Functional Characterisation of the SefA Protein of *Salmonella enterica* serovar Enteritidis

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ABSTRACT

This study was commenced as a continuation of earlier work which used sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)-fractionated proteins to define the antigens of an attenuated *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) strain, *S.* Enteritidis 11RX (11RX) able to stimulate *in vitro* proliferation of, and cytokine release from T cells harvested from 11RX-primed (C57BL/6 × BALB/c) F1 mice. A highly immunogenic 14 kDa protein was identified, it was purified to homogeneity and was free from LPS contamination. The protein was able to elicit delayed-type hypersensitivity reactions in 11RX-primed mice, and stimulated *in vitro* proliferation of, and cytokine release from T cells obtained from these animals. The protein is the mature form of the 16 kDa precursor polypeptide previously described, and was established to correspond to the structural subunit protein (SefA) of *S.* Enteritidis fimbriae (SEF14) described by other workers.

The role of SefA in the biology of *S.* Enteritidis was investigated. This necessitated cloning and sequencing the *sefA* gene which was accomplished using a cosmid library of the 11RX chromosome. To define the role of SefA in the pathogenesis of *S.* Enteritidis, *sefA* was mutated in the chromosome of strain 11RX and in a virulent *S.* Enteritidis strain 7314 by allelic exchange with a copy that has been inactivated by insertion of a nonpolar kanamycin resistance (*aphA-3*) cassette. The effect of this mutation on the abilities of the mutated *S.* Enteritidis strains to colonise the Peyer's patches and to invade the spleen was assessed in BALB/c mice, and *in vitro* by adherence and invasion of HeLa cells. The results obtained showed that 11RX colonised better and persisted longer in the Peyer's patches, but not in the spleens, of these mice when compared to its SefA-deficient counterpart. In contrast, no such difference was observed between the virulent strain 7314 and its SefA-deficient counterpart. These findings were

correlated with *in vitro* adherence and invasion of HeLa cells. Furthermore, there was no demonstrable role for SefA in the virulence of *S.* Enteritidis as assessed by LD_{50} measurements in BALB/c mice.

The role of SefA in protection of mice against challenge by the virulent strain 7314 was investigated. Strong, specific antibody responses were mounted against SefA in orally and intraperitoneally infected mice; T cells from mice intraperitoneally immunised with live 11RX organisms and recombinant *aroA S.* Typhimurium SL3261 strains expressing SefA also proliferated and released cytokines *in vitro* when cultured with this antigen. BALB/c mice vaccinated orally with SefA using various antigen delivery systems based on strain SL3261 showed between 60-70% level of protection when challenged with the virulent 7314 strain, compared to 20% level of protection with strain SL3261 alone. A SefA mutant of 11RX was also protective (70%), compared to its isogenic parent which induced 80% level of protection. The results indicate that SefA contributes to, but is not the only antigen involved in inducing protection in mice against virulent challenge.

T cell epitope mapping of SefA was carried out, using an analogous antigen, the FanC subunit protein of the K99 fimbriae of enterotoxigenic *E. coli*, as a model. A recombinant approach involving the fusion of carboxyl-terminal truncations of FanC to alkaline phosphatase was adopted. The results indicate that the B cell epitopes of FanC are likely to be continuous. T cell epitope analysis of the fusions by *in vitro* T cell proliferation assays did not indicate the presence of any immunodominant epitope. However, T cell epitope mapping of SefA, using a series of overlapping 16 amino acid synthetic peptides of SefA, indicated an immunodominant T cell epitope of SefA in a region corresponding to amino acids 55-61 of the protein.

The implications of, and the propositions arising from, the results presented in this study, as well as potential areas of further research, are discussed.

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 in the University library, being available for loan and photocopying.

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LIST OF ABBREVIATIONS

[^{3}H]-TdR: tritiated thymidine

11RX: *Salmonella* Enteritidis strain 11RX

7314: *Salmonella* Enteritidis strain 7314

A: adenine

A_{260}: absorbance at 260nm

A_{280}: absorbance at 280nm

A_{405}: absorbance at 405nm

A_{600}: absorbance at 600nm

Ab: antibody

Abs: antibodies

Ag(s): antigen(s)

Ap: ampicillin

APC: antigen-presenting cells

ATP: adenosine 5'-triphosphate

bp: base pair(s)

BSA: bovine serum albumin

C': complement

C: cytosine

C5: *Salmonella* Typhimurium C5

CBT: casamino acids, vitamin B1, tryptophan

CFU: colony forming units

CIAP: calf intestinal alkaline phosphatase

Cm: chloramphenicol

CM: culture medium

CMI: cell-mediated immunity

ConA: concanavalin A

cpm: counts per minute

CTL(s): cytotoxic T cell(s)

CTLL: cytotoxic T cell lymphoid line

D-PBS: Dulbecco's-PBS

dATP: 2'-deoxy adenosine 5'-triphosphate

dCTP: 2'-deoxy cytidine 5'-triphosphate

dGTP: 2'-deoxy guanosine5'- triphosphate

DIG-dUTP: digoxigenin-11-[2'-deoxy uridine 5'-triphosphate]

DIG: digoxigenin

DMSO: dimethyl sulfoxide

DNA: 2'-deoxyribonucleic acid

DNase: deoxyribonuclease

dNTP: deoxyribonucleoside triphosphate

DTH: delayed-type hypersensitivity

DTT: 1,4-dithiothreitol

dTTP: 2'-deoxy thymidine 5'-triphosphate

dUTP: deoxyuridine triphosphate

ECL: enhanced chemiluminescence

EDTA: ethylenediamine tetra-acetic acid

ELISA: enzyme-linked immunosorbent assay

EMEM: eagle's minimal essential medium

EtBr: ethidium bromide

Exo III: exonuclease III

F1: (BALB/c × C57BL/6) F1

F11RX: formalin-killed 11RX

FCS: foetal calf serum

FITC: fluorescein isothiocyanate

G: guanine

Gm: gentamycin

HBSS: hanks' balanced salt solution

HRP: horse-radish peroxidase

i.p.: intraperitoneal

i.v.: intravenous

IBP(s): intracellular bacterial pathogen(s)

IFN-γ: interferon-gamma

Ig: immunoglobulin

IL-2: interleukin 2

IPC(s): immune peritoneal cell(s)

IPTG: isopropyl-β-D-thiogalacto-pyranoside

kb: kilobase pairs

kDa: kiloDaltons

Km: kanamycin sulphate

LB: Luria Bertani

LPS: lipopolysaccharide

MAb: monoclonal antibody

MAbs: monoclonal antibodies

MEM: minimum essential medium

MHC: major histocompatibility complex

Minca: minimal casein

mRNA: messenger ribonucleic acid

NC: nitrocellulose

NK(s): natural killer cells

NMS: normal mouse serum

NPC: normal peritoneal cells

NRS: normal rabbit serum

NW: nylon wool

OVA: ovalbumin

P/B/A: PBS, BSA, Azide solution containing 10% normal rabbit serum

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PC(s): peritoneal cell(s)

PCR: polymerase chain reaction

PEG: polyethylene glycol-8000

PFA: paraformaldehyde

PhoA: alkaline phosphatase

POD: peroxidase

^{R}: resistant

RBS: ribosome binding site

Rif: rifampicin

RNA: ribonucleic acid

RNase: ribonuclease

rpm: revolutions *per* minute

^{S}: sensitive

S11RX: soluble 11RX antigen

SA5: *Salmonella* Enteritidis SA5

SA9: *Salmonella* Enteritidis SA9

SD: standard deviation

SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM: standard error of the mean

SHAM-FITC: sheep anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate

SHAM-HRP: sheep anti-mouse immunoglobulin conjugated to horse-radish peroxidase

SHAM: sheep anti-mouse immunoglobulin

Sm: streptomycin sulphate

t.s.: temperature sensitive

T: thymine

Tc: tetracycline

TE: Tris-EDTA

TEMED: N,N,N',N'-tetramethyl-ethylene-diamine

T_m: melting temperature

Tn: transposon

Tris: tris [hydroxymethyl] amino methane

Triton X-100: α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxypoly(oxy-1,2-ethanediyl)

Tween 20: polyoxyethylenesorbitan monolaurate

UV: ultraviolet

vol/vol: volume *per* volume

wt/vol: weight *per* volume

X-gal: 5-Bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside

X-pho: 5-Bromo-4-chloro-3-indolyl phosphate

XLD: xylose-lysine-desoxycholate

the family *Enterobacteriaceae* (Brenner, 1984), and are typically Gram-negative straight rods, 0.7-1.5 × 2.0-5.0 μm in size, with colonies generally 2-4 mm in diameter. They are usually motile by peritrichous flagella, and are facultatively anaerobic.

Most *Salmonella* strains will grow on defined media without special growth factors, and will produce gas from glucose, reduce nitrates to nitrites, utilise citrate as a sole carbon source, and produce hydrogen sulphide (H_2S) from triple-sugar iron agar. In addition, salmonellae do not produce deoxyribonuclease and lipase, are urease negative, indole negative, do not ferment sucrose, salicin, inositol and amygdalin, and they do not oxidatively deaminate phenylalanine and tryptophan, but usually give positive lysine and ornithine decarboxylase (Møller's) reactions (Le Minor, 1984). The G + C content of *Salmonella* DNA is 50-53 moles percent (by chemical analysis, T_m and buoyant density; Hill, 1966).

1.2.2 Nomenclature

The species concept in the genus *Salmonella* has evolved in four overlapping phases, with emphasis on clinical characteristics, antigenic specificities, biochemical properties and DNA relatedness reflecting the methodology and knowledge available at a particular time (Le Minor and Popoff, 1987). The Approved Lists (Skerman *et al.*, 1980) recognised five *Salmonella* species, namely *S. arizonae*, *S. choleraesuis*, *S. enteritidis*, *S. typhi* and *S. typhimurium,* as members of a single genospecies with *S. choleraesuis* as the type species. However, to avoid confusion in distinguishing between the single *Salmonella* species names and serotype names in clinical practice, Le Minor and Popoff in 1987 proposed that a single *Salmonella* species be given a name which had not been used earlier for a serotype. Therefore, it was suggested that the type species of the genus *Salmonella* be *S. enterica*, with strain CIP 60.62 (an H_2S-producing clone of strain LT2) as the type strain. The nomenclatural status of the names included in the Approved Lists was

subsequently modified as subjective synonyms of *S. enterica* subspecies *enterica* (Le Minor and Popoff, 1987). Accordingly, *S. enteritidis* is now *S. enterica* serovar Enteritidis, or *S.* Enteritidis, while *S. typhimurium* becomes *S. enterica* serovar Typhimurium, or *S.* Typhimurium.

1.2.3 Epidemiology

Salmonellae are pathogenic for humans and other animals, causing a spectrum of diseases which include enteric (typhoid) fever, gastroenteritis, septicaemia, and nontyphoid salmonellosis. These diseases are generally referred to as salmonellosis (Le Minor, 1984; Cohen and Tauxe, 1986). Some serovars of *Salmonella* are highly host-specific, rarely causing disease in more than one species. *S.* Typhi, for example, only infects humans, causing typhoid fever. Other serovars such as *S.* Typhimurium and *S.* Enteritidis have a broad host range and cause disease in many species. However, some serovars have specific animal resevoirs but frequently cause human infections. The human pathogens *S.* Heidelberg and *S.* Litchfield, for example, have primarily avian and reptilian reservoirs, respectively (Cohen and Tauxe, 1986). The serotyping of salmonellae by means of somatic and flagellar antigens, and subtyping by antibiotic sensitivity testing, biochemical reactions, phage typing and analysis of the plasmids they carry, have been useful in defining the epidemiology of salmonellosis.

The incidence of salmonellosis is fast becoming a global health problem. Apart from being a major cause of morbidity and mortality in humans and animals, it is also associated with considerable financial burden. For example, animal breeders incur costs from illnesses and deaths of their breeding stock of animals, restaurants and industry incur cost from lost business and lawsuits, and patients experience discomfort and direct and indirect costs (Cohen and Tauxe, 1986).

In the United Kingdom, there have been four dramatic rises in the incidence of human salmonellosis since data were first collected in the 1940s (Cooke, 1990). During the first peak in the 1950s, *S.* Typhimurium predominated, and the source of infection was attributed to infection in cattle and poultry. During the second peak between the late 1960s and the 1970s, serotypes other than *S.* Typhimurium were implicated, and the source of infection was traced to contaminated imported animal feed and associated with infection in poultry. During the late 1970s and early 1980s (the third peak), *S.* Typhimurium was again the main cause of salmonellosis, and this outbreak was attributed to infected cattle. Since 1985 however, the most recent increase in salmonellosis has been attributed to a huge rise in cases of *S.* Enteritidis [mostly phage type 4 (PT4)] (Rampling *et al.*, 1989; Stevens *et al.*, 1989), while *S.* Typhimurium cases increased less than two-fold, with the incidence of other *S.* Enteritidis phage types and other *Salmonella* serovars remaining unchanged (Cooke, 1990; Rodrigue *et al.*, 1990). This epidemic of salmonellosis in the United Kingdom due to *S.* Enteritidis PT4 has been associated with contaminated poultry and eggs, possibly due to infection of the ovaries (transovarian transmission) and oviduct (Timoney *et al.*, 1989), or alternatively due to faecal contamination and penetration of the egg shell by *Salmonella* organisms (Borland, 1975).

