Role of *sefD* and *sefR* in the biogenesis of *Salmonella enterica* serovar Enteritidis SEF14 fimbriae

James Alfons Desmond Botten

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Abstract

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is able to express a number of different fimbrial types, including SEF14 fimbriae. These fimbriae show a limited distribution within the species *Salmonella enterica*, confined to some members of the D1 serogroup, including: *S.* Dublin, *S.* Enteritidis and *S.* Pullorum. To date the specific role of SEF14 fimbriae in pathogenesis is unknown, however alternative proposals for its role have been made, including: macrophage internalisation, transovarian transmission and binding to inanimate surfaces. Along with its unknown role in pathogenesis, the mechanism by which SEF14 are regulated was also unknown at the commencement of this study. The SEF14 operon consists of four genes: *sefA* (major subunit); *sefB* (periplasmic chaperone); *sefC* (outer-membrane usher) and *sefD*, proposed to encode the major subunit of an unrelated fimbrial type designated SEF18. Analysis of the DNA immediately downstream of the SEF14 locus revealed a fifth ORF. BLASTN analysis of the nucleotide sequence revealed no significant similarities to any other sequences. However, BLASTX analysis of the predicted protein translation revealed that this ORF had significant protein identity to members of the AraC-like transcriptional regulator family, including some that are involved in the regulation of fimbriae. Based on these observations, this ORF was redesignated *sefR* (*S.* Enteritidis fimbriae regulator).

To assess the role of *sefR* in the regulation of SEF14 fimbriae, the *sefABCD* genes were cloned into a high copy number vector and examined for their ability to produce SEF14 fimbriae in *Escherichia coli* (*E. coli*) K-12. Western immunoblot and IEM analysis revealed that these genes conferred on *E. coli* K-12 the ability to express SefA and SEF14 fimbriae, although the fimbriae were not as extensive as observed in wild-type *S.* Enteritidis. However, expression of SEF14 fimbriae was not observed when the *sefABCD* genes were cloned into a low copy number plasmid. SEF14 fimbriae expression was restored when *sefR* was supplied *in trans*, suggesting that *sefR* had a role in upregulating SEF14 expression. *S.* Enteritidis with

a chromosomal mutation in *sefR* showed no detectable SefA or SEF14 fimbriae when examined by Western immunoblot, EIA or IEM. SEF14 expression was restored by supplying *sefR in trans*, confirming *sefR* as an activator of SEF14 fimbriae expression.

Prior to the commencement of this work the role of *sefD* in the biogenesis of SEF14 fimbriae, and its distribution within the Enterobacteriaceae family was also unclear. Previous work suggested that *sefD* was ubiquitous within the Enterobacteriaceae family. Dot blot and Southern hybridisation analysis revealed that, like the *sefABC* genes, *sefD* was actually limited to group D1 *Salmonella* strains. Similar analysis of *sefR* revealed a wider distribution, with apparent *sefR* homologues in both pathogenic *E. coli* O111:H$^-$ and *Shigella flexneri.* This suggested that there might be as yet uncharacterised *sefR*-like regulators in these bacteria.

Analysis of the *S.* Typhi CT18 genome revealed a non-functional SEF14 locus that was flanked by DNA with similarity to bacteriophage P4 DNA. Further analysis of 5' and 3' sequences revealed a region of ~23 kb that showed no similarity to any *E. coli* K-12 DNA and was flanked by DNA with similarity to the *leuX* and *mcrD* genes of *E. coli* K-12. *leuX* has been characterised as a site for DNA insertion mediated by bacteriophage P4. Therefore, it was proposed that the SEF14 locus in *S.* Typhi comprised part of a 23 kb pathogenicity island (PAI). Comparison of this putative PAI to the partially completed *S.* Enteritidis genome suggested that this region differs between these serovars, although the full extent of these differences remains to be determined.

Based on the fact that the distribution of the *sefABC* and *sefD* genes is the same, plus similarities in operon structure with other fimbrial loci such as the CS1 operon of enterotoxigenic *E. coli*, it was hypothesised that *sefD* encodes an initiator of SEF14 biogenesis. Thus, inactivation of *sefD* should result in a loss of SEF14 expression, with no observable surface expressed SefA. SEF14 fimbriae were not expressed by *S.* Enteritidis *sefD* mutants, however unlike the *sefR* mutants, examination of the *sefD* mutants by IEM revealed

anti-SefA reactive material localised at the bacterial cell surface. Supplying *sefD in trans* complemented this defect in SEF14 expression. Interestingly, *sefD* mutants complemented with plasmid borne *sefD* produced fimbriae of a slightly altered morphology compared to the wild type, suggesting that an increase in *sefD* over wild type levels has some effect on the biogenesis of SEF14 fimbriae. Hence, it was shown that *sefD* is required for production of SEF14 fimbriae with normal morphology, but is not required to initiate SEF14 biogenesis.

Based on both the data presented in this study, and previous knowledge of SEF14 fimbriae, a model for SEF14 biogenesis was proposed. Similar to other chaperone-usher type fimbriae, the periplasmic chaperone (SefB) delivers fimbrial subunits to the outer-membrane usher (SefC). Unlike these systems, the minor subunit (SefD) is not required to initiate fimbriae biogenesis, and is incorporated into the fimbriae at random intervals based on the stoichiometric ratio of major and minor subunits. Also, SefD most likely acts as a branching molecule, with multiple nucleation sites that are able to link individual fibres together, and initiate polymerisation of new branches within the same fibre. This model accounts for the highly branched and amorphous morphology of SEF14 fimbriae that is observed in wild type *S.* Enteritidis.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited into the University library, being made available for loan and photocopying.

James Alfons Desmond Botten, July 2001

This thesis is dedicated to my Grandfather

Alfons Hahnefeld

(13/9/1932 - 3/2/2001)

Without whom my life would have been all the poorer,

And to my wife Rochelle and my son Nathanael,

With whom my life is so much richer.

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Abbreviations

µg	microgram
µl	microlitre
µm	micrometre
A	adenine
aa	amino acid
Amp	ampicillin
ara	arabinose
ATP	adenosine 5'-triphosphate
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CFA	colonisation factor antigen
cyto	cytoplasm
dATP	deoxy adenosine nucleoside triphosphate
dCTP	deoxy cytosine nucleoside triphosphate
dGTP	deoxy guanosine nucleoside triphosphate
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dsDNA	double stranded DNA
DTT	diothiothreitol
dTTP	deoxy thymidine nucleoside triphosphate
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic-acid
EIA	ELISA inhibition assay
ELISA	Enzyme linked immuno-sorbent assay
EaggEC	enteroaggregative *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
g	gram
G	guanine

g/L	grams per litre
glu	glucose
h	hour
His_6 tag	6 residue histidine tag
IB	inclusion bodies
IM	inner membrane
i.p.	intra-peritoneal
IPTG	isopropyl-β-D-thiogalacto-pyranoside
Kan	kanomycin
kb	kilobase pairs
kDa	kilodalton
kV	kilovolts
LB	Luria-Bertani broth
LA	Luria-Bertani agar
LacZ	β-galactosidase
LR-PCR	Long Range polymerase chain reaction
M	molar
mA	milliamps
MBP	maltose binding protein
mg	milligram
min	minute(s)
ml	millilitre
mM	millimolar
MQ	milli-Q water
mRNA	messenger RNA
ng	nanogram
nm	nanometre
no.	number
nt	nucleotide
°C	degrees celsius
OD_{600nm}	optical density at 600 nm
oligo	oligonucleotide
OM	outer membrane
O/N	overnight
ORF	open reading frame

PAGE	polyacrylamide gel electrophoresis
Pap	pili associated with pyelonephritis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol-8000
peri	periplasm
pmol	picomole
R	resistant
RBS	Ribosome binding site
Rif	rifampicin
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	Room temperature
SAP	shrimp alkaline phosphotase
SDS	sodium dodecyl sulphate
sec	second(s)
SEM	standard error of the mean
S/N	supernatant
ssDNA	single stranded DNA
T	thymine
TBS	tris-buffered saline
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
Tris	Tris (hydroxymethyl) amino methane
tRNA	transport RNA
TTBS	Tween-20 plus tris-buffered saline
TTSS	Type III secretion system
U	unit(s)
UV	ultraviolet
V	volts
v/v	volume per volume
WC	whole cells
WM	whole membranes
w/v	weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

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Chapter 1: Introduction

1. *Salmonella*

Salmonella are Gram-negative, rod shaped bacteria belonging to the family Enterobacteriaceae. They are facultative anaerobes that are able to produce hydrogen sulphide (H_2S) from triple-sugar iron agar. This allows identification of *Salmonella* from other coliform bacteria as they form distinctive black colonies on this selective media. Most *Salmonella* are motile through the expression of peritrichous flagella, however the Gallinarum-Pullorum serotype is non-motile (Hook, 1990). *Salmonella* is able to infect the small intestine and induces a mild to severe gastroenteritis. Some serovars are also able to spread systemically and cause the more severe disease enteric fever.

Salmonella has been studied extensively since its discovery more than 100 years ago. Within this time, there has been a rise in the prevalence of *Salmonella* infection of humans and livestock (examined further in section 1.2) leading to a concomitant increase in the amount of *Salmonella* related research. A significant portion of this research had been directed towards understanding the molecular mechanisms of *Salmonella* pathogenesis (section 1.5). These mechanisms involve a measure of complexity that is increasingly understood as both our understanding of *Salmonella*, and our ability to investigate interactions between it and its host, improves with the use of new techniques.

While significant advances in our understanding of *Salmonella* have been made, many questions still remain. This study seeks to address some of those questions by focussing on the regulation and distribution of the SEF14 fimbrial operon of *Salmonella* Enteritidis.

1.1 *Salmonella* Nomenclature

Over 2000 serovars of *Salmonella* have been isolated and classified according to the Kaufman-White scheme of O- and H-antigen analysis (Kauffmann, 1954, Le Minor *et al*, 1982). This scheme utilises the large range of unique combinations of O- (somatic) and H-

(flagella) antigens in order to group isolated *Salmonella* into their various serovars. This large number of serovars has led to confusion with the nomenclature of *Salmonella* (for review see Old, 1992). Names have often been assigned with reference to the geographical site of identification (e.g. *S. adelaide*) or by some mistaken pathogenic effect (eg *S. choleraesuis* - once thought to be the causative agent of swine cholera) rather than with any reference to either genetic relatedness or species specificity. Improved techniques in the area of genetic analysis and comparison have revealed that there are only two *Salmonella* species, these being *Salmonella enterica* and *Salmonella bongori* with *S. enterica* further subdivided into seven subspecies, designated I, II, IIIa, IIIb, IV, VI and VII (Reeves *et al.*, 1989). Le Minor and Popoff (1987) proposed that all *Salmonella* strains take on subjective synonyms in addition to the *Salmonella enterica* designation (many of which had previously been used as species designation). For example *S. typhimurium* would be renamed to *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). This method of nomenclature makes sense from a genetic standpoint, although there has been some opposition to this system. If a *Salmonella* infection was diagnosed as being *Salmonella* enterica serovar Typhi rather than the common *Salmonella typhi*, there may be some confusion as to the urgency and type of treatment required. Also, researchers have long used the classical nomenclature and may be reluctant to change that with which they are comfortable. In keeping with advances in our knowledge of *Salmonella* genomics the convention proposed by Le Minor and Popoff (1987) will be used throughout this study such that *Salmonella enteritidis* becomes *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis).

1.2 Host Range and Adaptation

The host range of *Salmonella* is as widely varied as the large number of serotypes would suggest. *Salmonella* have been isolated and associated with disease in such diverse species as: cattle, pigs, rodents, poultry and other birds, dogs, sheep, horses and donkeys,

reptiles such as lizards and snakes, and higher mammalian species such as chimpanzees and humans (Bennet & Hook, 1959; Edwards and Bruner, 1943; Sojka and Wray, 1975; Sojka *et al.*, 1983; Sojka *et al.*, 1986; Wray *et al.*, 1981).

Some *Salmonella* serovars are able to infect a wide range of hosts e.g. *Salmonella* Enteritidis infects cattle, chickens and other birds, reptiles and humans, while others are restricted to a single host e.g. *Salmonella* Typhi is adapted to infect humans, causing the most severe form of human salmonellosis and has no known animal reservoir. Many serovars are found almost exclusively within a particular geographical region while others are found worldwide. This variation in location seems to suggest localised mutation and/or adaptation from an ancestor type strain that had previously seeded multiple geographical regions. How this variation might occur is examined in section 1.6. Of the *Salmonella* serovars identified in cases of human infection, >99% are represented by subspecies I serotypes with approximately 40% of these being *S.* Typhimurium and *S.* Enteritidis (PHLIS Surveillance Data, 1999).

Salmonella, with its large repertoire of host adapted and broad host range serovars, provides an excellent model for examining the phenomenon of host adaptation (for review see Kingsley and Bäumler, 2000). Host adaptation is commonly assumed to be the ability of a pathogen to cause disease only in the animal species to which the pathogen is adapted e.g. *S.* Typhi and infection of humans. However, while *S.* Dublin and *S.* Choleraesuis are not considered human adapted, since they do not circulate within human populations, they are able to cause disease in humans, albeit at low frequency (0.6% and 0.3% of isolates respectively, compared to 64% for *S.* Typhimurium; Sojka and Field, 1970). *S.* Dublin and *S.* Choleraesuis are considered to be cattle and swine adapted respectively, as they continually circulate within herds of these animals, occasionally passing on infections to humans. Therefore, host adaptation is more correctly described as the ability of a pathogen to circulate and cause disease within a particular host population (Kingsley and Bäumler, 2000). O-antigen polymorphism is one mechanism by which *Salmonella* is able to adapt to a particular

host (Reeves, 1992). Changes in O-antigen serotype would allow an infecting strain to survive within a host and through some as yet unknown genetic mechanism enter a balanced host-pathogen state. This would allow the bacteria to survive in the host without a significant reduction in bacterial load. Bacterial persistence would lead to increased rates of cross-infection between animals such that the entire herd becomes either immune to the dominant O-antigen serotype or becomes low level carriers of bacteria. These concepts of host adaptation are important when considering the epidemiology of various *Salmonella* outbreaks and how human interference in the stable state of host pathogen interactions can be detrimental, despite our good intentions (Rabsch *et al.*, 2000). An example of this is discussed with reference to *S.* Enteritidis in section 1.7.1

1.3 Epidemiology and Disease

Infection of humans by most *Salmonella* serovars (including *S.* Enteritidis) will generally lead to gastroenteritis, while a small group of serovars, including *S.* Typhi, cause the more severe enteric (typhoid) fever. *S.* Enteritidis enters a human host via the faecal/oral route, generally through contaminated food that has either not been correctly handled or cooked sufficiently. Contaminated shell eggs have been implicated as a primary source of *S.* Enteritidis, (St. Louis *et al.*, 1988). The very young, elderly and immuo-compromised individuals are the most susceptible to infection and symptoms associated with disease include severe headaches, abdominal cramps, diarrhoea, nausea, vomiting, fever and muscle aches. These symptoms usually last 4-7 days and appear 12-72 hours after infection, depending on dose of bacteria received. Some people have asymptomatic infections, but in both cases carriage and shedding of viable *S.* Enteritidis can persist for >5 weeks. A small proportion (up to 2%) will go on to be chronic carriers of *S.* Enteritidis and may develop chronic reactive arthritis (Wuorela and Granfors, 1998). More severe disease is rare and

includes septicaemia with associated enteric fever and fatal endocarditis (<0.1% of infections; Roll *et al.*, 1996; Shibusawa *et al.*, 1997; Pumberger and Novak, 2000; Sarria *et al.*, 2000).

Despite the large number of disease causing serovars, a subset of 7-12 *Salmonella* serovars (of which *S.* Enteritidis is a member) will account for the majority of infection at any given time, as determined by organisations such as the World Health Organisation (WHO) and the Centre for Disease Control (CDC). The cumulative number of reported cases of salmonellosis in the USA for the year 2000 was almost 37,000 (~13 cases /100,000 people; MMWR 49(51) Table II); it has been estimated that due to severe under reporting, the actual number of cases could exceed 3.5 million per year (Chalker and Blaser, 1988). In Europe it is estimated that there are 250 cases of Salmonellosis per 100,000 people each year (almost 2 million cases; WER 75:53-60). In Australia, the National Notifiable Diseases Surveillance System (NNDSS) recorded a total of 6085 cases of salmonellosis for the year 2000, which equates with approximately 31 cases of per 100,000 people.

A small but significant percentage (<0.1%) of all *Salmonella* infections will be fatal. While *Salmonella* does not cause the same morbidity and mortality as, for example, Enterotoxigenic *E. coli* (~800,000 deaths each year, Gaastra and Svennerholm, 1996), it is a significant problem in the Western world and results in major economic losses in terms of hospital costs, sick leave etc. These losses are compounded by the economic impact of livestock infections, which may lead to the slaughter of large numbers of livestock (cattle and poultry) along with the destruction of potentially infected foodstuffs (meat, eggs, dairy products). All these factors combine to make *Salmonella* an extremely important pathogen which will require much research before we fully understand the exact mechanisms of pathogenesis and find ways to limit or even negate the impact it has on humans.

1.4 *Salmonella* infection and Immune responses

S. Typhi is the causative agent of typhoid fever in humans, while *S.* Typhimurium causes a similar disease in mice, and as such is the accepted animal model for human typhoid fever (Eisenstein, 1999). As such the majority of work performed in the area of immunity to *Salmonella* has been done using the *S.* Typhimurium mouse model, which will be examined briefly here.

1.4.1 Overview of *Salmonella* Infection and Immunity

Salmonella infection begins with ingestion of virulent bacteria, followed by a transversal of the highly acidic lumen of the stomach, which *Salmonella* is able to survive by way of the acid tolerance response (Foster, 1995). Once past the stomach lumen, *Salmonella* penetrates the intestinal mucosa by invading specialised epithelial cells (M cells). M cells are found overlying the ileal Peyer's patches (Neutra *et al.*, 1996) and are required for sampling and delivery of processed antigen, including intracellular pathogens, to underlying lymphoid cells (Siebers and Finlay 1996).

Attachment to M cells is mediated by fimbriae (section 1.5.1.2) and is followed by the induction of specialised invasion genes located on pathogenicity islands (section 1.5.2), promoting the active uptake of *Salmonella* into the M cell. *Salmonella* is able to lyse M cells (Jones *et al.*, 1994), which requires the *Salmonella* virulence determinant SlyA (Daniels *et al.*, 1996; Watson *et al.*, 1999). At this point *Salmonella* gains access to lymphoid associated cells such as dendritic cells, which have been suggested to act as a vehicle for systemic dissemination (Sirard *et al.*, 1999), and to macrophages. Within macrophages, further pathogenicity islands are induced to promote bacterial survival and apoptotic death of the host cell. From this point *Salmonella* can enter the mesenteric lymph nodes and spread systemically via efferent lymph (Mäkelä and Hormaeche, 1997). The initial attachment and invasion of *Salmonella* occurs rapidly and is followed by a period of bacterial replication,

both locally and at systemic sites such as the spleen and liver (Mäkelä and Hormaeche, 1997). Interestingly, *S.* Typhimurium mutants that are unable to survive intracellularly show significant attenuation *in vivo* (Fields *et al.*, 1986b; Richter-Dahlfors and Finlay, 1997).

In mice, there appears to be a critical bacterial load (~10^8) of bacteria in excess of which mice cannot survive and succumb to secondary bacteraemia and endotoxic shock. In non-fatal infection, the bacterial load is restricted below this threshold, with infection persisting for several weeks (Mäkelä and Hormaeche, 1997, Eisenstein, 1999). During this time an adaptive immune response towards *S.* Typhimurium is generated, resulting in bacterial clearance and long lasting immunity to reinfection.

Immunity to *Salmonella* can be considered to have several interrelated facets i.e. non-specific or innate immunity, humoral immunity, cell mediated immunity and cytokine action. These aspects are briefly examined in the following section and the reader is directed to several reviews that expand on this area (Jones and Falkow, 1996; Lalmanach and Lantier, 1999; Mittrücker and Kaufmann, 2000).

1.4.1.1 Innate Immunity

A range of innate immune defences are employed to combat *Salmonella* infections and include antimicrobial peptides, complement, macrophages and neutrophils.

More than 40 antimicrobial agents have been discovered and include polymxins, defensins and melittin (Boman, 1995). These substances are small positively charged peptides that have various modes of action e.g. inhibition of nutrient uptake (Aspedon and Groisman, 1996) and lysis by disruption of bacterial outer and inner membranes (Boman 1995). Antimicrobial peptides form a significant part of the innate immune system since they constitute almost 7% of total protein in neutrophils and macrophages. The genetic disorder "specific granule deficiency" results in a lack of defensins, and people with this condition are prone to severe and recurrent microbial infections (Lehrer *et al.*, 1993). LPS and other

bacterial factors, including PhoP/Q regulated genes, are involved in resistance to these substances (Parra Lopez *et al.*, 1993; Scott *et al.*, 1999)

These antimicrobial substances are secreted/released by both neutrophils and macrophages, cells that have been shown to be important for limiting *Salmonella* infection. The importance of neutrophils was demonstrated by MacFarlane *et al* (1999a), who showed that blocking neutrophil influx into the spleen resulted in a 90% mortality of mice infected with a normally attenuated *S.* Typhimurium strain carrying an *aroA* mutation. The role of macrophages is more complex, due to the ability of *Salmonella* to interfere with both normal killing mechanisms and apoptosis.

It has been known for some time that *Salmonella* is able to survive and replicate in macrophages (Beaman & Beaman 1984; Fields *et al.*, 1986a, 1986b). Interestingly, the origin of the macrophage dictates how susceptible to killing a particular *Salmonella* serovar will be. For example, *S.* Typhimurium persists longer in murine than human macrophages, while the reverse is true for *S.* Typhi (Schwan *et al.*, 2000). This was in agreement with data obtained by Ishibashi and Arai (1996), who demonstrated the ability of *S.* Typhi to survive in human macrophages, but not in murine macrophages, while the reverse was true for *S.* Typhimurium. Further, different subpopulations of macrophages from a single host showed varying degrees of killing, with bone/splenic derived macrophages displaying less ability to kill *S.* Typhimurium than peritoneal macrophages (Buchmeier and Heffron 1989). In order to survive within macrophages, *Salmonella* subverts the normal vesicle trafficking pathways via the action of SpiC, preventing the fusion of *Salmonella*-containing phagosomes with lysosomes and endosomes (Buchmeier & Heffron, 1991; Uchiya *et al.*, 1999). This in turn prevents *Salmonella* from being exposed to antimicrobial agents, acidic pH, reactive oxygen intermediates and degredative enzymes found within these compartments.

The ability to induce apoptosis in macrophages is a significant part of *Salmonella* pathogenesis. Apoptosis, or programmed cell death, is a normal biological process, which

removes unwanted cells without the need for inducing an inflammatory response. This process is mediated by cysteine proteases (caspases; Thornberry and Lazebnik 1998). Through the action of the secreted effector protein SipB, *Salmonella* subverts the apoptotic process by activating caspase-1, resulting in both the death of the macrophage and activation of the pro-inflammatory cytokines IL-1β and IL-18 (Zychlinski and Sansonnetti, 1997; Navarre and Zychlinsky, 2000; Hersh *et al.*, 1999). The importance of SipB-induced macrophage apoptosis in *Salmonella* infection has been demonstrated by comparing oral and i.p. administration of *Salmonella* to normal and caspase$^{-/-}$ mice (Hersh *et al.*, 1999). *Salmonella* is able to colonise both mice equally well when introduced orally, however less systemic dissemination of *Salmonella* is observed in caspase$^{-/-}$ mice. Conversely no differences were observed in infection when *Salmonella* was introduced intraperitoneally. These results show the importance of apoptosis is promoting systemic dissemination of *Salmonella.*

Another innate host defence mechanism is the natural resistance associated macrophage protein-1 or NRAMP-1 (Ity; for review see Bellamy, 1999; Govoni and Gros, 1998). NRAMP-1 is localised to the phagosome membrane during phagocytosis (Gruenheid *et al.*, 1997) and has been characterised as a pH-dependant Mn^{++} transporter (Jabado *et al.*, 2000). This and other studies have suggested that NRAMP-1 causes a net efflux of Mn^{++} and other ions from the phagosome, thus inactivating bacterial metalloenzymes that are required for bacterial survival in the face of host defences.

1.4.1.2 Humoral immunity

The role of humoral immunity in *Salmonella* infection occurs at several levels, including opsonisation of bacteria, which in turn stimulates Fc mediated activation of macrophages and steric hindrance of surface proteins required for adherence or invasion. Passive transfer of immune serum to naïve mice was shown to be protective in inherently

resistant mice (i.e. $NRAMP^+$), but not in inherently susceptible mice (i.e. $NRAMP^-$; Eisenstein *et al.*, 1984). Transfer of both serum and T cells was required to confer protection against lethal challenge in inherently susceptible mice (Mastroeni *et al.*, 1993).

Experiments by Michetti *et al* (1992) used BALB/c mice injected with a hybridoma expressing *Salmonella*-specific secretory IgA to demonstrate protection against oral but not i.p. challenge with virulent *Salmonella*. Further experiments supported this result, showing that this monoclonal antibody prevented adherence of *Salmonella* to monolayers of polarized Madin-Darby canine kidney (MDCK) cells (Michetti *et al.*, 1994), suggesting that in this case protection was brought about by inhibition of *Salmonella* adherence to target cells.

As *Salmonella* does not live exclusively intracellularly during an infection there are several stages during which opsonisation of bacteria can result in enhanced killing, highlighting the importance of humoral immunity in *Salmonella* infections.

1.4.1.3 Cytokines and T cells

Several cytokines have been shown to be required for *Salmonella* clearance. IL-18 invokes a potent inflammatory response by stimulating the production of IFN-γ, TNFα and IL-1β. These cytokines in turn recruit cells (e.g. neutrophils) to the site of infection and activate macrophages, both of which are important in the early stages of resistance to *Salmonella* infection.

Interferon-γ (IFN-γ) plays an important role in immunity to intracellular pathogens such as *Salmonella* (Benbernou and Nauciel, 1994). One of its main functions is to activate macrophages, resulting in enhanced bacterial killing (Kagaya *et al.*, 1989). The importance of IFN-γ in *Salmonella* infection has been demonstrated by the fact that IFN-γ deficient mice are more susceptible to i.v. challenge with *S.* Typhimurium *aroA* mutant strain than are normal mice (Hess *et al.*, 1996). This result was essentially duplicated when Bao *et al* (2000) examined the susceptibility of IFN-γ deficient mice to oral challenge with a *S.* Typhimurium

aroA/aroD double mutant. The control mice displayed normal signs of sublethal *Salmonella* infection, including mild lesions of spleen, liver, mesenteric lymph nodes and Peyer's Patches. In contrast, the IFN-γ deficient mice displayed severe lesions of the same organs and high mortality. Similar results have been observed using MHC class II and CD4 knockout mice, which are both susceptible to infection with *aro* mutants of *S.* Typhimurium (Hess *et al.*, 1996). Taken together, these results suggest that IFN-γ/T-cell ($CD4^+$, Th1) mediated activation of macrophages is important as a means of *Salmonella* clearance. Interestingly, Hess *et al* (1996) showed no role for $CD8^+$ T-cells in *Salmonella* infection, as CD8 deficient mice were no more susceptible to infection with *S.* Typhimurium *aroA* than were normal mice, when the bacteria were administered intraperitoneally. Conversely, oral administration of *S.* Typhimurium *aroA/aroD* to normal mice resulted in an increase in both intraepithelial and lamina propria $CD8^+$ T-cells (Bao *et al.*, 2000), suggesting a role for $CD8^+$ T-cells in clearance of mucosal *S.* Typhimurium, but not in systemic clearance.

Infection by *Salmonella* and the host immune response is essentially a race for dominance, with the host trying to mount an effective immune response and *Salmonella* trying to adapt to that response. If the host is unable to mount an effective response, which includes all arms of the immune system, it will succumb to an overload of bacteria as mentioned in section 1.4.1. Should the defences be successful, *Salmonella* is cleared and immunity to reinfection is stimulated. The ability of *Salmonella* to use host defence mechanisms for its own purposes reveals the level of adaptation that it has undergone (Rhen *et al.*, 2000). The acquisition of genes that facilitate these adaptations is discussed in section 1.6.

1.5 Molecular Pathogenesis of *Salmonella*

1.5.1 Overview

Salmonella possesses a range of virulence determinants including lipopolysaccharide (LPS), pathogenicity islands, fimbriae, virulence plasmids, toxins and flagella. These will be discussed in the following sections.

The pathogenesis of *Salmonella* is a complex process, involving several genetic loci and molecular cross-talk between the host and invading bacteria. Much of the research into *Salmonella* pathogenesis has been centred on the *S.* Typhimurium/Mouse model of infection. In this model *Salmonella* invades and multiplies within M-cells of the Peyer's patches. Gaining access to this site first requires attachment to the target cell, which is most likely mediated by fimbriae (section 1.4.2.2). Once attachment occurs, *Salmonella* utilises a group of genes collectively referred to as the *inv-spa* locus or SPI-1 (*Salmonella* Pathogenicity Island-1). Expression of these genes provides the mechanism by which *Salmonella* forces its own uptake into the host cell (for review see Brumell *et al.*, 1999). From this point, several other genetic loci are brought into play, allowing both intracellular survival and subsequent bacterial dissemination. The following sections provide an overview of the various *Salmonella* virulence determinants and their role in *Salmonella* pathogenesis.

1.5.1.1 *Salmonella* enterotoxin (Stn)

The gene encoding *Salmonella* enterotoxin (*stn*) was isolated in *S.* Typhimurium by Chary *et al.*, (1993), with the 25 kDa gene product observed to promote fluid accumulation in rabbit ileal loops. Chopra *et al* (1994), found that Stn did not share any overall similarity to other enterotoxins such as cholera toxin. However they did observe a small region of similarity that correlated with an enterotoxin site involved in ADP-ribosylation of host cell proteins, perhaps indicating a conserved region within a family of enterotoxins.

A study by Prager *et al* (1995) examined the distribution of *stn* in 166 Enterobacteriaceal isolates including various *S. enterica* strains, *Shigella* sp, *E. coli*, *Klebsiella pneumoniae* etc. All of the *S. enterica* isolates, but not *S. bongori*, were found possess *stn*, as determined by PCR and gene probing. Of the genotypically *stn* positive isolates, only 45% were phenotypically positive, as indicated by cytotoxicity of cell free supernatants against MDCK cells.

Further studies have been performed in order to assess the function of Stn in *Salmonella* pathogenesis. Reeves-Darby *et al* (1995) found that Stn could induce changes in intestinal motility and substantial fluid production in rabbit ileal loops. Consistent with this result was the observation that, upon oral challenge of mice, the LD_{50} was greater for a *S.* Typhimurium *stn* mutant as compared to the parent strain (Chopra *et al.*, 1999). Complementation of the *stn* mutation restored the virulent phenotype. However, no role for Stn in calves could be found, since the secretory and inflammatory responses induced in bovine ileal loops by *S.* Typhimurium and *S.* Dublin was not significantly altered by introducing an *stn* mutation (Wallis *et al.*, 1999; Watson *et al.*, 1998). Therefore it appears that *stn* plays a host specific role in pathogenesis. Further studies, such as using bovine adapted *Salmonella* isolates in a mouse virulence assay, may confirm the host specific role of Stn.

1.5.1.2 Fimbriae

The ability of bacteria to cause disease first requires them to colonise and occupy a particular niche in their host suited to their individual physiology. Bacteria often achieve this by utilising fimbriae (or pili). The words fimbriae and pili are used interchangeably to describe thin hair-like appendages found on the surface of bacteria. However, the term 'pili' (from the Latin for 'hair' or 'hair-like') is generally reserved for the sex factor appendages (Mol and Oudega, 1996) while fimbriae (from the Latin for 'thread' or 'fibre') encompass the

remaining structures involved in adhesion. In general fimbriae will be used throughout this thesis, except where pili is the commonly used term, e.g. Pap pili.

The study of fimbriae has found that essentially all Gram-negative bacteria that have been examined, as well as several Gram-positive bacteria, possess at least one type of fimbriae (Mol and Oudega, 1996; Krogfelt, 1991). Elucidating the specific role of fimbriae in bacterial pathogenesis has been achieved by the construction of mutations in various fimbrial genes and assessing the impact this has on virulence. For example, uropathogenic *E. coli* have been shown to be significantly less virulent when genes of the Pap pili locus have been mutated (Roberts *et al.*, 1989). The basic role of fimbriae is to facilitate colonisation by 1) targeting bacteria to required growth sites e.g. Peyer's Patches of small intestine and 2) preventing bacteria from being expelled from the host by normal fluid flows within the body eg blood, stomach lumen, mucous membranes etc. This basic role is achieved by a variety of fimbrial types, which vary in their biogenesis, length, inter- and intra-molecular structure, stability and function. The large number of known fimbriae precludes a full review of all fimbrial types, and only those most relevant to this study will be examined in any detail, with comparison between *Salmonella*-specific and non-*Salmonella* fimbriae given where appropriate.

Salmonella sp. are known to possess genes for at least seven different fimbriae or fimbriae-like structures: Type I, Type IV, Curli-like fimbriae, Long polar fimbriae (*lpf*), Plasmid encoded fimbriae (*pef*), *Salmonella* atypical fimbriae (*saf*) and *Salmonella* Enteritidis Fimbriae (*sef*), although not all serovars possess the genes for all fimbrial types. This section outlines the different fimbriae that *Salmonella* is known to express, with respect to either their known or suspected roles in pathogenesis, and similarity to other fimbrial operons (as appropriate).

1.5.1.2.1 Type I Fimbriae

Type I fimbriae are a group of rigid, hollow fibres that are expressed peritrichously on the surface of *E. coli* and *Salmonella*. Type I fimbriae from *S.* Typhimurium were first characterised by Korhonen *et al* (1980) and have been found in all *S. enterica* subspecies but not in *S. bongori* (Boyd and Hartl, 1999). The expression of Type I fimbriae is regulated by phase variation (Eisenstein, 1981), which is discussed further in section 1.5.1.4.1.

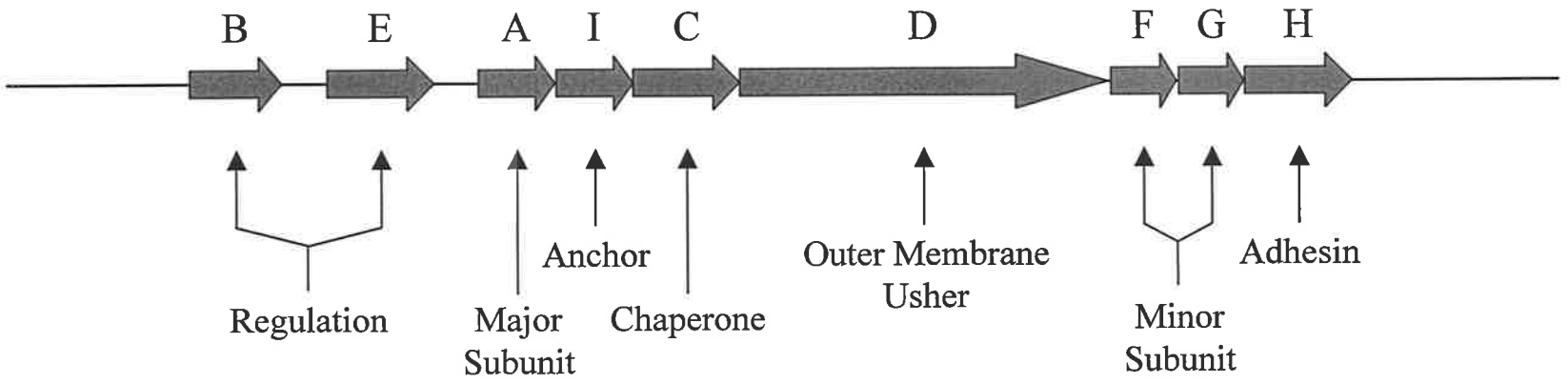
A comparison of the Type I loci from *E. coli* and *S.* Typhimurium reveals that four of the nine ORF's (*fimA*, *fimI*, *fimC*, *fimD*) share similar arrangement and functions. The remaining five genes (*fimB*, *fimE*, *fimF*, *fimG*, *fimH* in *E. coli* and *fimH*, *fimF*, *fimZ*, *fimY*, *FimW* in *Salmonella*) are arranged differently as shown in Figure 1.1.

The major subunit, FimA, forms the helical shaft of the fimbriae, which are 1-2 μm in length and 2-8 nm in diameter (Low *et al.*, 1996). FimC and FimD are the periplasmic chaperone and outer membrane usher respectively and are required for expression of Type I fimbriae (Jones *et al.*, 1993; Klemm and Christiansen, 1990; Klemm, 1992). FimI was first characterised as a fimbrin-like protein (Rossolini *et al.*, 1993) and later shown to be required for expression of Type I fimbriae and adherence of *E. coli* to INT407 cells (Boudeau *et al.*, 2001). The specific role and location of FimI within the fimbriae is not known, although Rossolini *et al.*, (1993) suggested that FimI might be the functional counterpart to PapH, which is involved in cell anchoring and length modulation of Pap pili (Baga *et al.*, 1987). The *E. coli* genes *fimB* and *fimE* are regulatory proteins (Klemm, 1986), as are the *Salmonella* *fimW* and *fimZ* genes (Tinker *et al.*, 2001; Yeh *et al.*, 1995). Interestingly, *fimY* constitutes a third regulator gene (Tinker and Clegg, 2000). FimF and FimG are minor fimbrial subunits, and along with FimH make up the distinctive fibrillar tip (2-3 nm) of Type I fimbriae in *E. coli*, with a 1:100 minor:major subunits ratio (Abraham *et al.*, 1988; Klemm and Christiansen, 1987; Krogfelt and Klemm 1988). FimF and FimG appear to modulate the number and length of expressed type I fimbriae (Russell and Orndorff, 1992). *Salmonella* also possesses *fimF*,

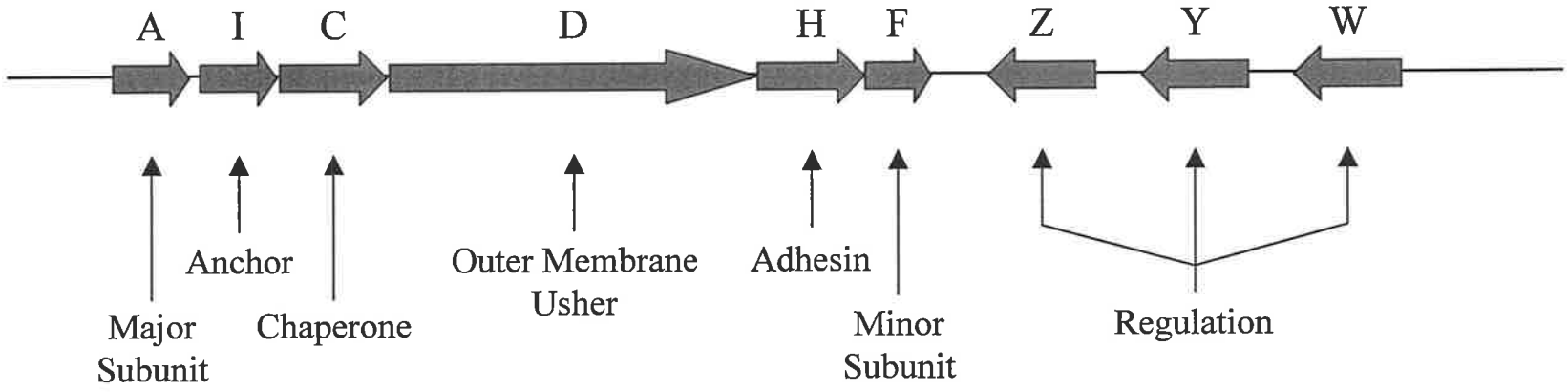
Figure 1.1: *Salmonella* and *E. coli fim* operons.

An arrow depicts each gene in the *fim* operon and their direction of transcription. The functions of the various genes are described in section 1.5.1.2.1. Adapted from Boyd and Hartl (1999).

E. coli



S. Typhimurium



which is assumed to encode a protein with the same function as *E. coli* FimF and FimG i.e. act as an adaptor between the main fimbrial shaft and the tip adhesion (Hancox *et al* 1997). FimH is a mannose binding adhesin; mutants in FimH still produce Type I fimbriae but show a marked decrease in adhesion to epithelial cells (Abraham *et al.*, 1988; Thankavel *et al.*, 1999), while incubation of bacteria expressing Type I fimbriae with D-mannose also results in a reduction in bacterial adhesion (Schaeffer *et al.*, 1980; Avots-Avotins *et al.*, 1981)

The importance of Type I fimbriae in colonisation has been demonstrated both *in vitro* and *in vivo*. Horiuchi *et al* (1992) showed that invasion of HeLa cells by *S.* Typhimurium was reduced in the presence of D-mannose, and that invasion was similarly decreased in *S.* Typhimurium that was unable to express Type I fimbriae. The same *S.* Typhimurium strain was used to demonstrate that other forms of adherence were occurring and that this secondary adherence contributed to the ability of *S.* Typhimurium to invade HeLa cells i.e. bacteria displaying D-mannose resistant adherence were better able to invade than those that displayed no adherence. Isaacson *et al* (1999) showed that a *S.* Typhimurium strain with a mutation in FimA displayed decreased ability to colonise the gut in mice and pigs. The fact that colonisation was not totally abolished is due to the expression of other adherence factors, which probably act in synergy with Type I fimbriae to maximise colonisation.

1.5.1.2.2 Type IV Pili

Type IV pili are expressed by a variety of bacterial species including: Enteropathogenic *E. coli* (EPEC), which expresses bundle forming pili (bfp); Enterotoxigenic *E. coli* (ETEC) expressing long pilus (longus) and colonization factor antigen III (CFA/III); *Neiserria* sp.; *Myxoccocus xanthus*; *Pseudomonas aeruginosa*; and *Vibrio cholerae* expressing toxin co-regulated pilus (TCP) (Darzins, 1994; Giron *et al.*, 1991, 1997; Herrington *et al.*, 1988; Taniguchi *et al.*, 1995; Tønjum and Koomey, 1997; Wu and Kaiser, 1995). Sohel *et al* (1993) showed that some *Salmonella* serotypes also possess gene

homologs of the bundle forming pili of ETEC, and demonstrated expression of these pili on the surface of *S.* Dublin. Type IV pili are divided into two classes, A and B, with Class B Type IV pili associated with human intestinal pathogens such as ETEC, EPEC, and *Vibrio cholerae* (Giron *et al.*, 1994, 1995; Strom and Lory, 1993). The biogenesis of Type IV pili utilises the general secretory pathway rather than specific genes encoded within the pilus operon (see section 1.5.1.3.2).

Stone *et al* (1996) showed that 14 genes of the *bfp* locus were sufficient for expression of bundle-forming pili, while Zhang and Donnenberg (1996) showed that chromosomal gene product DsbA, a periplasmic thiol:disulphide oxidoreductase (for review see Yu and Kroll, 1999) was required for stability of Bfp proteins. The various genes and their putative roles in BFP biogenesis is as follows: *bfpA* encodes the major pilin subunit, called bundlin (Donnenberg *et al.*, 1992); *bfpP* encodes a prepilin peptidase which converting pre-bundlin to its mature form (Zhang *et al.*, 1994), *bfpIJK* encode putative pilin subunits (Sohel *et al.*, 1996); *bfpD* and *bfpF* encode putative ATP-binding proteins (Anantha *et al.*, 1998; Bieber *et al.*, 1998; Donnenberg *et al.*, 1997) and *bfpE* encodes a potential cytoplasmic transmembrane protein (Donnenberg *et al.*, 1997). All of these genes have homologues in other Type IV pilus systems. *bfpG*, *bfpC*, *bfpU*, and *bfpL* do not have sequence homologues in other Type IV pilus systems, however mutations in *bfpG* and *bfpC* inhibit Type IV pili biogenesis (Anantha *et al.* 2000). *bfpB* encodes an outer membrane lipoprotein that is required for BFP biogenesis (Ramer *et al.*, 1996). Finally, *bfpH* is predicted to encode a lytic transglycosylase lipoprotein (Sohel *et al.*, 1996), but mutations in this gene have no effect on the expression of mature Type IV pili (Anantha *et al.* 2000).

The function of Type IV pili involves both adherence to specific target cell types to initiate infection, and also bacterial movement by retraction of attached fimbriae (for review see Wall and Kaiser, 1999; Kaiser, 2000). It has been demonstrated that *S.* Typhi expresses Type IVB pili and that these pili play a role in the invasion of human intestinal epithelial

(INT407) cells (Zhang *et al.*, 2000). Adherence and invasion was not abrogated by the inability to express Type IVB pili, highlighting the redundant nature of fimbriae expression in *Salmonella*.

1.5.1.2.3 Curli

Curli (or thin-aggregative fimbriae) were first described in *E. coli* by Olsen *et al* (1989) and in *Salmonella* by Collinson *et al* (1991), with the major subunits (CsgA and AgfA respectively) showing 74% identity and 86% similarity (Collinson *et al.*, 1991). Curli was found to have short, thin, irregular structures that are highly aggregated, forming an amorphous mat of cross-linked proteins, rather than the distinct fibres that other fimbriae display. Several other genes are required by curli for biogenesis. The *csgB/agfB* genes encode the nucleator protein, while *csgDEFG* encode a transcriptional activator (CsgD) and putative assembly factors (Hammar *et al.*, 1995). The loss of CsgG, an outer membrane lipoprotein, prevents the assembly of curli (Loferer *et al.*, 1997), while the specific roles of CsgE and CsgF are not known. Römling *et al* (1998) observed that the operon structure and regulation of curli in both *E. coli* and *S.* Typhimurium was highly conserved, with 70%-96% amino acid identity between the *csg* and *afg* genes. This high level of sequence identity correlated with the ability of *Salmonella* curli genes to complement mutations in *E. coli csg* genes (Römling *et al.*, 1998).

The assembly of these structures is also distinct from other fimbrial types, utilising the extracellular nucleation-precipitation pathway (section 1.5.1.3.3), with optimal expression of curli occurring at ambient temperatures (Arnqvist *et al.*, 1992; Dibb-Filler *et al.*, 1997). Curli fibres have been shown to mediate binding fibronectin (Olsen *et al.*, 1989; Collinson *et al.*, 1993) and are a major determinant in the formation of bacterial biofilms (Austin *et al.*, 1998; Vidal *et al.*, 1998; Prigent-Combaret *et al.*, 2000) which pose significant health risks in both

the food industry and in hospitals, due to colonisation of 'sterile' surfaces by pathogenic bacteria able to form biofilms (Kumar and Anand, 1998; Sedor and Mulholland, 1999).

1.5.1.2.4 Long Polar Fimbriae

Long polar fimbriae of *Salmonella* were first characterised by Bäumler and Heffron (1995) and, as the name suggests, are expressed in a polar fashion, rather than peritrichously as for other *Salmonella* fimbriae. The arrangement of the *lpfABCDE* genes was observed to be essentially the same as the *S.* Typhimurium *fimAICDHF* genes, with *lpfA* replacing *fimA* and *fimI*. The following identities were observed between the *lpf* and *fim* gene products: LpfA - 37% identical to FimA and 26% identical to FimI; LpfB - 39% identical to FimC; LpfC - 42% identical to FimD; LpfD - 25% identical to FimH and LpfE - 23% identical to FimF. The similar arrangement and high sequence identities suggested that the putative functions of the *lpfABCDE* genes were the same as their respective Fim counterparts (see section 1.5.1.2.1). Unlike the *fim* operon, the *lpf* operon lacked regulatory genes. Expression of long polar fimbriae is regulated by phase variation of the *lpfA* gene (Norris *et al.*, 1998), which is discussed further in section 1.5.1.4.1.

The distribution of *lpf* was assessed by Southern blot using a small DNA probe corresponding to 210 bp within the *lpfD* gene (Bäumler and Heffron, 1995). Under the conditions used, the DNA probe hybridized to *S.* Dublin, *S.* Enteritidis, *S.* Heidelberg and *S.* Typhimurium chromosomal DNA, but not to DNA from *S.* Arizona, *S.* Typhi or several *Shigella* and *E. coli* strains, suggesting that *lpf* might be limited to specific *Salmonella* serovars.

To assess the relative importance of long polar fimbriae in *Salmonella* pathogenesis, Bäumler *et al* (1996a) constructed an *lpfC* mutant, in order to disrupt expression of long polar fimbriae. No difference in virulence was observed when mice were infected i.p. with either the mutant strain or the virulent parent strain. However, mice were infected intragastrically

with the *lpfC* mutant, and 5-fold increase in lethal dose was observed. A 1:1 mutant:parent mixed infection of mouse ileal loops was performed, with less mutant bacteria recovered from Peyer's Patches, and no difference in numbers recovered from villous intestine. The same *lpfC* mutant was unable to adhere to and invade HEp-2 cells (Bäumler *et al.*, 1996b), but showed no effect in the attachment to and invasion of HeLa cells. Combined with the previous data, these results suggested that long polar fimbriae are involved in specific targeting and adhesion of *Salmonella* to cells of the Peyer's Patches.

1.5.1.2.5 Plasmid Encoded Fimbriae

Plasmid encoded fimbriae (*pef*) were first described by Friedrich *et al* (1993), with the *pef* genes located on a 7 kb fragment of the 90 kb *S.* Typhimurium virulence plasmid. Function for the PefA, PefB, PefC, PefD and PefI gene products (major fimbrial subunit, transcriptional regulator, outer membrane usher, periplasmic chaperone and negative regulator respectively) were assigned based on similarity to genes from other fimbrial operons including Pap pili, K88, K99 and S fimbriae (Friedrich *et al.*, 1993). Further studies have confirmed the function of PefI as a negative regulator (Nicholson and Low, 2000).

Southern hybridisation analysis of a range of *Salmonella* serovars revealed that the distribution of *pef* genes in *Salmonella* is restricted to two distantly related evolutionary lineages; the first including *S.* Enteritidis and *S.* Cholerasuis and the second including *S.* Typhimurium (Bäumler *et al.*, 1997a). This result was in contrast to the much wider distribution of the *lpf* operon (section 1.5.1.2.4), suggesting that acquisition of the *pef* operon was a relatively recent event. They also proposed that as *S.* Cholerasuis and *S.* Typhimurium are the most common *Salmonella* serovars isolated from swine (Schwartz, 1991), acquisition of *pef* fimbriae may have allowed *Salmonella* to exploit pigs as a new host. Interestingly, Woodward *et al*, (1996) showed that sera from chickens infected with *S.* Enteritidis was able to detect purified *pef* fimbriae by Western Blot. This indicated that *S.* Enteritidis expressed

pef fimbriae at some stage during infection. Whether *S.* Enteritidis obtained the *pef* operon from *S.* Cholerasuis, *S.* Typhimurium or from a third source remains to be determined.

Virulence studies were performed using an infant mouse model of infection to assess the role of *pef* fimbriae in *Salmonella* pathogenesis (Bäumler *et al.*, 1996c). A *S.* Typhimurium *pefC* mutant, which was unable to express *pef* fimbriae, caused significantly less fluid accumulation than the parent strain in mouse ileal loops, indicating that *pef* might play some role in pathogenesis. However, no difference in lethal dose was observed when infant mice were inoculated intragastically with either the *pefC* mutant or the parent *S.* Typhimurium. Also, expression of *pef* in *E. coli* did not confer the ability to cause fluid accumulation. Taken together these results suggest that *pef* is required for, but does not actually cause, fluid accumulation.

1.5.1.2.6 Other *Salmonella* Fimbriae

Salmonella possesses at least three other fimbriae, *saf*, *sef* and *stf*, which have either not been as extensively studied as other fimbriae (*stf*, *saf*) or show a more discreet distribution (*sef*).

1.5.1.2.6.1 *Salmonella* atypical fimbriae (*saf*)

saf, or *Salmonella* atypical fimbriae, were discovered by Folkesson *et al* (1999), and are located in a region of DNA that was present in *Salmonella* but not found in *E. coli*. Putative functions for the *safABCD* genes (fimbrial subunit, chaperone, outer membrane usher and fimbrial subunit respectively) were assigned on the basis of predicted amino acid sequence homology with known fimbrial gene products. SafA showed some weak sequence similarity to the Bor protein, which has been implicated in serum resistance (Barondess and Beckwith, 1990). SafB and SafC showed much stronger similarities to perplasmic chaperones and outer membrane ushers respectively, with nearly 50% identity over their entire sequence.

SafD was found to have 30% overall identity to both the AfaD and SefD fimbrial proteins (see section 1.9.1.4).

Folkesson *et al* (1999) also examined the distribution of the *safABC* genes in 198 isolates covering 55 different serovars. The *safB* and *safC* genes were found in all subspecies I *Salmonella* except *S.* Mbandaka, *S.* Rissen and *S.* Worthington and were not present in the single subspecies III isolate examined. Interestingly, *safA* was found in only 58% of the isolates. An examination of *S.* Typhi (*safA* negative) and *S.* Typhimurium (*safA* positive) *saf* sequences found that the *safB* and *safC* genes were 90 and 97% percent identical respectively, while the *safA* gene was only 44% identical. It was proposed that the lack of *safA* in some serovars was due to allelic variation rather than the absence of the gene.

There is no published evidence for either the expression of *saf* fimbriae or their role in *Salmonella* pathogenesis. However, unpublished observations by Folkesson *et al* (1999) showed that a mutation in *safA*, which would presumably prevent *saf* fimbrial expression, had no effect on virulence when mice were infected either orally or intra-peritoneally.

1.5.1.2.6.2 *Salmonella* Enteritidis fimbriae (*sef*)

The label *sef* fimbria has routinely been used to describe four different fimbriae in *S.* Enteritidis i.e. SEF14, SEF17, SEF18 and SEF21, with the numbers relating to the molecular weight of the major fimbrial subunit. These fimbriae are examined in detail in section 1.8.1.

1.5.1.2.6.3 *Salmonella* Typhimurium Fimbriae (*stf*)

Two independent studies identified a novel fimbrial operon in *S.* Typhimurium that was absent from *S.* Typhi (Emmerth *et al.*, 1999; Morrow *et al.*, 1999). The *stf* operon (*stfACDEFG* and *orf1*) showed an almost identical genetic organisation to the uncharacterised *yfc* operon from *E. coli* K-12, with predicted protein similarities ranging from 37% for *stfA/yfcV* to 64% for *stfD/yfcS*. Similarities were also observed between the *stf* genes and the

E. coli Pap, *S.* Typhimurium *fim* and *S.* Typhimurium *lpf* genes, which suggested that the *stf* operon encodes a novel Type I fimbriae. No evidence has been published showing either surface expression of *stf* or their role in *Salmonella* pathogenesis.

1.5.1.3 Models of Fimbrial biogenesis

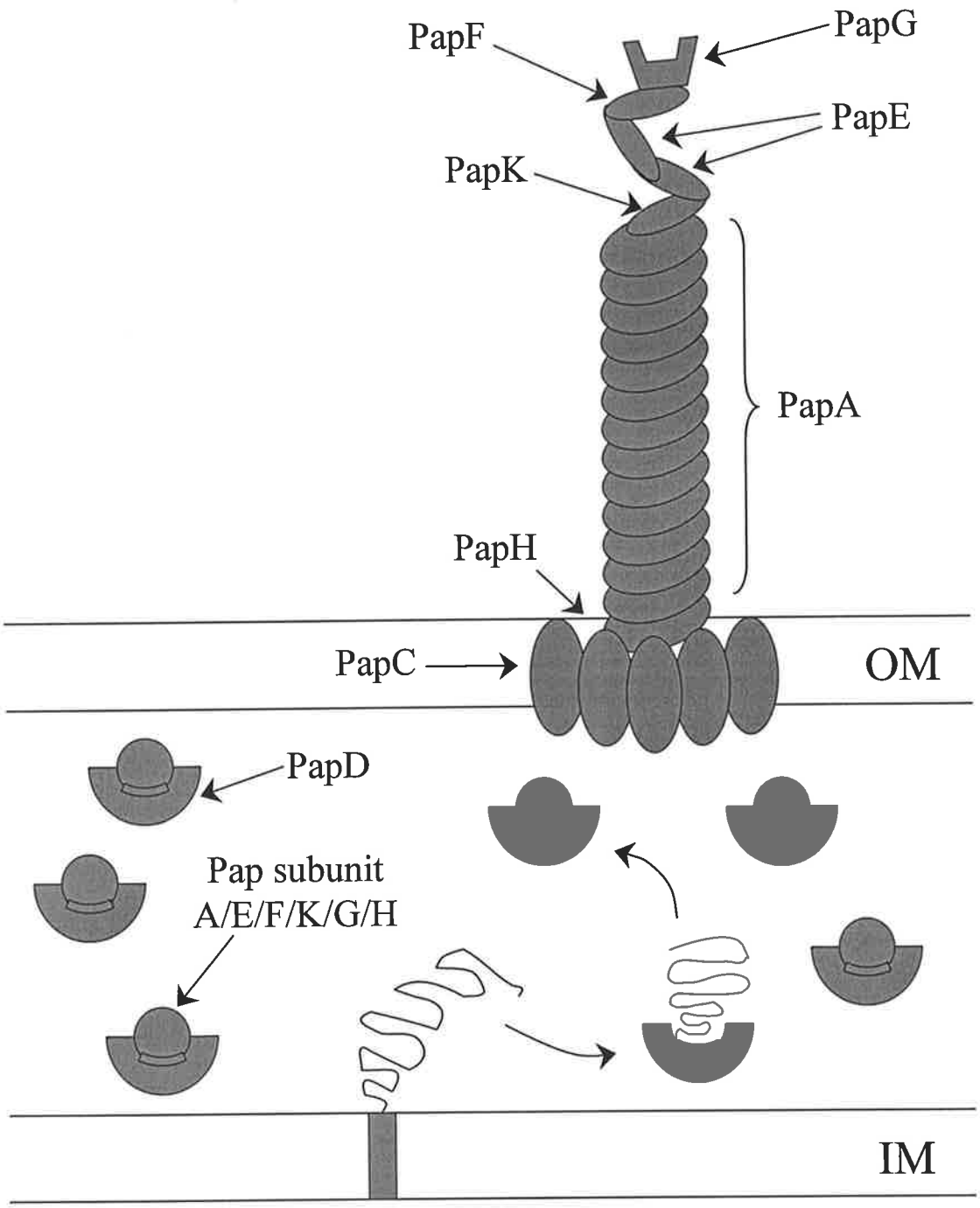
Fimbriae found in Enterobacteriaceae were previously categorised into four groups: Type I, II, III and others (for review see Clegg and Gerlach, 1987). Since then these groups have been refined as more detailed genetic and morphological studies have been performed. In a review by Soto and Hultgren (1999), fimbriae were grouped on the basis of similarities in assembly pathway, of which there were four main variants: the Chaperone usher pathway, which itself was further subdivided into: thick, rigid pili (e.g. Type 1 pili); thin, flexible pili (e.g. K88 pili), and atypical structures (e.g. aggregative adherence fimbriae I); the extracellular nucleation pathway (e.g. Curli); the general secretion pathway (e.g. Type IV pili); and the alternate chaperone pathway (e.g. CS1 pili). A specific example of each group is examined in more detail below.

1.5.1.3.1 Chaperone-usher pathway

The Chaperone-usher pathway constitutes the largest and best understood mechanism for fimbriae biogenesis. Type I fimbriae use this pathway (for review see Knight *et al.*, 2000; Schilling *et al.*, 2001), however the model system for this mechanism is the biogenesis of Pap pili, which has been extensively studied (for review see Sauer *et al.*, 2000a; Sauer *et al.*, 2000b; Thanassi *et al.*, 1998; Thanassi and Hultgren, 2000), and a schematic representation is shown in Figure 1.2. Essentially, the model involves binding of the pilus subunits (PapA, PapE, PapF, PapG, PapH and PapK; major pilin, minor pilin, adaptor pilin, adhesin, anchor and adaptor pilin respectively) by the chaperone (PapD), which delivers them to the outer membrane usher (papC), where polymerisation of the pilus occurs. PapI and PapB are

Figure 1.2: Chaperone-usher model of fimbrial biogenesis

Pap pili are assembled via the chaperone usher pathway. The Pap monomers are secreted into the periplasm and immediately bound by the chaperone PapD. The subunit-chaperone complex is delivered to the PapC usher complex and the pili are gradually formed by sequential addition of subunits. Biogenesis is terminated by addition of the anchor protein, PapH. IM = inner membrane and OM = outer membrane. Adapted from Soto and Hultgren (1999).


PapF
PapG
PapE
PapK
PapA
PapH
PapC
OM
PapD
Pap subunit
A/E/F/K/G/H
IM

involved in regulation of Pap pili expression (section 1.5.1.4.1) while PapJ is thought to be a second chaperone (Tennet *et al.*, 1990) but this has not been confirmed.

The effect of mutations in various pap genes has helped to reveal the function of individual Pap proteins in Pap pili biogenesis, and shown the critical role of PapD. *papD* null mutants do not express Pap pili due to the rapid degradation of PapA, PapE, PapF and PapG (Lindberg *et al.*, 1989), thus helping to confirm its role as a chaperone, which stabilises the pilin subunits in the periplasm. PapD is also required for the complete translocation of nascent pilin subunits into the periplasm (Jones *et al.*, 1997), which occurs in conjunction with an initial sec-dependant translocation step. PapD maintains the pilin subunits in a competent state via a β-zipper interaction between the G1 β-strand of PapD and a conserved C-terminal domain in the pilin subunit, with point mutations in invariant residues of the G1 β-strand abolishing the ability of PapD to form complexes with pilin subunits (Kuehn *et al.*, 1993; Soto *et al.*, 1998).

The *papC* gene was characterised by Norgren *et al* (1987), identifying it as encoding a possible transmembrane channel required for Pap pili expression. Studies by Dodson *et al* (1993) showed that complexes between PapD and the minor subunits PapE, PapF and PapG all bound to PapC with affinities that correlated with their position within the tip fibrillum. Conversely PapD complexes with PapA or PapK did not bind PapC. Further work by Saulino *et al*, (1998) revealed that the PapD-PapA complex did associate with PapC, but with a 16-fold lower affinity. These studies highlight the mechanism by which pilus subunits are assembled in the correct order.

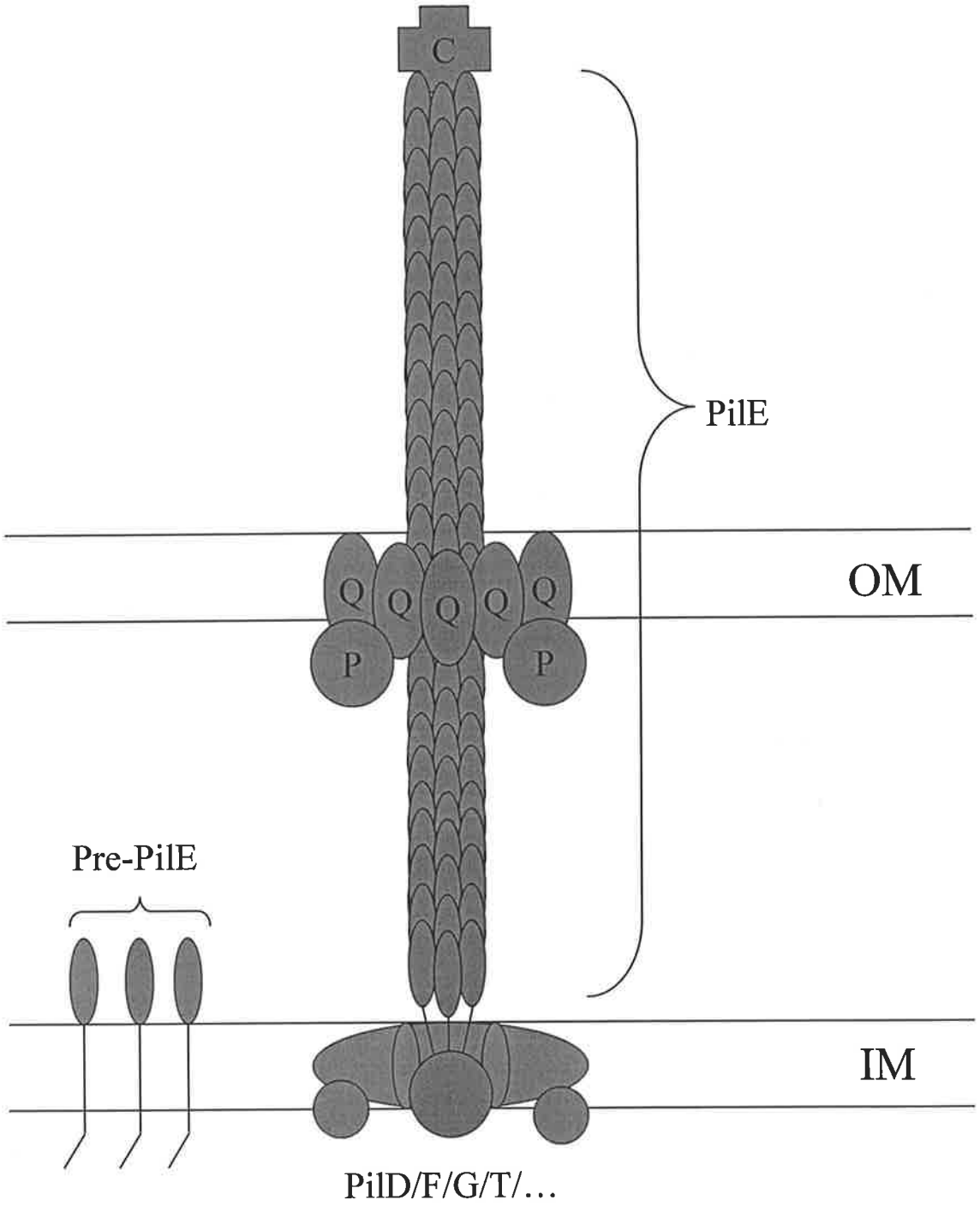
Local concentration of pilin subunits also has some effect on the composition of Pap pili. Over expression of PapK led to pili with shorter tips (Jacob-Dubuisson *et al.*, 1993) while overexpression of PapH led to shorter pili (Baga *et al.*, 1987). Thus there is a complex interplay between the chaperone, OM usher and the individual subunits that leads to the correct order and composition of Pap pili.

1.5.1.3.2 General Secretion Pathway (GSP)

Type IV pili are constructed utilising a mechanism that evolutionarily and functionally related to the main terminal branch of the general secretion pathway (see Figure 1.3; Pugsley, 1993). The example used here is based on the *pil* genes of *N. gonorrhoeae* (for review see Fernández and Berenguer, 2000), which are similar to the *bfp* genes described previously (section 1.5.1.2.2). The main difference between the biogenesis of Type IV pili and fimbriae produced by the chaperone-usher pathway is that Type IV assembly occurs in the absence of a chaperone, with assembly occurring via an inner membrane (IM) complex composed of the PilF, PilG and PilT proteins. It is not known whether other proteins are involved in this IM complex. PilF is thought to be either a kinase or ATPase that energises the polymerisation of the pili (Turner *et al.*, 1993). PilG has been proposed to stabilise and/or localise either PilD or PilF (Tønjum and Koomey, 1997) while PilT is a nucleotide binding protein that is thought to play a role in twitching motility (Wolfgang *et al.*, 1998). The pilus shaft is composed of PilE subunits, which are cleaved by the signal prepillin peptidase PilD (Lauer *et al.*, 1993), prior to incorporation into the pilus shaft. The adhesin, PilC is required for production of mature pili (Jonsson *et al.*, 1991; Rudel *et al.*, 1995), and is thought to target the growing pilus to the PilQ/PilP outer membrane complex. PilQ forms the OM pore by which the pili are exported to the surface (Drake and Koomey, 1995), while the lipoprotein PilP is thought to maintain the stability of this pore (Drake *et al.*, 1997). Another important difference between fimbriae produced by the general secretion pathway and the chaperone-usher pathway is that Type IV pili are produced in an energy dependant manner, and are anchored in the inner membrane while Type I and other chaperone-usher pathway assembled fimbriae are produced in an energy independent manner i.e. thermodynamically driven (Jacob-Dubuisson *et al.*, 1994) and are anchored in the outer membrane.

Figure 1.3: Fimbrial biogenesis via the general secretion pathway

Type IV pili are assembled via a modification of the general secretion pathway. An inner membrane complex composed of PilD, PilF, PilG, PilT and possibly other proteins provides the starting point for PilC, the tip located adhesin. Following PilC are multiple subunits of PilE. Pre-PilE is cleaved by PilD before being added to the pilus shaft. The growing pilus is guided and stabilised through the outer membrane by PilP and PilQ. This pathway of biogenesis allows for both extension and retraction of the pilus shaft, providing bacterial motility. IM = inner membrane and OM = outer membrane. Adapted from Soto and Hultgren (1999).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1.5.1.3.3 Extracellular nucleation-precipitation (ENP) pathway

Biogenesis of fimbriae by the extracellular nucleation-precipitation pathway is quite random compared with either Type I Fimbriae or Type IV pili, with Curli being the model system for this pathway (see Figure 1.4). Essentially the major fimbrin, CsgA, is secreted in a soluble form across the outer membrane, which is mediated by the outer membrane lipoprotein CsgG (Loferer *et al.*, 1997). CsgB is also secreted by CsgG, and serves as the nucleator, i.e. it provides a point for the CsgA subunit to precipitate (Bian and Normark, 1997, Hammar *et al.*, 1996), forming fimbriae by the addition of additional subunits to the distal rather than the proximal end of the fimbriae, the opposite of Chaperone-usher biogenesis. CsgB is also found distributed randomly throughout the fimbriae, and may serve to either introduce branching or cross linking in the fimbriae (Bian and Normark, 1997).

1.5.1.3.4 Alternate Chaperone pathway.

The mechanics of the alternate chaperone pathway (Figure 1.5) are essentially the same as the chaperone-usher pathway (section 1.5.1.3.1), however there is little or no sequence similarity between the chaperones from these two groups (for review see Sakellaris and Scott, 1998). The CS1 operon is comprised of four genes, *cooBACD*, encoding for the periplasmic chaperone, major fimbrial subunit, OM usher and minor subunit respectively. The chaperone (CooB) again appears to play the pivotal role in pili biogenesis, and has been shown to bind to the structural subunits CooA and CooD as well as the outer membrane protein CooC (Sakellaris *et al.*, 1996; Voegele *et al.*, 1997). A model for CS1 pili biogenesis was proposed by Sakellaris and Scott (1998). Each of the Coo proteins is translocated across the inner membrane via the Sec-pathway. CooB binds and stabilises the CooA, CooC and CooD proteins and the CooC-CooB complex localises to the outer membrane. The transfer of CooD from a CooB-CooD complex to the CooC-CooB complex initiates pili biogenesis. Pili

Figure 1.4: Extracellular nucleation-precipitation pathway

Curli fibres/thin aggregative fimbriae are assembled by this method. The major subunit, CsgA (circles), is secreted across the outer membrane, when surface located CsgB (squares) initiates polymerisation. CsgB also initiates fibre branching (open arrow). CsgG is required for the secretion of CsgA and CsgB. The requirement for other proteins (depicted by a question mark) is not yet known. IM = inner membrane and OM = outer membrane. Adapted from Soto and Hultgren (1999).

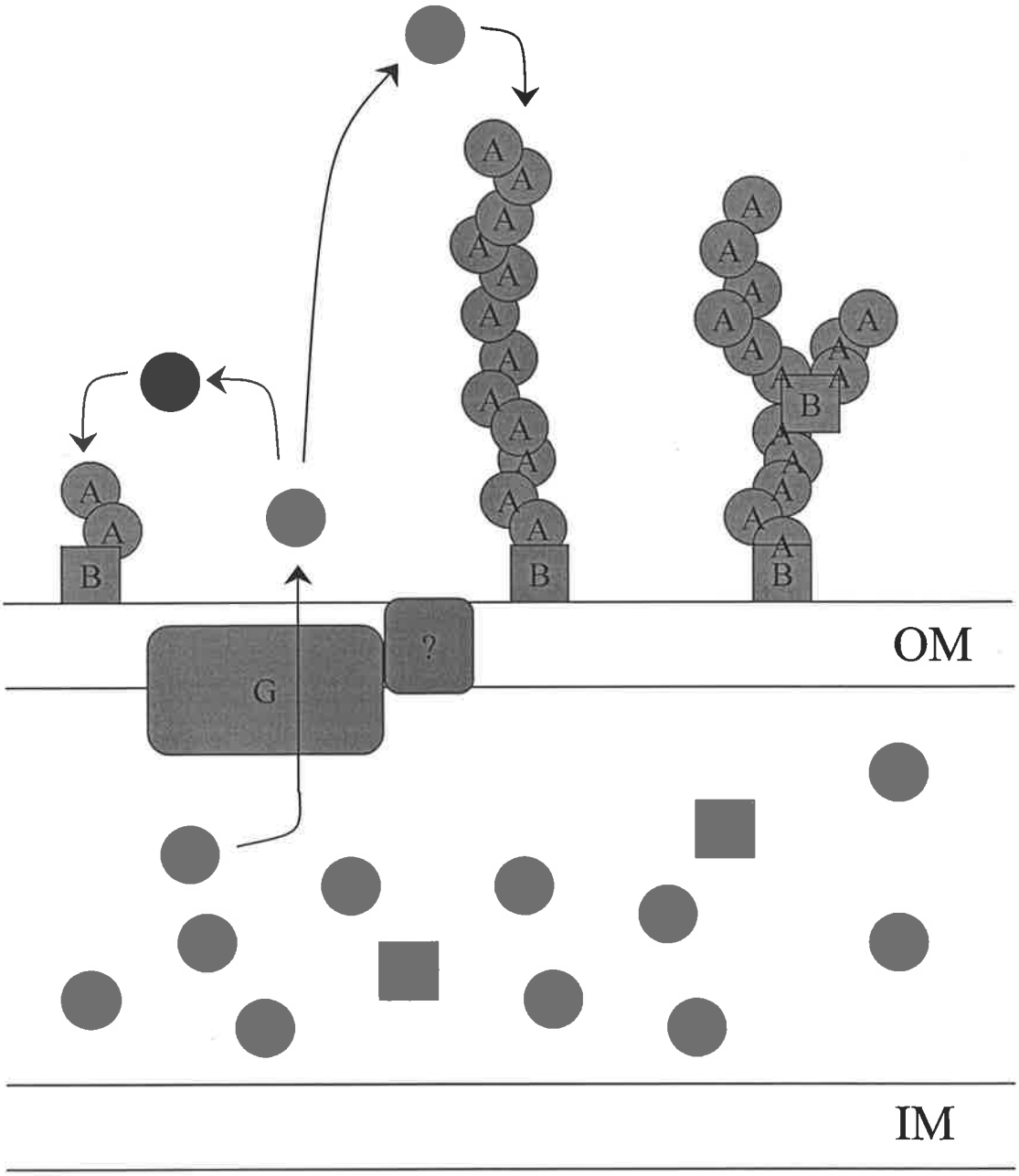
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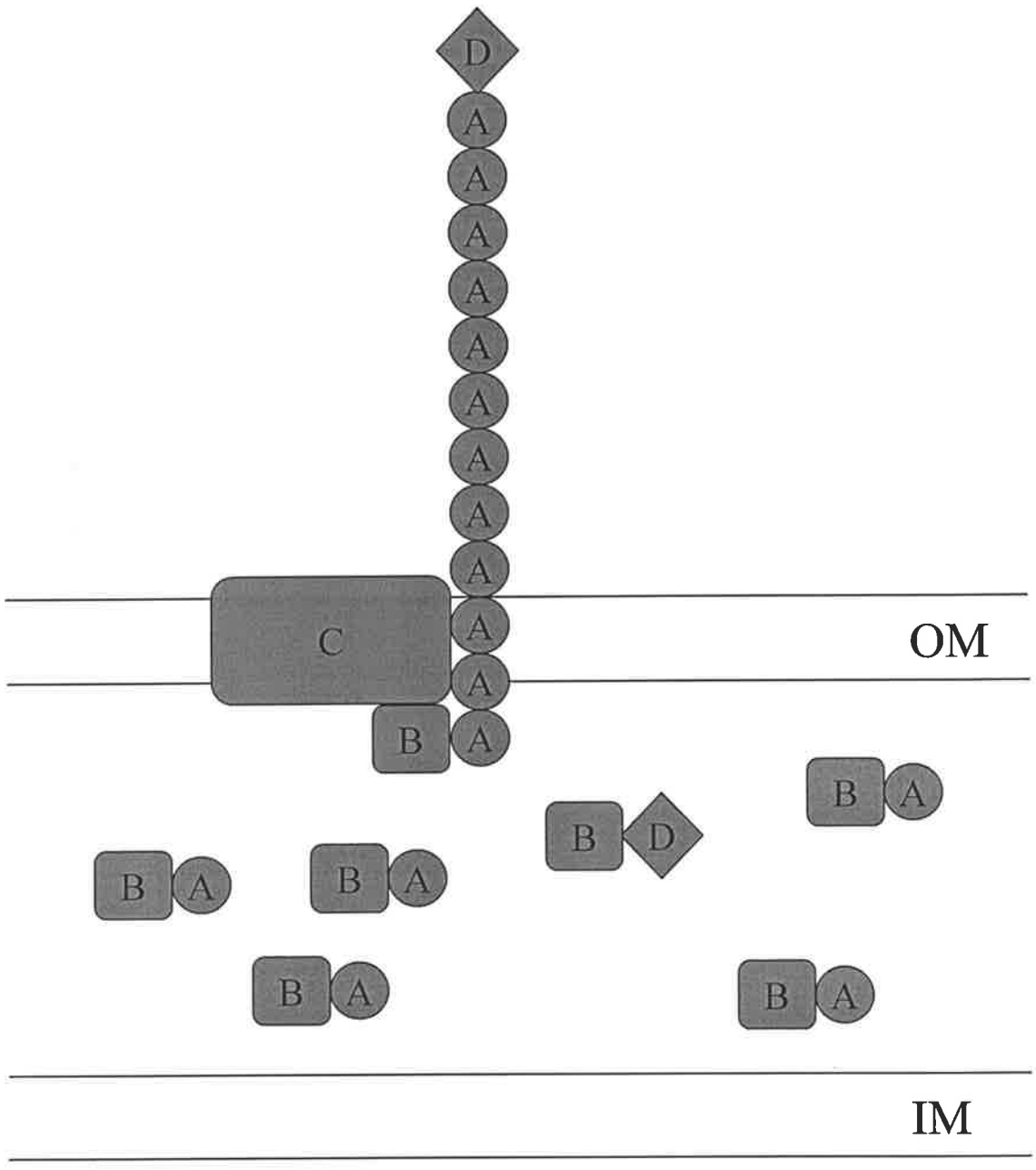
Figure 1.5: Alternate chaperone pathway.

This pathway is used for assembly of CS1 pili. It is similar to the Chaperone usher pathway. The fimbrial subunits, CooA and CooD are bound by the chaperone CooB. The subunit chaperone complex binds the outer membrane usher CooC, adding a further subunit to the growing fimbria. CooD is tip located and is required for initiation of fimbrial biogenesis. CooB is thought to bind and stabiles CooC when subunits are not being added. IM = inner membrane and OM = outer membrane. Adapted from Soto and Hultgren (1999).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are elongated by the repeated addition of CooA subunits to the growing pilus. The signal that terminates elongation is not yet known.

CS1 and related pili are primarily expressed by ETEC strains, however a putative homolog has been described in *S.* Typhi, designated the Typhi colonising factor (*tcf*) operon (Folkesson *et al.*, 1999). The predicted Tcf amino acid sequences displayed 31-38% identity to the Coo gene products, however *tcf* fimbriae expression has not yet been demonstrated.

1.5.1.4 Regulation of fimbrial expression

The regulation of fimbriae is in most cases tightly controlled, as fimbriae are only required at an early stage in pathogenesis in order to facilitate colonisation/invasion. Consequently, fimbrial expression is up-regulated in response to a particular set of environmental signals corresponding to the specific site where the bacteria will colonise/invade their host, and down regulated when they are no longer needed. This type of regulation prevents energy being wasted on the expression of fimbriae that are not needed. Environmental stimuli such as temperature, pH, osmolarity, local Ca^{++} concentration and oxygen tension (Göransson *et al.*, 1990; Hoe *et al.*, 1992; Lambert de Rouvroit *et al.*, 1992; Mekalanos, 1992; Edwards and Puente, 1998; Liles *et al.*, 1998; White-Ziegler *et al.*, 1998) provide the signals that ultimately translate, via the action of global regulators such as the histone-like protein H-NS, into up- or down- regulation of fimbriae expression. This section provides a brief overview of regulation of fimbrial expression, with emphasis on the interplay between H-NS and transcriptional regulators of fimbrial expression.

1.5.1.4.1 Phase Variation

Phase variation is the switching of expression (of fimbriae or other genes) from the phase ON to the phase OFF state and vice versa. At any one time, a sub-population of bacteria will be expressing virulence factors that are under phase-variable control, thus

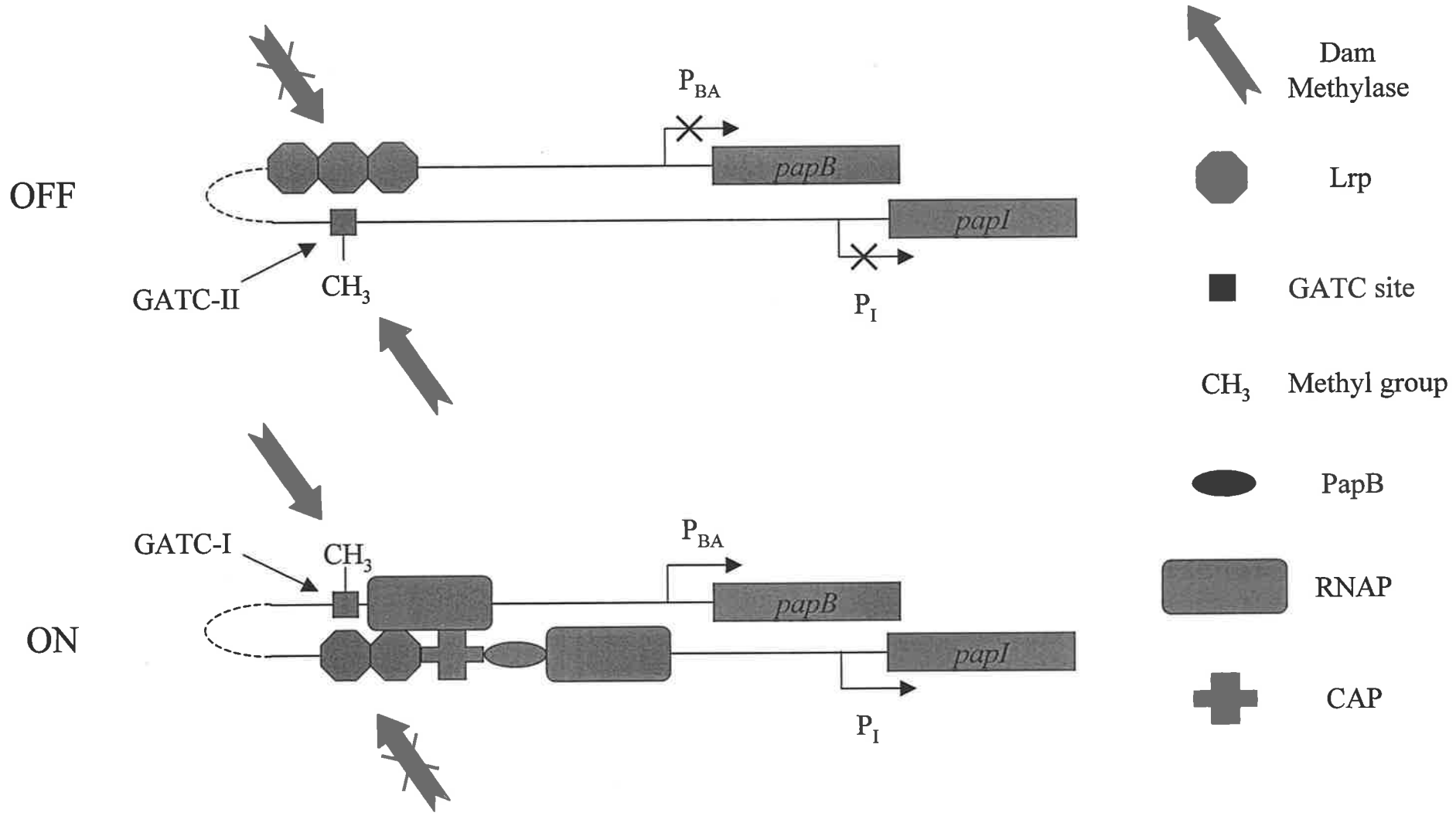
ensuring that at least some bacteria are able to respond to a change in environmental conditions. Phase variation occurs by four distinct mechanisms i.e. site specific recombination (Swansson and Koomey, 1989), general recombination or gene conversion (Swanson *et al.*, 1986), alteration of DNA methylation states (Braaten *et al.*, 1991; van der Woude *et al.*, 1992), and slipped strand mispairing within short repeats (Jonsson *et al.*, 1992; Willems *et al.*, 1990).

Several fimbriae of the Type I family are regulated by phase variation e.g. Pap pili, Type I fimbriae of *E. coli* and *Salmonella*, long polar fimbriae and plasmid encoded fimbriae (Norris *et al.*, 1998; van der Woude *et al.*, 1992; Nicholson and Low, 2000; Harel *et al.*, 2000; Long *et al.*, 1998). Pap pili are regulated by the alteration of DNA methylation sites (see Figure 1.6; van der Woude *et al.*, 1996). The global regulator Lrp (leucine responsive regulatory protein) is able to bind two areas with the regulatory region of the pap operon; GATC-I or GATC-II, acting as both a repressor and activator of Pap pili expression (van der Woude *et al.*, 1995). In Phase OFF, Lrp is bound to GATC-I, and GATC-II is methylated by DNA adenine methylase (Dam). This methylation prevents the binding of the Lrp-PapI complex to GACT-II, which is required for Pap pili expression. In Phase ON, the Lrp-PapI complex is bound to GATC-II, while GATC-I is methylated. In conjunction with catabolite gene activator protein (CAP; Weyand *et al.*, 2001), this leads to expression of Pap pili.

A study by Norris and Bäumler (1999) proposed that phase variation of long polar fimbriae might be a mechanism for evasion of cross-immunity between *Salmonella* serotypes. They showed that immunisation with *S.* Typhimurium *lpf* phase on bacteria elicited an immune response that selected against *S.* Enteritidis *lpf* phase on bacteria, yet the mice were not protected from challenge with *S.* Enteritidis, since the *lpf* phase off cells were able to evade cross-immunity.

Figure 1.6: Regulation of PapB and PapI expression by Phase variation.

Schematic representation of Pap regulatory region in phase OFF and phase ON configurations. Methylation of GATC-II by Dam methylase results in binding of Lrp to GATC-I, preventing expression from PapB and PapI (promoters P_{BA} and P_I respectively). Methylation of GATC-I allows binding of Lrp to GATC-II, recruitment of CAP, PapB and RNAP, leading to expression of both PapB and PapI. Adapted from van der Woude *et al* (1996).


OFF
ON
P_{BA}
papB
papI
P_I
GATC-II
CH_3
GATC-I
Dam Methylase
Lrp
GATC site
CH_3 Methyl group
PapB
RNAP
CAP

1.5.1.4.2 Protein-DNA interactions

Regardless of the signal that induces or represses fimbrial expression, the regulatory effect is brought about by protein-DNA and/or protein-protein interactions (or lack thereof) i.e. Rns from *Escherichia coli* represents a DNA binding protein that is required for the upregulation of CS1 pili (Caron *et al.*, 1989; Froehlich *et al.*, 1994a), while FimW represents a protein that interacts with FimY to negatively regulate Type I fimbrial expression (Tinker *et al.*, 2001).

There are a growing number of fimbriae that are recognised as being under the control of transcriptional regulators that belong to the AraC/XylS family of transcriptional regulators. There are more than 70 regulators of known function and a further 37 putative regulators of unknown function which also belong to this family (Gallegos *et al.*, 1997). They are characterised by significant amino acid homology within a 100 aa stretch of the protein containing a helix-turn-helix (H-T-H) DNA binding domain. Of those regulators with known function seven (AfrR, AggR, CfaD, CsvR, FapR and Rns, from various pathogenic *E. coli* and TcpN from *Vibrio cholerae*) have a direct regulatory effect on fimbriae production, and five others (InvF from *Salmonella typhimurium*, LcrF from *Yersinia pestis*, MxiE from *Shigella flexneri*, VirF from *S. dysentariae, S. flexneri, S. sonnei* and *Yersinia enterocolitica*) are involved in regulating operons directly involved in the pathogenesis of these organisms (Gallegos *et al.*, 1997).

1.5.1.4.2.1 Mechanism of action of AraC protein

AraC is the type protein of the AraC/XylS family and represents the most extensively studied member of this family. AraC comprises two functionally distinct domains, as shown by protein chimera studies (Bustos & Schleif, 1993). These domains are connected by a flexible linker region (Eustance *et al.*, 1994), which allows for the modular organisation of this protein. AraC effects DNA as a dimeric molecule, stabilised via binding of the N-

terminal regions (first 170 amino acids). The C-terminal end of the protein facilitates binding to specific regions of DNA via a well-characterised helix-turn-helix motif (Eustance *et al.*, 1994; Menon & Lee, 1990), and comprises the minimal activating portion of AraC. The crystal structure of AraC in complex with D-fucose has been determined (Soisson *et al.*, 1997), allowing insights into how AraC binds sugars. Crystal structures of AraC-like proteins MarA (Rhee *et al.*, 1998) and Rob (Kwon *et al.*, 2000) in complex with cognate DNA-binding sites revealed that although they belong to the same family, MarA and Rob bind DNA in different ways. Thus, care must be taken when making assumptions about regulators that show amino acid similarity to AraC-like regulators but may not have the same mechanisms of action.

A model representing the mechanism of action of the AraC protein was first proposed by Lobell & Schleif (1990), and modified by Niland *et al* (1996), which is represented in Figure 1.7. This model involves three regions of DNA (*ara*I_1, *ara*I_2 and *ara*O_2) within the P_{BAD} promoter region. In the normal inactive state, AraC binds the *ara*I_1 and *ara*O_2 sites, forcing a bend in the DNA, which inhibits transcription. Addition of arabinose and its subsequent binding to AraC protein induces a conformational change, which in turn induces a shift in the arrangement of the AraC dimer. This allows binding to the *ara*I_1 and *ara*I_2 sites, releasing the DNA bend and allowing transcription to proceed.

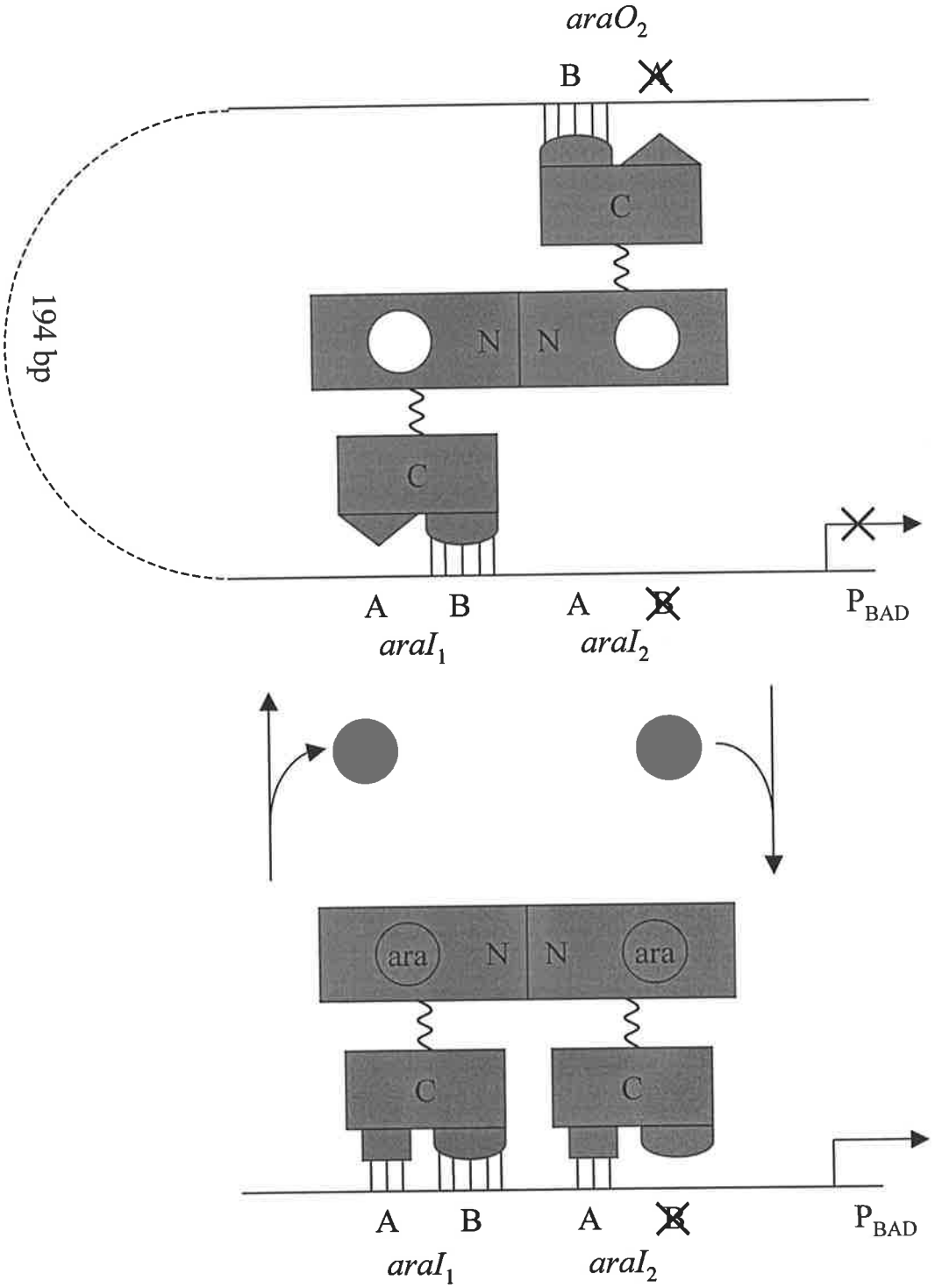
1.5.1.4.2.2 Role of H-NS in the repression of fimbrial expression

The histone-like-protein H-NS is a DNA binding protein that, when mutated, has pleiotropic effects on bacteria due to alterations in DNA topology (Hulton *et al.*, 1990; Hinton *et al.*, 1992). Due to the interplay between H-NS and DNA binding, H-NS has been implicated in regulating a wide range of bacterial processes.

