suggests that IcsA may not be exclusively targeted to the old pole of the bacterium, but is masked laterally by the length of the LPS O-antigen (Morona & Van Den Bosch, 2003). Thus highlighting the fact that a clear understanding of IcsA distribution and targeting is yet to be developed.". Add reference: Morona, R., Van Den Bosch, L. (2003). Multicopy *icsA* is able to suppress the virulence defect caused by the *wzz*(SF) mutation in *Shigella flexneri*. FEMS Microbiology Letters 221, 213 – 219.

p28 para 2: delete "but is detected only in *S. flexneri*, and is found almost exclusively in serotype 2a strains" and read "and is found almost exclusively in *S. flexneri* serotype 2a strains"

p34 para 2: delete "These include carriage" and read "These include absence from non-pathogenic strains of the same species, carriage"

p34 para 3. Insert after "Karaolis *et al.*, 1999)": "[although data relating to the production of phage particles has recently been contested (Faruque *et al.*, 2003)]". Add reference: Faruque, S. M., Zhu, J., Asadulghani, K. M. & Mekalanos, J. J. (2003). Examination of diverse toxin-coregulated pilus-positive *Vibrio cholerae* strains fails to demonstrate evidence for *Vibrio* pathogenicity island phage. Infection and Immunity 71, 2993 – 2999.

Chapter 2

p81 para 2. Insert after "Karaolis *et al.*, 1999)": "[although data relating to the production of phage particles has recently been contested (Faruque *et al.*, 2003)]". Add reference: Faruque, S. M., Zhu, J., Asadulghani, K. M. & Mekalanos, J. J. (2003). Examination of diverse toxin-coregulated pilus-positive *Vibrio cholerae* strains fails to demonstrate evidence for *Vibrio* pathogenicity island phage. Infection and Immunity 71, 2993 – 2999.

p84 para 3: delete "labelling and detection kit." and read "labelling and detection kit (Ausubel *et al.*, 1993)."

p85 para 2. Add after paragraph: "PCR conditions were as follows: 2 min at 94 °C followed by 30 cycles of [44 s at 94 °C, 40 s at 55 °C and 1 min – 3 min at 70 °C] with a final extension at 70 °C for 2 min."

p85 para 3: delete "transferred by conjugation" and read "transferred by conjugation (Ausubel *et al.*, 1993)"

p87 – p88. Delete paragraph entitled "Background" and insert section (without heading) after p82 para 2

Chapter 3

p111 para 3: delete "with only two PAIs shown to be mobilisable" and read "with only the *Vibrio* VPI and the *Salmonella* SaPI shown to be mobilisable (as discussed in Chapter 1 – Pathogenicity Islands)"

p113 para 1: delete "labelling and detection kit." and read "labelling and detection kit (Ausubel *et al.*, 1993)."

p114 para 2: delete "mobilising strain S17-1 (λpir) and the resulting strain, AL403 was used in overnight conjugations" and read "mobilising strain S17-1 (λpir) (Penfold & Pemberton, 1992). The resulting strain, AL403 was used in overnight conjugations (Ausubel *et al.*, 1993)"

p114 para 3. Add after paragraph: "PCR conditions were as follows: 2 min at 94 °C followed by 30 cycles of [44 s at 94 °C, 40 s at 55 °C and 1 min – 3 min at 70 °C] with a final extension at 70 °C for 2 min."

p120 para 3. Insert after "Karaolis *et al.*, 1999)": "[although data relating to the production of phage particles has recently been contested (Faruque *et al.*, 2003)]". Add reference: Faruque, S. M., Zhu, J., Asadulghani, K. M. & Mekalanos, J. J. (2003). Examination of diverse toxin-coregulated pilus-positive *Vibrio cholerae* strains fails to demonstrate evidence for *Vibrio* pathogenicity island phage. Infection and Immunity 71, 2993 – 2999.

Chapter 4

p128 para 2: delete" The SRL is lost from the *S. flexneri* 2a strain YSH6000 via three distinct but separate mechanisms involving deletion" and read "The SRL may be lost from the *S. flexneri* 2a strain YSH6000 via three distinct but separate mechanisms involving either deletion"

p130 para 3: delete "labelling and detection kit." and read "labelling and detection kit (Ausubel *et al.*, 1993)."

p131 para 1. Insert after first sentence: "PCR conditions were as follows: 2 min at 94 °C followed by 30 cycles of [44 s at 94 °C, 40 s at 55 °C and 1 min – 3 min at 70 °C] with a final extension at 70 °C for 2 min."