In Europe, salmonellosis in humans particularly due to infections with *S.* Enteritidis, is also a major problem. In some parts of Italy, *S.* Enteritidis infection has increased from about 3-4% of the total number of infections in the mid-1980s to more than 30% by 1990 (Binkin *et al.*, 1993). In Hungary, *S.* Enteritidis PT1 caused about 70% of human salmonellosis up to 1993 (Nagy *et al.*, 1993). In Germany, more than 100,000 cases of enteric infections were recorded for the first time in 1990, and the incidence is still increasing, with a yearly overall loss to the general economy estimated to be DM600 million. *S.* Enteritidis PT4 is also the predominant human isolate in Germany (Schulte, 1994). In Sweden, outbreaks of human salmonellosis have also been reported as far back

as 1976, mostly due to *S.* Typhimurium (Fonden *et al.*, 1976; Hakansson *et al.*, 1976). In Denmark, the incidence of salmonellosis in humans, poultry and cattle has also been documented, and *S.* Typhimurium and *S.* Enteritidis predominate. For instance, cases of human salmonellosis between 1988 and 1993 was due to the prevalence *S.* Typhimurium PT12, reflecting the increasing significance of pork as the source of infection (Wegener *et al.*, 1994). In the *S.* Enteritidis cases during the period of 1980-1990, PT1 was the most common type among isolates of poultry origin, followed by a PT4 strain. Isolates belonging to a PT8 strain were found exclusively in imported birds, while a PT4 strain predominated in the human isolates, as in most other Western European countries (Brown *et al.*, 1994).

In the United States of America and Canada, cases of salmonellosis have also steadily increased over the years, accounting for 10-15% of all instances of acute gastroenteritis in the United States (Rubin and Weinstein, 1977; Cohen and Tauxe, 1986). *S.* Typhimurium is the most frequently isolated serovar, and has been responsible for the largest foodborne outbreaks ever reported in both countries (Ryan *et al.*, 1987; Todd, 1989). Recent estimates of cases of salmonellosis in the USA range from 800,000 to 4 million per year, with an economic impact of about US$50 million (Cohen and Tauxe, 1986; Todd, 1990). *S.* Enteritidis accounts for 21% of all *Salmonella* infections in humans during this period, with most of these infections being caused by PT8 (Poppe *et al.*, 1993), and grade A shells have been implicated as the source of infection (St. Louis *et al.*, 1988). In Canada, *S.* Enteritidis also ranks second (20.3% in 1991) as the most commonly isolated serovar after *S.* Typhimurium, followed by *S.* Heildelberg (12.0%) and *S.* Hadar (10.9%; Lior and Khakhria, 1992). *S.* Enteritidis PT8 also predominates over other *S.* Enteritidis phage types, accounting for between 4.2 and 9.2% of all salmonellae isolated from humans between 1976 and 1989 (Khakhria *et al.*, 1991), and increasing to 12.5% in 1991 (Lior and Khakhria, 1992).

The incidence of salmonellosis has also been documented in Southern Africa (Durand *et al.*, 1990; Stadler and Nesbit, 1990), West Africa (Oboegbulem and Okoronkwo, 1990), and Central Africa (Gendrel *et al.*, 1994). An outbreak of salmonellosis in Italy was also traced to acquisition from North Africa (Lento, 1979).
The incidence of salmonellosis and the phage type distribution of isolates has also been reported in various countries in Asia, including Vietnam (Ricosse *et al.*, 1979), Indonesia (Sanborn *et al.*, 1979), and Japan (Kobayashi *et al.*, 1989).

In Australia, the incidence of salmonellosis has been documented for decades, and in the last three years there has been a slow increase in the incidence of *S.* Enteritidis, mostly of PT4 strains. This incidence has been attributable to acquisition from overseas, particularly from Europe and South East Asia and the usual sources are humans and egg-laying chickens. *S.* Enteritidis strains are now endemic in Australia and constitute the top 10 most common *Salmonella* isolates (Australian *Salmonella* Reference Centre, Institute of Medical and Veterinary Science, Adelaide, South Australia, 1994 Annual Report).

1.2.4 *Salmonella* pathogenesis

1.2.4.1 Introduction

Salmonellae are disseminated through oral ingestion of contaminated food or water. Upon entry, the bacteria penetrate the intestinal epithelium in membrane-bound vacuoles, from where they spread into deeper host tissues (Takeuchi, 1967). Evidence indicates that the organisms preferentially associate with the specialised membranous epithelial cells (M cells) within Peyer's patches of the distal ileum (Carter and Collins, 1974; Owen, 1977; Hohmann *et al.*, 1978; Clark *et al.*, 1994; Jones *et al.*, 1994), inducing membrane ruffles on the surface of the infected cell at the site of bacterial entry (Takeuchi, 1967; Kohbata *et al.*, 1986; Finlay and Falkow, 1990; Francis *et al.*, 1992; Ginocchio *et al.*, 1992; Clark *et al.*, 1994; Jones *et al.*, 1994). However, small numbers of salmonellae also localise in

columnar epithelial cells (Hohmann *et al.*, 1978; Kohbata *et al.*, 1986). Depending on the infecting serovar and the host infected, the bacteria may then be transported by macrophages to reticuloendothelial tissues such as regional lymph nodes, the liver and the spleen, where they multiply further. Alternatively, they may remain in the lamina propria of the intestinal epithelium (Carter and Collins, 1974; Hohmann *et al.*, 1978; Hook, 1985; Kohbata *et al.*, 1986). There is also evidence to suggest that splenic infections arise from a source other than the ileal Peyer's patches (Hohmann *et al.*, 1978), and the absorptive epithelium has been implicated as a possible source of infection (Finlay and Falkow, 1989).

1.2.4.2 *Salmonella*-host cell interactions

Salmonella-host cell interactions are believed to be specific, involving a number of bacterial determinants and host cell receptors (Moulder, 1985; Finlay and Falkow, 1989; Isberg, 1991; Falkow *et al.*, 1992, Ginocchio *et al.*, 1992; 1994) in a process termed microbial-directed endocytosis (Moulder, 1985), often involving the subversion of existing host cell signal transduction pathways (Bliska *et al.*, 1993).

A variety of tissue culture cell assays have been used as *in vitro* model systems to study *Salmonella*-host cell interactions, because such model systems mimic the invasion of enterocytes *in vivo*, allowing the identification of the genes and gene products involved in the interactions. Various studies indicate that efficient entry of *Salmonella* into tissue culture cells is multifactorial and complex, involving several chromosomal loci, most of which map between 58 and 60 min on the *Salmonella* chromosome (Finlay *et al.*, 1988; Elsinghorst *et al.*, 1989; Galán and Curtiss, 1989a; Ginocchio *et al.*, 1992; Lee *et al.*, 1992; Stone *et al.*, 1992; Altmeyer *et al.*, 1993; Collazo *et al.*, 1995; Mills *et al.*, 1995; Stone and Miller, 1995). It is now recognised that as much as 35-40 kb of DNA encompassing a contiguous region of *Salmonella* chromosome at centisome 63 encode determinants for

entry into mammalian cells (Mills *et al.*, 1995). Furthermore, a number of regulatory genes have been shown to be necessary for the survival of salmonellae in macrophages (Groisman *et al.*, 1989; Miller *et al.*, 1989; Fields *et al.*, 1989; Miller *et al.*, 1992; Behlau and Miller, 1993). For example, the PhoP (transcriptional activator) and PhoQ (sensor kinase) proteins constitute a two-component regulatory system that controls the coordinate synthesis and expression of many *Salmonella* proteins required for virulence and survival within macrophages (Miller *et al.*, 1989). In addition, the *phoP*-regulated gene products inhibit the processing and presentation of *S.* Typhimurium antigens as determined by an *in vitro* T cell-mediated immunity model (Wick *et al.*, 1995), ascribing a role for this locus in the inhibition of induction of specific, effective immunity. A *Salmonella* gene (*slyA*) encoding a cytolysin (salmolysin) has also been identified as necessary for virulence of salmonellae. This toxin facilitates the survival of these organisms within mouse peritoneal macrophages (Libby *et al.*, 1994). Similarly, a *Salmonella* cytotoxin that is firmly associated with the outer membrane has been proposed to have a possible role in damage and/or invasion of Chinese hamster ovary cells (Reitmeyer *et al.*, 1986). Although the inter-relationship between these factors is not fully understood, it has been observed that strains with mutations in these chromosomal loci and their regulatory genes are unable to invade cultured epithelial cells, cannot survive in macrophages, and are less virulent to mice (Fields *et al.*, 1986; Finlay *et al.*, 1988; Galán and Curtiss, 1989a, b; Miller *et al.*, 1989; Miller *et al.*, 1992; Stone and Miller, 1995).

In addition to the invasion loci and regulatory genes, it has been suggested that other bacterial components are necessary for invasion, survival and/or intracellular replication in eucaryotic cells. These include lipopolysaccharide (Mäkelä *et al.*, 1973; Jones *et al.*, 1982; Finlay *et al.*, 1988; Mroczenski-Wildey *et al.*, 1989), motility (Liu *et al.*, 1988; Curtiss *et al.*, 1993), and several loci in the virulence plasmid (Jones *et al.*, 1982; Helmuth *et al.*, 1985; Fields *et al.*, 1986; Lax *et al.*, 1993). However, the role of these

components in bacterial invasion varies between *Salmonella* serovars (Finlay and Falkow, 1989).

1.2.4.3 Intracellular multiplication

Salmonellae are internalised individually in membrane-bound vacuoles, and in some cases, they replicate extensively in epithelial cells, with a generation time of approximately 40-50 minutes (Yokoyama *et al.*, 1987; Finlay and Falkow, 1988, 1989). Individual vacuoles containing these bacteria appear to coalesce into large vacuoles, and after 12-24 hours of intracellular multiplication, most invaded cells have large vacuoles filled with salmonellae which lift off the infected monolayer without disrupting the eucaryotic cell membrane (Finlay and Falkow, 1988; Finlay and Falkow, 1989). How these bacteria obtain nutrients and other components necessary for intracellular multiplication has not been clearly defined, however, an *aroA* mutant of *S.* Choleraesuis has been defined which could invade and penetrate through polarised monolayers of Caco-2 and MDCK epithelial cells at normal levels, but was unable to multiply within these cells (Finlay *et al.*, 1991a). Likewise, a Tn*phoA* mutant in *S.* Choleraesuis has been described which transcytosed MDCK monolayers at normal levels, but was unable to multiply intracellularly (Finlay *et al.*, 1988).

Unlike *Shigella* species which escape from the phagosome into the cytoplasm where they multiply rapidly soon after uptake (Wassef *et al.*, 1989; Zychlinsky *et al.*, 1992; Perdomo *et al.*, 1994), the rate of intracellular multiplication of *Salmonella* in macrophages is slower and more variable because the bacteria must adjust to, and survive within, the acidified phago-lysosome. Thus, there is a lag period of several hours before salmonellae achieve optimal intracellular division rates (Finlay *et al.*, 1988; Finlay and Falkow, 1989). Salmonellae do not require endosome acidification for entry, intracellular growth, or escape from the endosome (Finlay and Falkow, 1988). Whereas some studies

indicate that *S.* Typhimurium does not inhibit phagosome-lysosome fusion in macrophages (Carrol *et al.*, 1979), other studies suggest that these organisms could actively inhibit this process, and appeared to divide within the unfused phagocytic vesicles (Buchmeier and Heffron, 1991). Ishibashi and Arai (1990a) demonstrated that fusion of phagosomes containing *S.* Typhimurium LT2 was impaired, whereas *S.* Typhi 1079 did not inhibit phagosome-lysosome fusion in murine macrophages. The inhibition of phagosome-lysosome fusion does not appear to play a critical role in the pathogenesis of *S.* Typhimurium, although it might contribute at least in part, to the survival of the organisms within macrophages (Buchmeier and Heffron, 1991; Ishibashi *et al.*, 1992). However, recombinant IFN-γ enhanced phagosome-lysosome fusion in *S.* Typhimurium-infected murine macrophages, leading to a slight decrease in phagocytosis and an enhanced killing of these organisms (Kagaya *et al.*, 1989; Ishibashi and Arai, 1990b). Data obtained from the studies by Fields *et al.* (1986) and Gahring *et al.* (1990) indicated that additional bacterial genes are required for survival of *Salmonella* within phagocytic cells, and that these organisms may have two entry pathways into macrophages - the classical phagocytic pathway, and a specific invasion pathway normally used for invasion of non-phagocytic mammalian cells.