H-NS alters DNA supercoiling in response to changes in both extracellular osmolarity (Harrison *et al.*, 1994) and temperature (Göransson *et al.*, 1990, White-Ziegler *et al.*, 1998).

Figure 1.7: Model of AraC binding of DNA

This figure is a representation of the model of Niland *et al* (1996) and is explained in section 1.5.1.4.2.1. AraC binds within the operator region of the *araBAD* promoter (P_{BAD}) at the $araI_1$, $araI_2$ and $araO_2$ recognition sequences. These sequences are made up of two short 'boxes', designated the A box and B box. Functional AraC is a dimeric molecule, with dimerisation occurring within the N-terminal region (N). The C-terminal region is separated from the N-terminal region by a flexible linker (wavy line), and in the absence of arabinose (ara), binds the AraC operator region at the B boxes of the $araI_1$ and $araO_2$ sites, with no apparent interaction with the A boxes. This pattern of binding (upper diagram) induces a bend in the DNA, which inhibits the binding of RNA polymerase. Thus no expression occurs from P_{BAD}, as represented by the crossed out arrow. When AraC binds arabinose (lower diagram), a conformational shift (triangle to squares) in the protein occurs. The interaction with the $araO_2$ B box is weakened, while interactions with the A boxes of $araI_1$ and $araI_2$ is strengthened. This change in conformation promotes RNA polymerase binding and expression from the P_{BAD} promoter. There is no apparent interaction of AraC with the $araO_2$ A box or the $araI_2$ B box (crossed out A and B respectively).


$araO_2$
B
C
194 bp
N
N
C
A
B
A
P_{BAD}
$araI_1$
$araI_2$
ara
N
N
ara
C
C
A
B
A
P_{BAD}
$araI_1$
$araI_2$

The alterations in DNA supercoiling can affect the binding of other proteins to the DNA, which in turn provides a regulatory mechanism for the expression of salt and/or temperature dependant virulence factors. An example of this is the temperature dependant regulation of Pap pili, which are expressed at 37°C but not at 26°C (Göransson *et al.*, 1990). H-NS has been shown to bind and obscure methylation sites in the Pap regulatory region at 23°C but not at 37°C (White-Ziegler *et al.*, 1998), thus inhibiting expression at the lower temperature.

H-NS has also been implicated in the negative regulation of other fimbriae. Nicholson and Low (2000) observed a 3-fold increase in *pefA* transcript in an *hns* mutant, indicating that H-NS acts as a repressor of *pefA* transcription. CFA/1 (Jordi *et al.*, 1992 - upregulated by CfaD), CS1 (Murphree *et al.*, 1997 - upregulated by Rns), Type 1 fimbriae (Dorman and Bhriain, 1992) and F1845 (White-Ziegler *et al.*, 2000) are also repressed by the action of H-NS. H-NS is antagonistic to non-fimbrial activator proteins including, AppY, which activates operons involved in energy metabolism (Atlung *et al.*, 1996) and VirB and VirF of Shigella and EIEC (Berlutti *et al.*, 1998; Tobe *et al.*, 1993; Dorman and Porter, 1998), which are involved in a virulence regulatory cascade.

A study by Harrison *et al* (1994) revealed that H-NS has a role in the virulence of *S.* Typhimurium. A mutation in *hns* resulted in an attenuated strain with altered colony morphology and increased DNA supercoiling. O' Byrne and Dorman (1994) showed that a mutation in *hns* resulted in enhanced transcription of the *spvR* regulatory gene (section 1.5.5).

1.5.2 *Salmonella* Pathogenicity Islands (SPI) and effector proteins

Pathogenicity islands (PAI's) have several defining characteristics, as outlined by Hacker *et al* (1997)

i. Carriage of virulence genes

ii. Presence in pathogenic strains; absence in less pathogenic strains of same or related species

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

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

v. Distinct genetic units, often flanked by direct repeats

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

vii. Presence of 'mobility' genes that are often cryptic (integrases, transposases, plasmid replication origins)

viii. Instability

Not all PAI's will conform to all these characteristics; some are particularly stable, while others may not be flanked by direct repeats. However PAI's by definition will possess at least some of these characteristics. PAI's are generally acquired horizontally from some external source, e.g. another bacteria (not necessarily the same species); a bacteriophage; naked DNA (see section 1.5). Pathogenicity islands are so named because their introduction and expression in a previously non-pathogenic strain will result in that strain becoming pathogenic. This does not necessarily imply that the strain becomes virulent - just that it possesses some pathogenic ability, such as adherence or invasion. An example of this is the introduction of *inv-spa* genes from *Salmonella* into *E. coli*, which allows *E. coli* to invade cells of the intestinal epithelium (Elsinghorst *et al.*, 1994).

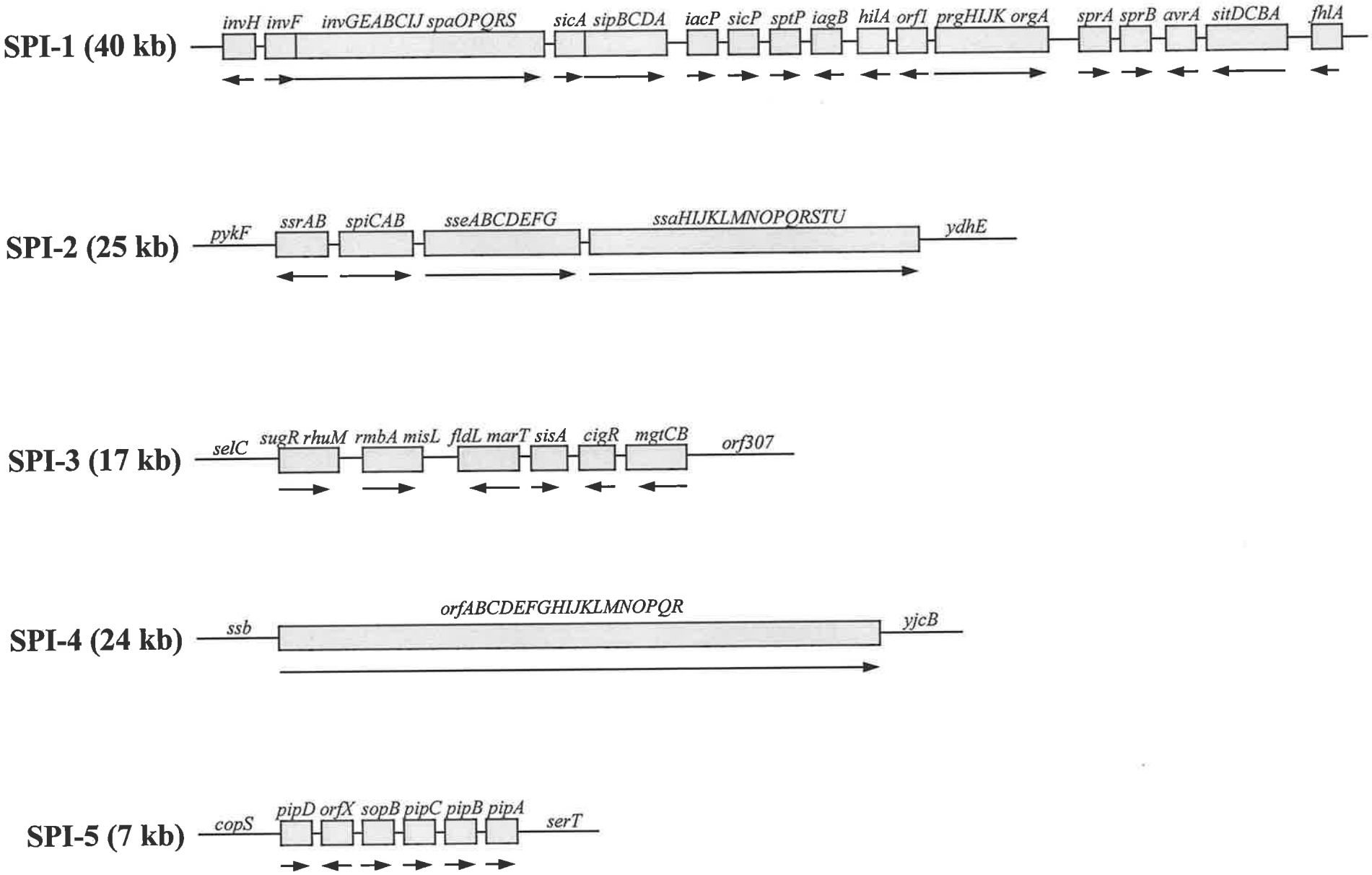
The *Salmonella* pathogenicity islands are highly complex systems with multiple structural, regulatory and secreted effector proteins (see Figure 1.8). As such only a brief outline of their role in *Salmonella* pathogenesis, using selected examples of well characterised genes, is given here.

1.5.2.1.1 SPI-1 and *Salmonella* invasion

Salmonella pathogenicity island SPI-1 is located at 63 centisomes (Cs) on the *S.* Typhimurium chromosome, has been extensively researched since discovery of several of the

Figure 1.8: Genetic arrangement of *Salmonella* pathogenicity islands.

This figure shows the relative arrangement of genes in each of the five recognized pathogenicity island of *Salmonella*. The size of each pathogenicity island (in kb) is indicated, and the arrows indicate the direction of transcription. The functions of specific genes are described in section 1.5.2. Adapted from Sirard *et al* (1999).


SPI-1 (40 kb)
invH invF invGEABCIJ spaOPQRS sicA sipBCDA iacP sicP sptP iagB hilA orf1 prgHIJK orgA sprA sprB avrA sitDCBA fhlA
SPI-2 (25 kb)
pykF ssrAB spiCAB sseABCDEFG ssaHIJKLMNOPQRSTU ydhE
SPI-3 (17 kb)
selC sugR rhuM rmbA misL fldL marT sisA cigR mgtCB orf307
SPI-4 (24 kb)
ssb orfABCDEFGHIJKLMNOPQR yjcB
SPI-5 (7 kb)
copS pipD orfX sopB pipC pipB pipA serT

genes it encodes by Galán and Curtiss (1989), and has since been implicated in *Salmonella* invasion of host cells (reviewed by: Darwin and Miller, 1999; Brumell *et al* 1999; Wallis and Galyov 2000). SPI-1 consists of more than 35 genes within a 40kb region that is absent from the *E. coli* K-12 genome (Mills *et al.*, 1995). This locus encodes genes that can be grouped according to their functional properties: genes required for expression of the secretion apparatus of a Type III secretion system (TTSS), such as InvC, InvE and InvG (Eichelberg *et al.*, 1994; Ginocchio *et al.*, 1992; Kubori *et al.*, 1998); genes encoding secreted effector proteins such as SipA, and SptP (Fu and Galán, 1998; Hueck *et al.*, 1995; Kaniga *et al.*, 1995; 1996); genes encoding molecular chaperones such as SicA (Kaniga *et al.*, 1995; Tucker and Galán, 2000); and regulatory proteins such as InvF and HilA (Eichelberg and Galán, 1999; Darwin and Miller, 1999; Bajaj *et al.*, 1995).

It has been known for a number of years that SPI-1 is absolutely required for *Salmonella* invasion into M cells, with mutations in a number of TTSS genes (eg InvA, InvG) resulting in an invasion deficient phenotype (Bäumler *et al.*, 1997b; Penheiter *et al.*, 1997). Interestingly, the effect of these mutations can vary depending on the model system used. For example, InvH is required for correct localisation of InvG, a major structural protein of the SPI-1 TTSS, and for secretion of the effector protein SipC (Daefler and Russel, 1998). In invasion studies, an *invH* mutation in *S.* Typhimurium (of human gastroenteritic origin) resulted in hypo-invasiveness for HEp-2 cells, but had no effect on invasion in a rabbit ileal loop model (Lodge *et al.*, 1995). In contrast, a *S.* Typhimurium (of bovine origin) *invH* mutant was recovered in lower numbers than the parent strain when using a bovine ileal loop model (Watson *et al.*, 1995). These results highlight the complexity of *Salmonella* invasion, and may even suggest some host adaptation between different isolates of the same species, although more comprehensive research is required to determine whether this is the case.

The ileal Peyer's patches of mice were shown to be the primary entry site for both *S.* Enteritidis (Carter and Collins, 1974) and *S.* Typhimurium (Hohmann *et al.*, 1978). This

observation was extended to show that the M cells within the Peyer's patches were the predominant target for invasion (Jones *et al.*, 1994; Penheiter *et al.*, 1997) and that SPI-1 was required for regulation of this process (Clark *et al.*, 1998).

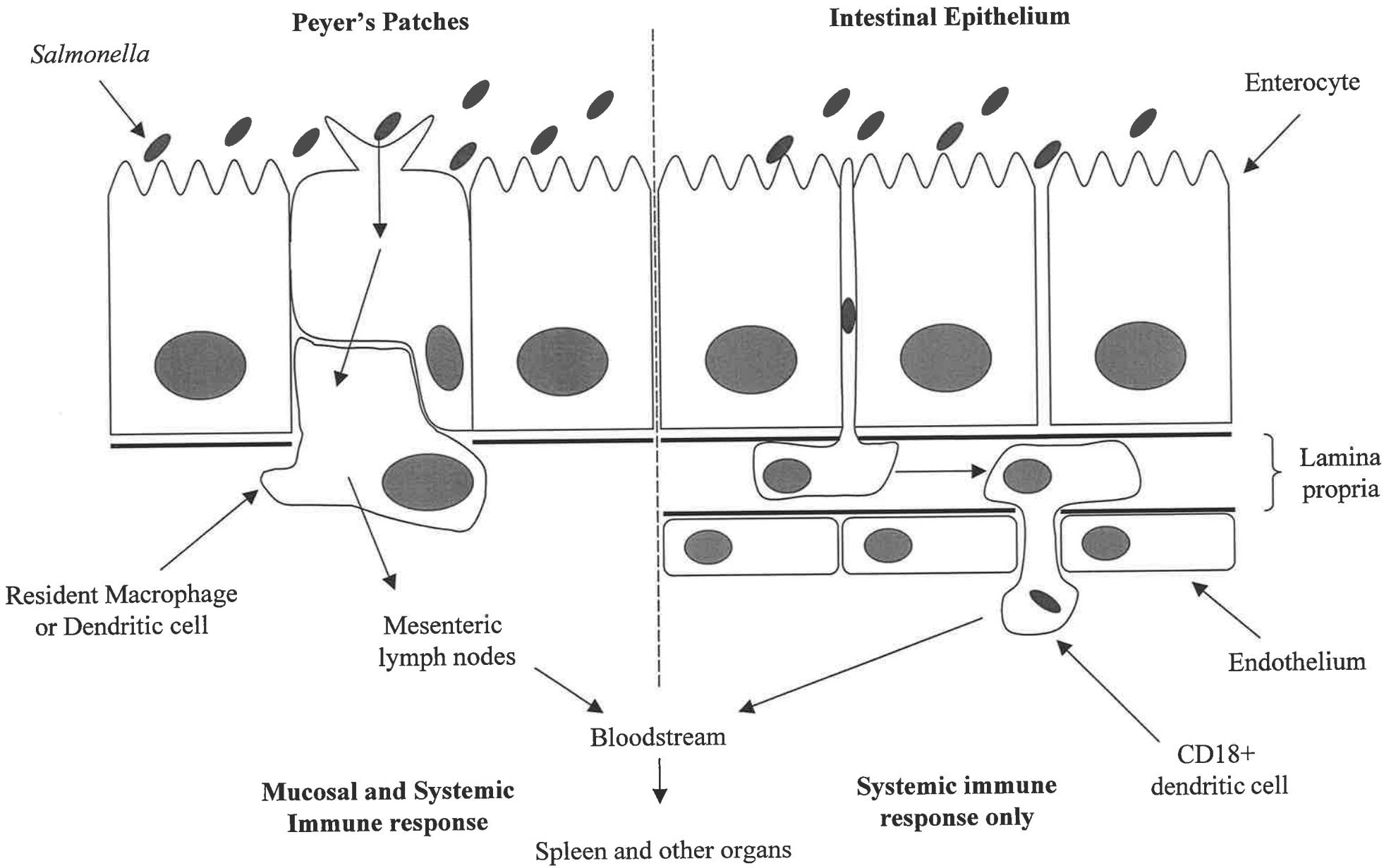
The expression and secretion of various effector proteins into the M-cell cytoplasm forces actin rearrangements and induces membrane ruffling (Francis *et al.*, 1993) in the target cell. SopE interacts with Rac and Cdc42, converting them to an activated GTP-bound form. These activated molecules stimulate membrane ruffling and facilitate bacterial internalisation (Hardt *et al.*, 1998). Once internalised, the effector protein SptP is secreted, and interacts with activated Rac and Cdc42 to stimulate their GTPase activity and convert them to inactive molecules (Fu and Galán, 1999). This in turn allows the membrane ruffles to be return to their normal morphology, while *Salmonella* continues to multiply within a *Salmonella* containing vacuole (SCV).

Salmonella invasion via M-cells leads to an interaction of *Salmonella* with subepithelial dendritic cells, which are efficient antigen presenting cells (Kelsall and Strober, 1996) and may play a crucial role in the mucosal immune response to *Salmonella* infection. However, these cells also present a potential vehicle for dissemination, as they are able to migrate to peripheral tissues (Stumbles *et al.*, 1999). A study by Penheiter *et al* (1997) suggested that non-invasive *Salmonella* were avirulent due to their inability to enter and destroy the M-cells of ileal Peyer's patches. However, a more recent study by Vazquez-Torres *et al* (1999) demonstrated that invasion deficient *S.* Typhimurium were able to disseminate systemically (via the spleen) by uptake into CD18-positive cells of the monocyte-macrophage lineage. This M-cell independent dissemination led to a specific IgG systemic immune response in the absence of either a significant mucosal IgA response or mucosal damage. A comparison of these two methods of invasion is shown in Figure 1.9.

Not all effector proteins secreted by SPI-1 TTSS are located within SPI-1. SopB, an effector in *S.* Dublin, is present on another pathogenicity island, SPI-5 (Wood *et al.*, 1998)

Figure 1.9: Alternate mechanism of *Salmonella* invasion.

This figure presents a schematic representation of two methods by which *Salmonella* are able to gain access to systemic sites. The left side represents the classic mode of entry, utilizing *inv* genes to gain access to M cells, migration to underlying APCs, and dissemination via the mesenteric lymph nodes and blood stream to the spleen/liver. This pathway generates both local mucosal and systemic immune responses. The right side represents a more recently discovered mechanism for *Salmonella* access to systemic sites. This mechanism does not require invasion genes, and depends on the active uptake of *Salmonella* by CD18+ phagocytes. These cells pass into the blood stream and then onto the spleen and liver. This pathway generates a systemic immune response only. Adapted from Vazquez-Torres *et al.*, (1999) and Vazquez-Torres and Fang (2000).


Peyer's Patches
Intestinal Epithelium
Salmonella
Enterocyte
Lamina propria
Resident Macrophage or Dendritic cell
Mesenteric lymph nodes
Endothelium
Bloodstream
CD18+ dendritic cell
Mucosal and Systemic Immune response
Systemic immune response only
Spleen and other organs

and has been shown to be involved in fluid accumulation in bovine ileal loops (Galyov *et al.*, 1997). SopE, localized near SPI-1 in *S.* Typhimurium (Cs 61), has been shown to have some role in membrane ruffling, since a *sopE* mutant does not induce membrane ruffling in cultured epithelial cells to the same extent as the wild type parent (Hardt *et al.*, 1998). However this mutant is still invasive and virulent in a mouse model of infection (Hardt *et al.*, 1998). This suggested that there is some functional redundancy in the action of secreted effectors, a notion that was demonstrated by Mirold *et al* (2001) who compared the invasion ability of various combinations of mutants in *sopB*, *sopE* and *sopE2*. A triple mutant in these genes was unable to induce membrane ruffling and was 100x less invasive, while single or double mutants showed less attenuation in these abilities.

1.5.2.1.2 SPI-2

The SPI-2 pathogenicity island (~25 kb) is responsible for the intracellular survival and proliferation of *Salmonella* in macrophages (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998) and is located at 30.5 - 31 Cs on the *S.* Typhimurium chromosome. SPI-2 encodes a second Type III secretion system which is distinct from that encoded by SPI-1 and consists of 31 genes (Hensel, 2000).

Several genes within this locus have been shown to be critical for *Salmonella* virulence (Klein & Jones, 2001; Ochman *et al.*, 1996, Shea *et al.*, 1996, 1999). It was demonstrated by Uchiya *et al* (1999) that the *spiC* gene encodes for a protein that inhibits the fusion of *Salmonella*-infected phagosomes with either endosomes or lysosomes and also interfered with normal trafficking of vesicles lacking resident *Salmonella*. While mutants in this gene were unable to prevent phagosome lysosome fusion and were attenuated for virulence, they were not defective for invasion, suggesting that SPI-1 and SPI-2 are independently expressed. The secreted effector SseD was shown to be required for systemic

disease in calves (Bispham *et al* 2001) as *S.* Dublin mutants in *sseD* were unable to induce systemic disease, however the calves still suffered from mild enteritis.

The secreted effector SifA is involved in the formation in epithelial cells of lysosomal glycoprotein containing filamentous membrane structures termed Sifs (Garcia del Portillo *et al.*, 1993; Stein *et al.*, 1996), and is important in intracellular survival and replication within murine macrophages (Brumell *et al.*, 2001). Beuzon *et al* (2000) postulated that SifA was required to maintain the *Salmonella* containing vacuole through the continual recruitment of Sifs, thus maintaining and environment conducive to replication.

Like SPI-1, SPI-2 secretes effector proteins that are not encoded with the SPI-2 locus e.g. SspH-1, and SspH-2 (Miao *et al.*, 1999), and SifA (Stein *et al.*, 1996).

1.5.2.1.3 SPI-3, SPI-4, and SPI-5

The roles for the SPI-3, SPI-4 and SPI-5 loci has not been as extensively researched as for SPI-1 and SPI-2, although they have been shown to be important in the virulence of *Salmonella*.

The SPI-3 loci, at ~17 KB, is much smaller than SPI-1 and -2, and is located at 82 CS on the *S.* Typhimurium chromosome, at the *selC* tRNA locus. It contains at least 10 ORFs including the *mgtBC* locus (Blanc-Potard *et al.*, 1999). This locus is activated in Mg^{++} limiting conditions via the PhoP/Q regulatory system (Garcia Véscovi *et al.*, 1996; Soncini *et al.*, 1996). *mgtB* encodes a P-type ATPase Mg^{++} transport protein, while *mgtC* encodes a protein of unknown function, which has been shown to be required for long term survival in macrophages (Blanc Potard & Groisman, 1997; Moncrief and Maguire, 1998). The remaining eight ORFs have unknown function, although MsiL and MarT show limited sequence similarity to AIDA-1 (responsible for diffuse adherence of diarrhoeagenic *Escherichia coli* O126:H27; Benz and Schmidt, 1992), and ToxR (transcriptional activator of cholera toxin;

Miller and Mekalanos, 1984), respectively. Further research is required to define the roles of the genes encoded by this region in *Salmonella* pathogenesis.

The SPI-4 locus was first described by Wong *et al* (1998). A small DNA locus mapping within this region (*ims98*) was found to be required for intramacrophage survival (Bäumler *et al.*, 1994). SPI-4 is somewhat larger than SPI-3 (~25 kb) and is located at 92 Cs in the *S.* Typhimurium chromosome. It encodes 18 putative ORFs (designated A-R), three of which (ORF-C, ORF-D and ORF-R) show similarity to proteins involved in the secretion of cytotoxic effectors; CyaE, (Gross, 1995); LipC, (Akatsuka *et al.*, 1997) and LktB, (Strathdee and Lo, 1989), respectively. These proteins are all members of Type 1 secretion systems (Salmond and Reeves, 1993), suggesting that SPI-4 might encode a Type I secretion system that secretes cytotoxic effectors into macrophages.

The SPI-5 locus was first characterised in *S.* Dublin by Wood *et al* (1998). It is the smallest characterised pathogenicity island of *Salmonella* to date (only ~7.5kb) and is located at approximately 20 Cs, located immediately downstream of the tRNA gene *serT*. This region was found to possess 5 major ORFs including the previously characterised SopB (Galyov *et al.*, 1997). PipC is thought to encode a chaperone for SopB, as these proteins have significant homology to the IpgD/IpgE and SigD/SigE effector/chaperone pairs of *Shigella* and *S.* Typhimurium (Allaoui *et al.*, 1993; Hong and Miller, 1998) respectively. Mutations in *pipC/D/A* or *sopB* all resulted in reduced virulence in *S.* Dublin as assessed by fluid and PMN accumulation within bovine ileal loops (Wood *et al.*, 1998). Woods *et al* (1998) also showed that mutants in *pipC/D/A* or *sopB* were all recovered in similar number from the liver and spleen of i.p. or orally infected mice. Together these results suggested that the genes of SPI-5 are involved in the development of enteritis but not systemic disease. Further studies in calves would need to be performed to whether this observation was simply due to the differences in the model systems used.

1.5.2.1.4 Regulation of SPI expression

SPI regulation is a multifactorial process, which involves both global regulators such as the PhoP/Q system (Behlau and Miller, 1993; Pegues *et al.*, 1995; Soncini *et al.*, 1996; Deiwick, *et al.*, 1999) and specific regulators such as HilA (Bajaj *et al.*, 1995; Lucas *et al.*, 2000) and InvF (Darwin and Miller, 1999; Eichelberg and Galán, 1999). The coordinated expression of specific SPI's via these regulatory systems ensures the correct timing of secretion for effector proteins, based on the current environmental conditions.

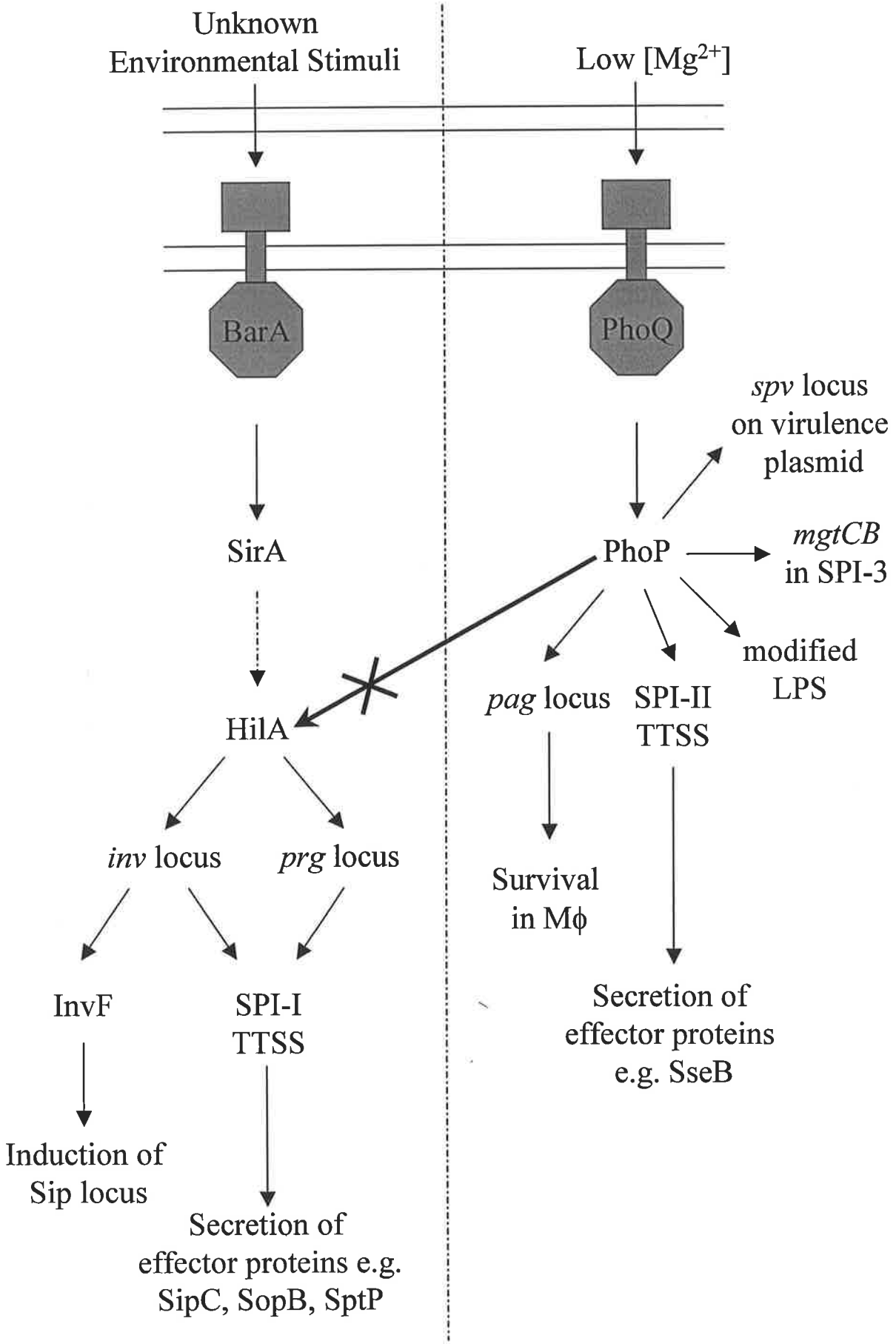
A schematic representation of the regulatory cascade is shown in Figure 1.10. The response regulator SirA is phosphorylated by the sensor kinase BarA (Altier *et al.*, 2000) in response to an as yet unknown environmental signal, and in turn activates the transcription of *hilA* (Johnston *et al.*, 1996; Ahmer *et al.*, 1999). The HilA regulator goes on to upregulate the expression of the *inv-spa* and *prg* genes, leading to assembly of the SPI1 TTSS. InvF, upregulated by HilA, activates the expression of the Sip effector proteins (Darwin and Miller, 1999), which promote the uptake of *Salmonella* into the host cell, where it resides in a vacuole. At this point there is a shift from expression of SPI-1 to SPI-2 genes, mediated by the PhoP/Q response regulator system. PhoP, induced by Mg^{++} limiting concentration (Garcia Véscovi *et al.*, 1996), becomes active within the vacuole and represses the transcription of *hilA* (Pegues *et al.*, 1995), which in turn prevents expression of the SPI-1 TTSS. Active PhoP induces expression of SPI-2 (Deiwick, *et al.*, 1999) and *pag* genes, promoting survival within macrophages (Cirillo *et al.*, 1998; Vazquez-Torres *et al.*, 2000).

1.5.3 Flagella

Prior to determining the role of SPI-1 in invasion and subsequent local versus systemic infection, the role of flagella in pathogenesis was somewhat controversial. A study by Lockman and Curtiss (1990) showed that wild-type and non-flagellated/non-motile mutants were equally virulent when mice were challenged via intraperitoneal injection. The

Figure 1.10: Extracellular and intracellular regulatory cascades

This figure depicts parts of the regulatory cascades that control expression of SPI-1 and SPI-2, which are crucial for the invasion and intracellular survival, respectively, of *Salmonella*. Within the extracellular milieu (left of the broken line), an unknown environmental signal is sensed by BarA, which phosphorylates SirA. Phosphorylated SirA, through an unknown mechanism and/or intermediate (represented by the dashed arrow), activates HilA, which in turn leads to the expression of the SPI-1 TTSS. This allows the coordinated expression of effector proteins that allow *Salmonella* to invade the target cell. Unlike the extracellular environment, the concentration of Mg^{++} encountered in intracellular vacuoles (right of the broken line) is low, which prompts the activation of the PhoP/Q regulatory cascade. This in turn leads to activation of SPI-2 along with a number of other virulence factors required for intracellular survival. Activation of PhoP/Q also results in repression of *hilA* transcription (represented by crossed out arrow), thus repressing expression of SPI-1. Adapted from Cotter and Miller (1998) and Groisman (1998).


Unknown
Environmental Stimuli
Low [Mg2+]
BarA
PhoQ
spv locus
on virulence
plasmid
SirA
PhoP
mgtCB
in SPI-3
modified
LPS
pag locus
SPI-II
TTSS
HilA
inv locus
prg locus
Survival
in Mϕ
InvF
SPI-I
TTSS
Secretion of
effector proteins
e.g. SseB
Induction of
Sip locus
Secretion of
effector proteins e.g.
SipC, SopB, SptP

virulence of *S.* Typhimurium, along with isogenic mutants lacking Type I fimbriae or both Type I fimbriae and flagella (double mutant), was examined by oral challenge of mice. It was observed that the virulence of the mutants lacking Type I fimbriae was similar to that of the parent strain, but the double mutant had significantly reduced virulence (Lockman and Curtiss, 1992). These studies revealed the requirement of flagella in facilitating colonisation of mice epithelium, with SPI-1 facilitating invasion. The role of flagella in facilitating close contact with epithelium was demonstrated by Jones *et al* (1992), who showed that a non-flagellated *S.* Typhimurium mutant was impaired in its ability to invade HEp-2 cells relative to wild type *S.* Typhimurium. The non-flagellated mutant was found to be as invasive as the wild type strain if centrifugation was used to bring the mutant into close contact with HEp-2 monolayers. This result revealed a clear distinction between colonisation and invasion in the pathogenesis of *S.* Typhimurium.

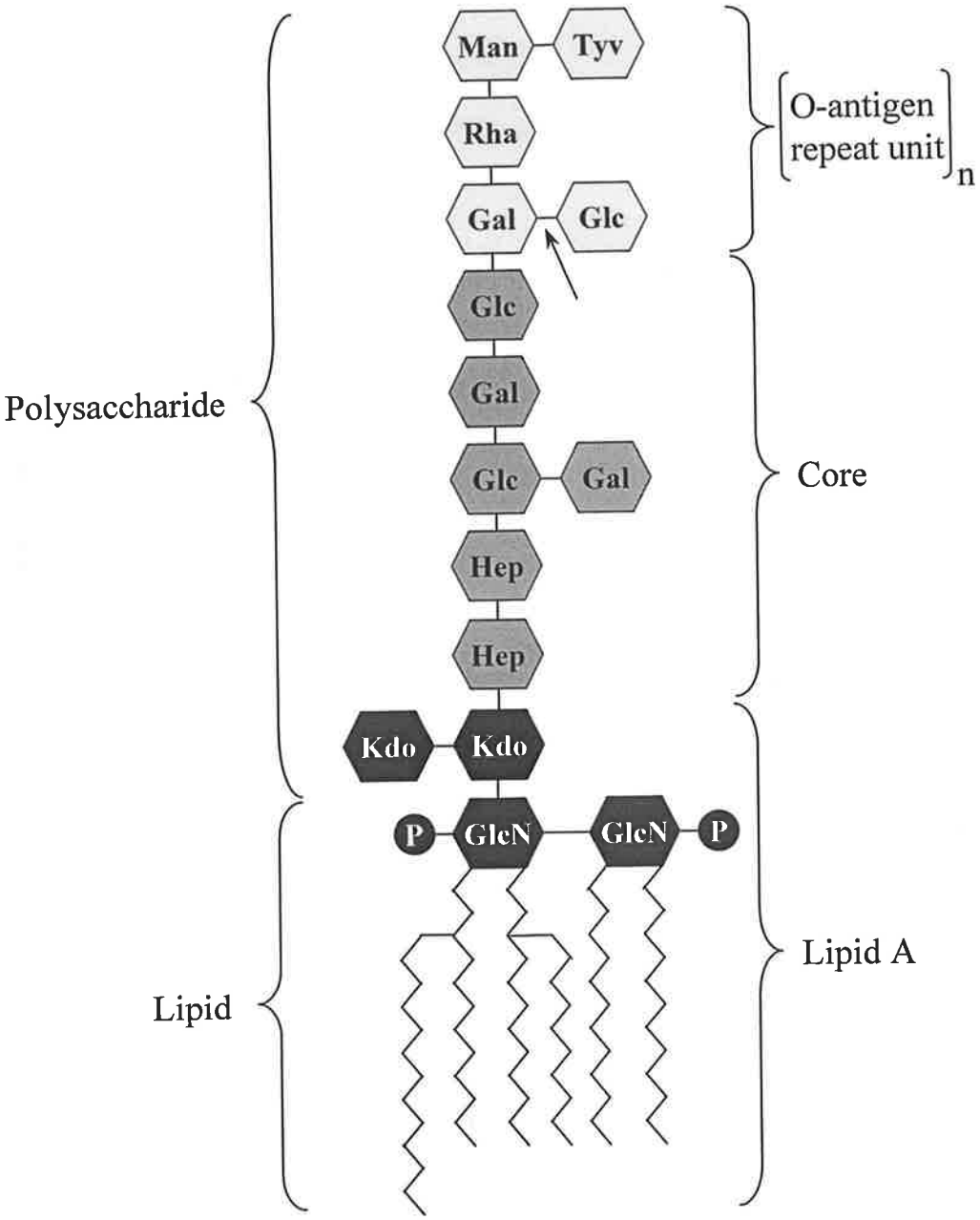
More recently, it has been demonstrated that *S.* Typhi flagella (STF) are able to induce secretion of high levels of pro-inflammatory cytokines (IL-1β and TNF-α) from human peripheral blood mononuclear cells (Wyant *et al.*, 1999), thus demonstrating a role for flagella in *Salmonella* pathogenesis outside of colonization. It has recently been shown that flagella are recognized by the innate immune system via Toll-like receptor 5 (TLR5), and induces the production of TNF-α (Hayashi *et al.*, 2001), thus providing a molecular basis for the results presented by Wyant *et al* (1999).

1.5.4 *Salmonella* lipolysaccharide (LPS)

Salmonella, like all Gram-negative bacteria, has an outer membrane that is primarily composed of lipopolysaccharide (LPS; see Figure 1.11). LPS consists of three distinct regions, which are: lipid A, core sugars and O-antigen. Lipid A is a hydrophobic glycophospoholipid anchor, which holds the entire LPS molecule into the outer leaflet of the outer membrane. Lipid A is covalently bound to the core sugars via a 3-deoxy-D-*manno*-

Figure 1.11: *S.* Enteritidis Lipopolysaccharide

Presented here is a schematic representation of *S.* Enteritidis lipopolysaccharide (LPS) showing the various functional regions. The Lipid A domain is comprised of the Lipid anchor, with associated phosphate groups, plus two Kdo sugars. The core region is comprised of a chain of 5 sugars, with a galactose sugar bound to the first glucose sugar. The O-antigen repeat unit is a trimeric sugar chain with an associated tyvelose sugar bound to the terminal mannose sugar. Note that glucosylation can occur at the primary galactose sugar of the O-antigen repeat (indicated by the arrow). The number of O-antigen repeats (n) ranges from 1 (very short chain), to ~20-30 (short chain), up to 70 (long chain). The various sugar molecules are as indicated. Hep = L-*glycero*-D-*manno*-heptose; Glc = D-glucose; GlcN = D-glucosamine; Gal = D-galactose; Kdo = 3-deoxy-D-*manno*-octulosonic acid; Man = D-mannose; Rha = L-rhamnose; P = phosphate; Tyv = tyvelose. Adapted from Raetz, (1996).


Man
Tyv
Rha
Gal
Glc
Glc
Gal
Glc
Gal
Hep
Hep
Kdo
Kdo
P
GlcN
GlcN
P
O-antigen repeat unit
n
Polysaccharide
Core
Lipid A
Lipid

octulosonic acid molecule. The core region acts as a bridge between the Lipid A and O-antigen components. *Salmonella* O-antigen varies between serovars, and, along with flagella antigens, forms the basis of the Kaufmann-White *Salmonella* serotyping scheme (Kauffmann, 1954). The O-antigen is composed of a number of repeat units, which in *Salmonella* can range from 1-70 (Peterson and McGroarty, 1985). In *S.* Enteritidis, each repeat unit is composed of a number of the trisaccharide mannose-rhamnose-galactose, with a tyvelose sugar joined to the mannose sugar by an $\alpha 1 \rightarrow 3$ linkage (Hellerqvist *et al.*, 1969). Additionally, there is non-stoichiometric glucosylation of the galactose sugar via an $\alpha 1 \rightarrow 4$ linkage (Hellerqvist *et al.*, 1969). Lysogenization of *S.* Choleraesuis by phage 14 (P14) led to an increase in the average length of the O-antigen chains (Nnalue *et al.*, 1990). This in turn conferred increased serum resistance, and as a consequence, increased bacterial virulence. Lysogenization of *S.* Typhimurium with the same phage did not result in an increase in O-antigen chain length, with no concomitant increase in serum resistance and virulence.

LPS is a dominant virulence factor in *Salmonella* infections; the lipid A moiety has for some time been considered a major contributor to septic shock, due to its endotoxic nature (Morrison and Ryan, 1987). Septic shock occurs due to the overstimulation of the immune system by lipid A and may ultimately lead to multi-organ failure and death (Schletter *et al.*, 1995). Ironically, bacterial lysis brought about by antibiotic therapy can exacerbate the problem, due to the massive release of LPS from the dead bacteria (Leeson *et al.*, 1994). A study by Khan *et al* (1998) showed a direct link between survival of infected mice and the endotoxicity of the lipid A component. Those mice that received a *Salmonella* strain expressing lipid A lacking a fatty acyl chain were better able to survive than those mice infected with the wild type parent. It was also shown that cytokine and inducible nitric oxide synthase (iNOS) responses both in vivo and in vitro were depressed in the lipid A mutant strain, indicating that the toxic effects of lipid A are probably mediated by a pro-inflammatory cytokine and/or iNOS response (Khan *et al*, 1998).

More recently, the mechanism by which LPS induces intracellular signaling was elucidated through the discovery of Toll-like receptor 4 (TLR4; reviewed by Beutler and Poltorak, 2001). In a complex cascade of events, LPS is bound by the LPS binding protein (Schumann *et al.*, 1990), which interacts with TLR4 in association with the accessory molecule MD-2, and in turn activates a number of intracellular signaling pathways, including the IκB kinase (IKK)-NFκB, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 pathways (see review by Guha and Mackman, 2001). TLR4 was shown to be responsible for LPS mediated intracellular signaling and inflammatory responses by showing that TLR4 deficient mice were hypo-responsive to LPS (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999; Qureshi *et al.*, 1999).

1.5.5 *Salmonella* virulence plasmids

Salmonella virulence plasmids (For review see Rotger and Casadesus, 1999) are found in non-typhoidal *Salmonella* serovars such as *S.* Typhimurium, *S.* Dublin, *S.* Enteritidis, *S.* Cholerasuis, *S.* Gallinarum and *S.* Pullorum (Guiney *et al.*, 1994; 1995). The predominant virulence factor on these plasmids is the *spv* locus, encoding the *spvRABCD* genes. The *spv* genes are required for systemic infection of host-adapted serovars in their corresponding animal hosts, and have been shown to increase bacterial growth rate in extra intestinal tissues (Barrow *et al.*, 1987; Chikami *et al.*, 1985; Danbara *et al*, 1992; Gulig and Curtiss, 1987; Gulig and Doyle, 1993; Heffernan *et al.*, 1987; Libby *et al.*, 1997; Wallis *et al.*, 1995). The *spv* genes are not limited to virulence plasmids but have been located on the chromosomes of subspecies II IIIa, IV, and VII isolates (Boyd and Hartl, 1998).

The expression of *spv* genes is induced by the intracellular microenvironment that *Salmonella* encounters within host cells e.g. macrophages (Fierer *et al.*, 1993; Rhen *et al.*, 1993). This nutrient limiting environment results in the upregulation of RpoS, which in turn upregulates of *spvR*, which itself upregulates the expression of *spvABCD* (Chen *et al.*, 1995).

The specific roles of the *spvABCD* genes are not yet known, but strains unable to express these genes show significantly reduced virulence (Libby *et al.*, 2000).

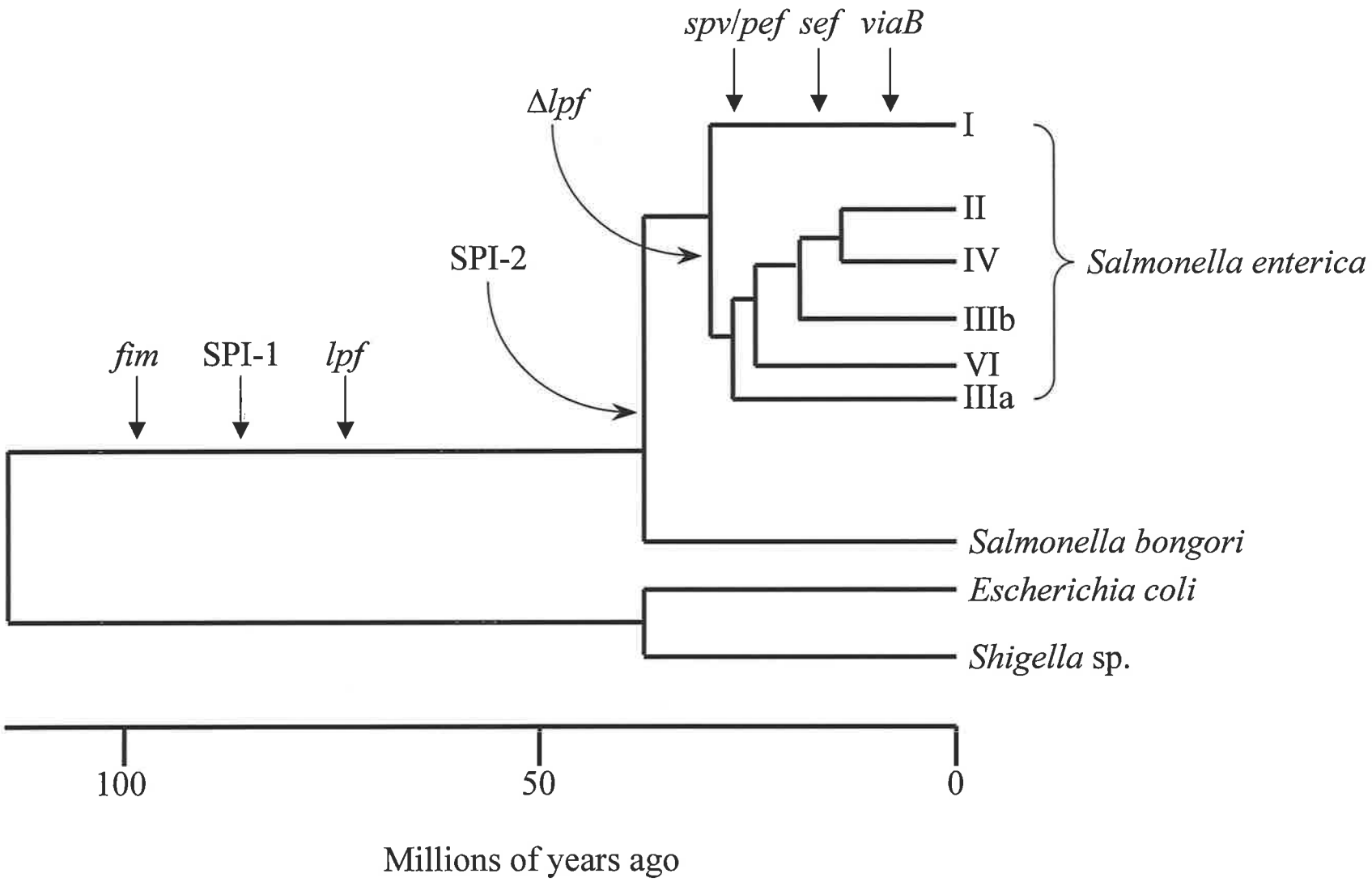
1.6 *Salmonella* evolution and acquisition of virulence determinants

As mentioned earlier (section 1.3), *Salmonella* is a significant human and animal pathogen. For this reason, many studies in the field of *Salmonella* research have focussed on the acquisition of virulence determinants i.e. the evolution of *Salmonella* as a pathogen. It is commonly believed that *Salmonella* and *E. coli* became two separate lineages from a single ancestor approximately 100-150 million years ago (Ochman and Wilson, 1987). From this point these bacteria have evolved into two distinct pathogens. Despite their differences, *Salmonella* and *E. coli* maintain a high level of sequence similarity over a large part of their genomes. The major sequence differences are clustered in "DNA-loops", of which there have been more than 30 (of 25 kb or more) discovered. These loops generally represent regions where insertions of DNA have occurred. Some of these insertions carry genes that are required for pathogenesis (eg SPI-1), and may themselves contain further insertions, giving then a 'mosaic-like' structure (eg SPI-2; Hensel *et al.*, 1999). The relationship between pathogenicity islands and microbial evolution is reviewed by Hacker and Kaper (2000).

In the process of diverging from *E. coli*, *Salmonella* has undergone many variations which give rise to the different serovars we see today. These variations between serovars include differences in fimbriae, O-antigen, flagella, antibiotic resistance, capsule, and nutrient utilisation genes, which together suggest a complex evolutionary history. Figure 1.12 shows an evolutionary pathway defining how different virulence factors come to be present in some *Salmonella* sp. but not others. Research into the acquisition of virulence determinants by *Salmonella* is not merely academic. Kingsley and Bäumler (2000) proposed that by understanding the mechanism by which *Salmonella* (and other pathogens) evolved, it might

Figure 1.12: Acquisition of virulence determinants by *Salmonella*.

The acquisition of virulence determinants by *Salmonella* has taken place in stages. Time flows from left to right, with zero representing the present day. The acquisition of SPI-2 marked a separation of *S. enterica* from *S. bongori*. The Δ*lpf* represents a deletion event, since only *Salmonella enterica* subspecies I possess these genes. Note also that not all *Salmonella enterica* subspecies I possess the *spv*/*pef*, *sef* or *viaB* virulence determinants. Abbreviations: *fim*, type I fimbriae; *lpf*, long polar fimbriae; *pef*, plasmid encode fimbriae; *sef*, *S.* Enteritidis fimbriae; SPI-1, *Salmonella* pathogenicity island 1; SPI-2, *Salmonella* pathogenicity island 2; *spv*, *Salmonella* plasmid virulence genes; *viaB*, Vi capsular antigen. Adapted from Bäumler *et al.*, (1997a)


spv/pef
sef
viaB
Δlpf
I
II
IV
IIIb
VI
IIIa
Salmonella enterica
SPI-2
fim
SPI-1
lpf
Salmonella bongori
Escherichia coli
Shigella sp.
100
50
0
Millions of years ago

be possible to accurately predict what sort of changes may arise in new pathogens, thus allowing intelligent and directed research into vaccine development.

1.6.1 Mechanisms leading to genomic variations

There are several mechanisms by which variation in bacterial genomes occur: point mutations, including nucleotide substitutions and frame-shift mutations, genetic rearrangements and horizontal gene transfer. These mechanisms, particularly the latter two, have allowed for significant variations between *Salmonella* serovars. Each of these mechanisms is examined below, with emphasis on work performed using *E. coli* and *Salmonella* as model systems.

1.6.1.1 Point Mutations

The rate of spontaneous point mutation (insertions, deletions, nucleotide substitutions and small inversions) for *E. coli* has been estimated to at 3-4 x 10^{-10}/nucleotide/generation or approximately 1.2 nucleotides per generation, and the number of generations per year has been estimated at ~200 (Drake, 1991); therefore over the ~100 million years since *E. coli* and *Salmonella* diverged from their common ancestor, a cumulative mutation total of a staggering 24 billion nucleotides (8 times the *E. coli* genome) may have potentially occurred. However, this rate of mutation is only an average over the entire genome; some regions of the chromosome appear more amendable to mutation than others. The majority of mutations will either be silent, conferring no selective advantage or disadvantage, or detrimental, such that they are selected against and not passed on due to the bacteria being outcompeted in the environment. Those mutations that are advantageous to the cell will generate variants that are better able to survive in the environment by taking advantage of their particular niche and surviving to pass on their DNA by vertical/parental transfer.

This high frequency of mutations has helped bacteria adapt and optimise their growth in their respective environments. Despite this, the closely related *E. coli* and *Salmonella enterica* do not have any distinguishable phenotypes that can be attributed to this point mutation process (Lawrence & Ochman 1997; 1998). The majority of point mutations have little or no effect on growth outside of their natural environment i.e. point mutations will enhance/depress growth within the current environment, but have little effect on growth in unrelated environments. For expansion into new ecological niches, changes in large regions of DNA need to occur simultaneously. This type of change is mediated by horizontal DNA transfer i.e. obtaining DNA from some non-parental source.

1.6.1.2 Genetic rearrangements and insertion sequences

This mechanism of DNA variation displays some significant changes within DNA that do not necessarily confer a phenotypic change. For example, there is a large inversion in the *S.* Enteritidis genome relative to the *S.* Typhimurium genome that involves ~18% of the chromosome spaning the replication terminus (Liu *et al.*, 1993), but the benefits of this inversion are not known.

Insertion sequence (IS) elements form a large family of mobile genetic elements that are loosely defined as small (<2.5 kb), phenotypically cryptic fragments of DNA that have a simple organisation, and are able to insert into multiple target sites (for review see Mahillon and Chandler 1998). The mobile nature of IS elements provide an important mechanism for genomic rearrangements and, coupled with horizontal transfer, allows for genomic rearrangements in new bacterial species. An interesting feature of insertion sequences is their ability to induce the expression of other genes, making some IS elements "mobile promoters". Examples of such IS elements include IS1 (Galas and Chandler, 1989), IS406 (Scordilis *et al.*, 1987), IS481 (DeShazer *et al.*, 1994) and IS1490 (Hubner and Hendrickson, 1997). This ability may provide bacteria with the means of altering gene expression to the point where a

selective advantage or disadvantage occurs, with advantages changes maintained by vertical transmission.

1.6.2 Horizontal DNA transfer and speciation

So how have bacteria been able to move into niches that they could not previously occupy, and over time become new species? Two related mechanisms are thought to exist. First is the selfish operon hypothesis (reviewed by Lawrence, 1997). Briefly, this mechanism allows for the sequential build up of operons over time into a single functional unit through the transfer of clustered genes (not grouped as an operon) to a recipient. Over time the (non-essential) DNA intervening the clustered genes is lost and the clustered genes become fused into a single unit i.e. an operon. Gene clusters such as these may confer the ability to occupy a new environmental niche and therefore grant a selective advantage to the bacteria. The second mechanism involves the transfer of those previously formed operons as single functional units into a naive recipient. These DNA fragments can exceed 40kb, and are able to confer new phenotypes in a single event. It is known that transfer of DNA can occur between widely separated taxonomic groups (Lawrence, 1997), providing a mechanism for speciation to take place.

For both mechanisms, the DNA that is clustered/transferred is not essential for growth and thus should theoretically be lost over time. However, clustering of genes into operons makes them amendable to horizontal transfer as a whole unit, thus allowing them to be maintained within a population, rather than lost due to genetic drift. Also, the novel functions that these operons provide may allow colonisation of previously uninhabitable niches, where there would be positive selection for these genes. It is believed that >3000 kb of DNA has been gained by *E. coli* since it diverged from *Salmonella* (Lawrence, 1997); of this large amount of foreign DNA, it is estimated that only 620 kb has been retained (Lawrence, 1997). Therefore it is hypothesised that *E. coli* has retained approximately 80-85% of its ancestral

DNA while the remaining 15-20% has been introduced by horizontal transfer events. These large DNA fragments correspond to the DNA-loops that were mentioned previously. Comparison of the *S.* Typhi and *S.* Typhimurium genomes revealed three DNA insertions present in *S.* Typhi but not in *S.* Typhimurium, the largest being ~118 kb (Liu and Sanderson, 1995). In vitro expression technology (IVET) revealed further sequence divergences between *E. coli* and *Salmonella* as well as between different *Salmonella* serovars (Conner *et al.*, 1998). These results highlight the dynamic nature of bacterial genomes and the importance of horizontal transfer in driving bacterial speciation (see Ochman *et al.*, 2000). The mechanisms by which horizontal DNA transfer occurs are described below (for review see Saunders *et al.*, 1999).

1.6.2.1 Natural transformation

Natural transformation is the uptake of non-cell associated (naked) chromosomal and plasmid DNA by naturally competent bacteria, and has been observed in all taxonomic groups including archaebacteria (for review see Lorenz and Wackernagel, 1994), suggesting that it is a significant method by which bacterial variation can occur. An excellent example of this mechanism is demonstrated by *Neisseria gonorrhoea* (reviewed by Fussenegger *et al.*, 1997). *N. gonorrhoea* absolutely requires the expression of Type IV pili for pathogenesis in humans. The major subunit is encoded by the *pilE* gene, while up to 15 silent variants (*pilS* gene) lacking the invariant N-terminal region of *pilE*, are found scattered throughout the chromosome. Recombination between *pilE* and *pilS* can occur within a single bacterium, but DNA can also be released into the extracellular environment upon spontaneous bacterial lysis, enabling other *N. gonorrhoea* bacteria to be transformed, potentially with DNA carrying *pilS* variants. Recombination with the resident *pilE* gene leads to antigenic variation in the expressed Type IV pili, allowing *N. gonorrhoea* to escape the host's adaptive immune responses.

1.6.2.2 Bacterial conjugation and membrane vesicles

Bacterial conjugation is the direct transfer of DNA from one bacterium to another, mediated by conjugative genes such as those found on the F-plasmid (Frost *et al.*, 1994). Expression of the F-pilus allows attachment of two bacteria, and subsequent pilus retraction brings the bacteria into intimate contact, allowing further conjugative genes to facilitate the transfer of DNA. Most bacterial taxa exchange DNA by conjugation (Smith *et al.*, 1993), and studies by Yamamoto *et al* (1984) showed that a virulence gene encoding plasmid from enterotoxigenic *E. coli* was able to replicate in a range of Enterobacteriaceal species, and the encoded virulence genes were expressed and functional. Balis *et al* (1996) found evidence that a conjugative R plasmid conferring ampicillin-resistance in clinical *S.* Enteritidis isolates was also present in commensal *E. coli* isolates from the same patients. These studies show that the transfer of virulence plasmids by conjugation provides a significant mechanism for bacterial variation and evolution of pathogenesis.

A more random method of horizontal DNA transfer is the release of DNA containing membrane vesicles. The ability of these DNA containing vesicles to transfer functional DNA was demonstrated in *N. gonorrhoeae* by Dorward *et al* (1989). Incubation of penicillin sensitive *N. gonorrhoeae* with membrane vesicles purified from a strain harboring an R-plasmid (encoding penicillin resistance). This resulted in efficient transfer of penicillin resistance (via the R-plasmid) to the sensitive strain. Similar observations were made by Yaron *et al* (2000). They demonstrated the ability of membrane vesicles obtained from the food-borne pathogen *E. coli* O157:H7 to transfer virulence genes into *E. coli* JM109 and *S.* Enteritidis. Both of these recipient strains were mildly cytotoxic for Vero cells, unlike *E. coli* O157:H7, which is highly cytotoxic due to the expression of chromosomal, plasmid and phage encoded virulence genes (*eaeA*, *hly*, *stx1* and *stx*2). After incubation with proteinase-K treated membrane vesicles, both *E. coli* JM109 and *S.* Enteritidis displayed similar

cytotoxicity as *Escherichia coli* O157:H7. This indicated the transfer of DNA was responsible for conferring the new phenotypes seen in the recipients.

1.6.2.2 Transducing Bacteriophages

The ability of bacteriophages to transfer DNA between bacteria has been known for many years, and constitutes a significant mechanism by which DNA transfer occurs. Many lysogenic phage stably integrate into host chromosomes, maintaining a quiescent state until induced to excise and replicate by some environmental factor such as UV-light. The integration / excision process is not always precise, and results in the acquisition of non-phage DNA. This DNA may be superfluous and result in totally non-productive sequences. However, this DNA may also be obtained such that infectious by replication incompetent phage particles are produced. These phage particles are able to infect and integrate into a new host chromosome, but have lost the ability to replicate. If the DNA obtained from the previous host encodes a functional virulence gene, a new virulent phenotype can potentially arise in the new host. Examples of phage encoded virulence determinants include: SopE and SodC in *S.* Typhimurium, carried by SopEϕ and *Gifsy*-2, respectively, (Figueroa-Bossi and Bossi, 1999; Mirold *et al.*, 1999); Cholera toxin in *Vibrio cholerae* carried by CTXϕ (Waldor and Mekalanos, 1996) and the *V. cholerae* toxin co-regulated pilis carried by VPIϕ (Karaolis *et al.*, 1999).

While bacteriophage are generally species specific, alterations in surface markers by separate events might lead to susceptibility to new bacteriophage types and thus lead to previously unavailable genetic variations. Bacteriophages often use highly conserved tRNA genes as targets for integration (Blanc-Potard and Groisman, 1997; Moss *et al.*, 1999; Rakin *et al.*, 2001), providing an explanation for the large number of pathogenicity islands that have been observed in close association with tRNA genes. An analysis of the molecular archaeology of the *E. coli* MG1655 genome revealed that more than 15 DNA insertions were

associated with tRNA genes (Lawrence and Ochman, 1998). The role of bacteriophage in the transfer of bacterial virulence factors, and targeting to tRNA genes has been extensively studied and will not be examined further here. (For review, see Cheetham and Katz, 1995; Hacker *et al.*, 1997; Miao and Miller, 1999; Dobrindt and Reid, 2000).

1.6.3 Whole genome sequencing and analysis of pathogenic bacteria

Whole genome sequencing of bacteria has revolutionised the research of pathogenic bacteria (for review see Weinstock, 2000). The availability of whole genomes has resulted in a situation where it is possible to quickly identify potential homologues to new genes, analyse variations between closely related bacteria and trace the possible movements of DNA in the evolution of present day pathogens. As of May 2001, more than forty bacterial genomes had been sequenced (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html), with the expectation that the next 2-4 years will see the completion of a further 100 genomes completed. These will not only include bacteria, but also genomes from lower eukaryotes such as fungi and protozoa. This wealth of sequence data will facilitate the rapid analysis of bacteria, with specific emphasis on determining the mechanisms of bacterial pathogenesis.

1.7 *Salmonella* Enteritidis

1.7.1 Epidemiology

Salmonella Enteritidis has become a significant worldwide pathogen in the last 30 years (Rodrigue *et al.*, 1990), with the increase in *S.* Enteritidis infections in the Western World linked to an attempt to reduce the burden of *S.* Gallinarum infections on the worldwide poultry industry (Rabsch *et al.*, 2000). Domestic and aquatic fowl are natural reserviors of *S.* Gallinarum (serotype O9,12:-:-), which does not cause significant disease in humans. However it has caused significant economic losses from poultry death. A test and slaughter approach was taken in order to eradicate *S.* Gallinarum from commercial flocks, culminating

in the eradication of *S.* Gallinarum from commercial flocks in the USA, England and Wales by the 1970s (Bullis, 1977; Sojka and Field, 1970). From that time onwards the incidences of *S.* Enteritidis (serotype O1,9,12:g,m:-) infections increased dramatically and was traced to ingestion of poultry products contaminated with *S.* Enteritidis. The slaughter of flocks seropositive for *S.* Gallinarum (ie O9,12 positive) abolished immunity to this O-antigen serotype, which also provided cross immunity against *S.* Enteritidis. This lack of herd immunity allowed *S.* Enteritidis to colonise flocks throughout the USA and Europe and become the dominant serotype. It was deduced that *S.* Gallinarum was effectively outcompeting *S.* Enteritidis and preventing it from taking a significant foothold in chicken flocks (Rabsch *et al.*, 2000). Contamination of eggs and other dairy products led to the aforementioned rise in *S.* Enteritidis infections, with a concurrent increase in burden on health costs.

1.7.2 *S.* Enteritidis 11RX

The *S.* Enteritidis strain 11RX, used throughout this work, is an attenuated *S.* Enteritidis strain that has undefined mutations leading to a rough colony morphology and is attenuated for virulence in mice (Ushiba *et al.*, 1959). 11RX has been used in a number of immunologically based studies. Early experiments demonstrated the ability of mice to resist tumour growth after immunisation with live 11RX (Hardy and Kotlarski, 1971; Tindle *et al.*, 1976). Also, this resistance could be recalled by i.p. administration of 11RX protein antigen preparations (Tindle *et al.*, 1976). The tumour resistance was shown to involve T cells by transferring T-cells from an 11RX primed mouse, and co-injecting them i.p. with 11RX protein antigen into a mouse prior to challenge with Ehrlich ascites tumour cells (Ashley *et al.*, 1977).

Other studies looked at the relationship between immunisation with 11RX and the role of thymus derived (T) cells in immunity to *S.* Enteritidis 11RX infection (Davies and

Kotlarksi, 1976), T-cell activation (Kotlarski *et al.*, 1989), and cytokine secretion by lymphocytes from 11RX infected mice (Attridge and Kotlarski, 1985). Proteins of 16-18 kDa were partially purified from 11RX and demonstrated the ability to stimulate *in vitro* proliferation in 11RX-primed T-cells (Vordermeier and Kotlarski, 1990). A further study found that one of these antigens was in fact the SefA protein of SEF14 fimbriae (Ogunniyi *et al.*, 1994).

1.8 *S.* Enteritidis pathogenesis

The pathogenesis of *S.* Enteritidis, and the virulence factors it expresses, is essentially the same as other *Salmonella* serovars (outlined in section 1.5) and will not be discussed further. However, as this thesis deals specifically with *S.* Enteritidis fimbriae, an overview of those specific to *S.* Enteritidis will be given below. Group D *Salmonellae*, including *S.* Enteritidis, possess genes for a fimbrial operon not found in other *Salmonella* serogroups (Turcotte and Woodward, 1993; Doran *et al.*, 1996). These genes are necessary for the expression of SEF14 fimbriae in *S.* Enteritidis, and will be examined in section 1.9.1.

1.8.1 *S.* Enteritidis specific Fimbriae

S. Enteritidis is known to express at least six distinct fimbriae: SEF14, SEF17, SEF18 and SEF21 (where SEF represents *Salmonella* Enteritidis Fimbriae), Lpf (Bäumler and Heffron, 1995; section 1.5.1.2.4) and Pef (Woodward *et al.*, 1996; section 1.5.1.2.5). A number of studies have been carried out to determine the role of these fimbriae in the pathogenesis of *S.* Enteritidis.

van der Velden *et al* (1998) demonstrated the value of multiple fimbrial expression in *Salmonella* virulence by showing a synergistic effect upon mouse LD_{50} values when comparing single versus multiple mutations in fimbrial expression in *S.* Typhimurium. Mutations in *fim*, *lpfC*, *pefC*, and *agfB* resulted in a maximum 4.8 fold attenuation for the *lpfC*

mutant, while a quadruple mutant showed greater than 26-fold attenuation. This is similar to results obtained by Dibb-Fuller *et al.*, (1999), who demonstrated an increasing attenuation of *S.* Enteritidis in 1-day-old chicks, as mutations were introduced sequentially into the SEF14, SEF17 and SEF21 fimbriae. Interestingly, *S.* Enteritidis mutant strains unable to express SEF14, SEF17, SEF21, Pef and Lpf was able to adhere to chicken gut explants as well as the wild type strain, however non-flagellate mutant strains were less adherent than the wild type (Allen-Vercoe and Woodward, 1999a). A comparison of the same mutants was performed by oral inoculation of 1-day-old chicks (Allen-Vercoe *et al* 1999b). When compared to wild type *S.* Enteritidis, the afimbriate mutant was recovered in similar number from livers and spleens. In contrast, the aflagellate mutant was recovered in significantly lower numbers than the wild type. This suggested that flagella, rather than fimbriae, were important in *S.* Enteritidis pathogenesis in chicks, although the possibility that further fimbriae are expressed in the afimbriate mutant cannot be discounted. Interestingly, a recent study by Townsend *et al* (2001) revealed that *S.* Typhi CT18 had the potential to express some 10 different fimbriae, with a further 4 fimbrial loci that could not be expressed due to accumulated mutations. This high number of fimbriae provides compelling evidence for a role for fimbriae in *Salmonella* pathogenesis.

Even though *S.* Enteritidis is able to express a range of fimbriae, its ability to express further fimbriae is not yet known. This possibility should not be discounted until the entire genome has been thoroughly analysed, which will be facilitated by the completion of the *S.* Enteritidis sequencing project (http://salmonella.life.uiuc.edu/).

1.8.1.1 SEF17

SEF17 fimbriae (or thin aggregative fimbriae) were first described by Collinson *et al* (1991), and show both sequence and functional similarity to Curli (see section 1.5.1.2.3). The expression of SEF17 has been correlated with binding to fibronectin (Collinson *et al.*, 1993)

and biofilm formation (Austin *et al.*, 1998), similar to that observed for Curli. These findings are significant, since the environments in which *S.* Enteritidis requires SEF17 expression are likely to differ significantly in temperature i.e. binding to fibronectin in a host would occur at 37°C, while biofilm formation could occur at a range of temperatures, both inside and outside of a host. Analysis of several *S.* Enteritidis strains by Dibb-Fuller *et al* (1997) revealed that the optimal expression temperature was 18°C, indicating that SEF17 is most likely required for biofilm formation outside of a host. However, two strains were identified in the same study that expressed SEF17 equally well at temperatures up to 42°C, suggesting that the global regulatory control that has been suggested for curli (Arnqvist *et al.*, 1992) was disrupted in these strains. A study by Woodward *et al* (2000) tested the adherence of *S.* Enteritidis to polystyrene microtitration plates. A wild type strain adhered well in this assay, however a mutant unable to express SEF17 fimbriae showed reduced adherence, further suggesting a role for SEF17 in biofilm formation.

Allen-Vercoe *et al* (1998) examined the ability of a *S.* Enteritidis mutant (*agfA*$^-$) that was unable to express SEF17 to colonise one-day old chicks. There was no observable difference in colonisation of spleens, liver or caeca between the mutant strain and its isogenic parent. This result supports the idea that SEF17 are required as an environmental survival mechanism, i.e. in biofilm formation, rather than as a virulence factor. This result was supported by Dibb-Fuller *et al* (1999), who observed that a similar mutation did not reduce the ability of *S.* Enteritidis to adhere to or invade INT-407 or Caco-2 monolayers. Interestingly, these results are in contrast with results obtained by Sukupolvi *et al* (1997), who observed that thin aggregative fimbriae promoted the binding of *S.* Typhimurium to mouse small intestinal epithelial cells. The differences in model used to determine the role of SEF17-like fimbriae is the most likely factor in these apparent discrepancies.

1.8.1.2 SEF18

SEF18 were described by Clouthier *et al* (1994) to be unusually long, thin, fimbriae-like structures in *S.* Enteritidis and *E. coli*, but were expressed as amorphous material on other bacterial strains such as *Klebsiella oxytoca* and *Providencia rettgeri*. The major subunit for SEF18 fimbriae is the *sefD* gene, which forms part of the SEF14 operon (see section 1.9.1). Recently a study by Edwards *et al* (2000) demonstrated a role for SefD in the pathogenesis of *S.* Enteritidis that is discussed in section 1.9.1.4.

1.8.1.3 SEF21

SEF21 are Type I fimbriae that were characterised in *S.* Enteritidis by Müller *et al* (1991). Genes involved in the regulation of type I fimbriae are briefly mentioned in section 1.5.1.2.1, however the gene *fimU* constitutes a further regulatory element. *fimU* is a tRNA(Arg) gene, identical to *fimU* in *S.* Typhimurium (Swenson *et al.*, 1994), and 96% identical to the *E. coli argU* homolog (Lindsey *et al.*, 1989). *fimU*, has been shown to be involved in the expression of both SEF21 and SEF14 fimbriae (Clouthier *et al.*, 1998), since a mutation in this gene abolishes expression of these two fimbriae. This observation correlates with the fact that SEF14 and SEF21 are expressed under similar environmental conditions, suggesting there is global control of both these fimbriae by the *fimU* gene.

Studies by Aslanzadeh and Paulissen (1990; 1992) suggested that type I fimbriae were important for adherence of *S.* Enteritidis to mouse small intestine epithelial cells, and antibodies specific for type I fimbriae were protective against a lethal dose of *S.* Enteritidis. More recently it has been shown that purified type I fimbriae or *S.* enteritidis expressing type I fimbriae were associated with rat ileal epithelium after oral administration, further indicating a role for type I fimbriae in the adherence and pathogenesis of *S.* Enteritidis (Ewen *et al.*, 1997).

1.9 SEF14 fimbriae

1.9.1 Genetic organisation & biogenesis

The SEF14 operon has been found to have an unusual genetic organisation. At the beginning of this thesis, four genes had been sequenced and assigned function. The genetic organisation of the SEF14 operon as it was known at that stage is shown in Figure 1.13. The operon consists of four genes; two are structural (*sefA* and *sefD*) while the remainder (*sefB* and *sefC*) are involved in fimbriae biogenesis. More recently, Collighan and Woodward (2001) published work which overlaps data presented here, revealing the presence of a fifth gene in the SEF14 locus, which they designated *sefE*.

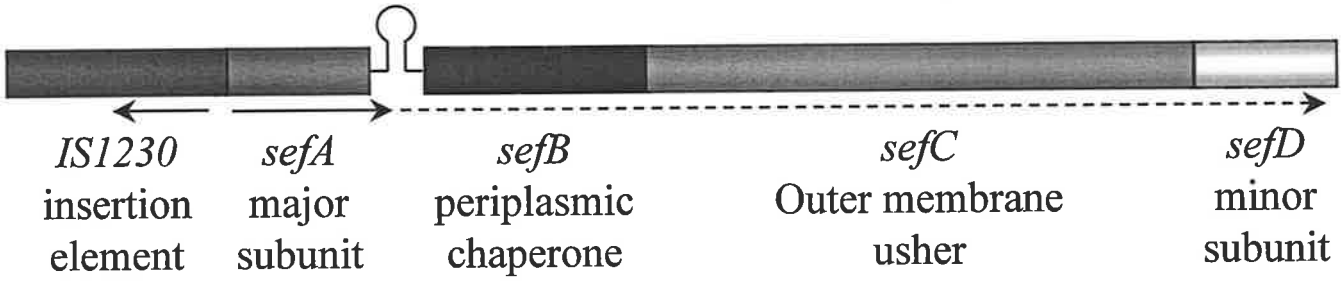
1.9.1.1 *sefA*

The *sefA* gene encodes for the major fimbrial protein SefA (Clouthier *et al.*, 1993; Turcotte and Woodward, 1993; Ogunniyi, 1996). However, the SefA protein was at first mistakenly identified as the major subunit of type I fimbriae (Feutrier *et al.*, 1986) in *S.* Enteritidis. Müller *et al* (1991) first described SEF14 fimbriae as being distinct from type I (SEF21) fimbriae, while both Clouthier *et al* (1993) and Turcotte & Woodward (1993) described the sequence of *sefA* at about the same time.

SefA has been shown to induce proliferation in T-cells harvested from *S.* Enteritidis 11RX-primed mice (Ogunniyi *et al.*, 1994). Further studies revealed that *S.* Enteritidis 11RX was better able to persist in Peyer's Patches and spleens than *S.* Enteritidis 11RX carrying a non-polar mutation in *sefA*. This result was in contrast to a similar comparison between the highly virulent *S.* Enteritidis strain 7314 and an isogenic mutant in *sefA*. No difference in virulence was observed between these two strains, suggesting that SefA probably does not have a role in *S.* Enteritidis pathogenesis (Ogunniyi *et al.*, 1997). Interestingly, immunisation with purified SefA has been shown to provide limited protection against challenge with virulent *S.* Enteritidis (Ogunniyi, 1994).

Figure 1.13: SEF14 operon

The SEF14 operon, as of the beginning of this work (1997), is made up of four genes. *sefA* and *sefD* represent fimbrial subunit proteins, with *sefA* being the major subunit of SEF14 fimbriae. *sefD* was thought to encode the major subunit of SEF18 fimbriae. *sefB* and *sefC* code for the periplasmic chaperone and outer membrane usher respectively. The stem-loop structure between the *sefA* and *sefB* genes is indicated. Clouthier *et al* (1993) hypothesized that the stem-loop structure located between *sefA* and *sefB* acts to regulate differential expression of the *sefA* and the *sefBCD* genes, as indicated by the dashed arrow. The IS1230 element has been sequenced and does not encode a functional transposase (Collighan and Woodward, 1997).


IS1230
insertion
element
sefA
major
subunit
sefB
periplasmic
chaperone
sefC
Outer membrane
usher
sefD
minor
subunit

The distribution of *sefA* has been addressed by several studies. Turcotte and Woodward (1993) showed that group D *Salmonella* strains *S.* Berta, *S.* Blegdam, *S.* Dublin, *S.* Enteritidis, *S.* Gallinarum, *S.* Moscow, *S.* Pullorum, *S.* Rostock, *S.* Seremban and *S.* Typhi were positive for *sefA* by DNA:DNA hybridisation. Of these strains only *S.* Blegdam, *S.* Dublin, *S.* Enteritidis, and *S.* Moscow were positive for surface expression of SefA by latex bead agglutination. No explanation for the disparity between the presence of *sefA* and the lack of surface expression of SefA was given. A more definitive study of the distribution of the sefA gene was carried out by Doran *et al* (1996) and involved the analysis of more than 700 *Salmonella* strains, comprising >100 different serotypes. They analysed these strains for the presence of *sefA* by DNA:DNA hybridisation and surface expression of SefA by Western and ELISA analysis. They found that *sefA* was limited to group D1 *Salmonella* strains, in agreement with previous studies. The following strains were tested and found to be positive for *sefA*; *S.* Berta, *S.* Dublin, *S.* Enteritidis, *S.* Gallinarum, *S.* Pullorum, *S.* Typhi. Of these strains all but *S.* Typhi were also found to be positive for the expression of surface expression of SefA. These results differed from the results obtained by Turcotte and Woodward (1993). While Doran *et al* (1996) did not test strains *S.* Blegdam, *S.* Moscow, *S.* Rostock and *S.* Seremban, which are considered minor serotypes, the positive SEF14 expression for *S.* Berta, *S.* Gallinarum and *S.* Pullorum which are predominantly chicken pathovars was in contrast to the lack of expression seen by the latex agglutination tests. Sequence analysis of *sefA* from *S.* Gallinarum and *S.* Pullorum have shown *sefA* to be identical in sequence to *sefA* from *S.* Enteritidis, precluding antigenic variation as the reason for their negative result. These more recent results by Doran *et al* (1996) are more likely to be accurate, given the more sensitive techniques used to detect SefA.

1.9.1.2 *sefB*

sefB is proposed to encode a periplasmic chaperone based on sequence similarity with other known chaperones sequences (Clouthier *et al.*, 1993). This has not been confirmed with experimental evidence. A SefB-MalE fusion was constructed and coexpressed with SefA by Clouthier *et al* (1998). Attempts were made to co-purify these proteins in a similar manner to other subunit/chaperone complexes were unsuccessful.

1.9.1.3 *sefC*

As with *sefB*, the function of *sefC* has been assigned on the basis of sequence similarity. *sefC* is proposed by Clouthier *et al* (1993) to encode an outer membrane protein (OMP), which provides a channel for the extrusion of SEF fimbriae and may also be an anchor for the mature fimbriae. Again this has not been confirmed with experimental evidence.

1.9.1.4 *sefD*

sefD encodes what is proposed to be a second fimbrial subunit, which is translationally coupled to *sefC*; the stop codon of *sefC* overlaps the start codon of *sefD* by a single adenine residue (Clouthier *et al.*, 1994). Circumstantial evidence exists to suggest that SefA and SefD are expressed independently to form two distinct fimbriae. SefA is known to be the major subunit for SEF14 fimbriae (see section 1.9.1.1), while immune sera specific for SefD was used in IEM experiments by Clouthier *et al* (1994) to demonstrate the expression of a fimbrial structure distinct from SEF14, and designated SEF18. This is the first operon of this type to be discovered and raises several questions that need to be addressed to fully understand the SEF14 operon. First, why are these two genes linked genetically in the same operon given that they appear to be expressed independently? Second, do they use the same fimbrial

machinery to be exported to the surface of the cell? And finally, what regulation strategy does *S.* Enteritidis use to allow such independent expression?

The distribution of the *sefD* gene was found to be at odds with that observed for *sefA*. *sefD* was observed to have an almost ubiquitous distribution amongst Enterobacteriaceal strains, as shown by Clouthier *et al* (1994). Both DNA dot-blot and antibody cross-reactivity were used to demonstrate that *sefD* was wide spread throughout most Enterobacteriaceae, and was also expressed by a significant number of those strains positive for the *sefD* gene. Further analysis of the *sefD* gene is required to determine its true distribution.

Studies by Edwards *et al* (2000) showed that SefD might have some role in the virulence of *S.* Enteritidis in mice. Non-polar *sefD* mutants gave higher I.P. and oral LD_{50} values as well as lower recovery of bacteria versus wild type from spleens and livers 4-5 days after oral inoculation. The mutant strain was outcompeted by the wild type strain when colonising spleens and livers in competition assays and were taken up less efficiently into macrophages than the wild type control. Edwards *et al* (2000) hypothesised that SefD could be surface expressed in the absence of *sefA*, as non-polar *sefA* mutants showed similar results to the wild type parent strain, and that SefA was not required for SefD function. This would explain previous results by Ogunniyi *et al* (1997) who constructed a non-polar *sefA* mutant and found no significant difference in virulence in mice. However this contradicts the results obtained by Rajashekara *et al* (2000). They constructed a polar mutation in *sefA*, which results in no *sefD* expression, and also found no difference as compared to the wild type strain in the invasion of chicken macrophages, or in the infection of SPF and normal chickens. The only difference they observed was a slightly lower recovery of the mutant strain from the liver and spleens on days 7 and 21 respectively, along with slightly reduced caecal shedding. They proposed that SEF14 may have some role in maintaining bacterial persistence in extra-intestinal tissues, but concluded that SEF14 did not have a major role in virulence. It is possible that host differences may account for these contradictory results, however the

relationship between SefD and SefA in the biogenesis of SEF14 fimbriae, and the role of SefD in the pathogenesis of *S.* Enteritidis, remains unclear.

1.9.2 Function

Since the discovery of SEF14 fimbriae, their role in *S.* Enteritidis pathogenesis has eluded definition. Several studies have found that there is no effect on virulence when *sefA* is inactivated, abolishing SEF14 expression (Thorns *et al.*, 1996; Ogunniyi *et al.*, 1997). As mentioned in section 1.6.5, inactivation of multiple fimbriae is required to observe major alterations in virulence. It is possible that the range of fimbriae that *S.* Enteritidis expresses possess the ability to functionally replace each other, and the subtle variations in pathogenesis/virulence in SEF14 mutants may not be detectable by current methods. A possible role for SEF14 fimbriae has been shown by Thiagarajan *et al* (1996). They showed that SEF14 might be involved in the binding of *S.* Enteritidis to chicken granulosa cells, an ability that was lost upon pre-incubation the granulosa cells with a 14 kDa fimbrial protein, which was not confirmed to be SefA. It was not confirmed whether this protein was actually SefA, and the authors noted the requirement for further study into this phenomenon. They also proposed that SEF14, along with other fimbriae, might be involved in transovarian transmission of *S.* Enteritidis, however this theory has not been investigated. A role not directly related to pathogenesis may have been revealed in a study by Woodward *et al* (2000). Examination of *S.* Enteritidis adherence to polystyrene microtitration plates revealed that the inability to express SEF14 fimbriae resulted in reduced adherence, however it is not known whether this effect translates to a reduced capacity for adherence in other systems.

Despite all the work that has been put into analysis of SEF14 function, no real role for SEF14 fimbriae has yet been discovered and will require further investigation to determine whether SEF14 is required for pathogenesis or for some as yet unknown function.

1.9.3 Regulation

SEF14 fimbriae are expressed at 37°C but not at 30°C or lower (Müller *et al.*, 1991), but the mechanism for this temperature-based regulation is not yet known. As mentioned in section 1.5.1.4.2.2, other fimbriae are known to be regulated in response to temperature, mediated by H-NS and a specific fimbrial regulatory protein. The only published information on SEF14 regulation is by Clouthier *et al.*, (1998), who demonstrated the loss of SEF14 expression upon mutation of the *fimU* gene, as mentioned in section 1.8.1.3. No further experimental evidence has been published for the regulation of SEF14 expression.

1.10 Aims of this study

Based on previous work that has been performed on both *sef* genes and SEF14 expression, several hypotheses were formulated in order to better define the nature of the SEF14 operon, and the biogenesis and regulation of SEF14 fimbrial expression. First, it was hypothesised that one or more genes downstream of *sefD* would be involved in the regulation of SEF14 fimbrial expression. Second, it was hypothesised that SefD was an integral part of SEF14 fimbriae, required for expression of SEF14 fimbriae. Third, it was hypothesised that the entire SEF14 operon constituted at least part of a pathogenicity island, given the limited distribution of the *sefA* gene in *Salmonella* sp. The specific aims of this thesis are summarised by the following points:

- To clone and characterise DNA downstream of *sefD*
- To determine the minimum required genes for SEF14 expression
- To determine the mechanism of SEF14 fimbriae regulation
- To analyse the distribution of *sefD* and downstream sequences in Enterobacteriaceae
- To determine whether the SEF14 operon constitutes part of a Pathogenicity island

Chapter 2: Materials and Methods

2.1 Chemicals and Reagents

All chemicals used were AnalaR grade. A list of chemicals and their suppliers is as follows: butan-2-ol, chloroform, Coomassie Brilliant Blue G250, ethanol, glacial acetic acid, glucose, glycerol, magnesium sulphate, methanol, perchloric acid, potassium acetate, propan-2-ol (isopropanol) and sucrose were from BDH Chemicals. Bovine serum albumin (fraction V), dithiothreitol (DTT), ethidium bromide, imidazole, L(+)-arabinose, magnesium chloride, Tris (Trisma base) and Tween-20 were obtained from Sigma. Calcium chloride, ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA), sodium chloride, sodium hydroxide and Triton X-100 were from Ajax chemicals, NSW, Australia.

Deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), isopropyl-β-D-thiogalacto-pyranoside (IPTG) and glycine were purchased from Roche. Nujol light mineral oil was from Perkin Elmer. Uranyl acetate was from Fluka. All other chemicals were from Ajax, BDH or Sigma. Milli-Q (MQ) water was produced by purifying water using a Milli-Q water purification system (Millipore Corp.) with a measured resistance to conductivity of 18 MΩ/cm.

Antibiotics (ampicillin, kanamycin sulphate, chloramphenicol) were purchased from Sigma. All other anti-microbial agents (dyes and detergents) were purchased from Glaxo, Calbiochem, Sigma Chemical Co., or BDH Chemicals Ltd.

Electrophoresis grade reagents and their suppliers were: acrylamide and ammonium persulphate (Bio-Rad), ultra pure N,N'-methylene bis-acrylamide and urea (BRL); high gelling temperature (HGT) agarose (Seakem); sodium dodecyl sulphate (SDS) and N,N,N',N'-Tetramethyl-ethylenediamine (TEMED) were purchased from Sigma.

2.2 Enzymes

Restriction endonucleases were purchased from New England Biolabs and used as per suppliers' instructions. Lysozyme was from Sigma. DNA modifying enzymes: T4 DNA ligase was from New England Biolabs, shrimp alkaline phosphatase (SAP) was from Roche Molecular Biochemicals and RNase A from Roche. Taq polymerase (Ampli Taq) was purchased from Perkin Elmer Cetus Corp. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Kirkegaard and Perry Laboratories Inc. Dye-labelled terminators for DNA sequencing were purchased from Perkin Elmer Applied Biosystems (Foster City, California).

2.3 Bacterial Strains and Plasmids

Cloning and expression vectors, as well as plasmids constructed in this study, are listed in Table 2.1. A list of bacterial strains used in this study is shown in Table 2.2.

2.4 Growth Media

Routine growth of bacteria was performed using Luria-Bertani broth (LB) which consisted of Bacto Tryptone (Difco) (10 g/l), Bacto Yeast Extract (Difco) (5 g/l) and NaCl (5 g/l) as described (Miller, 1972). Solidified media used included: Luria-Bertani agar (LA), which was LB with the addition of Bacto Agar (Difco) (15 g/l); Minimal agar (MA), which was M9 minimal medium (M9 salts supplemented with 200 µg/ml $MgSO_4$ and 0.5% (w/v) glucose) solidified with Bacto Agar (Difco) (15 g/l). CFA agar (CFA; Evans *et al*, 1979), consisting of Difco casamino acids (10 g/l), Bacto yeast extract (1.5 g/l), $MgSO_4.3H_2O$ (0.05 g/l) and $MnCl_2.4H_2O$ (0.005 g/l) was used for maximal expression of *sef* genes. Purity of *Salmonella* mutants was determined using XLD agar (Oxoid; 53 g/l). Working concentrations of ampicillin (Amp), kanamycin (Kan) and chloramphenicol (Cml) were 100 µg/ml, 50 µg/ml and 25 µg/ml, respectively, unless otherwise stated.

Table 2.1: Cloning vectors and plasmid constructs used in this study

Plasmid	Description	
pGEM-T	Amp^R PCR cloning vector	Promega
pBluescript-SK^+	Amp^R cloning vector	Stratagene
pBC-SK^+	Cml^R cloning vector	Stratagene
pBAD30	Arabinose inducible, Amp^R expression vector	Guzman *et al.*, 1995
pCACTUS-*mob*	Cml^R suicide vector with temperature sensitive replicon and *mob* region	Laboratory Collection, C. Clark
pET21a	Amp^R expression vector for constructing His_6 tagged proteins	Novagen
pMAL-c2X	Amp^R expression vector for constructing MBP protein fusions	New England BioLabs
pRS414	Amp^R cloning vector for construction of LacZ translational fusions	Simons *et al*, 1997
pWKS130	Kan^R low copy number cloning vector	Wang and Kushner, 1991
pRJ001	SSP-PCR fragment containing *sefR* in pGEM-T	This study (Section 3.2.3)
pRJ002	*sefR*-int fragment in pCACTUS-*mob*	This study (Section 4.2.1)
pRJ003	*Sph*I - *Sac*I *sefR* PCR product in pBAD-30	This study (Section 4.3.3)
pRJ004	LR-PCR fragment *sefABCD* in pBC-SK^+	This study (Section 4.4.1)
pRJ005	LR-PCR fragment *sefABCDR* in pBC-SK^+	This study (Section 4.4.1)
pRJ006	*Sph*I - *Sac*I subclone of *sefABCD* from pRJ004 into pWSK130	This study (Section 4.5.1)
pRJ007	Promoter + 149bp of *sefA* fused to *lacZ* in pRS414	This study (Section 4.6.1)
pRJ008	Promoter + 149bp of *sefA* fused to *lacZ* in pWSK130	This study (Section 4.6.1)
pRJ009	*sefD* in pMAL-c2X	This study (Section 5.2.1.1)
pRJ010	*sefD* in pET21a	This study (Section 5.2.3)
pRJ011	*sefABCΔD* subclone in pBC-SK^+	This study (Section 5.3.1.2)
pRJ012	*sefD*-int fragment in pCACTUS-*mob*	This study (Section 5.4)
pRJ013	*sefD* in pBAD30	This study (Section 5.4.4.1)

Table 2.2: Strains used in this study[a]

E. coli K-12	Description	Source/Reference
DH5	F$^-$, *recA*1, *endA*1, *hsdR*17 [r_k^- m_k^+], *supE*44, λ^-, *thi*1, *gyrA*, *relA*1	Bethesda Research Laboratories
DH5α	F$^-$, *endA*1, *hsdR*17 [r_k^- m_k^+], *supE*44, *thi*1, *recA*1, *gyrA* [Nal^R], *relA*1 Δ(*lac*IZYA-*arg*F), U169, *deoR* [ϕ 80*dlac*Δ[*lacZ*] M15]	Bethesda Research Laboratories
BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* DE3 (λ prophage carrying *proU*p-T7 RNA polymerase fusion)	Studier *et al.*, 1990
CC118	*recA*1, Δ(*ara*, *leu*), 7697 Δ*lac* X 74, Δ*phoA*20, *galE*, *galK*, *thi*, *rpoE*, *rpoB*, *argE* (Am)	Manoil and Beckwith, 1985
GJ1158	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (λ prophage carrying *lacUV*5-T7 RNA polymerase fusion)	Bhandari and Gowrishankar, 1997
RMJ001	DH5α + pRJ001	This study (Section 3.2.3)
RMJ004	DH5α + pRJ003	This study (Section 4.3.3)
RMJ009	DH5α + pBC-SK$^+$	This study (Section 4.4.2)
RMJ010	DH5α + pRJ004	This study (Section 4.4.2)
RMJ011	DH5α + pRJ005	This study (Section 4.4.2)
RMJ012	DH5α + pRJ006	This study (Section 4.5.1)
RMJ013	RMJ004 + pWSK130	This study (Section 4.5.2)
RMJ014	RMJ012 + pBAD30	This study (Section 4.5.2)
RMJ015	DH5α + pRJ003 + pRJ006	This study (Section 4.5.2)
RMJ016	DH5α + pRJ007	This study (Section 4.6.1)
RMJ017	DH5α + pRJ008	This study (Section 4.6.1)
RMJ018	CC118 + pRJ003	This study (Section 4.6.2)
RMJ019	CC118 + pRJ008	This study (Section 4.6.2)
RMJ020	CC118 + pRJ003 + pRJ008	This study (Section 4.6.2)
RMJ021	DH5α + pMAL-*sefD* (pRJ009)	This study (Section 5.2.1.1)
RMJ022	BL21(DE3) + pMAL-c2X	This study (Section 5.2.2)
RMJ023	BL21(DE3) + pMAL-*sefD* (pRJ009)	This study (Section 5.2.2)
RMJ024	GJ1158 + pMAL-*sefD* (pRJ009)	This study (Section 5.2.2)
RMJ025	DH5α + pET29a-*sefD* (pRJ010)	This study (Section 5.2.3)
RMJ026	BL21(DE3) + pET29a	This study (Section 5.2.3.4)
RMJ027	BL21(DE3) + pET29a-*sefD* (pRJ010)	This study (Section 5.2.3)

RMJ028	DH5α + pRJ011	This study (Section 5.3.1.2)
RMJ031	DH5α + pBAD30	This study (Section 5.4.4.1)
RMJ032	DH5α + pRJ013	This study (Section 5.4.4.1)

Salmonella	**Description**	**Source/Reference**
EX2000	LT2 *tryC2*, *metA22*, *Hl-bnml*, *H2-enx*, *fla-66*, *rpsL12*, *xyl-404met*, *E55-l*, *hsdSA29*, *ilv-452*, *hsdSB121*, *leu-3121*, *galE856*	R. Morona, University of Adelaide
11RX	*Salmonella* Enteritidis 11RX, avirulent rough mutant (serotype O1,9,12: flagellum antigen phase 1, g,m; flagellum antigen phase 2, -)	Ushiba *et al.*, 1959
7314	*Salmonella* Enteritidis 7314, virulent strain (serotype O1,9,12: flagellum antigen phase 1, g,m; flagellum antigen phase 2 [1,7])	P. Reeves
PE908	*sefA*::kan insertion mutant of 11RX	Ogunniyi *et al.*, 1997
PE910	*sefA*::kan insertion mutant of 7314	Ogunniyi *et al.*, 1997
RMJ002	*sefR*::pRJ002 insertion mutant of 11RX	This study (Section 4.2.3)
RMJ003	*sefR*::pRJ002 insertion mutant of 7314	This study (Section 4.2.3)
RMJ005	RMJ002 + pBAD30	This study (Section 4.3.4)
RMJ006	RMJ003 + pBAD30	This study (Section 4.3.4)
RMJ007	RMJ002 + pRJ003	This study (Section 4.3.4)
RMJ008	RMJ003 + pRJ003	This study (Section 4.3.4)
RMJ029	*sefD*::pRJ012 insertion mutant of 11RX (pRJ012)	This study (Section 5.4.1)
RMJ030	*sefD*::pRJ012 insertion mutant of 7314 (pRJ012)	This study (Section 5.4.1)
RMJ033	RMJ029 + pRJ013	This study (Section 5.4.4.2)
RMJ034	RMJ030 + pRJ013	This study (Section 5.4.4.2)

[a] Note that strains used only for dot- and Southern hybridisation analysis are listed in section 2.8.2

Plates for blue/white colour selection were LA supplemented with 40 µg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; 20 mg/ml solution in DMF) and 48 µg/ml isopropyl-β-D-thiogalactopyranoside (IPTG; 23.7 mg/ml solution in MQ; filter sterilised). All incubations were at 37°C unless otherwise specified, with broth cultures grown in 20 ml McCartney bottles and aerated at 150 - 200 rpm.

2.5 Maintenance of Bacterial Strains

Bacterial strains used routinely were stored as a suspension of freshly grown bacteria in glycerol (32% (v/v)) and Bacto-peptone (Difco; 0.6% (w/v)) at -70°C. Fresh cultures from glycerols were prepared by streaking a loopful of the glycerol suspension LA (with or without antibiotic as appropriate) followed by overnight incubation prior to use. Long-term storage was achieved by maintaining strains as lyophilised cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were transferred to 10 ml of LB and incubated with aeration overnight at the appropriate temperature. The remainder was streaked onto two LA plates (with or without antibiotic as appropriate) and incubated overnight at the required growth temperature. Single colonies were selected for further use.

2.6 Oligonucleotides

Oligonucleotides were purchased in lyophilised form from Geneworks (Adelaide, Australia) and resuspended to an optimal optical density (OD) in sterile MQ water. The oligonucleotides used in this study are listed in Table 2.3.

Table 2.3: Oligonucleotide primers used in this study

Primer #	Primer Sequence (5' → 3')	Description
M13 Forward	GGAAACAGCTATGACCATG	pBC-SK$^+$ (nt 826-808)
M13 reverse	GTAAAACGACGGCCAGT	pBC-SK$^+$ (nt 599-616)
2404	GGGGCATCTATTTAACAGAGAGGG	*sefD* (nt 165-188)
2433	GCCAGGATCCTGACTCCAGTTGG	*sefA* (nt 156-136) *Bam*HI underlined
2677	GCGGACTGTTGTAACTCA	pCACTUS-mob MCS (nt 3344-3361)
2678	GCGTTGGCCGATTCATTA	pCACTUS-mob MCS (nt 3490-3473)
2743	GACTGGCAACCAGATAATTC	*sefD* (nt 304-323)
2744	TTCTCCACCTATTCTTATCC	*sefD* (nt 303-284)
2933	CGGAATTCAAGCTTTCGCCCGCAGCA	*sefA* promoter region (nt 1-26) *Eco*RI underlined
3025	CGGAATTCATGAATCAGTATAATTCG	*sefD* (nt 1-18) *Eco*RI underlined
3026	CGGGATCCTTATTATAATTCAATTTC	*sefD* (nt 447-430) *Bam*HI underlined
3075	GGAATTCAGAGTTATAGGTGCGCCA	*sefD*/*sefR* intergenic region (nt 34-16 3' of *sefD*) *Eco*RI underlined
3076	GGAATTCAAAGCCCTACCTTTAAGA	*sefR* promoter region (nt 387-369 5' of *sefR*) *Eco*RI underlined
3096	ATGAATCAGTATAATTCG	*sefD* (nt 1-18)
3097	TTATAATTCAATTTCTGT	*sefD* (nt 447-430)
3125	CATATGAATCAGTATAATTCGTCA	*sefD* (nt 1-21) *Nde*I underlined
3126	GCGGCCGCTAATTCAATTTCTGTCGC	*sefD* (nt 444-427) *Not*I underlined
3190	ATGCATGCTATGACCGTATTACAAGT	*sefD* (nt 130-147) *Sph*I underlined
3191	ATGCATGCATCTGGTTGCCAGTCTTC	*sefD* (nt 318-300) *Sph*I underlined
3192	ATGCAGGCATCCTGGCATCCAGAA	*sefR* (nt 623-607) *Sph*I underlined
3193	ATGCATGCCAGATGAATTATTAAG	*sefR* (nt 208-224) *Sph*I underlined
3203	GAGCTCTAGGTGCGATTCC**ATG**TT	*sefR* (nt -13 to 5) *Sac*I underlined; start codon bold
3204	GCATGCTTGGCGCACCTATAACTC	*sefD*/*sefR* intergenic region (nt 18-1 3' of *sefR*) *Sph*I underlined
3209	CGAATTCAATGGAGTGTGCAAATGA	*sefC* (nt 2428-2445) *Eco*RI underlined
3210	GGCATGCAGAGTTATAGGTGCGCCA	*sefD*/*sefR* intergenic region (nt 34-16 3' of *sefD*) *Sph*I underlined
leuX-F	TCTTCCGGTTGGAGTTCT	*S.* Typhi genome (nt 4683293-4683310)
leuX-R	CCATTCACGAGCGATAGT	*S.* Typhi genome (nt 4684172-4684155)
IS1230-F	CTTCACCAACTCATCACC	*S.* Typhi genome (nt 4694787-4694804)
IS1230-R	ACTAGGATGGGCGCATTATC	*S.* Typhi genome (nt 4695442-4695423)
sefR-int	GCATCCTGGCATCTAGAA	*S.* Typhi genome (nt 4701515-4701532)
sefR-ext	CCTCCAGTGAATGACACC	*S.* Typhi genome (nt 4703224-4703207)
mcrD-F	GTATGAACACGGCGTCAC	*S.* Typhi genome (nt 4706038-4706055)
mcrD-R	CAGATGGTTATTCGCAGC	*S.* Typhi genome (nt 4707111-4707094)

2.7 Transformation, Electroporation and Conjugation

2.7.1 Preparation of Chemically Competent Cells

Bacteria were grown overnight in 10 ml of LB, subcultured 1/20 into fresh LB with the appropriate antibiotics and incubated with aeration until an OD_{600} of 0.5 was reached. The cells were cooled on ice for 5 min, then pelleted at 4°C in a bench centrifuge (Minor MSE bench centrifuge, 10 min, 4500 rpm) and resuspended in 10 ml of ice-cold solution α (30 mM KAc, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol). The bacteria were centrifuged again (Minor MSE bench centrifuge, 10 min, 4500 rpm) and resuspended in 1 ml of ice-cold solution β (10 mM MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol) and left on ice for 2 hr. The competent cells were aliquoted (100 µl) into 1.5 ml reaction tubes and either used immediately or stored at -70°C.

2.7.2 Chemical Transformation

Transformation was performed as a modification of the procedure described by Brown *et al*, (1979). Competent cells (100 µl) were thawed on ice for 10 minutes, mixed with required DNA and left on ice for a further 10 min. The cell/DNA mixture was heat shocked at 42°C for 1.5 min then cooled on ice for a further 10 min. This was followed by the addition of 900 µl of LB and incubation with aeration for 1 h at 37°C. Aliquots of the cultures were then plated directly onto selection plates. Addition of sterile buffer to competent cells was included as a negative control. Transformation controls consisted of competent cells plus pure cloning vector.

2.7.3 Preparation of Electro-competent cells

Electro-competent cells were prepared as follows. Bacteria were subcultured from overnight broths at 1/20 and grown to OD_{600} of 0.5. Bacteria were cooled on ice for 5 min, pelleted at 4°C (Minor MSE bench centrifuge, 10 min, 4500 rpm) and washed twice in 10 ml

of ice cold 10% (v/v) glycerol. Finally the bacteria were resuspended in 1 ml of ice cold 10% (v/v) glycerol, with aliquots (100 µl) stored in 1.5 ml reaction tubes at -70°C.

2.7.4 Electroporation

Electro-competent cells (100 µl) were thawed on ice, mixed with the required DNA and immediately transferred to a pre-chilled Gene Pulser™ cuvette (0.2 cm electrode gap, Bio-Rad). The cells were pulsed at 2.5 kV in a Bio-rad Gene pulser (25 µF, 200 Ω) giving time constants of 4.5-4.7 msec. Immediately after electroporation, cells were washed out of the cuvette with 1 ml of LB and transferred to a sterile McCartney bottle. After incubation at 30°C or 37°C for 60-90 min with agitation, 100 µl of neat and 1/10 diluted cells were plated onto LA containing the appropriate antibiotics. When introducing low copy number vector constructs, the remaining cells were transferred to a sterile 1.5 ml reaction tube, centrifuged at 15000 rpm for 1 min (Heraeus Biofuge 15) and supernatant was discarded. The cells were resuspended in 100 µl of sterile LB and plated onto LA containing the appropriate antibiotics.

2.7.5 Conjugation

Recipient and donor cells were grown overnight in LB (with appropriate antibiotics), centrifuged 4000 rpm, 10 min at RT (IEC Centra-4X centrifuge) and resuspended in 10 ml of LB (without antibiotics). Donor cells were mixed with recipient cells at a ratio of 1:10, centrifuged as before and resuspended in 1ml of fresh LB. The donor:recipient mix was applied to 0.2 µm filter discs (Type HA, Millipore) that had been previously overlaid onto LA without antibiotics. Conjugation plates were incubated at 37°C for 6 h. Bacteria were removed from the filters by vortexing in 1 ml of LB in a 20 ml McCartney bottle prior to plating 100 µl of neat or 1/10 dilutions onto the appropriate selective plates.

2.8 DNA isolation

2.8.1 Plasmid DNA isolation

Plasmid DNA for both general use and for sequencing was prepared by the following method. 1.5 ml of overnight culture was centrifuged (2 min, 14000 rpm, Eppendorf 5417R centrifuge, RT), the pellet resuspended in 300 µl of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1x 10^{-4} g/ml RNase A), and incubated at room temperature for 5 min. Bacteria were lysed by adding 300 µl of Solution II (0.2 M NaOH, 1% (w/v) SDS), followed by incubation at room temperature for 5 min. Protein, chromosomal DNA and high molecular weight RNA was precipitated by the addition of 300 µl of Solution III (5 M KAc, pH 4.8) and incubation for 10 min on ice. The cellular debris was pelleted (10 min, 14000 rpm, Eppendorf 5417R centrifuge, 4°C) and the supernatant transferred to a fresh 1.5 ml reaction tube. Chloroform (400 µl) was added to extract further protein and cell debris, and the tube mixed by inversion for 30 sec and centrifuged (15000 rpm, 3 min, Heraeus Biofuge 15). The upper aqueous layer was transferred to a fresh 1.5 ml reaction tube, ice-cold isopropanol (650 µl) was added and the sample incubated on ice for 10 min. DNA was pelleted (Eppendorf 5417R centrifuge, 14000 rpm, 5 min, 4°C), washed with 300 µl of 70% (v/v) ethanol and dried at 65°C for 10 min. Pellets were resuspended in 50 µl of sterile MQ water and stored at -20°C until further use.

2.8.2 Chromosomal DNA isolation

Bacteria from 18 h cultures grown in 10 ml LB (plus appropriate antibiotics) were centrifuged (10 min, 4000 rpm, RT, IEC Centra-4X centrifuge), resuspended in 3 ml saline and vortexed with an equal volume of Tris-buffered phenol for 2 min. The aqueous fraction was obtained by centrifugation (10 min, 4000 rpm, RT, IEC Centra-4X centrifuge) and transferred to a 20 ml McCartney bottle. The DNA was precipitated by the addition of 3 ml of isopropanol and rolling the bottle on a bench top for 2 min. The DNA was spooled from

the isopropanol/saline solution using a Pasteur pipette, washed in 70% ethanol and resuspended in 1 ml of sterile MQ. DNA was stored at 4°C.

Chromosomal preparations of the following strains were obtained from the Institute of Medical and Veterinary Science (IMVS): *Salmonella* Adelaide, *S.* Berta, *S.* Choleraesuis, *S.* Dublin, *S.* Enteritidis 1362, *S.* Enteritidis 6325, *S.* Enteritidis 8246, *S.* Enteritidis 9202, *S.* Enteritidis 9958, *S.* Gallinarum, *S.* Pullorum, *Legionella micdadei, Legionella pneumophila* and *Yersinia enterocolitica.* Chromosomal preparations of the following strains were obtained from the laboratory collection: *Escherichia coli* 1392-75 ($CS1^+$, $CS3^+$), *E. coli* 247425-1 ($CS1^+$, $CS3^+$), *E. coli* 248750-1 ($CS2^+$, $CS3^+$), *E. coli* PE563 (Serotype O157), *E. coli* PE834 (Serotype O26), *E. coli* PE890 (Serotype O111:H^-), *Salmonella* Dublin *aroA*, *S.* Stanley, *S.* Typhi Ty2, *S.* Typhimurium C5, *S.* Typhimurium LT2, *Shigella flexneri* (Serotype Y), *S. flexneri* 2457Ta (Serotype 2a), *S. flexneri* 2457Tb (Serotype 2a; spontaneous non-pigmented mutant of 2457Ta, Virulence plasmid negative), *S. flexneri* 736/89 (Serotype 2b), *Photorhabdus luminescens*, *Vibrio anguillarum*, *V. cholerae* non-O1, *V. Cholerae* 569B, *V. cholerae* O17, *V. cholerae* O139, *V. fluvialis* and *V. mimicus.*

2.9 Analysis and Manipulation of DNA

2.9.1 DNA Quantitation

DNA concentration was determined by measuring the absorption at 260 nm of a sample, with calculations made assuming that an OD_{260nm} of 1.0 is equal to 50 µg DNA/ml dsDNA (Miller, 1972).

2.9.2 Restriction Endonuclease Digestion of DNA

Restriction digests were performed in reaction buffers as recommended by the manufacturer. (0.1-0.5 µg) of plasmid DNA was incubated with 2 units of each restriction enzyme in a final volume of 20 µl, at 37°C, for 1.5 h. Restriction digest of chromosomal

DNA (~10-20 µg) was performed using 8 units of restriction enzyme in a final volume of 50 µl, incubated for 4 h at 37°C. Endonucleases were inactivated by heating at 65°C for 20 min, or 85°C for 20 min for enzymes with higher inactivation temperatures. Prior to loading on a gel, samples were mixed with a 1/10 volume of tracking dye (15% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, 0.1 mg/ml RNase A).

2.9.3 Agarose Gel Electrophoresis (AGE) of DNA Restriction Fragments and PCR products

Analysis of DNA digested with restriction endonuclease was carried out by electrophoresis at room temperature on horizontal, 0.8% or 1% (w/v) agarose gels (Seakem HGT) prepared in either 1x TBE buffer (67 mM Tris base, 22 mM boric acid and 2 mM EDTA, final pH 8.8) or 1x TAE buffer (40 mM Tris acetate, 2 mM EDTA). Gels were electrophoresed at 100-120 V for 1-3 h in the appropriate buffer. Chromosomal DNA was electrophoresed overnight at 20 V using 0.8% (w/v) TAE agarose gels. After electrophoresis, the gels were stained in distilled water containing 2 µg/ml ethidium bromide. DNA fragments were visualised using an ultraviolet transilluminator (UVT), and photographed with a Tracktel gel documentation video imager and thermal printer (Mitsubishi).

2.9.4 Calculation of Restriction Fragment Size

Restriction enzyme fragment sizes were calculated by comparing their relative mobility with that of *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA (Ratcliff *et al*, 1979). The fragment sizes (kilobases, kb) were as follows: 8.5, 7.35, 6.1, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36 and 0.09 kb.

2.9.5 Gel Extraction of DNA Fragments

DNA fragments (~2-5 µg) obtained by restriction endonuclease digestion for use in cloning procedures (from digests) were extracted from 1% (w/v) TAE-agarose gels using a QIAquick gel extraction kit (QIAGEN) as described by the manufacturer.

2.9.6 Dephosphorylation of DNA

Dephosphorylation of restriction endonuclease digested DNA was performed using shrimp alkaline phosphotase (SAP). 1-2 µg of digested DNA was added treated with 2 units of SAP in the presence of 1x dephosphorylation buffer (50 nm Tris-HCl, 0.1 mM EDTA, pH 8.5) in a total volume of 20 µl. The mixture was incubated for 15 min at 37°C for cohesive DNA ends, or 1 h at 37°C for blunt DNA ends, and the SAP inactivated by heating to 65°C for 20 min. The dephosphorylated DNA was then used directly in a ligation reaction, or stored at -20°C until further use.

2.9.7 Ligation of DNA Fragments

DNA modifying or restriction enzymes were heat inactivated for 20 min at 65°C or 85°C prior to ligation. Ligation reactions were performed using 1-2 units of T4 DNA ligase in 1x ligation buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5% (w/v) PEG 8000, 1 mM ATP and 1 mM DTT). Reactions were carried out in a volume of 20 µl, and incubated at 4°C for 16 hr. A vector DNA to insert DNA ratio of approximately 1:5 was routinely used.

2.9.8 DNA Cloning Procedures

2.9.8.1 *In vitro* Cloning

DNA for sub-cloning was prepared by restriction digest with the appropriate enzymes, and gel extraction as necessary. This was combined with similarly cleaved vector DNA that had been treated with SAP as necessary. These fragments were ligated as described

previously (section 2.9.7). The ligated DNA was then used directly for transformation of *E. coli* strains. Blue/white colour selection was used where possible. After isolation, plasmids were screened for the correct insert either by restriction enzyme digestion of isolated plasmid (section 2.8.1) or by PCR screening of small-scale lysates.

2.9.8.2 PCR Screening

Small scale lysates were prepared by resuspending a single colony in 100 µl of sterile MQ water followed by heating at 100°C for 5 min, and brief centrifugation (2 min, 14000 rpm, Heraeus Biofuge 15). 2-4 µl of the supernatant was used in a standard PCR reaction using the appropriate oligonucleotides. 10 µl of the PCR reaction was electrophoresed on 1% agarose gels in order to confirm the size of the cloned DNA.

2.10 Polymerase Chain Reaction (PCR) Protocol

2.10.1 Standard PCR Reaction

The protocol used for PCR is that described by Delidow (1993) for the generation of PCR products with cohesive ends. The PCR reaction was performed in reaction tubes (0.5 ml, Perkin Elmer Cetus) in a 50 µl volume containing Taq buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$), 2 mM final concentration of each deoxynucleoside triphosphate (dNTP), 100 pmol each primer, ~50-100 ng of plasmid template or genomic DNA and 2 U of Taq polymerase (Perkin Elmer Cetus). The reaction was overlaid with a drop of Nujol light mineral oil and subjected to 25 cycles of amplification (95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min per 1 kb of DNA template to be amplified, followed by a final extension at 72°C for 5 min) using a DNA thermal cycler (Perkin Elmer Cetus). Following PCR, the reaction was carefully removed from under the oil and 10 µl of the reaction product was analysed on a 1% agarose gel, the remainder purified using a QIAquick PCR purification kit (QIAGEN) according to manufacturer's instructions and stored at -20°C until further use.

2.10.2 Inverse PCR (iPCR)

Inverse PCR (iPCR) was used during this study to amplify fragments of DNA from the ends of known regions (Ochman *et al.*, 1988). 10 µg of bacterial chromosomal DNA was digested as described in section 2.9.2. Circularisation and ligation of the DNA fragment was performed by addition of 5 units of T4 DNA ligase in the presence of 1x Ligation buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.6 mM ATP, 10 mM DTT) in a final volume of 100 µl for 16 h at 4°C. Amplification of the iPCR fragment was then achieved using a 5 µl of circularised template in a standard PCR reaction, with the appropriate oligonucleotides reading out of the known gene fragment.

2.10.3 Single Specific Primer (SSP)-PCR

Amplification of unknown DNA flanking known DNA was achieved using the single specific primer (SSP)-PCR method (Shyamala and Ames, 1989). Template for these reactions was generated as follows. Chromosomal DNA and vector DNA were digested with the appropriate restriction enzyme (section 2.9.2). The vector DNA was SAP treated (section 2.9.6) and ligated with the digested chromosomal DNA as previously described (section 2.9.7). 5 µl of ligation reaction was used as template in PCR reactions. M13-forward and -reverse primers (Table 2.3) were paired with a specific gene primer for PCR amplification (section 2.10.1) of the target region. After amplification, PCR products were analysed by electrophoresis on 1% agarose gel. PCR products greater then 500 bp were cloned into the pGEM-T vector as recommended by the manufacturer (Promega; section 2.10.5) and analysed by sequencing with M13 forward and reverse primers.

2.10.4 Long Range PCR reaction

Long range PCR (LR-PCR) was performed using the Expand™ Long Template PCR System (Roche). Two master mixes were prepared in 0.5 ml reaction tubes on ice as follows;

Master mix 1 contained deoxyribonucleic acids at 10 mM each, 0.6 µM of appropriate oligonucleotide primers, chromosomal DNA template (0.4-0.8 µg), in 25 µl sterile MQ water. Master mix 2 consisted of 2x PCR buffer 3 (4.5 mM $MgCl_2$), 2.6 U of Taq/Pwo polymerase mix, in 25 µl sterile MQ water. The two master mixes were combined into a single thin-walled 0.5 ml reaction tube (Gene Amp, Perkin Elmer Cetus), briefly vortexed and overlaid with Nujol light mineral oil prior to amplification using the following conditions: 1 cycle - 2 min at 94°C; 10 cycles - 94°C for 10 sec, 55°C for 30 sec, 68°C for 1 min for each 1 kb of DNA template to be amplified; 15 cycles - 94°C for 10 sec, 55°C for 30 sec, 68°C for 1 min for each 1 kb of DNA template plus 20 sec extra per cycle; 1 cycle - 68°C for 7 min. 10 µl of the reaction was analysed on 1% agarose gel, prior to purification of appropriate product by gel extraction (section 2.9.5).

2.10.5 Cloning of PCR Products

PCR products were cloned using the pGEM-T and pGEM-T Easy vector systems (Promega). Ligation was carried out as described previously (section 2.9.7), using vector DNA and purified PCR product in a ratio of 1:3. The ligation mixture was then transformed into *E. coli* DH5α as described (section 2.7.2) and plated onto LA supplemented with Amp, X-gal and IPTG for blue/white colour discrimination of transformants. Positive (white) colonies were selected for screening by restriction digest and/or PCR.

2.10.6 Insertional mutagenesis

Mutations in *sef* genes were achieved by insertional mutagenesis using the temperature sensitive suicide vector pCACTUS-*mob*. Growth was at 30°C plus Cml unless otherwise stated. Internal fragments of the target gene were amplified by PCR (section 2.10.1) using chromosomal DNA as template. These fragments were cloned into the temperature sensitive suicide vector pCACTUS-*mob* (section 2.9.8.1). The suicide constructs were transformed

into SM10λpir (section 2.7.2) and then conjugated into the target strain (section 2.7.5). Exconjugants were grown to OD_{600} of 0.6 in LB supplemented with Cml at 30°C with shaking. At this point the bacteria were centrifuged (10min, 4000 rpm, RT, IEC Centra-4X centrifuge) and resuspended in fresh LB, and grown for 2 h at 42°C with shaking to promote insertion of the suicide construct into the chromosome. 5x 100 µl aliquots were plated onto LA supplemented with Cml and incubated for 18 h at 42°C. Any colonies that grew were considered potential insertion mutations. Colony purification was carried out at 37°C to ensure that free suicide plasmid constructs were lost, with strains screened for correct mutation by PCR (section 2.10.1) and Southern Hybridisation (section 2.12.2).

2.11 DNA Sequencing and Analysis

2.11.1 DNA Sequencing

DNA was sequenced (both strands) using Dye-terminator sequencing kits (Applied Biosystems) as described by the manufacturer. Briefly, DNA (1-2 µg) was added to a reaction containing the required oligonucleotide (3.2 pmol) and 8 µl of dye-terminator reaction mix made up to a final volume of 20 µl with sterile MQ water. Reactions were overlaid with mineral oil and subjected to 25 cycles (96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min). Sequencing reactions were precipitated by the addition of 50 µl 100% ethanol and 2 µl 3 M sodium acetate pH 5.2 and incubation for 2 h at -70°C. DNA was pelleted by centrifugation (20 min, 15000 rpm, Eppendorf 5417R, 4°C), washed in 70% (v/v) ethanol and allowed to dry at room temperature.

The dried DNA pellets were resuspended in 4.5 µl of loading buffer (83% deionised formamide, 8.3 mM EDTA, pH 8.0) and heated to 95°C for 2 min. Samples were electrophoresed on a 6% polyacrylamide-8M urea gel in an Applied Biosystems 373A or 377 DNA sequencer. Raw sequence data generated were analysed using Applied Biosystems Seq

Ed Program Version 6.0. The electrophoresis was carried out by the Molecular Pathology Sequencing Laboratory, Institute of Medical and Veterinary Science, Adelaide.

2.11.2 DNA Sequence Analysis

Sequence analysis was performed using the DNASIS program (Hitachi Software) to locate potential open reading frames (ORFs) and secondary structures such as stem-loops. Homology analysis of sequenced DNA and predicted amino acid sequences was performed using the BLASTN and BLASTX searchable databases respectively at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/) and the BLASTN searchable database at the Sanger Centre (http://www.sanger.ac.uk/Projects/S_typhi/blast_server.shtml). Multiple sequence alignments were carried out using Clustal X 1.8 (Thompson *et al.*, 1997). Signal peptide cleavage site predictions were carried out based on the constraints of von Heijne (1985) and the program SignalP Version 1.1 (Nielsen *et al*, 1997). Promoter analysis was performed using the Neural Network Promoter Prediction web site at http://www.fruitfly.org/seq_tools/promoter.html.

2.12 DNA hybridisation techniques

2.12.1 DNA Probe Generation

DNA Probes used for slot blot and Southern hybridisation were prepared as follows. Chromosomal DNA (~50-100 ng) was used as template in a PCR reaction containing 1x PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris pH 8.4, 50 mM KCl), of 20 µM dATP, dCTP, dGTP, and 19 µM dTTP, 1 µM DIG-11-dUTP, 100 pmol each oligonucleotide and 2 U Taq polymerase made up to 50µl with sterile MQ water. An unlabelled control reaction was also prepared using the standard PCR protocol (section 2.10.1). Samples of the labelled and control reactions were compared on a 1% agarose gel. As DIG-11-dUTP is larger than the dTTP which it replaces at random in the amplified PCR product, a probe was considered labelled if

it was observed to migrate slightly slower than the unlabelled control. The DIG labelled product was precipitated with 6 µl 4 M LiCl and 100 µl of cold ethanol at -20°C for 2 h. The labelled DNA probe was pelleted (15 min, 14000 rpm, Eppendorf 5417R centrifuge, 4°C), washed with 200 µl 70% (v/v) ethanol and dried at 65°C for 15 min. The DNA was resuspended in 50 µl of sterile MQ water. Labelled probes were further tested for successful dUTP-DIG incorporation by dotting 1 µl onto Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech) and developing as described (section 2.12.4). Successfully labelled probes were stored at -20°C and heated at 100°C for 10 min prior to adding to alternative pre-hybridisation fluid (5 x SSPE (Sambrook *et al.*, 1989), 7% SDS (w/v), 1% skim milk powder (w/v), 50% (v/v) formamide, 250 mg/ml herring sperm DNA).

2.12.2 Gel electrophoresis, Southern transfer and Probe Hybridisation

Digested chromosomal DNA (10-20 µg) samples were electrophoresed overnight on 0.8% agarose (w/v) TAE gels as per section 2.9.3. The gel was agitated in several volumes of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for one hour at RT, followed by agitation in several volumes of neutralising solution (1 M Tris-HCl pH 8.0, 1.5 M NaCl) for one hour. DNA was transferred to Hybond N^+ nylon membrane (Amersham Pharmacia Biotech) by overnight transfer at RT using capillary action as described by Sambrook *et al* (1989), with 10x SSC (1.5 M NaCl, 0.15 M Na citrate, pH 7.0) as the transfer buffer. After transfer DNA was fixed to the membrane by immersing in 0.4 M NaOH for 20 min. The membrane was then washed briefly in 5x SSC and treated with 20 ml of prehybridisation fluid for 2 h at 42°C prior to the addition of fresh DIG-labelled probe. Hybridisation was allowed to continue for ~18 h at 42°C. High stringency washes were performed as follows: two 5 min washes with 2x SSC containing 0.1% (w/v) SDS at RT followed by 2x 15 min washes with 0.2x SSC containing 0.1% (w/v) SDS at 65°C.

2.12.3 Dot blot Hybridisation

Dot blot analysis of chromosomal DNA was carried out using a vacuum equipped slot blotter (Hoefer Scientific Instruments). Hybond N^+ nylon membrane was cut to size and soaked in 10x SSC (1.5 M NaCl, 0.15 M Na citrate, pH 7.0) prior to assembly in the slot blotter. 10 µg of DNA was applied to the membrane while under vacuum. The slot blotter was disassembled after running the vacuum five minutes after adding the sample and the DNA fixed by immersing the filter in 0.4 M NaOH. The filter was then treated as for a standard Southern blot as outlined in section 2.12.2.

2.12.4 Colorimetric Detection of bound DIG-labelled Probe

Bound DIG-labelled probe was detected as follows: hybridised filters were washed briefly in Buffer 1 (0.1 M Tris pH 7.5, 0.15 M NaCl) and blocked by incubating in 5% skim milk (w/v) in Buffer 1 for 60 min at RT with agitation. A solution of anti-DIG antibody-conjugate (AP; Roche) diluted 1/5000 in 5% skim milk (w/v) in Buffer 1 was added and left to incubate for a minimum of 30 min at RT with agitation. Unbound Ab-conjugate was removed by washing twice for 10 min in Buffer 1. The filter was then incubated in 1x Buffer 3 (0.1 M Tris-HCl, 0.1 m NaCl, 0.05 M $MgCl_2$, final pH 9.5) for a minimum of 10 min and detected with 10 ml of freshly prepared colour solution (45 µl of nitroblue-toluidine (NBT; 75 mg/ml, Roche Molecular Biochemicals) and 35 µl of the substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP; 50 mg/ml, Roche Molecular Biochemicals) in 10 ml of Buffer 3). The filter was allowed to develop in the dark with no agitation and the reaction halted by removing the colour solution and adding 100 ml of 1x TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0).

2.13 Fimbriae Preparation

2.13.1 Small Scale Fimbrial preparation

Bacterial strains were grown to confluence overnight at 37°C on CFA agar plates (90 mm), containing the appropriate antibiotics where necessary. Cells were harvested in 1 ml of 1x PBS (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 1.15 g/L Na_2HPO_4, pH 7.4), and a sample retained for whole cell analysis as required. The remainder was heat shocked at 56°C with shaking for 20 min (Eppendorf Thermomixer model 5436). Bacterial cells were removed by centrifugation (10 min, 10000 rpm RT). The supernatant containing the heat-extracted fimbriae was collected and aliquots mixed with an equal volume of 2x SDS-sample buffer prior to analysis on a SDS-15% polyacrylamide gel. The remainder was stored at -20°C until further use.

2.13.2 Large Scale Fimbrial preparation

Large-scale preparation of fimbriae was performed by growing bacteria to confluence on large CFA agar plates (30 x 30 cm) with the appropriate antibiotics by plating 1 ml of an overnight culture incubated at 37°C. The cells were harvested in 10 ml of 1x PBS and the fimbriae dissociated from the cell surface by either 2 x 2 min treatments with a homogeniser (Ultra-Turrax Blender (Janke and Kunkel)) or by heating in a shaking water bath at 56°C for 20 min (orbital shaking bath, Paton Industries). The bacterial cells were then removed by centrifugation at 10000 rpm for 10 min at room temperature (Heraeus Biofuge 15) and the supernatant supplemented with 0.02% (w/v) sodium azide and stored at 4°C.

2.14 Protein Analysis

2.14.1 Sample preparation

Whole cell samples for protein analysis were prepared as follows, with OD_{600} values determined using a BioRad *SmartSpec*™ 3000. The OD_{600} of samples (1 ml) was measured

either directly (broth cultures) or from dilutions of harvested bacteria (section 2.13.1), with a conversion factor OD_{600} = 1.0 representing $1x10^9$ bacteria/ml. Samples were centrifuged (1 min, 14000 rpm, Eppendorf 5417R centrifuge) and resuspended in 50 µl MQ plus 50µl 2x SDS-sample buffer (0.125 M Tris pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.04% (w/v) Bromo-phenol blue) for each 0.5 OD units. 20-30 µl (corresponding to ~1 to $1.5x10^8$ bacteria) of sample was routinely used in SDS-PAGE analysis (section 2.14.3), unless otherwise specified.

2.14.2 Cell Fractionation

The cell fractionation procedure was a modification of that described by Morona *et al* (1995). Cells (50 ml) were grown in LB to mid-exponential phase at 37°C (OD_{600} of 0.6). Cells were pelleted in a Beckman JA-10 rotor, (7000 rpm, 10 min, 4°C) and resuspended in 1 ml of 20% (w/v) sucrose, 30 mM Tris-HCl, pH 8.1 then transferred to SM-24 tubes and chilled on ice. Cells were converted to sphereoplasts with 0.1 ml of 1 mg/ml lysozyme in 0.1 M EDTA, pH 7.3 for 30 min on ice. Cells were centrifuged as above and the supernatant collected (periplasmic fraction). The cell pellet was resuspended vigorously in 3 ml of 3 mM EDTA, pH 7.3 and freeze-thawed in an ethanol dry ice bath four times. Cells were lysed with a Branson Ultrasonifier (50% cycle, intermittent), and unlysed cells and inclusion bodies were removed by centrifugation in a Beckman JA-20 rotor (7000 rpm, 10 min, 4°C), and resuspended in 2 ml of MQ. The supernatant containing the membranes and the cytoplasm was centrifuged at 35000 rpm using a 50Ti or 80Ti rotor for 90 min at 20°C in a Beckman L8-80 ultracentrifuge. The supernatant (cytoplasmic fraction) was collected and the whole membrane pellet was resuspended in 1 ml MQ. 500 µl of Triton solution (4% Triton X-100, 2 mM $MgCl_2$, 50 mM Tris, pH 7.5) was added to an equal volume of the whole membrane sample that was vortexed intermittently for 30 min and kept on ice. The inner (Triton X-100, soluble) membrane fraction was separated from the outer (Triton X-100, insoluble) membrane

fraction by centrifugation at 35000 rpm for 90 min in a 50Ti or 80Ti rotor (Beckman L8-80) at 20°C. The pelleted outer membrane fraction was resuspended in 500 µl of MQ water. All fractions were placed at -20°C until further use. A sample of the fractions was mixed with an equal volume of 2x SDS-sample buffer and heated at 100°C for 5 min prior to electrophoresis on a SDS-15% polyacrylamide gel.

2.14.3 SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 5% stacking, 15% separating polyacrylamide gels using a modification of the procedure described by Lugtenberg *et al* (1975). Samples were diluted in 2x SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, and 30% (w/v) bromophenol blue) and then heated at 100°C for 5 min prior to loading. Samples were electrophoresed at 180 V for 2-3 h and the gels stained with Coomassie staining solution (0.1% (w/v) Coomassie Brilliant Blue G250, 10% (v/v) perchloric acid in distilled water (dH_2O)). Staining was performed for 3 h at room temperature with gentle agitation. Gels were de-stained with several changes of 5% (v/v) acetic acid at room temperature for 1-2 h with intermittent heating (1 min in a 700 W microwave oven) with agitation.

Protein molecular size markers (Pharmacia) were as follows: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Pre-stained molecular size markers (New England Biolabs) were: MBP-β-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-Lactoglobulin A (25kDa), lysozyme (16.5 kDa) and aprotinin (6.5 kDa)

2.14.4 Western Immunoblot Analysis

Proteins were transferred to nitrocellulose (Schleicher and Schuell, BA85) at 260 mA for 1.6 h in a Trans-blot cell (Bio-Rad) in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 5% (v/v) methanol (Towbin *et al.*, 1979). The membrane was incubated for 1 h in 5% skim milk powder in TTBS (0.05% (v/v) Tween-20, 20 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl) to block non-specific binding sites prior to overnight incubation in primary antiserum at room temperature with gentle agitation. Unbound antibody was removed by washing the filter (3x, 10 min) in TTBS before incubation with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (diluted 1/20,000 and 1/10,000 respectively in TTBS) for 2 h at RT. The membrane was washed (5x 5 min) with TBS (20 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl) prior to detection by enhanced chemiluminescence detection (ECL). Membranes were soaked in Chemiluminescence Blotting Substrate (POD; Roche) for 1 min in a transparent plastic bag. Membranes were exposed to X-ray film (Cronex) at room temperature for at least 30 sec, and up to 5 min. Films were developed in D19 developer (Kodak) for 2-5 min before being washed and fixed for 5 min in negative fixer (Kodak).

2.15 Protein Expression and Purification

2.15.1 MBP Tagged Protein expression

E. coli strains BL21(DE3) and GJ1158 harbouring pMAL-c2X-based expression constructs were grown at 37°C with aeration for 18 h in 10 ml of LB supplemented with Amp. Cells were centrifuged (10 min, 4500 rpm, Centra-4X IEC bench centrifuge), and the pellet resuspended in 10 ml of LB. 2 ml of this was subcultured into 100 ml of LB supplemented with Amp. The culture was grown with aeration at 37°C until the OD_{600} reached 0.5, and an aliquot retained for SDS-PAGE analysis. IPTG was then added to a final concentration of 0.5 mM, the culture grown for a further 3 h, and a second sample was retained for SDS-PAGE analysis (induced sample).

The induced cells were fractionated (section 2.14.2) to obtain the WM fraction. 500 µl of the WM fraction was solubilised by mixing with an equal volume of 0.1% (v/v) Triton X-100 in column buffer (20 mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA, 1mM azide). The mixture was gently agitated at RT for 1 h to ensure complete solubilisation of the membranes. The solubilised membranes were mixed with 200 µl of 50% (v/v) amylose resin (equilibrated with column buffer) with gentle agitation for 60 min at 4°C. After binding, the resin was washed twice with 1 ml column buffer, and briefly centrifuged (15 sec, 14000 rpm, Eppendorf 5417R centrifuge, 4°C). Bound protein was eluted with 2x 500 µl changes of column buffer supplemented with 10 mM maltose, with the resin gently agitated each time at RT for 15 minutes prior to centrifugation (1 min, 14000 rpm, Eppendorf 5417R centrifuge, RT). Samples (500 µl) were dialysed against column buffer O/N at RT and precipitated with TCA (final concentration 5% (w/v)). After centrifugation (5 min, 14000 rpm Eppendorf 5417R centrifuge, 4°C), the supernatant was removed, the pellet washed with 200µl of 100% ethanol and re-centrifuged. Finally, the supernatant was removed, the pellet was allowed to air dry and was resuspended in 50 µl of MQ and an equal volume of 2x SDS-sample buffer.

2.15.2 His_6 -Tagged protein expression

E. coli strain BL21(DE3) harbouring pET21 based expression constructs were grown at 37°C with aeration 18 h in 10 ml of LB supplemented with Amp. Cells were centrifuged (10 min, 4500 rpm, Centra-4X IEC bench centrifuge), and the pellet resuspended in 10 ml of LB. 2 ml of this was subcultured into 50 ml of LB supplemented with Amp. The culture was grown with aeration at 37°C until the OD_{600} reached 0.6, and an aliquot was retained for SDS-PAGE analysis (uninduced sample). IPTG was then added to a final concentration of 1 mM, the culture grown for a further 3 h, and a second sample was retained for SDS-PAGE analysis (induced sample). Alternatively, cultures were grown at 25°C, with the induction phase extended to 8 h. Cultures were centrifuged (10 min, 8000 rpm, Beckman JA-14 rotor) and

cells were fractionated (section 2.14.2) to extract the IB fraction. 2 ml of the IB fraction was solubilised in 8 ml of Buffer B (8 M urea, 100 mM NaH_2PO_4, 5 mM imidazole, 10 mM Tris-HCl, pH to 8.0 immediately before use), with insoluble material removed by centrifugation (5 min, 6000 rpm, Beckman JA-20.1 rotor), and the S/N retained. 200 µl of 50% (v/v) Nickel-NTA agarose resin (equilibrated with Buffer B) was added to the 8 ml of cleared S/N, and the IB/resin mixture was gently agitated at 4°C for 1 h in a 10 ml yellow capped tube (Sarstedt). The Ni-NTA resin was pelleted at 3500 rpm for 5 min (Heraeus Labofuge 400R) and the supernatant gently aspirated. The resin was washed twice in 4 ml of buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM imidazole, 10 mM Tris-HCl, pH to 6.3 immediately before use) and the S/N removed. The bound His-tagged proteins were eluted in 2x 500 µl of buffer D (8 M urea, 100 mM NaH_2PO_4, 5 mM imidazole, 10 mM Tris-HCl, pH to 5.9 immediately prior to use) and 2x 500 µl buffer E (8 M urea, 100mM NaH_2PO_4, 5 mM imidazole, 10 mM Tris-HCl, pH to 4.5 immediately prior to use). Elution samples were mixed with an equal volume of 2x SDS-sample buffer prior to electrophoresis on a SDS-15% polyacrylamide gel.

2.16 Polyclonal Antisera Preparation

2.16.1 Protein Preparation and Immunisation Schedule

New Zealand outbred white rabbits (female) were obtained from the Central Animal House, University of Adelaide. SDS-15% PAGE purified protein was used to immunise the rabbits. Briefly, inclusion body samples were electrophoresed on SDS-15% PAGE and stained with Coomassie Brilliant Blue G250, destained with 5% (v/v) acetic acid and the protein of interest was excised from the gel and re-electrophoresed to remove contaminating proteins. The protein band (corresponding to approximately 250 µg) was excised from the gel and emulsified with 0.5 ml of Freund's complete adjuvant and an equal volume of 1x PBS in an Ultra-Turrax Blender (Janke and Kunkel). At day 0 the rabbit was immunised subcutaneously with 10 injections of the protein homogenate at different sites (100 µl per

injection). The rabbit was boostered 3 times at day 14, day 41 and day 60 with 10 subcutaneous injections at different sites on the rabbit, with a protein homogenate in Freund's incomplete adjuvant. The rabbit was exsanguinated by cardiac puncture under anaesthesia 14 days after the last immunisation. The blood obtained was incubated at 37°C for 2 h, the clot removed by centrifugation (10 min, 1500 rpm, Centra-4X IEC bench centrifuge) and the serum stored at -20°C.

2.16.2 Absorption of immune serum

Rabbit antiserum was absorbed with live *E. coli* strain BL21 (DE3) harbouring plasmid vectors used in the specific cloning as well as *sefA* deficient *S.* Enteritidis strain PE908 (for absorbing anti-SefA antiserum only). 50 ml of overnight culture was centrifuged (10 min, 4500 rpm, Centra-4X IEC bench centrifuge) and resuspended in 5 ml of serum (to give ~10^{10} bacteria/ml) in the presence of 0.02% (w/v) sodium azide. Absorptions were carried out by incubating the serum at 37°C for 45 min with periodic inversion to resuspend settled bacteria. After each absorption, the bacteria were removed by centrifugation (10 min, 4500 rpm, Centra-4X IEC bench centrifuge). The process was repeated 6 times for each bacterial strain (12 absorptions in total), and after the final absorption the serum was passed through a 0.2 μm Millipore filter.

2.17 β-galactosidase assays

β-galactosidase activity of SefA-LacZ fusions was measured using a combination of two methods (Miller, 1972; Bignon *et al.*, 1993). Cultures were grown in CFA broth at 37°C with aeration for 16 h, cooled on ice and OD_{600} measured (Biorad SmartSpec™ 3000 Spectrophotometer). Cultures were diluted in ice-cold CFA broth such that all had OD_{600} values in the range of 0.550-0.850. In a microfuge tube, diluted culture (500 μl) was added to 500 μl of ice-cold Z buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1

mM $MgSO_4.7H_2O$, 50 mM β-mercaptoethanol adjusted to pH 7.0 and stored at 4°C). Cells were lysed by addition of 40 µl of chloroform and 20 µl drop of SDS (0.1% w/v) and vortexing for 10 seconds. Aliquots (100 µl) of the bacterial lysates were dispensed in triplicate into a 96 well microtitre tray. The tray was incubated at 28°C for 15 minutes and the reaction started by addition of 200 µl of 2-nitrophenyl-β-D-galactopyranoside (ONPG; Roche; 0.7 mg/ml in Z buffer) to each well, with mixing. Optical densities were recorded at 410 nm (O-nitrophenol colour change) and 570 nm (cell debris). Enzyme units were calculated for each strain at the time point when the OD_{410} first exceeded 0.6 using the following equation.

$$\beta\text{-galactosidase Units} = \frac{1000 \times (OD_{410} - 1.75 \times OD_{570})}{\text{Time} \times \text{Volume} \times OD_{600}}$$

2.18 ELISA (Enzyme Linked Immuno-Sorbent Assay) Inhibition Assays (EIA)

2.18.1 Standardising EIA Reagent Conditions

Reagent concentrations for optimal detection of SefA were determined as follows: 96 well flat-bottom trays (Nunc) were coated with duplicate aliquots (100 µl) of SefA protein (section 2.13.2) diluted in TSA buffer (25 mM Tris pH 7.5, 132 mM NaCl, 0.05% (w/v) sodium azide) and incubated at 4°C overnight. A negative control (TSA only) was also included. The coating solution was removed and the trays washed 3x by immersion in wash buffer (10 mM Tris pH 7.6, 300mM NaCl, 0.2% (v/v) Triton X-100) with buffer removed between washes by patting trays on paper towel. Trays were blocked with 200 µl blocking solution (1% (w/v) BSA, 0.05% (v/v) Tween-20 in 1x PBS) for 2 h at 37°C. The blocking solution was removed, and without washing, primary antibody (diluted in blocking solution) was added in 100 µl aliquots. This was allowed to incubate for 4 h at 37°C, followed by 3 washes in wash buffer. 100 µl of alkaline phosphatase conjugated goat anti-rabbit IgG (KPL;

diluted at 1/30,000) was added to the appropriate wells and the trays incubated at 4°C overnight. Following incubation, trays were washed 5x in wash buffer, followed by addition of substrate solution (1 mg/ml p-Nitrophenyl Phosphate (Sigma) in assay buffer (1 M diethanolamine, 0.05% (w/v) sodium azide, 1 mM $MgCl_2$, pH 9.8; 100 µl per well) and incubation for 4 h at 37°C. The OD_{405} (substrate) and OD_{600} (background) values were determined (Labsystems Multiskan *Ascent*) and the corrected OD_{405} plotted against the concentration of SefA (µg/ml) for each antibody concentration.

2.18.2 Sample Preparation for EIA

Bacterial samples were prepared for EIA analysis by growing the required strains on CFA supplemented with appropriate antibiotics and supplements, harvesting into 1 ml of 1x PBS with 0.5% (w/v) sodium azide and incubating for 1 h at 37°C. After incubation, the OD_{600} of each sample was determined and bacterial concentration adjusted to $2x10^{10}$ bacteria/ml. Samples were prepared immediately prior to use and were not stored in any way.

2.18.3 ELISA Inhibition Assays (EIA)

Surface expression of SefA was quantified by EIA, adapted from a method used by Hone *et al* (1988). Briefly, 5 µg/ml of SefA in TSA buffer was used to coat 96 well trays O/N at 4°C. Trays were washed in three changes of wash buffer and blocked with 200 µl of blocking solution for 2 h at 37°C. SefA standards were prepared by diluting stock solution to 10 µg/ml in blocking solution. 62.5 µl of standard was added in duplicate and serially diluted 1/5 (12.5 µl in 50µl blocking solution) across the tray (5 µg/ml SefA in first well). 75 µl of bacterial suspension were added in duplicate and serially diluted 1/3 (25 µl in 50 µl blocking solution) across the tray (10^9 bacteria in first well). 50 µl of primary antibody (1/15,000) was added to each well resulting in a final dilution of 1/30,000. Negative control wells received 100 µl of blocking solution only. After samples and primary antibody had been added, the

trays were mixed briefly (15 sec; 800 rpm, Flow Laboratories TiterTek) and incubated at 37°C for 4 hr. After incubation the trays were washed with three changes of wash buffer as before and 100 µl of Alkaline phosphatase conjugated goat anti-rabbit IgG (diluted at 1/30,000) was added and the tray incubated at 4°C O/N. The trays were washed with five changes of wash buffer and developed by addition of substrate solution and incubation for 4 h at 37°C. The OD_{405} (substrate) and OD_{600} (background) values were determined (Labsystems Multiskan *Ascent*). Corrected OD_{405} values of the standards were plotted against the log of the SefA concentration (Log_{10}[SefA]) in each well to generate a standard inhibition curve for each tray using GraphPad Prism (GraphPad Software Incorporated). For each bacterial sample, Log_{10}[SefA] values were calculated from two or more corrected OD_{405} values that fell within the linear range of the standard curve. The inverse log of these values were calculated, multiplied by the dilution factor to give the concentration of SefA per 10^9 bacteria, multiplied by 10 to give the concentration of SefA per 10^{10} and averaged to give the calculated SefA concentration per 10^{10} bacteria for that sample. Listed below are the concentrations of SefA and number of bacteria in each well after serial dilutions and addition of primary antibody.

Well	SefA standards (µg/ml)	Bacteria per well
1	5	$1x\ 10^9$
2	1	$3.3x\ 10^8$
3	0.2	$1.1x\ 10^8$
4	0.04	$3.7x\ 10^7$
5	0.008	$1.23x\ 10^7$
6	0.0016	$4.12x\ 10^6$
7	0.00032	$1.37x10^4$
8	0.000064	$4.57x10^5$

Samples that returned OD_{405} values above the linear range of the standard curve for that tray were considered to be negative i.e. no SefA was detectable. The level of detection for these assays was tray dependant i.e. the level of detection would vary according to the standard

curve for each tray. The level of detection for these assays was defined as the concentration of SefA that would give a 50% reduction in colour development, which in the absence of SefA was ~0.9 OD units and; the level of detection was calculated to be ~0.01 μg of SefA per 10^{10} bacteria.

2.19 Immunogold Electron Microscopy

Colloidin-coated copper-palladium grids (200 mesh, Centre for Electron Microscopy and Microstructure Analysis, Adelaide) were treated coat side down with 40 μl of poly-L-lysine (100 μg/ml) for 15 min, washed twice in MQ water (40 μl per wash) with excess liquid removed between each successive wash with pieces of Whatman 3MM chromatography paper. The grid was then placed onto a 40 μl suspension (~10^8 bacteria/ml) of bacterial cells grown on CFA agar, which had been carefully resuspended in 1x PBS, for 5 min. The grid was blocked with 40 μl of 1% (w/v) BSA in 1x PBS (1% BSA-PBS) before incubation on 40 μl of antiserum (diluted 1/10 in 1% BSA-PBS) for 15 min. The grids were washed a further two times in 1% BSA-PBS and incubated for 15 min on 20 μl of protein A-gold particles (20 nm; Amersham; diluted 1/40 in 1% BSA-PBS). The grids were washed twice in 1x PBS (40 μl), incubated on 20 μl of 1% (w/v) uranyl acetate (Fluka) for 30 sec followed by two washes with MQ. The grids were then inverted and allowed to dry. Samples were examined using a Phillips CM-100 transmission electron microscope at an acceleration voltage of 80 kV.

Chapter 3: Cloning and analysis of DNA 3' of the *sefD* gene

3.1 Introduction

The SEF14 operon of *S.* Enteritidis has been the subject of several studies over the past decade, with an emphasis on both its role in pathogenesis and the distribution of *sef* genes in *Salmonella* sp. and other Enterobacteriaceae (see section 1.9). Despite these studies, neither the role of SEF14 fimbriae in *S.* Enteritidis pathogenesis remains unclear, nor has the mechanism of regulation for SEF14 fimbriae been elucidated, although there has been some speculation on mechanisms involved (see section 1.9.3). The organisation of genes in the SEF14 operon, along with relevant restriction endonuclease sites, is shown in Figure 3.1. This chapter describes the cloning and characterisation of DNA immediately downstream of *sefD*, based on the hypothesis that the gene(s) involved in the regulation of SEF14 expression is located downstream of the *sefD* gene.

3.2 Cloning of DNA 3' of *sefD*

At the beginning of this project, the DNA downstream of *sefD* was unknown. In order to clone the DNA downstream of the *sefD* gene, two different methods were employed: inverse-polymerase chain reaction (iPCR; Ochman *et al*, 1988), and single specific primer PCR (SSP-PCR; Shyamala and Ames 1989).

The use of these methods is dictated by the availability of useful restriction sites within the target DNA area, in this case the *sef* genes. As outlined below, several sites were found and utilised during the cloning procedure.

3.2.1 Inverse-polymerase chain reaction

The iPCR method (Figure 3.2) involved digesting chromosomal DNA (section 2.10.2) with either *Hin*dIII or *Nde*I, which restrict at nt position 5015 within *sefD* and nt position 4605 within *sefC*, respectively. Once digested, the enzymes were inactivated and the DNA

Figure 3.1: Relevant restriction endonuclease sites within the SEF14 operon.

Restriction endonuclease sites relevant to this study are indicated above the DNA, while the solid and dashed arrows show the direction of transcription. The left-most *Hin*dIII site serves as nucleotide #1 and corresponds to the beginning of the submitted sequence for *sefA* (accession number X98516), incorporating 918 bp upstream of *sefA*, while the end of the *sefD* gene corresponds to nucleotide #5185.

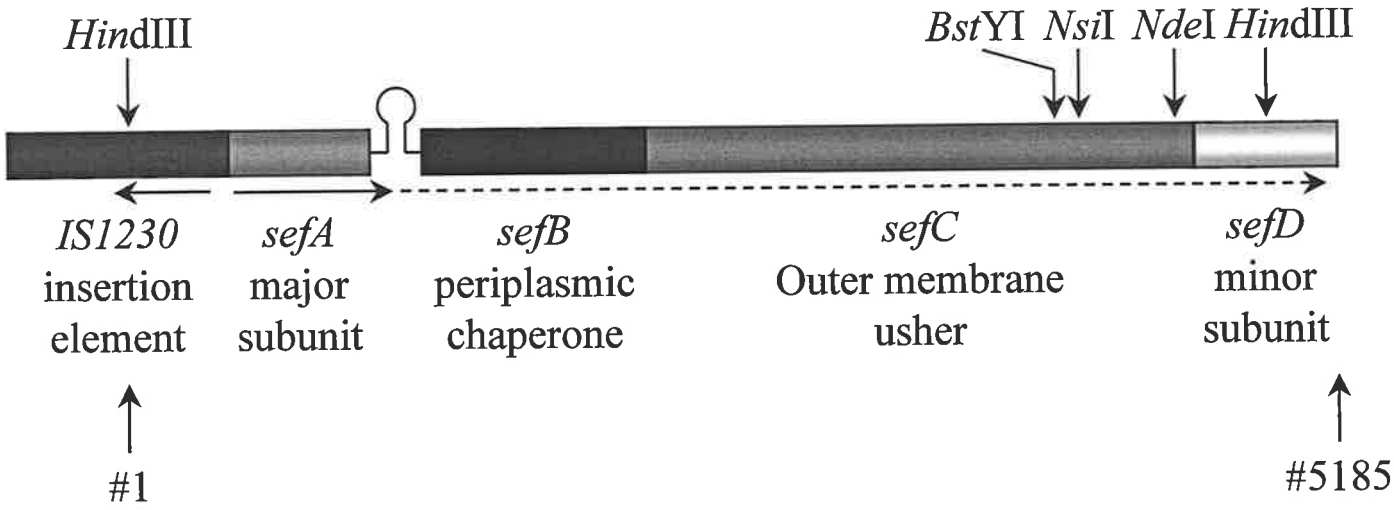
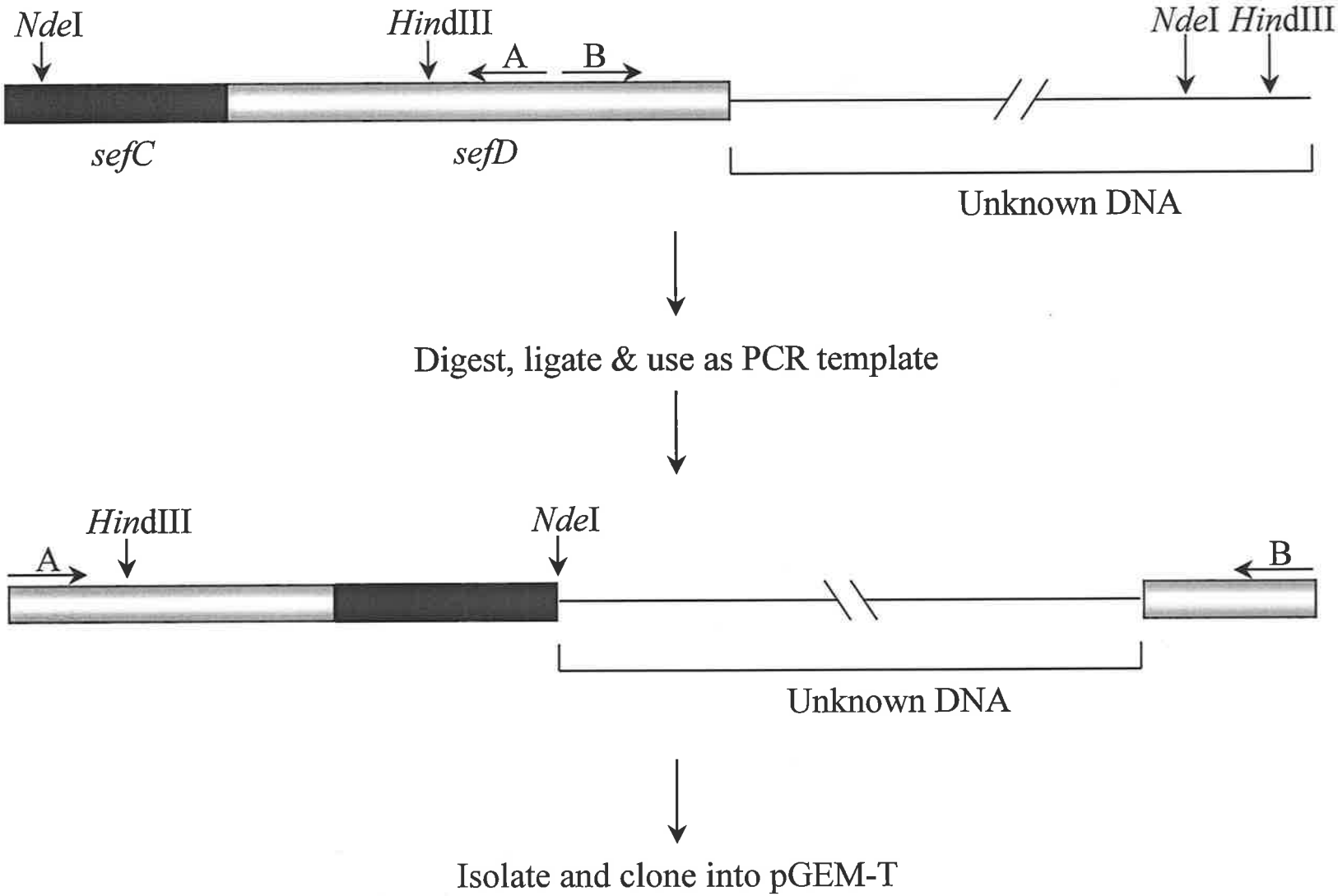

HindIII
BstYI
NsiI
NdeI
HindIII
IS1230
insertion
element
sefA
major
subunit
sefB
periplasmic
chaperone
sefC
Outer membrane
usher
sefD
minor
subunit
#1
#5185

Figure 3.2: Diagrammatic representation of the iPCR method.

This diagram shows an example of the iPCR method using chromosomal DNA digested with *Nde*I as an example. Digestion with *Nde*I gives rise to a DNA fragment containing a portion of *sefC*, the entire *sefD* gene as well as an unknown amount of DNA downstream of the *sefD* gene. This fragment is ligated to itself and serves as a template using primers as indicated by A and B. This PCR reaction gives rise to a PCR product flanked by portions of the *sefD* gene, which can be cloned and sequenced.


NdeI
HindIII
A
B
NdeI HindIII
sefC
sefD
Unknown DNA
Digest, ligate & use as PCR template
HindIII
NdeI
A
B
Unknown DNA
Isolate and clone into pGEM-T

ligated to itself in order to form closed circular chromosomal fragments, which would serve as PCR templates. In both cases the target DNA was amplified using primers 2743 and 2744 (Table 2.3), which bind within *sefD* such that they extend outwards from each other. PCR reaction conditions were designed to amplify DNA products up to 3 kb in length (Morona *et al*, unpublished observations; see section 2.10.1).

Samples of the PCR reactions were analysed by agarose gel electrophoresis and the results of this are shown in Figure 3.3. No PCR product was seen when using the template generated from *Hin*dIII digested DNA. This suggested the next *Hin*dIII site was further than 3 kb downstream of the site within *sefD*. A product of slightly greater than 720 bp was amplified when using template generated from DNA digested with *Nde*I. Based on the position of the *Nde*I restriction site within *sefC* and the positions of the primers within the *sefD* gene, it was calculated that approximately 150 bp of this fragment would actually be unique DNA downstream of the *sefD* gene, and as such would not be particularly useful in characterising this region.

3.2.2 Single specific primer-polymerase chain reaction

A schematic representation of the SSP-PCR method is depicted in Figure 3.4. This method involved digesting chromosomal and vector DNA such that they could be ligated together to produce templates for subsequent PCR reactions. These reactions utilised the specific chromosomal primer 2404 (Table 2.3), which binds the *sefD* gene at nt positions 165-188 (nt positions 4904-4927 relative to beginning of *sefA* promoter region). It was paired with the generic vector primers M13 forward or M13 reverse in two separate PCR reactions, with the expectation that only one of the two reactions would give rise to a PCR product (see section 2.10.3).

Analysis of the *sefC* and *sefD* genes using DNASIS failed to reveal any restriction enzyme sites that matched those found in the multiple cloning sites of pBluescript-KS+

Figure 3.3: Agarose gel electrophoresis of iPCR reactions.

iPCR was performed using primer 2743 and 2744 as per section 2.10.2, using template DNA restricted with *Hin*dIII or *Nde*I, and ligated as per section 2.9.7. 10 µl of each iPCR reaction was mixed with 3 µl of tracker dye and analysed by 1% TAE agarose gel (section 2.9.3). The contents of each lane are described in the legend below and the sizes of each marker fragment are shown.

M = *Eco*RI digested SPP1 Markers

1 = DNA digested with *Hin*dIII, amplified with primers 2743 and 2744

2 = DNA digested with *Nde*I, amplified with primers 2743 and 2744

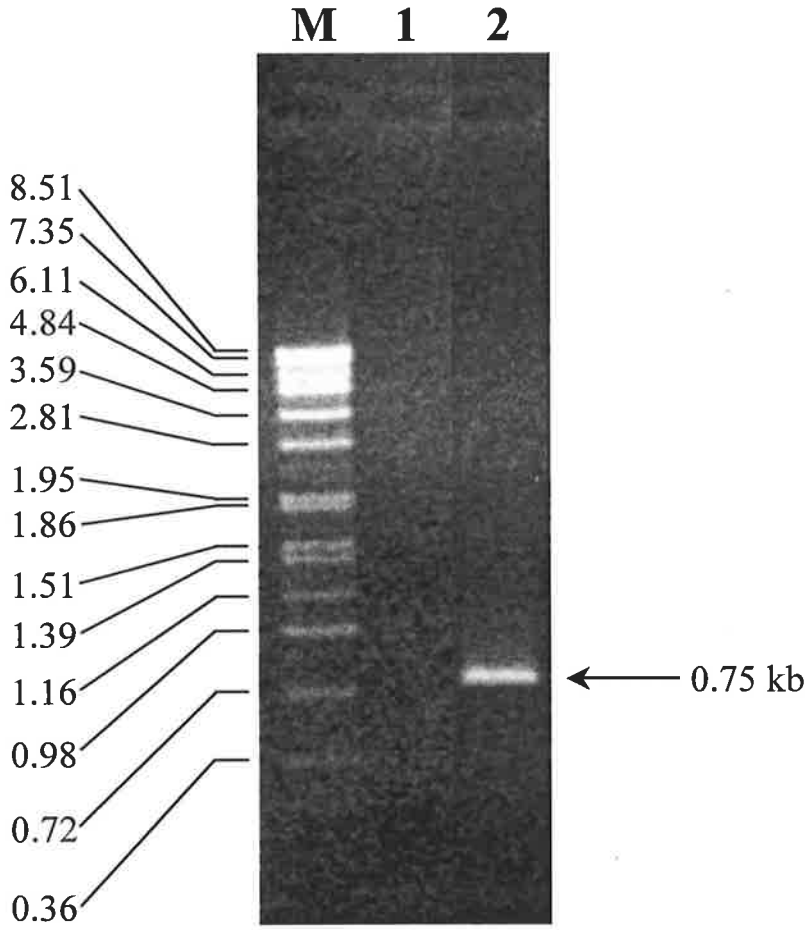
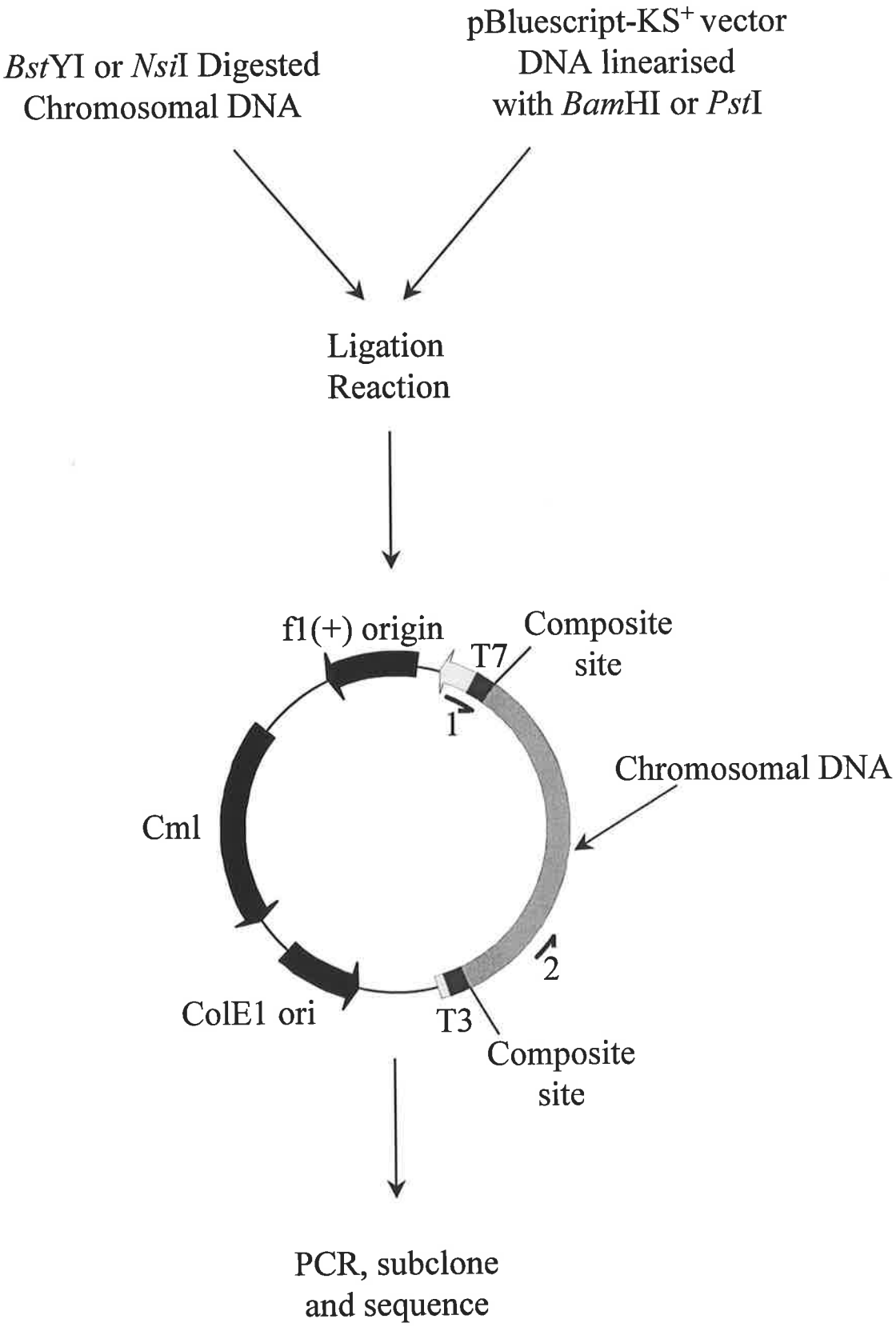

M
1
2
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
0.36
0.75 kb

Figure 3.4: Diagrammatic representation of the SSP-PCR method

This diagram shows the steps involved in generating template for the SSP-PCR method (section 2.10.3) using *Bst*YI/*Bam*HI and *NsiI*/*Pst*I digests. Primers sites 1 & 2 represent M13 vector priming site (either forward or reverse) and *sefD* primer 2404, respectively (Table 2.3). Composite sites refer to the joining of *Bst*YI/*Bam*HI and *NsiI*/*Pst*I digested fragments. PCR reactions were carried out as per section 2.10.1.


*Bst*YI or *Nsi*I Digested
Chromosomal DNA
pBluescript-KS$^+$ vector
DNA linearised
with *Bam*HI or *Pst*I
Ligation
Reaction
f1(+) origin
T7
Composite
site
1
Chromosomal DNA
Cml
2
ColE1 ori
T3
Composite
site
PCR, subclone
and sequence

(Stratagene). However, the sites for the enzymes *Bst*YI and *Nsi*I were located in this region and found to be suitable to use in this protocol as their cleavage sites have cohesive ends compatible with the common vector enzymes *Bam*HI and *Pst*I respectively. Both these enzymes cut within *sefC* (*Bst*YI at nt position 2076 and *Nsi*I at nt position 2190), with no sites within *sefD*. After digesting both the chromosomal and pBluescript-KS+ vector DNA with the appropriate enzymes, the fragments were ligated together to create the PCR templates. As mentioned above, these templates were used in two reactions each: both contained the *sefD* specific primer 2404 plus either M13 forward or reverse vector specific primers (Table 2.3). As for iPCR, reaction conditions were used such that products of up to 3 kb could be amplified, and samples of each PCR reaction were analysed by agarose gel electrophoresis.

Lanes 1, 2 and 3, 4 of Figure 3.5 show the reactions for *Bst*YI and *Nsi*I digested DNA, respectively. *Bst*YI digested DNA amplified with 2404 and M13 forward primers gave the least desirable results, with many bands, probably non-specific, seen on the gel. *Bst*YI digested DNA amplified with 2404 and M13 reverse primers resulted in two bands of ~800 bp and ~1.5 kb. *Nsi*I digested DNA amplified with 2404 and M13 forward resulted in a single strong band of ~720 bp. Finally, *Nsi*I digested DNA amplified with 2404 and M13 reverse primers resulted in a single band of ~1.7 kb, which was then cloned and sequenced as outlined below.

3.2.3 Cloning of 1.7kb fragment into pGEM-T Easy

The 1.7 kb PCR fragment obtained by SSP-PCR was purified (section 2.10.1) and then ligated into pGEM-T Easy (Promega; section 2.10.5). The ligation reaction was transformed into chemically competent DH5α (section 2.7.2), and grown on LA supplemented with Amp, IPTG and X-gal (section 2.4) to facilitate blue/white colour selection of transformants. Plasmid DNA was prepared from 24 white colonies and of these, only one showed the expected DNA band by both PCR (using M13 forward and reverse primers) and restriction

Figure 3.5: Agarose gel electrophoresis of SSP-PCR samples.

SSP-PCR was performed as per section 2.10.3, using template DNA digested with either *Bst*YI or *Nsi*I, and ligated with pBluescript-KS+ (Stratagene). 10 µl of each iPCR reaction was mixed with 3 µl of tracker dye and analysed by 1% TAE agarose gel (section 2.9.3). The contents of each lane are described in the legend below and the sizes of each marker fragment are shown.

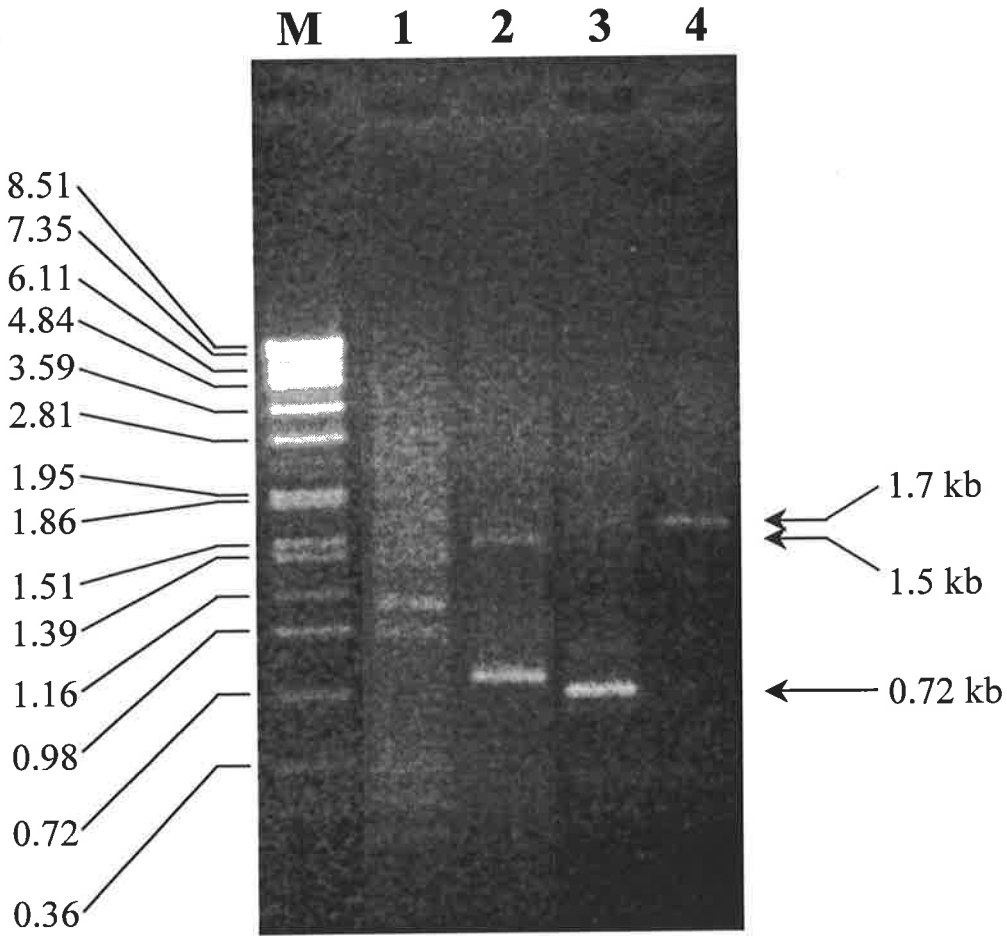
M = *Eco*RI digested SPP1 Markers

1 = DNA digested with *Bst*YI, amplified with primers #2404 and M13 Forward

2 = DNA digested with *Bst*YI, amplified with primers #2404 and M13 Reverse

3 = DNA digested with *Nsi*I, amplified with primers #2404 and M13 Forward

4 = DNA digested with *Nsi*I, amplified with primers #2404 and M13 Reverse


M
1
2
3
4
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
0.36
1.7 kb
1.5 kb
0.72 kb

digestion with *Eco*RI (data not shown); the plasmid construct was designated pRJ001, and the strain containing pRJ001 designated RMJ001.

3.3 Analysis of clone pRJ001

3.3.1 Sequence analysis

In order to determine the nature of the unique DNA obtained by SSP-PCR, sequencing of clone pRJ001 was performed as outlined in section 2.11.1 using the primers 2404 and M13 reverse (Table 2.3). Analysis of obtained DNA sequences was performed using DNASIS v2.5 (Hitachi Software Engineering Co.). Initial analysis of the sequence obtained using primer 2404 showed 283 bp of the 3' end of the *sefD* gene, with a single T to A substitution at position 377 of the published *sefD* sequence. An ATG start codon was found 51 bp downstream of the *sefD* stop codon, however 84 bp further downstream a TAA termination codon was found. Also, no significant RBS or -10 elements were found in the expected positions relative to the observed ATG codon. This suggested either that there were no ORFs beginning immediately downstream of *sefD*, or that if an ORF was present it was in the opposite orientation to the known SEF genes. The complementary sequence of this DNA fragment was also analysed for ORFs, however none of any significant size were found.

Analysis of sequence obtained using the M13 reverse primer revealed no significant ORFs. However, an interesting observation made about this sequence data was the presence of several ATG codons towards the 3' end (approximately 900 bp downstream of *sefD*). These ATG codons did not appear to be part of continuous ORFs, however they were found at the extreme end of the obtained sequence data. It was therefore hypothesised that one of these ATG codons was in fact a true start codon and that further sequence data would reveal an ORF beginning approximately 900 bp downstream of *sefD* and orientated in the opposite direction to the other *sef* genes.

To validate this hypothesis, new primers were designed from the sequence data obtained previously and used to sequence the full length of both strands of the 1.7 kb insert in clone pRJ001. The sequencing strategy and the primers (Table 2.3) used are shown in Figure 3.6. The individual sequences obtained were assembled into a contiguous fragment using Autoassembler v1.3.0 (Applied Biosystems).

Once assembled, the entire fragment was analysed for the presence of ORFs. The 5' region of the 1.7 kb insert in pRJ001 contained the 3' end of *sefD*, as expected. A new ORF was found just downstream of *sefD*, oriented in the opposite direction to the known SEF genes, which supported the proposed hypothesis. This ORF was found to be 813 bp in length and so was initially designated ORF813. Interestingly, restriction analysis of the completed sequence revealed an *Nde*I site 169 bp downstream from the end of *sefD*, confirming the analysis of the iPCR result obtained prior to sequencing (section 3.2.1).

3.3.2 Defining ORF813 and surrounding DNA.

After assembling the DNA fragments, the entire region was analysed for gene elements. A summary of these elements is shown relative to the DNA sequence in Figure 3.7. Note that the DNA sequence is presented in the opposite orientation relative to the *sefABCD* genes. An adenine residue 86 bp (nt position 345) upstream of the start codon (nt position 430) was predicted to be the most probable transcriptional start site (boxed residue in Figure 3.7) using predictive promoter analysis (section 2.11.2). A putative -10 TATA box (ATAATCA) was found at nt positions 330-336 inclusive, while the sequence at the -35 element site was TTAGATAT (nt positions 303-310 inclusive), matching 5/7 bases of the -35 consensus sequence (TTGACAT). Finally, a putative ribosome-binding site (RBS; AGGTGCG) was found at nt positions 418-424 inclusive.

The amino acid translation of ORF813 was determined using DNASIS, revealing a 271 aa polypeptide with a predicted molecular weight of 31.4 kDa. A summary of the amino

Figure 3.6: Sequencing strategy for clone pRJ001.

The labelled arrows represent individual sequencing reactions using the primers 2404, 2798, 2799, 2889, 2890, 2927 and M13 Reverse (Table 2.3). The *Nde*I site used in iPCR (section 3.2.1) is shown. The relative positions of *sefD*, ORF813 and vector DNA are indicated.

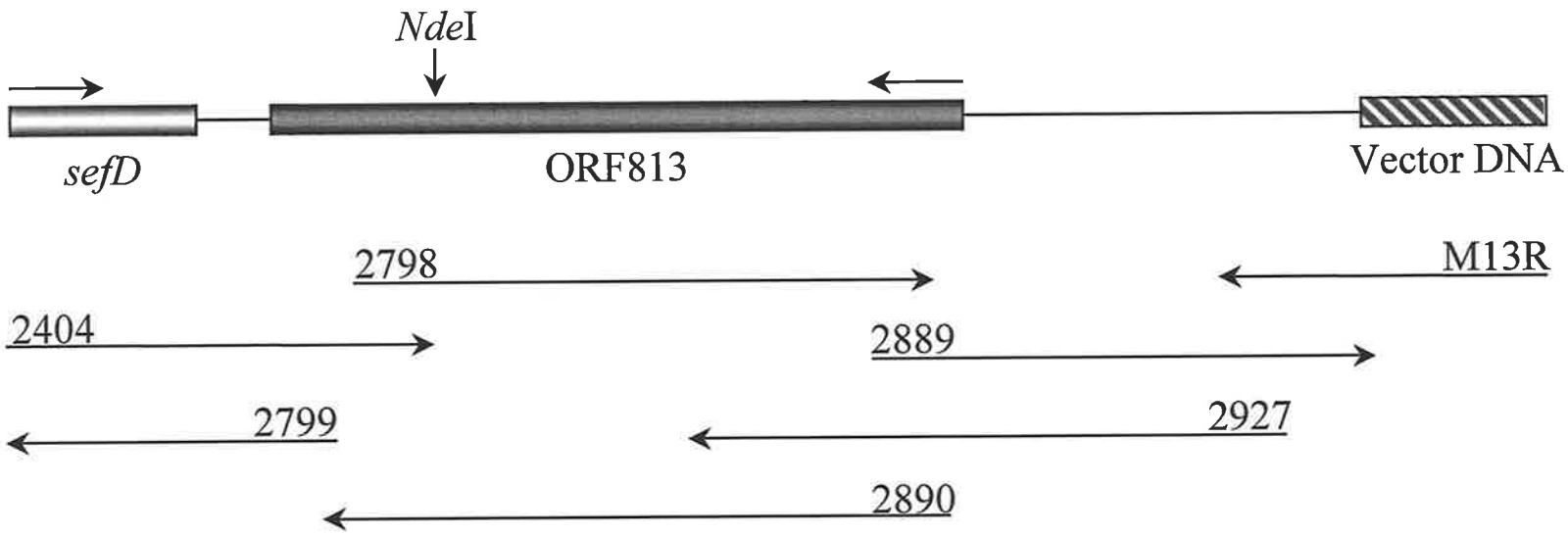

NdeI
sefD
ORF813
Vector DNA
2798
M13R
2404
2889
2799
2927
2890

Figure 3.7: Nucleotide and amino acid sequence from clone pRJ001

The nucleotide sequence presented here represents DNA that was sequenced from pRJ001, of which 1278 bp is DNA that has not previously been sequenced. The -35 (single underline; nt 303-310) and -10 elements (dotted underline; nt 330-336) are indicated. The boxed adenine residue at nt position 345 is the predicted to be the +1 transcriptional start site. The RBS is located at nt positions 418-424 (double underline). The aa sequence (nt 430-1242) is displayed by single letter code. The bold nucleotides (nt 1279-1488) represent the 3' end of the *sefD* gene. Positions 1 and 1488 represent nt positions 6462 and 4976 relative to the *Hin*dIII site upstream of *sefA* (See figure 3.1). The DNA sequence was submitted to GenBank and was assigned the accession number AF233854.

```
1    ATA TTC ACT TTC ATC AGA TTC ACT CGA GTA TTG GTT ATG TAT AAA GCC CTA CCT TTA AGA   60

61   CGT CAC ATC AAA GGC ATT GCA GTA TCC CCA TTC AAC ATT TAT ACG GAG AAT TTA AAA TCT  120

121  TAG GAG ATA GTC ATA TCT ATT TTT ATA GAT CCA ATC AAG CCT GCC CGG ACT GTA CAG TAC  180

181  CGG CAT TAA AAG AAA TAT CAC ATG GCT CTC CGT TGG ATA TAG ATG AAC ATA ATT AAT TGA  240

241  GCA TAA CGA AAC AAC AAC TCC TAT AAA GCA CTT ATA ACG ACC ACA CAT GGC TTA CAC TGC  300

301  ATT TAG ATA TTT TAA AAC ATC ACC TTC GGA TAA TCA TAT CTA ATA AAG TTA TTT TTA TAT  360
          -35                                  -10

361  CAT AAA ACA ATA CCT ACA ATA ATA TAA AAT CGG CTT CTG CAG TGA AGC GTG AAA ATT AGG  420

421  TGC GAT TCC ATG TTG AAA AAA AAC GCC ATA AAA ATA AAA CTA TAT CGT TAT GCT ATT TTA  480
1     RBS        M   L   K   K   N   A   I   K   I   K   L   Y   R   Y   A   I   L   17

481  CAT TCG AAA AAC TGT ATT GTT ACC ATT GAG AAC AAG TCA AAG CCA GAG GAA ATA AAA ATA  540
18    H   S   K   N   C   I   V   T   I   E   N   K   S   K   P   E   E   I   K   I   37

541  ACT AGA GGC AAC ATA GCC TTA ATA GAA AAA AAT ATA GAA GCC GTT GTG GAA ATT GAA TAT  600
38    T   R   G   N   I   A   L   I   E   K   N   I   E   A   V   V   E   I   E   Y   57

601  ATG GAT GAC ATT GAA TCA TTT GAC ATT ATT ACT TTG CCA GAT GAA TTA TTA AGT AGA GTT  660
58    M   D   D   I   E   S   F   D   I   I   T   L   P   D   E   L   L   S   R   V   77

661  TTA TGC TTA TTT GAG GCT TCT AAT TGC TCA GAA AGT TTA TCA CCA ATA CGC TAC AGA ACA  720
78    L   C   L   F   E   A   S   N   C   S   E   S   L   S   P   I   R   Y   R   T   97

721  TTT AGC GAT AAG GTT TTT ATT ATA ACC GAC AAT GGA ATT AAT GGA ATT TTA TTT GGA TAC  780
98    F   S   D   K   V   F   I   I   T   D   N   G   I   N   G   I   L   F   G   Y  117

781  TTA AAA AAG AGA AAA AAT AAC AAT AAT GAT ATT TAT GAA ATT GCC TGC TTA TTT TCA AAA  840
118   L   K   K   R   K   N   N   N   N   D   I   Y   E   I   A   C   L   F   S   K  137

841  GTG AAT AAT ATC GAG CAG CTA TAC ACA TCT CTG TGC ATT TCA GTC TCA CGT AGT TTT TCT  900
138   V   N   N   I   E   Q   L   Y   T   S   L   C   I   S   V   S   R   S   F   S  157

901  GAT ATT GTT AGA AAA ACA ATA GAT AAT GAC ATT TCA ACG AAA TGG AGA TTA AAA ACA TTA  960
158   D   I   V   R   K   T   I   D   N   D   I   S   T   K   W   R   L   K   T   L  177

961  TCC GAA AAA CTA AAT TTA TCG GAA GTG ACT ATC AGA AAA AAA CTT GAG AAT GAA AAT ACT 1020
178   S   E   K   L   N   L   S   E   V   T   I   R   K   K   L   E   N   E   N   T  197

1021 AAT TTT TAC AGA ATC TTT CTG GAT GCC AGG ATG CAA AAA GCA GCG CGT TTG GTG CTT GAT 1080
198   N   F   Y   R   I   F   L   D   A   R   M   Q   K   A   A   R   L   V   L   D  217

1081 AGC GAC ACC CAT ATT AAT AAA GTA TCA TAT GCC GTA GGA ATG TCA AGC GTA TCA TAT TTC 1140
218   S   D   T   H   I   N   K   V   S   Y   A   V   G   M   S   S   V   S   Y   F  237

1141 ATT AAA TTA TTT TCT GAC TAT TAT GGC TTA ACC CCA AAA CAA TTC CAT CTA AAA TAT AAG 1200
238   I   K   L   F   S   D   Y   Y   G   L   T   P   K   Q   F   H   L   K   Y   K  257

1201 CAT AGA AAT ACA GGA GAA AAA GCT GCA TTT ATG CTT TAT AAT TAA GAG TTA TAG GTG CGC 1260
258   H   R   N   T   G   E   K   A   A   F   M   L   Y   N   *                      271

1261 CAA TTT GCG CAC CCT TTA TTA TAA TTC AAT TTC TGT CGC ATA TAT GCT TAT TAA ATA TGT 1320

1321 GTC AAC AGG AAT GTC TCC ATT CCC AAA ATA ATA AAT ATT AAA TTC ATT AGT AAA ATC AGA 1380

1381 ATG AGA TAC CAT ACC AAT ACC TGA ATT ATC TGG TTG CCA GTC TTC TCC ACC TAT TCT TAT 1440

1441 CCT AAG CTT ATG GGC TGA GTT ATT CTG ACC TCT AAT AAT ATA TGC CCC                  1488
```

acid composition/codon usage is shown in Table 3.1. Of note is the high proportion (22.1% or 60 in total) of rare codons, defined by Wada *et al* (1992) as those used by *E. coli* at a frequency of <1%. The implications of this are discussed in section 3.5.

It was found that ORF813 terminated 33 bp from the end of the *sefD* stop site. Analysis of this small intergenic region for stem-loop and repeat elements revealed a single stem-loop structure containing 7 bp in the stem region with a 6 bp loop (see Figure 3.8). The dissociation energy was calculated to be -16.30 kcal/mol and it was thought that this structure acts as a bi-directional transcriptional terminator of the *sefABCD* and *sefR* mRNA transcripts.

3.3.3 Analysis of ORF813 sequence

Once the sequence of the unknown DNA in clone pRJ001 had been obtained, it was compared to nucleotide sequences lodged in the GenBank, EMBL, DDBJ and PDB databases by subjecting it to BLASTN analysis. While nt 1279-1488 matched exactly with the 3' end of the *sefD* gene as expected, no similarity to any other nucleotide sequences was found for the remainder of the sequence. The nucleotide sequence was then submitted for comparison against sequences in the GenBank, PDB, SwissProt, PIR and PRF databases using the BLASTX program, which compares the 6-frame translation of nucleotide sequence to protein sequences lodged in the databases. This analysis revealed a number of sequences showing significant similarity to ORF813. Table 3.2 presents a list of the proteins that were found to have significant similarity to ORF813, along with the percent identity/similarity to ORF813 and their proposed functions. The proteins with the greatest levels of similarity to ORF813 were all found to be regulators in the AraC-like transcriptional activator family, several of which have also been shown to be involved in the regulation of fimbriae. ORF813 encodes a new member of that family. Further evidence for this was found when the amino acid sequence of ORF813 was analysed using the Prosite (protein families and domains) database. ORF813 was found to possess the "AraC family signature" at amino acid positions 207-249.

Table 3.1: Amino acid composition of ORF813

This table shows a breakdown of the aa encoded by ORF813, presented as both the number of each aa and the percent of total aa's. A breakdown of the codons that are used and the number of aa's for each codon, with their percentage relative to the whole, are also shown. Note that the bold text signifies codons that are classified as rare (Kane, 1995; Wada *et al.*, 1992)

[1] Amino acids are described by the three and single letter codes

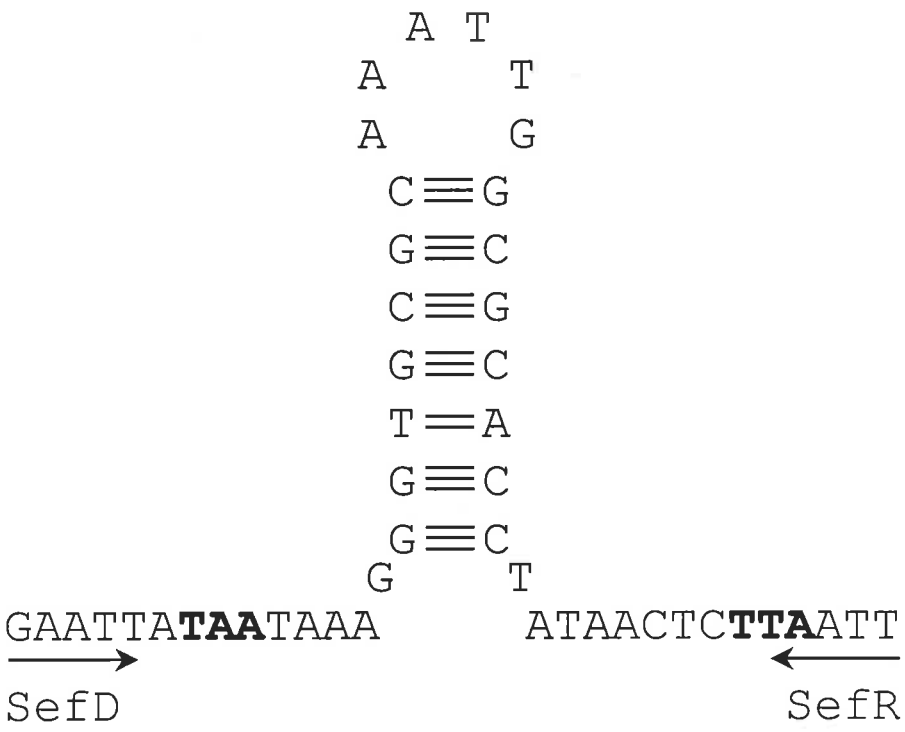
[2] Count refers to the number of aa's of a particular type

[3] Codon breakdown refers to the particular codons that are used to encode each aa

Amino Acid[1]	Count[2]	% of total amino acids	Codon breakdown[3]	Count[2]	% of total amino acids
Ala (A)	12	4.4	GCT	3	1.1
			GCC	6	2.2
			GCA	2	0.7
			GCG	1	0.4
Arg (R)	14	5.2	**AGA**	**9**	**3.3**
			AGG	**1**	**0.4**
			CGT	3	1.1
			CGC	1	0.4
Asp (D)	14	5.1	GAT	8	2.9
			GAC	6	2.2
Asn (N)	22	8.0	AAT	17	6.2
			AAC	5	1.8
Cys (C)	5	1.9	**TGT**	**1**	**0.4**
			TGC	**4**	**1.5**
Gln (Q)	3	1.1	CAA	2	0.7
			CAG	1	0.4
Glu (E)	18	6.6	GAA	13	4.8
			GAG	5	1.8
Gly (G)	7	2.5	GGC	2	0.7
			GGA	**5**	**1.8**
His (H)	4	1.5	CAT	4	1.5
Ile (I)	30	11.0	ATT	17	6.2
			ATC	3	1.1
			ATA	**10**	**3.7**
Leu (L)	27	9.9	CTT	3	1.1
			CTA	**4**	**1.5**
			CTG	2	0.7
			TTA	15	5.5
			TTG	3	1.1
Lys (K)	27	9.9	AAA	22	8.1
			AAG	5	1.8
Met (M)	5	1.8	ATG	5	1.8
Phe (F)	13	4.7	TTT	11	4.0
			TTC	2	0.7
Pro (P)	4	1.5	**CCA**	**4**	**1.5**
Ser (S)	24	8.8	**AGT**	**3**	**1.1**
			AGC	3	1.1
			TCT	4	1.5
			TCC	**1**	**0.4**
			TCA	**11**	**4.0**
			TCG	**2**	**0.7**
Thr (T)	14	5.2	ACT	4	1.5
			ACC	4	1.5
			ACA	**5**	**1.8**
			ACG	1	0.4
Trp (W)	1	0.4	TGG	1	0.4
Tyr (Y)	14	5.2	TAT	10	3.7
			TAC	4	1.5
Val (V)	13	4.8	GTT	5	1.8
			GTC	1	0.4
			GTA	3	1.1
			GTG	4	1.5
Stop	1	0.4	TAA	1	0.4
Totals (All)	271	100	-	271	100
Totals (rare)	-	-	-	60	22.1

Figure 3.8: Stem-loop structure separating the *sefD* and *sefR* genes.