The perception that salmonellae are facultative intracellular pathogens as suggested by Suter and Ramseier (1964) and by other workers has been questioned by Hsu (1989). Using a mouse typhoid model, Nakoneczna and Hsu (1980) observed an early infiltration of polymorphs with a conspicuous absence of macrophages at the site of *S.* Typhimurium infection. The predominance of polymorphs coincided with the most active phase of bacterial proliferation, similar to that observed with extracellular bacteria (Xu and Hsu, 1992). Bacterial invasion and replication was observed within hepatocytes, resulting in cellular degeneration, and this was followed by progressive and invasive bacterial proliferation in extracellular locations within sinusoids and in tissue lesions. In addition, a

consistently rapid destruction of salmonellae within inflammatory polymorphs and macrophages was also observed (Hsu, 1989). Similar observations were made by Lin *et al.* (1989). These findings suggest that the notion that salmonellae are facultative intracellular bacterial pathogens (IBPs) may be incorrect, and more work needs be done to clarify this issue.

1.2.4.4 Mechanisms of invasion by *Salmonella*

The underlying molecular and genetic mechanisms involved in the invasion of host cells by salmonellae are not fully defined as yet, but are being increasingly dissected. During the course of the present study, the work of various groups has led to a significantly better understanding of these mechanisms. It has been postulated that the entry of salmonellae into eucaryotic cells is the result of a multi-step process, culminating in host cell membrane ruffling and leading to internalisation of bacteria. It is believed that the first step involves the adherence of the bacteria to a host cell receptor(s). The bacteria subsequently release a soluble factor (Ginocchio *et al.*, 1994; Collazo *et al.*, 1995; Zierler and Galán, 1995), which triggers a signal transduction cascade within the host cell, leading to the internalisation of the bacteria. The events that trigger internalisation require a battery of bacterial proteins, including the components of a dedicated protein export system, termed type III, clustered together at the *inv* locus on the *Salmonella* chromosome (Galán and Ginocchio, 1994). One of these proteins, InvC (Eichelberg *et al.*, 1994), is an ATPase which presumably energises the transport system. Another component, InvG (Kaniga *et al.*, 1994), is an outer membrane-associated translocase. Apart from InvC and InvG, another *Salmonella* protein, InvJ, has also been shown to be involved in bacterial entry into cells and is secreted via the type III secretory pathway (Collazo *et al.*, 1995).

Contact of salmonellae with host cells results in the assembly of peritrichous appendages called invasomes (Ginocchio *et al.*, 1994). Invasome assembly requires a

functional type III secretion system, since mutations in *invC* or *invG* prevent invasome assembly and result in a drastic reduction of entry of salmonellae into host cells, indicating a close correlation between invasome assembly and the internalisation process. Furthermore, invasion-defective *invC* and *invG* mutants of *S.* Typhimurium do not secrete InvJ upon contact with live Henle-407 cells (Zierler and Galán, 1995).

The processes of adherence of salmonellae and the endocytic event leading to internalisation into the target cell appear to be independent of each other, and can be separated genetically. Mutations in *invA* or *invE*, for example, render salmonellae non-invasive, but do not affect the ability of the bacteria to attach to cultured epithelial cells (Galán and Curtiss, 1989a; Ginocchio *et al.*, 1992). However, it is unclear whether attachment has to be mediated by specific adhesins (fimbriae?) that target *Salmonella* organisms to a specific receptor(s), or if it is simply enough for the adhesins to bring *Salmonella* organisms to a critical distance from the eucaryotic cell in order to trigger endocytosis. Although it is known that (at least with *Yersinia enterocolitica*), attachment alone is necessary, but not sufficient for invasion (Miller and Falkow, 1988), it has been demonstrated that certain mutations that affect the ability of *Salmonella* organisms to attach to cultured cells render these organisms deficient in their ability to invade cells (Finlay *et al.*, 1988; Gahring *et al.*, 1990).

Although type 1 fimbriae of *S.* Typhimurium and *S.* Typhi have been shown to mediate their attachment to a variety of epithelial cells in culture (Jones and Richardson, 1981; Lockman and Curtiss, 1992; Curtiss *et al.*, 1993; Satta *et al.*, 1993), the role of these fimbriae in the invasion of eucaryotic cells and in virulence is somewhat controversial. For instance, it was shown that type 1 pili do not seem to have a role in invasion as indicated by the observation that the presence of mannose, a known inhibitor of type 1 pili, does not significantly alter the ability of *Salmonella* to enter epithelial cells (Tannock *et al.*, 1975; Jones and Richardson, 1981). Popoff (1991) demonstrated that in *S.*

Typhimurium, type 1 pili and mannose-resistant haemagglutinin played a marginal role in adhesion and invasion, and were not essential for full virulence expression. However, the findings of Satta *et al.* (1993) suggests that type 1 fimbriae might play a role in *S.* Typhi infection at the point of invasion of INT-407, HeLa and MDCK epithelial cells. It seems likely that the fimbriae mediate a close and stable interaction between the two cell surfaces, which in turn could be necessary for the invasion process to occur.

Recent evidence indicates that distinct fimbriae contribute to the tissue tropism of *S.* Typhimurium for the murine villous small intestine (Bäumler *et al.*, 1996a) and murine Peyer's patches (Bäumler *et al.*, 1996b). Whereas the *lpf* fimbrial operon of *S.* Typhimurium encoding for long polar fimbriae (Bäumler and Heffron, 1995) was shown to be necessary for the adhesion of these bacteria to HEp-2 cells, but not to MDCK, HeLa, or INT-407 cells (Bäumler *et al.*, 1996c), mutations in the fimbrial operons *fim* (Clegg and Swenson, 1994) and *pef* (Friedrich *et al.*, 1993), which encode for type 1 fimbriae and plasmid-encoded fimbriae, respectively, did not affect the ability of *S.* Typhimurium to adhere to the HEp-2 cells (Bäumler *et al.*, 1996c). These findings suggest that distinct fimbrial operons mediate the adhesion of *S.* Typhimurium to different epithelial cell lines *in vitro*, or tissues *in vivo*, based on the surface epitopes or receptors present on the various target cell types.

A gene, *invH*, has been identified in *S.* Choleraesuis and *S.* Typhimurium that is necessary for efficient adherence and entry of these organisms into cultured epithelial cells (Altmeyer *et al.*, 1993). This gene is present in all *Salmonella* serovars examined except *S.* Arizonae. Inactivation of the *invH* gene affected the attachment and/or invasion phenotypes of only a restricted group of *Salmonella* serotypes. The effect was more pronounced in the host adapted strains *S.* Gallinarum, *S.* Choleraesuis and *S.* Typhi than other strains of *Salmonella* with broader host ranges, such as strains of *S.* Enteritidis, and *S.* Typhimurium. On the basis of these observations, it has been hypothesised that

salmonellae have a number of alternative mechanisms which can help to dock the bacteria in critical proximity to the eucaryotic cell surface and allow other gene products to trigger the endocytic event (Altmeyer *et al.*, 1993).

1.2.4.5 Signal transduction and membrane ruffling during entry of *Salmonella*

The appearance of membrane ruffles on the surface of *S.* Typhi and *S.* Typhimurium-infected cells is accompanied by profound cytoskeletal rearrangements at the point of the bacterial-host cell contact (Kohbata *et al.*, 1986; Clark *et al.*, 1994; Jones *et al.*, 1994) and a number of cytoskeletal proteins, including actin, α-actinin, talin, tubulin, tropomyosin, and ezrin, accumulate at these sites (Finlay *et al.*, 1991b). Entry of *S.* Typhimurium into HeLa epithelial cells produces extensive aggregation of cell surface class I MHC heavy chains, β 2-microglobulin, fibronectin-receptors (α 5 β 1 integrin), and hyaluronate receptors (CD-44), and to a lesser extent, other cell surface proteins such as transferrin-receptor and Thy-1 (Garcia-del-Portillo *et al.*, 1994). The complement receptor type 1 and type 3 on murine macrophages have been identified as receptors for *S.* Typhimurium and *S.* Typhi, respectively (Ishibashi and Arai, 1990c). It is believed that actin is likely to play a key role in the formation of membrane ruffles because inhibitors of actin microfilament function block the entry of *Salmonella* organisms into cells (Kihlstrom and Nilsson, 1977; Buckholm, 1984; Finlay and Falkow, 1988; Garcia-del-Portillo *et al.*, 1994). Infection of cultured cells by *S.* Typhimurium is also accompanied by a marked increase in intracellular free calcium ($[Ca^{2+}]_i$) (Ginocchio *et al.*, 1992; Pace *et al.*, 1993), which possibly plays a role in the formation of membrane ruffles. This increase in $[Ca^{2+}]_i$ is critical for internalisation of the bacteria since Ca^{2+} chelators and antagonists of Ca^{2+} channels block *S.* Typhimurium internalisation and non-invasive bacterial mutants do not induce a $[Ca^{2+}]_i$ flux (Pace *et al.*, 1993).

It has been observed that prior to the events leading to membrane ruffling, cytoskeletal rearrangements and increase in $[Ca^{2+}]_i$, a number of host cell surface receptors are activated. This includes the epidermal growth factor receptor (EGFR). After EGFR activation, a signal transduction pathway follows; this involves the phosphorylation and activation of extracellular-regulated protein kinase and subsequent activation of phospholipase A_2, eventually leading to bacterial entry (Moolenar *et al.*, 1984; Kadowaki *et al.*, 1986; Rijken *et al.*, 1991; Galán *et al.*, 1992).

1.2.4.6 Environmental signals which influence entry of *Salmonella* into cells

There are various reports that a number of environmental cues, including oxygen tension, osmolarity, and/or growth state, influence to a great extent, the capacity of *Salmonella* organisms to enter mammalian cells (Ernst *et al.*, 1990; Galán and Curtiss, 1990; Lee *et al.*, 1992). These environmental conditions affect the degree of DNA supercoiling, which in turn influences the expression of the invasion gene *invA* (Galán and Curtiss, 1990). It therefore appears that the regulation of expression of the invasion genes in salmonellae may require the function of a series of gene products. This possibility is supported by the observation that a constitutive mutation in the *S.* Typhimurium *hil* (*h*yper-*i*nvasion *l*ocus) locus allows similar levels of entry when the organisms are grown under conditions of low (non-permissive) and high (permissive) oxygen tension, whereas a deletion encompassing this mutation was defective for entry (Lee *et al.*, 1992). The finding suggests that *hil* encodes an invasion factor or an activator of invasion factor expression that is essential for bacterial entry into mammalian cells. Recently, Véscovi *et al.* (1996) established that extracellular Mg^{2+} is the environmental signal that controls the PhoP/PhoQ regulon in *S.* Typhimurium by demonstrating that a mutation in the sensing domain of PhoQ resulted in an altered responsiveness to Mg^{2+}, and rendered these organisms avirulent.

1.3 Immunity to *Salmonella*

1.3.1 Introduction

The immune response to infections caused by salmonellae and other IBPs such as *Listeria monocytogenes*, or *Brucella abortus*, is complicated by the fact that these organisms can multiply within the cells whose normal function is to kill them (Collins, 1993). The precise mechanisms used by these pathogens to elude the host immune responses is not fully understood (Lowrie and Andrew, 1988), however, *S.* Typhimurium has several peptide-resistance loci that allow it to colonise successfully host tissues that are rich in antimicrobial peptides, and this property has been correlated with virulence (Fields *et al.*, 1989; Groisman *et al.*, 1992).

Resistance to infections by salmonellae has been shown to depend on a number of apparently unrelated factors, both of host and microbial origin, and involves a combination of humoral and cellular factors (Collins, 1993). From infection studies, it has been shown that normal mice infected intravenously with a sublethal dose of *S.* Typhimurium or *S.* Enteritidis exhibit a growth cycle comprising at least four distinct phases, extending over a period of weeks or even months. It has been demonstrated that this process can lead to solid immunity to rechallenge (Collins, 1974; 1993). The first phase, lasting for a few minutes to about an hour, involves the initial inactivation of as much as 90% of the challenge inoculum (innate immunity). This phase is followed by a phase of exponential bacterial growth in the reticuloendothelial system (RES) over the first week, and then, a plateau phase in which growth is suppressed. Finally, the bacterial population passes into a slow decline phase, eventually resulting in the clearance of the organisms.

1.3.2 Role of macrophages in immunity to *Salmonella*

It is generally believed that mononuclear phagocytes play an important role in the innate resistance of mice against infection by *Salmonella* organisms. The effectiveness of resident macrophages in this phase of infection depends on the genetic background of the host (Hormaeche *et al.*, 1985), and in the case of *S.* Typhimurium at least, resistance is under the control of the *Ity* gene which controls the growth rate of these organism in macrophages (Lissner *et al.*, 1983; O'Brien, 1986). Thus, macrophages harvested from C3H (Ity^r) mice are more bactericidal for *S.* Typhimurium than similar cells harvested from C57BL/6 (Ity^s) mice (Blumenstock and Jann, 1981). The extent of the activity of the macrophages is also greatly enhanced by the presence of specific opsonic antibodies and/or complement (Collins, 1971), and seems to be independent of functional T cells, at least in the Ity^r mice (O'Brien and Metcalf, 1982).

Macrophages have also been shown to diminish T cell responsiveness to *Salmonella* organisms. According to Deschenes *et al.* (1986), spleen cells from mice infected with live *S.* Typhimurium showed reduced responsiveness to T (and B) cell mitogens *in vitro*, and this activity was partially attributed to adherent cells. Similarly, Kotlarski *et al.* (1989) demonstrated that lymphoid cells harvested from mice infected with *S.* Enteritidis 11RX were unresponsive in *in vitro* proliferation assays unless most of the adherent cells were removed by passage through nylon-wool columns.

The activation of macrophages results in a different series of functional and biochemical changes which are not observed in resident macrophages. Besides greater ability to kill salmonellae, activated macrophages are able to inhibit intracellular multiplication of certain protozoa such as *Toxoplasma gondii* (Eisenhauer *et al.*, 1988), *Leishmania donovani* (Pappas and Nacy, 1983), and *Trypanosoma cruzi* (Hoff, 1975), and display an enhanced ability to secrete reactive oxygen metabolites (Murray *et al.*, 1985), reactive nitrogen intermediates (Nibbering *et al.*, 1991), and have an increased expression of Ia antigen (Koerner *et al.*, 1987).

According to Mackaness *et al.* (1966) and Collins (1974), activated macrophages are the most important defense mechanism against *S.* Typhimurium. Activation of macrophages by T cell-derived cytokines such as interferon gamma (IFN-γ) is believed to overcome infections by facultative intracellular pathogens (Mackaness, 1971), as inferred from the observation that mice that were infected with non-lethal doses of Bacillus Calmette Guérin (BCG), *Brucella* spp, or *L. monocytogenes*, were more resistant to infections by these bacteria, and this was accompanied by a decreased intracellular survival of the bacteria in macrophages (Mackaness, 1964; Blanden *et al.*, 1969). However, it has been reported that immunisation with an *S.* Typhimurium *aroA* mutant induces the activation of natural killer (NK) cells, which possibly have a role in the destruction of *S.* Typhimurium-infected cells (Garcia-Penarrubia *et al.*, 1989; Schafer and Eisenstein, 1992), resulting in the release of viable bacteria which can then become opsonised by antibodies before being ingested and killed by granulocytes. In addition, it has been shown that granulocytes rather than macrophages are responsible for the elimination of *S.* Typhimurium at primary foci of infection (Nakoneczna and Hsu, 1980; Wang *et al.*, 1988). These findings indicate that although macrophages become activated during a *S.* Typhimurium infection, there is some doubt that they are the single most important factor in the defense against a *Salmonella* infection.

1.3.3 Humoral immunity to *Salmonella*

Specific antibodies are believed to play a crucial role in immunity to *Salmonella* organisms, unlike the situation with other IBPs such as *L. monocytogenes* (Mackaness, 1971) or *Mycobacterium tuberculosis* (Lurie, 1942). The significance of the role of antibodies may depend on the route of infection. For example, Jenkin *et al.* (1964) demonstrated that specific antibodies play an important role in determining the resistance of mice to *S.* Typhimurium C5 (C5). Similarly, Rowley *et al.* (1968) showed that infection

of mice with an avirulent strain of *S.* Enteritidis (11RX), provided immunity to the normally virulent C5 strain, and that specific antibodies were important for this protection. Collins (1970) reported that when mice were immunised with a killed *Salmonella* vaccine, the antibodies elicited played a major role in the survival of mice following oral challenge with virulent salmonellae, and proposed that this was accomplished by a reduction in the number of bacteria which invaded gut tissues thereby prolonging dissemination of the organisms to the spleen and the liver. Davies and Kotlarski (1976) also demonstrated a requirement for antibodies as well as a cell-mediated immune response for survival of mice against high challenge doses of C5. When mice were preimmunised with alcohol-killed C5 followed by infection with live 11RX, they were able to survive large challenge doses of C5, whereas mice immunised with either live 11RX alone or killed C5 organisms alone were as susceptible as unimmunised animals (Collins, 1969a, b; Davies and Kotlarski, 1976). Furthermore, Matsui and Arai (1989) showed that a high level of protection, resulting from the induction of cell-mediated immunity and humoral responses, was obtained when mice were immunised with porin from *S.* Typhimurium LT2, and that both T cells and sera taken from such mice could transfer the protection against salmonellosis to normal, nonimmunised mice. A similar result was obtained by Muthukkumar and Muthukkaruppan (1993) with porin-lipopolysaccharide-immunised mice. The work of Michetti *et al.* (1992) showed that *in vivo* delivery of a single monoclonal secretory IgA directed against a carbohydrate epitope exposed on the surface of *S.* Typhimurium was sufficient to confer protection against oral challenge with the same bacteria. Presumably this occurs by immune exclusion at the mucosal surface, because the IgA did not confer any protection against intraperitoneal challenge. Quite recently, Peralta *et al.* (1994) showed that hen egg-yolk antibodies raised against the purified 14 kDa fimbriae of *S.* Enteritidis (SEF14) provided passive protection in orally infected mice, and suggested that the egg-yolk antibodies against these fimbriae may have played a significant role in

protection by minimising bacterial colonisation and invasion during the early stages of infection. Thus, it appears that since the oral route is a natural means of infection, antibodies are clearly important in protection.

1.3.4 Cell-mediated immunity to *Salmonella*

Until 1942, the importance of cell-mediated immunity in controlling infections with IBPs was only indirectly indicated by the observations that antibodies alone did not appear to provide effective immunity to these pathogens. In 1942, Lurie demonstrated the importance of cell-mediated immunity in resistance to *M. tuberculosis*. Subsequently, the importance of CMI was also established in *Listeria* infection when it was demonstrated that resistance to this organism could be transferred with lymphoid cells, but not with serum (Mackaness, 1971). In the case of *Salmonella* infections, both CMI and antibodies apppear to be essential for immunity, as shown by the observation that mice immunised with either killed vaccines alone or live vaccine strains of different serotype from that used for challenge were not protected against challenge with a virulent strain (Collins, 1969a, b; Davies and Kotlarski, 1976).

Further studies with *Listeria, Mycobacterium* and *Salmonella* organisms have established that CMI is required for effective clearance of these organisms (Lane and Unanue, 1972; Lefford, 1975; Davies and Kotlarski, 1976). More recently it has been shown that this involves cooperation between major histocompatibility complex (MHC) class-I restricted Lyt-2^+ T cells and MHC class II-restricted L3T4^+ T cells (Kaufmann *et al.*, 1985; Pedrazzini *et al.*, 1987; Pope and Kotlarski, 1994), although the Lyt-2^+ subset appears to be ultimately responsible for clearance of listeriae and salmonellae, especially during secondary infection (Bishop and Hindrichs, 1987; Mielke *et al.*, 1988; Pope *et al.*, 1994; Pope and Kotlarski, 1994). It is believed T cells are responsible for protection because following recognition of bacterial antigen, specific T cells release cytokines which

activate tissue macrophages, enhancing their bactericidal activity (Kaufmann and Hahn, 1982; Attridge and Kotlarski, 1985a). Animals remain resistant to secondary infection because the expanded population of recirculating sensitised T cells are relatively long-lived (North, 1975; North and Deissler, 1975), and can be activated quickly to release the cytokines needed to activate phagocytic cells.

The work of Davies and Kotlarski (1976) established the involvement of T cells in resistance to salmonellae. They demonstrated that thymectomy of mice led to a reduced clearance of the avirulent *S.* Enteritidis 11RX strain, and a failure to immunise against the highly virulent *S.* Typhimurium C5 strain. Although the phenotype of the T cells induced by immunisation with the 11RX strain was not established in that study, subsequent work by Attridge and Kotlarski (1985a, b) using adoptive transfer of cells from normal mice previously immunised with live 11RX, showed that cells mediating delayed-type hypersensitivity (DTH) reactivity induced by an antigenic extract of 11RX were nonadherent T cells of $Lyt1^+$ ($L3T4^+$) phenotype, and that the response was class II-restricted. They also showed that in the presence of specific antigen and adherent accessory cells, these T cells were induced to release macrophage activation factor (MAF) and interleukin-2 (IL-2) *in vitro*, which in turn maintained the proliferation of the T cells.

The phenotype of the T cells involved in protection against salmonellae was established by Nauciel (1990), who demonstrated that *in vivo* depletion of $CD4^+$ T cells by infusion of anti (α)-CD4 monoclonal antibody abolished the ability of mice to clear an infection of a temperature sensitive mutant of *S.* Typhimurium. In addition, *in vitro* treatment of immune T cells with α-CD4 antibodies and complement abrogated their capacity to transfer adoptive immunity. Resistance to reinfection was also reduced after treatment of mice with α-CD4 antibodies. However, *in vitro* treatment with α-CD8 and complement had little effect, suggesting that $CD4^+$ T cells, rather than $CD8^+$ T cells, are

responsible for mediating resistance to salmonellae. Recently, Pope *et al.* (1994) and Pope and Kotlarski (1994) obtained data which indicate that *Salmonella*-specific cytotoxic Lyt-2^+ ($CD8^+$) T cells were induced during infection of mice with *Salmonella* organisms. This response was enhanced following secondary infection and could be detected in peritoneal and spleen cell suspensions from immunised animals. This observation may be related to findings by other workers studying other IBPs which suggest that the $CD4^+$ T cells are actually "helpers", and that the $CD8^+$ T cells are the "effectors" ultimately responsible for bacterial clearance (Kaufmann *et al.*, 1985; Bishop and Hinrichs, 1987; Boom *et al.*, 1987; Kaufmann, 1988; Mielke *et al.*, 1988)

Considerable interest is being generated in a group of gamma-delta bearing T cells present in small, but significant, numbers in normal lung, skin and intestine and associated lymphoid organs where they may serve a sentinel function (Janeway *et al.*, 1988). These T cells recognise the stress proteins released by a number of cells (Born *et al.*, 1990), particularly those produced by *M. tuberculosis* and *S.* Typhimurium (Havlir *et al.*, 1991). The protective role of these gamma-delta T cells is not fully characterised or understood, but it is believed that they may be part of an early immune response against a number of important pathogens.

1.3.5 *Salmonella* immunogens and immunity

It is generally accepted that live vaccines of attenuated salmonellae are much more effective in generating sensitised T cells (CMI) and protection against parenteral or oral challenge than killed suspensions or subcellular extracts (Collins and Carter, 1974; George *et al.*, 1987). The explanations provided for this difference include the possibilities that live attenuated vaccines are able to elicit the appropriate type(s) of accessory cells which act as a focus for the interaction of T cells with foreign antigens, that the live vaccine strains modulate the immune response to ensure CMI is mounted, or that these organisms

provide a more intense antigenic stimulus in the form of protective sensitins (stress proteins). Alternatively, live vaccines may display important antigenic determinants that are otherwise denatured during the process of inactivation. The slow, steady release of these immunogens may lead to the development of CMI instead of the humoral response induced with killed vaccines.

Stress proteins are produced by the live organisms in response to the harsh growth conditions encountered within phagosomes (Young *et al.*, 1988a, b). These proteins can also be recovered from culture filtrates of *in vitro*-stressed organisms. There is some evidence that some of these proteins can act as "protective" antigens, and some have been shown to interact with sensitised T cells harvested from mice recovering from infection (Collins *et al.*, 1988; Vordermeier and Kotlarski, 1990a). Some of these stress proteins can also induce an effective CMI when they are presented with a suitable adjuvant, as shown with mycobacterial culture filtrate proteins (Hubbard *et al.*, 1992). However, some caution is needed in their use because they are highly conserved proteins, likely to share antigenic determinants with infected hosts. Indeed stress proteins have been implicated in autoimmune complications seen in some chronically infected patients (Young, 1990), and several of the heat-shock proteins react with T-cell clones prepared from arthritis patients who had been infected earlier with salmonellae (Life *et al.*, 1991). Volkman and Collins (1973) have shown that some *Salmonella*-infected rats develop a progressive type of polyarthritis resembling human rheumatoid arthritis, and Young (1990) suggested that, rather than being caused by tissue damage due to bacterial multiplication during the acute phase of systemic infection, these lesions may have been induced by stress proteins which interact with sensitised T cells within the synovial fluid.

The transfer of anti-*Salmonella* immunity with an infusion of purified T cells from immune mice into naive syngeneic recipients has been difficult to achieve using the murine model. This might be due in part to the high susceptibility of most mouse strains to *S.*

Typhimurium and *S.* Enteritidis challenge (Hormaeche *et al.*, 1985) compared to inbred rats which have been used with more convincing results (Hougen and Jensen, 1990). However, Paul *et al.* (1985) reported successful transfer of anti-*Salmonella* immunity in BALB/c mice infused with immune lymph node and peritoneal T cells harvested from *Salmonella*-infected syngeneic donors. The recipient mice resisted challenge with virulent *S.* Enteritidis both in terms of reduced growth within the liver and spleen, and by an increase in survival time.