A putative stem-loop structure was discovered between the *sefD* and *sefR* genes. The numbers indicate its position relative to the start of the *sefR* gene, while the numbers in brackets are the positions relative to the *Hin*dIII site at the start of the *sefA* gene. The stem is G+C rich and has a dissociation energy of -16.30 Kcal/mol, as calculated using DNASIS. The arrows indicate the direction of transcription for *sefD* and *sefR*, while their respective stop codons are shown in bold text.


A T
A T
A G
C G
G C
C G
G C
T A
G C
G C
G T
GAATTATAATAAA
ATAACTCTTAATT
SefD
SefR

Protein	Organism	Function	# of amino acids	Identity (%) ‡	Similarity (%) ‡	Accession no. §
AggR	EaggEC [1]	Transcriptional activator of aggregative adherence fimbria I	265	31	54	P43464
BfpT	EPEC [2]	positive regulatory protein of *bfpA*	274	28	51	AAB36830
CfaD	ETEC [3]	Transcriptional activator of the CFA/I adhesin	265	33	54	P25393
CsvR	ETEC	Coli surface virulence factor regulator	301	29	52	P43460
FapR	ETEC	Positive regulator of 987P pili	260	35	57	P23774
Rns	ETEC	Required for the expression of the CS1 and CS2 adhesins	265	32	53	P16114
VirF	*Shigella spp.*	Transcriptional activator of the *virB* gene	262	40	63	Q04248

Table 3.2: Proteins with similarity to ORF813

Proteins from the AraC-like family which displayed aa sequence similarity to ORF813 are listed here. % identity and % similarity are shown, along with the length of each peptide and their proposed functions.

[1] Enteroaggregative *E. coli*, [2] Enteropathogenic *E. coli*, [3] Enterotoxigenic *E. coli*

‡ Identity and similarity values were obtained by BLASTX analysis of the ORF813 aa sequence

§ GenBank accession numbers

Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) analysis revealed the presence of H-T-H domains within the region of amino acids 171-252, which are a feature of AraC-like proteins.

These results strongly suggested that ORF813 encoded for a regulator of the AraC family, and it was re-designated *sefR* (*Salmonella* Enteritidis Fimbriae Regulatory gene).

3.4 Comparison of SefR with AraC-like activators

3.4.1 Multiple alignments

The proteins that constitute the AraC family of transcriptional regulators all share a high degree of sequence similarity, primarily at the C-terminal end (a few sequences have this similarity at the N-terminus). A multiple alignment of SefR with the AraC-like proteins listed in Table 3.2 was performed using ClustalX v1.8 (Thompson *et al*, 1997) and is shown in Figure 3.9. There are 22 fully conserved amino acids (indicated by *), 19 of which fall within the C-terminal end of the sequences.

A consensus sequence for AraC-like regulators was first proposed by Gallegos *et al,* in 1993, by analysing a 99 amino acid region at the C-terminal end in 27 known AraC-like regulators. This consensus sequence was further refined (Gallegos *et al*, 1997) by comparing a more extensive list of 109 known AraC-like sequences, leading to a more statistically accurate representation of what the consensus should be. The consensus sequence was comprised of 17 key amino acids spread across a 75 amino acid region within the 99 amino acid conserved region. Figure 3.10 shows an alignment of this region from SefR and the proteins from Table 3.2, with AraC and the consensus amino acids included for comparison. Six of the 17 consensus amino acids are fully conserved in this group, while a further six are similar. A comparison of SefR directly to the consensus sequence reveals 11/17 conserved and 5/17 similar amino acids, supporting its inclusion in the AraC family.

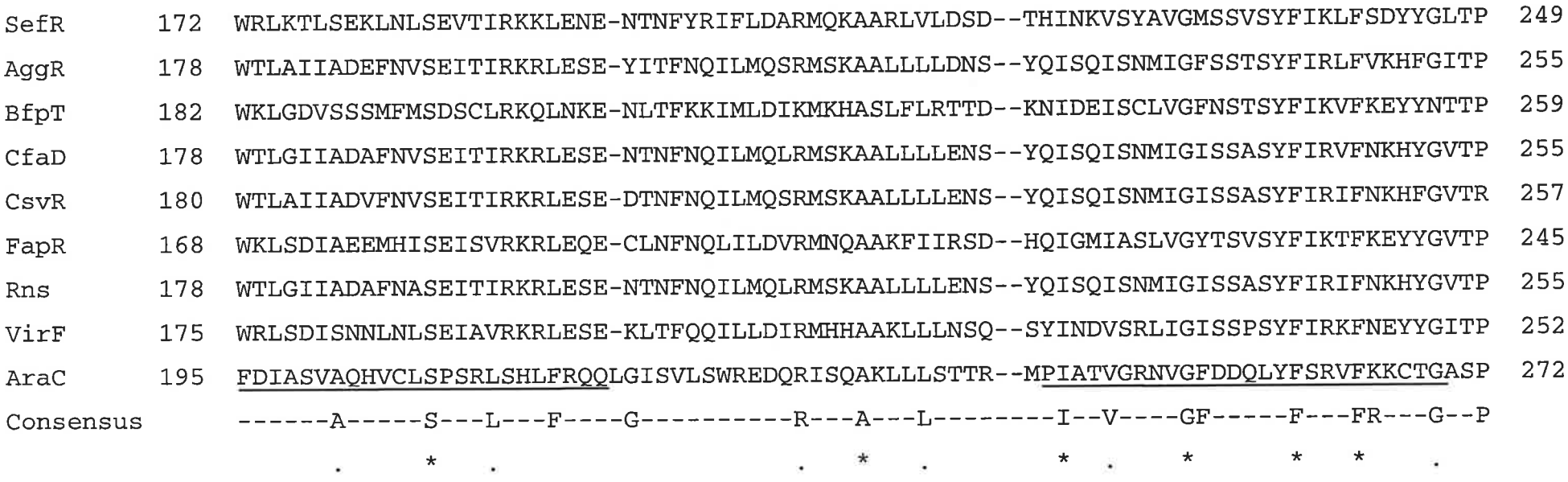
Figure 3.9: Clustal alignment of AraC-like sequences

The amino acid sequence of SefR was aligned with the amino acid sequences of the proteins from Table 3.2 using Clustal X 1.8 (Thompson *et al.*, 1997). The amino acid numbers are indicated. Fully conserved amino acids (22) are indicated by an asterix, while similar amino acids (38) are represented by a dot. The double underlined region (residues 207 - 249 in SefR) represents the AraC family signature as determined using the ScanProsite tool (http://au.expasy.org/tools/scnpsit1.html). The single underlined region (residues 172 - 252 in SefR), overlapping the AraC family signature, represents the AraC helix-turn-helix region as determined using the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

```
SEFR    1    -----MLKKNAIKIK---LYRYAILHS---KNCIVTIENKSKPEEIKITRGNIALIEKNI   49
AGGR    1    MKLKQNIEKEIIKINNIRIHQYTVLYT---SNCTIDVYTKE--GSNTYLRNELIFLERGI   55
BFPT    1    ---MLTSKKEMQSSENKQEENLALLLTNYISYQNIVIFTG---GNQFKIRNKKEFTEYTI   54
CFAD    1    MDFKYTEEKEMIKINNIMIHKYTVLYT---SNCIMDIYSEE--EKITCFSNRLVFLERGV   55
CSVR    1    MDFKYTEEKELIKINNVMIHKYTILYT---SNCILDISFGE--DKITCFNNRLVFLERGV   55
FAPR    1    ----------MKLKNIHLYNYVVIYT---KNCEIYINKGN--EQVYIPPRMVAIFEKNI   44
RNS     1    MDFKYTEEKETIKINNIMIHKYTVLYT---SNCIMDIYSEE--EKITCFSNRLVFLERGV   55
VIRF    1    --MMDMGHKNKIDIK-VRLHNYIILYA---KRCSMTVSSGN--ETLTIDEGQIAFIERNI   52

SEFR    50   E--AVVEIEYMDDIESFDIITLPDELLSRVLCLFEASNCSESLSPIRYRTFSDKVFIITD  107
AGGR    56   N-ISVRLQKKKSTVNPFIAIRLSSDTLRRLKDALMIIYGISKVDACSCPNWSKGIIVADA  114
BFPT    55   ESNSLFFLAKNTHWDMEIVGIDNSNPYRKIIIDDALIKLLHSISSDDSCYVKKKIFTANL  114
CFAD    56   N-ISVRIQKKILSERPYVAFRLNGDILRHLKNALMIIYGMSKVDTNDCRGMSRKIMTTEV  114
CSVR    56   N-ISVRIQKQKLTEKPYVAFRLNENVLRHLKNTLMIIYGMSKIDSCECRGVSRKIMTTEV  114
FAPR    45   S-FNIETIRKG-DGVLYESFDMKHELLTSLRRVIEPSVKFAAESYTNKRSFKERIFKVKS  102
RNS     56   N-ISVRMQKQILSEKPYVAFRLNGDMLRHLKDALMIIYGMSKIDTNACRSMSRKIMTTEV  114
VIRF    53   Q-INVSIKKSD-SINPFEIISLDRNLLLSIIRIMEPIYSFQHSYSEEKRGLNKKIFLLSE  110

SEFR    108  NGINGILFGYLKKRKNN---NNDIYEIACLFSKVNNIEQLYTSLCISVSRSFSDIVRKTI  164
AGGR    115  DDSVLDTFKSIDHNDD----SRITSDLIYLISKIENNRKIIESIYISAVSFFSDKVRNTI  170
BFPT    115  NEMQLNIVSNIITDIKYSGNNKKTFKILYLLSFFDDYNDIVNVILSASSKSIVDRVIKVI  174
CFAD    115  NKTLLDELKNINSHDD----SAFISSLIYLISKIENNEKIIESIYISSVSFFSDKVRNVI  170
CSVR    115  DKMLLNVLREMMGHHND--DSSFISALIYLISKIKCNDKIIESLYMSSITFFTDKVRGVI  172
FAPR    103  CSIVIDLFKRLKDNGSP--EFTAIYELAFLVSKCENPSMFAISLFSSVAVTFSERIVTLL  160
RNS     115  NKTLLDELKNINSHDN----SAFISSLIYLISKLENNEKIIESIYISSVSFFSDKVRNLI  170
VIRF    111  EEVSIDLFKSIKEMPFG---KRKIYSLACLLSAVSDEEALYTSISIASSLSFSDQIRKIV  167

SEFR    165  DNDISTKWRLKTLSEKLNLSEVTIRKKLENENTNFYRIFLDARMQKAARLVLDSDTHINK  224
AGGR    171  EKDLSKRWTLAIIADEFNVSEITIRKRLESEYITFNQILMQSRMSKAALLLLDNSYQISQ  230
BFPT    175  ELDISKNWKLGDVSSSMFMSDSCLRKQLNKENLTFKKIMLDIKMKHASLFLRTTDKNIDE  234
CFAD    171  EKDLSRKWTLGIIADAFNVSEITIRKRLESENTNFNQILMQLRMSKAALLLLENSYQISQ  230
CSVR    173  EKDLSRKWTLAIIADVFNVSEITIRKRLESEDTNFNQILMQSRMSKAALLLLENSYQISQ  232
FAPR    161  FSDLTRKWKLSDIAEEMHISEISVRKRLEQECLNFNQLILDVRMNQAAKFIIRSDHQIGM  220
RNS     171  EKDLSRKWTLGIIADAFNASEITIRKRLESENTNFNQILMQLRMSKAALLLLENSYQISQ  230
VIRF    168  EKNIEKRWRLSDISNNLNLSEIAVRKRLESEKLTFQQILLDIRMHHAAKLLLNSQSYIND  227

SEFR    225  VSYAVGMSSVSYFIKLFSDYYGLTPKQFHLKYKHRNTGEKAAFMLYN-------------  271
AGGR    231  ISNMIGFSSTSYFIRLFVKHFGITPKQFLTYFKSQ-------------------------  265
BFPT    235  ISCLVGFNSTSYFIKVFKEYYNTTPKKYNGVYSITQGTLP--------------------  274
CFAD    231  ISNMIGISSASYFIRVFNKHYGVTPKQFFTYFKGG-------------------------  265
CSVR    233  ISNMIGISSASYFIRIFNKHFGVTRSSFLIILKEDENVFATRQGNSSLTQLTCEFKHISG  292
FAPR    221  IASLVGYTSVSYFIKTFKEYYGVTPKKFEIGIKENLRCNR--------------------  260
RNS     231  ISNMIGISSASYFIRIFNKHYGVTPKQFFTYFKGG-------------------------  265
VIRF    228  VSRLIGISSPSYFIRKFNEYYGITPKKFYLYHKKF-------------------------  262

SEFR         ---------
AGGR         ---------
BFPT         ---------
CFAD         ---------
CSVR    293  GNRLNRCTE  301
FAPR         ---------
RNS          ---------
VIRF         ---------
```

Figure 3.10: Clustal alignment of the AraC consensus region

The C-terminal ends of SefR and the proteins from Table 3.2 were aligned using Clustal X 1.8 (Thompson *et al.*, 1997), shown here in descending order of similarity to SefR. The AraC and consensus sequences are included for comparison. The underlined regions delineate the two helix-turn-helix domains common to AraC-like proteins. Fully conserved amino acids (7/17) are indicated by an asterix, while similar amino acids (7/17) are represented by a dot. The dashes in the alignment represent gaps in sequence, but represent any amino acids in the consensus sequence.


SefR 172 WRLKTLSEKLNLSEVTIRKKLENE-NTNFYRIFLDARMQKAARLVLDSD--THINKVSYAVGMSSVSYFIKLFSDYYGLTP 249
AggR 178 WTLAIIADEFNVSEITIRKRLESE-YITFNQILMQSRMSKAALLLLDNS--YQISQISNMIGFSSTSYFIRLFVKHFGITP 255
BfpT 182 WKLGDVSSSMFMSDSCLRKQLNKE-NLTFKKIMLDIKMKHASLFLRTTD--KNIDEISCLVGFNSTSYFIKVFKEYYNTTP 259
CfaD 178 WTLGIIADAFNVSEITIRKRLESE-NTNFNQILMQLRMSKAALLLLENS--YQISQISNMIGISSASYFIRVFNKHYGVTP 255
CsvR 180 WTLAIIADVFNVSEITIRKRLESE-DTNFNQILMQSRMSKAALLLLENS--YQISQISNMIGISSASYFIRIFNKHFGVTR 257
FapR 168 WKLSDIAEEMHISEISVRKRLEQE-CLNFNQLILDVRMNQAAKFIIRSD--HQIGMIASLVGYTSVSYFIKTFKEYYGVTP 245
Rns 178 WTLGIIADAFNASEITIRKRLESE-NTNFNQILMQLRMSKAALLLLENS--YQISQISNMIGISSASYFIRIFNKHYGVTP 255
VirF 175 WRLSDISNNLNLSEIAVRKRLESE-KLTFQQILLDIRMHHAAKLLLNSQ--SYINDVSRLIGISSPSYFIRKFNEYYGITP 252
AraC 195 FDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRISQAKLLLSTTR--MPIATVGRNVGFDDQLYFSRVFKKCTGASP 272
Consensus ------A-----S---L---F----G----------R---A---L--------I--V----GF-----F---FR---G--P

3.4.2 Phylogenetic comparison of SefR with AraC-like proteins

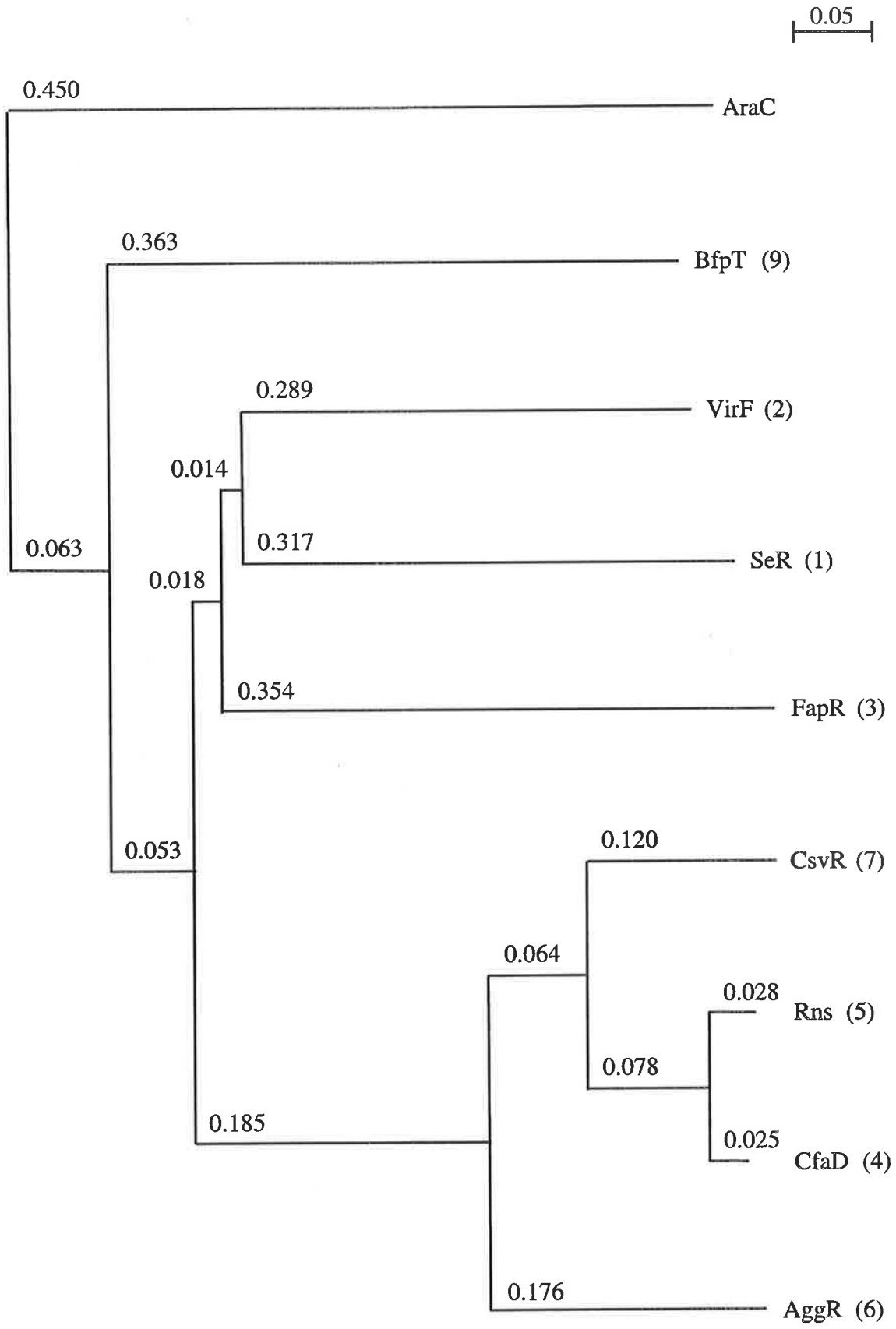
To compare the relationships between *sefR* and the AraC-like proteins listed in Table 3.2, a phylogenetic tree was constructed with NJplot (Perrière and Gouy, 1996), using the neighbour-joining method (Saitou and Nei, 1987). The tree is shown in Figure 3.11, and the branch lengths are indicated. As can be seen, AraC is an outlier in this group, which is not unexpected, as AraC plays no role in fimbrial regulation or virulence. BfpT is also more distantly related to the group, perhaps reflecting its role in regulating Type IV pili rather than the chaperone usher fimbriae, which the remainder regulate. *SefR* is paired with VirF, and lies on the same branch as FapR. Another significant grouping is the CsvR, Rns, CfaD branch. These proteins are involved in the regulation of the closely related CS1, CS2 and CFA/1 fimbriae.

3.5 Summary

Cloning and sequence analysis of DNA immediately downstream of the *sefD* gene revealed a previously uncharacterised ORF of 813 nucleotides (ORF813) that is transcribed in the opposite direction to the *sefABCD* genes. ORF813 was predicted to encode a 271 amino acid polypeptide, with a molecular weight of 31.4 kDa. Analysis of the region 5' of ORF813 (section 3.3.1) revealed various promoter elements including -10, -35 and ribosome binding sites, suggesting that ORF813 could be expressed. ORF813 was separated from *sefD* by a 33 bp gap that had the capacity to form a stem-loop structure. This stem-loop was hypothesised to act as a bi-directional terminator for both *sefD* and ORF813. ORF813 was analysed by both BLASTN and BLASTX, revealing significant similarity to regulator proteins of the AraC family. Alignment of ORF813 with these regulator proteins revealed a cluster of conserved amino acids in the C-terminal region, common to AraC-like proteins (Gallegos *et al.*, 1997). ORF813 was redesignated *sefR* to reflect its putative role as a S. Enteritidis Fimbriae Regulator and its similarity to other AraC-like regulators. The relatedness of SefR

Figure 3.11: Phylogenetic tree of SefR and related proteins

This tree was generated using Njplot (Perrière and Gouy, 1996), and depicts graphically the relationships between these AraC-like proteins. The branch lengths are indicated, with the scale bar representing 0.05 units (i.e. 0.05% divergence). The numbers in brackets next to each protein represents their ranking in terms of % sequence similarity to SefR (see Table 3.2), i.e. SefR is most similar, while AraC is the least similar.

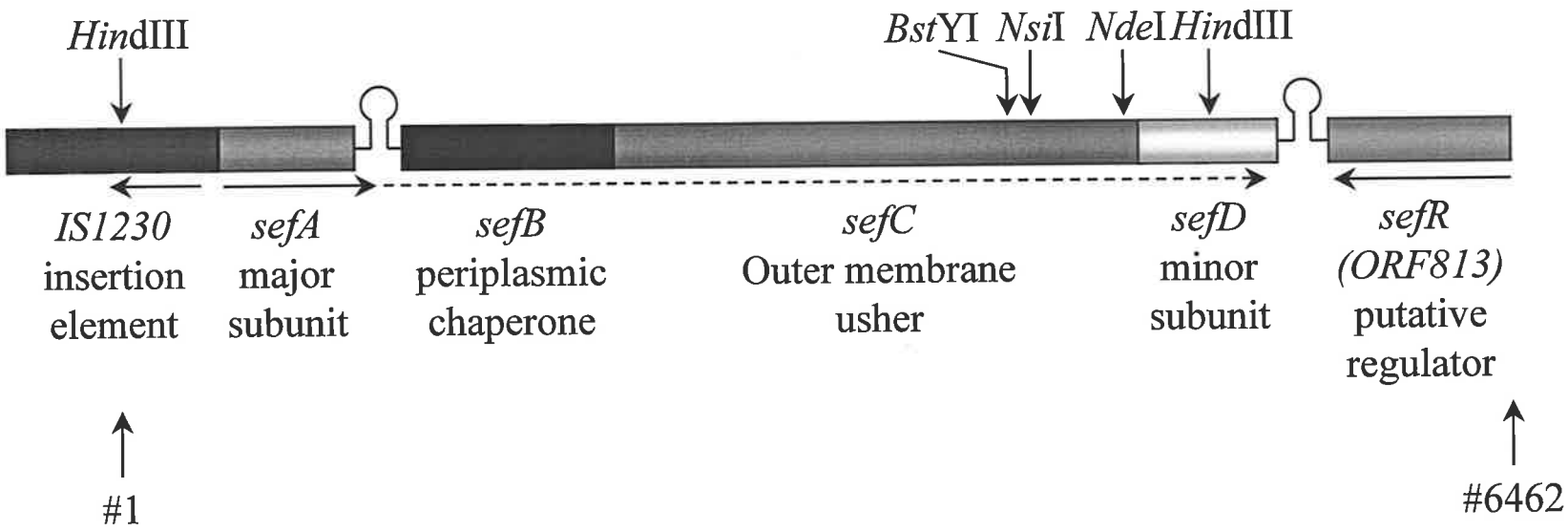

0.05
0.450
AraC
0.363
BfpT (9)
0.289
VirF (2)
0.014
0.317
SeR (1)
0.063
0.018
0.354
FapR (3)
0.053
0.120
CsvR (7)
0.064
0.028
Rns (5)
0.078
0.185
0.025
CfaD (4)
0.176
AggR (6)

to members of the AraC family (listed in Table 3.2) was shown graphically by a simple phylogenetic comparison (Figure 3.11). Interestingly, SefR is grouped with VirF, which is a plasmid encoded regulator that directly upregulates the *icsA* gene (Sakai *et al.*, 1988) and also the *virB* gene which in turn upregulates the *mxi-spa* TTSS (Adler *et al.*, 1989; Tobe *et al.*, 1991). It is not known if VirF is involved in the regulation of fimbriae, and has been shown that Rns, a regulator of CS1 and CS2 fimbriae (Caron *et al.*, 1989; Murphree *et al.*, 1997), can complement VirF function, but VirF cannot complement Rns function (Porter *et al.*, 1998).

The position of *sefR* relative to the entire SEF14 operon is shown in Figure 3.12. The identity of DNA 5' of the *sefR* gene is examined further in section 6.4.1.2. The observed similarity of SefR to known AraC-like regulators, some of which are involved in the regulation of fimbrial expression, argued for the inclusion of SefR within this group. It was hypothesised that SefR would act to increase the levels of expression of SefA and therefore SEF14 fimbriae. Further characterisation of the function of the predicted SefR protein is examined in the next chapter.

Figure 3.12: Revised SEF14 locus

This figure shows the relative position of the *sefR* gene to the *sefABCD* genes. Restriction endonuclease sites are indicated above the DNA, while the solid and dashed arrows show the direction of transcription. The dashed arrow indicates a potentially unstable region of mRNA lying downstream of the stem-loop structure located between the *sefA* and *sefB* genes. The second stem-loop structure between *sefD* and *sefR* is shown, and is hypothesized to act as a bi-directional terminator. The left-most *Hin*dIII site serves as nucleotide #1 and corresponds to the beginning of the submitted sequence for *sefA* (accession number X98516), while the end of the *sefR* gene corresponds to nucleotide #6462.


*Hin*dIII
*Bst*YI
*Nsi*I
*Nde*I
*Hin*dIII
IS1230 insertion element
sefA major subunit
sefB periplasmic chaperone
sefC Outer membrane usher
sefD minor subunit
sefR *(ORF813)* putative regulator
#1
#6462

Chapter 4: Characterisation of SefR and its role in the regulation of *sef* genes

4.1 Introduction

The previous chapter described how the cloning and sequencing of the *sefR* gene revealed that SefR was similar to regulator proteins from the AraC-family of transcriptional activators. It was hypothesised that when bacteria were grown under permissive conditions, the action of SefR would be to increase the production SEF14 fimbriae, by increasing the expression of *sefA*. Conversely, the absence of SefR activity would lead to a severe reduction in expression of *sefA*, and thus reduce SEF14 production. Therefore, the aim of this chapter was to functionally characterise the role of SefR in the production of SEF14 fimbriae by studying *sefA* expression.

4.2 Generation of an *S.* Enteritidis *sefR* mutant

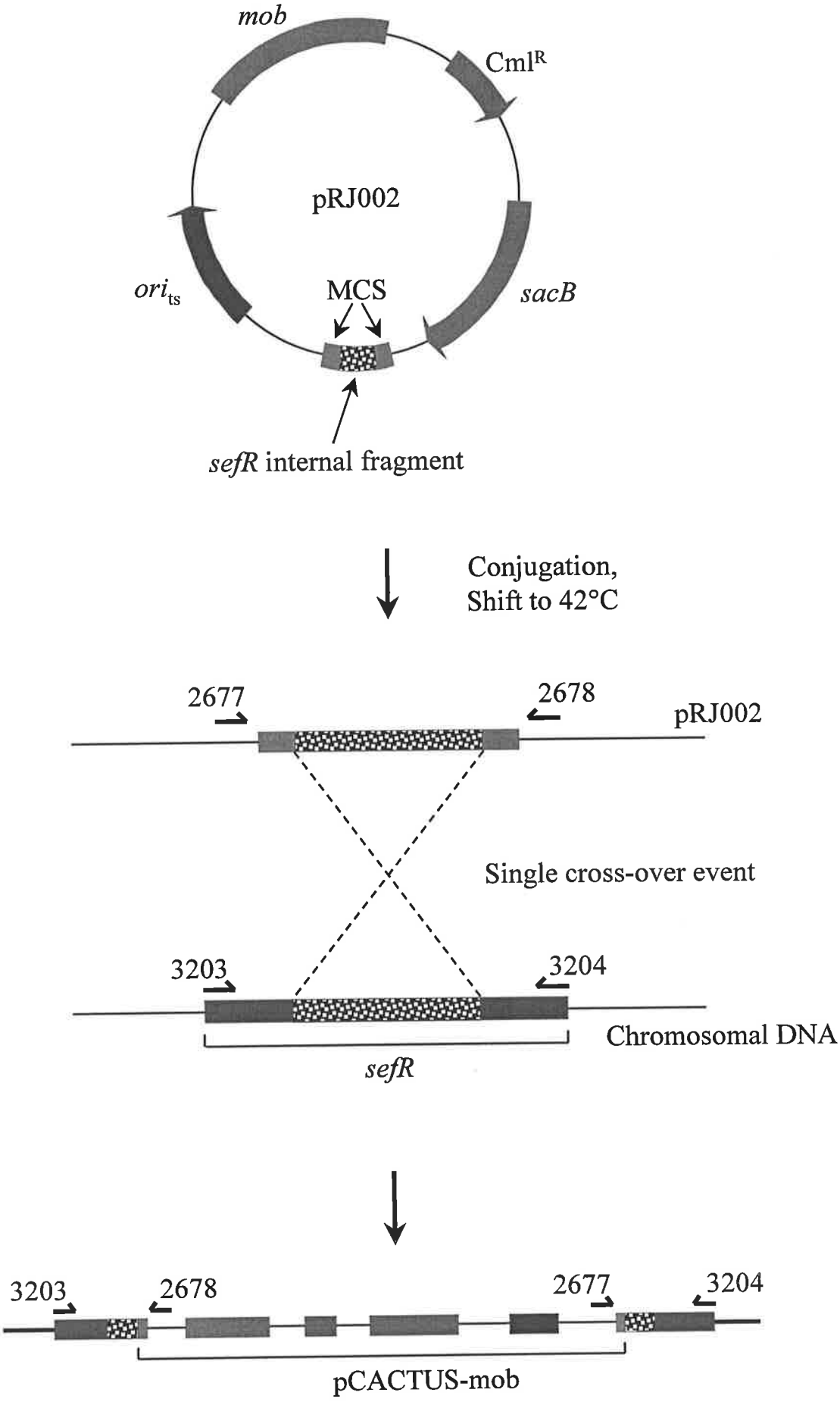
Characterisation of SefR action was first examined by constructing *sefR* null mutants in *S.* Enteritidis strains 11RX and 7314. These mutants were then investigated to determine if the absence of the putative activator protein would lead to a decrease in the levels of SEF14 expression.

4.2.1 Vector construction for single crossover insertion mutagenesis of *sefR*

Interruption of the *sefR* gene was performed by single crossover insertion mutagenesis. A small segment of internal *sefR* sequence was used to target the plasmid vector to the chromosomal *sefR* gene, thereby facilitating homologous recombination via a single crossover event, as outlined in Figure 4.1. This involved PCR amplification of 411 bp of internal *sefR* sequence (nt 214-625 of *sefR*, positions 5409 - 5821 relative to beginning of *sefA* promoter region), with primers 3192 and 3193, using chromosomal DNA from 11RX as template. Primers 3192 and 3193 coded for the restriction enzyme site *Sph*I, allowing the fragment to be cloned in the suicide vector pCACTUS-*mob*. The resulting construct, pRJ002

Figure 4.1: Chromosomal mutation of *sefR*

An internal fragment of *sefR* was PCR amplified (section 2.10.1) from 11RX chromosomal DNA (section 2.8.2) using primers 3192 and 3193 (see Table 2.3), both of which contained the *Sph*I restriction enzyme site, to give a 411 bp fragment. This fragment was digested with *Sph*I and ligated into similarly digested pCACTUS-*mob* and transformed into *E. coli* at 30°C. After selection with Cml, plasmid DNA was prepared (section 2.8.1) and screened by PCR using primers 2677 and 2678 (see Table 2.3), with the appropriate clone designated pRJ002. pRJ002 was electroporated into SM10λpir and then conjugated into the target strains 11RX and 7314. Single crossover recombination of pRJ002 was induced by growth at 42°C (section 2.10.6), with stable co-integrates selected by streaking single colonies for growth at 37°C with Cml. Screening of Cml^R exconjugants was performed using combinations of the indicated primers (i.e. 2677/3203; 2677/3204; 2678/3203 and 2678/3204). *mob* = mobilisation genes; Cml^R = chloramphenicol resistance gene; ori_{ts} = temperature sensitive origin of replication; *sacB* = levansucrase; MCS = multiple cloning site.


mob
Cml^R
pRJ002
ori_ts
MCS
sacB
sefR internal fragment
Conjugation,
Shift to 42°C
2677
2678
pRJ002
Single cross-over event
3203
3204
Chromosomal DNA
sefR
3203
2678
2677
3204
pCACTUS-mob

was electroporated into the donor strain SM10λpir, which facilitated conjugation into *S.* Enteritidis strains 11RX and 7314 as per section 2.7.5. After selection on minimal media + Cml, exconjugants were grown at 42°C (Section 2.10.6). Colonies that were able to grow at 42°C in the presence of Cml were considered potential co-integrates (i.e. chromosome::pRJ002), and were screened by PCR amplification as described below.

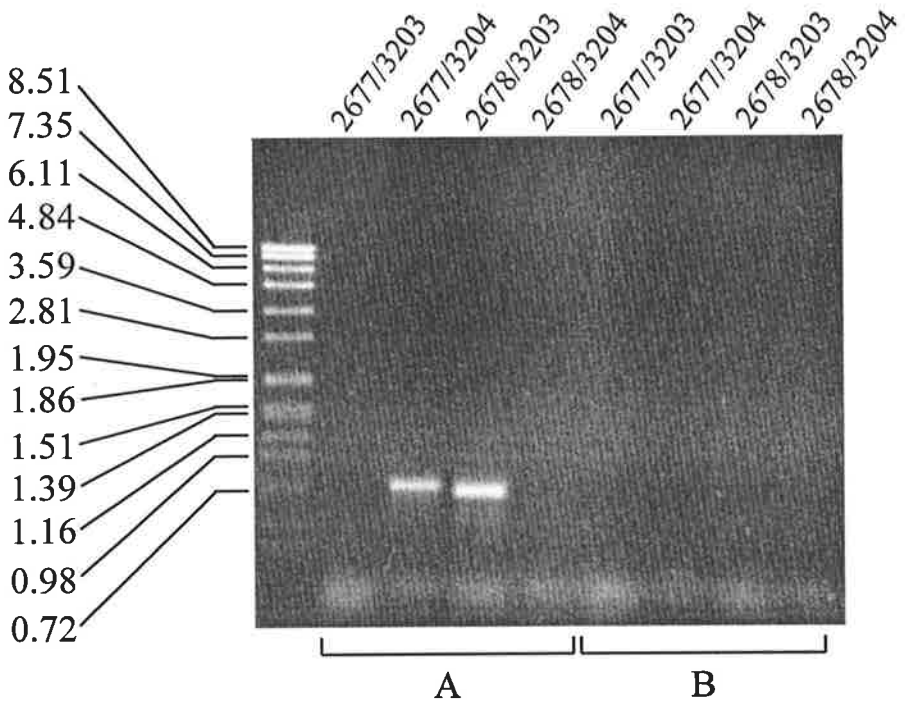
4.2.2 PCR screening of potential *sefR* mutants

Putative co-integrates were grown in LB supplemented with Cml and chromosomal DNA was prepared as per section 2.8.2. The chromosomal DNA was used as template for PCR analysis of the junctions between *sefR* and pCACTUS-*mob* using *sefR* primers 3203 and 3204 in combination with pCACTUS-*mob* primers 2677 and 2678, to give four different primer pairs. Based on the protocol outlined in Figure 4.1, it was expected that two of the four primer pairs would result in amplification of the target DNA while the remaining two would not. This would allow confirmation of vector insertion, while PCR reactions using *sefR* primers 3203 and 3204 or pCACTUS-*mob* primers 2677 and 2678 would show both the interruption of chromosomal *sefR* gene and confirm the absence of cytoplasmic pRJ002 that should have been lost during the mutagenesis process.

Figure 4.2 shows the results of PCR analysis of DNA from 2 exconjugants using the primer combinations 2677/3203; 2677/3204; 2678/3203 and 2678/3204. The first exconjugant (A) gave positive reactions using primers 2677/3204 and 2678/3203 (lanes 2 and 3), while reactions containing primers 2677/3203 and 2678/3204 were negative (lanes 1 and 4). This confirmed correct insertion of pRJ002 into these clones. The second exconjugant (B) was negative for all four reactions. Exconjugant A was also analysed in two control PCR reactions, using either *sefR* primers 3203 and 3204 or pCACTUS-*mob* primers 2677 and 2678. Both of these reactions were negative (data not shown), confirming interruption of *sefR* and loss of free pRJ002.

Figure 4.2: PCR analysis of exconjugants

Chromosomal DNA from putative *sefR* mutants were screened for insertion of pRJ002 into the *sefR* gene by PCR, using primer combinations 2677/3203, 2677/3204, 2678/3203 and 2678/3204 (See Figure 4.1). 10 µl of each PCR reaction was mixed with 3 µl of running buffer and electrophoresed on 1% TBE gel. The gel was stained and visualised as per section 2.9.3. Shown here are PCR reactions from 2 exconjugants, with the primer combination for each reaction indicated. Exconjugant A is positive for two of the four PCR reactions, indicating a single insertion of pRJ002 within *sefR*, while exconjugant B is negative for all four reactions, indicating no insertion of pRJ002 within *sefR*. *Eco*RI digested SPPI marker sizes are indicated below


2677/3203
2677/3204
2678/3203
2678/3204
2677/3203
2677/3204
2678/3203
2678/3204
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
A
B

4.2.3 Southern analysis of PCR positive, *sefR*::pRJ002 insertion mutants

Confirmation of *sefR* gene interruption was done by Southern analysis. Chromosomal DNA preparations of potential mutants were digested with *Nru*I, which does not have restriction sites in either pCACTUS-*mob* or *sefR*. Thus potential mutants probed with a full length *sefR* gene probe should give rise to a single band which is larger than that seen for the wild type. Figure 4.3A shows a comparison of wild type 11RX and 7314 with their putative *sefR*::pRJ002 insertion mutants. 11RX and 7314 show a single band that is >10 kb, while the respective mutant strains show a band that is larger again. This difference in size is due to the insertion of plasmid pRJ002, adding almost 8 Kb of extra DNA. The same clones, digested with *Nru*I, were also probed with a CAT gene probe (Figure 4.3B). As expected, both 11RX and 7314 were negative for CAT, while the putative mutants were positive, displaying a band the same size as that seen when probing with *sefR*. This confirmed the mutation of *sefR* in these strains, which were designated RMJ002 and RMJ003, respectively.

4.3 Characterisation of the effects of the *sefR* mutation

It was hypothesised that *sefR*::pRJ002 insertion mutation would result in a decrease in the production of SEF14 fimbriae. This was to be assessed both qualitatively and quantitatively. SefA expression was detected using a polyclonal antisera (Ogunniyi, 1996) raised in rabbits immunised with formalin-fixed 11RX which had been grown for maximal SEF14 expression.

4.3.1 Absorption of anti-11RX antiserum

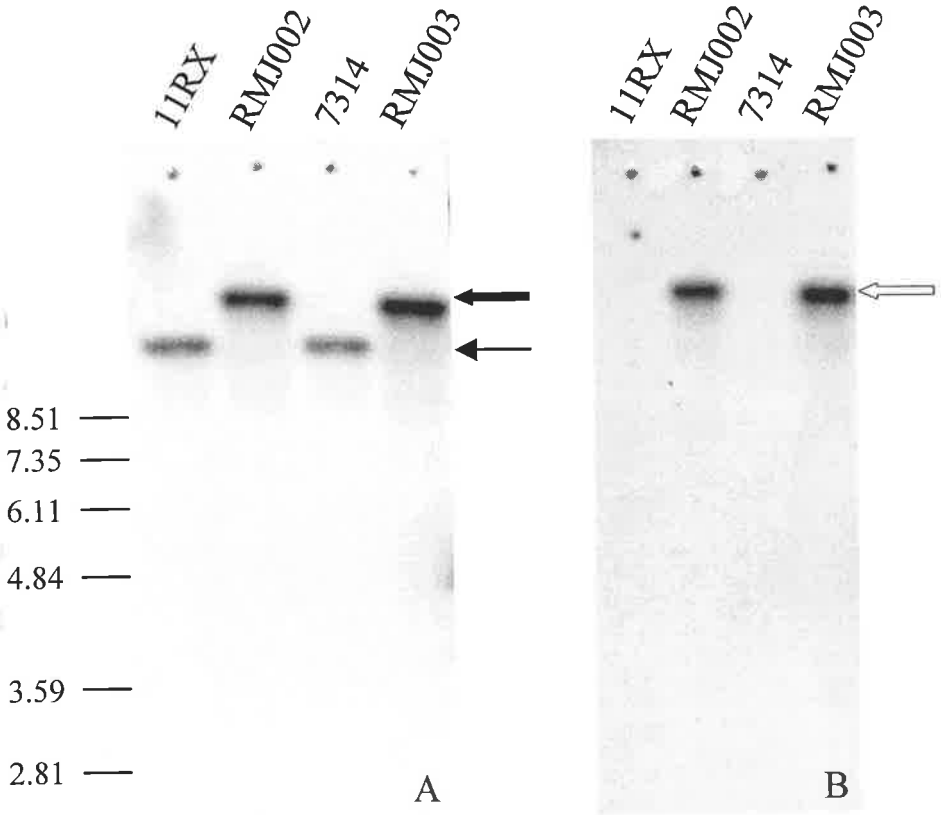
Prior to use, non-SefA activity was removed by absorbing the anti-serum several times against both *E. coli* K-12 and PE908 (Ogunniyi, 1996), an 11RX strain that has an insertion mutation in *sefA*. Unabsorbed and absorbed anti-11RX sera were tested against whole cell and heat-shock supernatants. Figure 4.4 (Panel A) shows a Western immunoblot of whole

Figure 4.3: Southern analysis of Putative insertion mutants

Panel A: Chromosomal DNA was digested with *Nru*I and electrophoresed overnight on a 0.8% TAE gel. The DNA was transferred to nylon membrane and probed with a full-length *sefR* gene probe (section 2.12.1). Lanes 1-4 represents strains 11RX, RMJ002, 7314 and RMJ003 respectively. The thin arrow represents uninterrupted *sefR* gene in 11RX and 7314. The thick arrow represents *sefR* gene interrupted by pCACTUS-*mob*.

Panel B: Chromosomal DNA was digested with *Nru*I and electrophoresed overnight on a 0.8% TAE gel. The DNA was transferred to nylon membrane and probed with a full-length CAT (chloramphenicol acetyl transferase) gene probe (section 2.12.1). Lanes 1-4 represent strains 11RX, RMJ002, 7314 and RMJ003 respectively. The hollow arrow highlights the presence of the CAT gene from pCACTUS-*mob* on a band of approximately the same size as that seen in the *sefR* probed DNA. This indicated that pCACTUS-*mob* had inserted into the *sefR* gene.

*Eco*RI digested SPP1 marker sizes (kb) for both blots are indicated.


11RX
RMJ002
7314
RMJ003
11RX
RMJ002
7314
RMJ003
8.51
7.35
6.11
4.84
3.59
2.81
A
B

cells and heat-shocked supernatant from 11RX grown for maximal SEF14 production and probed with unabsorbed sera. A wide range of reactivity was observed, and a potential SefA specific band at the expected position is indicated. Figure 4.4 (Panel B) shows the same samples probed with anti-11RX polyclonal sera after absorption (anti-SefA [adsorbed]). It can clearly be seen that the absorption has removed all background antibodies, leaving antibodies reactive with SefA. This serum was termed anti-SefA [adsorbed].

4.3.2 Analysing the effect of *sefR*::pRJ002 insertion mutation on SefA expression

S. Enteritidis strains 11RX and 7314 and their respective *sefR*::pRJ002 insertion mutants RMJ002 and RMJ003 were compared for production of SEF14 fimbriae. The strains were grown on CFA agar with appropriate antibiotics and harvested in PBS. A sample of cells was retained for whole cell analysis and the remainder was heat shocked at 56°C to release fimbriae into the supernatant (section 2.13.1). Samples were analysed by SDS-15% PAGE and Coomassie blue staining, and Western immunoblotting.

Figure 4.5A shows a Coomassie stained SDS-15% polyacrylamide gel of the 11RX parent strain and strain RMJ002. SefA was observed in the supernatant fraction of 11RX (Panel A, Lane 2), however it was difficult to detect SefA in the whole cell fraction (Panel A lane 1). No SefA was apparent in the whole cell or supernatant fractions of strain RMJ002 (Panel A lanes 3 & 4). This data was confirmed by Western immunoblot using anti-SefA [adsorbed]. SefA was observed in both whole cell and supernatant fractions of 11RX but none was seen in the corresponding samples of RMJ002 (Figure 4.5B). Similar results were obtained in a comparison of 7314 and RMJ003 (data not shown).

4.3.3 Cloning of *sefR* into pBAD30 and identification of SefR

The *sefR* gene was placed under arabinose inducible control. PCR was used to amplify the *sefR* gene using 11RX chromosomal DNA as the template and oligonucleotides

Figure 4.4: Western immunoblot using unabsorbed and absorbed anti-11RX serum

11RX was grown on CFA agar and heat shock supernatant (S/N) and whole cell (WC) samples prepared (sections 2.13.1 and 2.14.1). WC and S/N samples, representing ~10^9 bacteria, were electrophoresed on SDS-15% PAGE and then analysed by Western immunoblot using unabsorbed anti-11RX serum (panel A; 1/1000) and anti-SefA [absorbed] (panel B; 1/1000). Absorption was performed against both *E. coli* DH5α and PE908. This resulted in all activity except that specific for SefA (hollow arrow) being removed. Molecular weight standards (kDa; section 2.14.3) are indicated.

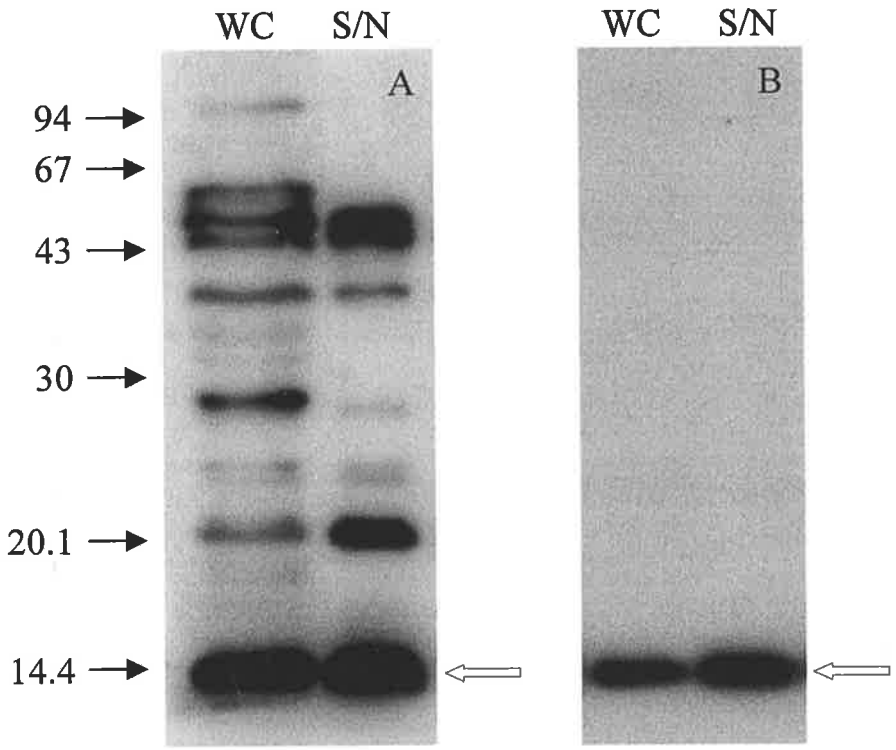
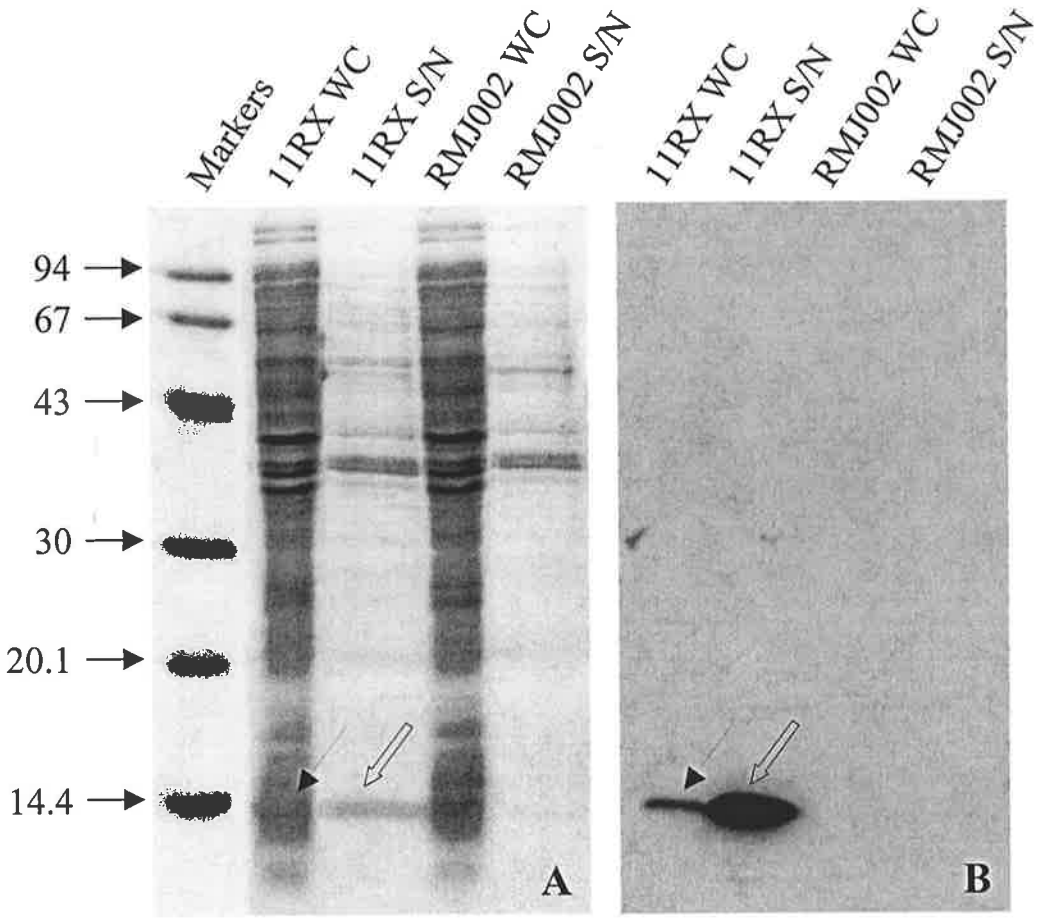

WC
S/N
A
WC
S/N
B
94
67
43
30
20.1
14.4

Figure 4.5: Comparison of SefA expression from 11RX and RMJ002.

Strains 11RX and the *sefR*::pRJ002 insertion mutant RMJ002 were grown on CFA agar and whole cell (WC) and heat shock supernatant (S/N) samples from each were prepared (sections 2.13.1 and 2.14.1). Samples representing ~10^8 bacteria were electrophoresed by SDS-15% PAGE and either stained with Coomassie Blue (A) or transferred to nitrocellulose and Western immunoblotted (B) with 1/1000 anti-SefA [adsorbed]. The thin arrow shows the location of SefA in WC samples, while the hollow arrow represents SefA in S/N samples. No SefA was observed in any of the RMJ002 samples. Molecular weight standards (kDa; section 2.14.3) are indicated.


Markers
11RX WC
11RX S/N
RMJ002 WC
RMJ002 S/N
11RX WC
11RX S/N
RMJ002 WC
RMJ002 S/N
94
67
43
30
20.1
14.4
A
B

3203 and 3204, which incorporated *Sac*I and *Sph*I restriction sites, respectively. Primer 3203 also incorporated the putative RBS of *sefR*. The PCR product was purified, digested with *Sac*I and *Sph*I, and ligated to similarly digested pBAD30 (Guzman *et al*, 1995). The ligated DNA was transformed into *E. coli* K-12, and grown on LA plus Amp, supplemented with 0.2% (w/v) glucose to repress expression of SefR. Transformants were screened by plasmid isolation (section 2.8.1) and digest with *Sac*I and *Sph*I. This resulted in construction of the plasmid clone pRJ003 (Strain RMJ004). The outline of this cloning procedure is shown in Figure 4.6

To visualise SefR, strain RMJ004 was grown in LB plus Amp supplemented with either 0.2% (w/v) glucose or 0.2% (w/v) arabinose, and also on LA plus Amp with the same supplements. An aliquot of each broth culture was centrifuged and resuspended in 1x PBS, while bacteria grown on agar were harvested directly into 1x PBS. All four samples were adjusted to the same OD600 and samples prepared for electrophoresis (section 2.14.1). The samples were analysed by SDS-15% PAGE (section 2.14.3). Figure 4.7 shows the Coomassie-stained gel. A band with an apparent molecular size of ~31 KDa was observed in the arabinose induced bacteria but not in those grown in the presence of glucose (indicated by the asterix). This band was very close to the predicted molecular weight of SefR (31.4 KDa).

4.3.4 Complementation of *sefR* mutation

To assess whether plasmid pRJ003 was able to functionally complement the *sefR* insertion mutation, plasmid pRJ003 was first electroporated into EX2000, a *Salmonella* restriction$^-$, modification$^+$ strain (Table 2.2), to protect the plasmid DNA from the restriction system in 11RX and 7314. After plasmid extraction, the DNA was electroporated into RMJ002 and RMJ003, giving rise to strains RMJ007 and RMJ008. Controls strains were generated by the same method, substituting pBAD30 for pRJ003 to give strains RMJ005 and RMJ006. All four strains (RMJ005, RMJ006, RMJ007 and RMJ008) were grown on CFA

Figure 4.6: Cloning of *sefR* into pBAD30

Cloning of the *sefR* gene into pBAD30 was achieved by PCR amplification of *sefR* using primers 3203 and 3204 (Table 2.3), which incorporated *Sph*I and *Sac*I restriction enzyme sites, respectively. The *sefR* PCR product was digested with *Sph*I and *Sac*I, and ligated into similarly digested pBAD30. After ligation the DNA was transformed into *E. coli* DH5α (section 2.7.2). Plasmid DNA from transformants was screened by restriction digest with *Sph*I and *Sac*I. This resulted in construction of plasmid pRJ003 (strain RMJ004)

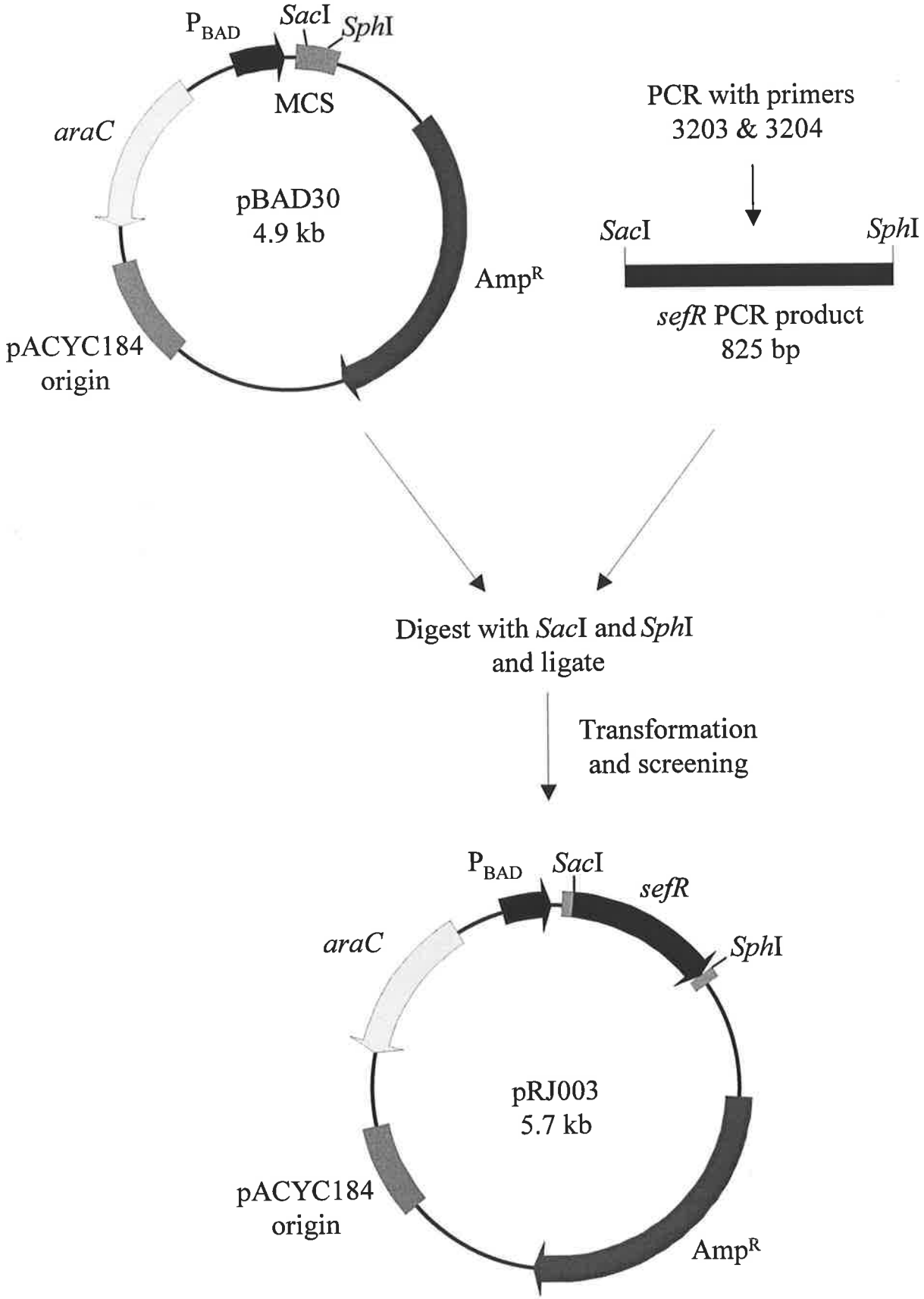
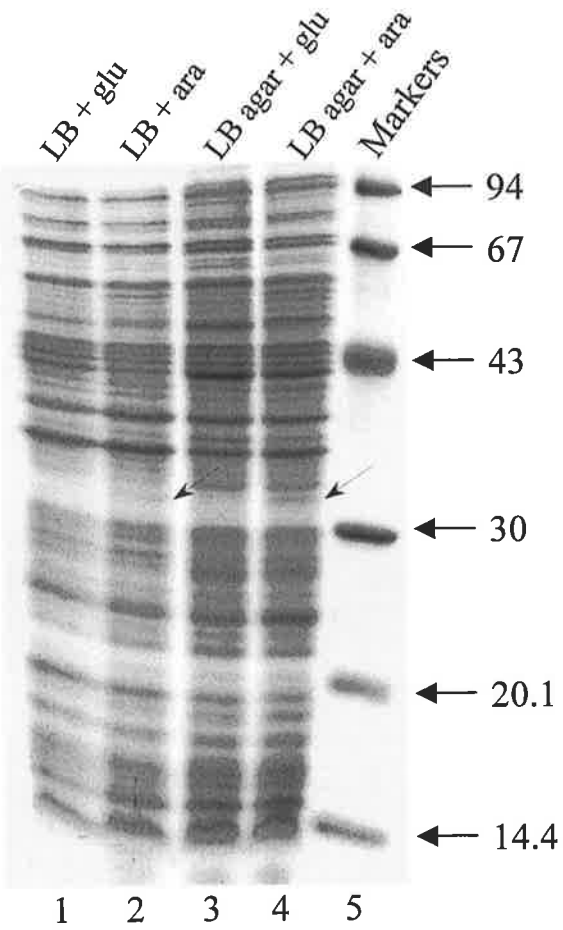

P BAD
SacI
SphI
MCS
araC
pBAD30
4.9 kb
Amp R
pACYC184
origin
PCR with primers
3203 & 3204
SacI
SphI
sefR PCR product
825 bp
Digest with SacI and SphI
and ligate
Transformation
and screening
P BAD
SacI
sefR
araC
SphI
pRJ003
5.7 kb
pACYC184
origin
Amp R

Figure 4.7: Detection of SefR

Strain RMJ004 was grown in CFA broth or on CFA agar supplemented with either 0.2% (w/v) glucose or 0.2% (w/v) arabinose. Whole cell samples were prepared as per section 2.14.1 and analysed by SDS-15% PAGE and Coomassie blue staining (section 2.14.3). Lanes 1-4 represent CFA broth + 0.2% (w/v) glucose, CFA broth + 0.2% (w/v) arabinose, CFA agar + 0.2% (w/v) glucose and CFA agar + 0.2% (w/v) arabinose respectively. The two arrows show a protein with an apparent molecular weight of ~31 kDa, present in lanes 2 & 4, and absent from lanes 1 & 3. Molecular weight standards (kDa; section 2.14.3) are indicated.


LB + glu
LB + ara
LB agar + glu
LB agar + ara
Markers
94
67
43
30
20.1
14.4
1
2
3
4
5

agar with the appropriate selection in the presence of either 0.2% (w/v) glucose or 0.2% (w/v) arabinose. All cultures were harvested into 1x PBS (section 2.13.1) and adjusted to the same OD_{600}. A sample of each suspension was mixed with an equal volume of 2x SDS-sample buffer (whole cell sample). The remainder was heat shocked, centrifuged and the supernatant retained (section 2.13.1). Both whole cell and supernatant samples were analysed by SDS-15% PAGE and Western immunoblotting. Figure 4.8A shows the Coomassie blue stained gel, with the supernatant sample from RMJ007 grown in 0.2% (w/v) arabinose showing a significant 14 kDa protein, corresponding to the size of SefA (Lane 8). However, it was difficult to detect the 14 kDa protein in the whole cell sample (Lane 7). No 14 kDa protein (SefA) was detected in samples from RMJ005 or in RMJ007 when grown in glucose. Confirmation of these results was obtained by Western analysis as shown in Figure 4.8B. The supernatant sample from RMJ007 grown with arabinose was strongly positive for SefA, while the whole cell sample was also positive for SefA. No SefA was detected in supernatant or whole cells when the control strain RMJ005 was grown with arabinose. Similar results were obtained in a comparison of RMJ006 with RMJ008 (data not shown). Clearly, the *sefR* gene supplied *in trans* is able to restore production of SefA, as evidenced by the large band of SefA protein seen by both Coomassie staining and Western immunoblotting.

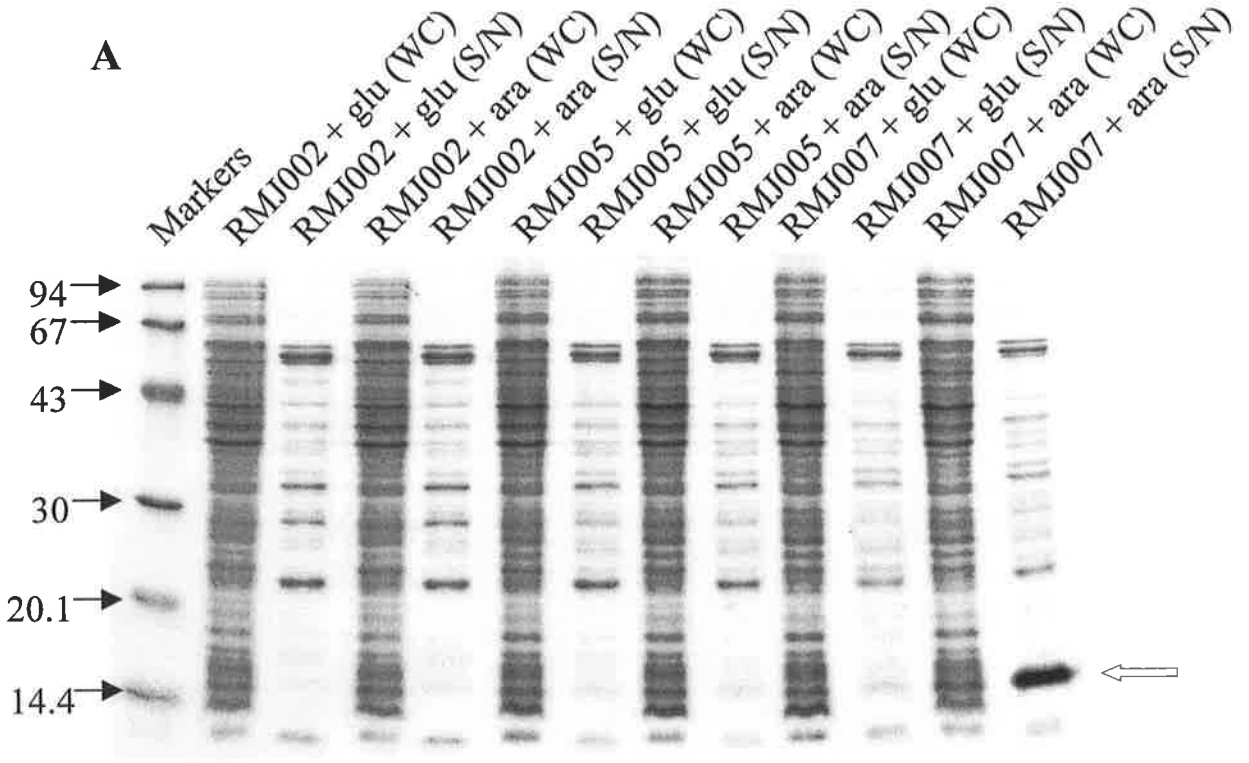
4.3.5 IEM analysis of SEF14 expression by *S.* Enteritidis *sefR* mutant and *sefR* complemented mutant

To visualise the effect of the *sefR*::pRJ002 insertion mutation on SEF14 expression, strains 11RX, 7314 and their respective *sefR* mutant strains RMJ002 and RMJ003 were grown on CFA agar and their respective complemented mutants RMJ007 and RMJ008 were grown on CFA agar supplemented with 0.2% (w/v) arabinose. Each strain was prepared for IEM as per section 2.19. The results are shown in Figure 4.9. 11RX and 7314 both expressed fimbriae, with approximately 1 in 10 and 1 in 20 bacteria respectively positive for SEF14

Figure 4.8: Complementation of *sefR* mutation

Panel A: The *sefR*::pRJ002 insertion mutant RMJ002, RMJ005 (RMJ002 + pBAD30) and RMJ002 carrying the SefR expressing plasmid pRJ003 (strain RMJ007) were grown on CFA agar supplemented with the appropriate antibiotics and either 0.2% (w/v) glucose or 0.2% (w/v) arabinose. Whole cell and heat shock supernatant samples were prepared (sections 2.14.1 and 2.13.1) and analysed by SDS-15% PAGE and Coomassie blue staining (A; section 2.14.3). The arrow designates the expressed SefA band. Molecular weight standards (kDa; section 2.14.3) are indicated.

Panel B: The *sefR*::pRJ002 insertion mutant RMJ002, RMJ005 (RMJ002 + pBAD30) and RMJ002 carrying the SefR expressing plasmid pRJ003 (strain RMJ007) were grown on CFA agar supplemented with the appropriate antibiotics and either 0.2% (w/v) glucose or 0.2% (w/v) arabinose. Whole cell and heat shock supernatant samples were prepared (sections 2.14.1 and 2.13.1) and analysed by SDS-15% PAGE and Western immunoblotting. 1/1000 anti-SefA [adsorbed] was used to detect SefA, as indicated by the arrow. Molecular weight standards (kDa; section 2.14.3) are indicated.


A
Markers
RMJ002 + glu (WC)
RMJ002 + glu (S/N)
RMJ002 + ara (WC)
RMJ002 + ara (S/N)
RMJ005 + glu (WC)
RMJ005 + glu (S/N)
RMJ005 + ara (WC)
RMJ005 + ara (S/N)
RMJ007 + glu (WC)
RMJ007 + glu (S/N)
RMJ007 + ara (WC)
RMJ007 + ara (S/N)
94
67
43
30
20.1
14.4

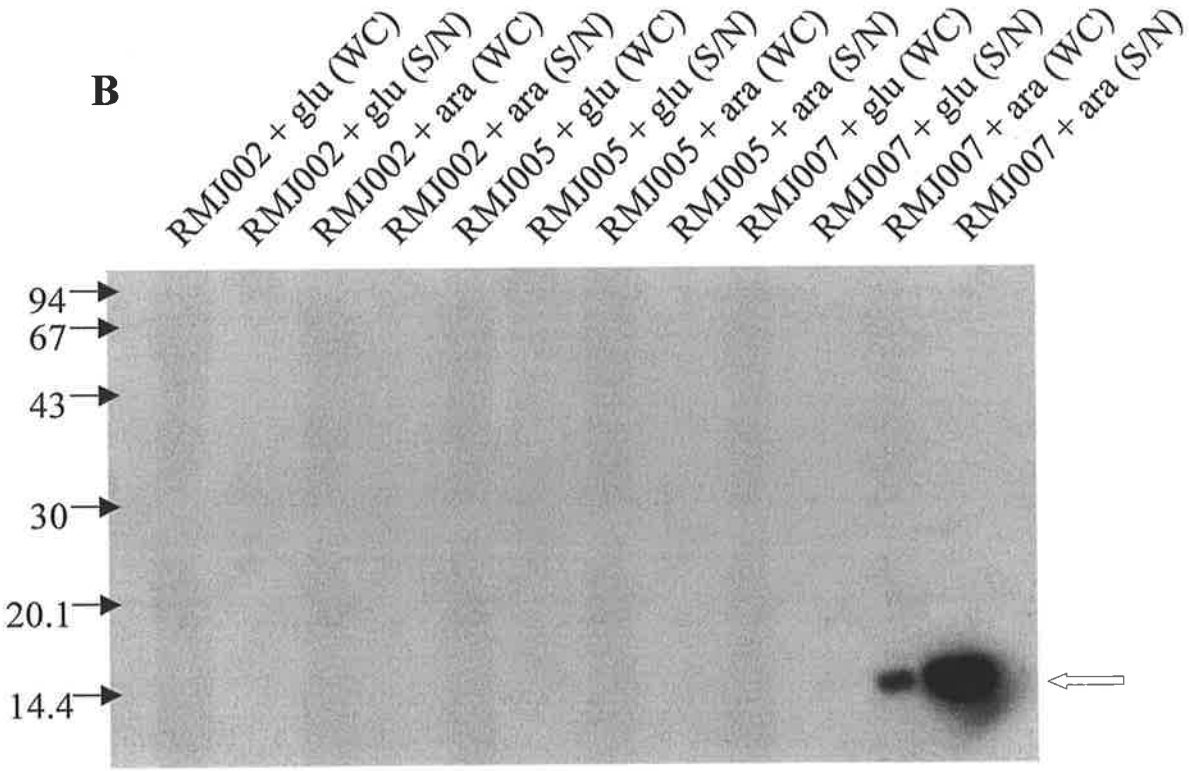
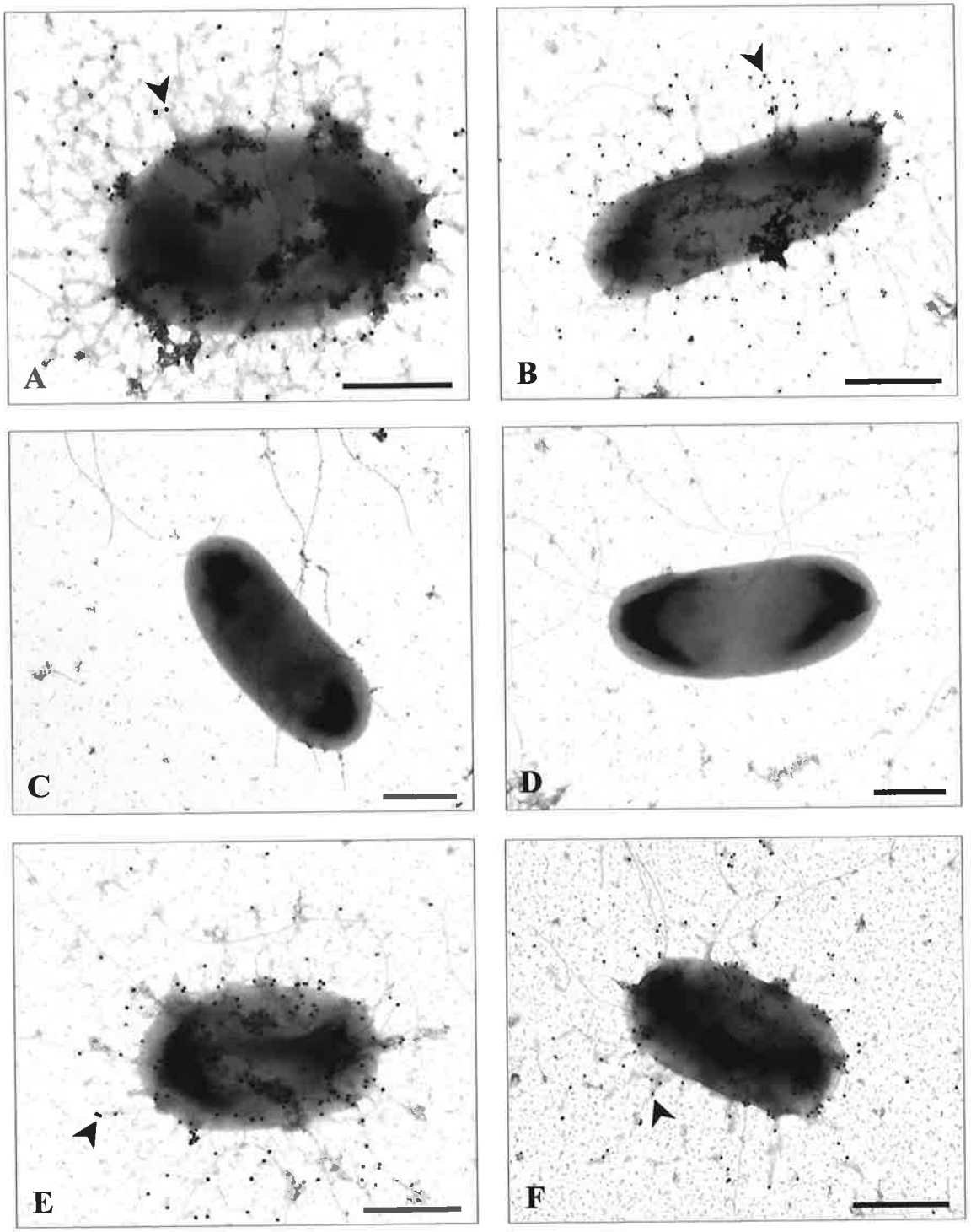

B
RMJ002 + glu (WC)
RMJ002 + glu (S/N)
RMJ002 + ara (WC)
RMJ002 + ara (S/N)
RMJ005 + glu (WC)
RMJ005 + glu (S/N)
RMJ005 + ara (WC)
RMJ005 + ara (S/N)
RMJ007 + glu (WC)
RMJ007 + glu (S/N)
RMJ007 + ara (WC)
RMJ007 + ara (S/N)
94
67
43
30
20.1
14.4

Figure 4.9: IEM of *S.* Enteritidis *sefR* mutant and complemented mutant strains

The strains 11RX, 7314, and their respective *sefR* mutant strains RMJ002 and RMJ003 were grown on CFA agar and their respective complemented mutants RMJ007 and RMJ008 were grown on CFA agar supplemented with 0.2% (w/v) arabinose. Bacteria were prepared for immunoelectron microscopy (section 2.19), using 1/50 anti-SefA [adsorbed]. The samples are displayed here as follows: Panel A = 11RX, Panel B = 7314, Panel C = RMJ002, Panel D = RMJ007, Panel E = RMJ007 and Panel F = RMJ008. Arrowheads indicate examples of gold particles bound to fimbriae. Scale bar represents 0.5 μm.


A
B
C
D
E
F

fimbriae (Panels A and B). No fimbriae were observed on either mutant strain (Panels C and D). As expected from data presented in the previous section (4.3.4), both complemented mutants expressed fimbriae (Panels E and F).

4.4 Defining minimal coding region for SEF14 fimbriae

It is already known that the *sefA* gene is essential for SEF14 expression (Ogunniyi, 1996) and as shown here, that *sefR* contributes to this expression. It is not known however if further genes outside of the SEF14 locus are required for expression of SEF14 fimbriae. To address this issue, two plasmid constructs containing clones of the SEF14 locus were generated; one containing the entire SEF14 locus (i.e. *sefABCD* and *sefR*) to determine whether SEF14 expression would occur in the absence of any other *S.* Enteritidis-specific expression factors, and the second with just *sefABCD* to determine if *sefR* was essential.

4.4.1 PCR amplification and cloning of the *sef* locus

The amplification of the SEF locus (*sefABCD* and *sefABCD / sefR*) was achieved using long range-PCR (LR-PCR). Three primers were used for this process. The 5' primer, 2933, was designed to bind upstream of the *sefA* gene, such that the native SEF14 promoter would be incorporated into the construct and expression of SEF14 genes should initiate from this promoter. Primer 3075 was designed to bind the 3' end of the *sefD* gene (giving rise to *sefABCD* in combination with primer 2933). Primer 3076 was designed to bind upstream of the putative RBS of the *sefR* gene to allow expression of this gene from the *lacZ* promoter in the plasmid vector. All three primers incorporated an *Eco*RI restriction enzyme site to facilitate cloning into the target vector, pBC-SK$^+$ (Stratagene). The cloning strategy is shown in Figure 4.10. LR-PCR was carried out as per section 2.10.4 and samples of each reaction analysed by agarose gel electrophoresis. Figure 4.11 shows 5.1 kb and 5.9 kb bands, respectively, as expected for these reactions, after purification by gel extraction (Section

Figure 4.10: Cloning of *sefABCD* and *sefABCDR* into pBC-SK$^+$

LR-PCR products spanning the *sefABCD* and *sefABCDR* genes (represented by A, B, C, D and R respectively) were digested with *Eco*RI and ligated into similarly digested pBC-SK$^+$. After transformation into DH5α, plasmid DNA was prepared (section 2.8.1) and screened by separate *Eco*RI and *Bam*HI digests. Using this strategy, plasmids pRJ004 and pRJ005 (strains RMJ010 and RMJ011 respectively) were constructed. Plasmid promoters T7 and *lacZ* are indicated.

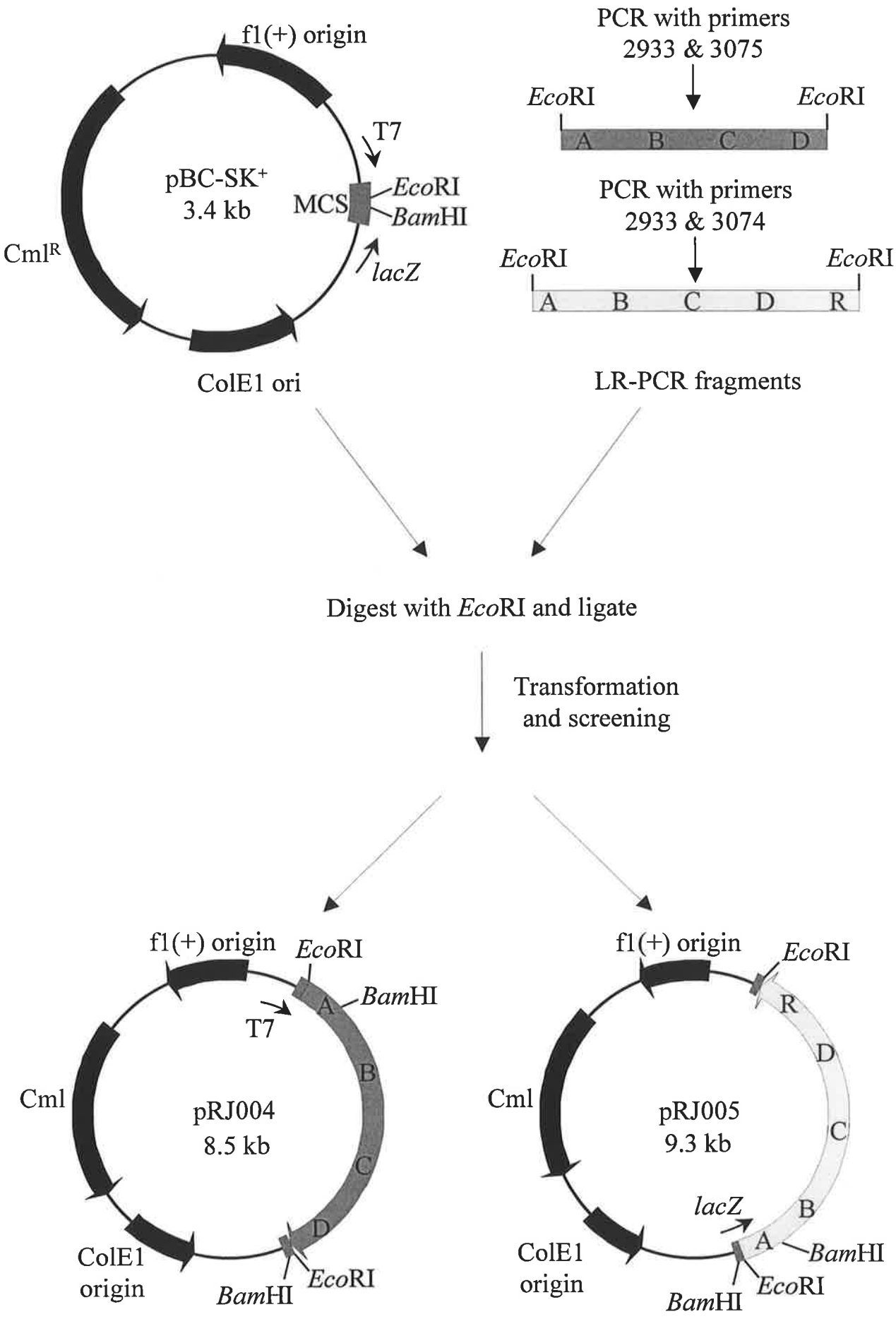
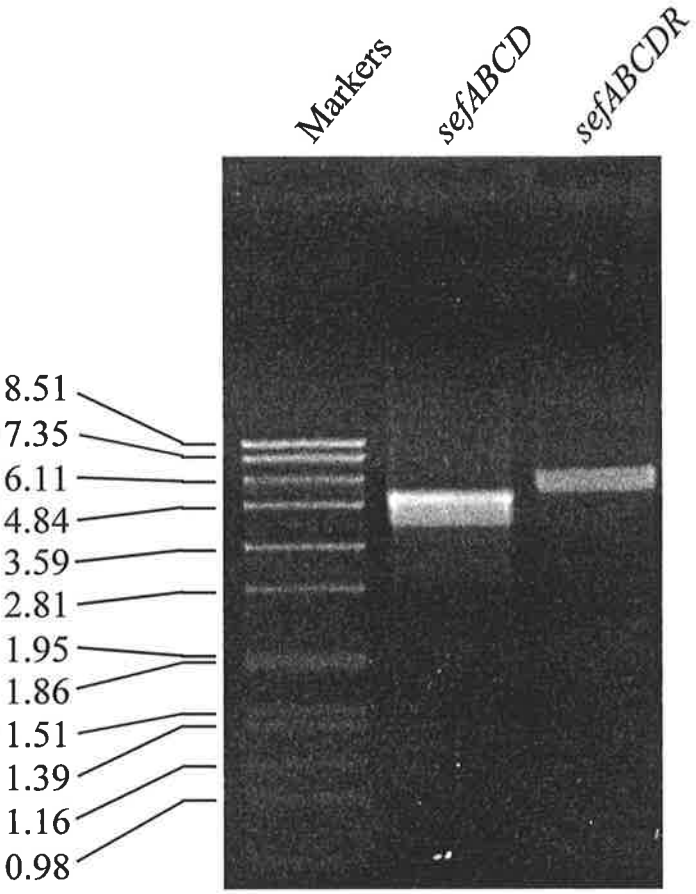

f1(+) origin
T7
pBC-SK+
3.4 kb
MCS
EcoRI
BamHI
CmlR
lacZ
ColE1 ori
PCR with primers
2933 & 3075
EcoRI
EcoRI
A
B
C
D
PCR with primers
2933 & 3074
EcoRI
EcoRI
A
B
C
D
R
LR-PCR fragments
Digest with EcoRI and ligate
Transformation
and screening
f1(+) origin
EcoRI
BamHI
T7
A
B
C
D
Cml
pRJ004
8.5 kb
ColE1
origin
BamHI
EcoRI
f1(+) origin
EcoRI
R
D
C
B
A
BamHI
EcoRI
lacZ
Cml
pRJ005
9.3 kb
ColE1
origin
BamHI

Figure 4.11: LR-PCR of *sefABCD* and *sefABCDR*

Primer 2933 was used in combination with primers 3075 and 3076 to amplify the *sefABCD* and *sefABCDR* gene fragments, respectively. This resulted in amplification of bands of the expected sizes (5.1 kb and 5.9 kb, respectively). The bands were purified by gel extraction and a sample analysed on a 0.8% TAE gel. Lane 1 represents the *sefABCD* fragment while lane 2 is the *sefABCDR* fragment. *Eco*RI digested SPP1 DNA size markers are indicated.


Markers
sefABCD
sefABCDR
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98

2.9.5). An aliquot of each PCR product was digested using *Eco*RI and ligated into similarly digested pBC-SK$^+$. After ligation *E. coli* DH5α was transformed and grown on blue/white selective media (section 2.7.2). Plasmid DNA from transformants was analysed by *Eco*RI digestion. The plasmids that presented the expected restriction pattern were further digested with *Bam*HI to determine their orientation relative to the Lac/T7 promoters of pBC-SK$^+$, with approximately 50% of isolates analysed had the *sefABCD* insert with *sefA* proximal to the T7 promoter. One of these was designated pRJ004 (strain RMJ010). However, all isolates possessing the *sefABCDR* fragment were found to have the *lacZ* promoter proximal to the 5' end of *sefA*, and the T7 promoter proximal to the 5' end of *sefR*. One of these was designated pRJ005 (strain RMJ011). It was hoped that both constructs would have their respective inserts orientated with the T7 promoter proximal to the 5' end of the *sefA* gene such that any comparison of expression of SefA would be due solely to the effects of SefR on the *sefA* promoter, rather than endogenous expression from the *lacZ* promoter. Attempts to force clone the *sefABCDR* fragment by subcloning into pBC-SK$^+$ such that the 5' end of *sefA* was adjacent to the T7 promoter, were unsuccessful. It is possible that no clones of *sefABCDR* with *sefR* proximal to the *lacZ* promoter were obtained because expression of SefR from the *lacZ* promoter was sufficiently high that expression of SefA/SEF14 became deleterious to cell growth.

4.4.2 Analysis of SefA production by RMJ010 and RMJ011

Strains RMJ010 and RMJ011 and the control strain RMJ009 (*E. coli* DH5α containing pBC-SK$^+$ only) were compared for growth on both LA and CFA agar. On either medium, RMJ010 grew as well as RMJ009. RMJ011, containing pRJ005, grew as well as RMJ010 and RMJ009 on LA, but grew poorly on CFA agar. Similarly, when grown on LA and harvested into 1x PBS, all three strains were evenly suspended in solution. The same result was observed for the control strain when grown on CFA agar. However, when RMJ010 and

RMJ011 were grown on CFA agar and harvested into 1x PBS, cellular aggregates formed. This is indicative of SEF14 expression, which has been shown to mediate aggregation (Woodward *et al.*, 2000). These results are summarised in Figure 4.12A. Strains RMJ009, RMJ010 and RMJ011 were grown on both LA and CFA agar and heat shock supernatants prepared (section 2.13.1). Samples of each were electrophoresed on a SDS-15% polyacrylamide gel, which was stained with Coomassie blue (section 2.14.3). No SefA was observed in any strain after growth on LA (data not shown). Figure 4.12B shows the results of analysing samples from bacteria grown on CFA agar. As expected, the control strain did not express any SefA, while RMJ010 and RMJ011 were both positive for SefA. This result was confirmed by Western immunoblot using anti-SefA [adsorbed] (data not shown).

An important conclusion drawn from these results was the observation that *sef*A expression in *E. coli* K-12 can occur in the absence of SefR expression. The use of a high copy number vector most likely circumvented the requirement for SefR. It is likely that excessive SefA expression may impair *E. coli* K-12 growth.

4.4.3 Analysis of minimal expression clones by IEM

To determine whether RMJ010 and RMJ011, containing pRJ004 and pRJ005 respectively, were able to express of SefA in the form of SEF14 fimbriae on the bacterial surface, they were grown on CFA agar along with the negative control strain RMJ009 and 11RX as a positive control. These strains were prepared for immunoelectron microscopy as per section 2.19. Figure 4.13 shows electron micrographs of these strains. The negative control strain RMJ009 did not express any fimbriae (Panel A). Approximately 1 in 10 11RX bacteria displayed SEF14 fimbriae (Panel B). This compared with approximately 1 in 200 RMJ010 and RMJ011 bacteria displaying SEF14 fimbriae (Panels C and D respectively). Large aggregates of non-cell associated SEF14 fimbriae were also observed in the sample containing RMJ011 (Panel E). The morphology of expressed fimbriae was essentially the

Figure 4.12: SefA production by RMJ010 and RMJ011

A: The strains RMJ009 (pBC-KS+), RMJ010 (pRJ004) and RMJ011 (pRJ005) were grown on LB and CFA agar and their relative growth recorded. Whole cells (WC) samples were prepared (section 2.14.1) and any aggregation was recorded.

[a] Growth: ++ represents >75% coverage of agar plate; + represents 50-75% coverage of agar plate; ± represents < 50% coverage of agar plate;

[b] Aggregation: + represents aggregation; - represents no aggregation.

B: Heat shock supernatants of the strains RMJ009 (pBC-KS+), RMJ010 (pRJ004) and RMJ011 (pRJ005) grown on CFA agar were analysed by SDS-15% page and Coomassie blue staining (section 2.14.4). The arrow indicates a SefA band present in S/N samples of RMJ010 and RMJ011 but not in RMJ009. Molecular weight standards (kDa; section 2.14.3) are indicated.

A

Strain	Medium	Growth[a]	Aggregation[b]
RMJ009 (pBC-SK$^+$)	LB	++	-
RMJ009 (pBC-SK$^+$)	CFA	++	-
RMJ010 (pRJ004)	LB	++	-
RMJ010 (pRJ004)	CFA	+	+
RMJ011 (pRJ005)	LB	++	-
RMJ011 (pRJ005)	CFA	±	+

B

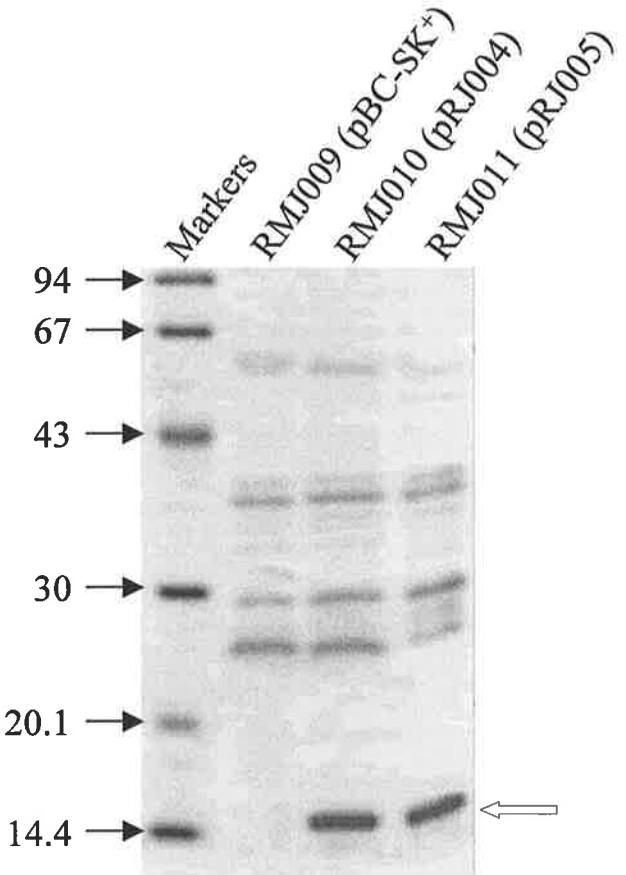
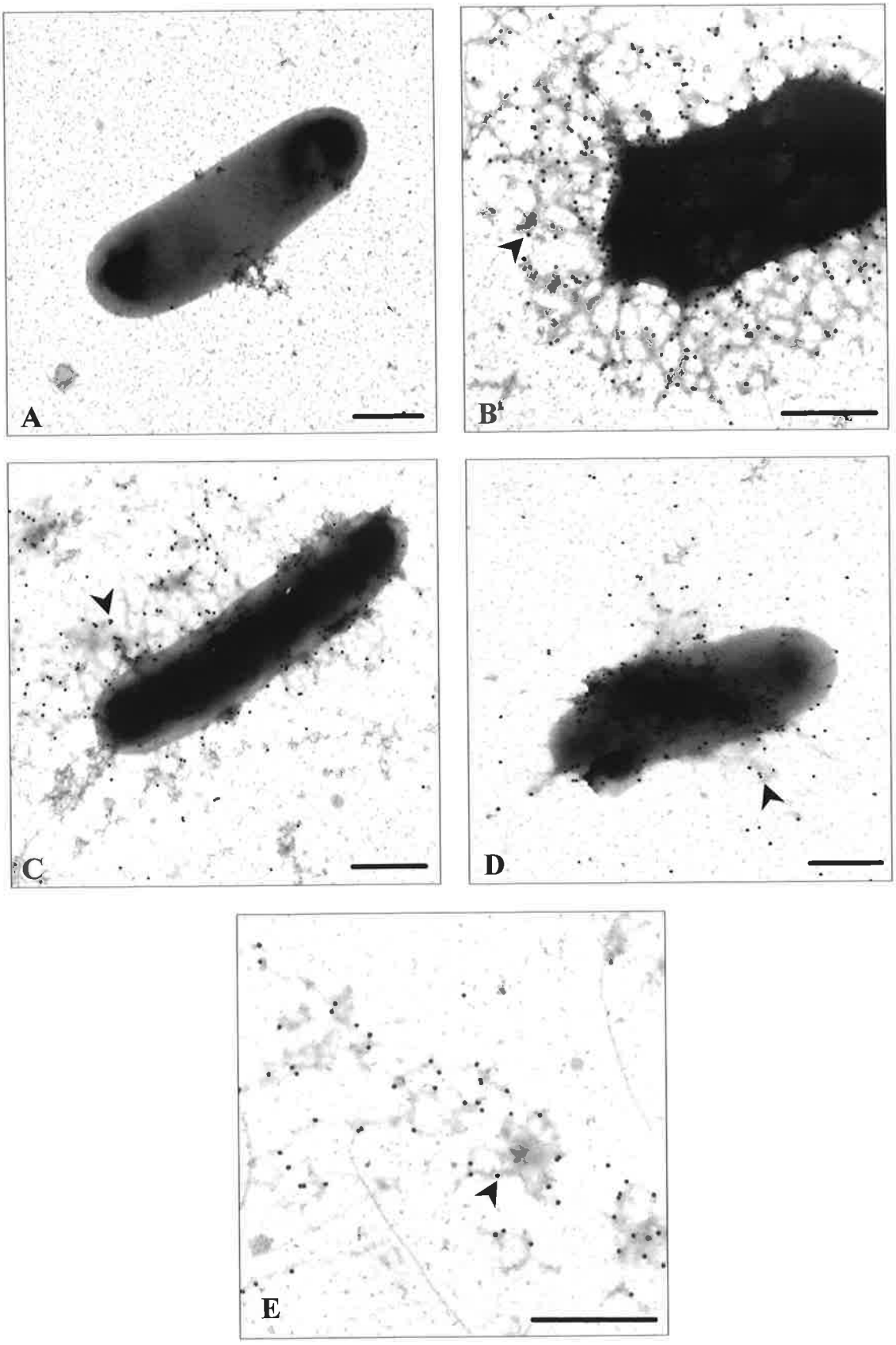


Figure 4.13: IEM analysis of SEF14 production by minimal *sef* fragments

The strains 11RX, RMJ009 (control), RMJ010 (pRJ004) and RMJ011 (pRJ005) were grown on CFA agar and prepared for immunoelectron microscopy as per section 2.19, using 1/50 anti-SefA [adsorbed]. The control strains 11RX and RMJ009 were positive and negative respectively, for SEF14 expression as expected (Panels A and B). RMJ010 (Panel C) and RMJ011 (Panel D) were also positive for SEF14 expression. Panel E shows a cell free aggregate of SEF14 fimbriae that was observed in samples prepared from RMJ010 and RMJ011. Arrowheads indicate examples of gold particles bound to fimbriae. Scale bar represents 0.5 μm.


A
B
C
D
E

same for all positive samples, however *E. coli* K-12 with *sefA* carrying minimal plasmids pRJ004 and pRJ005 rarely presented the same amount of fimbriae seen in the 11RX sample.

This result confirmed the role of the *sefABCD* genes in the expression of SEF14 fimbriae, and showed also that *sefR* is not critical for expression, at least in a high copy plasmid number environment. In order to better simulate the wild type situation, further analysis of minimal clones was performed in a low copy vector background. It was hypothesised that in this low copy number environment, the role of SefR would become more critical in expression of the *sefABCD* operon. In order to circumvent the problem of trying to express *sefR* from the same plasmid construct, a two-plasmid system was devised, such that *sefR* was provided *in trans* on an inducible vector, with the *sefABCD* genes present on the low copy vector pWSK130.

4.5 Two-plasmid system for indirect analysis of SefR activity

4.5.1 Subcloning of *sefABCD* into pWSK130

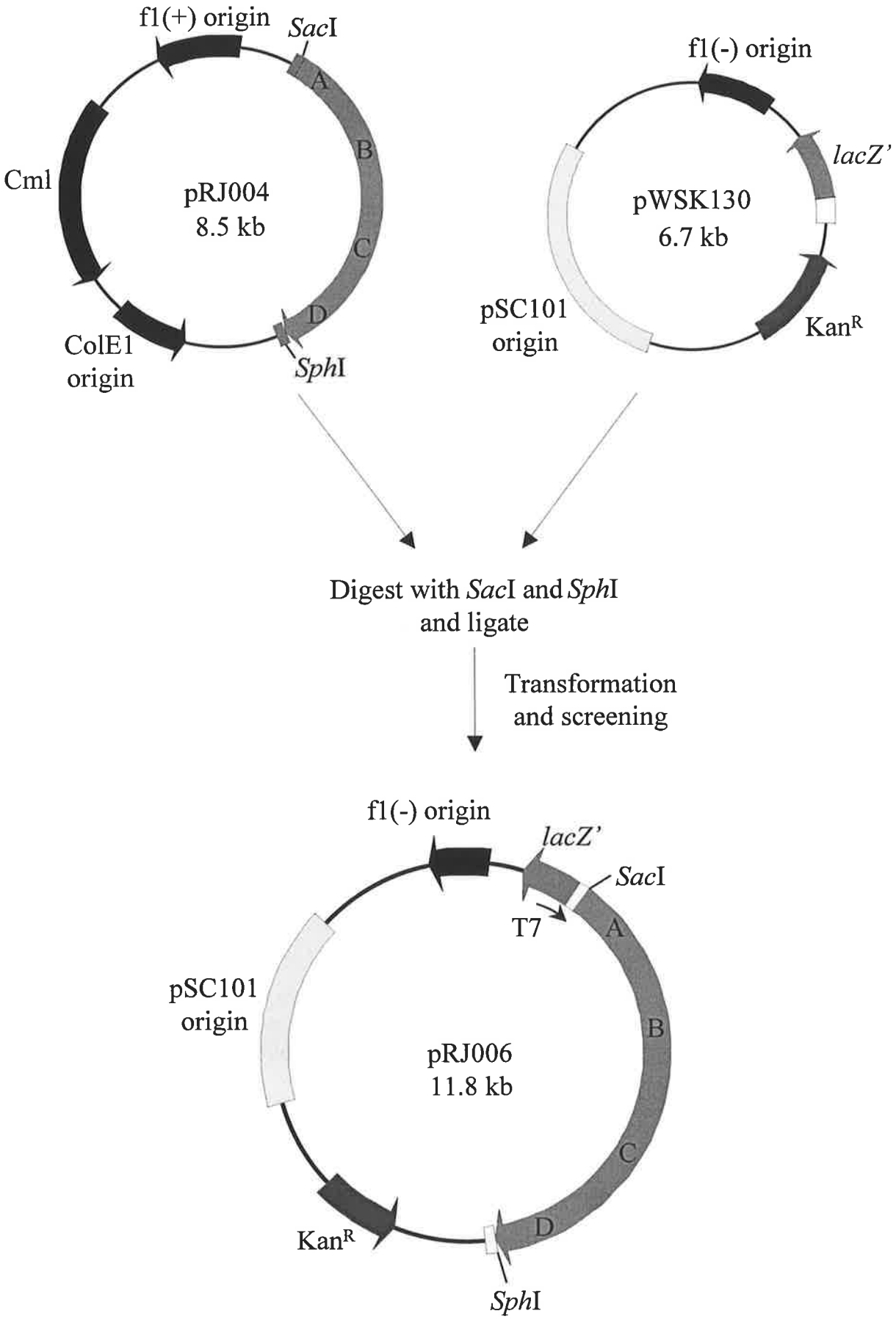
Figure 4.14 depicts the strategy used to subclone the *sefABCD* genes into pWSK130. Clone pRJ004 was digested with *SacI* and *SphI* to liberate the *sefABCD* containing fragment, which was ligated into similarly digested pWSK130. After ligation and transformation of *E. coli* DH5α, plasmid DNA isolates were screened and the appropriate plasmid construct designated pRJ006 (strain RMJ012).

4.5.2 Analysis of *sefABCD/sefR* two plasmid system for SEF14 expression

To assess the ability of SefR to induce expression of SefA, plasmids pRJ003 and pRJ006 were sequentially transformed into *E. coli* DH5α, producing strain RMJ015. To ensure that production of SefA was due solely to *sef* genes, the plasmid control strains RMJ013 and RMJ014 were produced by transforming strains RMJ004 and RMJ012 with plasmids pWSK130 and pBAD30 respectively.

Figure 4.14: Subcloning of *sefABCD* into pWSK130

The *sefABCD* genes (represented by A, B, C and D, respectively) were liberated from pRJ004 by restriction digest with *Sph*I and *Sac*I, subcloned into similarly digested pWSK130 and transformed into *E. coli* DH5α. Plasmid DNA from transformants was screened by restriction digest and PCR. A positive construct was selected and designated pRJ006 (strain RMJ012).


f1(+) origin
SacI
A
B
C
D
SphI
Cml
pRJ004
8.5 kb
ColE1
origin
f1(-) origin
lacZ'
pWSK130
6.7 kb
pSC101
origin
Kan^R
Digest with SacI and SphI
and ligate
Transformation
and screening
f1(-) origin
lacZ'
SacI
T7
A
B
C
D
pSC101
origin
pRJ006
11.8 kb
Kan^R
SphI

Strains DH5α, RMJ004, RMJ012, RMJ013, RMJ014, and RMJ015 were grown on either CFA agar alone, or on CFA agar supplemented with 0.2% (w/v) glucose or 0.2% (w/v) arabinose. All strains grew normally on all media types except RMJ015 (pRJ003 + pRJ006), which grew poorly in the presence of 0.2% (w/v) arabinose. To test for SefA expression, bacteria were harvested in PBS and samples of whole cell (WC) and heat-shocked supernatant (S/N) were analysed by SDS-15% PAGE and Coomassie blue staining (Section 2.14.1).

Figure 4.15 shows Coomassie blue stained gels of the above samples. As expected, the host strain DH5α without any plasmids showed no evidence of SefA production when grown with either glucose or arabinose (Gel A lanes 1-6). Strains RMJ004 (pRJ003; panel A lanes 7-12) and RMJ012 (pRJ006; panel B lanes 1-6) also failed to produce SefA, discounting any plasmid effects in the production of SefA. The negative control strains RMJ013 (pRJ004 + pWSK130; panel B lanes 7-12) and RMJ014 (pRJ006 + pBAD30; panel C lanes 1-6) also failed to produce SefA. The lack of SefA expressed by RMJ012 was interesting as it validated the hypothesis that plasmid copy number was responsible for the expression of SefA by strain RMJ010 in the absence of *sefR*. Strain RMJ015 failed to express SefA when grown without arabinose (gel C lanes 7-10). However, when the media was supplemented with arabinose (gel C lanes 11 & 12), SefA was present in both the WC and S/N samples.

Western immunoblot analysis of these samples using anti-SefA serum confirmed the results observed by SDS-15% PAGE and Coomassie blue staining. Only RMJ015 grown in the presence of arabinose was positive for expression of SefA, as shown in Figure 4.16. All other samples were negative for SefA (not shown).

4.6 Construction of vectors for quantification of SefR activity

The results presented thus far have shown that SefR is required to upregulate the production of SefA in *E. coli* K-12. A method was devised to quantify the *sefA* expression.

Figure 4.15: Coomassie stained PAGE analysis of SefA expression.

Strains DH5α, RMJ004 (pRJ003), RMJ012 (pRJ006), RMJ013 (pRJ003 + pWSK130), RMJ014 (pRJ006 + pBAD30), and RMJ015 (pRJ003 + pRJ006) were grown on CFA agar, and in the presence of either 0.2% glucose or 0.2% arabinose. Cells were harvested in PBS and a sample heat shocked to release expressed fimbriae (Section 2.13.1). 40µl samples of heat shocked supernatants (S/N) and whole cells (WC; equivalent of ~10^8 cells) were analysed by SDS-15% PAGE and Coomassie blue staining. The arrow indicates expressed SefA. The Table shown below represents the samples analysed. Molecular weight standards (kDa; section 2.14.3) are indicated.

Lane	Panel A	Panel B	Panel C
1	DH5α (S/N)	RMJ013 (S/N)	RMJ014 (S/N)
2	DH5α (WC)	RMJ013 (WC)	RMJ014 (WC)
3	DH5α (S/N) + glu	RMJ013 (S/N) + glu	RMJ014 (S/N) + glu
4	DH5α (WC) + glu	RMJ013 (WC) + glu	RMJ014 (WC) + glu
5	DH5α (S/N) + ara	RMJ013 (S/N) + ara	RMJ014 (S/N) + ara
6	DH5α (WC) + ara	RMJ013 (WC) + ara	RMJ014 (WC) + ara
7	RMJ004 (S/N)	RMJ012 (S/N)	RMJ015 (S/N)
8	RMJ004 (WC)	RMJ012 (WC)	RMJ015 (WC)
9	RMJ004 (S/N) + glu	RMJ012 (S/N) + glu	RMJ015 (S/N) + glu
10	RMJ004 (WC) + glu	RMJ012 (WC) + glu	RMJ015 (WC) + glu
11	RMJ004 (S/N) + ara	RMJ012 (S/N) + ara	RMJ015 (S/N) + ara
12	RMJ004 (WC) + ara	RMJ012 (WC) + ara	RMJ015 (WC) + ara

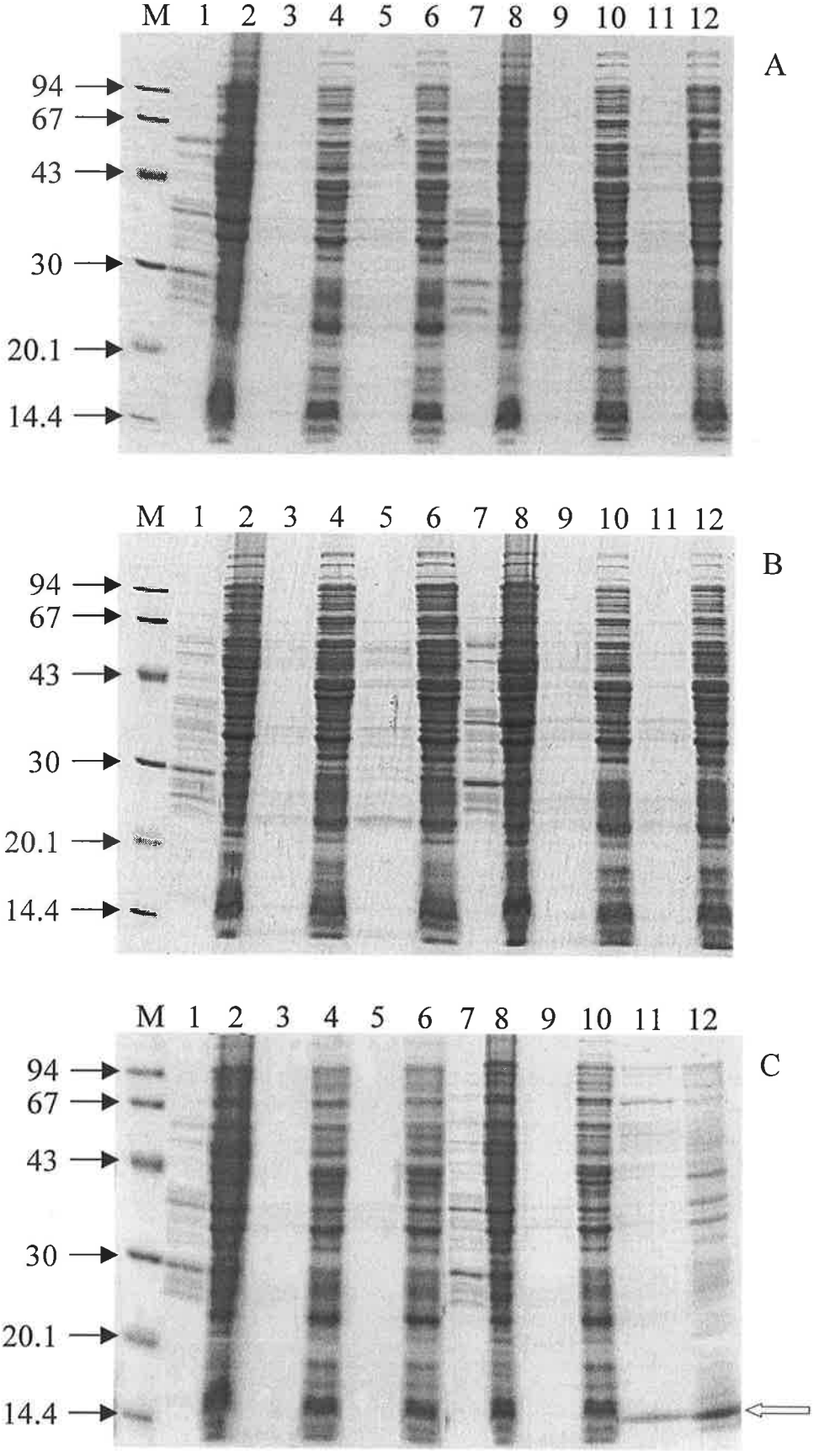
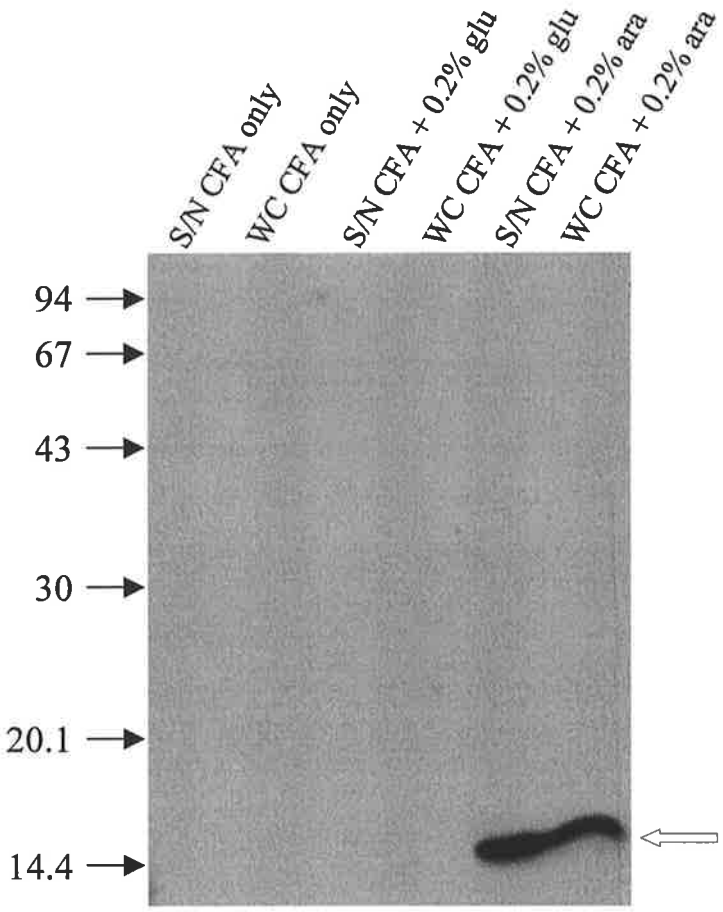

M 1 2 3 4 5 6 7 8 9 10 11 12
94
67
43
30
20.1
14.4
A
M 1 2 3 4 5 6 7 8 9 10 11 12
94
67
43
30
20.1
14.4
B
M 1 2 3 4 5 6 7 8 9 10 11 12
94
67
43
30
20.1
14.4
C

Figure 4.16: Western analysis of SefA production by strain RMJ015

Strain RMJ015, containing the *sefR* expression construct pRJ003 and the low copy *sefABCD* construct pRJ006, was grown on CFA agar and CFA agar supplemented with either 0.2% glucose or 0.2% arabinose. Samples of whole cell (WC) and heat shock supernatants (S/N) (section 2.13.1; equivalent of ~10^8 cells) were electrophoresed on an SDS-15% PAGE gel, transferred to nitrocellulose membrane and Western immunoblotted using anti-SefA [adsorbed] at 1/1000 (section 2.14.4). Molecular weight standards (kDa; section 2.14.3) are indicated


S/N CFA only
WC CFA only
S/N CFA + 0.2% glu
WC CFA + 0.2% glu
S/N CFA + 0.2% ara
WC CFA + 0.2% ara
94
67
43
30
20.1
14.4

The *sefR* expression vector pRJ003 was co-transformed with a *sefA*::*lacZ* reporter construct (see section 4.6.1 below) to facilitate this quantitation.

4.6.1 Construction of *sefA'*::*lacZYA* fusion

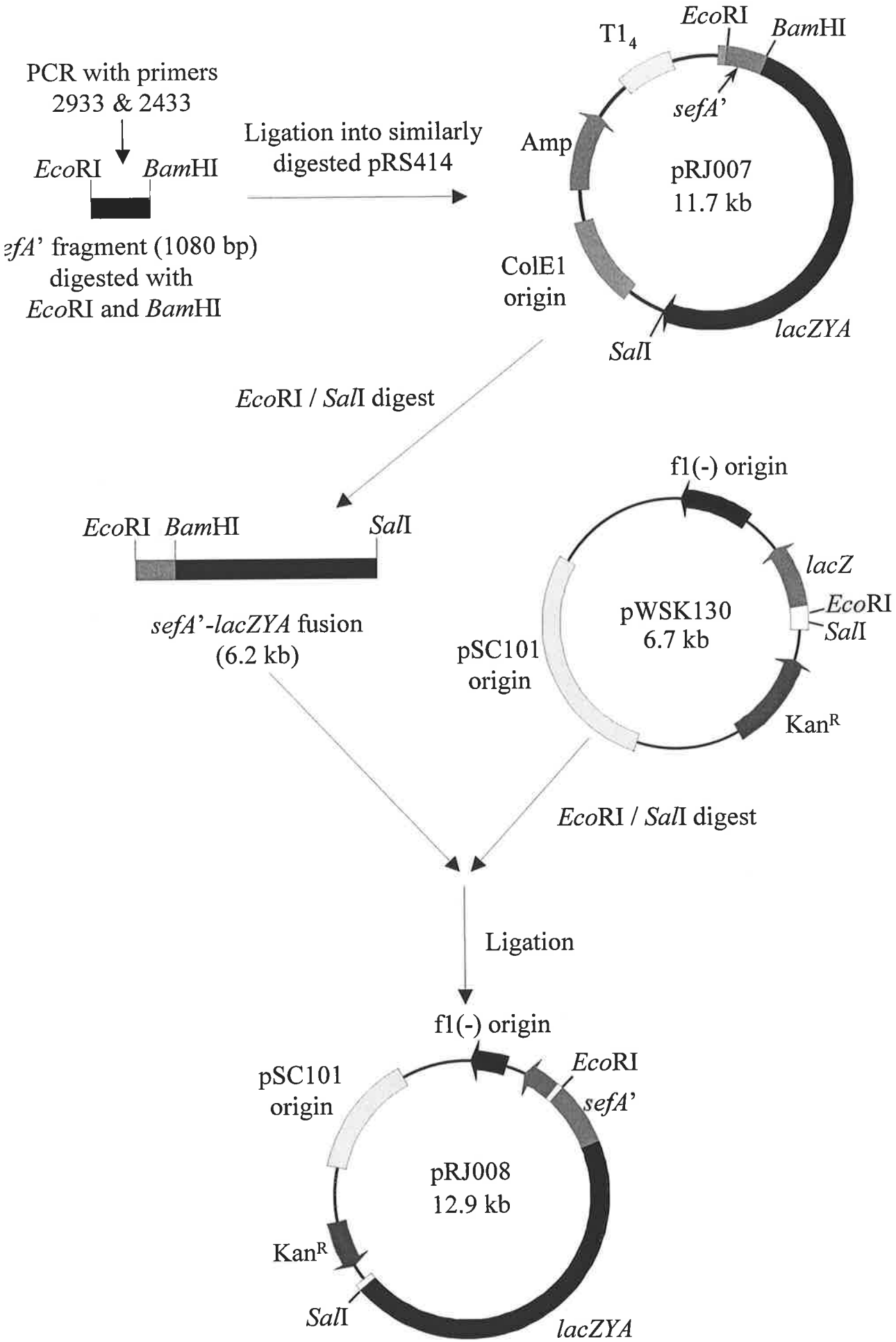
A *sefA'*::*lacZYA* fusion was constructed by PCR amplification of *sefA* from 11RX chromosomal DNA using primer 2933, such that the *sefA* promoter was incorporated as a target for SefR, and primer 2433 which bound at position 156-136 within the *sefA* gene. This primer bound such that it encompassed the *Bam*HI restriction site at nucleotide position 149 of *sefA*. The *sefA'* PCR product was digested with *Eco*RI and *Bam*HI and ligated into similarly digested pRS414 (Simons *et al*, 1997). After transformation and screening, the correct construct was designated pRJ007 (strain RMJ016). pRJ007 was subsequently digested with *Eco*RI and *Sal*I to liberate the *sefA'*::*lacZYA* fusion. This fragment was ligated into similarly digested pWSK130, and the ligation reaction transformed into DH5α. Transformants were screened by restriction digest and the appropriate construct designated pRJ008 (strain RMJ017). Figure 4.17 shows the cloning strategy, which produced a fusion between the first 49 aa of SefA and the LacZ protein, under the control of the *sefA* promoter.

4.6.2 Analysis of *sefA*::*lacZ* expression

Plasmids pRJ003 and pRJ008 were sequentially transformed into the target strain CC118 (Table 2.1) to give rise to the strains RMJ018, RMJ019 and RMJ020 (with pRJ003, pRJ008, and pRJ003 + pRJ008, respectively). These clones were tested for growth in CFA broth in the presence of either 0.2% (w/v) glucose or 0.2% (w/v) arabinose to assess whether these sugars would have any impact on growth rate. It was observed that RMJ020 did not grow in this concentration of arabinose, perhaps due to a toxic effect of SefA-LacZ expression. RMJ020 was further tested with 0.1% (w/v), 0.05% (w/v), 0.01% (w/v) and 0.002% (w/v) arabinose. Growth improved with decreasing arabinose concentration, however

Figure 4.17: Construction of *sefA'*::*lacZYA* fusion vector pRJ008

The construction of pRJ008 is shown. The *sefA'* fragment was generated by PCR amplification from 11RX chromosomal DNA using primers 2933 and 2433 (2.10.1), followed by restriction digest with *Eco*RI and *Bam*HI (section 2.9.2). This fragment was ligated into similarly digested pRS414 (Simons *et al*, 1987), resulting in a fusion of the first 49 amino acids of *sefA* to the 9th amino acid of the LacZ protein. Following transformation of DH5α, plasmid DNA was screened and the appropriate plasmid construct designated pRJ007. The *sefA'*::*lacZYA* fusion was then subcloned into pWSK130 by digestion of pRJ007 with *Eco*RI and *Sal*I, and ligation into similarly digested pWSK130. After transformation of DH5α, plasmid DNA was screened by restriction digest and the appropriate clone of pWSK130 containing *sefA'*::*lacZYA* was designated pRJ008 (Strain RMJ017).


PCR with primers
2933 & 2433
EcoRI
BamHI
sefA' fragment (1080 bp)
digested with
EcoRI and BamHI
Ligation into similarly
digested pRS414
T1 4
EcoRI
BamHI
sefA'
Amp
pRJ007
11.7 kb
ColE1
origin
SalI
lacZYA
EcoRI / SalI digest
EcoRI BamHI
SalI
sefA'-lacZYA fusion
(6.2 kb)
f1(-) origin
lacZ
EcoRI
SalI
pWSK130
6.7 kb
pSC101
origin
Kan R
EcoRI / SalI digest
Ligation
f1(-) origin
pSC101
origin
EcoRI
sefA'
pRJ008
12.9 kb
Kan R
SalI
lacZYA

growth at 0.01% (w/v) was slower than that observed for control strains. Growth in 0.002% (w/v) arabinose was the same as the control strains.

Whole cell samples of control strains RMJ018 and RMJ019 grown with 0.2% (w/v) glucose or 0.2% (w/v) arabinose as well as RMJ020 grown in 0.2% (w/v) glucose and 0.01% (w/v) or 0.002% (w/v) arabinose were electrophoresed on a SDS-15% polyacrylamide gel and stained with Coomassie blue (Figure 4.18). A protein with an apparent molecular weight of 31 kDa is likely to be overexpressed SefR from RMJ018 grown with arabinose (lane 2). The putative SefR protein is absent when the same clone is grown in the presence of glucose (lane 1). A protein with an apparent molecular weight greater than 94 kDa is present in RMJ020 grown in 0.01% arabinose (lane 6) and absent when E012 was grown in 0.2% glucose (lane 5) or 0.002% arabinose (lane 7). The size of this protein corresponds to the estimated 120 KDa SefA'::LacZ fusion protein. Interestingly, a band corresponding to the SefR protein was not seen in lanes 6 or 7, despite being present in lane 2. The same samples were analysed by Western immunoblot using an anti-SefA antisera, however the sera was not able to detect the expressed SefA'::LacZ protein. This was not unexpected as the SefA'::LacZ fusion protein only contained the first 49 amino acids of SefA, which may not contain the specific epitopes that anti-SefA [adsorbed] antiserum detects.

4.6.3 LacZ assay to assess *sefA* promoter activity

β-galactosidase assays were performed as per section 2.17 to assess the effect of SefR on *sefA* promoter activity in strain RMJ020 (CC118 containing plasmid pRJ003 and pRJ008). Strains CC118, RMJ018 (CC118 containing pRJ003) and RMJ019 (CC118 containing pRJ008) were used as negative controls. These four strains were grown in CFA broth in the presence of either 0.2% (w/v) glucose or 0.01% (w/v) arabinose. The positive control strain DH5 was grown in LB with or without 1mM IPTG. Table 4.1 presents a summary of the results obtained from three separate experiments. The positive control strain DH5 showed a

Figure 4.18: Detection of SefA'-LacZ' fusion protein.

Expression of the SefA-LacZ fusion was assessed by growing strains RMJ018, RMJ019 and RMJ020 (as described in section 4.6.2) in CFA broth supplemented with either 0.2% glucose or 0.2% arabinose. Whole cell (WC) samples (section 2.14.1), representing ~10^8 bacteria, were analysed by SDS-15% PAGE and Coomassie Blue staining. The samples are indicated above their respective lanes. A protein corresponding to the molecular weight (31.4 kDa) of SefR is designated by the thin arrow, similar to that seen previously in Figure 4.7 lane 4. The hollow arrow designates a protein of mass between 83 and 175 kDa, which corresponds to the estimated 120 kDa SefA'-LacZ fusion protein. Pre-stained molecular weight standards (kDa; section 2.14.3) are indicated.

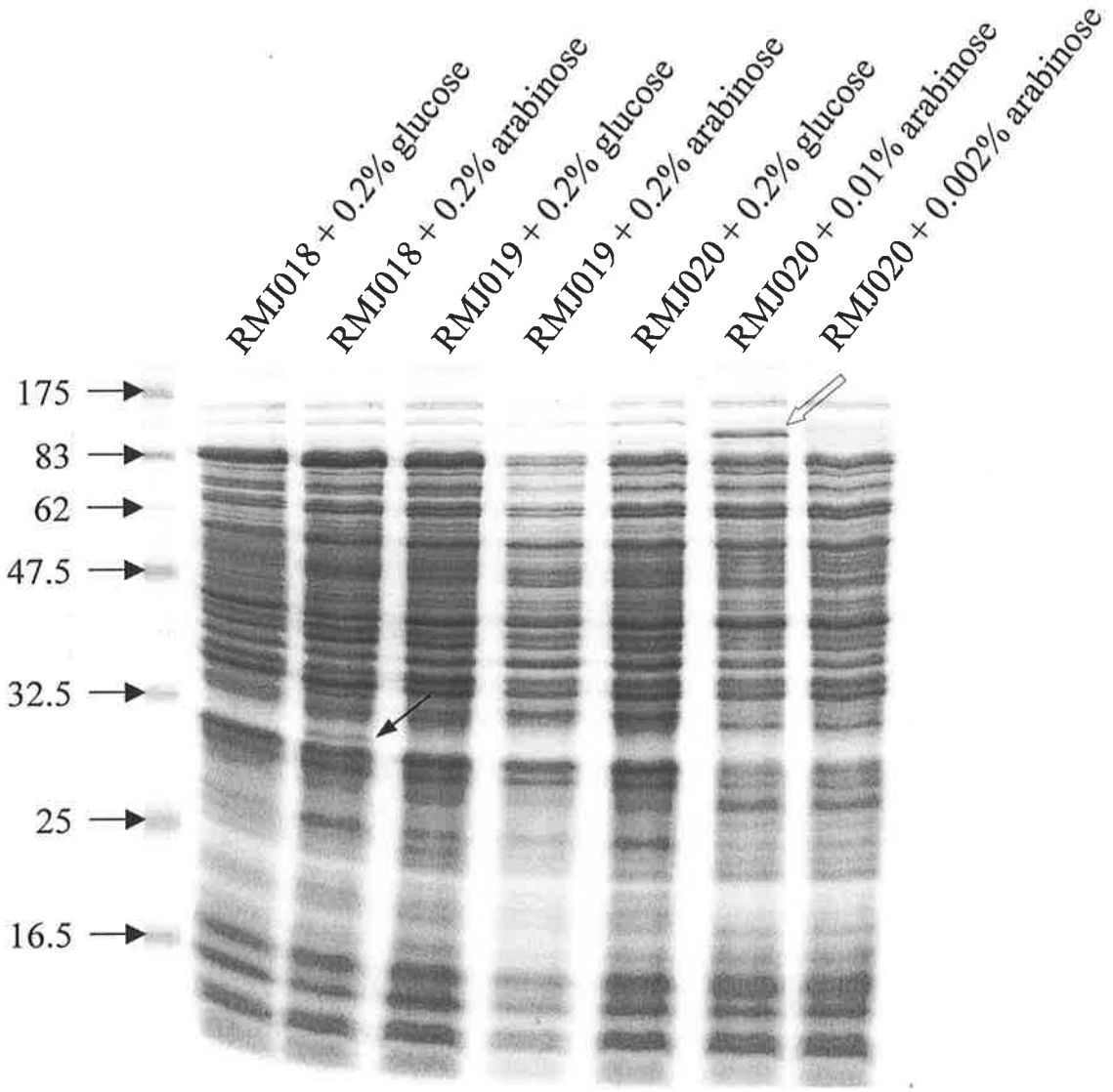

RMJ018 + 0.2% glucose
RMJ018 + 0.2% arabinose
RMJ019 + 0.2% glucose
RMJ019 + 0.2% arabinose
RMJ020 + 0.2% glucose
RMJ020 + 0.01% arabinose
RMJ020 + 0.002% arabinose
175
83
62
47.5
32.5
25
16.5

Table 4.1: β-galactosidase assay to detect activity from *sefA* promoter.

A series of β-galactosidase assays were performed (section 2.17) to determine the effect of SefR on expression from the *sefA* promoter. Strains DH5, CC118, RMJ018 (pRJ003), RMJ019 (pRJ008) and RMJ020 (pRJ003 + pRJ008) were grown in CFA broth supplemented with either 0.2% (w/v) glucose or 0.01% (w/v) arabinose and assayed for β-galactosidase activity. The results shown here represent three independent experiments, with samples assayed in triplicate.

[a] β-galactosidase Units were determined using the formula in section 2.17.

[b] The strains listed here are described in Table 2.1 and section 4.6.2

[c] Fold induction represents the β-galactosidase units in inducing conditions divided by the β-galactosidase units in non-inducing conditions.

[d] Values are presented as the mean plus or minus the standard error.

Strain[b]	Genes	β-galactosidase Units[a] -IPTG	β-galactosidase Units[a] +IPTG	Fold induction[c]
DH5 (positive control)	*lacZYA*	22 ± 3.4	2506 ± 54.7[d]	114
		glu (0.2%)	**ara (0.2%)**	
CC118 (negative control)	*lacZ*⁻	14 ± 1.1	22 ± 1.0	1.6
RMJ018 (pRJ003)	*sefR*	22 ± 2.2	33 ± 5.2	1.5
RMJ019 (pRJ008)	*sefA'::lacZ*	25 ± 6.2	7 ± 1.9	0.28
RMJ020 (pRJ003 + pRJ008)	*sefR* + *sefA'::lacZ*			
Experiment 1		16 ± 4.1	221 ± 5.5	14
Experiment 2		14 ± 5.9	1042 ± 66.0	74
Experiment 3		14 ± 1.5	641 ± 42.9	46

114-fold increase in β-galactosidase activity when grown in the presence of IPTG. The control strains CC118, RMJ018 and RMJ019, grown with either 0.2% (w/v) glucose or 0.01% (w/v) arabinose and showed levels of β-galactosidase activity similar to DH5 grown without IPTG. A similar level of β-galactosidase activity was observed for RMJ020 when grown with 0.2% (w/v) glucose. When RMJ020 was grown with 0.2% (w/v) arabinose, the level of β-galactosidase activity varied for each assay, with increases of 14-, 74- and 46-fold. This variation occurred despite bacteria being grown under identical conditions for each assay. It was thought that the slower growth of RMJ020 in the presence of arabinose might have been accompanied by cell lysis, which in turn would lead to errors in the estimations of bacterial numbers. These variations would then impact on the calculations for β-galactosidase activity. Despite the variation in activity, it is clear from these results that SefR is able to upregulate expression of SefA, presumably from the *sefA* promoter.

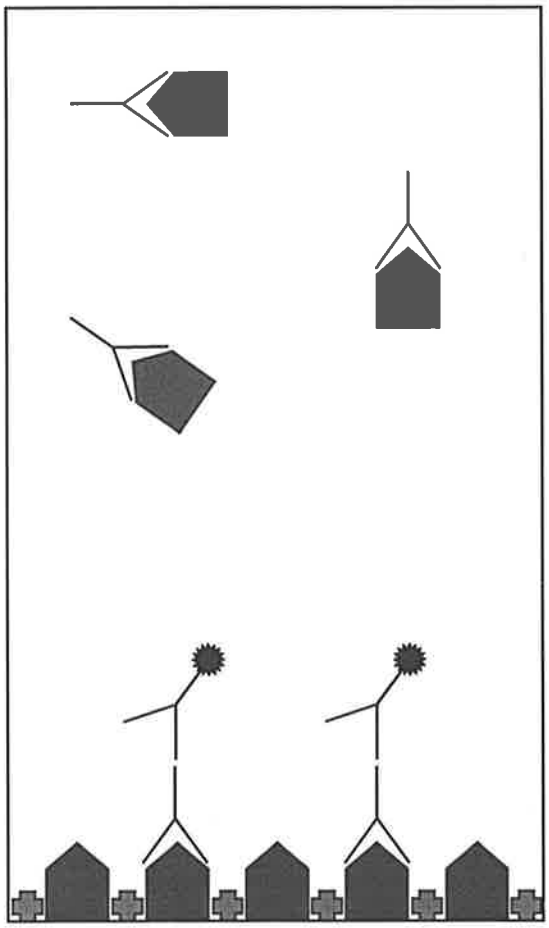
4.7 Development of an ELISA inhibition assay (EIA) for quantitation of SefA production

Due to the variation in the β-galactosidase system used above, an ELISA inhibition assay (section 2.18) was developed to quantify the levels of surface expressed SefA, using clones constructed previously (Sections 4.3.4 and 4.5.2). This assay was adapted from a method used by Hone *et al* (1988), and simply required optimisation of antibody and sensitising protein (SefA) concentrations. This method would also allow analysis of 11RX and mutant strains without the need for integrating β-galactosidase fusions into the chromosome.

An outline of the ELISA inhibition assay is presented in Figure 4.19. By coating sample wells with a known concentration of SefA, and combining the sample to be assayed with a known concentration of 1° antibody in the coated well, there is competition between the two sources of SefA for 1° antibody. Therefore, increasing concentration of SefA in a

Figure 4.19: EIA protocol

Schematic representation of the EIA protocol (section 2.18.3). ELISA trays were coated with 5 μg/ml of SefA and blocked with a 1% BSA (w/v) solution in PBS. Samples were diluted as necessary before adding to the appropriate wells and serially diluting 1/3 across the tray using the BSA solution. This was followed by addition of the 1°Ab and incubation at 37°C. During this time any SefA in the sample would competitively inhibit binding of 1° Ab to the tray-associated SefA. After incubation, the sample-1°Ab mix was removed and HRP-conjugated 2°Ab added. Colour detection of bound 2°Ab was performed using di-sodium p-Nitrophenyl Phosphate (0.9 mg/ml) and reading samples at OD_{405} and OD_{600} to measure substrate and background values, respectively.



SefA protein (5µg/ml)

1% BSA (w/v)

Anti-SefA antibody

Goat-anti-Rabbit HRP conjugate

sample leads to progressively less 1° antibody available for binding to the SefA bound to the well. This in turn leads to a reduction in the amount of coloured end product. By this method, the amounts of SefA in a sample could be determined, as compared to a set of SefA standards of known concentration.

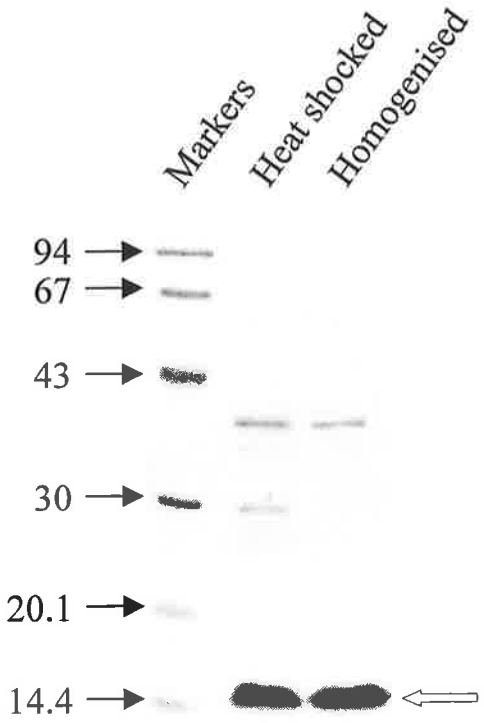
A standard curve was included with each assay tray, providing an internal control for all samples, which would take into account variations in washing between trays. The standard curve was determined by plotting the mean of duplicate OD_{405} values for each concentration of SefA against the Log_{10} of the dilution required to give that concentration. This results in a sigmoidal curve, with the straight portion representing the most sensitive and therefore the most accurate part of the curve. Those OD_{405} values for each sample that fell within this region were used to determine the concentration of SefA for that sample. Those samples with OD_{405} values that fell outside the upper range of the standard curve were considered to have no detectable SefA. The level of detection for these assays was defined as the concentration of SefA that would give a 50% reduction in colour development, which in the absence of SefA was ~0.9 OD units and the level of detection was calculated to be ~0.01 μg of SefA per 10^{10} bacteria.

4.7.1 Preparation of SefA protein stock to use in ELISA

SefA was used as the competitive agent bound to the ELISA tray in known concentrations for EIA quantitation of SefA. As shown in section 4.4.1, strain RMJ011 expresses large amounts of SefA, and this strain was used to produce a SefA stock. RMJ011 was grown on 30 x 30 cm CFA agar plates and fimbriae were obtained either by heat shock (section 2.13.1) or homogenisation of bacteria (2.13.2). A sample of each supernatant was analysed by SDS-15% PAGE and Coomassie blue staining, as shown in Figure 4.20. Both samples showed large amounts of SefA (arrow), however volume analysis using the Quantity One program (BioRad) revealed that SefA obtained by homogenisation constituted ~93% of

Figure 4.20: SDS-PAGE analysis of purified SefA

Samples of supernatants from heat shocked and homogenised RMJ011 grown in the presence of 0.2% arabinose were analysed by SDS-15% PAGE and Coomassie Blue staining, and the proportion of SefA was determined by volume analysis using Quantity One (BioRad). S/N from ~10^8 cells was loaded for each sample, with the SefA band indicated. Lane 1 shows protein obtained by heat shock, with SefA representing 86% of the total protein. Lane 2 shows the protein obtained by homogenisation, with SefA representing 93% of the total protein. Molecular weight standards (kDa; section 2.14.3) are indicated.


Markers
Heat shocked
Homogenised
94
67
43
30
20.1
14.4

the total protein, compared with ~86% for that obtained by heat shock. To minimise non-specific protein interactions during analysis, the supernatant obtained by homogenisation was used as a SefA protein stock. BCA protein estimation of the SefA protein stock showed that the total protein concentration was ~2 mg/ml or ~1.86 mg/ml SefA.

4.7.2 ELISA to determine reagent concentration

Several assays were performed using various combinations of SefA concentrations and anti-SefA dilutions (section 2.18.1) and it was found that a SefA concentration of 5 µg/ml and anti-SefA [adsorbed] diluted to 1/30,000 gave the best time vs OD_{420} ratio (data not shown); these values were used for all subsequent EIA experiments. Several starting concentrations and dilutions of SefA were assayed to determine the optimal conditions for the standard curve by which the concentration of SefA in unknown samples would be determined. Starting concentrations ranged from 10 µg/ml down to 0.05 µg/ml, with serial dilutions performed at 1/2, 1/5 or 1/10. It was found that using 5 µg/ml SefA as the starting concentration and serially diluting 1/5 gave optimal results for the standard curve (data not shown). These values were used for all subsequent EIA experiments. The level of detection for these assays was calculated to be < 0.01 µg per 10^{10} bacteria.

4.8 Quantitation of SefA by EIA analysis

4.8.1 Quantitation of SefA in *sefR*::pRJ002 insertion mutants.

It was shown previously that a mutation in *sefR* led to the loss of SefA expression (section 4.3.2). However, it is possible that the *sefR*::pRJ002 insertion mutant strains RMJ002 and RMJ003 are still producing SefA at a level not detectable by Western immunoblotting. Therefore, the mutant strains were compared with the parent strains 11RX and 7314 for their ability to produce SefA. All strains were grown on CFA agar, and samples were prepared by fixing bacteria in sodium azide (section 2.18.2). The concentration of SefA

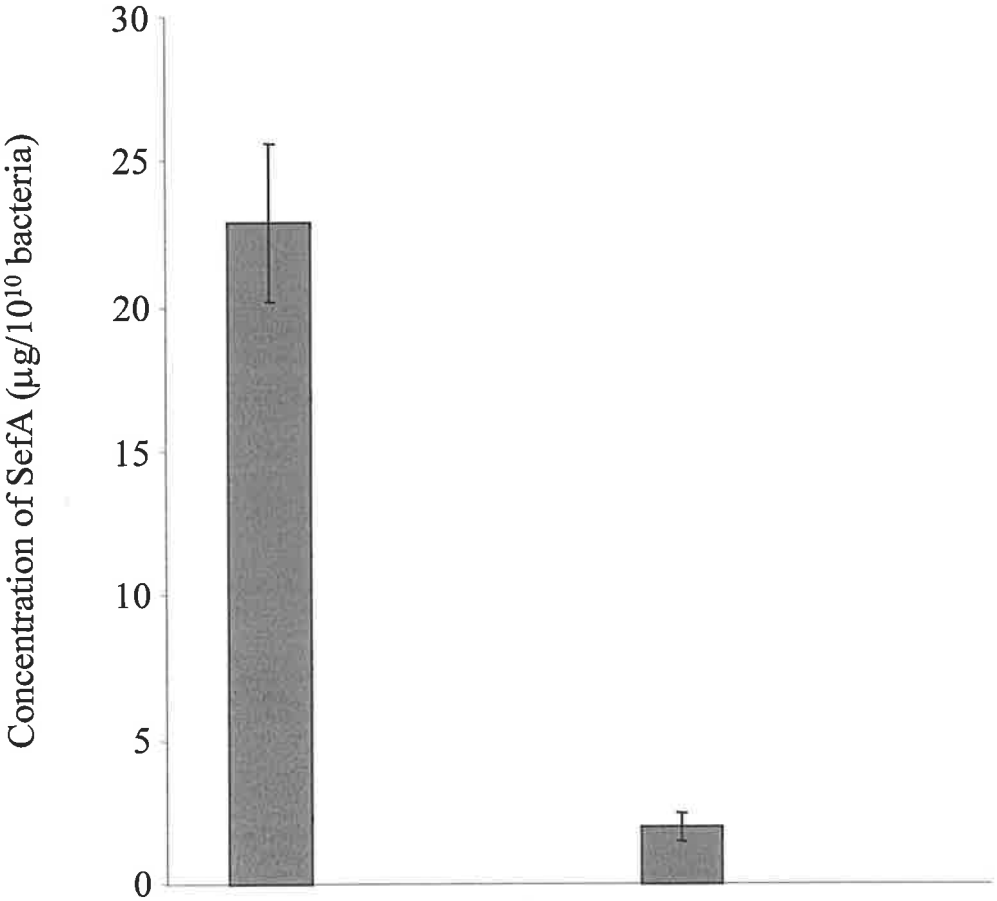
for each strain was determined by EIA analysis as per section 2.18.3. Figure 4.21 shows a histogram comparing the concentration of SefA for each strain. 11RX and 7314 both produced SefA as expected, although an 11-fold difference in concentration was observed (22.9 vs 2.0 $\mu g/10^{10}$ bacteria respectively). Interestingly, SefA was not detectable in either RMJ002 or RMJ003 (i.e. < 0.01 μg SefA per 10^{10} bacteria). This correlated with the observation that SefA is not detectable in *sefA*::kan mutants of 11RX and 7314 (Ogunniyi *et al.*, 1997). This result reinforces the notion that SefR is required for expression of SefA and further, suggests that under the conditions used, no other factor compensates for the loss of SefR.

4.8.2 Quantitation of SefA relative to increasing levels of SefR

It was shown previously in strain RMJ020 (containing the *sefR* expression construct pRJ003 and the *sefA'*::*lacZ* construct, pRJ008) that decreasing arabinose concentration (from 0.2% (w/v) to 0.002% (w/v)) resulted in improved growth (section 4.6.2). It has also been shown that SefR upregulates the expression of SefA (section 4.3.4). From these two observations it was hypothesised that SefR regulates SefA expression in a concentration dependant manner. To test this hypothesis, strain RMJ015 (containing the plasmids pRJ003 and pRJ006) was grown on CFA agar supplemented with either 0.2% (w/v) glucose or 0.1% (w/v), 0.05% (w/v), 0.01% (w/v) or 0.002% (w/v) arabinose. The pattern of growth of RMJ015 in the various arabinose concentrations, i.e. increasing arabinose concentration leading to decreased growth, was consistent with that seen for RMJ020 used in the β-galactosidase assays. Bacterial samples were prepared (section 2.18.2) and analysed by EIA (Section 2.18.3). Figure 4.22 summarises the results obtained for this experiment. As expected, there was no detectable SefA when strain RMJ015 was grown in the presence of glucose. The concentrations of SefA varied when grown in different concentrations of arabinose. The lowest concentration of SefA (299 $\mu g/10^{10}$ bacteria) was seen when RMJ015

Figure 4.21: Quantitation of SefA production by wild-type and *sefR* mutant strains

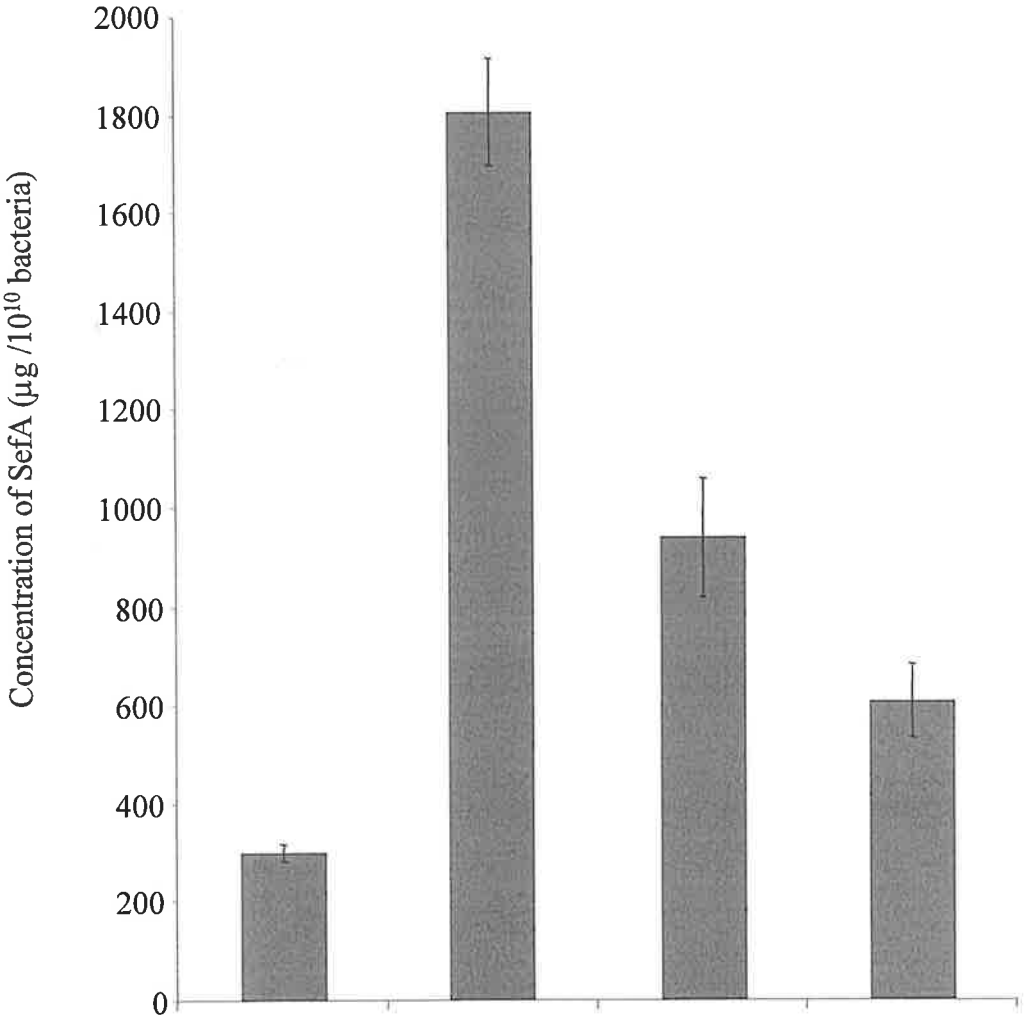
Strains 11RX and 7314 were compared with their respective *sefR* mutants, RMJ002 and RMJ003 for the amount of SefA they produced. All strains were grown on CFA agar, samples prepared by fixing in sodium azide (section 2.18.2) and analysed by EIA (section 2.18.3). Presented here is the concentration of SefA in $\mu g/10^{10}$ bacteria ± standard error calculated from three separate experiments. nd = no SefA detected (i.e. < 0.01 µg of SefA per 10^{10} bacteria).



[SefA] µg/10^{10} bacteria	23 ± 2.68	nd	2 ± 0.48	nd

Figure 4.22: Quantitation of SefA production by RMJ015.

Strain RMJ015 (containing plasmids pRJ003 and pRJ006) was grown on CFA agar supplemented with either 0.2% (w/v) glucose or 0.1% (w/v), 0.05% (w/v), 0.01% (w/v) or 0.002% (w/v) arabinose. Bacteria were prepared as per section 2.18.2 and analysed by EIA (section 2.18.3). Presented here is the concentration of SefA in $\mu g/10^{10}$ bacteria ± standard error calculated from three separate experiments. nd = no SefA detected (i.e. < 0.01 μg of SefA per 10^{10} bacteria). The fold increase is presented relative to sample grown with 0.002% (w/v) arabinose.



Growth condition	0.002%	0.01%	0.05%	0.1%
[SefA] µg/10^{10} bacteria	299 ± 17.3	1805 ± 106.2	939 ± 119.7	608 ± 76.5
Fold-increase	1.0	6.0	3.1	2.0

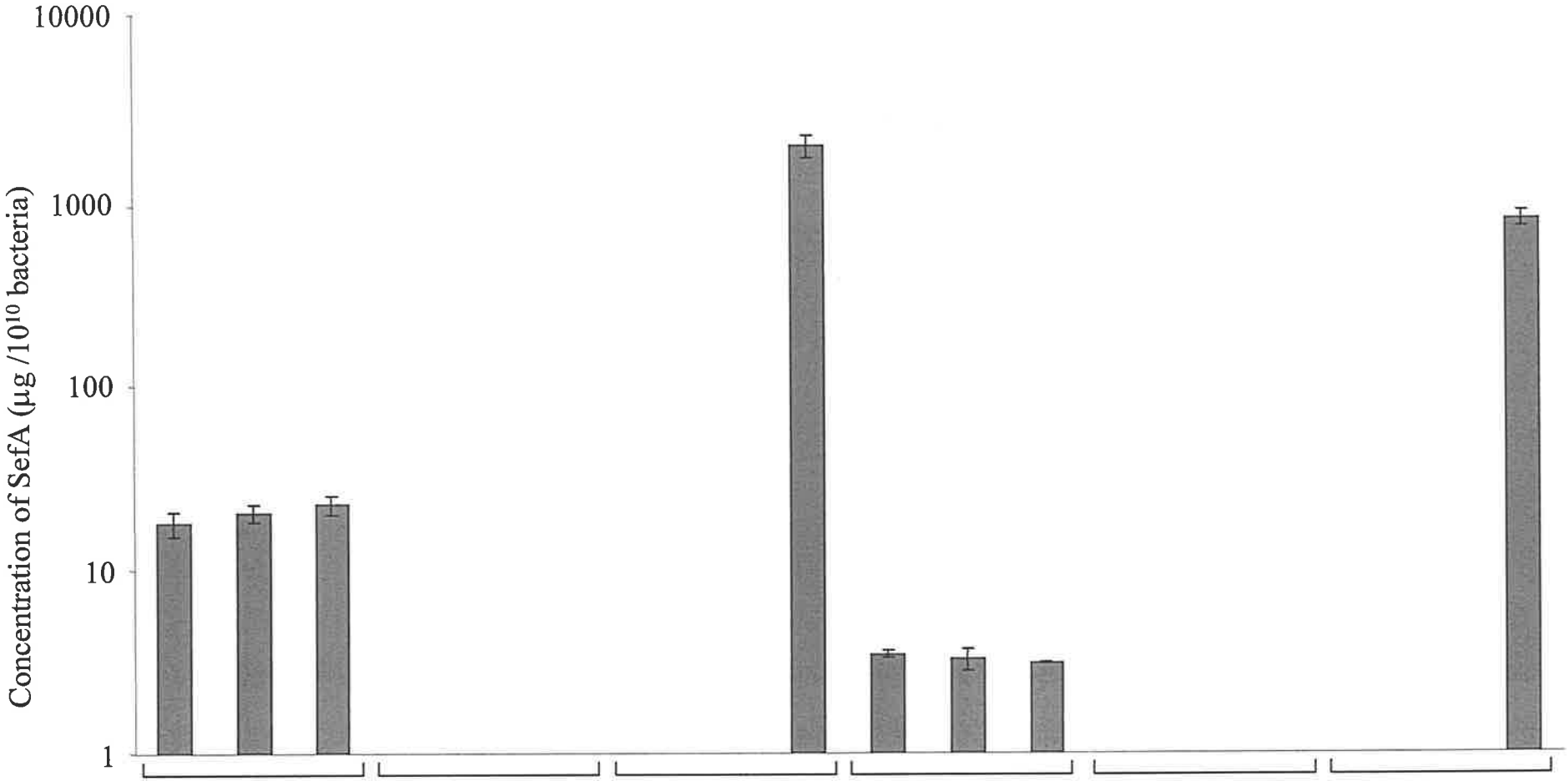
was grown in 0.002% (w/v) arabinose. When the concentration of arabinose was increased to 0.01%, the level of SefA increased (1805 μg/10^{10} bacteria). However, when the level of arabinose was increased further, to 0.05% and 0.1%, the level of SefA decreased (939 μg and 608 μg/10^{10} bacteria, respectively). Therefore, increasing the level of SefR increases the production of SefA up to a point, from which the concentration of SefA declines with increasing SefR levels. This suggested that there was an optimal level of SefR, beyond which further SefA expression has deleterious effects on growth, resulting in an apparent decrease in SefA expression.

4.8.3 Quantitation of SefA in complemented *sefR* mutants

To compare the level of SefA production in wild type, *sefR* mutant and complemented mutant *S.* Enteritidis strains, 11RX, 7314, the *sefR*::pRJ002 insertion mutants RMJ002 and RMJ003 and the sefR complemented mutants RMJ007 (RMJ002 + pRJ003) and RMJ008 (RMJ003 + pRJ003) were analysed for surface expressed SefA by EIA analysis. These strains were grown on either CFA agar alone or CFA agar supplemented with either 0.2% (w/v) glucose or 0.01% (w/v) arabinose, and samples prepared for EIA analysis as per section 2.18.2. A summary of the results is presented in Figure 4.23. The level of SefA produced by 11RX and 7314 was similar to that observed previously (17 to 22 μg/10^{10} bacteria; section 4.8.1) when grown on CFA agar only, or on CFA agar with either 0.2% (w/v) glucose or 0.01% (w/v) arabinose. As expected, no SefA was detected in RMJ002 or RMJ003 when grown under any conditions. Similarly, no SefA was detected when strains RMJ007 and RMJ008 when grown on CFA agar only or in the presence of 0.2% (w/v) glucose. When RMJ007 was grown in the presence of 0.01% (w/v) arabinose, a 97-fold increase (1959 μg/10^{10} bacteria) in the level of SefA over the wild type was found. Interestingly, a 240-fold increase (785 μg/10^{10} bacteria) in the level of SefA was observed for RMJ008 grown in 0.01% (w/v) arabinose.

Figure 4.23: Quantitation of SefA in complemented *sefR* mutants

The concentration of SefA produced by the *sefR* (pRJ003) complemented mutant strains RMJ007 and RMJ008 were compared to that of their respective wild type (11RX and 7314) and *sefR* mutant strains (RMJ002 and RMJ003). All strains were grown on CFA agar alone, and on CFA agar supplemented with either 0.2% glucose or 0.01% arabinose. Bacteria were prepared as per section 2.18.2 and analysed by EIA (section 2.18.3). Presented here is the concentration of SefA in $\mu g/10^{10}$ bacteria ± standard error calculated from three separate experiments. Note that this graph has a log scale, to allow comparison of low and high concentrations of SefA. nd = no SefA detected (i.e. < 0.01 μg of SefA per 10^{10} bacteria).



Strain	11RX			RMJ002			RMJ007			7314			RMJ003			RMJ008		
Growth condition	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara
[SefA] µg/10^{10} bacteria	18 ± 2.7	20 ± 2.3	22 ± 2.7	nd	nd	nd	nd	nd	1959 ± 268	3.4 ± 0.2	3.2 ± 0.4	3.1 ± 0.1	nd	nd	nd	nd	nd	785 ± 78

4.8.4 Analysing effects of temperature on SefA expression

It is known that SEF14 expression does not occur at temperatures less than 30°C (Müller *et al.*, 1991; Walker *et al.*, 1999). The mechanism by which this temperature regulation occurs has not been established. Given that SefR appears to act in a concentration dependant manner (section 4.8.2) it was hypothesised that the repression of SEF14 production at 25°C would be circumvented by the presence of SefR supplied in multiple copies by the vector construct pRJ003, as determined by EIA.

To test this hypothesis, strains RMJ007 (11RX *sefR* [pRJ003] complemented mutant) and RMJ015 (DH5α containing plasmids pRJ003 and pRJ006) were grown on CFA agar supplemented with 0.2% (w/v) glucose and 0.2% (w/v) or 0.01% (w/v) arabinose respectively, at 25°C and 37°C. Both strains showed a reduction in growth when grown in the presence of arabinose, relative to growth at the same temperature in the presence of glucose. The reduction in growth was greater at 37°C than at 25°C. Analysis of these strains by EIA (section 2.18.3) showed no detectable SefA when either strain was grown in the presence of 0.2% glucose, as expected. Interestingly, EIA analysis also revealed that reduced growth inversely correlated with the production of SefA. The production of SefA was 14-fold and 24-fold lower for RMJ007 and RMJ015 respectively at 25°C. These results are summarised in Table 4.2 and demonstrated that in this system, the expected repression of SefA production could be partially overcome by supplying an increased level of SefR *in trans* from pRJ003.

4.8 Summary

The results presented in this chapter have revealed several key features regarding the SEF14 locus and its regulation. First, SefR acts to positively upregulate the expression of *sefA*, as shown by mutagenesis and complementation of the *sefR* gene (section 4.3.4). This result was supported by the induction of β-galactosidase activity in a SefA'::LacZ fusion in the presence of SefR (Section 4.6.3). The upregulation in SefA production led to an increase

Table 4.2: Growth and level of SefA production at 25°C and 37°C

The strains RMJ007 (RMJ002 [11RX *sefR*::pRJ002 carrying pRJ003) and RMJ015 (DH5α carrying pRJ003 and pRJ006) were compared for their ability to express SefA at both 25°C and 37°C. Both strains grown on CFA agar supplemented with either 0.2% (w/v) glucose or 0.01% (w/v) arabinose at 25°C or 37°C, and their relative growths recorded. Samples were prepared (2.18.2) and analysed by EIA (section 2.18.3). This table presents the relative growth of each strain and concentration of SefA ($\mu g/10^{10}$ bacteria ± standard error) is presented for each growth condition, and represents data obtained from three separate experiments. nd = no SefA detected (i.e. < 0.01 μg of SefA per 10^{10} bacteria).

[a] Growth: +++ represents 100% coverage of agar plate; ++ represents >75% coverage of agar plate; + represents 50-75% coverage of agar plate; ± represents < 50% coverage of agar plate;

Strains	Temperature	Additive	Growth[a]	[SefA]
RMJ007	25	glu 0.2%	++	nd
	25	ara 0.2%	++	113 ± 7.9
	37	glu 0.2%	+++	nd
	37	ara 0.2%	+	1567 ± 214.1
RMJ015	25	glu 0.2%	++	nd
	25	ara 0.01%	+	61.6 ± 6.2
	37	glu 0.2%	+++	nd
	37	ara 0.01%	±	1499 ± 63.3

in the expression of SEF14 fimbriae, as demonstrated by IEM analysis of both *S.* Enteritidis *sefR* mutant strains (RMJ002 and RMJ003), and mutant strains complemented with the *sefR* expressing construct pRJ003 (strains RMJ007 and RMJ008) (section 4.3.5). This in turn demonstrated a direct link between SefR expression and SEF14 biogenesis. Regulation by SefR was also shown to occur in a concentration dependant manner (section 4.8.2). Too little SefR resulted in less than maximal expression of SefA, while excess SefR reduced the apparent amount of SefA expressed, as compared to maximal expression. It is unclear whether this was due to some negative feedback effect, or that cell growth was adversely affect by overproduction of SefA, with excess SefA degraded before it could be detected.

While it has been known that the regulation of SEF14 fimbriae is temperature dependant, results presented here provide a mechanism for this regulation. It was demonstrated that the expression of SefA through SefR provided *in trans* could occur at non-permissive temperatures for SefA production, although at a much reduced level when compared to permissive conditions. It was interesting that this temperature dependant expression was similar for both the complemented *S.* Enteritidis *sefR* mutant strains (RMJ007 and RMJ008), and the *E. coli* K-12 SEF14 expression strain RMJ015, suggesting that a common mechanism may be involved. This factor is most likely to be the histone-like protein H-NS, which has been shown to play a role in the regulation of expression in a number of fimbriae systems (Jordi *et al.*, 1992; Morschhauser *et al.*, 1993; White-Ziegler *et al.*, 2000) and is present in both *E. coli* and *Salmonella*.

While SefR was shown to be required for the expression of SefA in the wild-type environment (i.e. single copy number), the *sefABCD* genes alone were sufficient for the production of SEF14 fimbriae, if provided in high copy number in *E. coli* K-12 (strains RMJ010 and RMJ011). This was probably due to a combination of factors, including a basal level of expression from the SefA promoter that is independent of SefR action, expression

from a vector promoter and the presence of multiple copies due to the high copy number vectors used.

Cell free aggregates of SEF14 fimbriae were observed in IEM samples of strains RMJ010 and RMJ011. These are an indication of either mechanical shearing of fimbriae during sample preparation or that the fimbriae were shed due to the lack of some outer membrane stabilizing factor in *E. coli*.

Finally, the apparent expression of SefA varied between the attenuated S. Enteritidis strain 11RX, and the highly virulent strain 7314. The major difference between these strains is in their lipopolysaccharide (LPS), with 11RX containing an undefined mutation leading to a rough phenotype, while 7314 has a smooth phenotype. It is possible that steric hindrance by LPS prevents the accurate detection of all surface expressed SefA in 7314, leading to an underestimation of SefA concentration. It is also possible that 7314 is inherently impaired in expression of SefA relative to 11RX. Analysing the level of SefA expression from a range of different *S*. Enteritidis strains would be a means of determining the 'normal' level of SefA expression. The implications of the results presented in this chapter are examined in more detail in section 7.4.

Chapter 5: Characterisation of the role of *sefD* in biogenesis of SEF14 fimbriae

5.1 Introduction

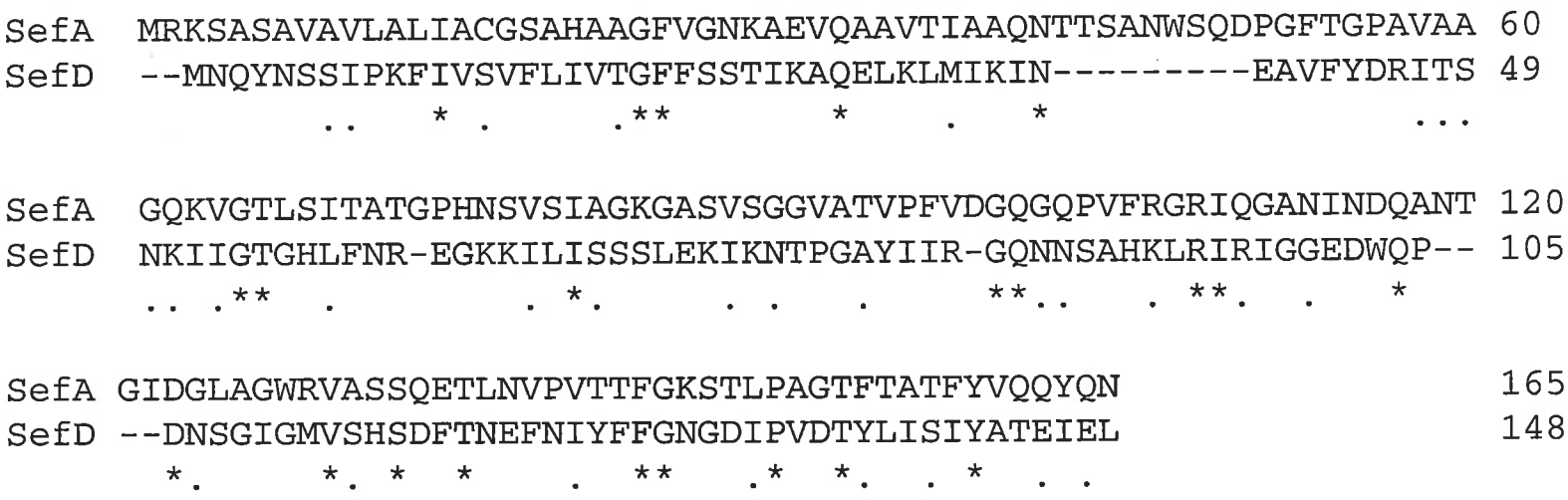
At the beginning of this project the role of the SefD protein in the biogenesis of SEF14 fimbriae was somewhat controversial. In 1994, Clouthier *et al* provided evidence to suggest that SefD constituted the major subunit of SEF18 fimbriae, a structure that was considered distinct from SEF14 fimbriae. This was an interesting result, given the close association of the *sefA* and *sefD* genes within the SEF14 operon, and was considered to be the first encounter with a fimbrial operon that encoded two distinct fimbrial structures.

Clouthier *et al* (1994) generated antisera specific for SefD by immunising rabbits with a purified MBP-SefD fusion protein, and used this serum in immunoelectron microscopy (IEM) analysis of various Enterobacteriaceal strains. Their analysis revealed that SefD appeared to be expressed by all the bacterial species examined, although the morphology varied between species.

However, some details make this result appear less than conclusive. First, it was stated by Clouthier *et al* (1994) that they were unable to purify SEF18 fimbriae. Thus no direct evidence was available to show that SEF18 fimbriae were exclusively composed of SefD subunits. Second, since that study there has been other studies that have demonstrated the limited distribution of the *sefABC* genes (Turcotte and Woodward, 1993, Doran *et al.*, 1996). This contradicted the apparent widespread distribution that was found for both the *sefD* gene and SefD protein. Given that the *sefC* and *sefD* genes overlap at their stop and start codons respectively, it seems unlikely that *sefABC* are limited in distribution while *sefD* is widespread. This line of inquiry is discussed further in Chapter 6, where the distribution of both *sefD* and *sefR* is examined. Third, when the SefA and SefD protein products are compared in a clustal alignment, they show a 14% amino acid identity and 20% similarity (Figure 5.1), which is similar to other fimbrial systems when comparing major and minor subunits, e.g. CsfA/CsfD (CS5 pili), CooA/CooD (CS1 pili), PapA/PapG (Pap pili) and

Figure 5.1: Alignment of predicted amino acids sequences of SefA and SefD

The predicted amino acid sequences of SefA and SefD were determined using DNAsis and aligned using ClustalW (1.5). The Gonnet Protein weight matrix series was used for the alignment. Identical amino acids are denoted by an asterix while the dots represent similar amino acids. These proteins were calculated to be 14% identical and 20% similar.


SefA MRKSASAVAVLALIACGSAHAAGFVGNKAEVQAAVTIAAQNTTSANWSQDPGFTGPAVAA 60
SefD --MNQYNSSIPKFIVSVFLIVTGFFSSTIKAQELKLMIKIN---------EAVFYDRITS 49
SefA GQKVGTLSITATGPHNSVSIAGKGASVSGGVATVPFVDGQGQPVFRGRIQGANINDQANT 120
SefD NKIIGTGHLFNR-EGKKILISSSLEKIKNTPGAYIIR-GQNNSAHKLRIRIGGEDWQP-- 105
SefA GIDGLAGWRVASSQETLNVPVTTFGKSTLPAGTFTATFYVQQYQN 165
SefD --DNSGIGMVSHSDFTNEFNIYFFGNGDIPVDTYLISIYATEIEL 148

FimA/FimH (type I pili) (Duthy 2000, Girardeau and Bertin, 1995; Sakellaris and Scott, 1998). This suggested that SefA and SefD probably constitute two subunits of the same fimbriae rather than subunits for two distinct fimbriae.

Thus, it was hypothesised that SefD was essential for the biogenesis of SEF14 fimbriae, acting as an initiator of fimbriae biogenesis. The examination of this hypothesis took several approaches, including the construction of chromosomal mutations and of minimal SEF14 operon clones. Attempts were also made to over-express SefD for the purpose of generating polyclonal antiserum, in order to further characterise the role of SefD in the biogenesis of SEF14 fimbriae.

5.2 Over-expression of SefD

5.2.1 Signal sequence analysis of SefD

The mechanism by which surface expressed fimbrial proteins are exported out of the cytoplasm to the cell surface varies, depending on which particular mechanism is used. Some fimbriae use the standard sec-dependant pathway while others are secreted in a sec-independent manner (section 1.5.1.3.1). Those that are sec-dependant, which include the majority of chaperone-usher group of fimbriae, all possess a standard signal sequence which targets them to the periplasm from where the chaperone can bind and target them to their specific usher (For review see Pugsley, 1993; Sauer *et al.*, 2000a).

The mechanism by which SefD is exported to the surface of a host cell is not known, and so becomes important when considering how best to over-express the protein. Generating an N-terminal fusion may result in purification of truncated protein, while a C-terminal fusion may result in over-expressed protein that is secreted into the periplasm, where it is subject to cleavage by periplasmic proteases.

To address this issue, the amino acid sequence of SefD was analysed for the presence of a standard signal sequence, which would then dictate whether this region of aa sequence

would be amplified along with the rest of the *sefD* gene. The web based analysis program, SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP/) was used for this analysis. A putative cleavage site between the alanine (29) and glutamine (30) residues was found. It was not known whether this region was actually cleaved prior to secretion, and as such all clones were amplified to include this region.

The initial over-expression of SefD was performed using the pMAL-c2X expression vector, with fusion of SefD to Maltose Binding Protein (MBP) by cloning the *sefD* gene immediately downstream of the *malE* gene. This vector would allow expression of MBP-SefD fusion protein that remains cytoplasmic as the signal sequence for MBP is specifically deleted in this vector. This method had previously been performed by Clouthier *et al* (1994), who used the expressed protein to generate polyclonal anti-SefD serum. This polyclonal serum was found to react with surface expressed proteins from a wide range of bacterial species, as determined by Western blot analysis. This method was repeated here as expression of the fusion protein had been demonstrated, and should therefore be reproducible.

5.2.1.1 PCR amplification and cloning of *sefD* into pMAL-c2X

To generate a SefD over-expressing construct, *sefD* was PCR amplified (section 2.10.1) using chromosomal DNA prepared from 11RX as a template, and *sefD* specific oligonucleotide primers 3025 and 3026 (Table 2.3), which contained *Eco*RI and *Bam*HI restriction sites respectively. The *sefD* PCR product and the expression vector pMAL-c2X were digested with *Eco*RI and *Bam*HI (section 2.9.2), the DNA fragments ligated (section 2.9.7) and the ligation reaction transformed into DH5α (section 2.7.1). Putative clones were selected by blue/white colour selection (section 2.10.5), plasmid DNA was prepared (section 2.8.1) and screened by restriction digest with *Eco*RI and *Bam*HI. The correct plasmid construct was designated pRJ009 (strain RMJ021), and had the *sefD* gene cloned such that it was translationally fused to the C-terminal end of the Maltose binding protein (MBP),

allowing expression of MBP-SefD fusion protein. Figure 5.2 shows a schematic representation of the cloning procedure.

5.2.2 Expression of SefD-MBP fusion proteins

Expression of the MBP-SefD fusion from pRJ009 was accomplished by transformation into the *E. coli* strain BL21(DE3) (Studier *et al.*, 1990) to give strain RMJ023. The expression vector pMAL-c2X was separately introduced into the same expression strain (giving strain RMJ022) to serve as a control for the expression process. Expression from pMAL-c2X alone results in a 50.8 kDa MBP-LacZα fusion protein. Strains RMJ022 and RMJ023 were grown, induced and whole cell samples prepared (section 2.15.1). Uninduced and induced samples were electrophoresed on SDS-15% polyacrylamide gels and either stained with Coomassie brilliant blue (Figure 5.3A) to visualise total protein, or subjected to Western immunoblot analysis using anti-MBP antibody (Figure 5.3B). Lanes 1 and 2 show the uninduced and induced samples respectively for the control strain RMJ022. The filled arrow indicates over-expressed MBP-LacZ protein which migrated to ~50 kDa, as expected. Lanes 3 and 4 represent uninduced and induced samples respectively of RMJ023. The unfilled arrow indicates the over-expressed MBP-SefD fusion protein. The fusion protein migrated at ~60 kDa, which was ~16-18 kDa larger then MBP alone. This size difference matches the predicted 16.3 kDa of the predicted SefD protein. Interestingly, the level of expression of MBP-SefD is far lower than that of MBP. Because of this, plasmid pRJ009 was transformed into strain GJ1158 (Bhandari and Gowrishankar, 1997), a NaCl induced T7 expression system, in an attempt to improve the level of MBP-SefD expression. The resulting strain, RMJ024, was grown and induced as before and samples analysed by polyacrylamide gel electrophoresis. Despite the change in host strain, the level of MBP-SefD seen after induction were less than that observed when expressing in BL21(DE3) (data not shown), therefore strain RMJ023 was used for all further expression analysis.

Figure 5.2: Cloning strategy to construct an MBP-SefD translational fusion

Cloning of the *sefD* gene into pMAL-c2X was achieved by PCR amplification (section 2.10.1) of *sefD* using primers 3025 and 3026 (Table 2.3), which incorporated *Eco*RI and *Bam*HI restriction sites respectively. After amplification, the *sefD* PCR product was digested with *Eco*RI and *Bam*HI, and ligated into similarly digested pMAL-c2X. After ligation the DNA was transformed into *E. coli* DH5α (section 2.7.2). Plasmid DNA from transformants was screened by restriction digest with *Eco*RI and *Bam*HI. This resulted in construction of plasmid pRJ009 (strain RMJ021)

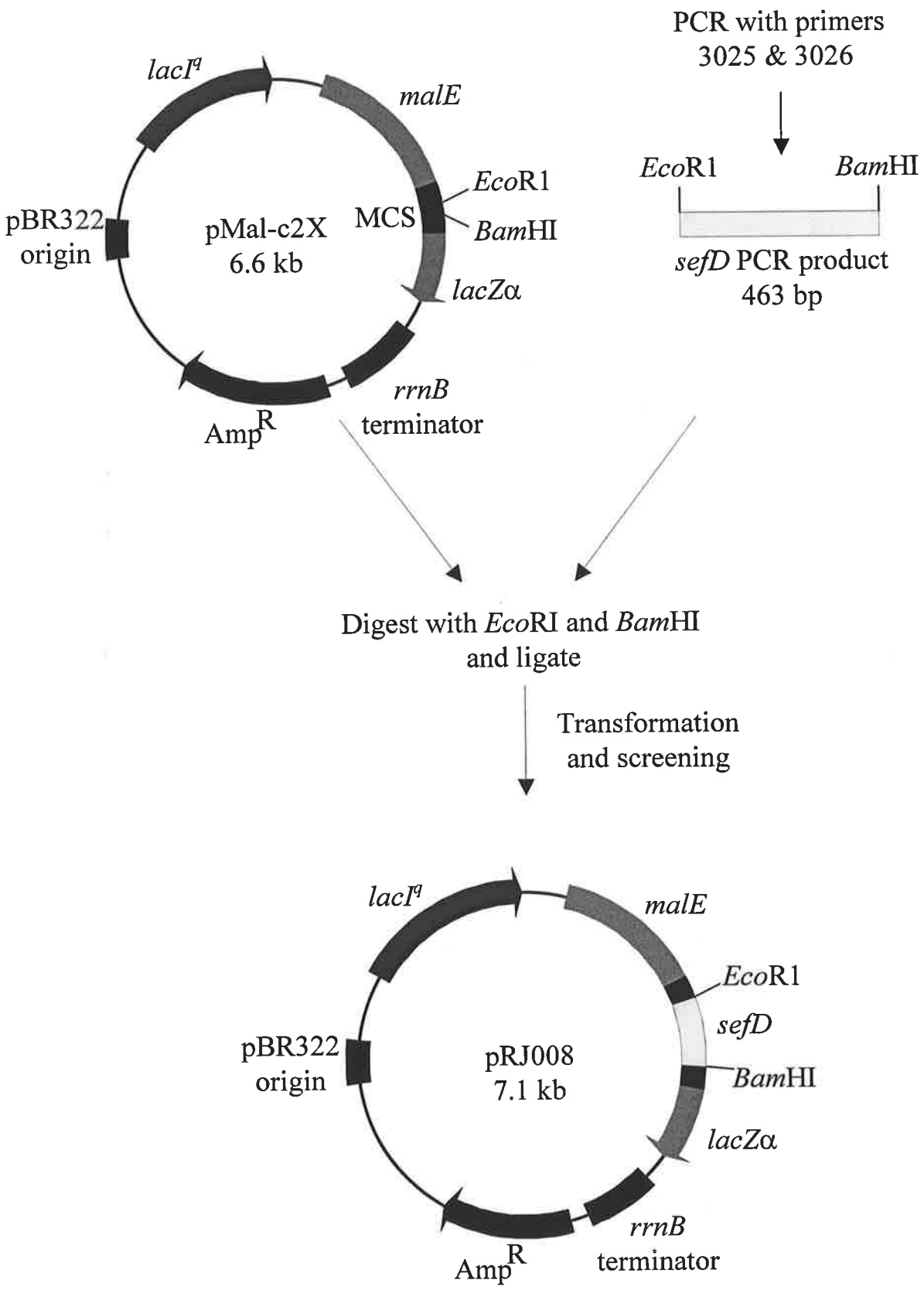
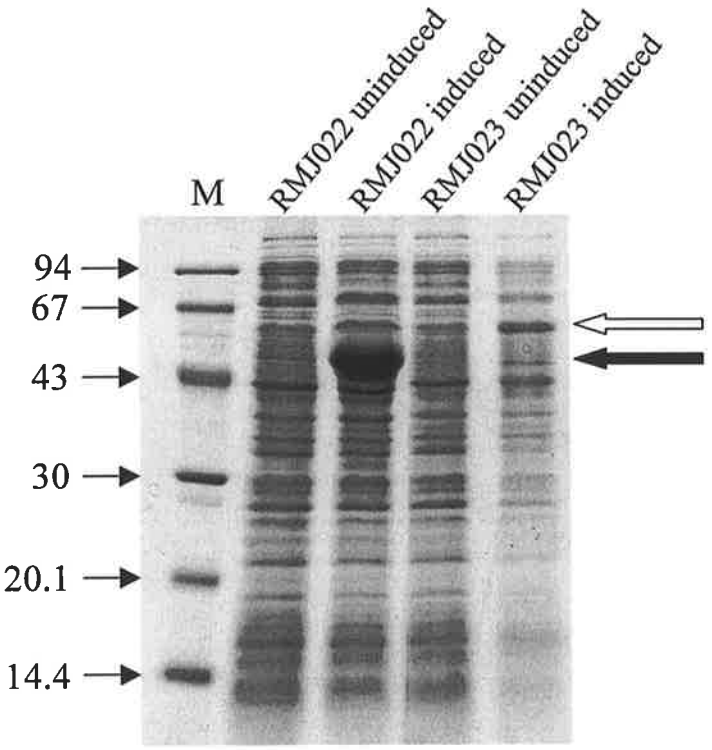

PCR with primers
3025 & 3026
EcoR1
BamHI
sefD PCR product
463 bp
lacIq
malE
EcoR1
BamHI
lacZα
rrnB
terminator
AmpR
pBR322
origin
pMal-c2X
6.6 kb
MCS
Digest with EcoRI and BamHI
and ligate
Transformation
and screening
lacIq
malE
EcoR1
sefD
BamHI
lacZα
rrnB
terminator
AmpR
pBR322
origin
pRJ008
7.1 kb

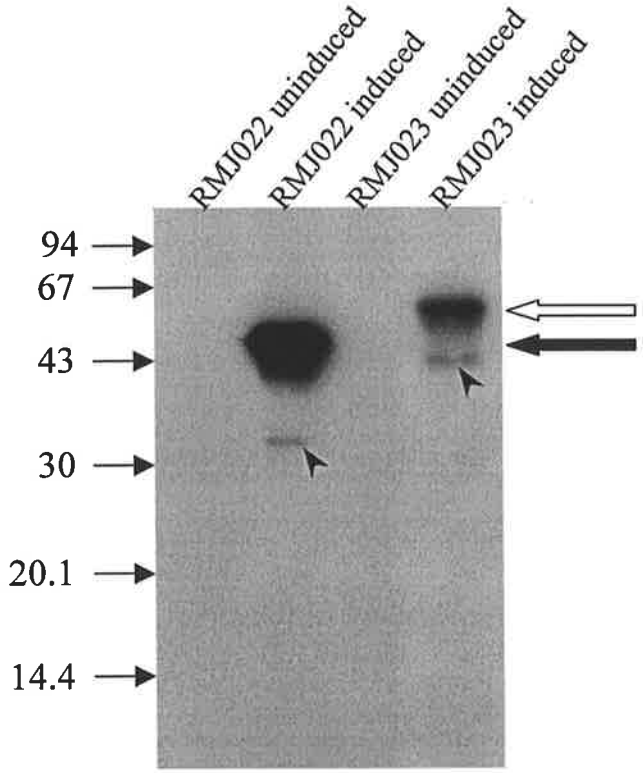
Figure 5.3: Over-expression of MBP-SefD fusion product

MBP-LacZ and MBP-SefD fusion proteins were expressed from RMJ026 (BL21(DE3) + pMAL-c2X) and RMJ027 (BL21(DE3) + pRJ009), respectively (section 2.15.1). Whole cell samples (section 2.14.1), representing ~10^8 bacteria, were analysed by gel electrophoresis on SDS-15% polyacrylamide gels. Gels were either stained with Coomassie blue (A) or transferred to nitrocellulose membrane for Western immunoblotting with anti-MBP (B). The open arrow indicates expressed MBP-SefD, while the filled arrow indicates MBP alone. The arrowheads indicate apparent breakdown products. Molecular weight markers (kDa) are indicated.

A


M
RMJ022 uninduced
RMJ022 induced
RMJ023 uninduced
RMJ023 induced
94
67
43
30
20.1
14.4

B


RMJ022 uninduced
RMJ022 induced
RMJ023 uninduced
RMJ023 induced
94
67
43
30
20.1
14.4

5.2.2.1 Fractionation of MBP-SefD expressing clones

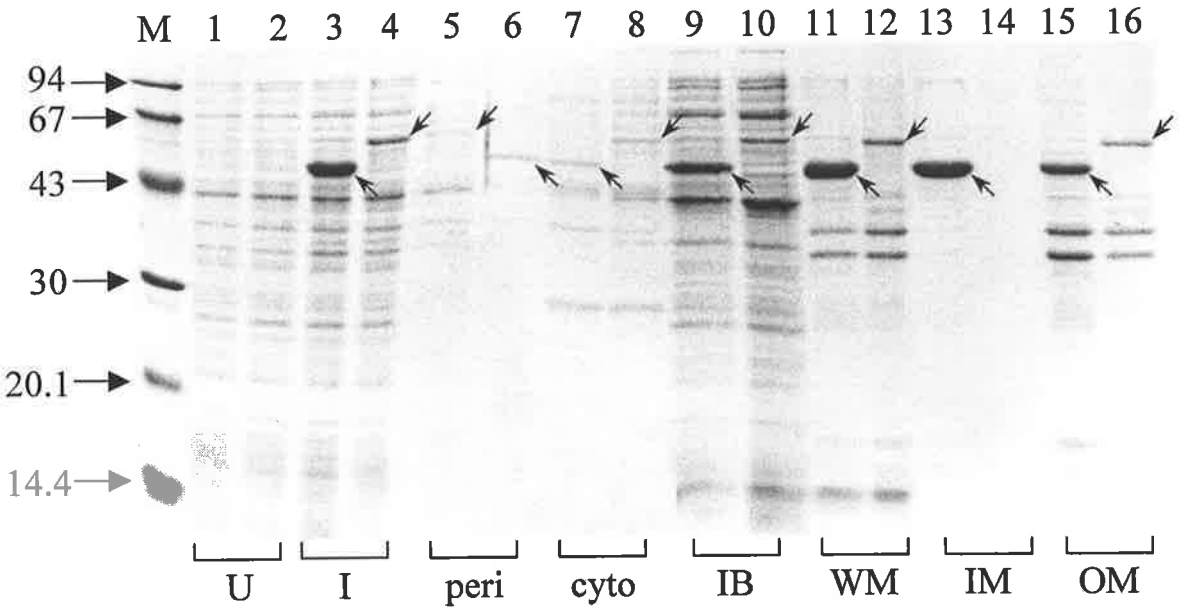
Once the expression of MBP-SefD from pRJ009 had been established, the bacteria were fractionated in order to determine the subcellular localisation of the expressed fusion protein. 100 ml of induced RMJ022 and RMJ023 was harvested and separated into seven fractions as per section 2.14.2: whole cell (WC); periplasm (peri); cytoplasm (cyto); inclusion body (IB); whole membrane (WM); inner membrane (IM) and outer membrane (OM). These samples were then analysed by electrophoresis on a SDS-15% polyacrylamide gel followed by Coomassie blue staining (Figure 5.4). Significant amounts of MBP and MBP-SefD were localized to the IB and membrane fractions, with lesser amounts found in the P and C fractions. As MBP has no signal sequence, it was thought that perhaps localisation to these membrane fractions represented displacement from inclusion bodies and subsequent association with the membrane during the fractionation process, rather than specific targeting to the membrane. Given that the MBP-SefD fusion was present in inclusion bodies, attempts were made to improve its solubility by growing and inducing the bacteria at 30°C, a method that has been documented to reduce the formation of inclusion bodies for some over-expressed proteins (Bishai *et al.*, 1987). The results of this expression were no different than that observed when RMJ023 was grown at 37°C (data not shown) and thus the original expression protocol was used prior to the purification process.