There is some controversy regarding the nature of the "protective" antigens (and their epitopes) that are produced by salmonellae. For instance, while some investigators have shown an increased protection in mice immunised with outer membrane proteins, others have found porin preparations from *S.* Typhimurium to be adequately protective (Udhayakumar and Muthukkaruppan, 1987a, b; Isibasi *et al.*, 1988; Matsui and Arai, 1989, 1990). These proteins were recognised by T cells of previously immunised donors (Young *et al.*, 1988b; Vordermeier and Kotlarski, 1990a). As these immunogens become better defined and cloned, there is an increasing likelihood that new, more effective recombinant vaccines for human use can be developed (Collins, 1993).

1.4 *Salmonella* Enteritidis

1.4.1 Introduction

As outlined in Section 1.2.3, *S.* Enteritidis is an invasive enteropathogen commonly associated with nontyphoid salmonellosis in humans and poultry infections (Cohen and Gangarosa, 1978; O'Brien, 1986; St. Louis *et al.*, 1988; Humphrey *et al.*, 1989; Rampling *et al.*, 1989; Cooke, 1990; Rodrigue *et al.*, 1990). The incidence of salmonellosis due to *S.* Enteritidis has increased dramatically worldwide in recent years. It has been established that antigen-specific T cell-mediated immunity is required for resistance to this organism,

as is the case with other salmonellae and other IBPs such as listeriae and mycobacteria (see Section 1.3.4).

1.4.2 Fimbriae of *S.* Enteritidis

There is considerable interest in fimbriae because they are potential immunogens against many pathogenic bacteria that colonise epithelial cell surfaces of animals, and also because they mediate specific attachments to the host cell surface in the early stages of infection (Duguid *et al.*, 1966; Duguid and Old, 1980; Pearce and Buchanan, 1980; Tramont and Boslego, 1985; Korhonen *et al.*, 1990; Krogfelt, 1991). *S.* Enteritidis is now known to produce a range of distinct fimbrial types, namely: SEF14 (Feutrier *et al.*, 1986; Thorns *et al.*, 1990), SEF17 (Collinson *et al.*, 1991), SEF18 (Clouthier *et al.*, 1994) and the type 1 fimbriae SEF21 (Müller *et al.*, 1991), with the following nomenclature: SefA, AgfA, SefD, and FimA refer to the fimbrins; *sefA, agfA, sefD*, and *fimA* refer to the genes encoding the fimbrins of the SEF14, SEF17, SEF18, and SEF21 fimbriae, with subunit sizes of 14, 17, 18 and 21 kDa, respectively. Whereas SEF14 is limited in distribution to members of group D salmonellae (Thorns *et al.*, 1990; Turcotte and Woodward, 1993), SEF17 (Doran *et al.*, 1993), SEF18 (Clouthier *et al.*, 1994), and SEF21 (Clegg and Gerlach, 1987) are widely distributed among the *Enterobacteriaceae*.

The fimbrial genes which encode fimbrial proteins are generally clustered in large, 7-9 kb operons, for example, the *pap* operon of uropathogenic *E. coli* (Hultgren and Normark, 1992), the *fim* operon of type 1 fimbriae of *E. coli* (Orndorff and Falkow, 1984), and the *fae* and *fan* operons of the K88ab and K99 fimbriae of porcine and bovine enterotoxigenic *E. coli*, respectively (De Graaf, 1990). However, the corresponding operons for SEF14, SEF17, SEF18 and SEF21 have not yet been fully characterised. The SEF14 gene cluster is unique in that it is the first example in the *Enterobacteriaceae* of a gene cluster that encodes two fimbrin-like proteins, SefA and SefD, which are assembled

into two, distinct cell-surface structures, SEF14 and SEF18, respectively (Clouthier *et al.*, 1994).

SEF14 filaments are thin, measuring less than 5 nm in diameter. The filaments have a kinked conformation and they become entangled with each other to give an amorphous, matted appearance. The fimbriae do not agglutinate any of bovine, equine, ovine, murine, rabbit or human group A erythrocytes in the presence or absence of D-mannose and are not expressed at 30°C or below (Thorns *et al.*, 1990; Müller *et al.*, 1991). SEF17 filaments are also thin (3-4 nm in diameter), flexible, extremely insoluble and aggregative. They are produced at 30 and 37°C, are difficult to purify from cells by conventional methods (Collinson *et al.*, 1991) and also bind to fibronectin (Collinson *et al.*, 1993). SEF18 fimbriae, on the other hand, are often found concentrated at the junction of two adjacent cells or found between cells. The fimbriae appear on *S.* Enteritidis and *E. coli* as unusually long, thin fimbriae-like structures, and in other bacterial species they are expressed as an amorphous material (Clouthier *et al.*, 1994). SEF21 fimbriae are morphologically similar to the type 1 fimbriae produced by other genera of the *Enterobacteriaceae* (Duguid and Old, 1980; Müller *et al.*, 1991). They are rigid structures, 7 nm in diameter and about 100 nm in length. The protein subunits are non-covalently linked around a hollow core, giving a channelled appearance under the electron microscope. The fimbriae are characterised by their ability to bind to mannose and mannose derivatives, and fimbriated bacteria can be detected *in vitro* by their ability to mediate agglutination of a range of eucaryotic cells, including guinea pig erythrocytes and yeast cells (Duguid *et al.*, 1966).

1.4.3 Previous studies with *S.* Enteritidis 11RX

Over the years, this Department has been investigating the mechanisms responsible for the nature of T cell-mediated immunity induced by infections with the attenuated *S.*

Enteritidis 11RX strain. 11RX is a rough strain derived from the virulent *S.* Enteritidis strain 11,and lacks the O and the H antigens of the parent (Ushiba *et al.*, 1959). The median lethal dose of 11RX, determined after an intravenous or intraperitoneal challenge of F_1 (BALB/c × C57BL/6) mice, is approximately 2×10^6 organisms (Davies, 1975; Ashley, 1976).

It has been demonstrated that infection of mice with 11RX immunised against challenge with the isogenic virulent strain (Ushiba *et al.*, 1959), and also provided a high degree of protection against lethal infection with *S.* Typhimurium C5, *S.* Paratyphi C, and *L. monocytogenes* (Rowley *et al.*, 1968). Protection was due, in part, to an increase in the bactericidal activity of macrophages (Saito *et al.*, 1960), with further evidence indicating that this resistance was only expressed in the presence of antibacterial antibodies (Rowley *et al.*, 1968). Subsequent work by Davies and Kotlarski (1974; 1976) and later by Attridge and Kotlarski (1985a, b), established that live 11RX organisms generate T cell-mediated immunity which is required for resistance to C5 infection, showed that the responding T cells responsible for DTH were mainly of the $Lyt1^+$ ($L3T4^+$) phenotype, and that the response was class II-restricted. Later, Kotlarski *et al.* (1989) showed that intraperitoneal injection of F_1 mice with live, but not killed 11RX induced *Salmonella*-specific $L3T4^+$ T cells able to proliferate and release cytokines when cultured *in vitro* with formalin-killed and soluble antigenic preparations of 11RX, and to transfer DTH to normal mice.

As an extension of those studies, Vordermeier and Kotlarski (1990a) used proteins fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to define antigens of 11RX able to stimulate T cells from 11RX-primed F_1 mice by a "T-cell Western blot" technique (Abou-Zeid *et al.*, 1987; Lamb *et al.*, 1988). The results obtained from that study indicated that T-cell stimulating antigens predominated in the region containing proteins of approximately 16 kDa, but were also present in the 24-, 34-, and 50- to 60-kDa regions. The responding T cells were again mainly of the $L3T4^+$

phenotype. The highly immunogenic 16 kDa protein was partially purified, and was shown to be able to elicit DTH reactivity, and stimulate *in vitro* proliferation of, and cytokine release from, T lymphocytes obtained from 11RX-primed F_1 mice, to levels comparable to those induced by the whole organism (Vordermeier and Kotlarski, 1990b).

1.4.4 Aims of this thesis

Vordermeier and Kotlarski (1990b) suggested that to gain a better understanding of the pathways of antigen processing in the induction of T-cell responses to *Salmonella*, purified antigens, preferably of relative low molecular weight, are required, thereby reducing the number of different antigenic determinants being studied. Preliminary studies reported by these workers suggested that the highly immunogenic 16 kDa protein material of *S.* Enteritidis 11RX might be a suitable candidate antigen. The work reported in this thesis is an extension of these preliminary observations. The aim was to purifying this protein to facilitate its detailed characterisation, including the definition of its T cell epitopes. In addition, the role of this protein in the pathogenesis of *S.* Enteritidis and its significance in protection against challenge by virulent *S.* Enteritidis, was also investigated.

It was anticipated that results from these investigations would lead to a better understanding of immunity to, and pathogenesis of *S.* Enteritidis, would contribute to the rational design of vaccines against this organism, and assist in devising vaccine strategies for other salmonellae.

CHAPTER 2

Materials and Methods

2.1 Animals

Sex-matched, 8- to 10-week old BALB/c and (BALB/c x C57BL/6) F1 (F1) mice of both sexes were used throughout this study. Semi-lop-eared rabbits provided the normal rabbit serum which was used as the source of complement (C'). All animals were obtained from and housed at the Central Animal House of the University of Adelaide, South Australia.

2.2 Tissue culture media and reagents

Hanks' balanced salt solution (HBSS) supplemented with 100 IU of penicillin per ml and 100 μg of streptomycin per ml was used to harvest and prepare most lymphoid cell suspensions.

RPMI 1640 (Difco) with 2 mM glutamine, 0.1 mM β-mercaptoethanol, 5 μg of indomethacin per ml, 10% (vol/vol) heat-inactivated foetal calf serum (FCS, Flow laboratories), with or without antibiotics (see above) was used for most cell culture work and is referred to throughout as culture medium (CM).

Eagle's minimal essential medium (EMEM, Gibco) containing 0.1 mM β-mercaptoethanol, 26 mM sodium bicarbonate ($NaHCO_3$) and 2 mM glutamine was used to dilute tritiated thymidine ([^{3}H]-TdR).

Minimum essential medium (MEM, Gibco) containing Earle's salts, L-glutamine and non-essential amino acids, and supplemented with 26 mM $NaHCO_3$, 10% FCS, with or

without 5 IU penicillin per ml and 5 μg streptomycin per ml, was used to maintain HeLa cells for *in vitro* adherence and invasion studies.

Phosphate-buffered saline (PBS, pH 7.4) was prepared by dissolving 0.139 M NaCl, 0.0027 M KCl, 0.008 M Na_2HPO_4, and 0.0015 M KH_2PO_4 in Milli-Q water, the pH adjusted, and sterilised by autoclaving. Dulbecco's-PBS (D-PBS) was prepared by adding $CaCl_2$ and $MgCl_2.6H_2O$ to PBS to a final concentration of 0.9 mM and 0.5 mM, respectively.

PBS/bovine serum albumin (BSA, Cytosystems)/sodium azide (NaN_3) (P/B/A) solution was prepared by dissolving 1 g BSA and 1 g NaN_3 in 1 litre of PBS and supplementing this solution with 10% normal rabbit serum that had been heat inactivated at 56°C for 20 minutes. This was used for the preparation of cell suspensions and antibody (Ab) dilutions for immunofluorescence labelling of lymphocytes.

Paraformaldehyde (PFA) was prepared as a 1% solution in sterile PBS using a steaming waterbath to dissolve the PFA. The solution was sterilised by filtration through a 0.22 μM Millex-GS filter unit (Millipore) and stored at 4°C. It was used to resuspend immunofluorescently-labelled lymphocytes to be analysed on the FACScan (Becton Dickinson).

Giemsa buffer (pH 7.0) was prepared by dissolving 500 mg of a mixture of Na_2HPO_4 (5.447 g) and KH_2PO_4 (4.75 g) in 1 litre of Milli-Q water.

Anti-bleach for immunofluorescence microscopy was prepared by dissolving 100 mg of paraphenylenediamine in 1 ml of absolute ethanol. The mixture was vortexed, and clarified by a brief centrifugation. The solution was stored at -20°C until needed. Mowiol mounting medium (Calbiochem) was prepared as follows: 400 mg of Mowiol and 1 g of glycerin were dissolved in 1 ml of Milli-Q water for 2 h at room temperature. To this mixture, 2 ml of 200 mM Tris-HCl (pH 8.5) was added, and the mixture was heated at

50°C in a water bath until fully dissolved. The solution was then clarified by centrifugation at 5,000 × *g* for 15 min and stored at 4°C in a sealed tube until needed. Before use, anti-bleach was added to the medium at a 1/50 dilution.

Milli-Q water was deionised water purified using a Milli-Q water purification system (Millipore) with a measured resistance to conductivity of 18 MΩ/cm.