5.2.2.2 Purification of SefD-MBP fusion protein

Once expression of MBP-SefD had been established, it was tested for its ability to be bound by an amylose resin for subsequent purification. Whole membrane fractions were used for this purification procedure for several reasons. First, amylose resin is not compatible with harsh denaturants such as 8M urea or 6M guanidine hydrochloride, which would have been required to fully solubilise the IB that were observed. Second, the membrane fraction had the highest ratio of MBP-SefD:contaminating protein, thus increasing the proportion of MBP-

Figure 5.4: Fractionation of strains RMJ022 and RMJ023

Strains RMJ022 (BL21(DE3) + pMAL-c2X) and RMJ023 (BL21(DE3) + pRJ009) were grown, induced and fractionated as per section 2.14.2. Samples representing ~10^8 bacteria were separated on a SDS-15% polyacrylamide gel and stained with Coomassie blue (section 2.14.3). The various fractions are as follows: uninduced whole cell (U); induced whole cell (I); periplasm (P); cytoplasm (C); inclusion body (IB); whole membrane (WM); inner membrane (IM) and outer membrane (OM). The upper arrows indicated MBP-SefD, while the lower arrows indicates MBP. The odd numbered lanes contain samples from RMJ022 while the even numbered lanes contain samples from RMJ023 (Note however that the Periplasmic samples are reversed with respect to the other samples). Molecular weight markers (kDa) are indicated.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
94
67
43
30
20.1
14.4
U
I
peri
cyto
IB
WM
IM
OM

SefD for any given volume. Triton X-100 was used to solubilise the membrane fractions (section 2.15.1). Solubilised whole membranes from induced RMJ022 and RMJ023 were applied to amylose resin; after binding, the resin was washed twice, and bound protein eluted with 10 mM maltose. Figure 5.5 lanes 1 and 2 shows the sample supernatants from RMJ022 and RMJ023, respectively, after incubation with amylose resin. Significant amounts of MBP-LacZ and MBP-SefD were present, indicating minimal binding to the amylose resin. The subsequent wash and elution fractions all showed very low amounts of proteins, with no apparent specific binding or elution of MBP or MBP-SefD. It is possible that the Triton X-100 used to solubilse the membranes was inhibiting binding to the amylose resin, which has been noted for some, but not all, MBP fusion proteins (pMAL expression manual pg 33, 45).

5.2.2.3 Crude antigen production and Rabbit immunisation

As purification of MBP-SefD fusion was unsuccessful using affinity to amylose resin, it was decided to obtain antigen directly from Coomassie stained SDS-15% polyacrylamide gels (section 2.14.3), using the whole membrane fraction from induced RMJ023 as the source of MBP-SefD. The prepared antigen was used to immunise a female New Zealand outbred white rabbit (section 2.16.1). After immunisation and boosting the antiserum obtained was absorbed extensively against RMJ022 (section 2.16.2) to remove cross-reactive antibodies prior to use in further analysis.

5.2.2.4 Characterisation of Rabbit Sera

Absorbed anti-MBP-SefD antiserum was initially diluted at 1/1000 prior to use in Western immunoblot analysis of MBP-SefD expressing samples. Figure 5.6A shows a Western immunoblot of uninduced and induced whole cell samples from RMJ0222 and RMJ023. Significant activity against both MBP-LacZ and MBP-SefD was observed, along with some background activity. Significant breakdown of the MBP-SefD fusion protein was

Figure 5.5: Purification of MBP-LacZ and MBP-SefD

Purification of MBP-LacZ and MBP-SefD was performed using solubilised whole membrane fractions (section 2.15.1) from induced RMJ022 (BL21(DE3) + pMAL-c2X) and RMJ023 (BL21(DE3) + pRJ009). The solubilised membranes were applied to amylose resin, incubated for 1 h at 4°C and the supernatant removed (Supernatant post binding [PB]). The resin was washed twice in column buffer (Wash 1 [W1] & Wash 2 [W2]). After washing, bound MBP-SefD was eluted twice by gentle agitation for 15 minutes with column buffer containing 10 mM maltose (Elution 1 [E1] & Elution 2 [E2]). Samples were precipitated with 5% (w/v) TCA, washed with 100% ethanol and resuspended in SDS-sample buffer prior to loading on SDS-15% polyacrylamide gel, followed by staining with Coomassie brilliant blue. The odd numbered lanes contain samples from RMJ022 while the even numbered lanes contain samples from RMJ023. Molecular weight standards (kDa; section 2.14.3) are indicated.

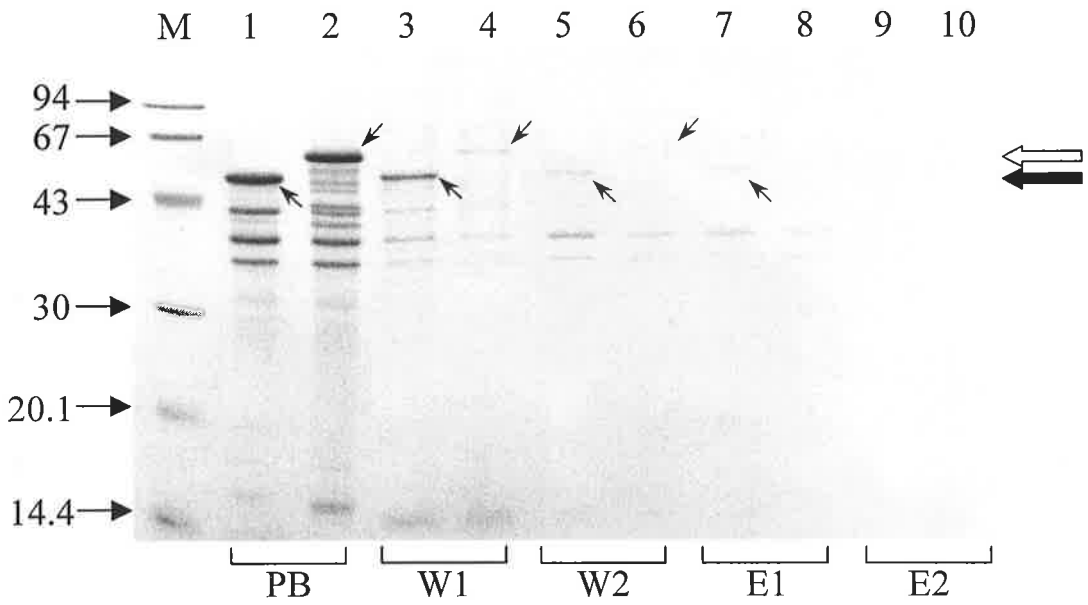
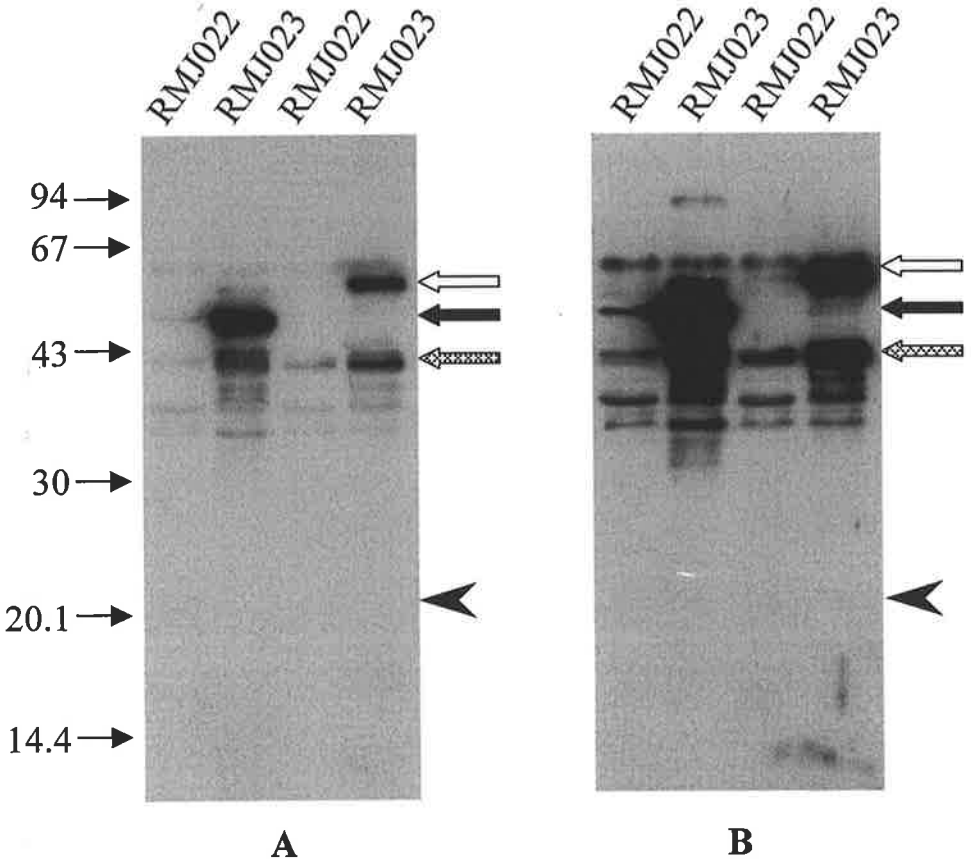

M
1
2
3
4
5
6
7
8
9
10
94
67
43
30
20.1
14.4
PB
W1
W2
E1
E2

Figure 5.6: Characterisation of anti-MBP-SefD sera

Whole cell samples from uninduced and induced RMJ022 (BL21(DE3) + pMAL-c2X) and RMJ023 (BL21(DE3) + pRJ009) were resuspended in a 1:1 mixture of 1x PBS and 2x SDS-sample buffer and boiled prior to loading ~30µl (10^8 bacteria) onto SDS-15% polyacrylamide gel (section 2.14.3) and transfer to nitrocellulose membranes (section 2.14.4). The nitrocellulose membranes were subjected to Western immunoblotting using adsorbed anti-MBP-SefD antiserum at both 1/1000 (Panel A) and 1/200 (Panel B). The open arrow indicates MBP-SefD, the filled arrow indicates MBP alone and the stippled arrow indicates breakdown products. The arrowhead represents the approximate position of the expected SefD band. Molecular weight standards (kDa; section 2.14.3) are indicated.


RMJ022
RMJ023
RMJ022
RMJ023
94
67
43
30
20.1
14.4
A
RMJ022
RMJ023
RMJ022
RMJ023
B

also observed, as evidenced by the presence of the 42.5 kDa MBP band. The SefD portion of MBP-SefD should also be present as a cleavage product, but it was not detected. This suggested that the sera only contained low-level anti-*sefD* antibodies. Therefore, the same samples were analysed by Western immunoblotting using 1/200 diluted sera (Figure 5.6B). Unfortunately, no protein band of the expected size was observed, and the level of anti-MBP reactivity had increased substantially. From this it was concluded that the anti-MBP-SefD serum contained no anti-SefD antibodies, and that a significant humoral immune response was occurred to the MBP potion of the MBP-SefD fusion protein. As this method for generating anti-SefD antiserum was unsuccessful, a different method was used.

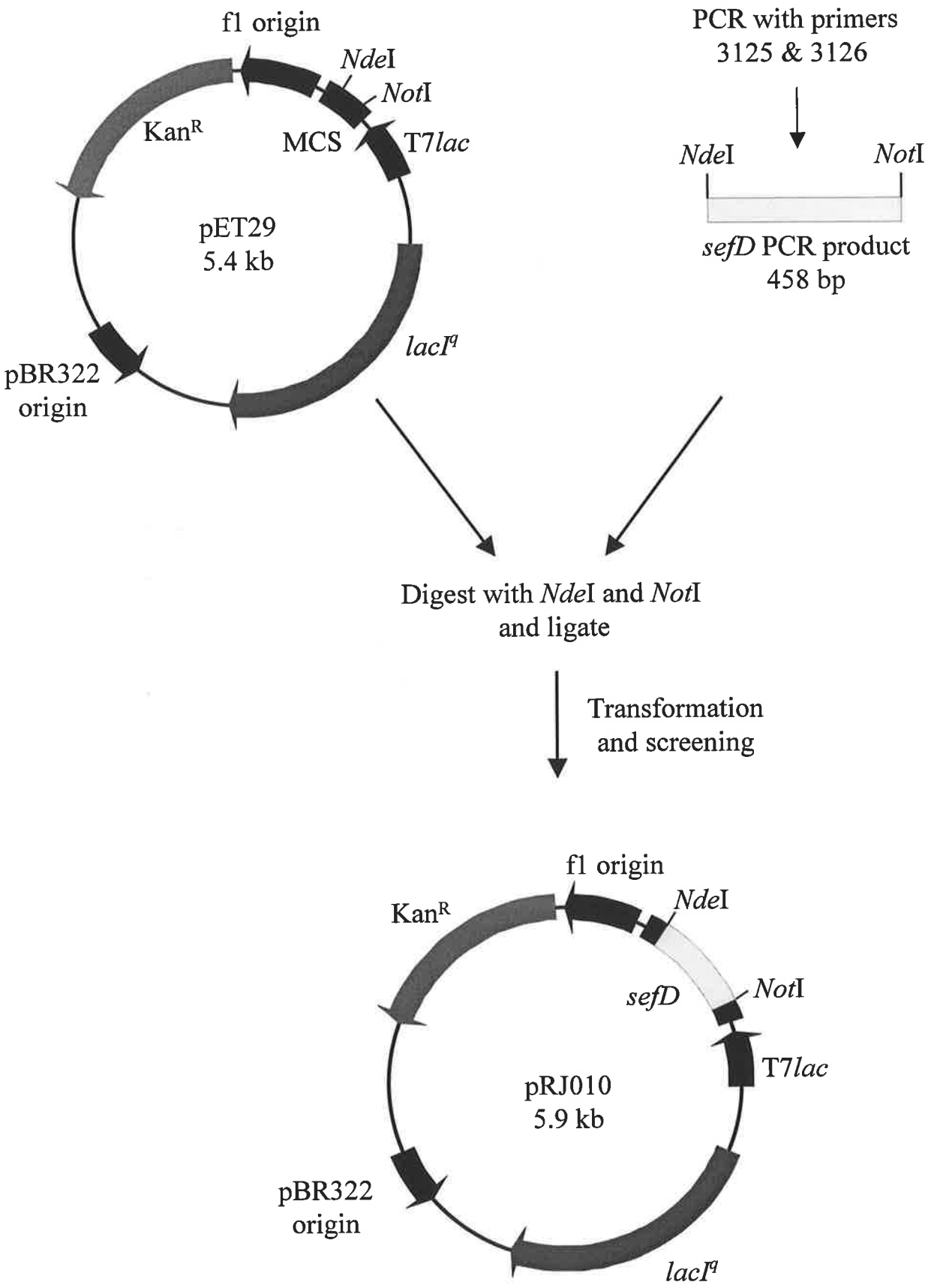
5.2.3 Construction of *sefD*-His tag fusions using pET-21a expression vector

In order to circumvent the difficulties observed when using the pMAL expression system, a 6xHis-tagged system, using the pET range of vectors (Novagen), was used. Both N-terminal and C-terminal fusions were examined. N-terminal fusions were attractive due to the higher levels of expression they normally provide over C-terminal fusions. However, it is not uncommon to have premature termination of the expressed protein, such that only a certain percentage of the purified protein is actually full length. When purifying C-terminal fusions, essentially all protein that is purified is full length. For this reason, as well as the possibility that SefD might be cleaved at the previously predicted cleavage site, the pET-21a expression vector, a C-terminal expression system, was chosen.

The SefD gene was PCR amplified (section 2.10.1) using primers 3125 and 3126 (Table 2.3), which contained the restriction enzyme sites *Nde*I and *Not*I. These primers bound to the *sefD* gene such that the entire gene, minus the stop codon and including signal sequence, was amplified. The 447 bp PCR product and pET-21a were digested with *Nde*I and *Not*I, ligated (section 2.9.7) and electroporated into *E. coli* DH5α (section 2.7.4). Plasmid DNA was prepared (section 2.8.1) and screened by restriction digest with *Nde*I and *Not*I. The

Figure 5.7: Cloning of *sefD* into pET-21a

The *sefD* gene was PCR amplified from 11RX chromosomal DNA using oligonucleotides 3125 and 3126 that incorporated *Nde*I and *Not*I restriction sites respectively. The amplified *sefD* PCR product was digested with the *Nde*I and *Not*I, and ligated into similarly digested pET-21a, such that the His_6 tag was in frame with the 3' end of *sefD*. After transformation into DH5α (section 2.7.2), plasmid DNA was prepared (section 2.8.1) and screened by restriction digest with *Nde*I and *Not*I. This resulted in plasmid construct pRJ010.


f1 origin
NdeI
NotI
Kan^R
MCS
T7lac
pET29
5.4 kb
lacI^q
pBR322
origin
PCR with primers
3125 & 3126
NdeI
NotI
sefD PCR product
458 bp
Digest with NdeI and NotI
and ligate
Transformation
and screening
f1 origin
Kan^R
NdeI
sefD
NotI
T7lac
pRJ010
5.9 kb
pBR322
origin
lacI^q

construction process is shown in Figure 5.7, and the resulting plasmid construct was designated pRJ010 (strain RMJ025). Due to the position of the *Not*I site relative to the His_6 tag, a total of 17 additional amino acids were added to the 3' end of the *sefD* gene, increasing the theoretical molecular weight from 16.3 kDa to 18.1 kDa (Figure 5.8). pRJ010 was transformed (section 2.7.2) into the strain BL21(DE3), giving rise to strain RMJ027.

5.2.3.1 Expression of SefD-C-His_6 from pRJ010

Initially, strain RMJ027 was grown for 18 h then subcultured and induced with IPTG at 37°C (section 2.15.1). A very low level of pre-SefD-C-His_6 expression was observed by SDS-15% PAGE and Coomassie blue staining (Figure 5.9A). Since it is has been reported that growing bacteria at 30°C (or lower) may increase the overall level of protein expression (Bishai *et al.*, 1987), RMJ027 was grown at 25°C for 8 h, induced with IPTG and allowed to express over a 16 h period. Significantly more pre-SefD-C-His_6 was observed using this method, when compared to growth at 37°C (Figure 5.9B). Western analysis of samples from expression at both 25°C and 37°C, using a pool of three anti-His_6-tag monoclonal antibodies (Qiagen), was able to detect a band of ~18 kDa that corresponded with the calculated molecular mass of SefD plus the extra 17 aa added to the C-terminal end. Significantly more pre-SefD-C-His_6 was detected at 25°C than at 37°C, as expected. Interestingly, a slightly smaller band was noted in the 25°C induced sample, with an estimated size of 15 kDa. This size corresponds to pre-SefD-C-His_6 without the predicted SefD signal sequence, calculated to be 14.9 kDa.

5.2.3.2 Fractionation of clones expressing SefD-C-His_6

Once expression conditions had been established, cells were induced and harvested for fractionation as per section 2.14.2. The subcellular fractions were analysed by SDS-15% PAGE followed by staining with Coomassie blue (Figure 5.10A), or transfer to nitrocellulose

Figure 5.8: Nucleotide and amino acid sequence of the 3' end of pre-SefD-C-His_6.

The nucleotide sequence and amino acid translation of pre-SefD-C-His_6 is shown. The His_6-tag is italicised while the intervening amino acids added during construction of pRJ010 are shown in bold text.

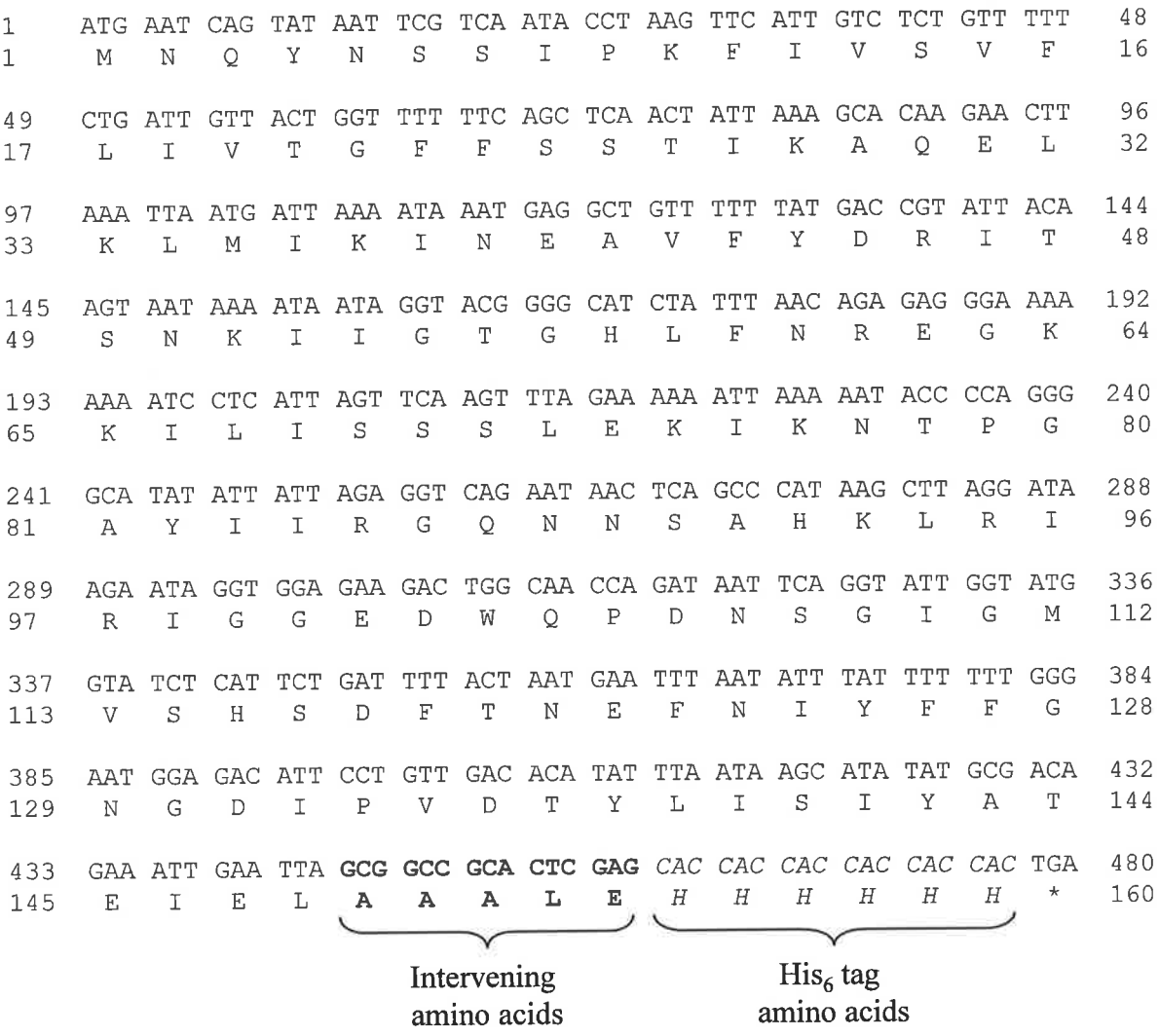
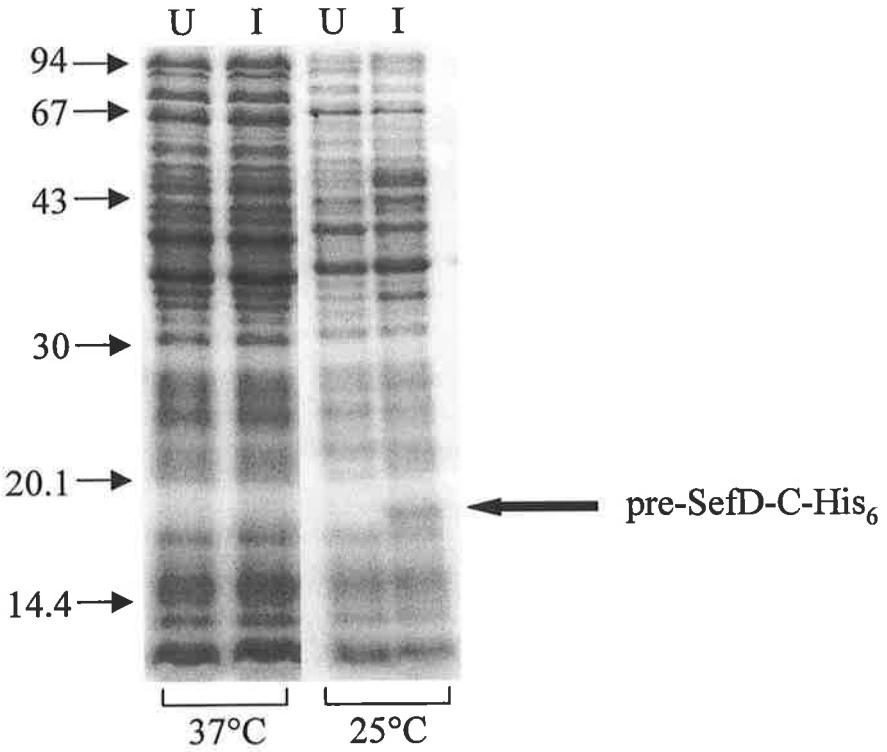

1 ATG AAT CAG TAT AAT TCG TCA ATA CCT AAG TTC ATT GTC TCT GTT TTT 48
1 M N Q Y N S S I P K F I V S V F 16
49 CTG ATT GTT ACT GGT TTT TTC AGC TCA ACT ATT AAA GCA CAA GAA CTT 96
17 L I V T G F F S S T I K A Q E L 32
97 AAA TTA ATG ATT AAA ATA AAT GAG GCT GTT TTT TAT GAC CGT ATT ACA 144
33 K L M I K I N E A V F Y D R I T 48
145 AGT AAT AAA ATA ATA GGT ACG GGG CAT CTA TTT AAC AGA GAG GGA AAA 192
49 S N K I I G T G H L F N R E G K 64
193 AAA ATC CTC ATT AGT TCA AGT TTA GAA AAA ATT AAA AAT ACC CCA GGG 240
65 K I L I S S S L E K I K N T P G 80
241 GCA TAT ATT ATT AGA GGT CAG AAT AAC TCA GCC CAT AAG CTT AGG ATA 288
81 A Y I I R G Q N N S A H K L R I 96
289 AGA ATA GGT GGA GAA GAC TGG CAA CCA GAT AAT TCA GGT ATT GGT ATG 336
97 R I G G E D W Q P D N S G I G M 112
337 GTA TCT CAT TCT GAT TTT ACT AAT GAA TTT AAT ATT TAT TTT TTT GGG 384
113 V S H S D F T N E F N I Y F F G 128
385 AAT GGA GAC ATT CCT GTT GAC ACA TAT TTA ATA AGC ATA TAT GCG ACA 432
129 N G D I P V D T Y L I S I Y A T 144
433 GAA ATT GAA TTA GCG GCC GCA CTC GAG CAC CAC CAC CAC CAC CAC TGA 480
145 E I E L A A A L E H H H H H H * 160
Intervening amino acids
His6 tag amino acids

Figure 5.9: Expression of pre-SefD-C-His_6 fusion protein

Pre-SefD-C-His_6 fusion protein was expressed in strain RMJ027 by growth in media supplemented 1mM IPTG as per section 2.15.2, at both 25°C (A) and 37°C (B). 30 µl samples of uninduced (U) and induced (I) whole cell samples (~ 10^8 bacteria) were electrophoresed on SDS-15% polyacrylamide gels and either stained with Coomassie blue (A and B), or transferred to nitrocellulose for Western immunoblotting with 1/1000 of a mixture of three anti-His monoclonal antibodies (C). The solid arrows indicate pre-SefD-C-His_6 protein. The unfilled arrow designates putative SefD-C-His_6 i.e. pre-SefD-C-His_6 without the signal sequence. Molecular weight standards (kDa; section 2.14.3) are indicated.

A


U
I
U
I
94
67
43
30
20.1
14.4
pre-SefD-C-His6
37°C
25°C

B

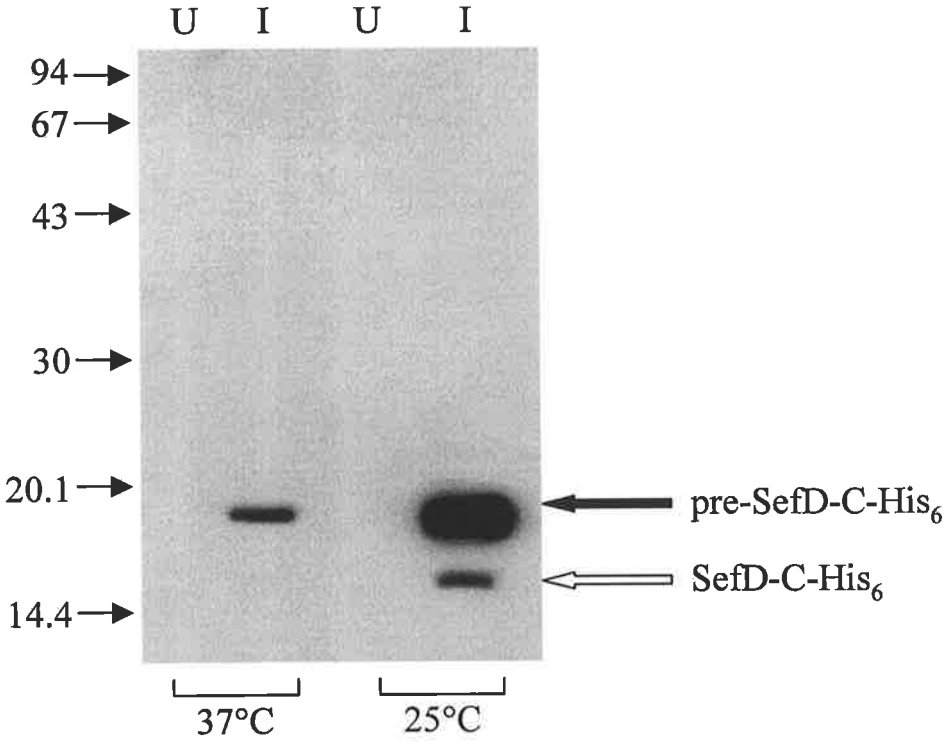
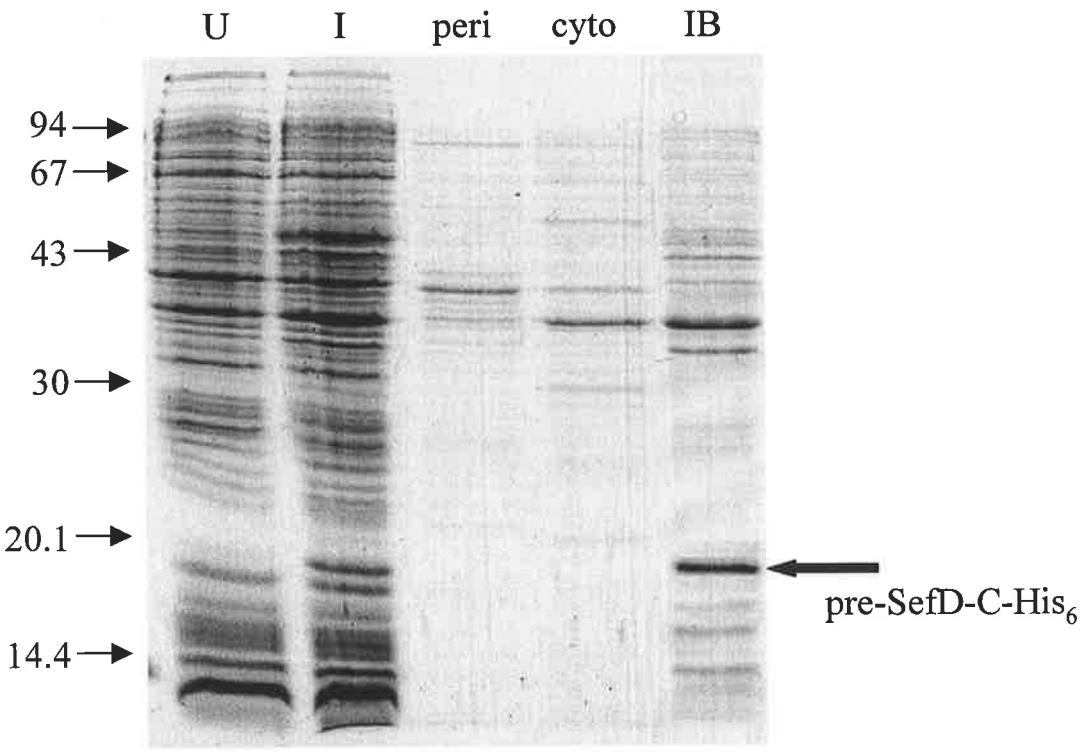

U
I
U
I
94
67
43
30
20.1
14.4
pre-SefD-C-His6
SefD-C-His6
37°C
25°C

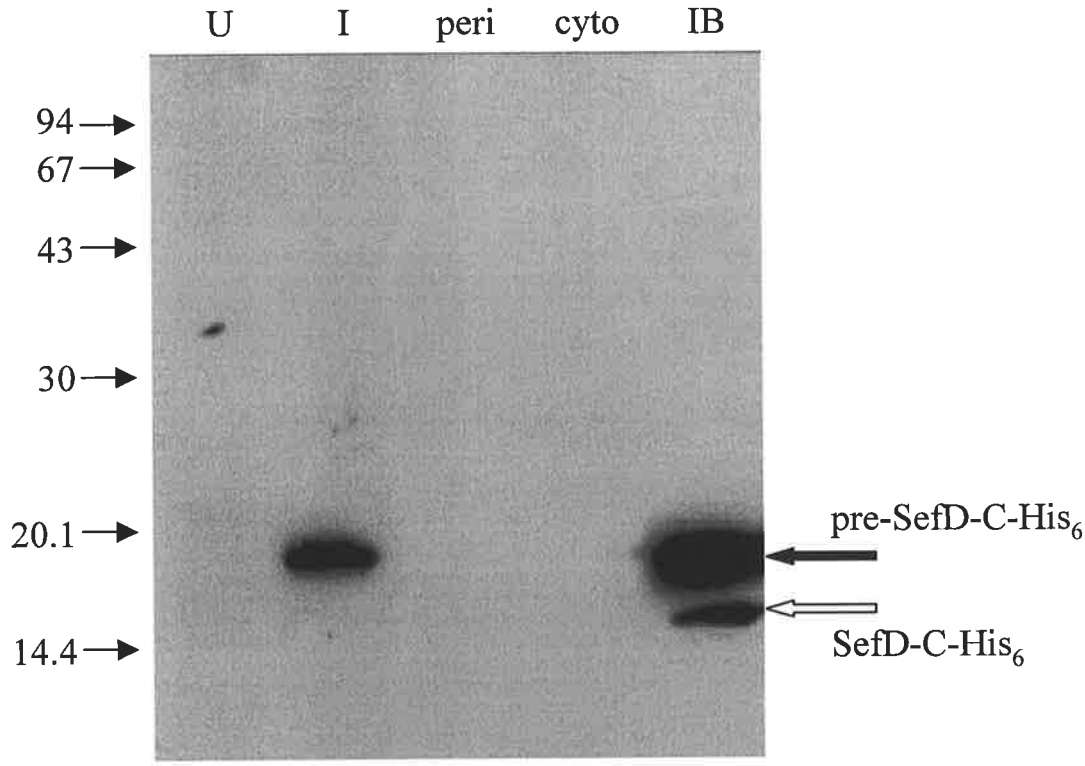
Figure 5.10: Fractionation of RMJ027

SefD-His_6 fusion protein was expressed in strain RMJ027 by growth in media supplemented 1mM IPTG as per section 2.15.2. After induction, bacteria were harvested and separated into the following fractions: uninduced whole cell (U); induced whole cell (I); periplasm (peri); cytoplasm (cyto) and inclusion bodies (IB) as per section 2.14.2. Samples were diluted ½ in 2x SDS-sample buffer, electrophoresed on SDS-15% polyacrylamide gels (2.14.3) and either stained with Coomassie blue (A) or transferred to nitrocellulose prior to Western immunoblotting (section 2.14.4) using 1/1000 of a mixture of three anti-His_6 monoclonal antibodies (B). The solid arrow indicates pre-SefD-C-His_6 protein while the open arrow designates putative SefD-C-His_6. Molecular weight standards (kDa; section 2.14.3) are indicated.

A


U
I
peri
cyto
IB
94
67
43
30
20.1
14.4
pre-SefD-C-His6

B


U
I
peri
cyto
IB
94
67
43
30
20.1
14.4
pre-SefD-C-His6
SefD-C-His6

and Western immunoblot (Figure 5.10B) using 1/1000 anti-His_6-tag monoclonal. Similar to that seen for the MBP expression system, pre-SefD-C-His_6 was observed to form inclusion bodies.

5.2.3.3 Attempted purification of the SefD-C-His_6 tagged fusion protein

Purification of SefD-C-His_6 was attempted according to standard protocols (Qiagen) for the purification of proteins trapped in inclusions bodies (section 2.15.2). Strain RMJ027 (BL21(DE3) + pRJ010) was induced by growth in 1mM IPTG and fractionated to obtain the IB's. The IB's were solubilised under denaturing conditions using 8 M urea, and debris removed by centrifugation. The cleared S/N was applied to Nickel-NTA (Qiagen) resin. After washing and eluting, samples of the various fractions were electrophoresed on a SDS-15% polyacrylamide gel and stained with Coomassie Blue (Figure 5.11). The purification process appeared to be unsuccessful as a significant amount of pre-SefD-C-His_6 was present in the lysate after incubation with the Nickel-NTA resin. Also, no pre-SefD-C-His_6 was seen in any of the elution samples. A sample of the resin after elution showed no evidence of pre-SefD-C-His_6. Western immunoblot using a pool of anti-His_6 monoclonal antibodies confirmed that pre-SefD-C-His_6 was only present in the lysate after incubation with the resin and in the first wash solution (not shown).

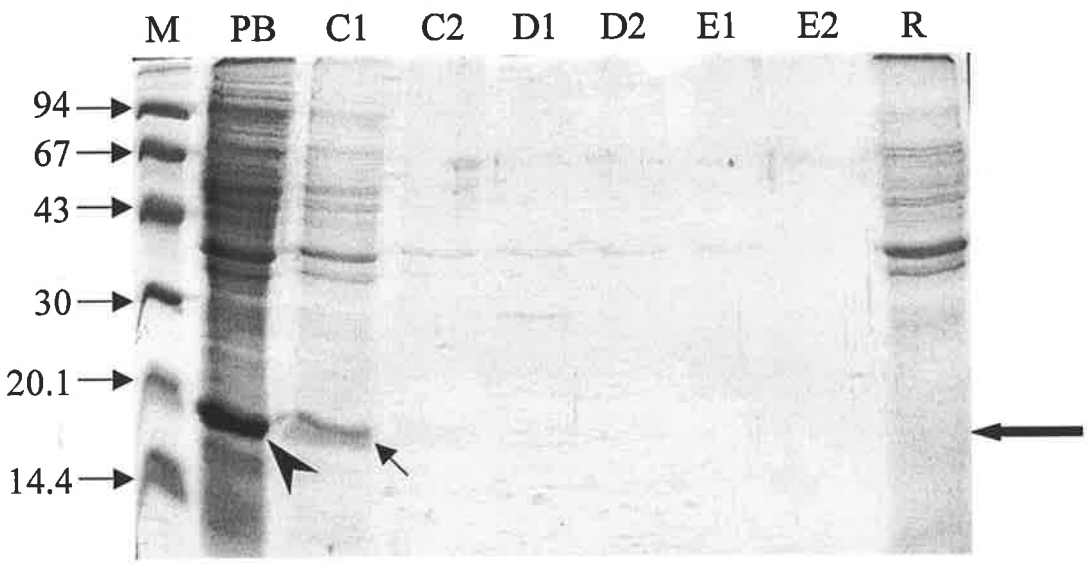
It is possible that the denaturation buffer was not fully dissociating the inclusion bodies, or that the His_6-tag was being shielded from binding to the resin by inappropriate folding of the SefD protein.

5.2.3.4 Rabbit immunisation using His-tagged *sefD*

While SefD could not be purified using the standard protocol, the large amount of protein sequestered in inclusion bodies was used for immunisation of rabbits in the same manner as performed for MBP-SefD fusion. As pre-SefD-C-His_6 lacked the large MBP

Figure 5.11: Batch purification of pre-SefD-C-His_6 from RMJ027

Strain RMJ027 was grown, induced and inclusion bodies obtained for use in purification (section 2.15.2). The inclusion bodies were solubilized in Buffer C, containing 8 M urea, and the debris removed by centrifugation. The S/N was applied to a 50% (v/v) solution of Ni-NTA resin and allowed to bind with mixing at 4°C for 1 h. A sample of the S/N after binding (AB) was retained for analysis. The Ni-NTA resin was washed 2x in Buffer C (C1 and C2) and the bound protein eluted with two washes of buffer D (D1 and D2) followed by 2 washes in Buffer E (E1 and E2). A sample of the resin after elution (R) was also retained for analysis of residual bound protein. Each of the samples were separated on a SDS-15% polyacrylamide gel which was stained with Coomassie Blue (section 2.14.3) The arrowhead shows a significant amount of pre-SefD-C-His_6 present in the lysate after binding to the resin, while the arrow shows SefD-C-His_6 present in the first wash solution. No pre-SefD-C-His_6 was observed in any other sample. Molecular weight standards (kDa; section 2.14.3) are indicated.


M
PB
C1
C2
D1
D2
E1
E2
R
94
67
43
30
20.1
14.4

fusion, it was hoped that the majority of immunogenic epitopes present were due to SefD rather than a competing protein. As done previously, samples of pre-SefD-C-His_6 containing IB fraction from induced RMJ027 were electrophoresed on a SDS-15% polyacrylamide gel, excised and re-electrophoresed prior to preparation as per section 2.16.1. After immunisation and boosting the antiserum obtained was absorbed extensively against RMJ026 (BL21(DE3) containing pET-21a only; section 2.16.2) to remove cross-reactive antibodies prior to use in further analysis.

5.2.3.4.1 Characterisation of pre-SefD-C-His_6 sera

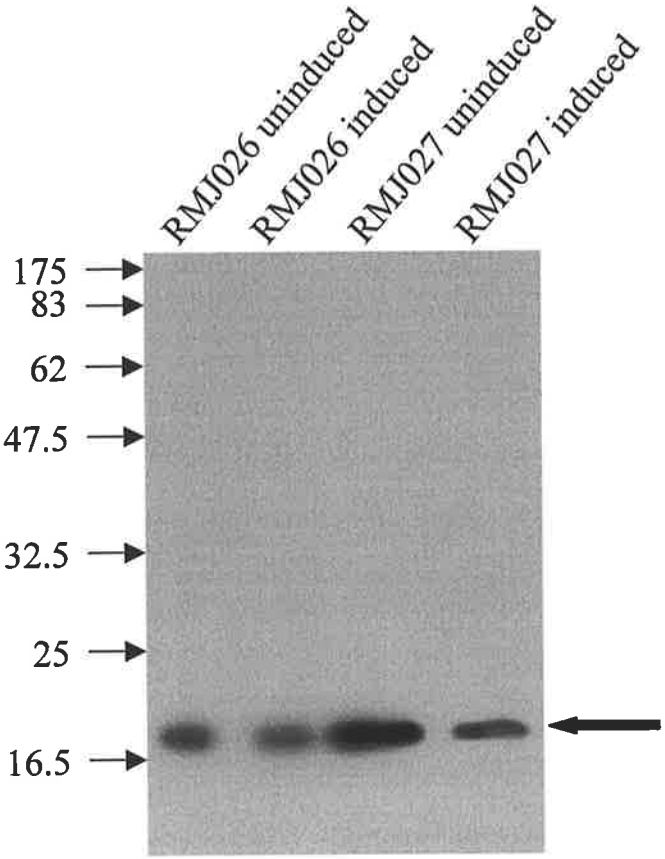
The absorbed rabbit serum (1/1000), obtained by immunisation with SDS-15% PAGE extracted pre-SefD-C-His_6, was used to analyse the following whole cell samples: RMJ026 (pET-21a) and RMJ027 (pRJ010) uninduced, and RMJ026 and RMJ027 induced with 1 mM IPTG. Figure 5.12 shows the results of this analysis, with all four samples showing a single positive band at the size expected for pre-SefD-C-His_6. This suggested that an unknown protein was present in the excised polyacrylamide fragment used to obtain SefD-His fusion protein. It appears that an immune response was mounted against the unknown protein, resulting in "SefD-His" being detected even in samples that should have been negative. Therefore this second attempt to produce an anti-SefD antiserum was also unsuccessful.

5.3 Molecular analysis of the role of *sefD*/SefD

While the generation of anti-SefD serum was unsuccessful (see section 5.5), the role of SefD in SEF14 fimbriae biogenesis was investigated indirectly by examining the effects of SefD protein on surface expression of SefA, in the form of SEF14 fimbriae.

Figure 5.12: Characterisation of anti-SefD-His antiserum.

Strain RMJ027 (BL21(DE3) pRJ010) was grown, induced by addition of 1 mM IPTG and samples of uninduced (U) and induced (I) whole cells solubilized in 2x SDS-sample buffer. The control strain RMJ026 (containing pET-21a only) was similarly treated. Samples representing ~10^8 bacteria were separated on a SDS-15% polyacrylamide gel (section 2.14.3) and then transferred to nitrocellulose for Western immunoblotting with a 1/1000 dilution of adsorbed anti-pre-SefD-C-His_6 antiserum (section 2.14.4). The arrow indicates the expected position of pre-SefD-His_6 fusion protein. Prestained molecular weight markers (section 2.14.3; kDa) are indicated


RMJ026 uninduced
RMJ026 induced
RMJ027 uninduced
RMJ027 induced
175
83
62
47.5
32.5
25
16.5

5.3.1 Construction of *sefABC* cutdown clone

The initial experiments to analyse the role of SefD was to compare the ability of two minimal *sef* operon constructs, *sefABCD* and *sefABCΔD*, to express SEF14 fimbriae from high copy number vectors. It was hypothesised that using high copy number vectors would circumvent the requirement for *sefR* (see section 4.4.2), and that the deletion of *sefD* would result in a lack of SEF14 fimbriae expression.

5.3.1.2 *Hin*dIII cutdown and sub cloning

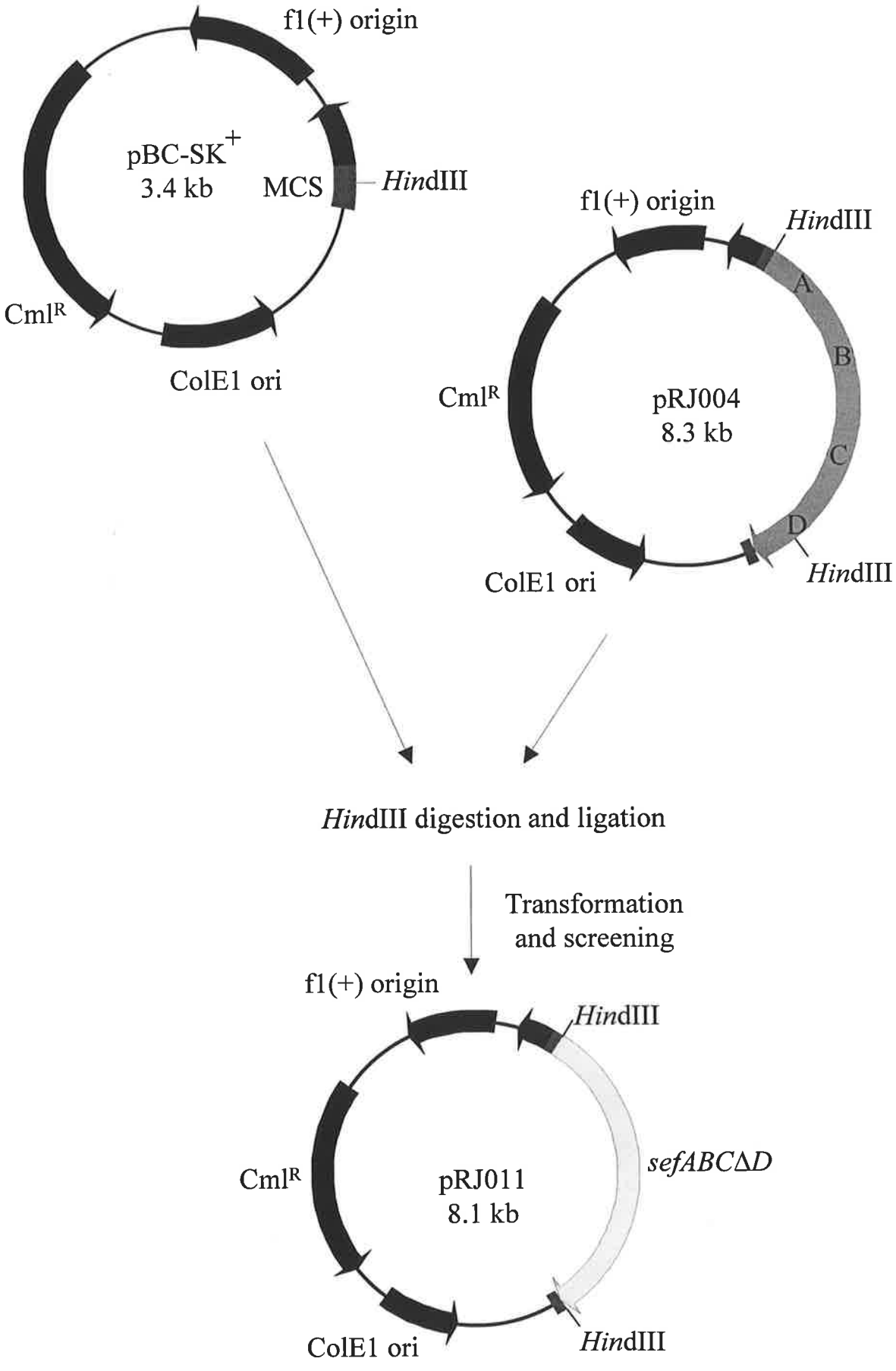
The *sefABCΔD* construct was derived by subcloning a *Hin*dIII fragment from pRJ004 into pBC-SK$^+$ (Figure 5.13). *Hin*dIII restrictions sites were present at the beginning of the *sefA* promoter region and at position 277 within *sefD* (position 5015 relative to the beginning of the SEF14 operon - see Figure 3.1). Thus digesting with *Hin*dIII would give *sefABC* and the first 277 bp of *sefD*, resulting in the fragment *sefABCΔD*. The restriction fragments were separated on a 1% agarose gel and the 4.8 Kb *Hin*dIII-*Hin*dIII fragment was purified by gel extraction. The purified fragment was ligated into pBC-SK$^+$ that had been digested with *Hin*dIII and treated with shrimp alkaline phosphatase to prevent vector recircularisation, and the ligation reaction transformed into competent DH5α. After selection for Cml^R, transformants were screened by restriction digestion with *Hin*dIII and the correct plasmid construct was designated pRJ011 (strain RMJ028).

5.3.1.3 Comparison of SefA expression from strains RMJ010 and RMJ028

It was hypothesised that strains RMJ010 (containing pRJ004) and RMJ028 (containing pRJ011) would both possess the ability to express SefA, however the ability to produce fimbriae would be impaired in RMJ028 due to the lack of *sefD*, leading to an accumulation of SefA in the cytoplasm/periplasm. To test this hypothesis whole cell and heat shock supernatant samples were prepared from strains RMJ010 and RMJ028 after growth on

Figure 5.13: Subcloning of *SefABCΔD*

Plasmid pRJ004, containing the *sefABCD* operon, was digested with the restriction enzyme *Hin*dIII (section 2.9.2), liberating the *sefABCΔD* fragment. This fragment was purified by gel extraction (section 2.9.5) and ligated into *Hin*dIII digested pBC-SK$^+$. After transformation into DH5α (section 2.7.2) and selection for Cml^R, plasmid DNA was prepared and screened by digestion with *Hin*dIII, and the correct construct was designated pRJ010.


f1(+) origin
pBC-SK+
3.4 kb
MCS
HindIII
CmlR
ColE1 ori
f1(+) origin
HindIII
A
B
C
D
CmlR
pRJ004
8.3 kb
ColE1 ori
HindIII
HindIII digestion and ligation
Transformation
and screening
f1(+) origin
HindIII
CmlR
pRJ011
8.1 kb
sefABCΔD
ColE1 ori
HindIII

CFA agar. These samples were solubilized, electrophoresed on a SDS-15% polyacrylamide gel, and then transferred onto nitrocellulose membrane for Western immunoblot analysis. Figure 5.14 shows the results of this analysis. It was observed that an abundance of SefA was present in both the whole cell and heat shock supernatant samples of strain RMJ010. It seems that sufficient basal expression from the *sefA* promoter, combined with a high copy number vector, accounts for the apparent non-requirement of SefR in the expression of SefA in RMJ010.

A very low level of SefA was observed in both whole cell and heat shock supernatant samples from RMJ028. This was unexpected for two reasons. First, it was hypothesised that the level of SefA expression from this construct would be the same as from RMJ010, as they were essentially the same construct, except for the 3' end of *sefD*. Second, it was hypothesised that there would be an accumulation of SefA within the cytoplasm/periplasm, with no SefA present in the heat shock supernatant. It is likely that the SefA present in the heat shock supernatants is present due to periplasmic leakage rather than expression of fimbriae on the surface of the bacteria. However this does not explain the reduced SefA expression in strain RMJ028 relative to strain RMJ010.

Immunoelectron microscopy was performed to compare the surface expression of SEF14 fimbriae on 11RX with RMJ010 and RMJ028. 11RX expressed extensive amorphous fimbriae as expected (Figure 5.15A) while RMJ009 (DH5α containing pBC-SK$^+$) showed no evidence of SEF14 fimbriae (Figure 5.15B). RMJ010 expressed fimbriae of similar length and morphology as 11RX (Figure 5.15C) while RMJ028 showed no evidence of SEF14 fimbriae expression (Figure 5.15D). These observations suggested that *sefD* is required for the biogenesis of SEF14 fimbriae. However in conjunction with Western immunoblotting (section 2.14.4), it seems that RMJ028 is unable to produce SEF14 fimbriae due to some inability to express amounts of SefA similar to RMJ010 rather than a direct effect from the lack of SefD. For this reason, it was felt that construction of a chromosomal mutation in the

Figure 5.14: SefA expression in strains RMJ010 and RMJ028

Whole cell (WC) and heat shock supernatant (S) samples were prepared from 11RX, DH5α, RMJ010 and RMJ028 grown for maximal SefA expression on CFA medium (section 2.13.1). Samples representing ~10^8 bacteria were solubilized in 2x SDS-sample buffer and electrophoresed on a SDS-15% polyacrylamide gel (section 2.14.3) and transferred to nitrocellulose for Western immunoblotting (section 2.14.4) using absorbed anti-SefA antiserum (1/1000). Molecular weight standards (kDa; section 2.14.3) are indicated.

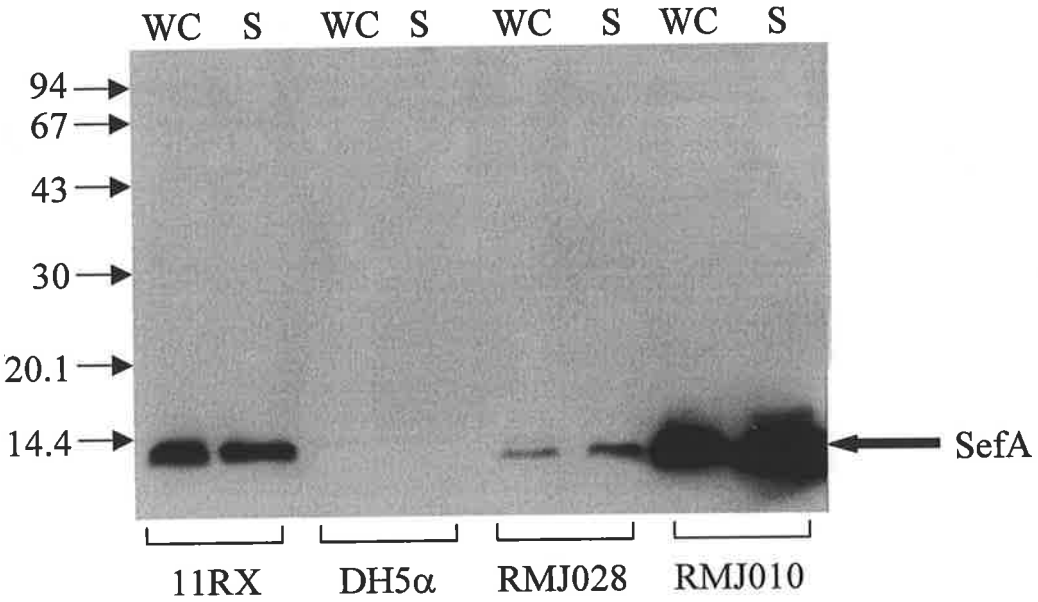
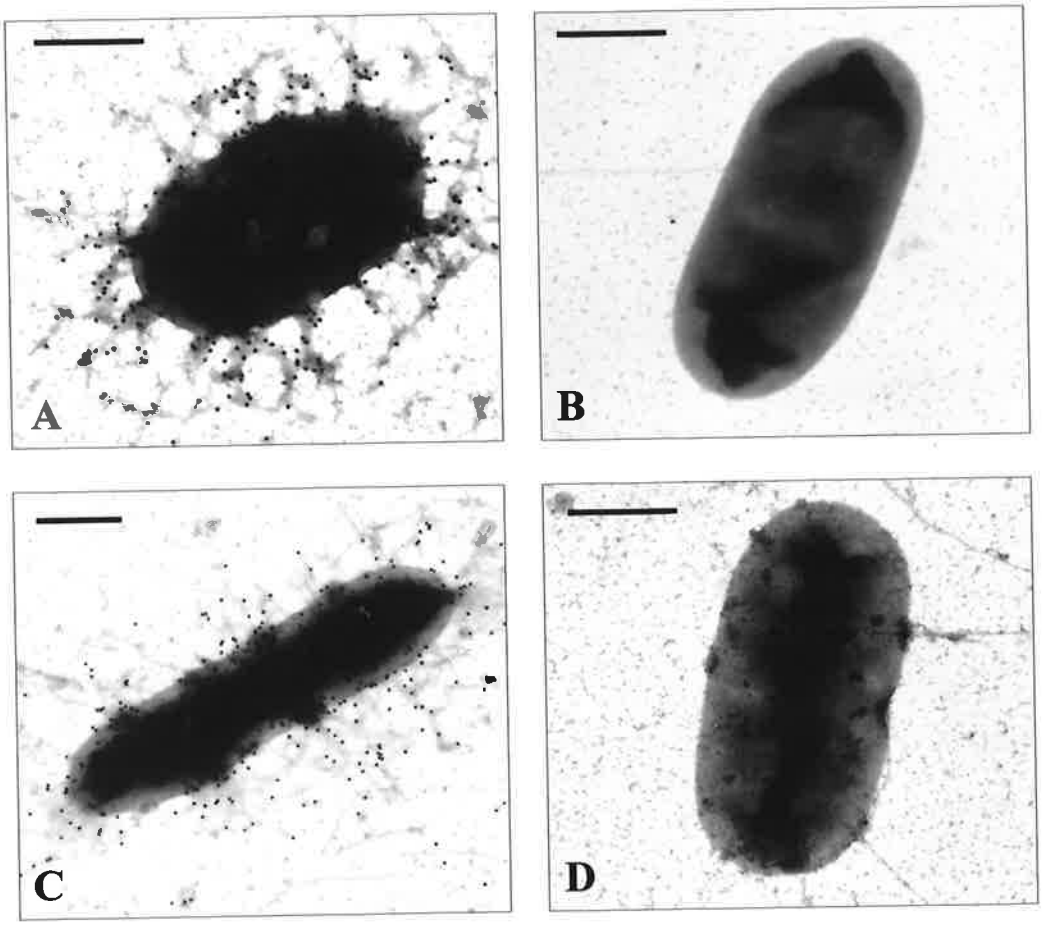

WC
S
WC
S
WC
S
WC
S
94
67
43
30
20.1
14.4
SefA
11RX
DH5α
RMJ028
RMJ010

Figure 5.15: Effect of *sefD* deletion on SEF14 fimbriae expression

Strains 11RX (A), DH5α (B), RMJ010 (containing pRJ004) (C) and RMJ028 (containing pRJ011) (D) were grown to confluence on CFA agar to induce maximal SEF14 fimbriae expression (section 2.13.1). A suspension of each strain was made in 1x PBS and incubated with poly-L-lysine treated Copper-Palladium electron microscopy grids (section 2.19). SEF14 fimbriae were detected by incubation with absorbed anti-SefA antiserum (1/50) followed by protein A gold conjugated to 20 nm diameter gold particles (1/40). The grids were stained with the 1% uranyl acetate stained prior to visualising. Scale bar represents 500 nm.


A
B
C
D

sefD gene of *S.* Enteritidis would be more suited to defining the role of SefD in the biogenesis of SEF14 fimbriae.

5.4 Construction and analysis of *sefD* chromosomal mutation

Construction of a *sefD* chromosomal mutation was achieved in the same manner as that of *sefR*, i.e. by insertional inactivation. Briefly, an internal fragment (nt 130-318, positions 4868-5056 relative to beginning of *sefA* promoter region) of the *sefD* gene was PCR amplified using the primers 3190 and 3191 (see table 2.3), both of which contained an *Sph*I restriction enzyme site. This resulted in the amplification of a 188 bp fragment, which was cloned into the *Sph*I site of pCACTUS-*mob* (see Figure 5.16). The resulting construct, pRJ012, was electroporated into the donor strain SM10λpir, which facilitated conjugation into *S.* Enteritidis strains 11RX and 7314 as per section 2.7.5. After selection on minimal media + Cml, exconjugants were grown at 42°C (Section 2.10.6). Colonies that were able to grow at 42°C in the presence of Cml were considered potential co-integrates (i.e. chromosome::pRJ012), and were screened by PCR amplification as described below.

5.4.1 PCR and Southern hybridisation analysis of putative *sefD* mutants

PCR analysis of putative *sefD*::pCACTUS-*mob* insertion mutants was performed using the primers which flanked the pCACTUS-*mob* multiple cloning site (2677 and 2678) and primer which bind the ends of the *sefD* gene (3096 and 3097). The primer combinations 2677/3096, 2677/3097, 2678/3096 and 2678/3097 were used to screen chromosomal DNA to ensure the correct insertion of the construct. Figure 5.17 shows an example of a positive insertion mutant analysed using these primers. As can be observed, only two of the four reactions gave positive results, as was expected.

Putative *sefD* mutant strains RMJ029 (parent strain 11RX) and RMJ030 (parent strain 7341) were analysed by Southern hybridisation. Chromosomal DNA was extracted and

Figure 5.16: Chromosomal mutation of *sefD*

An internal fragment of *sefD* was PCR amplified (section 2.10.1) from 11RX chromosomal DNA (section 2.8.2) using primers 3190 and 3191 (see Table 2.3), both of which contained the *Sph*I restriction enzyme site, to give a 188 bp fragment. This fragment was digested with *Sph*I and ligated into similarly digested pCACTUS-*mob* and transformed into *E. coli* at 30°C. After selection with Cml, plasmid DNA was prepared (section 2.8.1) and screened by PCR using primers 2677 and 2678 (see Table 2.3), with the appropriate clone designated pRJ012. pRJ012 was electroporated into SM10λpir and then conjugated into the target strains 11RX and 7314. Single crossover recombination of pRJ012 was induced by growth at 42°C (section 2.10.6), with stable co-integrates selected by streaking single colonies for growth at 37°C with Cml. Screening of Cml^R exconjugants was performed using combinations of the indicated primers (i.e. 2677/3096; 2677/3097; 2678/3096 and 2678/3097). *mob* = mobilisation genes; Cml^R = chloramphenicol resistance gene; ori_{ts} = temperature sensitive origin of replication; *sacB* = levansucrase; MCS = multiple cloning site.

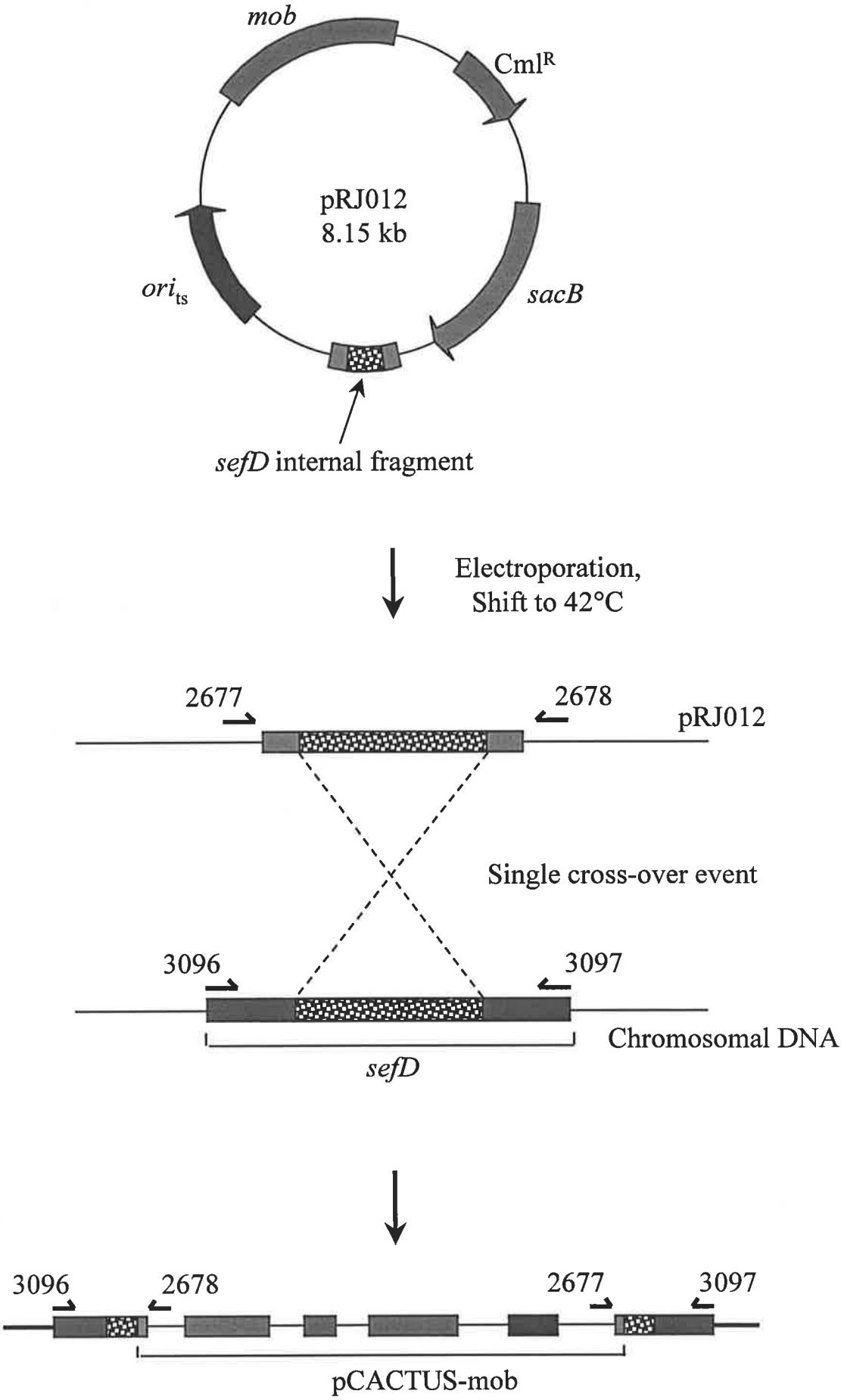
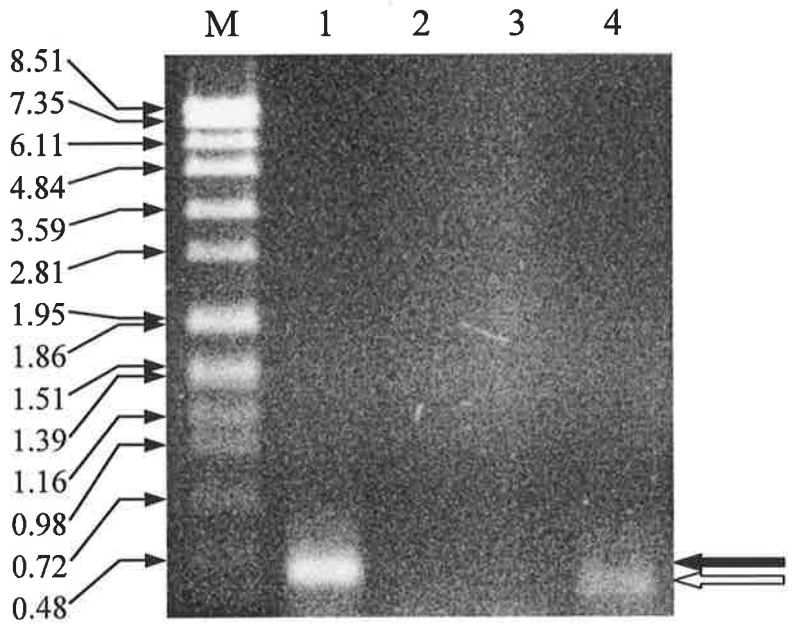

mob
Cml^R
pRJ012
8.15 kb
ori_ts
sacB
sefD internal fragment
Electroporation,
Shift to 42°C
2677
2678
pRJ012
Single cross-over event
3096
3097
Chromosomal DNA
sefD
3096
2678
2677
3097
pCACTUS-mob

Figure 5.17: PCR analysis of putative *sefD* mutant.

PCR analysis (section 2.10.1) was performed on the putative 11RX *sefD* mutant strain RMJ029 and analysed by electrophoresis on 1% TAE agarose gel (section 2.9.3). The primers used were as follows: 2677/3096 (lane 1), 2677/3097 (lane 2), 2678/3096 (lane 3) and 2678/3097 (lane 4). The filled and unfilled arrows designate positive PCR reactions using primers 2677/3096 and 2678/3097 respectively. Similar results were obtained for PCR analysis of putative 7314 *sefD* mutant strain RMJ030. Size of *Eco*RI digested SPP-1 markers (kb) is indicated.


M
1
2
3
4
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
0.48

digested with *Nru*I. Both the mutant and parent strains were analysed using full-length DIG-labelled *sefD* and CAT gene probes. The results of this analysis are shown in Figure 5.18. When probed with *sefD*, the parent strains had only a single band of >10 kb, while the mutant had a significantly larger band, indicating the insertion of vector construct pRJ012. When probed with the CAT gene, only the mutant strains were positive, with a band the same size as seen when probing with *sefD*, again indicating that construct pRJ012 had inserted into the *sefD* gene.

5.4.2 Western immunoblot analysis of chromosomal *sefD* mutants

Having confirmed the mutation within the *sefD* gene, various strains were compared for their ability to express SEF14 fimbriae. 11RX, 11RX-*sefA*::Kan and RMJ029 were grown on CFA medium to induce maximal SEF14 expression and heat shock supernatants were prepared (section 2.13.1). Samples of heat shock supernatants equivalent to ~10^8 bacteria were electrophoresed on a SDS-15% polyacrylamide gel which was subjected to Western transfer using anti-SefA antiserum (1/1000; Figure 5.19). The results of this analysis were somewhat unexpected. While 11RX and 11RX-*sefA*::Kan showed the presence and absence of SefA respectively, strain RMJ029 was also positive for SefA. A similar result was observed when comparing 7314 with RMJ030 (not shown). To explain this result, two hypotheses were proposed, firstly, the *sefD* mutation has no effect on the surface expression of SEF14 fimbriae, i.e. SefD is not an initiator protein. Alternatively, SefD is required for SEF14 biogenesis, however periplasmic leakage during heat-shock released SefA that had been expressed but not exported to the bacterial surface. In order to distinguish between these two possible scenarios, further analysis of RMJ029 and RMJ030 was performed using EIA and IEM.

Figure 5.18: Confirmation of *sefD* mutation by Southern analysis.

Chromosomal DNA was prepared from putative *sefD* mutant strains RMJ029 and RMJ030 and their respective parent strains 11RX and 7314 (section 2.8.2). The DNA was digested with *Nru*I and electrophoresed on 1% TAE agarose gel (section 2.9.3). The DNA was transferred to nylon membrane (section 2.12.2) and incubated with full-length DIG-labelled probes (section 2.12.1) of either *sefD* (Panel A) or CAT (Panel B). The solid arrow represents wild type *sefD* positive fragment, while the unfilled arrow represents the same fragment with pCACTUS-*mob* insertion. The stippled arrow represents CAT positive fragments present in the mutant, but not the parent strains. Sizes of *Eco*RI digested SPP-1 markers are indicated

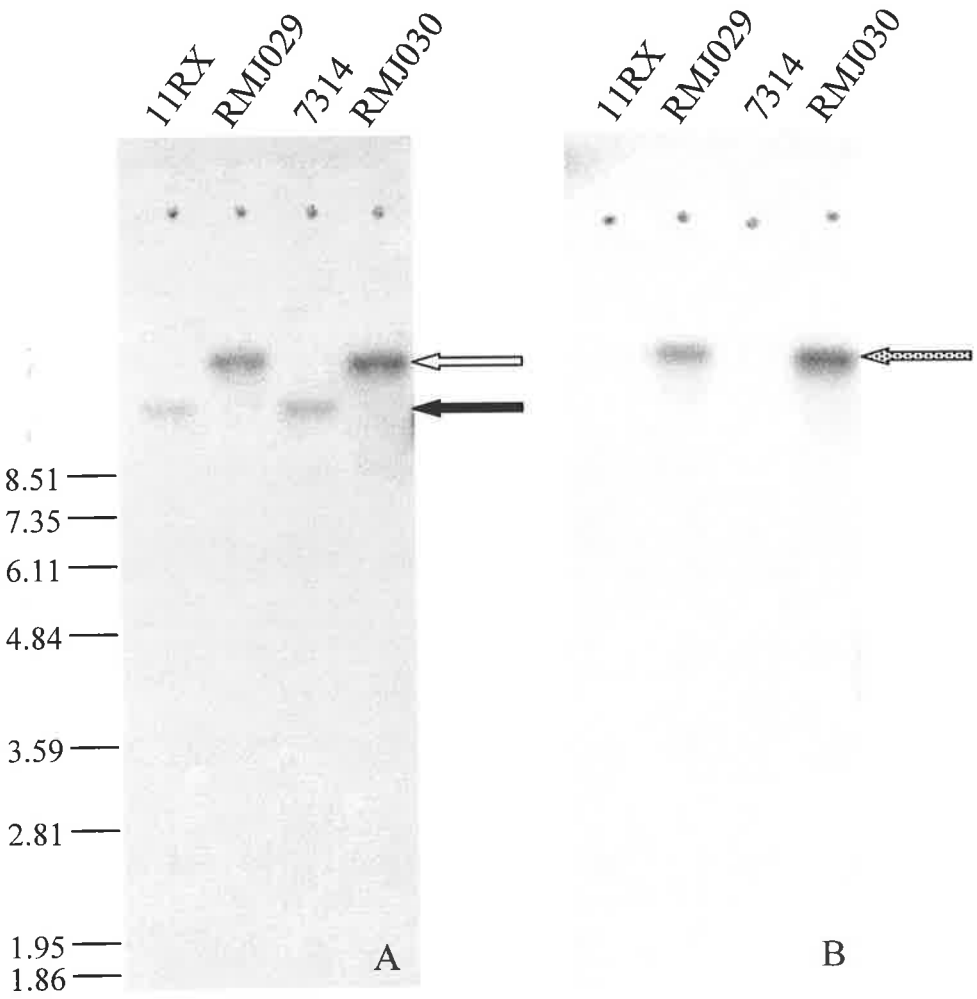
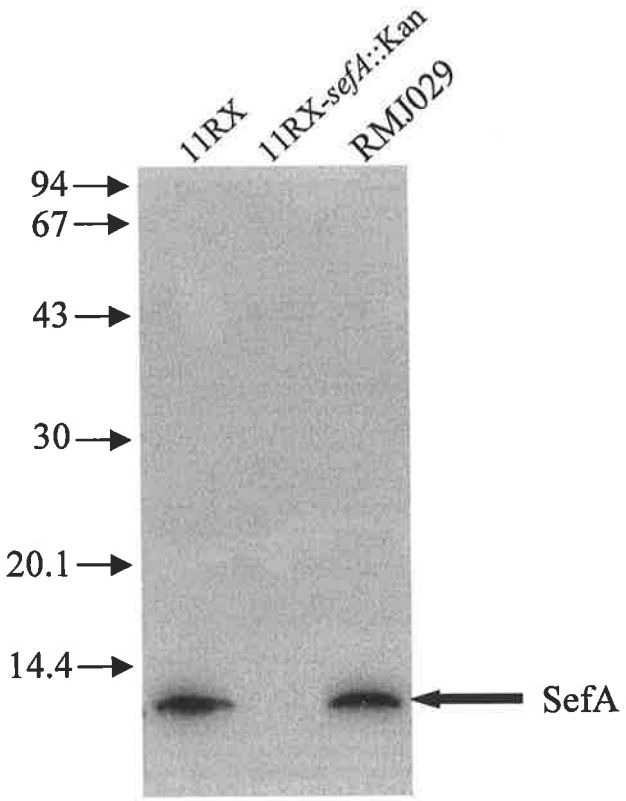

11RX
RMJ029
7314
RMJ030
11RX
RMJ029
7314
RMJ030
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
A
B

Figure 5.19: Effect of *sefD* mutation on SEF14 fimbriae expression.

Strains 11RX, 11RX-*sefA*::Kan and RMJ029 were grown for maximal SEF14 expression on CFA agar, harvested and heat shock supernatants prepared (section 2.13.1). Samples were diluted ½ in 2x SDS-sample buffer, and ~30 µl (representing the equivalent of ~10^8 bacteria) was electrophoresed on SDS-15% polyacrylamide gel (section 2.14.3) and transferred to nitrocellulose prior to Western immunoblotting (section 2.14.4) using 1/1000 anti-SefA antiserum. The arrow designates the SefA positive band observed in both 11RX and the *sefD* mutant strain RMJ029. Molecular weight standards (kDa; section 2.14.3) are indicated.


11RX
11RX-sefA::Kan
RMJ029
94
67
43
30
20.1
14.4
SefA

5.4.3 EIA and IEM analysis of chromosomal *sefD* mutants

It was hypothesised (section 5.1) that the *sefD* gene plays a critical role in the biogenesis of SEF14 fimbriae, in a similar manner to *cooD* in CS1 fimbriae. Mutants in *cooD* are unable to produce CS1 fimbriae, as determined by Froehlich *et al* (1994b), indicating that CooD may play a role as an initiator protein. Given that the SEF14 and CS1 operons share similar arrangements (Sakellaris and Scott, 1998), it was thought that the corresponding genes i.e. *sefD* and *cooD* might have similar function, despite the lack of nucleotide or protein homology.

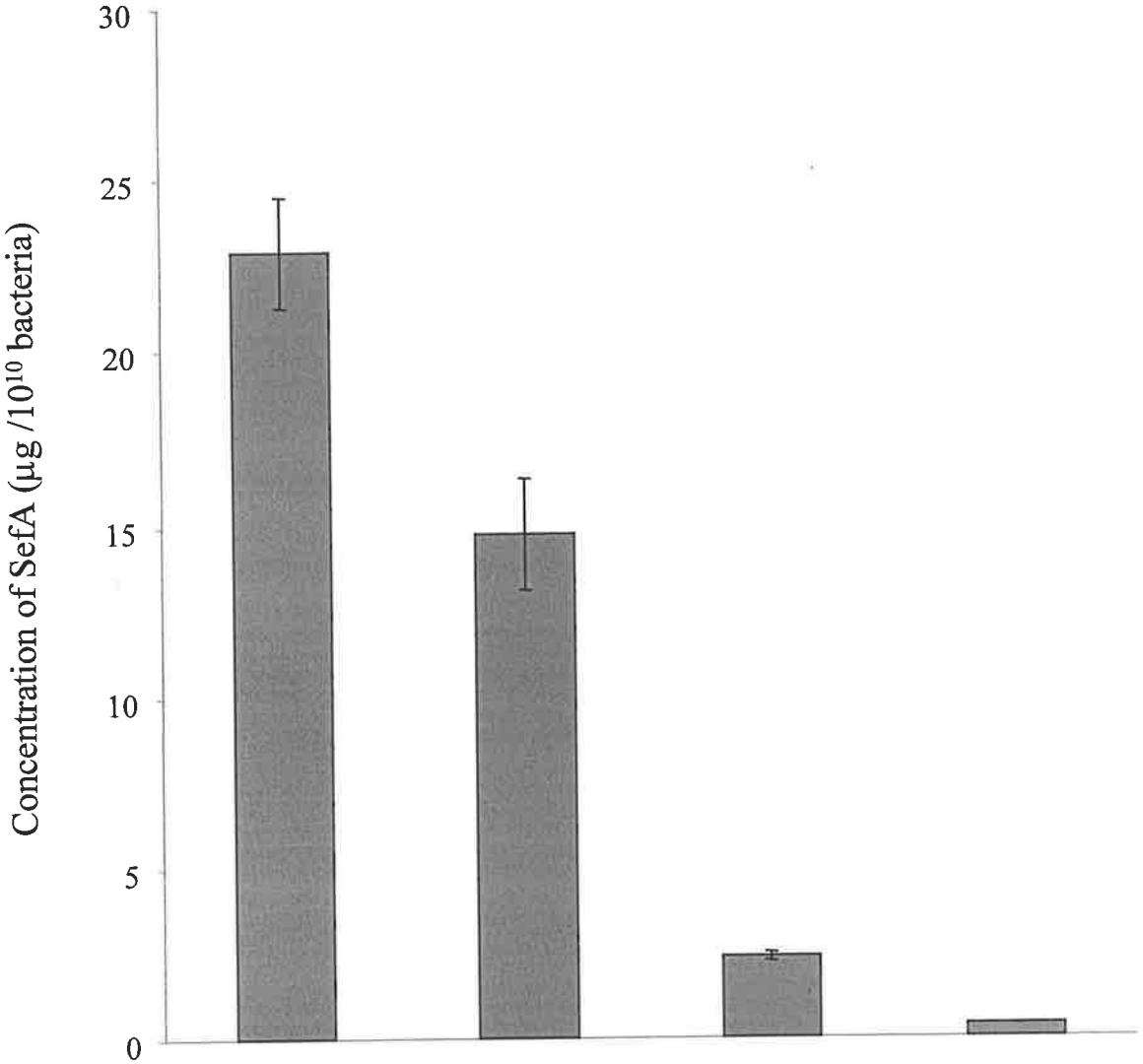
The effect of an absence of *sefD* on the surface expression of SefA in the form of SEF14 fimbriae was examined by EIA and IEM, using anti-SefA sera. It was expected that no SefA would be detected by either method since the lack of initiator protein, i.e. SefD, would prevent export of SefA to the surface of the cell.

5.4.3.1 EIA analysis

EIA was carried out on the *sefD* insertion mutants RMJ029 and RMJ030 and their respective parent strains 11RX and 7314, after growth on CFA agar at both 25°C and 37°C. As expected, no SefA was detected in any of the bacteria grown at 25°C. The parent strains 11RX and 7314 produced amounts of SefA similar to that seen in previous experiments (section 4.8). The mutant strains unexpectedly still possessed surface expressed SefA, however the amount was approximately 65% of that observed for 11RX (22.86 ± 1.6 μg /10^{10} 11RX vs 14.66 ± 1.6 μg /10^{10} RMJ029), and only 14% of that observed for 7314 (2.36 ± 0.12 μg /10^{10} 7314 vs 0.325 ± 0.03 μg /10^{10} RMJ030) as shown in Figure 5.20. The difference between 11RX and RMJ029, and 7314 and RMJ030 suggested that the lack of functional SefD does indeed have some effect on the biogenesis of SEF14 fimbriae.

Figure 5.20: Effect of *sefD* mutation on SEF14 fimbriae expression

The *sefD* mutant strains RMJ029 and RMJ030 and their respective parent strains 11RX and 7314 were grown for maximal SEF14 fimbriae expression by growth on CFA agar. Samples were prepared as per section 2.18.2, and analysed by EIA (section 2.18.3). Shown here are the concentrations of SefA, in $\mu g/10^{10}$ bacteria. Data represents the mean of three independent experiments. Error bars represent the calculated SEM.



Strain	11RX	RMJ029	7314	RMJ030
[SefA] µg/10^{10} bacteria	22.86 ± 1.6	14.66 ± 1.6	2.36 ± 0.12	0.33 ± 0.03

5.4.3.2 IEM analysis

In order to visualise the differences in SEF14 fimbriae production between the *sefD* mutants (RMJ029 and RMJ030) and their parent strains (11RX and 7314, respectively), IEM analysis was performed (section 2.19) and is shown in Figure 5.21. 11RX produced SEF14 fimbriae as an amorphous tangle of fimbriae, without real distinct fibres being observed, while 7314 expressed fimbriae of similar morphology, but less were extensive. The less extensive expression of fimbriae by 7314 correlates with the reduced level of SefA detected by EIA, as compared with 11RX. In contrast, the mutant strains RMJ029 and RMJ030 did not show extensive tangles of fimbriae, with gold particles located either at, or very close to, the cell surface, binding to anti-SefA reactive 'stubs'.

5.4.4 Complementation of *sefD* mutation

To determine whether the observed effects were due to the *sefD* mutation, a plasmid carrying the *sefD* gene that could be induced to express in the presence of arabinose was constructed to trans-complement the *sefD* mutation.

5.4.4.1 Construction of inducible *sefD* expression vector

The method for construction of a *sefD* complementing vector is shown in schematic form in Figure 5.22. The *sefD* gene was PCR amplified using primers 3209 and 3210 (Table 2.3), which incorporated *Eco*RI and *Sph*I restriction enzyme sites respectively. Primer 3209 bound 18 bp upstream of the *sefD* start codon, incorporating the putative *sefD* RBS (ATGGAGT). The *sefD* PCR product was digested with *Eco*RI and *Sph*I and ligated to similarly digested pBAD30 (Guzman *et al.*, 1995). After transformation into DH5α and selection with Amp, PCR and restriction enzyme analysis of mini-prep DNA was used to confirm correct insertion of *sefD* gene, resulting in the construction of plasmid pRJ013. This construct was then retransformed into DH5α, resulting in strain RMJ032.

Figure 5.21: IEM analysis of *sefD* mutant strains RMJ029 and RMJ030

The *sefD* mutant strains RMJ029 and RMJ030 and their respective parent strains 11RX and 7314 were grown for maximal SEF14 fimbriae expression by growth on CFA agar. Suspensions of each strain were prepared (section 2.19) and applied to poly-L-lysine treated Copper-Palladium grids. After washing, the grids were incubated in a 1/50 dilution of anti-SefA antiserum followed by incubation with protein A gold conjugated to 20 nm diameter gold particles (1/40). The grids were then stained 1% uranyl acetate. Panels A, C, E and G represent 11RX, RMJ029, 7314 and RMJ030 visualised at 13,500x magnification, while panels B, D, F and H represent 19,000x magnification of the same bacteria. The arrowheads indicate gold particles associated with SEF14 fimbriae. Scale bars represent 500 nm.

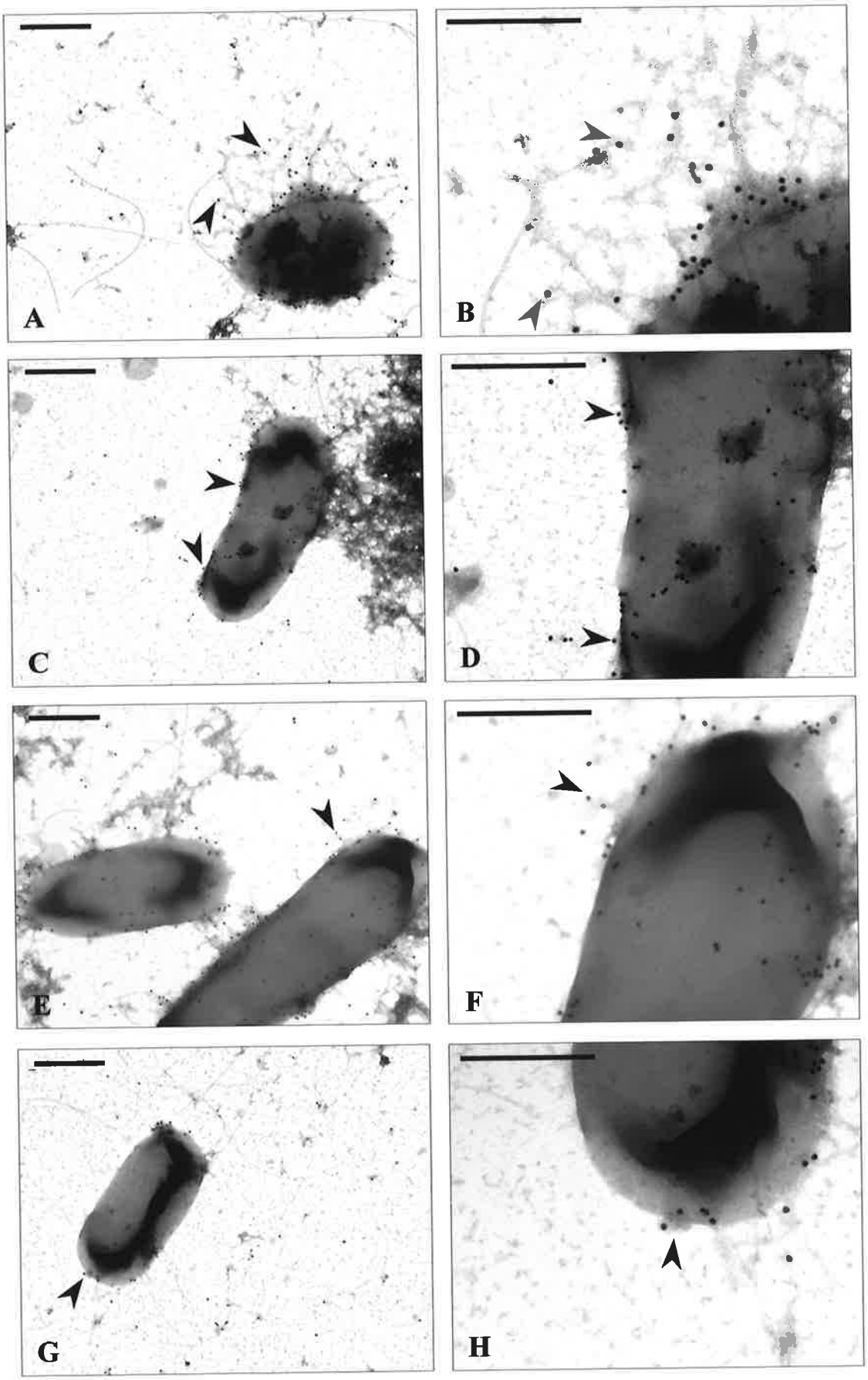
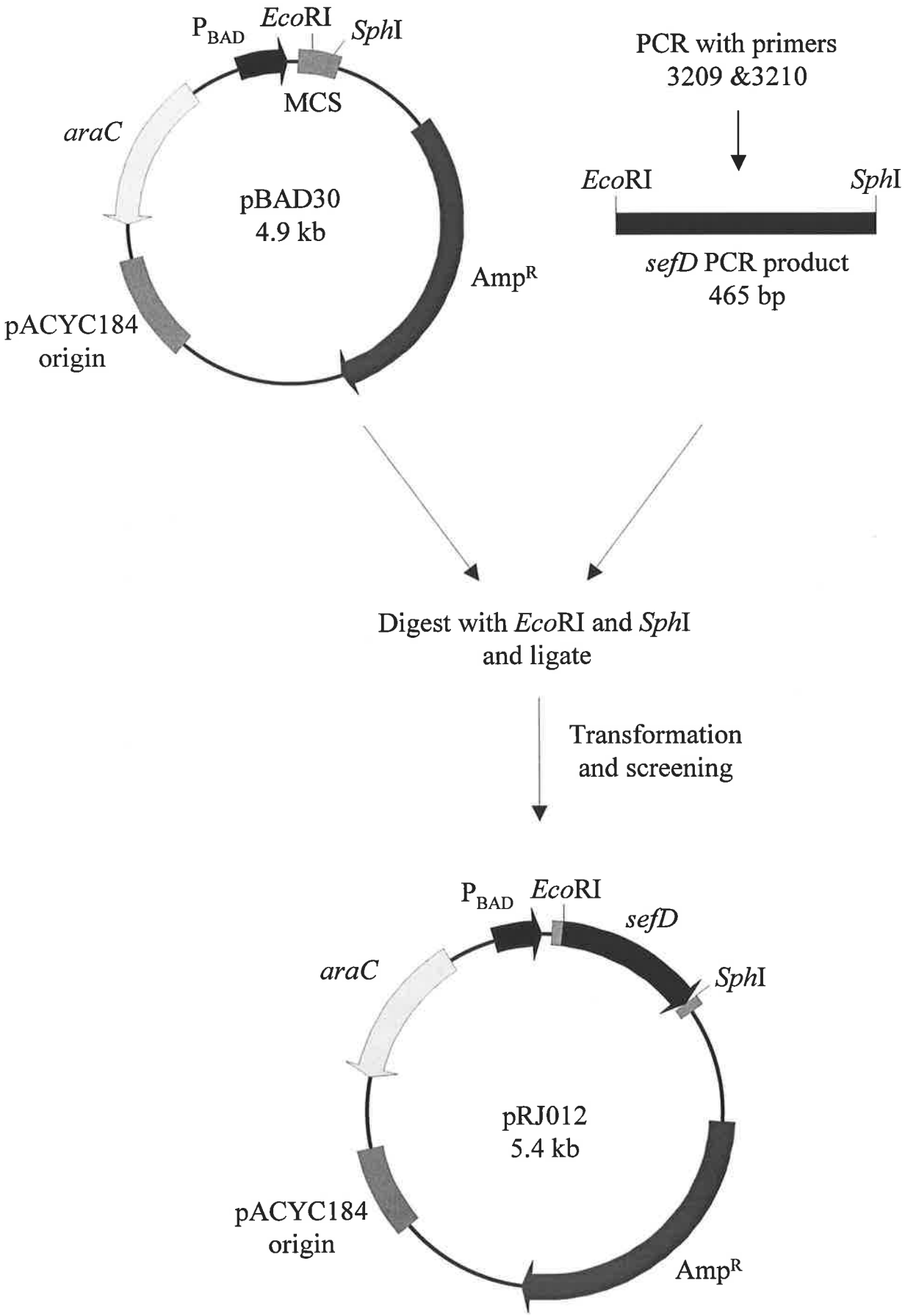

A
B
C
D
E
F
G
H

Figure 5.22: Construction of *sefD* complementing vector

The *sefD* gene was PCR amplified (section 2.10.1) from 11RX chromosomal DNA using oligonucleotides 3209 and 3210 that contained *Eco*RI and *Sph*I restriction enzyme sites, respectively. Primer 3209 incorporated the putative *sefD* RBS (ATGGAGT) found at the extreme 3' end of the *sefC* gene. The amplified *sefD* PCR product (465 bp) was digested with the *Eco*RI and *Sph*I enzymes and ligated into similarly digested pBAD30 (Guzman *et al* 1995) to place *sefD* under the control of the P_{BAD} promoter. After transformation into DH5α and selection with Amp, plasmid DNA was extracted (section 2.8.1) and screened by restriction digest with *Eco*RI and *Sph*I. This resulted in construction of plasmid pRJ012.