2.3 Bacteriological media

2.3.1 CBT medium

CBT (Casamino acids, vitamin B1, tryptophan) agar was used to grow *S.* Enteritidis organisms for maximal SEF14 fimbriae production. The medium was freshly prepared before use by adding 50 ml of filter-sterilised minimal salts [K_2HPO_4, 70 g/litre; KH_2PO_4, 30 g/litre; $(NH_4)_2SO_4$, 10 g/litre (pH 7.5)], 12.5 ml 20% Casamino Acids, 5 ml of 50% glucose, 5 ml of 1% $MgSO_4$, 5 ml of 2 mg/ml tryptophan, and 0.5 ml of 1-mg/ml vitamin B1, to 450 ml of sterile molten water agar (7.5 g of BBL granulated agar in 450 ml of deionised water). Aliquots of 25 ml or 250 ml of this agar were poured onto either sterile 90 mm x 14 mm plastic Petri dishes (Techno-Plas, South Australia) or glass trays (290 mm^2) using aseptic technique, allowed to set, dried, and used the same day.

2.3.2 Luria Bertani (LB) broth and LB agar

LB broth was prepared by dissolving 10 g tryptone (Difco), 5 g yeast extract (Difco), and 5 g NaCl in deionised water to 1 litre, and sterilised by autoclaving. LB agar was prepared accordingly, with the addition of 15 g agar before autoclaving.

2.3.3 Nutrient agar

Nutrient agar consisted of 10 g Lab-Lemco powder (Oxoid), 10 g peptone (Oxoid), 5 g NaCl, and 15 g agar in 1 litre of deionised water. It was sterilised by autoclaving.

2.3.4 Terrific broth

This was prepared by adding 100 ml of a sterile (autoclaved) salts solution consisting of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4, to 900 ml sterile broth consisting of 12 g tryptone, 24 g yeast extract and 4 ml (vol/vol) glycerol.

2.3.5 Minimal casein (Minca) medium

Minca medium (Guinée *et al.,* 1976) was used to cultivate K99-expressing clones of *E. coli* for maximal production of FanC subunit and its peptides from FanC-PhoA fusions. The medium consisted of 1.36 g KH_2PO_4, 10.1 g Na_2HPO_4. $2H_2O$, 1 g glucose, 1 ml trace salts solution, 1 g Casamino acids, and 12 g agar, in 1 litre of deionised water. The pH of the medium was 7.5. The trace salts solution contained, per litre: 10 g $MgSO_4$. $7H_2O$, 1 g $MnCl_2$. $4H_2O$, 135 mg $FeCl_3$. $6H_2O$, and 400 mg $CaCl_2$. $2H_2O$.

2.3.6 XLD medium

Xylose-Lysine-Desoxycholate (XLD) medium was prepared by resuspending 53 g of dried medium (Oxoid, CM469) in 1 litre of deionised water, and heating with frequent agitation until boiling. The medium was allowed to cool to 50°C, and then poured into Petri dishes, as usual.

2.4 Enzymes

Deoxyribonuclease I (DNase I), Ribonuclease A (RNase A) and lysozyme were purchased from Sigma. Pronase and proteinase K were purchased from Boehringer-Mannheim.

All restriction endonucleases were purchased from either Amersham, Boehringer-Mannheim, New England Biolabs, Pharmacia or Progen and used according to the manufacturers' recommendations.

Other DNA modifying enzymes were purchased from the following suppliers: T4 DNA polymerase and T4 DNA ligase from Amersham and Progen; DNA polymerase I, Klenow fragment of DNA polymerase I and calf intestinal alkaline phosphatase (CIAP) from Boehringer-Mannheim.

Taq polymerase (Ampli Taq) was purchased from Perkin Elmer Cetus Corporation, rabbit anti-bacterial alkaline phosphatase (PhoA) was from 5 Prime→3 Prime, while goat anti-rabbit horse-radish peroxidase (HRP) and goat anti-mouse HRP were purchased from Kirkegaard & Perry Laboratories Inc. (KPL). Sequencing kits using either dye-labelled primer or dye-labelled terminators were purchased from Applied Biosystems.

2.5 Antibiotics

Ampicillin (Ap), gentamicin (Gm), kanamycin sulphate (Km), rifampicin (Rif), and streptomycin sulphate (Sm) were purchased from Sigma, tetracycline (Tc) and chloramphenicol (Cm) were purchased from Calbiochem. All other anti-microbial agents (dyes, detergents and antibiotics) were purchased from Sigma Chemical Co., BDH Chemicals Ltd., Glaxo, or Calbiochem.

Antibiotics were added to broth and solid media at the following final concentrations: Ap, 50 μg per ml; Cm, 25 μg per ml; Km, 50 μg per ml; Rif, 200 μg per ml; Gm, 50 μg per ml; and Tc, 8 μg per ml.

2.6 Chemicals and reagents

Most chemicals used were AnalaR grade. Phenol, polyethylene glycol-8000 (PEG), and sucrose were supplied by BDH Chemicals. Glycogen, Tris (Trisma base) were from Boehringer-Mannheim. Caesium chloride (Cabot) was technical grade. Mineral oil (Primol 352) was purchased from ESSO. Ethylenediamine tetraacetic acid, disodium salt (EDTA, AnalaR analytical grade), 25% glutaraldehyde solution (UNILAB), 37% formaldehyde solution (UNIVAR) and D.P.X. neutral mounting medium were from Ajax Chemicals.

The following electrophoresis grade reagents were obtained from the sources indicated: acrylamide and ammonium persulphate, from Bio-Rad; ultra pure N,N'-methylene bis-acrylamide and urea, from BRL; and N,N,N',N'-tetramethyl-ethylene-diamine (TEMED), from Sigma.

The four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP) were purchased from Boehringer-Mannheim. Adenosine-5'-triphosphate, sodium salt (ATP), herring sperm DNA, cytochalasin D and dithiothreitol (DTT) were purchased from Sigma. X-pho (5-Bromo-4-chloro-3-indolyl phosphate), X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) and IPTG (isopropyl-β-D-thiogalacto-pyranoside) were purchased from Boehringer-Mannheim or 5 Prime→3 Prime. X-pho and X-gal were dissolved in dimethyl formamide, IPTG was dissolved in Milli-Q water, and were used at a concentration of 40 μg/ml in agar preparations.

Digoxigenin (DIG) DNA labelling and detection kits were purchased from Boehringer-Mannheim.

Detergents: Sarkosyl, from Ciba Geigy; Tween 20, from Sigma; sodium dodecyl sulphate (SDS) and Triton X-100 from BDH Chemicals.

2.7 Plasmids and cloning vectors

The bacterial plasmids and cloning vectors used in this study are listed in Table 2.1.

Table 2.1 Bacterial plasmids and cloning vectors

Plasmid/Vector	Markers	Reference
pBluescript SK+	Ap^R	Stratagene
pBluescript KS^+	Ap^R	Stratagene
pCACTUS	Cm^R, $repA_{ts}$	C. A. Clark, Unpublished
p*cI857*	Km^R	Remaut *et al.* (1983)
pET-17b	Ap^R	Novagen
pEV41b	Ap^R	Pohlner *et al.* (1993)
pFK99	Ap^R	De Graaf *et al.* (1984)
pGP1-2	Km^R	Tabor and Richardson (1985)
pPM2101	Ap^R, Tc^R	Sharma *et al.* (1989)
pPM3500	Ap^R	C. A. Clark, Unpublished
pUC18K	Ap^R, Km^R	Vieira and Messing (1982); Ménard *et al.* (1993)
pUC1318	Ap^R	Kay and McPherson (1987)
pYZ100	Ap^R	Y. Zheng-Xin, M.P.I, Tübingen

2.8 Oligodeoxynucleotides

The oligodeoxynucleotides used in this work (Table 2.2) were synthesised using reagents purchased from Applied Biosystems (ABI) or Ajax Chemicals (acetonitrile). Synthesis was performed on an Applied Biosystems 381A DNA synthesiser and oligodeoxynucleotides were routinely of sufficient purity to require no further purification.

Table 2.2 Oligodeoxynucleotides

Name or number[a]	Sequence[b]	Description of use
#565	5'-ACGCAGAGCGGCAGTCTG-3'	Sequencing junctions of *fanC::phoA* fusions (Section 2.14.8) Hoffman and Wright (1985)
#571	5'-GCTGGCTTTGTTGGTAACAAAGCAGAGGTT-3'	Sequencing (Section 2.14.7.1); amplification of *sefA* along with #572
#572	5'-TGAGTTATGTGGACCAGTAGCAGTAATGCT-3'	Amplification of *sefA* along with #571 (Section 2.14.11.2)
#708	5'-GAGTATATTAGCATCCGC-3'	Sequencing region upstream of *sefA*
#746	5'-TAATACGACTCACTATAGGG-3'	T7 promoter primer (ABI)*; Amplfication of *fanC::phoA* clones along with #779
#779	5'-ATGGAGAATCCATATGAAAAAAACACTGCT-3'	Amplification of *fanC::phoA* clones along with #746
#808	5'-TGTCCGAATGCTAATAGTTG-3'	Amplification of *sefA* along with #809
#809	5'-CTGCTGAACGTAGAAGGTCG-3'	Amplification of *sefA* along with #808
#816	5'-GTTCAACTCTTTACACCACC-3'	Sequencing (Section 2.14.7.3)
#929	5'-GGAGATTTTGTCATATGCGTAAATCAGCAT-3'	Amplification of *sefA* along with #954
#954	5'-TTTGCATGGAAGCTTAAAACCTATC-3'	Amplification of *sefA* along with #929
#2138	5'-GCCGCAGCCATCCCAATGCG-3'	Sequencing of IS*3*-like element upstream of *sefA*
#2141	5'-CACAACATCCCCATTAATAAGTGG-3'	Sequencing of IS*3*-like element upstream of *sefA*
#2142	5'-GGATCATCCCACTTATTAATGGG-3'	Sequencing of IS*3*-like element upstream of *sefA*
M13-Rev	5'-CAGGAAACAGCTATGACC-3'	Dye primer sequencing (ABI)*
M13-20 primer	5'-GTAAAACGACGGCCAGT-3'	Dye primer sequencing (ABI)*
YZ020	5'-GGATCCATATGCGTAAATCAGCA-3'	Amplification of *sefA* along with primer pYZ021
YZ021	5'-GCAGGTCGACTTAAATTAGTTTTGAT-3'	Amplification of *sefA* along with primer pYZ020

[a] Primer numbers are those designated to a primer in the laboratory catalogue.
[b] Cleavage sites for restriction endonucleases *Nde*I, *Hin*dIII and *Sal*I are underlined.

2.9 Bacterial strains and antigens

2.9.1 Bacterial strains and their maintenance

The bacterial strains used throughout this work, and their characteristics, are listed in Table 2.3.

Table 2.3 Bacterial strains

Escherichia coli K-12 strains

Strains	Genotype/Phenotype and other characteristics	Source (reference)
AAEC189	F$^-$, *endA1, hsdR17* (r_k^-,m_k^+), *supE44, thi1, Δ(lacZYA-argF) U169, Δfim, recA56,* λ$^-$	I. Blomfield (Blomfield *et al.*, 1991a)
BL21	F$^-$, *dcm, ompT,* ($r_B^-m_B^-$), *gal*	Grodberg and Dunn (1988)
CC118	F$^-$, *araD139, Δ(ara-leu)7697, ΔlacX74, phoAΔ20, galE, galK, thi, rpsE, rpoB, argEam, recA1,* λ$^-$	J. Beckwith (Manoil and Beckwith, 1985)
C75a	HfrC, *tonA22, pho64*^{a}, *ompF627*(T2^R), *relA1, pit10, spoT1,* λ$^-$	B. Bachmann (A. Garen, unpublished)
DH1	F$^-$, *gyrA96, recA1, relA1, endA1, thi1, hsdR1, supE44,* λ$^-$	B. Bachmann
DH5	F$^-$, *deoR, recA1, endA1, hsdR17*(r_k^-,m_k^+), *supE44, thi1, gyrA96, relA1,* λ$^-$	BRL*
DH5α	F$^-$,ϕ*80dlacZΔM15*, Δ(*lacZYA-argF*), *U169, deoR, recA1, endA1, hsdR17*(r_k^-,m_k^+), *supE44, thi1, gyrA96, relA1,* λ$^-$	BRL*
DS410	F$^-$, *minA, minB, rpsL*	D. Sherratt
E2136	λ *cI857*	J. Pohlner (Earhart *et al.*, 1979)
ECC219	*his, ilv, rpsL, galK*$_{am}$, *pglΔ8 (bio-uvrB), ΔH1 htrA63::mini* Tn*10*	Zuber *et al.*, 1987; C. A. Clark (This laboratory)
G206	HfrC, *tonA22, phoB62, ompF627*(T2^R), *relA1, pit10, spoT1,* λ$^-$	B. Bachmann (Kreutzer *et al.*,1975)
KS474	F$^-$, *ΔlacX74, galE, galK, rpsL*(Sm^R), *ΔphoA*(*Pvu*II), *degP4::*Tn*5*, Km^R	J. Beckwith (Strauch and Beckwith, 1988)
S17-1	RP4-2-Tc::Mu-Km::Tn7/*pro,* res$^-$, mod$^+$, *recA*	U. Priefer
UT5600	F$^-$, *ara14 leuB6, azi6, lacY1, proC14, tsx67, Δ(ompT-fepC)266, entA403, trpE38, rfbD1, rpsL109, xyl5, mtl1, thi1*	J. Pohlner (Elish *et al.*, 1988)
χ2866	F$^-$, *lacY1, glnV44, galK2, galT22,* λ$^-$, *metB1, uvrA1, hsdR2*	R. Curtiss III (Jacobs *et al.*, 1986)
χ2875	F$^-$, *lacY1, tsx465, lon9, glnV44, galK2, galT22,* λ$^-$, *metB1, uvrA1, hsdR2*	R. Curtiss III (Jacobs *et al.*, 1986)

*BRL=Bethesda Research Laboratories

Table 2.3 (continued)

Salmonella strains

Strains	Genotype/Phenotype and other characteristics	Source (reference)
S. Enteritidis 11RX	No somatic antigen; flagella antigen phase1: g,m; flagella antigen phase 2: -; phagetype 7	(Ushiba *et al.*, 1959)
S. Enteritidis 7314	Somatic antigen: O1,9,12; flagella antigen phase1: g,m; flagella antigen phase 2: [1,7]; phagetype RDNC-03	P. Reeves
S. Typhimurium C5	Prototroph	(Hormaeche, 1979)
S. Typhimurium SL3261	*aroA554::*Tn*10*, *his*, Tc^R	R. A. Strugnell (Hoiseth and Stocker, 1981)
SA9	*S.* Enteritidis 11RX (*thyA*, pEVX24)	Bertram *et al.* (1994); Morona *et al.* (1994)
SA5	*S.* Enteritidis 11RX (*thyA*, pBTAH)	Bertram *et al.* (1994) Belfort *et al.* (1983)

Vibrio cholerae strain	Genotype/Phenotype and other characteristics	Source (reference)
569B	Sm^R, motile	K. Bhaskaran

For long-term storage, lyophilized cultures of each strain were prepared by suspension of several loopfuls of overnight growth on solid medium in a small volume of sterile skim milk. About 200 µl aliquots of this suspension were dispensed into sterile 0.25 × 4 inch freeze drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilised in a freeze-drier. After the vacuum was released, the cotton wool plugs were pushed well down the ampoule and a constriction was made just above the level of the plug. The ampoules were evacuated to a partial pressure of 30 microns and then sealed at the constriction without releasing the vacuum. Finally the ampoules were labelled and stored at 4°C. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were then transferred to 10 ml broth and incubated with shaking overnight at the appropriate

temperature. The other half was streaked onto LB or CBT agar plates and incubated overnight at the appropriate growth temperature. Antibiotics were added to the medium when appropriate. If the colony form was uniform, single colonies were selected and picked off the plates for subsequent storage or use. Short-term storage of strains in routine use was as a suspension of freshly grown bacteria in 15% (vol/vol) glycerol and 0.6% (wt/vol) peptone at -20°C. Fresh cultures were prepared by streaking a loopful of the glycerol suspension onto an LB or CBT agar plate (with or without antibiotic(s) as required) and incubating overnight before use.

2.9.2 Bacterial antigens and their preparation

For the preparation of bacterial antigens (Ags), overnight cultures of bacteria were prepared by picking single colonies from either CBT agar or LB agar and growing them overnight for 15 h at 37°C with shaking in 10 ml of CBT broth or LB broth. The overnight cultures were diluted 1/10 in the same broth and grown to exponential phase by incubating for 2½ h with shaking at 37°C to give between 2-4 $\times$ 10^9 live bacteria per ml. The only exception was in the preparation of temperature sensitive strains and their recombinants which were grown routinely at 28°C. The bacteria were then washed twice in cold PBS and diluted in PBS to the required concentrations for intraperitoneal (i.p.) immunisation or oral infection.

Formalin-killed organisms were prepared by washing 100 ml of exponential-phase cultures of each bacterial strain twice in PBS and resuspending the organisms in 10 ml of PBS before the addition of formalin to a final concentration of 1% (vol/vol) and incubation for 1 h at 37°C with occasional shaking. The sterility of each preparation was ascertained by plating 200 µl samples onto CBT or LB agar plates without antibiotics and incubating overnight at 30°C or 37°C. The dry weight of each formalin-killed bacterial preparation

was determined to allow dilutions to a concentration of 1 mg per ml, and 100 µl aliquots of each suspension were stored at -20°C. These preparations were used at 1 µg per ml and 10 µg per ml for most *in vitro* proliferation and IL-2 assays, respectively. The T-cell mitogen concanavalin A (ConA, Pharmacia) was used at 300 ng, 1 µg and 2 µg per ml in most *in vitro* proliferation and IL-2 assays.

Soluble 11RX Ag (S11RX) was prepared as described by Ashley *et al.* (1974) and used in proliferation assays at a final concentration of 10 µg per ml.

2.10 Immunological methods

2.10.1 Immunisations

For intraperitoneal immunisations, F1 and BALB/c mice were injected with 200 µl bacterial suspensions containing 1×10^5 and 5×10^5 organisms, respectively, delivered using a 1 ml syringe fitted with a 26G × 1/2 (0.45 × 13 mm) needle (Becton Dickinson).

2.10.2 Lymphoid cell suspensions

Peritoneal lymphoid cells from immunised mice (IPCs) and from normal (nonimmunised) mice (NPCs) were harvested by washing out the peritoneum of each mouse with 5 ml HBSS. The peritoneal cell suspensions from each group of mice were pooled, centrifuged at 1500 rpm for 5 min in a bench centrifuge (Econospin, Sorvall Instruments), washed once in HBSS, resuspended in CM, counted and adjusted to the appropriate cell concentration in CM.

Spleen cell suspensions were prepared as described by Attridge and Kotlarski (1984) and adjusted to the working cell concentration in CM.

2.10.3 Adherence to nylon wool columns

Nylon wool (NW) columns were prepared by gently stuffing 620 mg of well-teased NW (Pacific Diagnostics) into each 10 ml plastic syringe to the half-way mark, and sterilising these by autoclaving. Adherent cells were removed by passage on NW columns essentially as described by Attridge and Kotlarski (1985a). This was carried out by carefully loading no more than 1×10^8 cells in 2.5 ml warm CM onto each column. The columns were incubated at 37°C for 1 h in an atmosphere of 5-10% CO_2 in air, and the nonadherent cells were washed off with 10-12 ml warm CM into a sterile 50 ml centrifuge tube (Falcon 2070). After pooling similar cell suspensions, they were washed, counted and adjusted to the required cell concentration in CM.

2.10.4 *In vitro* proliferation assays

Cells were resuspended in CM and set up in quadruplicate in flat-bottom 96 well trays (Falcon 3072 or Linbro 76 033 05) at a density of 1×10^5 IPC with 2×10^4 NPC and cultured with each antigenic preparation in a final volume of 200 µl in each well. Cultures were incubated for 3 days at 37°C in a sealed box gassed with 10% CO_2, 7% O_2 and 83% N_2 before adding 50 µl of EMEM containing 1 µCi (37 kBq per well) of [^{3}H]-TdR (Amersham). To "pulse-label" the dividing cells, the cultures were incubated for an additional 4 h at 37°C in an atmosphere of 5-10% CO_2 in air and then harvested onto glass fibre discs (Titertek) with a Flow cell harvester (Flow Laboratories). The amount of radioactivity incorporated by the cells was measured in a Beta counter (Beckman LS 6000TA) using standard procedures. Results are expressed as cpm (mean ± SEM) for each quadruplicate set of cultures.

2.10.5 T-cell phenotype analysis

To determine the phenotype(s) of proliferating cells, 2 ml cultures consisting of 2×10^6 IPC and 4×10^5 NPC were set up in 24-well tissue culture trays (Corning 25820) with the appropriate Ags and incubated as described for *in vitro* proliferation assays. Aliquot samples of the cells were then treated with T-cell and T-cell subset-specific monoclonal antibodies (MAbs) Thy 1.2 (TIB 107), L3T4 (TIB 207) and Lyt 2.2 (TIB 150) from the Departmental collection and sheep anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (SHAM-FITC; Silenus Laboratories, Hawthorn, Australia) to label the cells on a FACScan (Becton Dickinson) as described by Ashley and Kotlarski (1987), and Kotlarski *et al.* (1989).

2.10.6 Interferon Gamma (IFN-γ) assays

Nylon-wool fractionated peritoneal cells of immunised animals were cultured with normal peritoneal cells and stimulating Ags in 24-well trays (Corning 25820) at the same concentrations as above. After 48 h incubation, sample supernatants were assayed for IFN-γ production by ELISA using the biotinylated rat anti-mouse IFN-γ monoclonal antibody (MAb) [Pharmingen, San Diego, California, USA] according to the manufacturer's protocol. Results are calculated as IFN-γ units/ml using an IFN-γ standard.

2.10.7 Interleukin-2 (IL-2) assays

The IL-2-dependent cytotoxic T cell line (CTLL) was used to determine IL-2 levels in CM supernatants from Ag-stimulated T cells. The supernatants assayed were harvested after 24 h incubation of 1-ml cultures containing 1×10^6 IPC, 4×10^5 NPC and the Ags being analysed. The CTLL cells were set up at 5×10^3 per well with serial two-fold dilutions of these CM supernatants in 0.2-ml volumes and incubated for 24 h. [^{3}H]-TdR

(37 kBq/well) was added for the last 4 h of incubation and the amount of radioactivity incorporated was measured as described in Section 2.10.4. Results are expressed as IL-2 units per ml calculated as described by Attridge and Kotlarski (1985a). Alternatively, 24 h culture supernatants from Ag-stimulated T-cells were assayed for IL-2 activity by measuring the extent to which they could maintain the proliferation of 3-day concanavalin A (Con A)-stimulated T-cell blasts. Blast proliferation was measured by [^{3}H]-TdR incorporation and the results expressed as IL-2 units per ml.

2.10.8 Serology

This was performed by the ELISA method using 96-well round-bottom microtitre trays (Costar 2595), with 5 μg/ml of either purified SEF14 or proteinase K-treated 11RX LPS as the sensitising Ags and sera obtained from orally-immunised mice. Goat anti-mouse IgA and IgG conjugated to alkaline phosphatase (KPL) were used to detect the antibodies (Abs) to SEF14 and LPS. Ab titres were determined as the reciprocal of the $\log_{10}$ dilution of the sera giving an absorbance of 0.4 above the background at 405nm.

2.10.9 DTH assays and measurement of footpad swelling

Freshly harvested nylon-wool fractionated IPCs and cells harvested from 3-day *in vitro* cultures of fractionated IPCs were tested for their ability to transfer delayed-type hypersensitivity (DTH) to normal mice. The cells were suspended to the required concentrations (5×10^5 in 50 μl) in CM without FCS and antibiotics, and were injected alone, or with formalin killed 11RX (F11RX, 2.5 μg) or the purified SEF14 (1 μg) into the right hind footpads of recipient mice. The thickness of both hind feet of the injected mice was measured at 24 and 48 h by using dial gauge calipers accurate to 50 μm (Attridge and Kotlarski, 1985b; Boom *et al.*, 1987). To compare the antigenic activity of the purified

SEF14 preparation with whole 11RX organisms *in vivo*, groups of three 11RX-immunized mice were injected into one hind footpad with F11RX (2.5 μg/mouse) or purified SEF14 (1 μg/mouse). The footpad thicknesses of both hind feet were measured 24 and 48 h later and the feet were examined at 72 h.

2.10.10 Preparation and absorption of anti-11RX serum

Mice were immunized by i.p. injection of 1×10^5 live 11RX organisms, followed by three weekly i.p. injections of 500 μg of F11RX starting four weeks later. The mice were bled a week after the last injection.

The serum obtained was absorbed extensively with *S.* Typhimurium C5 and *E. coli* K-12 strains DH1, S17-1, and AAEC189. Before use the bacterial cells were harvested and washed three times in physiological saline (0.85% [wt/vol] NaCl in deionised water), and each cell pellet was resuspended at 5×10^{10} cells per ml in 2 ml of serum. The absorption was repeated seven times with each strain by alternating incubation at 37°C for 6 h in the presence of 0.0002% NaN_3, with incubation at 4°C overnight, using fresh live bacteria for each absorption step. After the last absorption, serum was dispensed into 0. 4-ml aliquots and stored at -100°C.