P BAD
EcoRI
SphI
MCS
araC
pBAD30
4.9 kb
Amp R
pACYC184
origin
PCR with primers
3209 &3210
EcoRI
SphI
sefD PCR product
465 bp
Digest with EcoRI and SphI
and ligate
Transformation
and screening
P BAD
EcoRI
sefD
araC
SphI
pRJ012
5.4 kb
pACYC184
origin
Amp R

To assess if pRJ013 was able to produce SefD, strain RMJ032 was grown on CFA agar in the presence of either 0.01% (w/v) glucose or 0.01% (w/v) arabinose; strain RMJ031 (DH5α + pBAD30) was used as a negative control. Figure 5.23 shows a Coomassie blue stained SDS-15% polyacrylamide gel of whole cell lysates from these strains. Unlike expression of SefR from pRJ003 (Figure 4.18, section 4.6.2), no obvious SefD band was observed, however this does not discount the possibility that SefD was expressed at low levels.

5.4.4.2 Complementation of *sefD* mutation

The ability of pRJ013 to provide *sefD in trans* and complement the chromosomal *sefD* mutation in strains RMJ029 and RMJ030 was assessed. Plasmid pRJ013 was electroporated into EX2000 prior to introduction into the *sefD* mutants RMJ029 and RMJ030, giving rise to strains RMJ033 and RMJ034, respectively.

5.4.4.2.1 Western immunoblot of complemented *sefD* mutants

Heat shock supernatants were prepared (section 2.13.1) from RMJ029 and RMJ033 grown on either CFA + 0.01% (w/v) glucose or CFA + 0.01% (w/v) arabinose. Samples were analysed by Western immunoblotting using anti-SefA antiserum to detect whether there was any difference in the expression of SEF14 fimbriae (Figure 5.24). As was expected from previous results (section 5.4.3) both strains expressed SefA under all conditions, and to similar levels as seen previously when grown on CFA alone. With these stains there were some apparent variations, i.e. there appeared to be less SefA expressed when RMJ029 is grown in the presence of arabinose (lane 2) as compared to glucose (lane 1) while RMJ033 appears to express more SefA when grown in the presence of arabinose (lane 4) as compared to glucose (lane 3). The latter observation is consistent with complementation of *sefD* mutation leading to improved surface expression of SEF14 fimbriae. Similar results were

Figure 5.23: Expression of SefD from pRJ012

Strains RMJ031 (DH5α + pBAD30) and RMJ032 (DH5α + pRJ013) were grown for 18 h in CFA broth supplemented with either 0.01% (w/v) glucose (glu) or 0.01% (w/v) arabinose (ara). Whole cell lysates (representing ~10^8 bacteria) of each strain were solubilised in 2x SDS-sample buffer (section 2.14.1), electrophoresed on an SDS-15% polyacrylamide gel and stained with Coomassie blue (section 2.14.3). The arrow indicates the expected size of SefD. Molecular weight standards (kDa; section 2.14.3) are indicated.

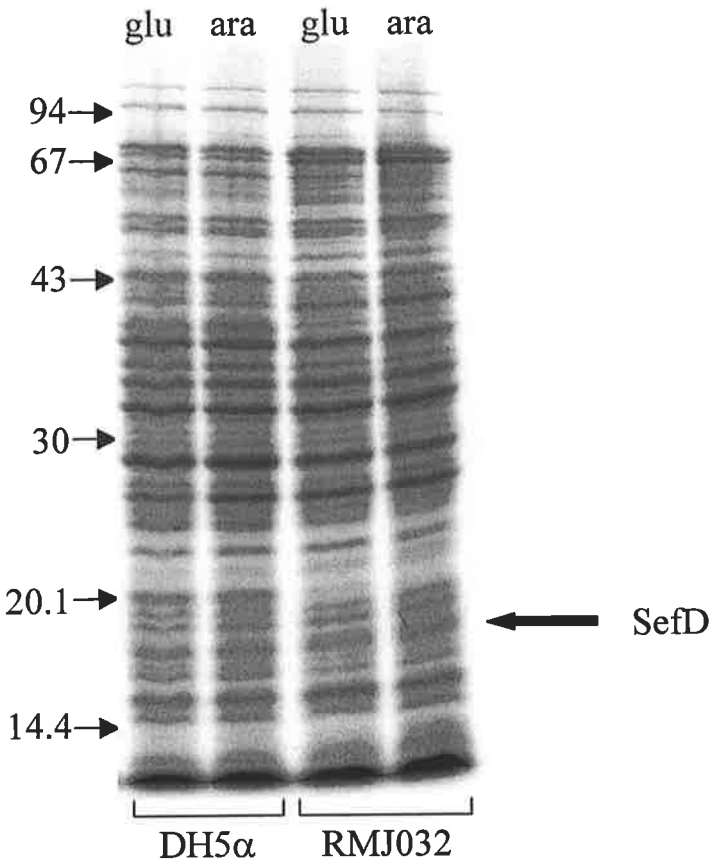
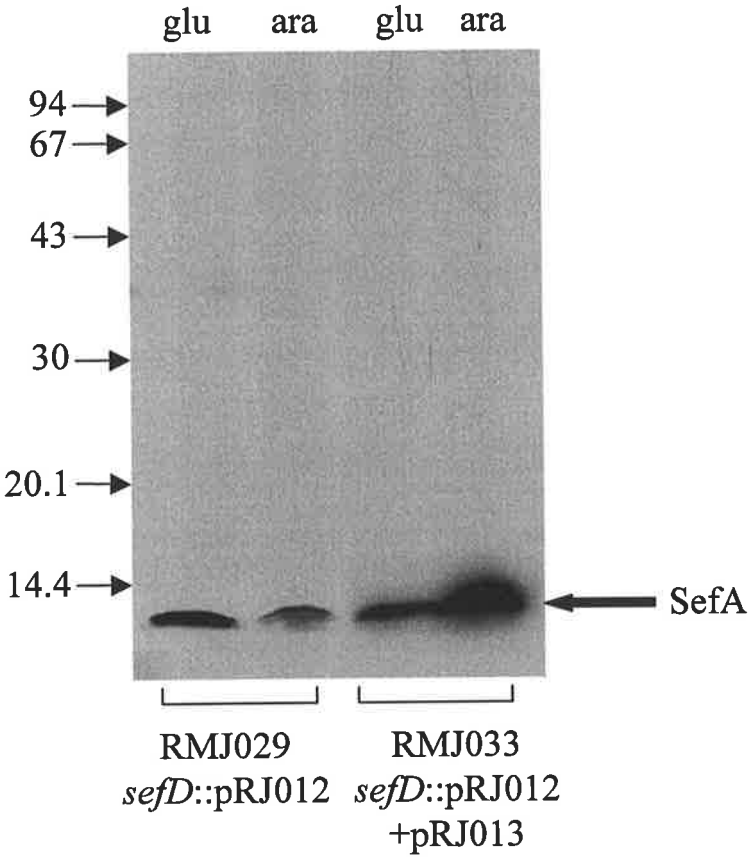

glu
ara
glu
ara
94
67
43
30
20.1
14.4
SefD
DH5α
RMJ032

Figure 5.24: Complementation of the chromosomal *sefD* mutation by pRJ012

Strains RMJ029 and RMJ033 (containing pRJ013) were grown for maximal SefA expression on CFA medium supplemented with either 0.01% (w/v) glucose or 0.01% (w/v) arabinose. Bacteria were harvested and heat shock supernatant samples prepared (section 2.13.1). Samples (representing ~10^8 bacteria) were electrophoresed on a SDS-15% polyacrylamide gel and transferred to nitrocellulose prior to Western immunoblotting using absorbed anti-SefA antiserum (1/1000). The arrow indicates the position of SefA. Molecular weight standards (kDa; section 2.14.3) are indicated.


glu
ara
glu
ara
94
67
43
30
20.1
14.4
SefA
RMJ029
sefD::pRJ012
RMJ033
sefD::pRJ012
+pRJ013

observed when comparing RMJ030 and RMJ034 (not shown). To support these observations, SefA expression was further examined in these strains by EIA and IEM analysis.

5.4.4.2.2 EIA analysis of complemented *sefD* mutants

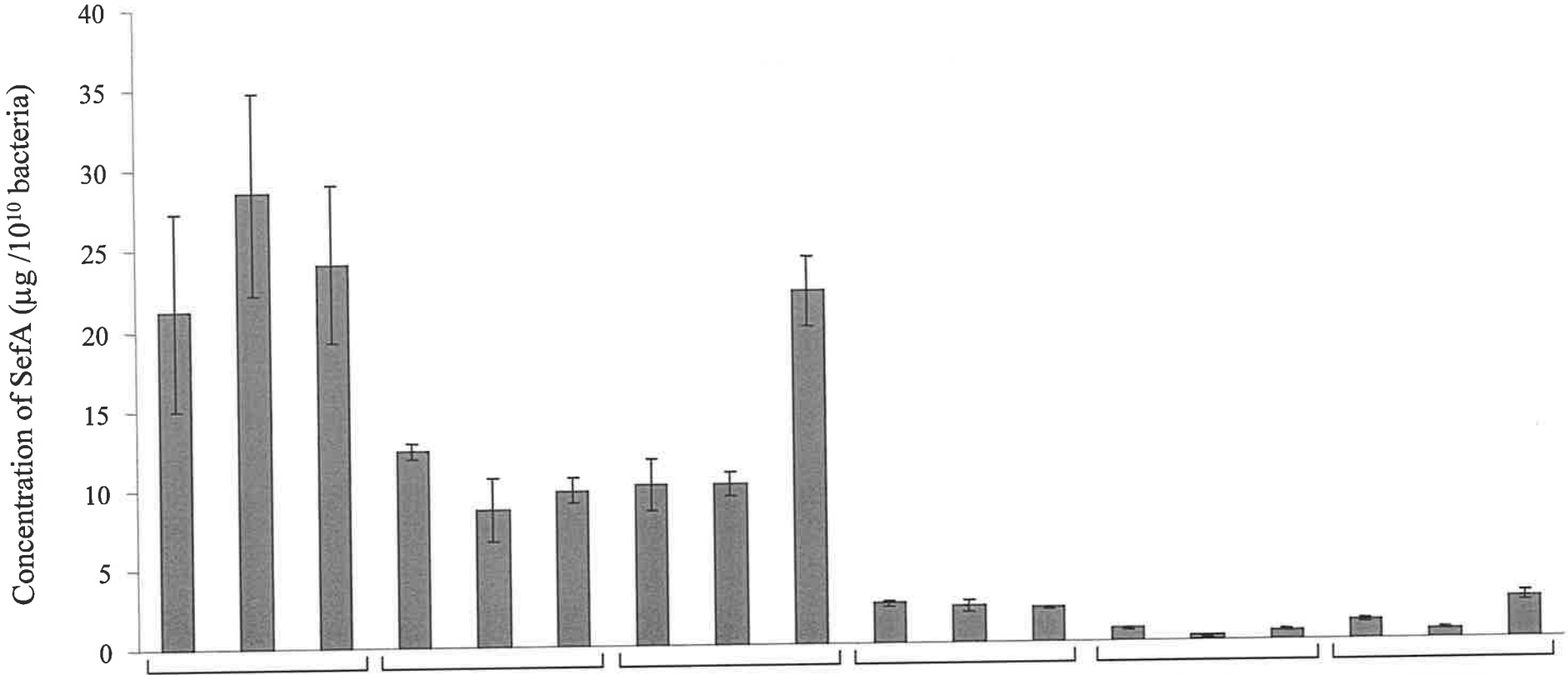
11RX, 7314, RMJ029, RMJ030, RMJ033 and RMJ034 were grown on CFA agar alone or CFA agar supplemented with either 0.01% (w/v) glucose or 0.01% (w/v) arabinose. After growth, bacterial samples were prepared and analysed by EIA (section 2.18.2). Figure 5.25 shows the results of EIA to measure SefA production. 11RX and RMJ029 produced similar amounts of SefA to that observed previously (section 5.4.3.1), with the presence of glucose or arabinose having no significant effect on the level of SefA expression. RMJ033 expressed similar amounts of SefA (10.1 ± 0.7 $\mu g/10^{10}$ bacteria) compared with RMJ029 (8.6 ± 1.9 $\mu g/10^{10}$ bacteria) when grown under non-permissive conditions for *sefD* expression i.e. in presence of glucose. However when grown under inducing conditions, i.e. in presence of arabinose, the levels of SefA returned to that observed for the parent strain 11RX (22 ± 2.2 $\mu g/10^{10}$ bacteria vs 24 ± 5.0 $\mu g/10^{10}$ bacteria respectively), showing that pRJ013 is able to provide SefD and complement the chromosomal mutation. Proportionately similar results were observed when comparing 7314 with RMJ030 and RMJ034. This result supports the hypothesis that *sefD* plays an essential role in the biogenesis of SEF14 fimbriae.

5.4.4.2.3 IEM analysis

Visual analysis of the complemented *sefD* mutant strains RMJ033 and RMJ034 was carried out by IEM (section 2.19), and compared to the parent strains 11RX and 7314, and also to the *sefD* mutant strains RMJ029 and RMJ030 (Figures 5.26 and 5.27). 11RX and 7314 produced the expected amorphous fimbriae, while the mutant strains displayed the limited surface expression as observed previously (Figure 5.26 C and D and 5.27 C and D). Also as observed earlier, the presence of glucose or arabinose seemed to have not observable

Figure 5.25: Analysis of surface expression of SefA by complemented strains

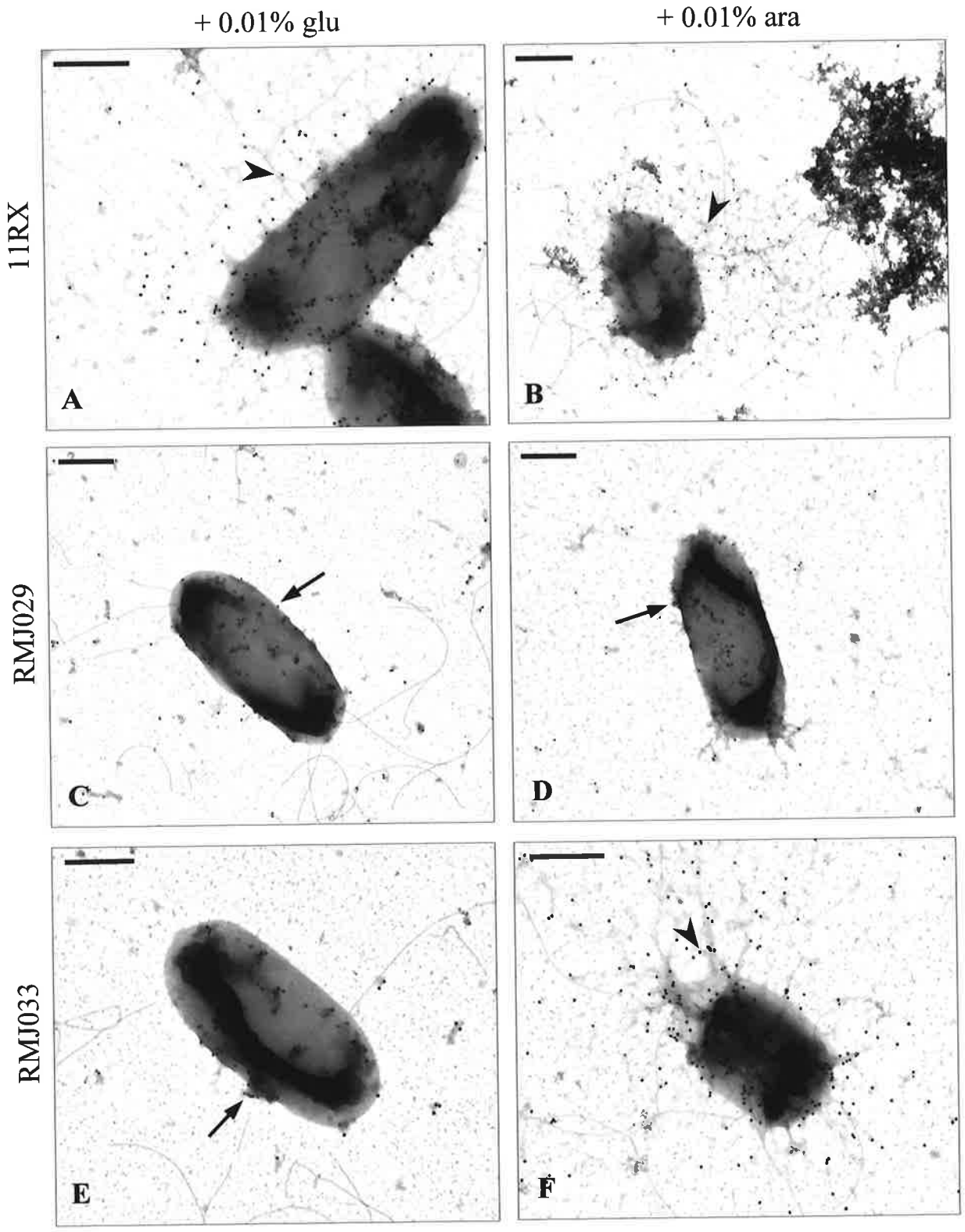
Strains 11RX and 7314, their respective *sefD* mutants (RMJ029 and RMJ030) and *sefD* complemented mutants (RMJ033 and RMJ034 (each containing pRJ013)) were grown on CFA agar alone or CFA agar supplemented with either 0.01% (w/v) glucose or 0.01% (w/v) arabinose. Samples were prepared (section 2.18.2), and analysed by EIA (section 2.18.3). Shown here are the concentrations of SefA, in $\mu g/10^{10}$ bacteria. Data represents the mean of three independent experiments. Error bars represent the calculated SEM.

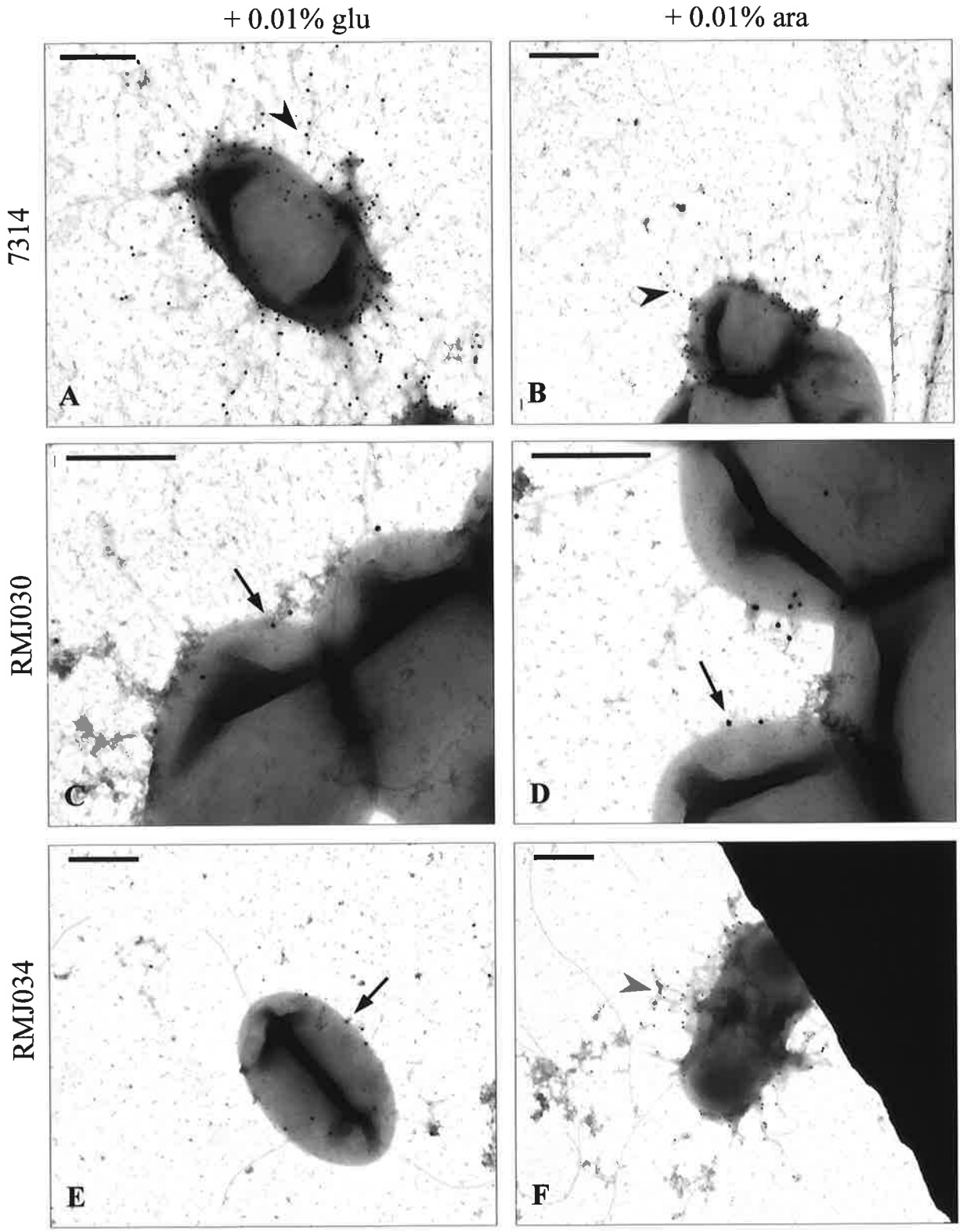


Strain	11RX			RMJ029			RMJ033			7314			RMJ030			RMJ034		
Growth condition	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara	-	0.01% glu	0.01% ara
[SefA] µg/10^{10} bacteria	21 ± 6.2	28 ± 6.3	24 ± 5.0	12.4 ± 0.51	8.6 ± 1.9	9.8 ± 0.84	10.1 ± 1.6	10.1 ± 0.7	22 ± 2.2	2.4 ± 0.17	2.2 ± 0.43	2.1 ± 0.03	0.7 ± 0.1	0.22 ± 0.05	0.56 ± 0.07	1.1 ± 0.1	0.53 ± 0.08	2.6 ± 0.3

Figure 5.26: Complementation of *sefD* mutation in RMJ029

Strains 11RX (A & B), RMJ029 (C & D) and RMJ033 (E & F) were grown (18 h) for maximal SEF14 fimbriae expression on CFA medium supplemented with either 0.01% glucose (A, C, E) or 0.01% arabinose (B, D, F). A suspension of each strain was made in 1x PBS and incubated with poly-L-lysine treated Copper-Palladium electron microscopy grids (section 2.19). SEF14 fimbriae were detected by incubation with absorbed anti-SefA antiserum (1/50) followed by protein A gold conjugated to 20 nm diameter gold particles (1/40). The grids were stained with the 1% uranyl acetate stained prior to visualising. Arrowheads indicate normal fimbriae (panels A, B & F) while arrows indicate truncated fimbriae (panels C, D & E). Scale bars represent 500 nm.


+ 0.01% glu
+ 0.01% ara
11RX
RMJ029
RMJ033
A
B
C
D
E
F


+ 0.01% glu
+ 0.01% ara
7314
RMJ030
RMJ034
A
B
C
D
E
F

effect on fimbrial expression. When RMJ033 and RMJ034 were grown in presence of glucose i.e. non-permissive for *sefD* expression, the fimbriae morphology was the same as observed for their respective mutant strains (Figures 5.26E and 5.27E). However, when grown in media supplemented with arabinose, RMJ033 and RMJ034 expressed fimbriae with similar morphology and abundance to that seen in the parental strains, indicating that pRJ013 was able to provide SefD *in trans* to complement the chromosomal *sefD* mutation, and restore full expression of SEF14 fimbriae. Therefore, SefD is required for the biogenesis of SEF14 fimbriae, and is probably a structural component, given the localisation of SefA at the cell surface in the SefD mutant strains.

5.5 Summary

This focus of this chapter was to characterise the role of SefD in the biogenesis of SEF14 fimbriae, with the specific hypothesis that SefD acts as an initiator of SEF14 expression. In order to detect the expression of SefD in the context of SEF14 fimbriae, attempts were made to produce anti-SefD antiserum. *sefD* was successfully cloned into two different expression vectors: pMAL-c2X and pET-21a, which contain MBP and His_6 tags respectively to facilitate purification. These *sefD* expression constructs (pRJ009 and pRJ010) were successfully used to express the fusion proteins MBP-SefD and pre-SefD-C-His_6 respectively, as assessed by Coomassie stained SDS-15% polyacrylamide gel and Western immunoblotting using monoclonal antibodies directed against the MBP and His_6 tags. Fractionation and SDS-15% PAGE analysis of the expression strains RMJ023 (pMAL-c2X + *sefD*) and RMJ027 (pET-21a + *sefD*) after induction showed that both MBP-SefD and pre-SefD-C-His_6 formed inclusion bodies. The IB fraction from each strain was used as a source of protein for purification of SefD, however in both cases the purification procedure was unsuccessful. MBP-SefD and pre-SefD-C-His_6 were SDS-15% PAGE extracted from IB fractions to remove the bulk of the contaminating proteins, and used as antigen for

immunisation of rabbits. In the case of MBP-SefD as immunising antigen, a significant response was observed against the MBP portion, however no reactivity to SefD was observed. In the case of pre-SefD-C-His_6, a protein of the expected size was observed by Western immunoblot analysis of induced RMJ027, however the same band was also observed in the control strain RMJ026. This result was most likely due to the presence of a contaminating protein in the antigen preparation used for immunisation of the rabbit. Thus in both cases the production of specific anti-sefD antiserum was unsuccessful, and alternative methods were used to examine the role of SefD in SEF14 biogenesis.

Previously (section 4.4.3) it was observed that the *sefABCD* genes, in the context of a high copy number vector (pRJ004), were able to elaborate SEF14 fimbriae in a DH5α background. To examine the effect of a deletion of the 3' end of *sefD*, *Hin*dIII sites were used to subclone a *sefABCΔD* fragment into the same vector background (pRJ011). This clone was examined for SefA expression alongside strains 11RX and RMJ010 (containing pRJ004) by Western immunoblotting and IEM. It was observed that strain RMJ028 (containing pRJ011) did not appear to express the same amount of SefA as RMJ010. This reduced expression may have been caused by some instability in the mRNA, since removing the 3' end of *sefD* also removed the stem-loop structure between *sefD* and *sefR* (section 3.3.2). This instability would lead to reduced expression of both sefB and sefC, perhaps accounting for the reduced expression/export of SefA to the cell surface. IEM analysis revealed that RMJ028 was unable to elaborate SEF14 fimbriae, which would support a role for SefD in the biogenesis of SEF14 fimbriae. However, it was unclear whether this was due to the apparent decreased expression of SefA for the reasons mention above, or due to the physical absence of the SefD protein.

As creating *sefR* mutants in *S.* Enteritidis was successful in demonstrating a role for SefR in the regulation of SEF14 fimbriae expression, a similar mutation was introduced into the *sefD* gene. This mutation had an interesting and unexpected effect on SEF14 expression. Instead of abolishing expression, as was hypothesised, the level of expression was reduced as

determined quantitatively by EIA. Also the apparent length of fimbriae, as observed by IEM, was also reduced. The *sefD* mutant strains RMJ029 and RMJ030 (11RX and 7314 *sefD* mutants respectively) showed predominately cell surface associated SefA expression, with no mass of amorphous fimbriae observed in the parent strains. The implications of this observation in terms of other fimbrial systems is discussed further in the general discussion (section 7.5)

This study presents the first morphological examination of a *sefD* mutant, and revealed that the biogenesis of SEF14 fimbriae is probably more complex than the small number of genes would suggest. The implications of these results are discussed further in chapter 7.

Chapter 6: Analysis of the distribution of *sefR* and *sefD* genes

6.1 Introduction

Previous studies on the distribution of *sef* genes in Enterobacteriaceae have presented conflicting results, leading to an ambiguous interpretation of the true distribution of these genes. Hybridisation with *sefA* (Turcotte and Woodward, 1993; Doran *et al.*, 1996), *sefB* (Doran *et al.*, 1996) or *sefC* (Doran *et al.*, 1996) specific gene probes have shown that these genes are limited to a subset of the group D1 *Salmonellae*, which argues for their recent acquisition, presumably by horizontal transfer. This is in contrast to a study carried out by Clouthier *et al* (1994) who showed that *sefD* was ubiquitous within the Enterobacteriaceae family, as determined by dot blot hybridisation and antibody cross-reactivity. This result implied that *sefR*, linked to *sefD* by a short segment of 33 bp (see Figure 3.8, section 3.3.2), might have a similar distribution. However, the available sequence data (Clouthier *et al.*, 1993; Clouthier *et al.*, 1994; this study section 3.3) give no indication as to whether these genes were acquired separately i.e. in two different transfer events before or after the *sefABC* genes had been acquired, or whether the *sefABCDR* genes were previously linked and acquired as a single unit by *S.* Enteritidis. The close association of *sefC* and *sefD* (i.e. overlapping stop and start codons) suggests they were acquired simultaneously with *sefA* and *sefB*, despite the published data (Clouthier *et al.*, 1994; Doran *et al.*, 1996) showing *sefC* and *sefD* have quite different distributions. Whether *sefR* was also acquired in the same event or during a separate event is not known.

It was hypothesised that *sefD* was not ubiquitous, but rather an integral part of the SEF14 operon. It was also hypothesised that *sefR* would display the same pattern of distribution as the *sefABCD* genes, confirming *sefR* as part of the SEF14 loci as a single genetic unit that has been recently acquired, in evolutionary terms, by an ancestor of the group D1 *Salmonellae*. In attempting to define the possible sequence of events that led to the

acquisition of the current SEF14 operon, the distributions of both *sefD* and *sefR* were compared using both dot blot and Southern hybridisation techniques.

6.2 Hybridisation studies to determine distribution of *sefD* and *sefR*

6.2.1 Analysis of *sefD* gene distribution by dot blot hybridisation

40 Enterobacteriaceal strains were analysed for the presence of a *sefD* gene homolog using the dot-blot method in order to test the reproducibility of the results obtained by Clouthier *et al* (1993). The only difference in experimental protocol was the use of a full-length *sefD* gene probe (positions 4739-5185) labelled with DIG, rather than ^{32}P-labelled probes. The dot blot filter probed with *sefD* is shown in Figure 6.1. Even though the variety of bacterial species analysed is smaller than that examined previously, it is clear that not all of the bacteria tested posses a *sefD* gene homolog. The group D1 *Salmonellae* serovars *S.* Berta (A8), *S.* Dublin (A10, B1), various *S.* Enteritidis strains (B2-B8), *S.* Gallinarum (B9), *S.* Pullorum (B10) and *S.* Typhi (C2) were positive for *sefD*, which matches the limited distribution pattern reported for the *sefABC* genes (Turcotte and Woodward, 1993; Doran *et al.*, 1996).

6.2.2 Analysis of *sefD* gene distribution by Southern hybridisation

A subset of those bacteria analysed by dot-blot were subjected to Southern hybridisation analysis. Chromosomal DNA from 16 of the 40 strains was extracted (section 2.8.2), digested with *Bam*HI and electrophoresed as described in section 2.9.3. Southern transfer was then carried out (section 2.12.2), followed by probing with the DIG-labelled *sefD* used for the dot-blot procedure. The Southern hybridisation filter is shown in Figure 6.2. Of the strains tested, only the *S.* Enteritidis strains 11RX and 7314 (Figure 6.2, lanes 5 & 6) and *S.* Typhi (Figure 6.2, lane 8) were positive for *sefD*, with bands of ~8.5 and >10 kb respectively. This result was consistent with the data obtained by dot-blot hybridisation.

Figure 6.1: Detection of *sefD* by dot-blot hybridisation

Forty strains, mainly from the Enterobacteriaceae family, were tested for the presence of *sefD* by dot-blot hybridisation as per section 2.12.3. The source of the strains is listed in Table 2.2. The *sefD* probe used was a full-length gene probe (447 nt) labelled with DIG (section 2.12.1). High stringency conditions were used to minimise false positive results. The Table below lists the strains (and their coordinates) used in this analysis

A1	*Escherichia coli* K-12
A2	*Escherichia coli* O26
A3	*Escherichia coli* O157
A4	*Escherichia coli* O111:H$^-$
A5	*Legionella micdadeai*
A6	*Legionella pneumophila*
A7	*S.* Adelaide
A8	*S.* Berta
A9	*S.* Choleraesuis
A10	*S.* Dublin

C1	*S.* Stanley
C2	*S.* Typhi Ty2
C3	*S.* Typhimurium C5
C4	*S.* Typhimurium LT2
C5	*Shigella flexneri* PE577
C6	*Shigella flexneri* 2457Ta
C7	*Shigella flexneri* 2457Tb
C8	*Shigella flexneri* 736/89 RKI 92
C9	*Escherichia coli* 1392-75
C10	*Escherichia coli* 247425-1

B1	*S.* Dublin *aroA*
B2	*S.* Enteritidis 11RX
B3	*S.* Enteritidis 7314
B4	*S.* Enteritidis 1362
B5	*S.* Enteritidis 6325
B6	*S.* Enteritidis 8246
B7	*S.* Enteritidis 9202
B8	*S.* Enteritidis 9958
B9	*S.* Gallinarum
B10	*S.* Pullorum

D1	*Escherichia coli* 248750-1
D2	*Vibrio anguillarum*
D3	*Vibrio fluvialis*
D4	*Vibrio mimicus*
D5	*Vibrio cholerae* non-O1
D6	*Vibrio cholerae* 569B
D7	*Vibrio cholerae* O17
D8	*Vibrio cholerae* O139
D9	*Yersinia Enterocolitica*
D10	*Photorhabdus luminescens*

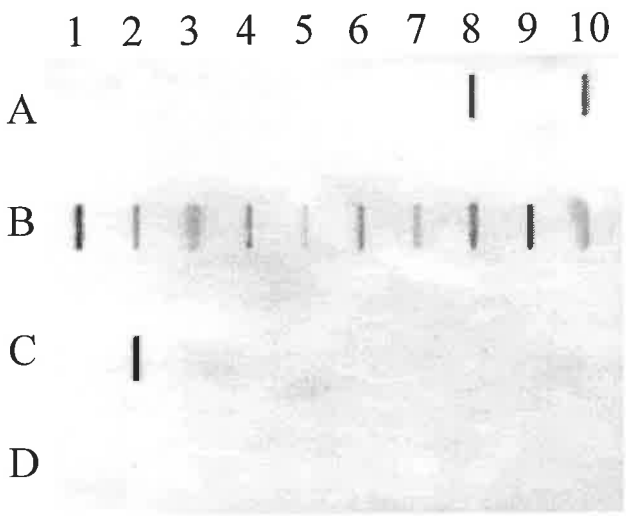
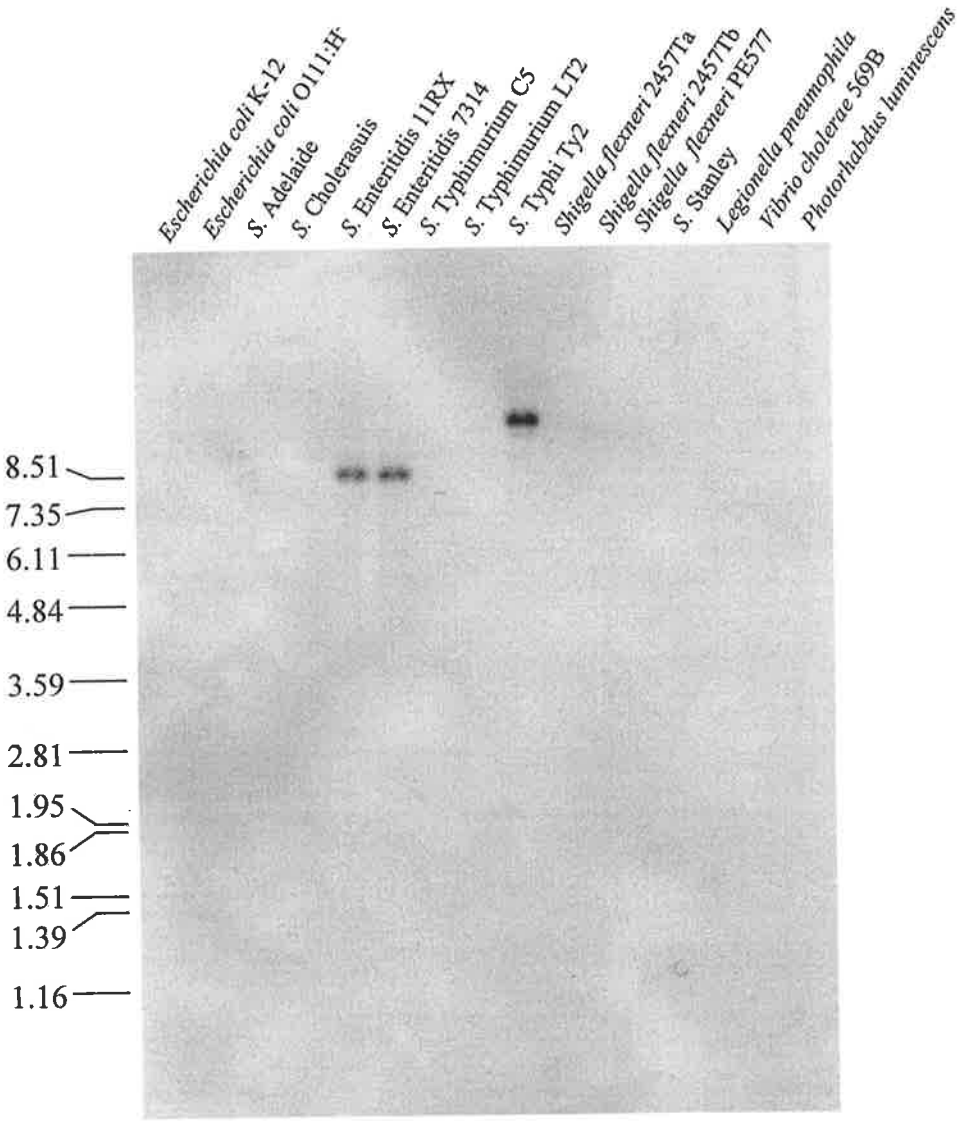

1 2 3 4 5 6 7 8 9 10
A
B
C
D

Figure 6.2: Detection of *sefD* by Southern hybridisation

Sixteen of the forty strains tested by dot-blot were re-analysed by Southern hybridisation as per section 2.12.2. Chromosomal DNA was digested with *Bam*HI, electrophoresed on a 0.8% TAE gel and transferred to nitrocellulose. The *sefD* probe used for dot-blot analysis was used here (Figure 6.1). The strains examined are listed above their respective lanes. *Eco*RI digested SPPI DNA markers (kb) are indicated.


Escherichia coli K-12
Escherichia coli O111:H-
S. Adelaide
S. Cholerasuis
S. Enteritidis 11RX
S. Enteritidis 7314
S. Typhimurium C5
S. Typhimurium LT2
S. Typhi Ty2
Shigella flexneri 2457Ta
Shigella flexneri 2457Tb
Shigella flexneri PE577
S. Stanley
Legionella pneumophila
Vibrio cholerae 569B
Photorhabdus luminescens
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16

6.2.3 Analysis of the *sefR* gene by dot-blot and Southern Hybridisation

Based on the results obtained for *sefD* distribution, it was expected that *sefR* would be similarly distributed. In order to test this hypothesis, both dot-blot and Southern hybridisation was carried out on the same bacterial strains, using a full-length *sefR* DIG-labelled gene probe (nt 5218 to 6031). The results of the dot-blot hybridisation are shown in Figure 6.3. All strains that had previously tested positive for *sefD* by this method (Figure 6.1) were also positive for *sefR*, indicating that there is some link between these two genes. However, several *E. coli* strains (Figure 6.3 A1-A4, C9-C10 & D1) and several *Shigella flexneri* strains (Figure 6.3 C5-C8), previously shown to be negative for *sefD* (Figure 6.1), tested positive for *sefR*.

This seemingly anomalous distribution of *sefR* was confirmed by Southern hybridisation analysis of strains previously examined using a *sefD* DIG probe. *S.* Enteritidis strains 11RX and 7314 (Figure 6.4, lanes 5 & 6) and *S.* Typhi (Figure 6.4, lane 8) were positive for *sefR*, with *Bam*HI restriction fragment of similar size to those observed in Figure 6.2, indicating that *sefD* and *sefR* were located on the same fragment as expected. In addition, *E. coli* O111:H$^-$ (Figure 6.4 lane 2) and the *Shigella flexneri* strains 2457Ta and 2457Tb displayed multiple bands that hybridised the *sefR* probe, ranging in size from ~8.5 kb to ~2 kb. This suggested either the presence of multiple *sefR* homologues in these strains or that *Bam*H1 sites were present within the *sefR* gene sequence, leading to different sized DNA fragments that were bound by the *sefR* gene probe. The significance of this observation is examined in section 7.3.

Interestingly, DNA from *E. coli* K-12 did not bind the *sefR* probe, in contrast to the positive result obtained by dot-blot hybridisation (Figure 6.3 A1). This suggested that the dot-blot method is susceptible to false positive results; this may explain the prior observation by Clouthier *et al* (1994) that *sefD* was ubiquitous throughout the Enterobacteriaceae.

Figure 6.3: Detection of *sefR* by Dot-blot hybridisation

Forty strains, mainly from the Enterobacteriaceae family, were tested for the presence of *sefR* by dot-blot hybridisation as per section 2.12.3. The source of the strains is listed in Table 2.2. The *sefR* probe used was a full-length gene probe (813 nt) labelled with DIG (section 2.12.1). High stringency conditions were used to minimise false positive results. The Table below lists the strains (and their coordinates) used in this analysis

A1	*Escherichia coli* K-12
A2	*Escherichia coli* O26
A3	*Escherichia coli* O157
A4	*Escherichia coli* O111:H$^-$
A5	*Legionella micdadeai*
A6	*Legionella pneumophila*
A7	*S.* Adelaide
A8	*S.* Berta
A9	*S.* Choleraesuis
A10	*S.* Dublin

B1	*S.* Dublin *aroA*
B2	*S.* Enteritidis 11RX
B3	*S.* Enteritidis 7314
B4	*S.* Enteritidis 1362
B5	*S.* Enteritidis 6325
B6	*S.* Enteritidis 8246
B7	*S.* Enteritidis 9202
B8	*S.* Enteritidis 9958
B9	*S.* Gallinarum
B10	*S.* Pullorum

C1	*S.* Stanley
C2	*S.* Typhi Ty2
C3	*S.* Typhimurium C5
C4	*S.* Typhimurium LT2
C5	*Shigella flexneri* PE577
C6	*Shigella flexneri* 2457Ta
C7	*Shigella flexneri* 2457Tb
C8	*Shigella flexneri* 736/89 RKI 92
C9	*Escherichia coli* 1392-75
C10	*Escherichia coli* 247425-1

D1	*Escherichia coli* 248750-1
D2	*Vibrio anguillarum*
D3	*Vibrio fluvialis*
D4	*Vibrio mimicus*
D5	*Vibrio cholerae* non-O1
D6	*Vibrio cholerae* 569B
D7	*Vibrio cholerae* O17
D8	*Vibrio cholerae* O139
D9	*Yersinia Enterocolitica*
D10	*Photorhabdus luminescens*

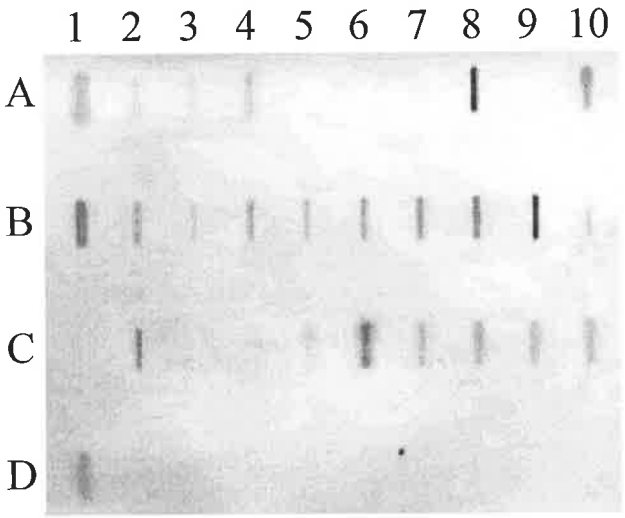
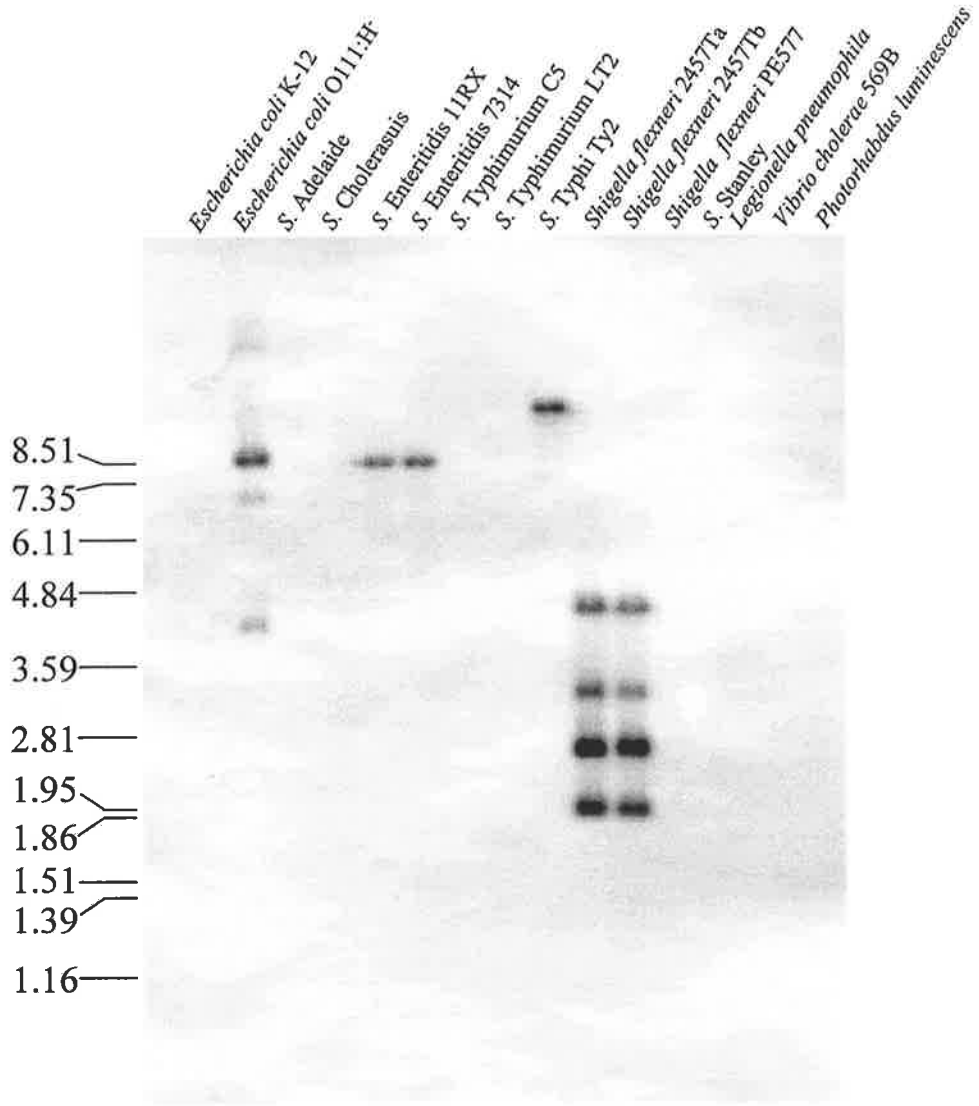

1 2 3 4 5 6 7 8 9 10
A
B
C
D

Figure 6.4: Detection of *sefR* by Southern hybridisation

Sixteen of the forty strains tested by dot-blot were re-analysed by Southern hybridisation as per section 2.12.2. Chromosomal DNA was digested with *Bam*HI, electrophoresed on a 0.8% TAE gel and transferred to nitrocellulose. The *sefR* probe used for dot-blot analysis (Figure 6.3) was used here. The strains examined are listed above their respective lanes. *Eco*RI digested SPPI DNA markers (kb) are indicated.


Escherichia coli K-12
Escherichia coli O111:H-
S. Adelaide
S. Cholerasuis
S. Enteritidis 11RX
S. Enteritidis 7314
S. Typhimurium C5
S. Typhimurium LT2
S. Typhi Ty2
Shigella flexneri 2457Ta
Shigella flexneri 2457Tb
Shigella flexneri PE577
S. Stanley
Legionella pneumophila
Vibrio cholerae 569B
Photorhabdus luminescens
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16

6.3 Comparison of *S.* Enteritidis and *S.* Typhi *sef* genes

S. Enteritidis and *S.* Typhi have been shown to possess both *sefD* and *sefR* genes by dot-blot and Southern hybridisation techniques. The two genes resided on an approximately 8.5 kb *Bam*HI restriction fragment in *S.* Enteritidis. A similar observation was made for *S.* Typhi, however the *sef* gene positive restriction fragment was larger (>10 kb) than that observed for *S.* Enteritidis, suggesting that restriction fragment length polymorphism had taken place.

Given that the *sefD* and *sefR* genes were located on the same restriction fragment in *S.* Enteritidis and *S.* Typhi, it was hypothesised that the arrangement of the SEF14 operon in these bacteria would be the same. The following sections deal with the analysis of *sef* sequences from the *S.* Typhi genome and their comparison to the *sef* sequences of *S.* Enteritidis.

6.3.1 *S.* Typhi *sef* gene sequences

The *S.* Typhi genome posted on the Sanger centre website (www.sanger.ac.uk) was sequenced from strain CT18 and is 4,809,036 bp in length. This strain was isolated from a typhoid patient in Cho Quan Hospital, Ho Chi Minh City, Vietnam, and is a highly pathogenic, multiple drug resistant strain. The genome sequence was arranged such that its start site corresponded to the start of the *E. coli* K-12 genome.

Retrieval of *sef* gene sequences from the *S.* Typhi CT18 genome was accomplished by submitting the *sefABCDR* genes (accession numbers X98516, L11009, L11010, U07129 and AF233854 respectively) of *S.* Enteritidis to the *S.* Typhi BLAST server (http://www.sanger.ac.uk/Projects/S_typhi/blast_server.shtml). This revealed the positions of the *sef* genes within the *S.* Typhi genome, which were found to be position 4696023 (beginning of *sefA* promoter region) to position 4702566 (beginning of *sefR* promoter region)

or 6544 bp. This differed slightly to the 6462 bp that comprised the *S.* Enteritidis *sef* operon, i.e. 82 bp difference. The significance of this difference is examined in more detail below.

6.3.2 Clustal Alignment of *S.* Enteritidis and *S.* Typhi *sef* genes

Once the *S.* Typhi *sef* gene equivalents were obtained from the *S.* Typhi genome database, they were compared to their *S.* Enteritidis counterparts by Clustal analysis (Thompson *et al.*, 1997) of the coding regions. The alignments are shown in Figures 6.5 to 6.9, with the precent similarity for each gene listed in the respective figure legends. This analysis highlights the high level of sequence similarity across the entire *sef* region.

6.3.2.1 Analysis of point mutations in *S.* Typhi *sef* genes

Table 6.1 summarises all the nucleotide changes between the two sets of *sef* genes, as well as showing the resulting effects of those changes on the amino acid sequence. Interestingly, only $sefB_{St}$ and $sefC_{St}$ were found to have full length coding potential, which correlates with the inability of *S.* Typhi to express SEF14 fimbriae, as assessed by latex bead agglutination (Turcotte and Woodward, 1993).

The most significant amino acid change in the $sefA_{St}$ sequence was the loss of an adenine residue at nt position 248, resulting in a frame-shift mutation and premature stop codon at nt position 271. This results in a shortened peptide of only 90 aa compared with 167 aa for *S.* Enteritidis SefA.

The $sefB_{St}$ gene was found to have only three point mutations. It is not known if either the Phe_7 to Cys_7 or Arg_{10} to Ile_{10} changes (hydrophobic to hydrophilic, basic to hydrophobic, respectively) would result in a non-functioning chaperone. However, given that these changes are from one type of amino acid to another, it is possible they may lead to conformational changes in the tertiary structure, resulting in a lack of function.

Figure 6.5: Alignment of *S.* Enteritidis and *S.* Typhi *sefA* coding region

A Clustal alignment of *S.* Enteritidis (Se) and *S.* Typhi (St) *sefA* genes was performed as per section 2.11.2. These sequences were 98.9% identical (493/498 matches). The most significant change was at position 248, resulting in a frame shift mutation and premature stop codon at nucleotide position 271. The truncated product was reduced from 167 to 90 aa. The remaining changes were point mutations, which are summarised in Table 6.1. Nucleotides 1-497 correspond to nucleotide positions 4496957-4497453 in the S. Typhi genome. Note: red text highlights nucleotide differences.

```
Se ATGCGTAAATCAGCATCTGCAGTAGCAGTTCTTGCTTTAATTGCATGTGGCAGTGCCCAC 60
St ATGCGTAAATCAGCATCTGCAGTAGCAGTTCTTGCTTTAATTGCATGTGGCAGTGCCCAC 60
   ************************************************************

Se GCAGCTGGCTTTGTTGGTAACAAAGCAGAGGTTCAGGCAGCGGTTACTATTGCAGCTCAG 120
St GCAGCTGGCTTTGTTGGTAACAAAGCAGAGGTTCAGGCAGCGGTTACTATTGCAGCTCAG 120
   ************************************************************

Se AATACAACATCAGCCAACTGGAGTCAGGATCCTGGCTTTACAGGGCCTGCTGTTGCTGCT 180
St AATACAACATCAGCCAACTGGAGTCAGGATCCTGGCTTTACAGGGCCTGATGTTGCTGCT 180
   ************************************************* **********

Se GGTCAGAAAGTTGGTACTCTCAGCATTACTGCTACTGGTCCACATAACTCAGTATCTATT 240
St GGTCAGAAAGTTGGTACTCTCAGCATTACTGCTACTGGTCCACATAACTCAGTATCTATT 240
   ************************************************************

Se GCAGGTAAAGGGGCTTCGGTATCTGGTGGTGTAGCCACTGTCCCGTTCGTTGATGGACAA 300
St GCAGGTAA-GGGGCTTCGGTATCTGGTGGTGTAGCCACTGTCCCGTTCGTTGATGGACAA 299
   ******** ***************************************************

Se GGACAGCCTGTTTTCCGTGGGCGTATTCAGGGAGCCAATATTAATGACCAAGCAAATACT 360
St GGACAGCCTGTTTTCCGTGGGTATATTCAGGGAGCCAATATTAATGACCAAGCAAATACT 359
   **********************  ************************************

Se GGAATTGACGGGCTTGCAGGTTGGCGAGTTGCCAGCTCTCAAGAAACGCTAAATGTCCCT 420
St GGAATTGACGGGCTTGCAGGTTGGCGAGTTGCCAGCTCTCAAGAAACGCTAAATGTCCCT 419
   ************************************************************

Se GTCACAACCTTTGGTAAATCGACCCTGCCAGCAGGGACTTTCACTGCGACCTTCTACGTT 480
St GTCACAACCTTTGGTAAATCGACCCTGCCAGCAGGTACTTTCACTGCGACCTTCTACGTT 479
   ********************************** ************************

Se CAGCAGTATCAAAACTAA 498
St CAGCAGTATCAAAACTAA 497
   ******************
```

Figure 6.6: Alignment of *S.* Enteritidis and *S.* Typhi *sefB* coding region

A Clustal alignment of *S.* Enteritidis (Se) and *S.* Typhi (St) *sefB* genes was performed as per section 2.11.2. These sequences were 99.5% identical (737/740 matches). Only three point mutations were noted between these sequences, which are listed in Table 6.1. Nucleotides 1-741 correspond to nucleotide positions 4497575-4498314 in the S. Typhi genome. Note: red text highlights nucleotide differences.

```
Se ATGTATATTTTGAATAAATTTATACGTAGAACTGTTATCTTTTTCTTTTTTTGCTACCTT 60
St ATGTATATTTTGAATAAATGTATACGTATAACTGTTATCTTTTTCTTTTTTTGCTACCTT 60
   ******************* ******** ********************************

Se CCAATTGCTTCTTCGGAAAGTAAAAAAATTGAGCAACCATTATTAACACAAAAATATTAT 120
St CCAATTGCTTCTTCGGAAAGTAAAAAAATTGAGCAACCATTATTAACACAAAAATATTAT 120
   ************************************************************

Se GGCCTAAGATTGGGCACTACACGTGTTATTTATAAAGAAGATGCTCCATCAACAAGTTTT 180
St GGCCTAAGATTGGGCACTACACGTGTTATTTATAAAGAAGATGCTCCATCAACAAGTTTT 180
   ************************************************************

Se TGGATTATGAATGAAAAAGAATATCCAATCCTTGTTCAAACTCAAGTATATAATGATGAT 240
St TGGATTATGAATGAAAAAGAATATCCAATCCTTGTTCAAACTCAAGTATATAATGATGAT 240
   ************************************************************

Se AAATCATCAAAAGCTCCATTTATTGTAACACCACCTATTTTGAAAGTTGAAAGTAATGCG 300
St AAATCATCAAAAGCTCCATTTATTGTAACACCACCTATTTTGAAAGTTGAAAGTAATGCG 300
   ************************************************************

Se CGAACAAGATTGAAGGTAATACCAACAAGTAATCTATTCAATAAAAATGAGGAGTCTTTG 360
St CGAACAAGATTGAAGGTAATACCAACAAGTAATCTATTCAATAAAAATGAGGAGTCTTTG 360
   ************************************************************

Se TATTGGTTGTGTGTAAAAGGAGTCCCACCACTAAATGATAATGAAAGCAATAATAAAAAC 420
St TATTGGTTGTGTGTAAAAGGAGTCCCACCACTAAATGATAATGAAAGCAATAATAAAAAC 420
   ************************************************************

Se AACATAACTACGAATCTTAATGTGAATGTGGTTACGAATAGTTGTATTAAATTAATTTAT 480
St AACATAACTACGAATCTTAATGTGAATGTGGTTACGAATAGTTGTATTAAATTAATTTAT 480
   ************************************************************

Se AGGCCTAAAACTATAGACTTAACGACAATGGAGATTGCAGATAAATTAAAGTTAGAGAGA 540
St AGGCCTAAAACTATAGACTTAACGACAATGGAGATTGCAGATAAATTAAAGTTAGAGAGA 540
   ************************************************************

Se AAAGGAAATAGTATAGTTATAAAGAATCCAACATCATCATATGTGAATATTGCAAATATT 600
St AAAGGAAATAGTATAGTTATAAAGAATCCAACATCATCATATGTGAATATTGCAAATATT 600
   ************************************************************

Se AAATCTGGTAATTTAAGTTTTAATATTCCAAATGGATATATTGAGCCATTTGGATATGCT 660
St AAATCTGGTAATTTAAGTTTTAATATTCCAAATGGATATATTGAGCCATTTGGATATGCT 660
   ************************************************************

Se CAATTACCTGGTGGAGTACATAGTAAAATAACTTTGACTATTTTGGATGATAACGGCGCT 720
St CAATTACCTGGTGGAGTACATAGTAAAATAACTTTGACTATTTTGGATGATAACGGCGCT 720
   ************************************************************

Se GAAATTATAAGAGATTATTAG 741
St GAAATTATAAGAGATTATTAA 741
   ********************
```

Figure 6.7: Alignment of *S.* Enteritidis and *S.* Typhi *sefC* coding region

A Clustal alignment of *S.* Enteritidis (Se) and *S.* Typhi (St) *sefC* genes was performed as per section 2.11.2. These sequences were 99.7% identical (2437/2445 matches). *sefC,* along with *sefB* was the only gene that had full length coding potential. An interesting feature of the *S.* Typhi sequence was a 72 bp insert made up of multiple repeats of TTGGTG. The function of this repeat region is not yet known. All other mutational alterations are listed in Table 6.1. Nucleotides 1-2517 correspond to nucleotide positions 4498332-4500848 in the S. Typhi genome. Note: red text highlights nucleotide differences.

```
Se ATGAAGAAAACCACAATTACTCTATTTGTTTTAACCAGTGTATTTCACTCTGGAAATGTT 60
St ATGAAGAAAACCACAATTACTCTATTTGTTTTAACCAGTGTATTTCACTCTGGAAATGTT 60
   ************************************************************

Se TTCTCCAGACAATATAATTTCGACTATGGAAGTTTGAGTCTTCCTCCCGGTGAGAATGCA 120
St TTCTCCAGACAATATAATTTCGACTATGGAAGTTTGAGTCTTCCTCCCGGTGAGAATGCA 120
   ************************************************************

Se TCTTTTCTAAGTGTTGAAACGCTTCCTGGTAATTATGTTGTTGATGTATATTTGAATAAT 180
St TCTTTTCTAAGTGTTGAAACGCTTCCTGGTAATTATGTTGTTGATGTATATTTGAATAAT 180
   ************************************************************

Se CAGTTAAAAGAAACTACTGAGTTGTATTTCAAATCAATGACTCAGACTCTAGAACCATGC 240
St CAGTTAAAAGAAACTACTGAGTTGTATTTCAAATCAATGACTCAGACTCTAGAACCATGC 240
   ************************************************************

Se TTAACAAAAGAAAAACTTATAAAGTATGGGATCGCCATCCAGGAGCTTCATGGGTTGCAG 300
St TTAACAAAAGAAAAACTTATAAAGTATGGGATCGCCATCCAGGAGCTTCATGGGTTGCAG 300
   ************************************************************

Se TTTGATAATGAACAATGCGTTCTCTTAGAGCATTCTCCTCTTAAATATACTTATAACGCG 360
St TTTGATAATGAACAATGCGTTCTCTTAGAGCATTCTCCTCTTAAATATACTTATAACGCG 360
   ************************************************************

Se GCTAACCAAAGTTTGCTTTTAAATGCACCATCTAAAATTCTATCTCCAATAGACAGTGAA 420
St GCTAACCAAAGTTTGCTTTTAAATGCACCATCTAAAATTCTATCTCCAATAGACAGTGAA 420
   ************************************************************

Se ATTGCTGATGAAAATATCTGGGATGATGGCATTAACGCTTTTCTTTTAAATTACAGAGCT 480
St ATTGCTGATGAAAATATCTGGGATGATGGCATTAACGCTTTTCTTTTAAATTACAGAGCT 480
   ************************************************************

Se AATTATTTGCATTCTAAGGTTGGAGGAGAAGATTCATACTTTGGTCAAATTCAACCTGGT 540
St AATTATTTGCATTCTAAGGTTGGAGGAGAAGATTCATACTTTGGTCAAATTCAACTTGGT 540
   ************************************************************ ****

Se TTTAATTTTGGTCCCTGGCGGCTAAGGAATCTATCATCTTGGCAAAACTTGTCAAGCGAA 600
St TTTAATTTTGGTCCCTGGCGGCTAAGGAATCTATCATCTTGGCAAAACTTGTCAAGCGAA 600
   ************************************************************

Se AAAAAATTTGAATCAGCATATATTTATGCTGAGCGAGGTTTAAAAAAAATAAAGAGCAAA 660
St AAAAAATTTGAATCAGCATATATTTATGCTGAGCGAGGTTTAAAAAAAATAAAGAGCAAA 660
   ************************************************************

Se CTAACAGTTGGGGACAAATATACCAGTGCAGATTTATTCGATAGCGTACCATTTAGAGGC 720
St CTAACAGTTGGGGACAAATATACCAGTGCAGATTTATTCGATAGCGTACCATTTAGAGGC 720
   ************************************************************

Se TTTTCTTTAAATAAAGATGAAAGTATGATACCTTTCTCACAGAGAACATATTATCCAACA 780
St TTTTCTTTAAATAAAGATGAAAGTATGATACCTTTCTCACAGAGAACATATTATCCAACA 780
   ************************************************************

Se ATACGTGGTATTGCGAAAACCAATGCGACTGTAGAAGTAAGACAAAATGGATACTTGATA 840
St ATACGTGGTATTGCGAAAACCAATGCGACTGTAGAAGTAAGACAAAATGGATACTTGATA 840
   ************************************************************
```

```
Se TATTCTACTTCAGTCCCCCCCGGGCAATTCGAGATAGGTAGAGAACAAATTGCTGATCTT 900
St TATTCTACTTCAGTCCCCCCCGGGCAATTCGAGATAGGTAGAGAACAAATTGCTGATCTT 900
   ************************************************************

Se GGTGTTGGGGTTGG------------------------------------------------ 914
St GGTGTTGGTGTTGGTGTTGGTGTTGGTGTTGGTGTTGGTGTTGGTGTTGGTGTT 960
   ******** *****

Se ---------------------------GGTTCTTGATGTTAGCATTTATGAAAAAAATGGG 948
St GGTGTTGGTGTTGGTGTTGGTGTTGGGGTTCTTGATGTTAGCATTTATGAAAAAAATGGG 1020
                               **********************************

Se CAGGTCCAAAACTATACAGTGCCATATTCAACTCCTGTATTATCTTTGCCTGATGGATAT 1008
St CAGGTCCAAAACTATACAGTGCCATATTCAACTCCTGTATTATCTTTGCCTGATGGATAT 1080
   ************************************************************

Se TCTAAATATAGTGTAACTATTGGTAGATACAGGGAGGTTAACAATGATTATATCGATCCT 1068
St TCTAAATATAGTGTAACTATTGGTAGATACAGGGAGGTTAACAATGATTATATCGATCCT 1140
   ************************************************************

Se GTTTTTTTTGAAGGGACTTATATATATGGTCTGCCTTATGGGTTTACTTTATTTGGTGGA 1128
St GTTTTTTTTGAAGGGACTTATATATATGGTCTGCCTTATGGGTTTACTTTATTTGGTGGA 1200
   ************************************************************

Se GTGCAATGGGTAAATATTTATAATTCATATGCCATAGGCGCAAGTAAAGATATTGGTGAG 1188
St GTGCAATGGGCAAATATTTATAATTCATACGCCATAGGCGCAAGTAAAGATATTGGTGAG 1260
   ********** ****************** ******************************

Se TATGGTGCTCTGTCTTTTGACTGGAAAACATCTGTTTCGAAGACTGATACATCCAATGAA 1248
St TATGGTGCTCTGTCTTTTGACTGGAAAACATCTGTTTCGAAGACTGATACATCCAATGAA 1320
   ************************************************************

Se AATGGTCATGCATATGGGATTAGATACAATAAAAATATCGCTCAGACAAACACCGAAGTA 1308
St AATGGTCATGCATATGGGATTAGATACAATAAAAATATCGCTCAGACAAACACCGAAGTA 1380
   ************************************************************

Se TCTTTGGCTAGTCATTACTATTATTCGAAAAATTATAGAACTTTTTCTGAAGCAATTCAT 1368
St TCTTTGGCTAGTCATTACTATTATTCGAAAAATTATAGAACTTTTTCTGAAGCAATTCAT 1440
   ************************************************************

Se AGTAGCGAGCATGATGAATTTTACGATAAAAATAAGAAATCAACAACCTCTATGTTATTA 1428
St AGTAGCGAGCATGATGAATTTTACGATAAAAATAAGAAATCAACAACCTCTATGTTATTA 1500
   ************************************************************

Se AGTCAGGCATTAGGATCTCTGGGTTCTGTTAACTTAAGCTACAATTATGATAAATATTGG 1488
St AGTCAGGCATTAGGATCTCTGGGTTCTGTTAACTTAAGCTACAATTATGATAAATATTGG 1560
   ************************************************************

Se AAACATGAAGGTAAAAAATCAATAATTGCTAGTTATGGGAAGAATTTAAATGGTGTTTCG 1548
St AAACATGAAGGTAAAAAATCAATAATTGCTAGTTATGGGAAGAATTTAAATGGTGTTTCG 1620
   ************************************************************

Se TTATCGCTTTCATATACGAAAAGTACATCAAAGATTAGTGAAGAAAATGAAGATTTATTC 1608
St TTATCGCTTTCATATACGAAAAGTACATCAAAGATTAGTGAAGAAAATGAAGATTTATTC 1680
   ************************************************************
```

```
Se AGTTTTCTACTCAGTGTACCTTTGCAAAAACTTACAAATCATGAAATGTATGCTACATAT 1668
St AGTTTTCTACTCAGTGTACCTTTGCAAAAACTTACAAATCATGAAATGTATGCTACATAT 1740
   ************************************************************

Se CAAAACTCATCCTCTTCAAAGCATGATATGAATCATGATTTAGGTATTACTGGTGTGGCA 1728
St CAAAACTCATCCTCTTCAAAGCATGATATGAATCATGATTTAGGTATTACTGGTGTGGCA 1800
   ************************************************************

Se TTTAATAGCCAATTGACATGGCAAGCAAGAGGGCAAATAGAAGATAAATCGAAAAATCAA 1788
St TTTGATAGCCAATTGACATGGCAAGCAAGAGGGCAAATAGAAGATAAATCGAAAAATCAA 1860
   *** ********************************************************

Se AAGGCTACATTTTTAAATGCTTCTTGGCGAGGTACTTATGGGGAGATCGGAGCAAACTAT 1848
St AAGGCTACATTTTTAAATGCTTCTTGGCGAGGTACTTATGGGGAGATCGGAGCAAACTAT 1920
   ************************************************************

Se AGTCATAATGAAATAAATCGTGATATTGGGATGAATGTTTCTGGTGGGGTGATTGCTCAT 1908
St AGTCATAATGAAATAAATCGTGATATTGGGATGAATGTTTCTGGTGGGGTGATTGCTCAT 1980
   ************************************************************

Se TCATCAGGAATTACGTTTGGTCAGAGTATATCGGATACTGCTGCACTGGTAGAGGCTAAA 1968
St TCATCAGGAATTACGTTTGGTCAGAGTATATCGGATACTGCTGCACTGGTCGAGGCTAAA 2040
   ************************************************ *********

Se GGTGTAAGTGGGGCAAAAGTTCTGGGCCTACCAGGTGTTAGAACCGATTTTAGAGGCTAT 2028
St GGTGTAAGTGGGGCAAAAGTTCTGGGCCTACCAGGTGTTAGAACCGATTTTAGAGGCTAT 2100
   ************************************************************

Se ACAATATCCAGTTATCTTACTCCATATATGAATAACTTCATATCTATAGATCCAACAACG 2088
St ACAATATCCAGTTATCTTACTCCATATATGAATAACTTCATATCTATAGATCCAACAACG 2160
   ************************************************************

Se TTACCAATAAATACGGATATTAGGCAAACTGATATTCAAGTAGTTCCTACCGAAGGTGCT 2148
St TTACCAATAAATACGGATATTAGGCAAACTGATATTCAAGTAGTTCCTACCGAAGGTGCT 2220
   ************************************************************

Se ATTGTAAAAGCTGTATATAAAACAAGCGTGGGTACTAATGCATTAATTAGAATTACAAGA 2208
St ATTGTAAAAGCTGTATATAAAACAAGCGTGGGTACTAATGCATTAATTAGAATTACAAGA 2280
   ************************************************************

Se ACAAATGGAAAGCCACTAGCTCTTGGCACAGTTCTTTCACTTAAGAATAATGATGGAGTA 2268
St ACAAATGGAAAGCCACTAGCTCTTAGCACAGTTCTTTCACTTAAGAATAATGATGGAGTA 2340
   ************************ ***********************************

Se ATCCAATCAACATCTATTGTTGGCGAAGATGGTCAGGCATATGTATCTGGATTGTCAGGA 2328
St ATCCAATCAACATCTATTGTTGGCGAAGATGGTCAGGCATATGTATCTGGATTGTCAGGA 2400
   ************************************************************

Se GTGCAAAAATTAATCGCTTCGTGGGGGAATAAGCCCTCCGATACTTGTACAGTTTTTTAC 2388
St GTGCAAAAATTAATCGCTTCGTGGGGGAATAAGCCCTCCGATACTTGTACAGTTTTTTAC 2460
   ************************************************************

Se TCTCTTCCCGATAAAAACAAAGGTCAGATTAGCTTTTTAAATGGAGTGTGCAAATGA 2445
St TCTCTTCCCGATAAAAACAAAGGTCAGATTAGCTTTTTAAATGGAGTGTGCAAATGA 2517
   ********************************************************
```

Figure 6.8: Alignment of *S.* Enteritidis and *S.* Typhi *sefD* coding region

A Clustal alignment of *S.* Enteritidis (Se) and *S.* Typhi (St) *sefD* genes was performed as per section 2.11.2. These sequences were 98% identical (438/447 matches). The most significant variation between these sequences is the absence of an ATG start codon in the *S.* Typhi sequence. There are also a number of other mutational alterations that are listed in Table 6.1. Nucleotides 1-446 correspond to nucleotide positions 4500845-4501290 in the S. Typhi genome. Note: red text highlights nucleotide differences

```
Se ATGAATCAGTATAATTCGTCAATACCTAAGTTCATTGTCTCTGTTTTTCTGATTGTTACT 60
St ---AATCAGTATAATTCGTCAATACCTAAGTTCATTGTCTCTGTTTTTCTGATTGTTACT 60
     ************************************************************

Se GGTTTTTTCAGCTCAACTATTAAAGCACAAGAACTTAAATTAATGATTAAAATAAATGAG 120
St GGTTTTTTCAGCTCAACTATTAAAGCACAAGAACTTAAATTAATGATTAAAATAAATGAG 120
   ************************************************************

Se GCTGTTTTTTATGACCGTATTACAAGTAATAAAATAATAGGTACGGGGCATCTATTTAAC 180
St GCTGTTTTTTATGACCGTATTACAAGTAATAAAATAATAGGTACGGGGCATCTATTTAAC 180
   ************************************************************

Se AGAGAGGGAAAAAAAATCCTCATTAGTTCAAGTTTAGAAAAAATTAAAAATACCCCAGGG 240
St AGAGAGGGAAAAAAA-TCCTCATTAGTTCAAGTTTAGAAAAAATTAAAAATACCCCAGGG 239
   *************** ********************************************

Se GCATATATTATTAGAGGTCAGAATAACTCAGCCCATAAGCTTAGGATAAGAATAGGTGGA 300
St GCATATATTATTAGAGGTCAGAATAACTCAGCCCATAAGCTTAGGATAAGAATAGGTGGA 299
   ************************************************************

Se GAAGACTGGCAACCAGATAATTCAGGTATTGGTATGGTATCTCATTCTGATTTTACTAAT 360
St GAAGACTGGCAACCAGATAATTCAGGTATTGGTATGGTATCTCATTCTGATTTTACTAAT 359
   ************************************************************

Se GAATTTAATATTTATTTTTTTGGGAATGGAGACATTCCTGTTGACACATATTTAATAAGC 420
St GAATTTAATATTTATTATTTTGGGAATGGAAACATTCCCGTTGATACCTATTTAATAAGC 419
   **************** ************* ******* ***** ** ************

Se ATATATGCGACAGAAATTGAATTATAA 447
St ATATATGCGACAGAAATTGAATTATAA 446
   ***************************
```

Figure 6.9: Alignment of *S.* Enteritidis and *S.* Typhi *sefR* coding region

A Clustal alignment of *S.* Enteritidis (Se) and *S.* Typhi (St) *sefR* genes was performed as per section 2.11.2. These sequences were 97.2% identical (793/816 matches). The *S.* Typhi *sefR* sequence is truncated relative to the *S.* Enteritidis sequence due to a frame shift mutation at position 143. Also of interest was the observation that *sefR* had the greatest number of point mutations out of all the *sef* genes. These are listed in Table 6.1. Nucleotides 1-815 correspond to nucleotide positions 4502138-4501324 in the S. Typhi genome. Note: red text highlights nucleotide differences

```
Se ATGTTGAAAAAAAACGCCATAAAAATAAAACTATATCGTTATGCTATTTTACATTCGAAA 60
St ATGCTGAAAAAAAACGCCATAAAAATAAAACTATATCGTTATGCTATTTTACATTCGAAA 60
   *** ************************************************************

Se AACTGTATTGTTACCATTGAGAACAAGTCAAAGCCAGAGGAAATAAAAATAACTAGAGGC 120
St AACTGTATTATTACCATTAAGAACAAGTCAAAGCCAGAGGAAATAAAAATAACTAGAGGC 120
   ********* ******** ****************************************

Se AACATAGCCTTAATAGAAAAAAATATAGAAGCCGTTGTGGAAATTGAATATATGGATGAC 180
St AACATAGCCTTAATAGAAAAAA-TATAGAAGCTGTTGTGGAAATTGAATATATGGATGAT 179
   ********************** ********* ***************************

Se ATTGAATCATTTGACATTATTACTTTGCCAGATGAATTATTAAGTAGAGTTTTATGCTTA 240
St ATTGAATCATTTGATATTATTACTTTACCAGATGAATTATTAAGTAGAGTTTTATGCTTA 239
   ************** *********** *********************************

Se TTTGAGGCTTCTAATTGCTCAGAAAGTTTATCACCAATACGCTACAGAACATTTAGCGAT 300
St TTTGAGGCTTCTAATTGCTCAGAAAGTTTATCACCAATACGCTACAGAACATTTAGCGAT 299
   ************************************************************

Se AAGGTTTTTATTATAACCGACAATGGAATTAATGGAATTTTATTTGGATACTTAAAAAAG 360
St AAGGTTTTTATTATAACCGACAGTGGAATTAATGGAATTTTATTTGAATATTTAAAAAAG 359
   ********************** ********************** *** *********

Se AGAAAAAATAACAATAATGATATTTATGAAATTGCCTGCTTATTTTCAAAAGTGAATAAT 420
St AGAAAAAATAACAACAACGATATTTATGAAATTGCCTGTGTATTTTCAAAAGTGAATAAT 419
   ************** ** ********************  *********************

Se ATCGAGCAGCTATACACATCTCTGTGCATTTCAGTCTCACGTAGTTTTTCTGATATTGTT 480
St ATCGAGCAGCTATACACATCTCTGTGCATTTCAGTCTCACGTAGTTTTTCTGATATTGTT 479
   ************************************************************

Se AGAAAAACAATAGATAATGACATTTCAACGAAATGGAGATTAAAAACATTATCCGAAAAA 540
St AGAAAAACAATAGATAATGACATTTCAACAAAATGGAGATTAAAAACATTATCCGAAAAA 539
   ****************************** ******************************

Se CTAAATTTATCGGAAGTGACTATCAGAAAAAAACTTGAGAATGAAAATACTAATTTTTAC 600
St CTAAATTTATCAGAAGTGACTATCAGAAAAAAACTTGAGAATGAAAATACTAATTTTTAC 599
   *********** ************************************************

Se AGAATCTTTCTGGATGCCAGGATGCAAAAAGCAGCGCGTTTGGTGCTTGATAGCGACACC 660
St AGAATCCTTCTAGATGCCAGGATGCAAAAAGCAGCGCGTTTGGTACTTGATAGCGACACA 659
   ****** **** ********************************* **************

Se CATATTAATAAAGTATCATATGCCGTAGGAATGTCAAGCGTATCATATTTCATTAAATTA 720
St CATATTAATAAAGTATCATATGCCGTAGGAATGTCAAGCGTATCATATTTCATTAAGTTA 719
   ************************************************************ ***

Se TTTTCTGACTATTATGGCTTAACCCCAAAACAATTCCATCTAAAATATAAGCATAGAAAT 780
St TTTTCTGACTATTATGGTTTAACCCCAAAACAATTCCATCTAAAATATAAGCATAGAAAT 779
   ***************** ******************************************

Se ACAGGAGAAAAAGCTGCATTTATGCTTTATAATTAA 816
St ACAGGAGAAAAAGCTGTATTTATGCTTTATAATTAA 815
   ***************** ********************
```

Table 6.1: List of point mutations in *S.* Typhi *sef* genes

This table outlines all the mutations within the *sef* genes of *S.* Typhi, along with the effect on amino acid composition for each mutation. The most significant mutations were those point mutations that resulted in premature termination of the *sefA*, *sefD* and *sefR* genes.

Gene	Nucleotide position	Codon change	Effect of Mutation
sefA$_{St}$	170	GCT→GAT	Ala→Asp
	248	AAA→AA-	Frame shift (Premature stop codon at position 271)
	322/323	CGT→TAT	Arg→Tyr
	456	GGG→GGT	Gly→Gly
sefB$_{St}$	20	TTT→TGT	Phe→Cys
	29	AGA→ATA	Arg→Ile
	741	TAG→TAA	No effect (within stop codon)
sefC$_{St}$	536	CCT→CTT	Pro→Leu
	909	GGG→GGT	Gly→Gly
	914		12 copies of TTGGTG (remains in frame)
	1139/1211	GTA→GCA	Val→Ala
	1158/1230	TAT→TAC	Tyr→Tyr
	1732/1804	AAT→GAT	Asn→Asp
	1959/2031	GTA→GTC	Val→Val
	2233/2305	GGC→AGC	Gly→Ser
sefD$_{St}$	196	AAA→AA-	Frame shift (premature stop codon at position 214)
	378	TTT→TAT	Phe→Tyr
	392	GAC→AAC	Asp→Asn
	400	CCT→CCC	Pro→Pro
	406	GAC→GAT	Asp→Asp
	409	ACA→ACC	Thr→Thr
sefR$_{St}$	4	TTG→CTG	Leu→Leu
	70	GTT→ATT	Val→Ile
	79	GAG→AAG	Glu→Lys
	143	AAT→A-T	Frame shift (Premature stop codon at position 148)
	153	GCC→GCT	Ala→Ala
	180	GAC→GAT	Asp→Asp
	195	GAC→GAT	Asp→Asp
	207	TTG→TTA	Leu→Leu
	323	AAT→AGT	Asn→Ser
	347	GCA→GAA	Gly→Glu
	351	TAC→TAT	Tyr→Tyr
	375	AAT→AAC	Asn→Asn
	378	AAT→AAC	Asn→Asn
	399	TGC→TGT	Lys→Lys
	400	TTA→GTA	Leu→Val
	510	ACG→ACA	Thr→Thr
	552	TCG→TCA	Ser→Ser
	607	TTT→CTT	Phe→Leu
	612	CTG→CTA	Leu→Leu
	645	GTG→GTA	Val→Val
	660	ACC→ACA	Thr→Thr
	717	AAA→AAG	Lys→Lys
	738	GGG→GGT	Gly→Gly
	797	GCA→GTA	Ala→Val

sefC$_{St}$ is the only *sef* gene to incorporate an insertion of DNA relative to the *S.* Enteritidis sequence. At nt position 914 is a 72 bp insertion composed of 12 copies of the repeat TGTTGG. Despite this insertion, an open reading frame is maintained, allowing a full length SefC$_{St}$ to be produced. The *S.* Enteritidis *sefC*$_{Se}$ and *sefD*$_{Se}$ genes overlap at the stop and start codons i.e. TGATG. In the *S.* Typhi sequence, the ATG has been lost such that *sefD*$_{St}$ has no start codon. This does not lead to a read through from *sefC*$_{St}$, as the next nucleotide after those that are missing is an adenine, therefore maintaining the TGA stop codon for *sefC*$_{St}$, while preventing *sefD*$_{St}$ expression.

As well as the lack of a start codon, the *S.* Typhi *sefD*$_{St}$ gene was also found to have a frame-shift mutation. The *S.* Enteritidis *sefD*$_{Se}$ gene has a string of 8 adenine residues at nt positions 189-196 inclusive, while the *S.* Typhi equivalent is lacking one of these nucleotides. This would lead to a premature termination at amino acid position 71 (nt position 214), compared to the 148 aa for full length SefD$_{Se}$.

The *sefR*$_{St}$ gene from *S.* Typhi was found to have by far the largest number of mutational alterations relative to the *S.* Enteritidis sequence. A total of 23 point mutations were found, along with a further mutation that results in the truncation of the *sefR*$_{St}$ gene product to just 48 amino acids (nucleotide position 145), as compared to the 271 aa for full length *S.* Enteritidis SefR. A string of adenine residues at nt positions 137-143 is present in *sefR*$_{Se}$, but the *S.* Typhi sequence lacks one of these residues, leading to the premature termination.

Overall, there are 43 point mutations within the coding regions of the *sef* genes, not including the multiple repeat insertion within *sefC,* that represent a 0.0066% change in the *sef* sequences. Of these, 3 have led to premature terminations in the coding regions of the *S.* Typhi *sefA*, *sefD* and *sefR* genes, resulting in the complete disruption of SEF14 fimbriae expression. While the 72 bp insert accounts for the majority of the 82 bp differences in

sequence length between *S.* Enteritidis and *S.* Typhi *sef* sequences, the remaining 10 bp differences is due to differences in the DNA upstream of *sefA*, within the IS1230 element.

6.4 Analysis of DNA flanking the SEF14 operon

The SEF14 operon, along with the attending insertion element IS1230, fulfils some of the criteria outlined by Hacker *et al* (1997) for the definition of a PAI i.e. that genes encode for (putative) virulence functions; the %G+C is much lower relative to the rest of the genome (~35% vs ~52%); limited distribution within related species (present only in group D1 Salmonellae; high level of sequence conservation between *S.* Enteritidis and *S.* Typhi); at least one mobility element (IS1230) closely associated with the genes in question. For these reasons it was hypothesised that either 1) the SEF14 operon is a single mobile unit that along with IS1230 constitutes a PAI; or 2) the SEF14 operon is only part of a larger PAI. It was thought that the second hypothesis was more likely due to the small size of the putative *sef* PAI; the majority of characterised PAI, with the notable exception of SPI-5 are larger than 10 kb (Wood *et al.*, 1998). Also, no mobility elements eg insertion sequences, phage DNA etc, were found flanking the *sefR* gene suggesting that DNA in this region may be part of a larger PAI. In order to determine which of these hypotheses was correct, an analysis of DNA flanking the SEF14 operon was undertaken.

As the *S.* Typhi chromosome sequence had recently been completed, and the sequence similarity between the *sef* genes of *S.* Typhi and *S.* Enteritidis was so high, the assumption was initially made that the DNA flanking the SEF14 operon in *S.* Typhi would be essentially the same as the corresponding region in *S.* Enteritidis, at least to the point where the entire putative PAI had originally inserted. It would be more likely that an ancestor to the group D *Salmonella*, to which *S.* Typhi and *S.* Enteritidis belong, acquired the SEF14 operon (or a larger PAI) and then went on to diverge into the distinct serovars, rather than the various serovars of groups D1 obtaining the SEF14 operon (or a larger PAI) independently.

This line of reasoning also assumed that this region flanking the SEF14 operon had not changed substantially since its introduction into the *S.* Typhi / *S.* Enteritidis ancestor. To address this assumption, *S.* Typhi DNA flanking the SEF14 operon was compared with the partially completed *S.* Enteritidis genome sequence (see section 6.5.1). *S.* Enteritidis contigs that showed similarity to the *S.* Typhi DNA were re-analysed by BLASTN to confirm their identity and positions relative to *S.* Typhi DNA.

It is possible that there are significant DNA changes within this region, especially if it is susceptible to recombination, and therefore it is difficult to be 100% certain that the DNA flanking the *sef* operon in these two serovars is the same, however without fully sequencing this region, which is currently being undertaken by Maloy *et al* (www.salmonella.org), this method provides the best means currently available to analyse this region, and can be easily updated as more *S.* Enteritidis sequence becomes available.

6.4.1 BLASTN analysis of DNA flanking the *S.* Typhi SEF14 operon

Using the location of the ends of the *S.* Typhi *sef* operon (section 6.3.1), 30 kb of DNA sequences flanking each end (i.e. positions 4666023 to 4696022 and 4702567 to 4732566 in the *S.* Typhi genome) were subjected to BLASTN analysis (www.ncbi.nlm.nih.gov/BLAST) to ascertain the identify of putative genes within these regions. The significance of observed DNA similarity was considered on an individual basis, rather than applying an arbitrary cut-off which might exclude short but important sequences.

6.4.1.1 DNA 5' of *sefA*

BLASTN analysis of the DNA upstream of *sefA* revealed sequence similarities which could be broadly classified into one of two groups: *E. coli* chromosomal DNA in the region of 1.4 kb to 17.6 kb (Region 1) and bacteriophage-related DNA sequences in the region ~17.6 kb to 29 kb (Region 2). The similarity to *E. coli* K-12 DNA in Region 1 correlated to sequences

from 92.8 to 00.1 minutes (GenBank accession #U14003), and included 89% identity with the *valS* gene (GenBank accession #X05891). Sequences in region 2 showed up to 90% identity to sequences from bacteriophage ϕP4. Between these two large sections was a segment of DNA that showed 97% identity to the 5' end of the leucine tRNA (*leuX*; GenBank accession #S68105) gene of *E. coli* K-12, which had previously been associated with the PAI II of Uropathogenic *E. coli* 536 (O6:K15:H31; Blum *et al.*, 1994). Finally, the segment from 29 kb to 30 kb shows 98% identity to the 3' end of IS1230 (GenBank accession #U91789). The various regions of DNA sequence similarity are shown in schematic form in Figure 6.10, and summarised in Table 6.2.

6.4.1.2 DNA 5' of *sefR*

BLASTN analysis was also performed on the 30 kb of DNA 5' of the *sefR* gene, with the observations are summarised in Figure 6.11 and Table 6.3. Within the first 4 kb of this fragment, similarity to several *Salmonella* virulence associated genes was observed. These included up to 96% identity with parts of the 14.7 kb *pef* region of the *S.* Enteritidis virulence plasmid (GenBank accession #AB041905), 97% identity to the 5' end of the complement resistance gene *rck* (GenBank accession #M76130), 95% identity with the *dlt* gene of *S.* Typhi (GenBank accession #X94325) and 97% identity with the gene of *dlp* S. Enteritidis (GenBank accession #X94326), respectively. The remaining DNA sequence showed similarity to *E. coli* K-12 chromosomal DNA of the region from 92.8 to 00.1 minutes (GenBank accession #U14003). DNA sequences in the 5' end of this region showed limited identity to the *E. coli* K-12 *mcrD* gene (GenBank accession #X55662).

6.4.1.3 Defining the boundary of the putative SEF14 PAI in *S.* Typhi

Analysis of DNA sequences flanking the SEF14 operon revealed similarity to *E. coli* K-12 chromosomal DNA [in the region of 98.1 to 00.1 minutes]. This similarity ended at

Figure 6.10 BLASTN analysis of 30 kb *S.* Typhi DNA 5' of *sefA*.

This figure shows a representation of the results obtained by BLASTN analysis of the 30 kb of DNA sequence 5' of the *sefA* gene. Region 1 and Region 2 indicate areas of sequence similarity to *E. coli* K-12 chromosomal DNA and bacteriophage-related DNA sequences respectively, and are separated by the *leuX/attP* region (striped area). The extreme 3' end showed homology to the 3' end of IS1230, as expected. Scale bar represents 3 Kb.

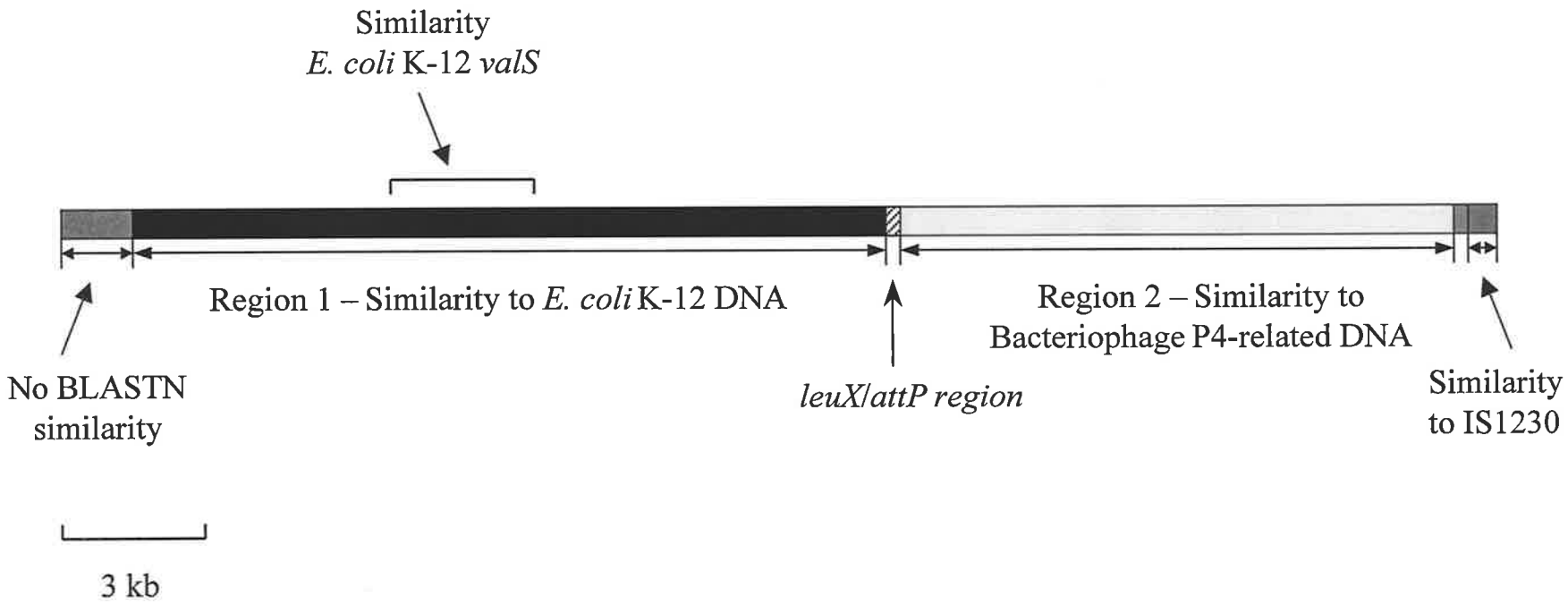

Similarity
E. coli K-12 valS
Region 1 – Similarity to E. coli K-12 DNA
Region 2 – Similarity to
Bacteriophage P4-related DNA
No BLASTN
similarity
leuX/attP region
Similarity
to IS1230
3 kb

Table 6.2: Summary of DNA identities 5' of *sefA* in *S.* Typhi.

The DNA identities shown in Figure 6.10, determined by BLASTN analysis, are listed here. The DNA position (nt) is presented relative to the *sefA* gene, with position 1 corresponding to 30,000 nt upstream of the SefA gene. Identities refer to the number of identical nt relative to the total number of nt compared, with the percentage shown in brackets. The GenBank accession # from each sequence that had similarity to the DNA 5' of *sefA* in *S.* Typhi is also shown.

Description	DNA position	DNA sequence identities	GenBank accession #
E. coli K-12 *valS* gene for valyl-tRNA synthetase	7076 to 10074	2696/2999 (89%)	X05891
E. coli K-12 chromosomal region from 92.8 to 00.1 minutes	1405 to 17666	Direct matches = 9754/11364 (86%) Total matches = 9754/16261 (60%)	U14003
Bacteriophage P4 *attP*/integrase region	17647 to 19029	90% (1256/1386)	X05947
Bacteriophage P4 alpha gene and cis replication region crr.	25866 to 28688	877/1010 (86%) 496/603 (82%) 394/467 (84%) 130/152 (85%) 97/114 (85%) 2346/3063 (77%)	X05623
S. Enteritidis IS1230 (3' end)	29401 to 30000	588/600 (98%)	U91789

Figure 6.11 BLASTN analysis of 30 kb *S.* Typhi DNA 5' of *sefR*.

This figure shows a representation of the results obtained by BLASTN analysis of the 30 kb of DNA sequence 5' of the *sefR* gene. Region 1 had sequences with similarity to *Salmonella* virulence determinants, which extended for ~4 kb upstream of the *sefR* gene. Region 2 had sequences with similarity to *E. coli* K-12 chromosomal DNA, and to the *E. coli* K-12 *mcrD* gene. Scale bar represents 3 Kb.

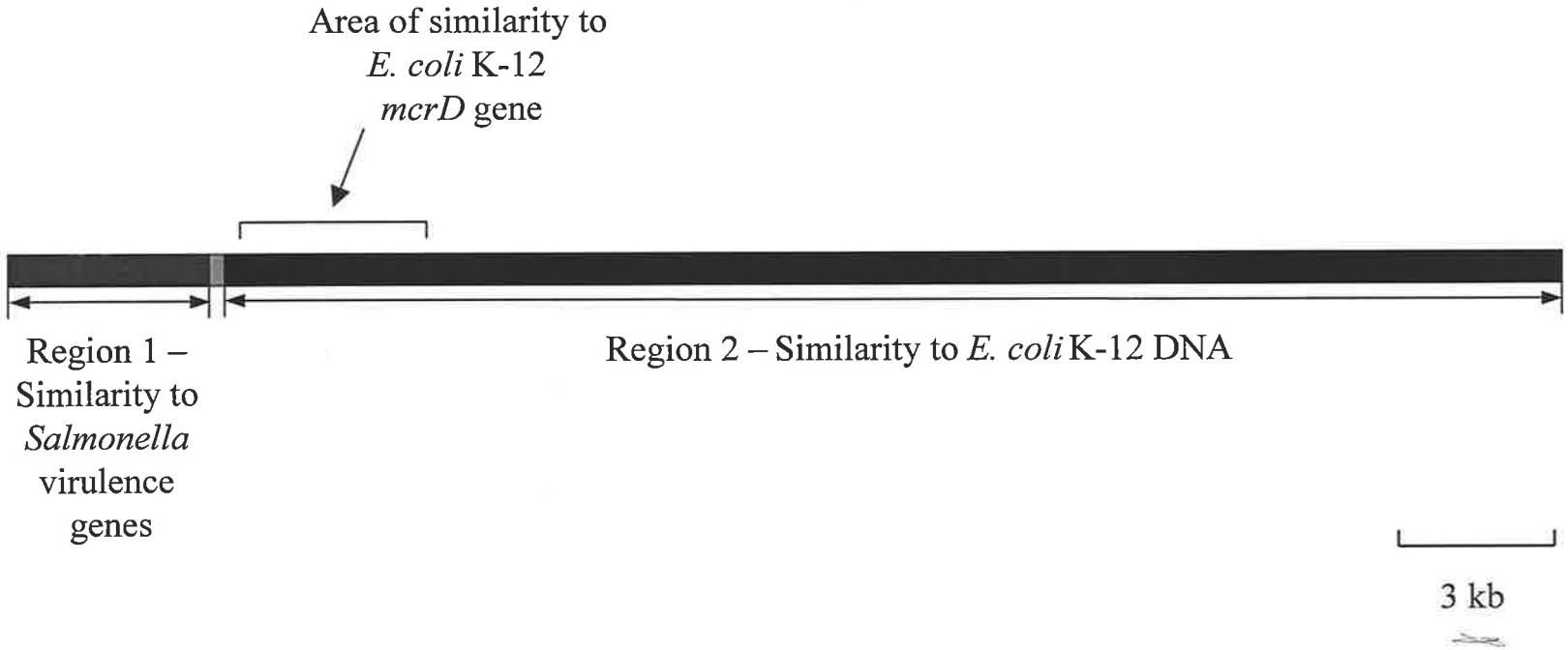

Area of similarity to
E. coli K-12
mcrD gene
Region 1 –
Similarity to
Salmonella
virulence
genes
Region 2 – Similarity to E. coli K-12 DNA
3 kb

Table 6.3: Summary of DNA identities 5' of *sefR* in *S.* Typhi.

The DNA similarities shown in Figure 6.11, determined by BLASTN analysis, are listed here. The DNA position (nt) presented relative to the end of the *sefR* gene. Identities refer to the number of identical nt relative to the total number of nt compared, with the percentage shown in brackets. The GenBank accession # from each sequence that had similarity to the DNA 5' of *sefR* in *S.* Typhi is also shown.

Description	DNA position	DNA sequence identities	GenBank Accession #
S. Enteritidis virulence plasmid, 14.7 kb *pef* region	145-986 1024-2637 2718-2794	91% (771/846) 96% (1564/1614) 96% (74/77)	AB041905
S. Typhi *dlt* gene	798-1724	98% (917/926)	X94325
S. Enteritidis *dlp* gene	798-986 1024-1724	90% (171/189) 95% (666/701)	X94326
S. Typhimurium *rck* gene		97% (914/935)	M76130
E. coli K-12 *mcrD* gene	3982-4084 4124-4232 4283-4328 6812-6977 7241-7363 7478-7632 7656-7706	82% (85/103) 82% (90/109) 93% (43/46) 79% (132/166) 82% (101/123) 80% (125/155) 94% (48/51)	X55662

position 4683682 (13274 bp upstream of the *sefA* coding region) of the *S.* Typhi CT18 chromosomal sequence (http://www.sanger.ac.uk/Projects/S_typhi/blast_server.shtml) and did not resume until position 4706416 (4277 bp upstream of the *sefR* coding region); this corresponded to a total of 22733 bp that does not show any similarity to the *E. coli* K-12 chromosome. A 30 kb fragment of *S.* Typhi DNA spanning this region is shown in Figure 6.12, displaying the SEF14 operon in context with flanking *E. coli* K-12 sequences. Similarity within the 22733 bp fragment can be broken down into three major areas: area 1 has DNA sequence with similarity to bacteriophage-related DNA (section 6.4.1.1), area 2 is the SEF14 loci itself, flanked by IS1230 insertion element, and area 3 contains DNA sequence with similarity to various *Salmonella* virulence determinants, including *rck* and *pef* genes (section 6.4.1.2). The DNA sequences 5' of area 1 and 3' of area 3 represent similarity to *E. coli* K-12 chromosomal DNA. The SEF14 locus constitutes almost 30% of the DNA sequence that is flanked by *E. coli* K-12 chromosomal DNA sequences, and supports the hypothesis that the SEF14 operon is part of a larger PAI.

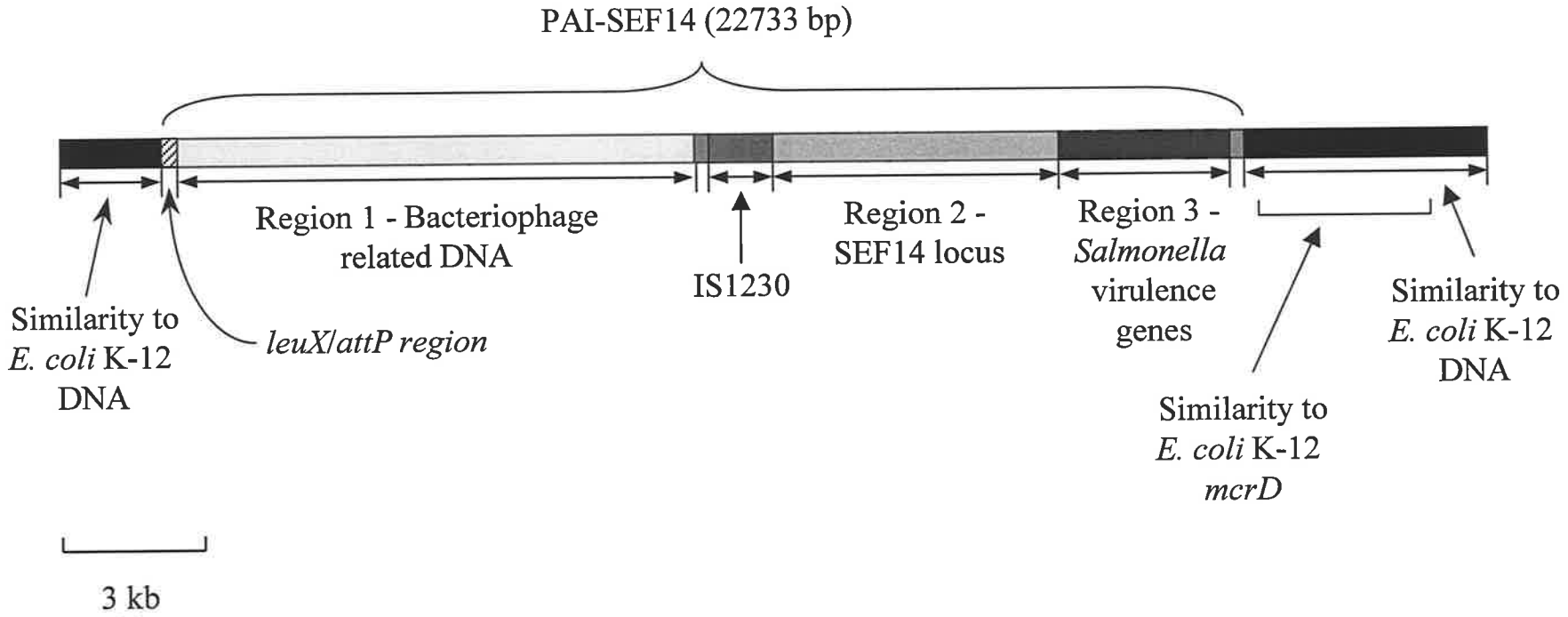
6.4.2 Comparison of *S.* Typhi DNA with corresponding *E. coli* DNA

6.4.2.1 Comparison of *leuX* and *mcrD* positions in *E. coli* K-12 and *S.* Typhi

Given that the putative SEF14 PAI was located between two known *E. coli* genes, an attempt was made to analysed the corresponding region of DNA between these genes on the *E. coli* genome. The positions of *leuX* and *mcrD* genes in *E. coli* K-12 are at nt 4493973 to nt 4494057 and nt 4573163 to nt 4574425, respectively. The exact position where the *E. coli* K-12 and *S.* Typhi sequences diverged was determined by alignment of the respective DNA sequences. Divergence within the *leuX* gene took place at nt 4683682/4494038 (*S.* Typhi/*E. coli* K-12), and at nt 4706548/4574275 (*S.* Typhi/*E. coli* K-12) within *mcrD*. These values correspond to DNA fragment lengths of 22866 bp and 80237 bp for *S.* Typhi and *E. coli* K-12, respectively.

Figure 6.12: *S.* Typhi SEF14 operon in context with flanking DNA

This figure shows a representation of the SEF14 operon of *S.* Typhi in context with flanking DNA. Regions of similarity as determined by BLASTN analysis are defined by the horizontal arrows. Scale bar represents 3 Kb.


PAI-SEF14 (22733 bp)
Region 1 - Bacteriophage related DNA
IS1230
Region 2 - SEF14 locus
Region 3 - *Salmonella* virulence genes
Similarity to *E. coli* K-12 DNA
leuX/attP region
Similarity to *E. coli* K-12 DNA
Similarity to *E. coli* K-12 *mcrD*
3 kb

6.4.2.2 Identity of *E. coli* DNA between *leuX* and *mcrD*

The identity of DNA between *leuX* and *mcrD* genes in the 80 kb *E. coli* region was determined from the microbial genomes *E. coli* K-12 website (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=Genome&gi=115), and confirmed by BLASTN analysis. DNA sequence within this region contained genes encoding for: Type 1 fimbriae (*fim*; GenBank accession #X51655, Z37500, X05672), ferric citrate transport (*fec* GenBank accession # M26397, M20981, M63115) and hexuronate degradation (*uxu* GenBank accession # D13329). Sequence similarity to a number of insertion elements, including IS1F (GenBank accession #X52538), IS2 (GenBank accession #V00610), IS4 (GenBank accession #J01733) and IS30 (GenBank accession #X00792) was also found. Interestingly, DNA sequences extracted from the *E. coli* K-12 genome varied slightly with the DNA sequences obtained by BLASTN analysis of this region (Table 6.4). As expected from hybridisation analysis (section 6.2), no similarity to sequences from the putative SEF14 PAI was observed within this region.

6.4.2.3 Comparison of %G+C content of *S.* Typhi and *E. coli* fragments

The %G+C content of DNA has been used as an indicator of whether a specific region of DNA might have a separate origin from the rest of the chromosome. As would be expected, some regions are naturally rich or deficient in G+C residues, but segments of external origin may not have had sufficient time, in evolutionary terms, to become normalised to the host genome G+C ratio (Lawrence and Ochman, 1997). For this reason, a PAI often possess a significantly different %G+C content as compared to the average host %G+C, and this difference is used as one of the criteria for defining a new PAI (see section 1.5.2).

The total %G+C values for the DNA between *leuX* and *mcrD* was slightly less for *S.* Typhi as compared to *E. coli* (45.7% vs 49.3%). When compared to the %G+C for their respective genomes (52.1% vs 50.8%), it can be seen that the *E. coli* fragment is slightly

Table 6.4: Summary of DNA identities within *leuX/mcrD* region of *E. coli* K-12

A summary of genes found by BLASTN analysis within the 80 kb *leuX/mcrD* region of *E. coli* K-12 is shown. The DNA position (nt) presented relative to the end of the overlap within the leuX gene, and ends at the beginning of the overlap in the *mcrD* gene. The numbers in brackets correspond to the regions outlined in Figure 6.13. Identities refer to the number of identical nt relative to the total number of nt compared, with the percentage shown in brackets. The GenBank accession # of the listed genes is also presented.

Description	DNA sequence identities	GenBank Accession #
Multiple IS elements (4)		
- IS1F	100% (881/881)	X52538
- IS2	99% (1325/1327)	V00610
- IS4	99% (1419/1426)	J01733
- IS30	100% (1221/1221)	X00792
fec genes (5)	99% (4824/4842)	M26397
	99% (2632/2644)	M20981
	100% (4074/4074)	M63115
fim genes (6)	99% (3451/3480)	X51655
	95% (2510/2625)	Z37500
	99% (2035/2050)	X05672
uxu genes (7)	99% (4532/4539)	D13329

lower, while the ~23 kb *S.* Typhi fragment was 6.3% less %G+C rich than the chromosome as a whole.

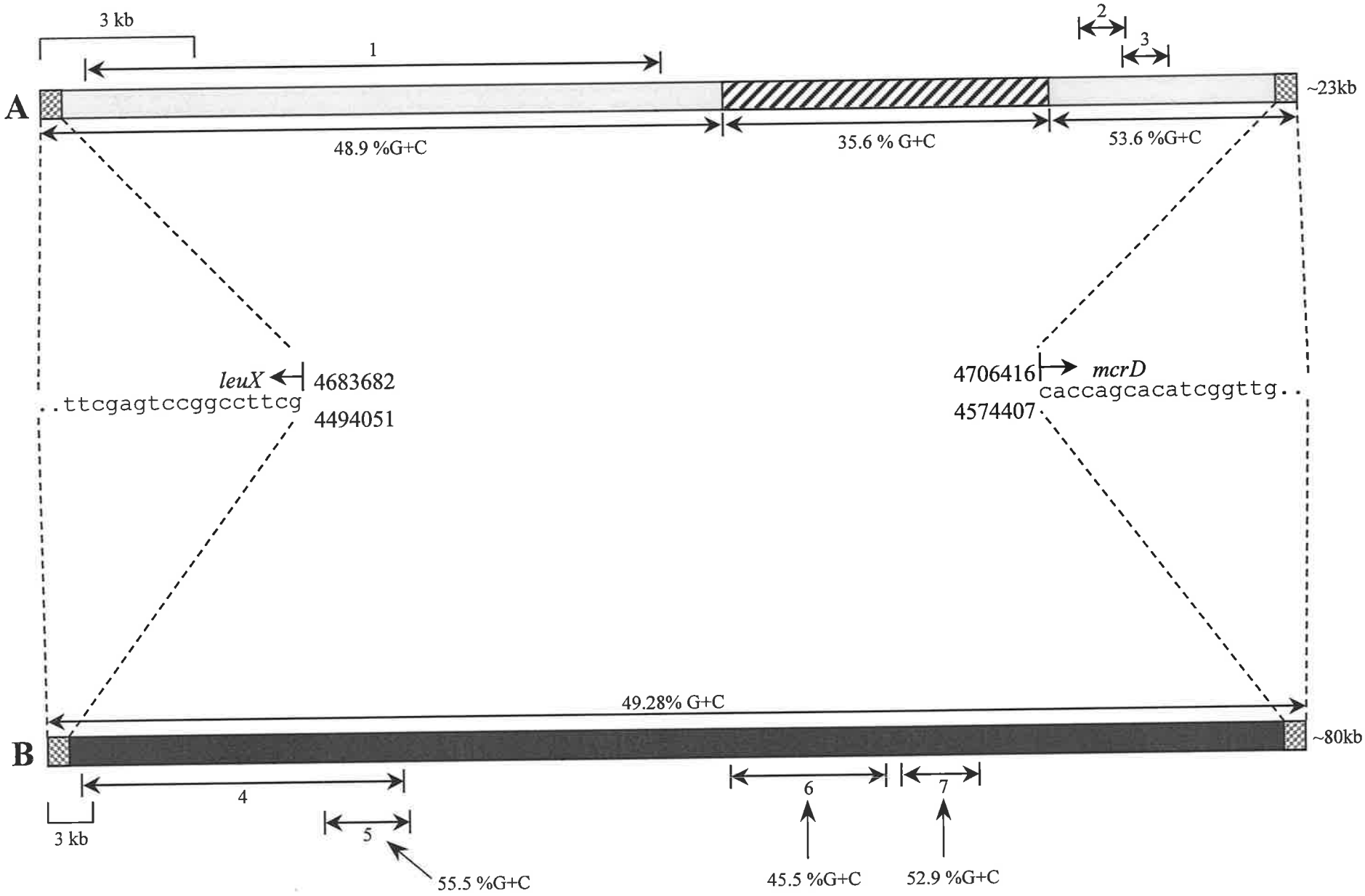
The differences become more pronounced when comparing different regions within each fragment. The DNA between *leuX* and the start of the *sefA* gene has a %G+C of 48.9%, the SEF14 operon has a %G+C of 35.6%, and the DNA between the start of the *sefR* gene and the *mcrD* gene is 53.6%. This observation implies that the DNA in this region has a mosaic-like structure, with different fragments of DNA obtained and integrated at different times. Similarly for the *E. coli* fragment, different regions show different %G+C values. Interestingly, the different operons within this region have quite different %G+C values, i.e. the *fim* genes (45.5%) have a %G+C lower than the genomic average (50%) while the transport (*fec* genes; 55.5%) and metabolic (*uxu* genes; 52.9%) genes are higher than the genomic average. The presence of IS elements within the 5' end also lends weight to the hypothesis that this region of the genome has been accumulated in large sections, rather than a gradual accumulation of new operons as suggested by Lawrence and Roth (1996). A comparison of the putative SEF14 PAI with the corresponding region in *E. coli* is shown in Figure 6.13, highlighting the various regions of varying %G+C content.

6.5 Attempts to confirm similarity of *S.* Enteritidis and *S.* Typhi DNA

Having confirmed the presence of a putative PAI in *S.* Typhi and compared it to the *E. coli* K-12 sequence, attempts were made to confirm the identity of the corresponding *S.* Enteritidis sequence. Initially, BLASTN analysis was performed on *S.* Enteritidis contigs obtained from comparing *S.* Typhi DNA against the incomplete *S.* Enteritidis genome sequence (3.6 of 4.5 Mb sequenced; www.ncbi.nlm.nih.gov/Microb_blast/credits/592.html, last updated 23/12/2000). This would give some indication as to the nature of *S.* Enteritidis within this region. Also, PCR and Southern analysis were performed on the four junction regions in the *S.* Typhi sequence i.e. *leuX*, *mcrD*, IS1230 and *sefR*. These experiments would

Figure 6.13: Comparison of *S.* Typhi and *E. coli* DNA between *leuX* and *mcrD* genes.

Comparison of DNA from *S.* Typhi (A) with DNA from *E. coli* K-12 (B). The ends of each DNA fragment (stippled boxes) represent regions of identity between *S.* Typhi and *E. coli* sequences, corresponding to *leuX* and *mcrD* genes of *E. coli*, while the intervening region between the stippled boxes represents unique DNA from each species. The expanded DNA sequences within the broken lines show part of the sequence identity between the *E. coli* K-12 *leuX/mcrD* genes and *S.* Typhi DNA. The numbers 4683682 and 4706416 and the numbers 4494051 and 4574407 represent corresponding nucleotide positions from the *S.* Typhi and *E. coli* K-12 genomes respectively for each DNA fragment. The *S.* Typhi fragment contains the SEF14 operon (diagonal shading). The DNA flanking the SEF14 operon shows similarity to virulence associated genes and sequences of bacteriophage P4. The *E. coli* K-12 region is ~3.4x larger than the corresponding *S.* Typhi region. The numbered regions represent similarity to the following genes, as summarised in Table 6.4. 1) Bacteriophage related DNA sequences (Phage P4 and Retronphage R73); 2) *S.* Typhi *dlt* gene (Rodriguez-Pena *et al.*, 1997), *S.* Enteritidis *dlp* gene (Edwards *et al.*, unpublished); 3) *S.* Typhimurium *rck* gene (Heffernan *et al.*, 1997); 4) multiple insertion elements, including IS1, IS2, IS4, and IS30; 5) ferric citrate (*fec*) transport genes; 6) type 1 Fimbriae (*fim*) genes; 7) hexuronate degradation (*uxu*) genes. Scale bars representing 3kb are shown next to each fragment, and the %G+C content of various regions are indicated.


3 kb
1
2
3
A
~23kb
48.9 %G+C
35.6 % G+C
53.6 %G+C
leuX
4683682
..ttcgagtccggccttcg
4494051
4706416
mcrD
caccagcacatcggttg..
4574407
49.28% G+C
B
~80kb
4
5
6
7
3 kb
55.5 %G+C
45.5 %G+C
52.9 %G+C

reveal whether *S.* Enteritidis possessed these junctions and also whether there was any RFLP between the two serovars. It is to be expected that in all cases, the incomplete nature of the *S.* Enteritidis genome precludes any definitive conclusions being drawn, however this type of analysis can give clues as to the nature of any rearrangements and/or insertions that may be present.

6.5.1 Comparison of *S.* Typhi DNA against the *S.* Enteritidis genome sequence

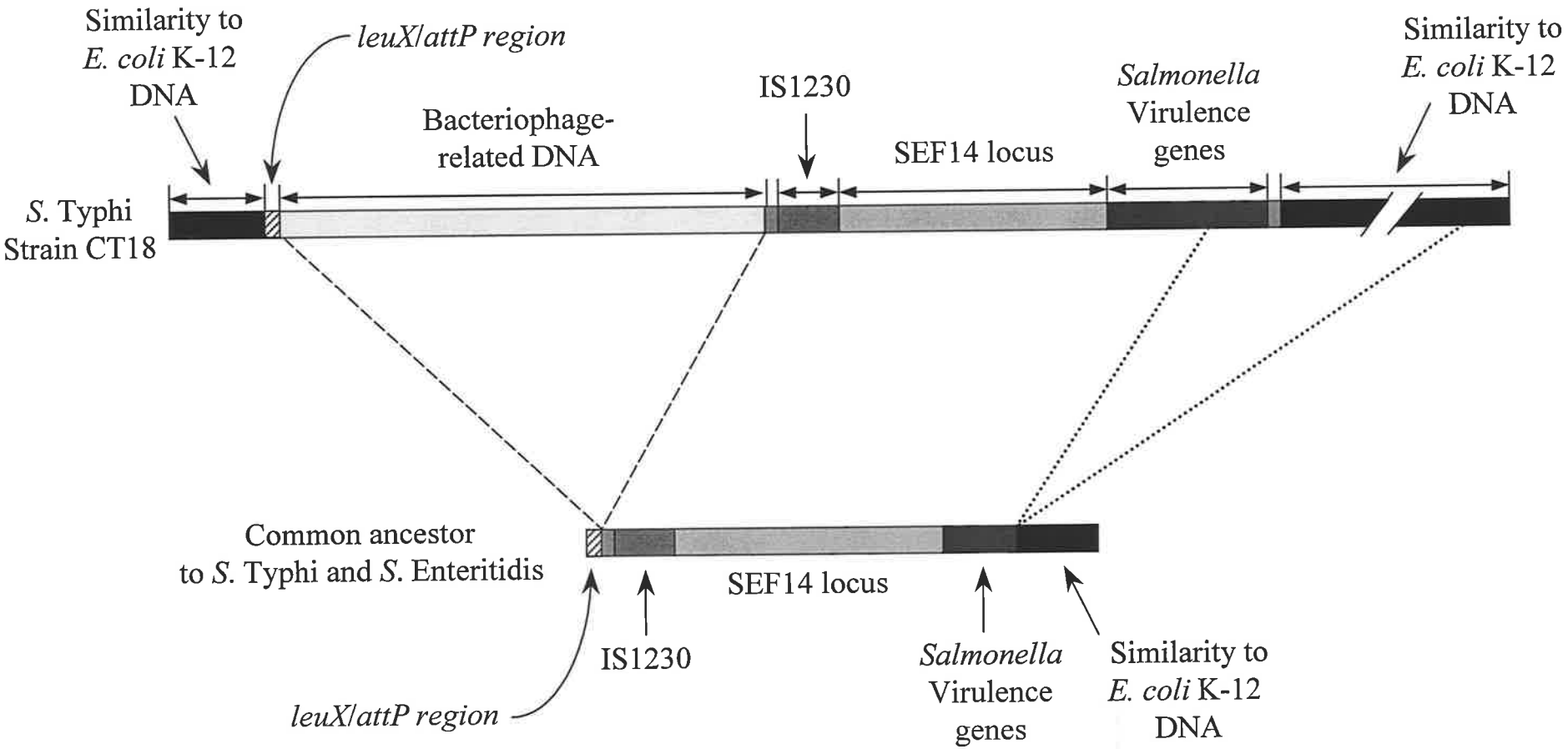
6.5.1.1 BLASTN analysis of DNA 5' of *sefA* and of *sefR*

BLASTN comparison of 30 kb of *S.* Typhi DNA 5'of *sefA* against the incomplete *S.* Enteritidis genome sequence revealed that this region is similar to the *S.* Typhi DNA sequence with one important difference. Contig 2321 showed similarity to the *leuX* junction region, along with similarity to IS1230, but did not have similarity to any of the DNA in between. This 'missing' region in *S.* Enteritidis corresponds to the bacteriophage P4 insertion in *S.* Typhi. Therefore it is possible that this phage inserted or replaced DNA after the acquisition of the SEF14 operon, marking a divergence between *S.* Typhi and *S.* Enteritidis.

The same analysis was then carried out for the 30 kb DNA sequence 5' of *sefR*. While several *S.* Enteritidis contigs showed >90% similarity to this sequence, all matched the corresponding region within the *S.* Typhi fragment, except for one. Contig 2257 showed sequence similarity to *S.* Typhi DNA in two widely spaced regions i.e. 1644 to 2809 nucleotides and 14008 to 15233 nucleotides upstream of *sefR,* suggesting that an insertion may have occurred in this region in *S.* Typhi. However this cannot be confirmed without further sequencing of this region in the *S.* Enteritidis genome. Figure 6.14 shows a diagrammatic representation of the possible events that occurred during the divergence of *S.* Typhi and *S.* Enteritidis from a common ancestor.

Figure 6.14: Possible DNA insertions in the *S.* Typhi genome

This diagram shows two possible insertion events that may have occurred after *S.* Enteritidis and *S.* Typhi had diverged from their common ancestor. DNA 5' of the SEF14 locus contains an insertion of ~11 kb of bacteriophage related DNA (indicated by dashed lines). The putative insertion 3' of the SEF14 locus is approximately 12 kb in size, and corresponds to parts of the regions with similarity to *Salmonella* virulence genes and *E. coli* K-12 DNA (indicated by the dotted lines).


Similarity to
E. coli K-12
DNA
leuX/attP region
Bacteriophage-
related DNA
IS1230
SEF14 locus
Salmonella
Virulence
genes
Similarity to
E. coli K-12
DNA
S. Typhi
Strain CT18
Common ancestor
to S. Typhi and S. Enteritidis
SEF14 locus
IS1230
leuX/attP region
Salmonella
Virulence
genes
Similarity to
E. coli K-12
DNA

6.5.2 PCR and Southern hybridisation analysis

To confirm and extend the data obtained by computer analysis, PCR and Southern hybridisation analysis on DNA from *S.* Enteritidis and *S.* Typhi was performed. Chromosomal DNA was used as template for PCR amplifications of four junction regions i.e. at the *leuX*, *mcrD*, IS1230 and *sefR* regions as marked in Figure 6.15. These PCR reactions were carried out using primers *mcrD*-F & *mcrD*-R; *leuX*-F & *leuX*-R; *sefR*-int & *sefR*-ext and IS1230-int & IS1230-ext respectively (see Table 2.3). Figure 6.16 shows the results of these reactions. As the primers were designed from *S.* Typhi DNA, all four primer pairs were able to amplify the desired products using *S.* Typhi DNA as template. However, only primer pair *sefR*-int and *sefR*-ext was able to amplify DNA from *S.* Enteritidis, giving a faint PCR product. This result suggested that *S.* Enteritidis DNA was sufficiently different in these regions to prevent, or in the case of the *sefR* region, greatly reduce, primer binding. This in turn prevented or greatly diminished the amplification of the expected PCR products.

Southern hybridisation analysis was carried out to determine if the PCR products amplified from S. Typhi DNA could bind specific S. Enteritidis sequences. DIG-labelled PCR probes were generated using *S.* Typhi DNA as template and the *mcrD*-F & *mcrD*-R; *leuX*-F & *leuX*-R; *sefR*-int & *sefR*-ext and IS1230-int & IS1230-ext primers (section 2.12.1). The restriction enzyme *Nco*I was selected for digestion of chromosomal DNA from *S.* Enteritidis and *S.* Typhi, as this would result in DNA fragments of <10 kb in the region where the DIG-Labelled probes would bind. The expected band sizes were approximately 3 kb for the *leuX* probe; 8 kb and 9 kb for the IS1230 probe (due to an *Nco*I site within the probe region), 8 kb for the *sefR* probe, and 4 kb for the *mcrD* probe. After electrophoresis on 1% (w/v) TAE agarose, the DNA was transferred and probed under high stringency conditions (section 2.12.2) and probe binding detected as per section 2.12.4. The results of this analysis are presented in Figure 6.17. It was observed that of the four probes used, all four hybridised to *S.* Typhi DNA as expected. The bands sizes were ~ 3 kb, 8 and 9 kb, 8kb, and 4 kb, for the

Figure 6.15: Expected PCR products in 30 kb region containing putative SEF14 PAI

The relative positions of the expected PCR products (thick black lines) are shown. Also displayed are the positions of six *Nco*I (N) sites within this region. The probe expected to bind within the IS1230 junction region spans one of these sites. The scale represents number of kb within the fragment, while the putative PCR products are indicated as follows:

A = *leuX* junction (primers *leuX*-F, *leuX*-R, expected size 873 bp)

B = IS1230 junction (primers IS1230-int, IS1230-ext, expected size 655 bp)

C = *sefR* junction (primers *sefR*-int, *sefR*-ext, expected size 1709 bp)

D = *mcrD* junction (primers *mcrD*-F, *mcrD*-R, expected size 1073 bp)

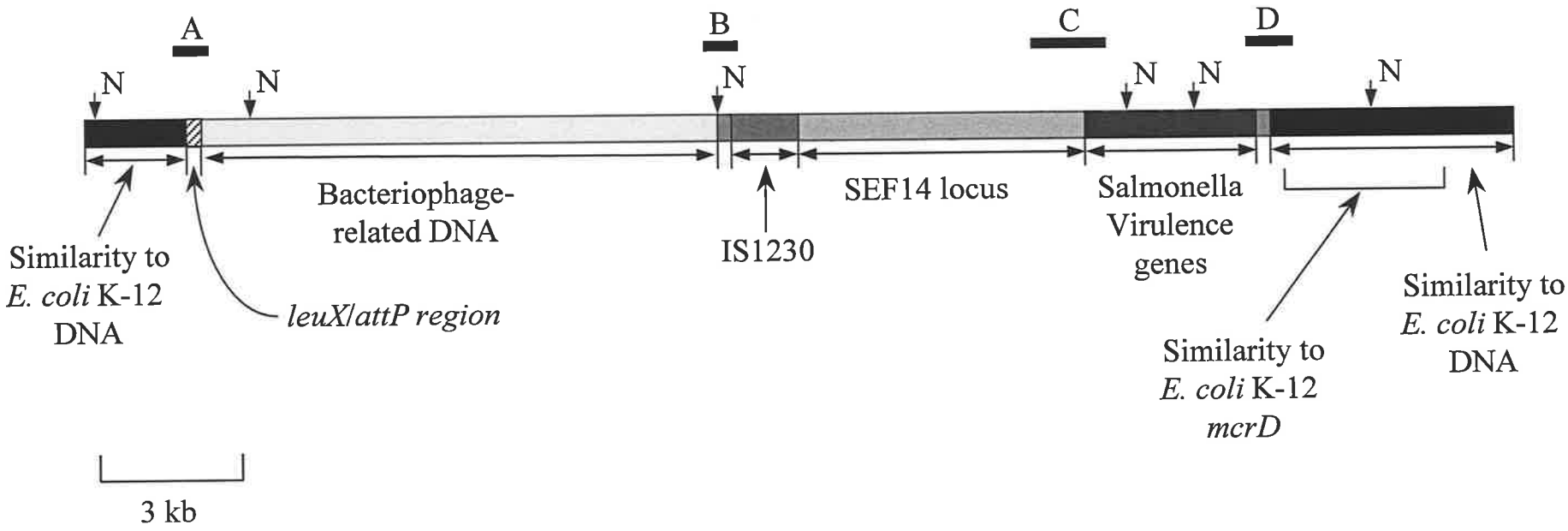

A
B
C
D
N
N
N
N
N
N
Similarity to
E. coli K-12
DNA
leuX/attP region
Bacteriophage-
related DNA
IS1230
SEF14 locus
Salmonella
Virulence
genes
Similarity to
E. coli K-12
mcrD
Similarity to
E. coli K-12
DNA
3 kb

Figure 6.16: PCR analysis of junction regions

PCR reactions were performed (section 2.10.1) using *S.* Typhi (St) and *S.* Enteritidis (Se) chromosomal DNA as template. The oligonucleotide primers used were *mcrD*-F & *mcrD*-R; *leuX*-F & *leuX*-R; *sefR*-int & *sefR*-ext and IS1230-int & IS1230-ext (see Table 2.3) respectively, and are indicated above the appropriate lanes. 10 µl of each reaction was mixed with 3 µl loading buffer and electrophoresed on 1% (w/v) TAE gel for 1 hour (section 2.9.3). The numbers on the left hand side represent the size (kb) of *Eco*RI digested SPPI markers.

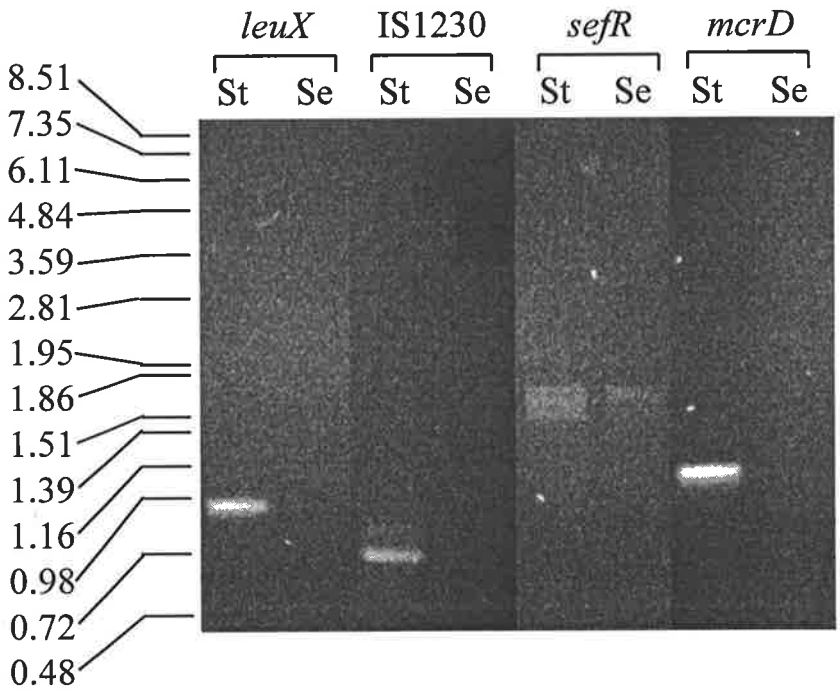
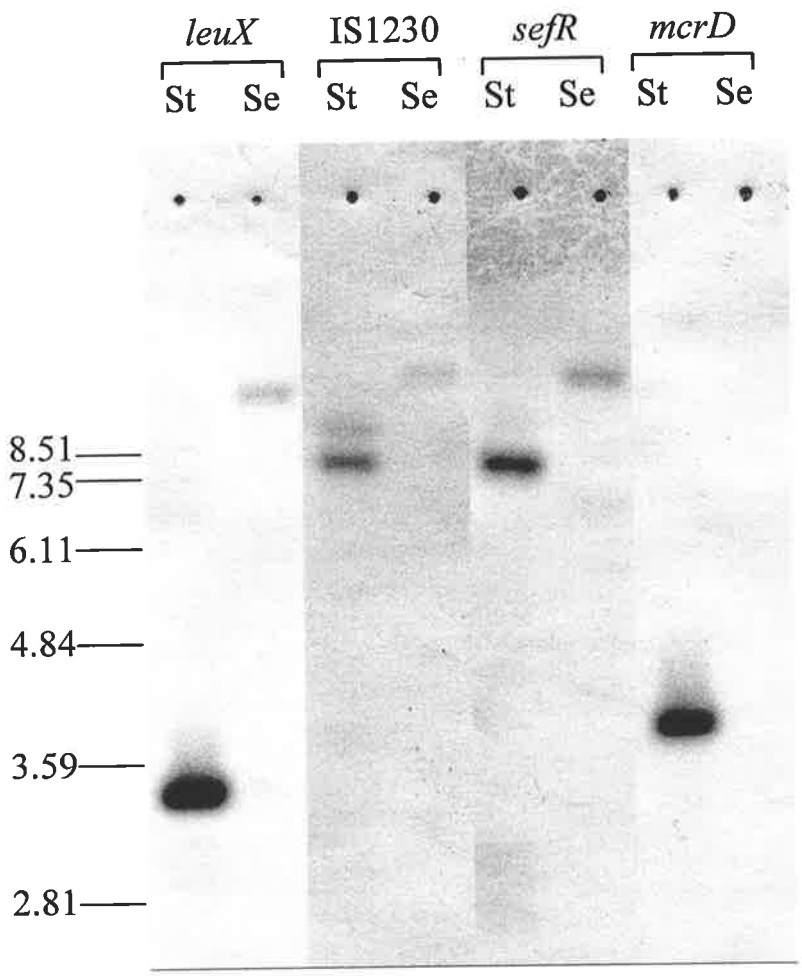

leuX
IS1230
sefR
mcrD
St
Se
St
Se
St
Se
St
Se
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
0.48

Figure 6.17: Southern hybridisation using "junction" probes

Samples of *S.* Typhi and *S.* Enteritidis DNA were digested with *Nco*I, separated by gel electrophoresis on 0.8% (w/v) TAE gel and transferred to nylon membranes as per section 2.12.2. After transfer the DNA was hybridised under high stringency conditions with DIG-labelled probes (section 2.12.1) spanning the *leuX* (873 nt), IS1230 (655 nt), *sefR* (1709 nt), and *mcrD* (1073 nt) "junction" regions (see Figure 6.16). The numbers on the left hand side represent the size (kb) of *Eco*RI digested SPPI markers.


leuX
IS1230
sefR
mcrD
St
Se
St
Se
St
Se
St
Se
8.51
7.35
6.11
4.84
3.59
2.81

leuX, IS1230, *sefR* and *mcrD* probes respectively, which matched the expected sizes. Only three of the probes i.e. *leuX*, IS1230 and *sefR* showed positive hybridisation to *S.* Enteritidis DNA. These bands were similar in size and all were >10 kb. The absence of positive hybridisation to *S.* Enteritidis DNA using the *mcrD* probe correlated with an observation made previously in section 6.5.1.1, where an *S.* Enteritidis contig (2257) showed similarity to DNA flanking the region where *mcrD* is located in *S.* Typhi, but lacked similarity in the region where *S.* Typhi DNA had similarity to the *mcrD* gene. This lack of hybridisation gives further evidence for either an insertion of DNA in this region in *S.* Typhi or a replacement/deletion of this region in *S.* Enteritidis.

6.6 Summary

The dot-blot and Southern hybridisation results presented in this chapter (sections 6.2.1 & 6.2.2) show that the distribution of the *sefD* gene is limited to group D1 *Salmonella* serovars, consistent with the distribution of the *sefABC* genes (Doran *et al.*, 1996). Conversely, dot-blot and Southern hybridisation analysis revealed a wider distribution for *sefR* (section 6.2.3). *sefR* was detected in both *S.* Enteritidis and *S.* Typhi (Figure 6.4), with the same sized *Bam*HI restriction fragment to that observed when examining *sefD* distribution (Figure 6.2). This was expected given the close association of these two genes, as shown in Chapter 3. In contrast to *sefD*, putative *sefR* homologues were also detected in *E. coli* O111:H$^-$ and *Shigella flexneri* strains (Figure 6.3). Unlike *S.* Enteritidis and *S.* Typhi, which displayed a single positive *sefR* signal, *E. coli* and *Shigella flexneri* displayed multiple hybridisation signals of quite different sizes (Figure 6.4). This may simply be due to restrictions sites being present within the probed gene, or it may be that the *sefR* homologue is present in multiple copies in these strains. The implications of these results are discussed further in section 7.3.

S. Typhi has previously been shown to possess the *sefA* gene but is unable to express SEF14 fimbriae (Turcotte and Woodward, 1993). With the availability of the complete genome sequence of *S.* Typhi it was possible to compare the SEF14 locus from *S.* Enteritidis and *S.* Typhi (section 6.3.2 onwards). This comparison revealed a number of nucleotide changes (listed in Table 6.1) relative to the *S.* Enteritidis sequence. Several of these result in the premature termination of the *sefA*, *sefD* and *sefR* genes, thus explaining why *S.* Typhi is unable to express SEF14 fimbriae. The observed nucleotide changes in *sefB* and *sefC*, including the insertion of 12 copies of the direct repeat TGTTGG within *sefC* (Figure 6.7), do not result in premature termination of either these genes, which could theoretically produce full-length proteins. Whether these proteins are expressed is not known.

Analysis of DNA flanking the SEF14 locus in *S.* Typhi revealed a region of 22733 bp that showed no similarity to *E. coli* chromosomal sequences, which in turn was flanked by DNA with significant identity to DNA from the region of 92.8-00.1 minutes on the *E. coli* K-12 chromosome. This observation, combined with the presence of DNA sequences with similarity to bacteriophage related DNA and *Salmonella* virulence genes in the regions flanking the SEF14 locus, supported the hypothesis that the SEF14 locus formed part of a PAI. This 22.7 kb *S.* Typhi PAI was flanked by the *leuX* gene, which has been implicated as a hot-spot for DNA insertion mediated by Bacteriophage P4 (Blum *et al.*, 1994), and further supports the above hypothesis.

Chapter 7: Discussion

7.1 Introduction

The SEF14 fimbria is one of a number of fimbriae expressed by the clinically and economically important bacterial pathogen *Salmonella enterica* serovar Enteritidis, which is responsible for significant morbidity and mortality in both humans and livestock (section 1.3). A number of studies have shown that all *S.* Enteritidis strains, regardless of their source, express SEF14 fimbriae (Turcotte and Woodward, 1993; Doran *et al.*, 1996). Such consistent expression suggests a role in pathogenesis, however experimental infection of mice (Thorns *et al.*, 1996; Ogunniyi *et al.*, 1997) and chickens (Thorns *et al.*, 1996; Dibb-Fuller *et al.*, 1999; Rajashekara *et al.*, 2000) with mutants unable to express SEF14 fimbriae shows that they have no significant difference in virulence when compared to the isogenic parent strain. More recently, Edwards *et al* (2000) showed that SEF14 fimbriae, and in particular the minor subunit, SefD, might play some role in attachment to peritoneal macrophages. They also showed that non-polar *sefA* insertion mutants were as virulent as the parental strains, while non-polar *sefD* insertion mutants were reduced in their ability to be internalised by peritoneal macrophages, and to colonise the livers and spleens of mice. Thus the role of SEF14 fimbriae in pathogenesis remains unclear. Coupled with the uncertainty of the role of SEF14 fimbriae in pathogenesis is our lack of knowledge as to the regulation and biogenesis of these fimbriae. It is known that SEF14 fimbriae are regulated in response to temperature, with expression occurring at 37°C but not at 30°C or lower (Müller *et al.*, 1991), however the mechanism for this regulation has not been elucidated. Passing reference has been made by Clouthier *et al* (1998) and Edwards *et al* (2000) to a gene downstream of the SEF14 operon, however no experimental studies characterising this putative gene have been published.

This study sought to identify and characterise the gene(s) directly involved in the regulation of SEF14 expression as well as to assess the role of the minor subunit (*sefD*) in the biogenesis of SEF14 fimbriae. Furthermore, the distribution of these genes was examined,

with the expectation that they would display the same limited distribution that has been observed for the *sefA* (Turcotte and Woodward, 1993) and *sefABC* genes (Doran *et al.*, 1996). Characterisation of these aspects of SEF14 fimbriae may lead to more effective experimental design for determining the role of SEF14 *in vivo*.

7.2 Initial characterisation of *sefR*

As mentioned above, several studies have made some reference to a gene downstream of *sefD*, alternatively designated *sefE* (Clouthier *et al.*, 1998; Collighan and Woodward, 2001) or *sefR* (this study; Edwards *et al.*, 2000). Suggestions were made that this gene might have some regulatory effect on SEF14 fimbrial expression, however no evidence for this hypothesis has been published.

During this study, the characterisation of DNA downstream of *sefD* was achieved by first cloning the DNA using SSP-PCR method. Sequence analysis of the 1.7 kb PCR product revealed an ORF, designated ORF813, which was transcribed in the opposite direction to the *sefABCD* genes, and separated from *sefD* by an intergenic region of 33 bp. A small stem-loop structure (dissociation energy of -16.3 kcal/mol [ca. -68.2 kJ/mol]) was located within this intergenic region. This structure is less complex than that located between the *sefA* and *sefB* genes (Clouthier *et al.*, 1993), which possessed a dissociation energy of -37.4 kcal/mol (ca. -156.4 kJ/mol). Clouthier *et al* (1993) hypothesised that the stem-loop structure between *sefA* and *sefB* might act to stabilise the *sefA* portion of the polycistronic *sefABCD* transcript, by protecting it from 3'-5' endonucleolytic digestion (Petersen, 1992), while at the same time reducing the level of expression of *sefBCD* (Higgins *et al.*, 1982), thus regulating the levels of *sefA* expression relative to the *sefBCD* genes. Extending this hypothesis, the stem-loop between *sefD* and ORF813 might act to stabilise the *sefBCD* portion of the transcript, ensuring that sufficient *sefBCD* is expressed to produce normal amounts of SEF14 fimbriae. Sequences closely matching the -10 and -35 promoter element consensus sequences were

located upstream of ORF813 in the expected positions, indicating this gene was probably not part of a larger operon.

ORF813 was examined by BLASTN analysis (http://www.ncbi.nlm.nih.gov/BLAST/), however no similarity to any other nucleotide sequences was found. BLASTX analysis, comparing a 6-frame translation of the submitted nucleotide sequence against peptide sequences, revealed significant similarity to a number of proteins belonging to the AraC family of transcriptional regulators. Not surprisingly, six of the seven proteins with significant similarity to the translated ORF813 sequence were involved in the regulation of fimbrial expression (i.e. AggR, BfpT, CfaD, CsvR, FapR, Rns). This observation made it fairly likely that ORF813 was in fact a transcriptional regulator of SEF14 expression. The AraC family of transcriptional regulators (section 1.5.1.4.2) consists of more than 100 different regulators, and have been used by Gallegos *et al* (1997) to describe a consensus sequence within the C-terminal region. An alignment of translated ORF813 with the protein sequences of significant similarity, and the type protein AraC, showed that 7/17 residues were identical with the consensus while a further 7/17 were similar. Comparison of translated ORF813 with the consensus alone revealed 11/17 identical and 5/11 similar residues. Based on this data, ORF813 was considered to be a transcriptional regulator of the AraC family, and redesignated *sefR* to reflect its role as a *S*. Enteritidis Fimbriae Regulator. The SEF14 locus was updated to include the *sefR* gene, as well as the intervening stem-loop structure (Figure 3.8).

7.3 The SEF14 operon and distribution of *sefD* and *sefR* genes

Previous studies analysing the distribution of the *sefABC* and *sefD* genes presented a confusing picture, with *sefABC* limited to group D1 *Salmonella* serovars (Doran *et al.*, 1996), while *sefD* was ubiquitous throughout *Salmonella* and also present in other Enterobacteriaceae (Clouthier *et al.*, 1994). These results were also inconsistent with the

close association of the *sefC* and *sefD* genes (overlapping stop and start codons). Therefore, it was hypothesised that in fact *sefD* would be confined to the same small group of *Salmonella* serovars as was *sefABC*, and that *sefR* would also show the same distribution. It was shown by Southern analysis that this hypothesis held true for *sefD*, but not for *sefR*. Unlike the *sefABCD* genes, which show a very limited distribution, the s*efR* gene was found to be present in a larger group of closely related bacteria (e.g. *Shigella*, *E. coli*; section 6.2.3), but not in less related bacteria such as *Legionella* and *Vibrio cholerae*. Also multiple hybridisation signals observed for *E. coli* O111:H$^-$ and *Shigella flexneri* suggest multiple copies of the same or similar sequence. These signals may correspond to as yet uncharacterised AraC-like fimbrial regulators which raises the possibility that both *E. coli* and *S. flexneri* possess as yet uncharacterised fimbrial operons, a possibility further substantiated by a recent study of the *S.* Typhi genome. Towsend *et al* (2001) analysed the *S.* Typhi genome with the aim of locating all possible fimbrial operons. Incredibly, 14 distinct operons were discovered, of which only 5 (Type IV, *fim*, *saf*, *sef* and *tcf*) had previously been identified and/or characterised (Rossolini *et al.*, 1993; Doran *et al.*, 1996; Folkesson *et al.*, 1999; Zhang *et al.*, 2000). Similar analysis of the *E. coli* O111:H$^-$ and *S. flexneri* genomes is required to reveal the presence of potentially uncharacterised fimbrial operons, which would have major implications in altering our understanding of the role of unknown fimbriae in the pathogenesis of these organisms.

Analysis of *S.* Typhi DNA revealed that *S.* Typhi possesses a complete SEF14 locus (i.e. *sefABCDR* genes), however three of the five genes (*sefA*, *sefD* and *sefR*) contained mutations that render them non-functional. This suggests that SEF14 fimbriae expression is under positive selection in *S.* Enteritidis and thus gene function is maintained, while in *S.* Typhi the need for them has been lost, and mutations have accumulated leading to inactive genes. This in turn provides an explanation as to why Turcotte and Woodward (1993) were unable to detect expression of SEF14 fimbriae by *S.* Typhi. Further analysis of DNA up- and

down-stream of the *sef* genes revealed evidence that the SEF14 locus constitutes part of a PAI, with approximately 23 kb of DNA not registering any sequence similarity with *E. coli* K-12 DNA and flanked by DNA with sequence similarity to *E. coli* K-12 *leuX* and *mcrD* genes. The presence of the *leuX* gene was significant as this gene has been implicated as an insertion site for bacteriophage P4 (Pierson and Kahn, 1987), a process that has been implicated in the formation of PAIs (see section 1.6.2.2). The corresponding region in *E. coli* was approximately 80 kb and contained multiple insertion elements and the *fim* genes. It is possible that this region is an insertional hotspot, providing a location where insertions occur more rapidly than in other regions, allowing divergence of bacteria from ancestral strains.

7.4 SefR regulates SEF14 expression

Despite having defined the *sefR* gene as a putative regulator of SEF14 expression, initial experiments showed that the *sefABCD* genes alone, when cloned into the high copy number vector pBC-SK$^+$, were sufficient for the production of SEF14 fimbriae in *E. coli* K-12, independent of *sefR*. These fimbriae appeared to have similar morphology to those observed in 11RX (section 4.4.3). It is probable that there is a basal level of *sef* expression independent of *sefR*, which, when multiplied by several hundred (corresponding to the number of vector copies), gives the appearance that *sefR* is not required for SEF14 expression. However, the high copy number vector system used in these experiments obviously does not reflect the single copy found on the *S.* Enteritidis chromosome. Therefore, a mutation in the chromosomal *sefR* gene was generated in *S.* Enteritidis strains 11RX and 7314 (section 4.2), which resulted in a total loss of SEF14 expression as assessed by Western immunoblot and IEM.

To confirm the role of SefR in upregulating SEF14 expression, the *sefR* gene was cloned into an inducible vector system and overexpressed. A protein product closely matching the predicted molecular weight of SefR (31.4 kDa) was observed by on SDS-15%

polyacrylamide gel stained with Coomassie Blue. Overexpressed SefR was able to induce a 14- to 74-fold increase in the level of β-galactosidase activity from a *sefA-lacZ* reporter construct. This result correlated with an observed increase in SefA expression from *sefR* mutant strains when *sefR* was supplied *in trans* on a complementing vector. Visualisation of complemented bacteria by IEM (section 4.3.5) showed that the complemented mutants were able to express SEF14 fimbriae, however the expression did not appear as extensive as the wild type. This may have been due to an increased level of fimbriae biogenesis, leading to a greater number of shorter fimbriae. Given the irregular nature of the SEF14 fimbrial structure, it is difficult to determine if this is the case. Alternatively, excess *sefR* may lead to an increase in the ratio of SefA to SefD, although given that the *sefABCD* genes are expressed as a single transcript, this explanation seems unlikely, as the increases in SefA and SefD would most likely be proportional.

Therefore, it was shown that SefR was absolutely required for detectable SEF14 expression in *S.* Enteritidis strains 11RX and 7314. This is similar to results observed for other fimbrial regulators such as Rns, which is required for expression of CS1 pili (Caron *et al.*, 1989), and CfaD, which is required for expression of CFA/I fimbriae (Savelkoul *et al.*, 1990). A recent study by Munson *et al* (2001) showed that there appears to be a class of transcriptional activator proteins, including the prototype Rns as well as VirF, AggR, CfaD and CsvR, that activate transcription through binding to regions both up- and down-stream of the transcriptional start site. Given that *sefR* shows significant aa identity with these proteins, it is likely that SefR is a member of this class. DNA binding analysis would need to be carried out in order to determine the exact location of SefR binding upstream of *sefA*.

7.5 *sefD* required for normal expression of SEF14 fimbriae

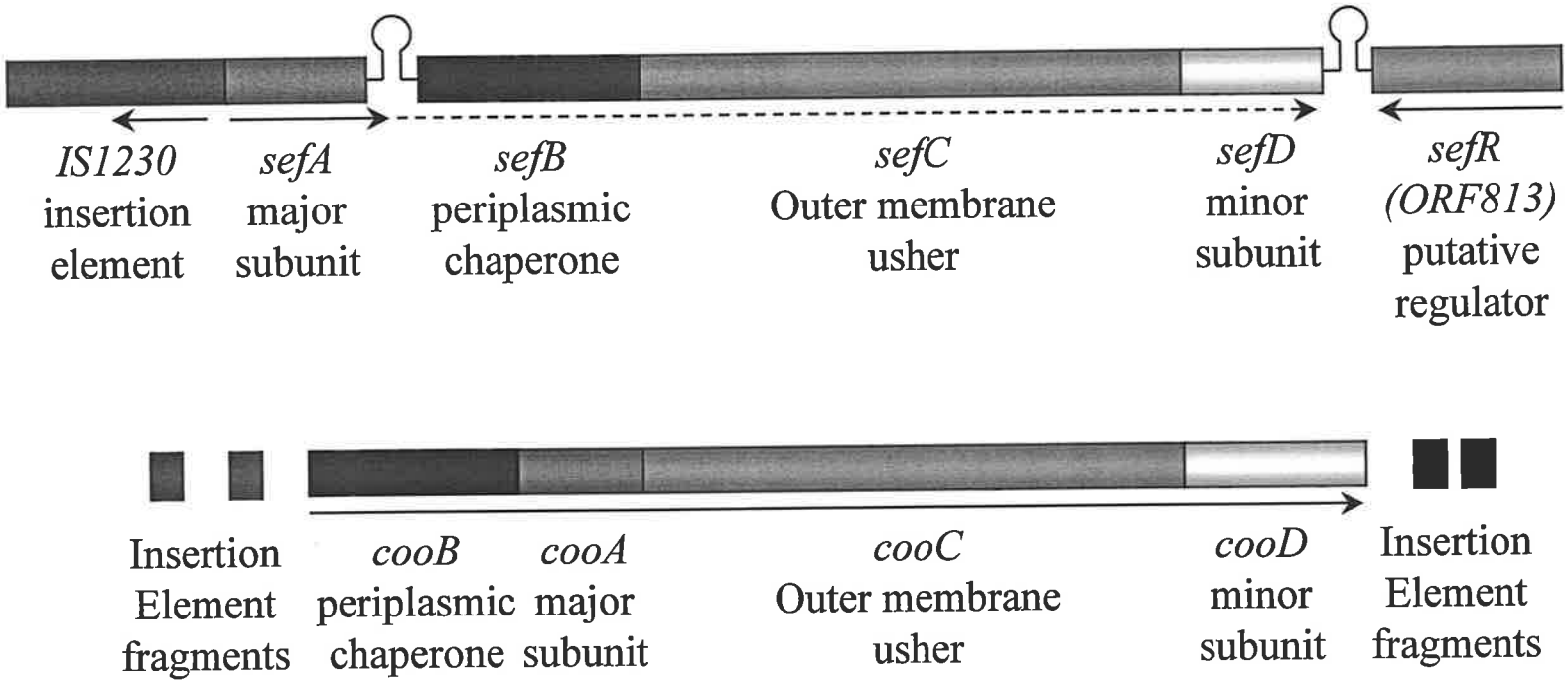
Prior to the commencement of this work, the role of *sefD* in the biogenesis of SEF14 fimbriae was unknown. Based on the organisational similarity between the SEF14 and CS1

loci (Figure 7.1), and the observation that a mutation in the minor subunit CooD resulted in a loss of CS1 expression (Froehlich *et al.*, 1994b; Sakellaris *et al.*, 1996), we hypothesised that SefD acts as an initiator protein, and a mutation in *sefD* would lead to a loss of SEF14 expression. A similar proposal (that SefD is a tip located adhesin, required for both the initiation of SEF14 biogenesis, and translocation of SefA across the cell membrane) was put forward recently by Edwards *et al* (2000). Their hypothesis was based on the observation that both a polar mutation in *sefA* (disrupting *sefD* expression) and a non-polar mutation in *sefD* attenuate the growth of *S.* Enteritidis in mice, while a non-polar mutation in *sefA* had no effect on virulence. This correlated with a study by Ogunniyi *et al* (1997), who also showed that a non-polar mutation in *sefA* did not affect the virulence of *S.* Enteritidis strain 7314 in mice. Therefore, SefD is required for virulence, and by extension constitutes the functional portion of SEF14 fimbriae. They also proposed a model for the biogenesis of SEF14 fimbriae, based on the chaperone usher model (Soto and Hultgren, 1999). Using this model, the absence of *sefA* would lead to the formation of a fibrillar structure, composed of one or more SefD molecules, which can functionally substitute for SEF14 fimbriae. However, no experimental evidence for these putative fibrillar structures, or the effect of the *sefA* and *sefD* mutations on SEF14 biogenesis, was presented.

In order to address this discrepancy, *sefD* mutants were constructed in *S.* Enteritidis strains 11RX and 7314, in the same manner as that used to construct *sefR* mutations in these strains. The effect of this mutation on surface expression of SEF14 fimbriae was examined by EIA and IEM. Unlike the *sefR* mutation, which led to a complete loss of SEF14 expression, mutations in *sefD* seemed to alter the ability to produce SEF14 fimbriae without inhibiting export of *sefA* to the cell surface. The level of *sefA* expressed on the surface was reduced by ~35%, as determined by EIA (section 5.4.3.1). Immunogold labelling and IEM analysis of *sefD* mutants revealed the presence of surface associated SefA, termed SEF14 "stubs", rather than complete SEF14 fimbriae, which does not fit with the model proposed by

Figure 7.1: Comparison of SEF14 and CS1 loci

This figure highlights some of the similarities and differences between the SEF14 and CS1 loci. The similarities include 4 genes encoding major and minor subunits, periplasmic chaperone and outer membrane usher. Also, both loci have remnants of IS elements flanking the 5' end, while the CS1 loci also has 3' flanking IS elements. The SEF14 and CS1 loci are regulated by the AraC-like transcriptional activators SefR and Rns, respectively. However, *sefR* is located immediately downstream of *sefD*, while *rns* is located on a virulence plasmid. Other differences include swapped orientation between the major subunit and periplasmic chaperone, the absence of a stem-loop structure to give differential expression of the structural genes, and the lack of regulator gene immediately downstream of the minor subunit gene. The arrows indicate the direction of transcription; the dashed arrow indicating either reduced read through or decreased relative stability. The stem loop structures are shown between the *sefA-sefB* and *sefD-sefR* genes. Adapted from Murphree *et al.*, 1997 and Sakellaris & Scott, 1998


IS1230
insertion
element
sefA
major
subunit
sefB
periplasmic
chaperone
sefC
Outer membrane
usher
sefD
minor
subunit
sefR
(ORF813)
putative
regulator
Insertion
Element
fragments
cooB
periplasmic
chaperone
cooA
major
subunit
cooC
Outer membrane
usher
cooD
minor
subunit
Insertion
Element
fragments

Edwards *et al* (2000). Evidence for these 'stubs' has been proposed by Clouthier *et al* (1993). *E. coli*, harbouring a vector carrying the *sefABC* genes, was examined by IEM, and showed surface structures that were described as 'blebs'. However the data presented by Clouthier *et al* (1993) did not show a clear difference between SEF14 fimbriae expressed from wild type *S.* Enteritidis and anti-SefA reactive blebs expressed from *E. coli* K-12. This difference is clearly shown in *S.* Enteritidis strains 11RX and 7314 (section 5.4.3.2). The loss of *sefD* was confirmed as the cause for the formation of these anti-SefA reactive 'stubs', since providing *sefD in trans* from an inducible vector complemented the chromosomal mutation, and restored both the level of *sefA* expression to wild type levels (section 5.4.4.2.2) and surface expression of SEF14 fimbriae. Interestingly, the morphology of SEF14 fimbriae in the complemented mutants was slightly different to those observed in the wild type. The wild type SEF14 fimbriae form a web-like tangle of fibres (Figure 5.26, 11RX), with spaces visible between the fimbriae. In contrast, SEF14 fimbriae expressed by the *sefD*-complemented mutant (Figure 5.26, RMJ033) are clumped together, with little or no visible space between individual fibres. This clumping may have been due to the presence of excess SefD, the effect of which is discussed below.

Having shown that *sefD* is not an essential initiator protein then raises the issue of whether either SefA or SefD acts as the adhesin for SEF14 fimbriae. There exists significant sequence similarity (65%) between SefA and the adhesin of the recently characterised ETEC non-fimbrial adhesin CS22 (Pichel *et al.*, 2000), while *sefD* shows limited similarity to the AfaD invasin, a protein that promotes internalisation of bacteria into HeLa cells (Jouve *et al.*, 1997). Thus, on the basis of sequence similarity, SefA and SefD might be an adhesin and an invasin, respectively. Further experiments, including identification of potential binding sites in either SefA and/or SefD, followed by site directed mutagenesis of these sites would help to identify which subunit is the adhesin.

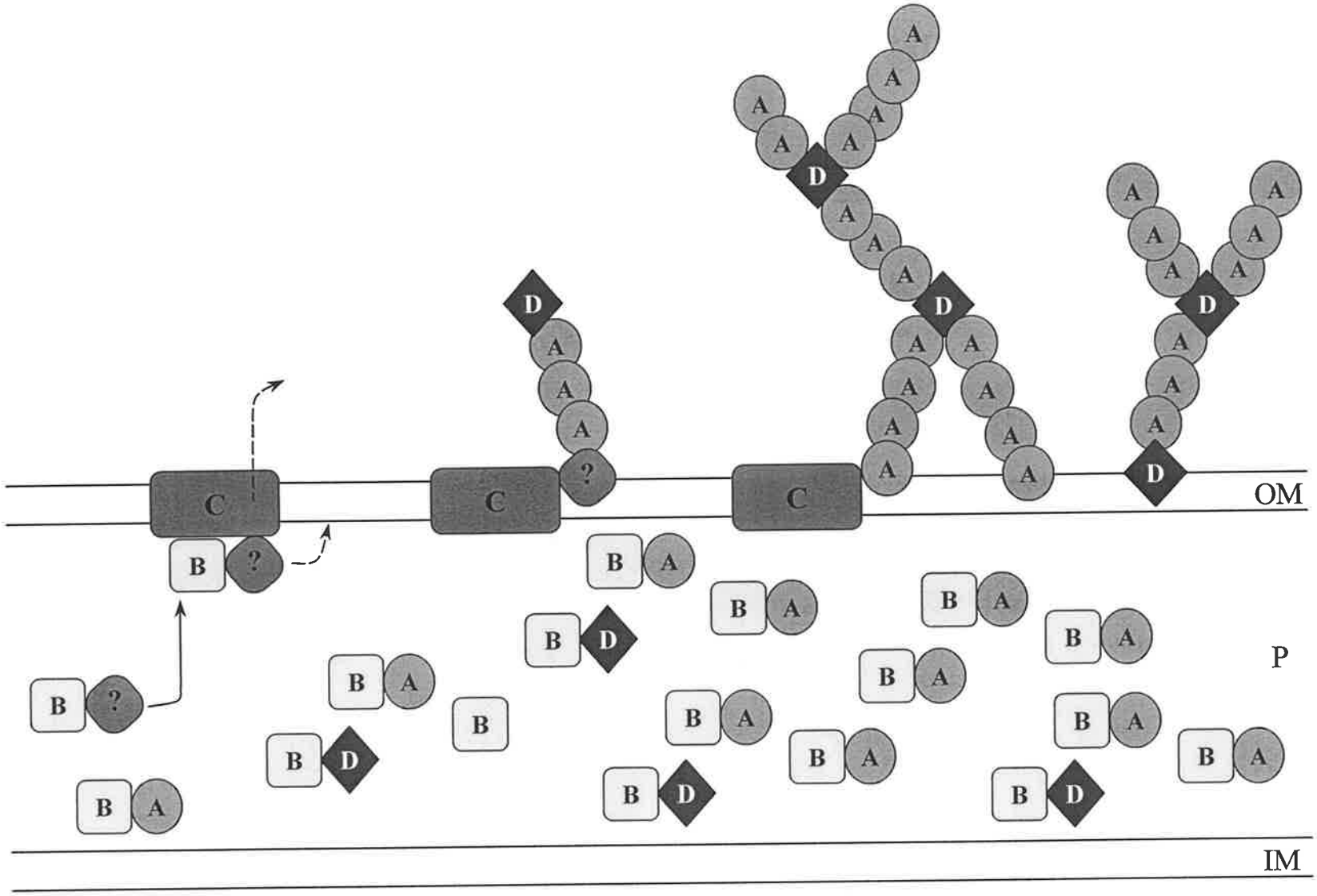
7.6 Model for SEF14 fimbriae biogenesis

There are several lines of evidence that present themselves when attempting to describe a model for the biogenesis of SEF14 fimbriae. At face value, it would be easy to assume that SEF14 fimbriae have a mechanism for biogenesis similar to the classic chaperone-usher model, into which class CS1 fimbriae belong. The SEF14 and CS1 loci share similar operon structure, including major and minor subunit, periplasmic chaperones and outer-membrane ushers; both loci are positively regulated by an AraC-like transcriptional regulator, and the level of identity (~14%) between the major and minor subunits is comparable to other chaperone-usher type fimbriae (section 5.1). Despite these similarities, there are some significant differences. First there are no significant similarities in DNA or protein sequence between these two loci. Second, the morphology of SEF14 is amorphous, more akin to the random structure of curli fibres (Olsen *et al.*, 1989) than the ordered, rigid pili of CS1. Finally, it has been shown in this study that a chromosomal mutation in *sefD* does not result in the complete loss of surface *sefA* (major subunit) expression, unlike that observed in *cooD* mutants (Froehlich *et al.*, 1994; Sakellaris *et al.*, 1996), which are unable to express CS1 pili.

Based on this list of similarities and differences, a model for the biogenesis of SEF14 fimbriae has been proposed (Figure 7.2). This model combines features of both the chaperone-usher (section 1.5.1.3.1) and extracellular-nucleation (section 1.5.1.3.3) pathways. In this model, both *sefA* and *sefD* are able to initiate fimbrial biogenesis, as evidenced by the presence of SefA on the surface of *sefD* mutant bacteria. Similar to the chaperone usher pathway, the chaperone is required for the transport of both SefA and SefD to the outer membrane usher SefC. As the levels of *sefBCD* transcript are much lower than *sefA* transcript due to differential mRNA stability/transcription, the proportion of SefA is much higher than that of SefD within individual fibres, resulting in SefD being scattered randomly within each fibre. This model closely matches that proposed by Duthy (2000), in a model for the

Figure 7.2: A model for SEF14 biogenesis

This figure shows a model for the biogenesis of SEF14 fimbriae based on data from this study as well as observations by other groups (see section 7.6). The SefB (periplasmic chaperone) and SefC (outer membrane usher) proteins behave in a manner similar to that observed for the standard chaperone-usher pili systems. Since *sefD* does not initiate fimbriae biogenesis as in other systems, *sefA* or *sefD* could be the first subunit (indicated by the question mark). SefB transports the major and minor subunits (SefA and SefD respectively) to SefC, where they are exported to the cell surface. At this point it is unknown whether the subunits are attached to the base of the growing fimbria, with cross-linking occurring after polymerisation or if both subunits are secreted to the surface where *sefD* acts as a nucleator to induce fimbria polymerisation. In either case, *sefD* most likely acts to induce branching within and cross-linking between fimbriae, possibly at multiple sites. This leads to the amorphous appearance of SEF14 fimbriae on the surface of *S.* Enteritidis. The overproduction of *sefD* induces excess cross-linking and branching, since the ratio of SefD to SefA increases, with the result that the SEF14 fimbriae appear to clump together. A, B, C and D represent the SefA, SefB, SefC and SefD proteins, respectively. IM and OM represent the inner- and outer-membranes, respectively, while P represents the periplasm.


A
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biogenesis of CS5 pili. Similar to CS1, CS5 pili are distinct, rigid structures composed primarily of the major subunit CsfA, with the minor subunit CsfD randomly distributed throughout the pilus shaft based on the stoichiometric ratio of the two subunits in the periplasm. Unlike CS1 or CS5 pili, SEF14 fimbriae are not rigid structures and appear to be linked together in a manner proposed for curli fibres i.e. the extracellular-nucleation pathway (Soto and Hultgren, 1999). In this pathway, the minor subunit is the nucleation protein, which initiates extension of the subunits from the end of the curli fibre. Again, the minor subunit is also randomly distributed throughout the curli fibre, however in this case branching can be initiated from these minor subunits (Bian and Normark, 1997). A comparison of CsgB (Hammar *et al.*, 1995), the nucleator for *E. coli* curli, and SefD subunits reveals no significant sequence similarity, however this does not preclude a similar function. Bian and Normark (1997) showed by IEM that the CsgB nucleator is present at the cell surface in the absence of the major subunit CsgA. Therefore, it is conceivable that SefD performs a similar function to CsgB, promoting the polymerisation of SefA subunits as well as initiating branching of fibres. However, the observation that an increase in the levels of SefD promotes an apparent clumping of SEF14 fimbriae due to excess branching is not observed in the curli system; an increase in the level of CsgB leads to an increase in the level of CsgB within the curli fibres without altering the morphology with respect to wild type fibres (Bian and Normark, 1997). While the data presented in this study advances our knowledge of the possible role of *sefD* in the biogenesis of SEF14 fimbriae, more work needs to be done in order to clarify the exact nature of this role.

7.7 Concluding remarks and future direction

This study has sought to answer some of the questions relating to SEF14 fimbriae, in particular, how their expression is regulated (Chapter 4), what role SefD plays in biogenesis (Chapter 5), and why the apparent distribution of the *sefABC* and *sefD* genes was vastly

different (Chapter 6). In addressing these three areas, a number of significant results were obtained. First, regulation of SEF14 fimbriae occurs via the AraC-like regulator SefR, which is absolutely required by wild type bacteria for the expression of SEF14 fimbriae (section 4.8.1). Second, the loss of *sefD* does not prevent the export of SefA to the cell surface (section 5.4.3), showing that SefD is not required to initiate biogenesis of SEF14 fimbriae. However, the *sefD* gene is limited to those strains that possess the *sefABC* genes (section 6.2), and is required for normal expression of SEF14 fimbriae (section 5.4.4).

The difficulties in characterising the role of SEF14 fimbriae in pathogenesis are most likely complicated by the fact that SEF14 fimbriae defy classification into any current class of fimbriae. Similar to a number of fimbrial systems, the *sefABCD* genes are positively regulated by an AraC-like regulator protein. Unlike those same systems, the loss of the minor subunit does not inhibit export of the major subunit to the cell surface. The biogenesis of SEF14 fimbriae appears at face value to occur by the chaperone-usher pathway, yet their morphology is more related to curli, produced by the extracellular-nucleation pathway.

There are a number of issues that need to be addressed in order to better understand the regulation and function of SEF14 fimbriae. It would be useful to define the actual binding site of SefR, perhaps by gel shift analysis and DNase I foot printing. This would support the inclusion of SefR into the Rns-like class of transcriptional regulators proposed by Munson *et al* (2001). Analysis of a range of Enterobacteriaceae genomes for fimbrial operons might reveal the presence of 'SEF14-like' fimbrial operons, which in turn may lead to further discoveries into the role of SEF14 fimbriae in pathogenesis. Defining the location of SefD within SEF14 fimbriae, along with structural analysis and site-directed mutagenesis would allow confirmation of SefD as a nucleator protein, and/or an adhesin. The culmination of these further studies would lead to a defining of the exact role of SEF14 fimbriae in pathogenesis, and perhaps providing insights into why *S.* Enteritidis is such an effective and successful pathogen.

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Corrigenda (changes underlined as indicated)

Abstract

3rd page, line 3: with plasmid-borne *sefD*
3rd page, line 4: over wild-type levels
3rd page, line 5: Hence, it was
3rd page, line 15: observed in wild-type

Table of Contents

1st page, line 6: 1.4 *Salmonella* Infection and Immune responses
1st page, line 9: 1.4.1.2 Humoral Immunity
1st page, line 23: 1.5.1.2.6.3 *Salmonella* Typhimurium fimbriae (*stf*)
4th page, line 14: 2.10.6 Insertional Mutagenesis
4th page, line 20: 2.12.2 Gel Electrophoresis, Southern Transfer and Probe Hybridisation

Abbreviations

SAP - Shrimp alkaline phosphatase

Chapter 1

Page 1, line 3: *Salmonella* spp. are
Page 1, line 5: identification of *Salmonella* spp. from
Page 1, line 7: *Salmonella* spp. are motile
Page 1, line 8: Most *Salmonella* are able to infect the small intestine and induces
Page 1, line 11: The *Salmonella* genus has been
Page 1, line 20: by focussing on
Page 1, line 23: "Over 2000 serovars of *Salmonella*" should read "Over 2000 *Salmonella* serovars"
Page 2, line 22: range of *Salmonella* spp. is
Page 2, line 23: *Salmonella* spp. have been
Page 3, line 4: range of hosts, e.g. *Salmonella*
Page 3, line 5: while others serovars are
Page 3, line 6: a single host, e.g. *Salmonella*
Page 3, last line: which *Salmonella* spp. are able
Page 4, line 17: and immuno-compromised
Page 4, line 21: on the dose
Page 5, line 8: MMWR 49(51) refers to Morbidity and Mortality Weekly, Volume 49 issue 51
Page 5, line 10: cases of salmonellosis per
Page 5, line 11: abbreviation WER – see references under WER
Page 5, line 18: sick leave ~~etc~~ and so on. These
Page 5, line 21: make *Salmonella* spp ~~an~~ extremely important pathogens
Page 6, line 16: located on Pathogenicity Islands (section
Page 6, last line: *Salmonella* occurs rapidly and are followed
Page 7, line 1: systemic site, such as
Page 7, line 4: In mice, there appears to be a critical load of bacteria ($\sim 10^8$) in excess of
Page 7, line 18: include polymixins
Page 12, line 6: Typhimurium/mouse model
Page 12, line 8: which is most likely
Page 12, line 10: which is most likely
Page 13, line 2: *Shigella* spp,
Page 13, line 3: were found to possess
Page 14, line 5: fimbriae (Mol and Oudega
Page 14, line 12: mucous membranes ~~etc~~.
Page 14, line 21: fimbriae (*sef*)
Page 14, line 22: that *Salmonella* spp are known
Page 16, line 23: Girón *et al*
Page 17, line 4: cholerae (Girón *et al*
Page 17, line 12: which converts pre-bundlin
Page 18, line 7: Curli were found

Page 20, line 1: and a 5-fold
Page 21, line 17: The genes encoding *Salmonella* atypical fimbriae (*saf*), were
Page 24, line 2: (Tennent *et al.*, 1990)
Page 24, line 3: various *pap* genes
Page 25, line 2: that is evolutionarily
Page 26, line 17: minor subunit, respectively
Page 27, line 18: of fimbrial expression
Page 28, line 12: areas within the regulatory
Page 28, line 21: Typhimurium *lpf* Phase ON
Page 28, line 22: Enteritidis *lpf* Phase ON
Page 28, line 23: since the *lpf* Phase OFF
Figure 1.6 legend, line 2: region in Phase OFF and Phase ON
Page 30, line 5: AraC-like proteins,
Page 30, line 6: (Kwon *et al.*, 2000), in complex
Page 30, line 13: Niland *et al* (1996), and is
Page 31, line 22: Hacker *et al* (1997).
Page 32, line 25: chromosome, has been
Page 32, line 12: external source, e.g. another bacterium
Page 32, line 13: or naked DNA
Page 32, line 24: SPI-1, ~~is~~ located
Page 32, line 25: chromosome, has
Page 33, line 13: mutations in a number
Figure 1.9 legend, line 2: figure presents a
Page 35, line 4: However, this
Page 35, line 7: the invasive ability
Page 35, line 13 of *Salmonella* spp in macrophages
Page 35, line 22: phagosome-lysosome
Page 36, line 4: containing, filamentous should read containing filamentous
Page 36, line 7: *Salmonella*-containing
Page 36, line 8: maintaining and environment
Page 36, line 9: encoded within the
Page 36, line 12: SPI-5 loci have not
Page 36, line 13: those of SPI-1 and SPI-2
Page 36, line 15: at ~17 kb
Page 37, line 14: approximately 20 Cs, ~~located~~ immediately
Page 37, line 16: The *pipC* gene is thought
Page 37, line 24: calves are needed to determine whether
Page 38, line 2: global regulator, such
Page 38, line 4: Deiwick *et al.*, 1999), and specific
Page 38, line 8: cascade is shown
Page 38, line 14: uptake of the *Salmonella* bacterium into
Page 39, line 6: of mouse epithelium
Page 39, line 21: *Salmonella* spp., like all Gram-negative bacteria, have an outer
Page 40, line 24: with the wild-type parent
Page 40, line 25: both *in vivo* and *in vitro* were depressed
Page 41, line 12: plasmids (for review
Page 41, line 13: *Salmonella* serovars, such as
Page 41, line 17: in extra-intestinal tissues
Page 41, line 23: the *Salmonella* bacterium encounters within host cells, e.g.
Page 41, last line: upregulates *spvR*
Page 42, line 5: have focused on
Page 42, line 6: determinants, i.e.
Page 42, line 9: distinct pathogenic groups.
Page 42, line 15: them a mosaic
Page 42, line 16: (2000).
Page 42, line 22: *Salmonella* spp. but
Page 44, line 14: chromosome spanning the

Page 45, line 1: with advantageous changes
Page 45, line 19: them amenable to
Page 45, last line: Therefore, it is
Page 46 & 47: *N. gonorrhoeae* / *N. gonorrhoea* should read *N. gonorrhoeae*
Page 47, lines 14, 15: "DNA containing" should read "DNA-containing"
Page 47, line 16: (1989) following incubation of
Page 48, line 10: infectious but replication
Page 49, line 23: However, it has
Page 50, last line: thymus-derived
Page 52, line 5: Lpf were able
Page 52, line 8: compared to wild-type
Page 52, line 9: similar numbers from
Page 54, line 21: fimbriae or *S.* Enteritidis expressing
Page 55, last line: (Ogunniyi, 1994).
Page 56, line 14: positive for ~~the~~ ~~expression~~ ~~of~~ surface
Page 57, line 6: complexes which were
Page 57, line 18: immune serum for SefD
Page 58, line 10: higher i.p. and
Page 58, line 11: recovery of mutant bacteria
Page 58, line 12: by the wild-type strain
Page 58, line 13: assays and was taken
Page 58, line 18: However, this contradicts
Page 60, line 16: of a Pathogenicity Island, given
Page 60, line 17: *Salmonella* spp.
Page 60, line 22: sequences in members of the family Enterobacteriaceae
Page 60, last line: Pathogenicity Island

Chapter 2

Page 64, line 10: ice for 2 h.
Page 64, line 14: for 10 min~~utes~~
Page 66, line 4: "1.5 ml of overnight" should read "An aliquot (1.5 ml) of overnight"
Page 67, line 23: "0.1-0.5 µg of plasmid DNA" should read "Plasmid DNA (0.1-0.5 µg)"
Page 67, last line: for 1.5 h.
Page 69, line 7: "1-2 µg of digested DNA" should read "Digested DNA (1-2 µg)"
Page 69, line 7: added then treated
Page 69, line 18: 16 h. A vector
Page 70, line 8: "2-4 µl of the supernatant" should read "An aliquot (2-4 µl) of the supernatant"
Page 71, line 3: "10 µg of bacterial chromosomal DNA" should read "bacterial chromosomal DNA (10 µg)"
Page 71, line 16: "5 µl of ligation reaction" should read "An aliquot of the ligation reaction (5 µl)"
Page 72, line 9: "10 µl of the reaction" should read "An aliquot of the reaction (10 µl)"
Page 73, line 5: "5x 100 µl aliquots" should read "Aliquots (5x 100 µl)"
Page 75, line 18: After transfer, DNA
Page 76, line 5: "10 µg of DNA" should read "Chromosomal DNA (10 µg)"
Page 78, line 3: plus 50 µl of 2x
Page 78, line 5: "20-30 µl (corresponding to approximately 1 to 1.5 x 10^8 bacteria) of sample was" should read "Aliquots of 20-30 µl, corresponding to approximately 1 to 1.5 x 10^8 bacteria, were"
Page 78, line 22: "500 µl of Triton solution" should read "Triton solution (500 µl)"
Page 79, line 22: β-lactoglobulin
Page 79, last line: kDa).
Page 81, line 1: abbreviation WM refers to Whole Membrane
Page 81, line 1: "500 µl of the WM fraction" should read "The WM fraction (500 µl)"
Page 81, line 13: with 200 µl of
Page 81, line 18: aeration for 18 h
Page 81, line 20: "2 ml of this was" should read "An aliquot (2ml) of this was"
Page 82, line 1: abbreviation IB refers to Inclusion bodies
Page 82, line 1: "2 ml of the IB fraction" should read "The IB fraction (2 ml)"

Page 82, line 4: abbreviation S/N refers to supernatant
Page 82, line 4: "200 µl of 50% (v/v) Nickel-NTA agarose resin (equilibrated" should read "50% (v/v) Nickel-NTA agarose resin (200 µl; equilibrated"
Page 83, line 9: specific cloning, and also with *sefA* deficient
Page 83, line 10: "50 ml of overnight culture" should read "Overnight culture (50 ml)"
Page 84, last line: "100 µl of alkaline phosphatase conjugated goat anti-rabbit IgG (KPL" should read "Alkaline phosphatase conjugated goat anti-rabbit IgG (100µl, KPL"
Page 85, line 19: "62.5 µl of standard" should read "The SefA standard (62.5 µl)"
Page 85, line 20, 21: "75 µl of bacterial suspension" should read "Aliquots of bacterial suspension (75 µl)"
Page 85, line 22: "50 µl of primary antibody (1:15,000)" should read "Primary antibody (50 µl, diluted 1:15,000)"
Page 86, line 2: 37°C for 4 h.
Page 86, line 3: of alkaline phosphatase
Page 86, line 11: values was calculated

Chapter 3

Page 88, line 6: ~~neither~~ the role
Page 88, line 15: the *sefD* gene from strain 11RX, two
Page 93, line 8: *sefABCD* and ORF813 mRNA
Figure 3.8 legend, lines 1, 2, 3 and 6: *sefR* should be replaced with ORF813
Table 3.2, line 4: Positive regulatory protein
Figure 3.11: SefR

Chapter 4

Page 97, line 8: Therefore, the aim
Page 97, line 13: and 7314 (see Table 2.2).
Figure 4.1 legend, line 2: *sefR* was amplified by PCR, not "was PCR amplified"
Figure 4.3 legend, line 1: analysis of putative insertion
Figure 4.3 legend, line 10: The unfilled arrow
Page 105, line 17: express ~~of~~ SefA in the form
Figure 4.13 legend, line 4: strains RMJ009 and 11RX were
Figure 4.16 legend, last line: indicated.
Figure 4.16 legend, line 7: (section 2.14.4). The unfilled arrow indicates SefA protein. Molecular
Figure 4.17 legend, line 3: amplification from
Page 109, line 11: estimated 120 kDa
Page 109, line 14: using ~~an~~ anti-SefA antiserum, however the serum was
Page 111, line 15: units ~~and~~; the level
Page 112, line 15: ~~The level of detection for these assays was calculated to be <0.01 µg per 10^{10} bacteria.~~
Figure 4.22, line 7: The fold-increase
Figure 4.23, line 2: The concentrations of
Table 4.2 legend, last line: of agar plate.
Page 116, lines 5, 11, 14: dependent
Page 116, line 9: affected by
Page 117, line 7: attenuated *S*. Enteritidis

Chapter 5

Page 118, line 8: purified maltose-binding protein (MBP)-SefD fusion
Page 119, line 16: majority of the chaperone
Page 119, line 19: host cell is no known
Page 120, lines 7, last line: delete "Maltose Binding Protein"
Page 122, line 16: different from those observed
Figure 5.4 legend, line 9: the periplasmic samples
Page 123, line 9: used to solubilise the membrane
Page 123, line 22: From RMJ022 and
Figure 5.5 legend, line 1: MBP-LacZ (filled arrow) and MBP-SefD (unfilled arrow) was
Figure 5.6 legend, line 4: loading ~30 µl

Page 124, line 3: that the serum only
Page 124, line 8: had occurred to the MBP portion
Figure 5.7: pET29 should read pET-21a
Figure 5.9 legend, line 5: blue (A), or transferred
Figure 5.9 legend, line 6: antibodies (B).
Figure 5.11 legend, line 10: Coomassie blue (section
Figure 5.12 legend, line 9: indicated.
Page 128, line 2: SefD were to compare
Page 128, line 9: *Hin*dIII restrictions sites
Page 128, line 13: the 4.8 kB *Hin*dIII
Page 131, line 10: for their ability
Figure 5.18 legend, line 9: indicated.
Page 132, line 10: anti-SefA serum.
Page 133, line 6: but were less extensive.
Page 133, line 22: DNA were used
Page 135, line 9: level of SefA
Page 135, last line: to have no observable
Page 136, line 5: abundance to those observed in the
Page 137, line 7: alternative methods were
Page 137, line 22: reasons mentioned above
Page 138, line 1: determined quantitatively

Chapter 6

Page 139, line 5: Hybridisations with
Page 140, line 5: Forty Enterobacteriaceae strains
Page 140, line 11: tested possess a *sefD*
Figure 6.3 legend, line 6: analysis.
Page 143, line 7: with the percent similarity
Figure 6.8 legend, last line: differences.
Figure 6.9 legend, last line: differences.
Page 145, line 2: differences are due
Page 145, line 8 salmonellae;
Page 145, line 14: elements, eg insertion sequences or phage DNA ~~etc~~,
Page 147, line 12: observations ~~are~~ summarised
Page 147, line 16: with the *dlp* gene of *S.* Enteritidis
Figure 6.11 legend, last line: represents 3 kb.
Table 6.3 legend, line 4: with the ratio shown
Figure 6.12 legend, last line: represents 3 kb.
Page 148, line 18: to analyse the corresponding
Table 6.4 legend, line 6: with the ratio shown
Page 152, line 3: Typhi were performed.
Page 153, line 22: restrictions sites

Chapter 7

Page 156, line 21: the levels of
Page 157, line 24: throughout all *Salmonella* serovars,
Page 158, line 6: (e.g. *Shigella* spp., *E. coli*
Page 158, line 7: bacteria, such as *Legionella* spp. And
Page 159, line 14: similar morphology to the morphology of fimbriae
Figure 7.1 legend, last line: Scott, 1998.
Page 162, line 4: from wild-type
Page 162, line 11: different to the morphology of SEF14 fimbriae observed

References

Page 168, line 19: analysis of *lpfABCDE*, a
Page 168, line 26: the *lpf* fimbrial
Page 169, line 5: mutations in *invA* and *lpfC* on

Page 175, line 6: Girón, J. A.
Page 177, line 20: *Infect Immun* **61**:5321-5326
Page 179, line 9, 12, 14, 16: Giron, J. A. should read Girón, J. A.
Page 180, line 14: **Gulig, P.A., and Curtiss, R. L. R.**
Page 182, line 6: for *Vibrio cholerae*
Page 183, line 6: *Burkholderia cepacia* AC1100
Page 185, line 8: homology to the *Shigella* IpaD
Page 186, line 3: Three *fim* genes
Page 188, line 25: variation of *Neisseria gonorrhaeae* FA1090
Page 188, line 28: *Microbiol Rev*
Page 191, line 26: regulation of *pef* expression
Page 193, line 22: **Pierson, L. S. R., and**
Page 195, line 5: genome of *Bacillus subtilis* phage
Page 199, line 25: JAMA.
Page 200, line 10: missing details *Enzymol.* **185**:60-89.
Page 200, line 22: gene *fimU* affects
Page 202, line 9: *Sci U S A.* 76**(9)**:4350-4.
Page 203, lines 4, 7, 9: pap should read Pap
Page 204, lines 15, 17: stn should read *stn*
Page 204, line 17: *invH*, but not
Page 205, line 2: pap should read Pap
Page 205, line 8: variation in *Bordetella pertussis*:
Page 205, line 12: in *Neisseria gonorrhaeae*.