Prior to storage, the effectiveness of the absorption was assayed by colony and Western blotting (immunoblotting) with the nonabsorbed serum and normal mouse serum as primary Ab controls. The secondary Ab used was sheep-anti-mouse $F(ab)_2$ horse radish peroxidase (SHAM-HRP; Amersham). Bound Abs were detected using the enhanced chemiluminescence kit (ECL, Boehringer-Mannheim).

2.10.11 Preparation of anti-SEF14 monoclonal antibodies

Five 8-week old female BALB/c mice were primed for production of Abs to the SEF14 protein by injecting 100 μg of purified SEF14 in Freund's complete adjuvant at the base of the tail of each mouse. After 2 weeks, mice received a booster intravenous dose of 100 μg of the same Ag. They were given a final boost a week later with the same amount of Ag and sacrificed 4 days after tertiary immunisation.

Spleen cells and myeloma (X63) cells were prepared and fused as described by Oi and Herzenberg (1980) using spleen cells at 2×10^7 per ml and X63 cells at 4×10^6 per ml (5:1 ratio) in serum-free CM in the presence of PEG to promote the fusion of the cells. Cells were plated in 96-well flat bottomed trays (Falcon 3072; Becton Dickinson) using 100 μl of the cell mixture resuspended in hypoxanthine, aminopterin, and thymidine (HAT)-supplemented medium per well, and the trays were incubated at 37°C in an atmosphere of 5-10% CO_2 in air. The medium was replenished at appropriate intervals and apparent fusions were transferred to 24-well trays and maintained with fresh medium as required. Supernatants of positive hybrids producing Abs to SEF14 were identified by ELISA, colony (dot) blotting and reactions in Western blots. Positive hybrids were subsequently cloned and expanded by limiting dilution. Each hybrid supernatant was concentrated 20-fold in an Amicon ultrafiltration apparatus by positive pressure using a PM10 membrane. These served as primary Abs in the immunoblotting assays.

The immunoglobulin (Ig) class of each positive hybrid supernatant was determined by the double immunodiffusion technique (Ouchterlony, 1968) using appropriate anti-mouse Ig Abs (Sigma, ISO-2).

2.10.12 Immunofluorescence microscopy

This procedure was adapted from Klauser *et al.* (1990) with minor modifications. All reactions were performed at room temperature as follows. (i) Sterile washed cover slips (13 mm in diameter) were placed in the wells of a 24-well tissue culture tray (Corning 25820) and each coverslip was treated with 100 µl of poly-L-lysine (100 µg per ml) for 5 min. (ii) The coverslips were washed with PBS and replaced with 100 µl of a suspension of 11RX organisms previously adjusted to 1×10^8 bacteria per ml in PBS. (iii) The volume in each well was made up to 500 µl with PBS and the bacterial suspension was centrifuged at 500 rpm for 5 min in a Labofuge 400R (Heraeus Instruments). (iv) About 450 µl of the supernatant in each well was aspirated and the bacteria in each well were fixed to the coverslip with 450 µl of a solution of 0.4% (vol/vol) glutaraldehyde and 2.0% (vol/vol) formaldehyde in PBS for 20 min. (v) This was followed by three PBS washes using 500 µl PBS per wash. (vi) Unoccupied binding sites on the coverslips were blocked with 300 µl of a 1% solution of FCS in PBS for 5 min. (vii) Each Primary Ab was diluted in 10% FCS in PBS and 20 µl added to the coverslips in a humidified atmosphere for 1 h. (viii) After incubation, the primary Ab was aspirated and each well was washed as in step (v). (ix) Unreacted sites were blocked as in step (vi) and the coverslips were incubated with a 1/100 dilution of goat anti-mouse immunoglubulins (IgA, IgG, and IgM) conjugated to fluorescein isothiocyanate (FITC), for 30 min. (x) The coverslips were washed as in step (v) with 10 min between each wash, and then inverted onto a glass slide to which a drop of mounting medium (25% glycerol in PBS, containing a 1/50 dilution of anti-bleach) had been added. (xi) Excess mounting medium was aspirated and each coverslip was sealed with nail polish. Preparations were observed under an Olympus BH-2 fluorescence microscope with a 100× (oil immersion) objective.

2.11 Transformation and electroporation of strains

2.11.1 Preparation and transformation of competent *E. coli* strains

Transformation was performed essentially according to the method described by Brown *et al.* (1979). *E. coli* K-12 strains were made competent for transformation with plasmid DNA as follows: an overnight shaken culture (in LB broth) was diluted 1:20 into fresh LB broth and incubated with shaking until the culture reached an A_{650} of 0.6 (approximately 4×10^8 cells per ml). The bacterial cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in half volume of cold 100 mM $MgCl_2$, centrifuged again and resuspended in a tenth volume of cold 100 mM $CaCl_2$. The bacterial cells were allowed to stand for 1 h on ice before addition of DNA. Competent cells (200 µl) were then mixed with 20 µl DNA [made up in 1× Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and kept on ice for a further 30 min. The cell/DNA mixture was heat-shocked at 42°C for 2 min, and 1 ml LB was added before incubation with shaking at 37°C for 1 h. The bacterial cell suspension was then plated onto selection plates directly or concentrated by centrifugation and plated. Bacterial cells in sterile buffer were included as a control. For the transformation of plasmids with a temperature-sensitive replicon, the heat-shock procedure was carried out at 30°C for 3 min and subsequent incubations were carried out at 30°C for 2-3 h with or without shaking. For long-term storage, sterile glycerol was added to the transformation-competent bacteria to a final concentration of 30% (vol/vol). The mixture was dispensed into sterile 1.5 ml microcentrifuge tubes in 200 µl volumes, quickly frozen on dry ice for 5 min, and transferred to -70°C. It was established that bacterial cells remained viable with no apparent loss of competence for 4 months.

2.11.2 Preparation of electrocompetent *Salmonella* strains

Preparation of *Salmonella* strains for electroporation was performed essentially as described in the Pulse Controller Instruction Manual (Bio-Rad). Briefly, 100 ml fresh LB broth was inoculated with 10 ml of an overnight broth culture of *Salmonella*. The culture was grown at 37°C with vigorous shaking until a density reading of A_{600} of 0.5 to 0.8 was achieved. Before harvesting, the bacterial cells were chilled on ice for 15 min and then centrifuged in a cold rotor at 4,000 × *g* for 15 min. The supernatant was drained away as much as possible and the pellet was resuspended in 100 ml ice-cold water and centrifuged as before. The last step was repeated, but with 50 ml ice cold water. The resultant pellet was then resuspended in 20 ml of ice-cold 10% glycerol, centrifuged as before and resuspended in a final volume of 2 ml of ice-cold 10% glycerol. Cells were quickly frozen in dry ice in 100 µl aliquots in 1.5 ml polypropylene tubes, and stored at -70°C. The cells were stable for about six months under these conditions.

2.11.3 Electro-transformation and plating

Frozen bacteria were gently thawed at room temperature and placed on ice immediately after thawing. About 1 to 2 µg of DNA dissolved in Milli-Q water were added to the cell suspension, mixed thoroughly and allowed to stand for 1 min. The cell-DNA mixture was transferred carefully to a chilled sterile 0.2 cm electroporation cuvette; this was then placed in a chilled safety chamber fitted onto the Gene Pulser apparatus previously set at 25 µF, 2.50 kV and 400 Ω as advised by Dr Yan Zheng-Xin (Max-Planck Institute for Biology, Tübingen, Germany). The mixture was pulsed once, producing a time constant of 9.1 msec. After electroporation, each cell suspension was made up to 1 ml in LB broth, incubated at 30 or 37°C for 2 to 3 h without shaking, and then plated onto selective medium.

2.12 DNA extraction procedures

2.12.1 Small scale plasmid isolation

Small scale plasmid isolation procedures were based on the alkaline lysis method of Birnboim and Doly (1979). A 1. 5 ml aliquot of a bacterial culture grown overnight was transferred to a microcentrifuge tube, pelleted by centrifugation for 1 min in a microfuge, and resuspended in 100 µl of a solution of 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA and lysozyme at a final concentration of 4 µg per ml. After 5 min incubation at room temperature, 200 µl of a solution of 1% (wt/vol) SDS and 200 mM NaOH was added and the mixture incubated on ice for 5 min. After the addition of 150 µl 3 M potassium acetate pH 4.8 and a 15 min incubation on ice, cellular debris was removed by centrifugation (5 min, microfuge). Supernatants from *endA*$^+$ strains were then extracted once with an equal volume of Tris-EDTA saturated phenol (pH 7.5) and once with a chloroform, isoamyl alcohol (24:1) mixture. Extracts from *endA* strains were mixed with a half volume of 7.5 M ammonium acetate, kept on ice for a further 10 min, centrifuged for 3 min (Biofuge 15) and the supernatant collected. In all cases plasmid DNA was then precipitated by the addition of an equal volume of 2-propanol followed by storage on ice for 10 min. The plasmid DNA was pelleted by centrifugation (15 min, Biofuge 15), the pellet was washed with 70% (vol/vol) ethanol, and dried *in vacuo* (Speedi Vac SC 110, Savant). The pellet was resuspended in 100 µl of Milli-Q water and stored at -20°C.

Plasmid DNA to be used for dye-terminator sequencing (Applied Biosystems) was further purified as follows. The pellet obtained after the last ethanol wash was resuspended in 32 µl of Milli-Q water, and 8.0 µl of 4 M NaCl was added with mixing, followed by addition of 40 µl of autoclaved 13% PEG_{8000}. After thorough mixing, the sample was placed on ice for 30 min and then centrifuged at 15,000 rpm for 15 min at 4°C in a microfuge. The resultant pellet was rinsed with 500 µl of ice-cold 70% (vol/vol)

ethanol, dried *in vacuo*, resupended in 20 µl of Milli-Q water and stored at -20°C until needed. Alternatively, plasmids to be used in sequencing reactions were prepared using the plasmid mini-kit (QIAGEN), following the procedure outlined in the Qiagen plasmid handbook.

2.12.2 Large scale plasmid isolation

Large scale plasmid isolation was performed by the three step alkaline lysis method (Garger *et al.*, 1983). Bacterial cells from a litre culture (LB broth) were harvested (8,700 × *g*, 10 min, 4°C, JA-10, Beckman J2-MI) and resuspended in 24 ml of a solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA. Freshly prepared lysozyme (4 ml at 20 mg/ml in the above solution) was mixed with the cell suspension and kept at room temperature for 10 min. Next, 56 ml of 1% (wt/vol) SDS in 0.2 M NaOH was added before placing on an ice/water slurry for 5 min. After the addition of 28 ml of 3 M potassium acetate (pH 4.8), the sample was kept on an ice/water slurry for a further 15 min before removing the cellular debris by centrifugation at 27,200 × *g* for 10 min at 4°C in a JA-20 rotor (Beckman J2-MI). The DNA in the supernatant fraction was then extracted with an equal volume of a mixture of Tris-saturated phenol (pH 7.5), chloroform, and isoamyl alcohol (25:24:1) and centrifuged at 3,000 × *g* for 10 min at 4°C in a JA-20 rotor. Plasmid DNA was precipitated from the aqueous phase by the addition of 0.6 volumes of 2-propanol at room temperature for 10 min and then collected by centrifugation at 39,200 × *g* for 15 min at 4°C in a JA-20 rotor. After washing in 70% (vol/vol) ethanol, the pellet was dried *in vacuo* and resuspended in 4.8 ml of a solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. CsCl was then added to a density of 1.8 g/ml, followed by 0.8 ml of 10 mg/ml ethidium bromide and the mixture placed under 8 ml CsCl (density of 1.47 g/ml) in a mixture of 10 mM Tris-HCl (pH 8.0) and 1mM

EDTA into 13 × 38 mm Beckman quickseal polyallomer tubes with a capacity of 3.9 ml each. The tubes were completely filled with the 1.47 g/ml CsCl solution, sealed, and centrifuged at 354,000 × *g* for 4 h, at 20°C in a TLN 100 rotor (Beckman Optima TLX Ultracentrifuge). After centrifugation, supercoiled DNA was removed by side puncture of the tubes with a 19 gauge needle attached to a 1 ml syringe, and ethidium bromide removed by several extractions using isoamyl alcohol. CsCl was removed by dialysis against three changes of 5 litres of 1× TE at 4°C. DNA was then precipitated by adding an equal volume of 2-propanol and a one-tenth volume of 3 M sodium acetate (pH 5.2) and the mixture was kept on ice for 10 min. DNA was collected by centrifugation (15 min, microfuge), washed with 70% (vol/vol) ethanol, dried *in vacuo*, resuspended at a concentration of 1 μg/μl in 1× TE buffer and stored at 4°C until needed.

2.13 Preparation of *S.* Enteritidis and *E. coli* genomic DNA

Genomic DNA from either *S.* Enteritidis or *E. coli* was prepared according to Manning *et al.* (1986) as follows. Bacterial cells from a 20 ml shaken overnight culture were pelleted in a bench centrifuge for 10 min and washed once with TES buffer (50 mM Tris-HCl (pH 8.0) 5 mM EDTA, and 50 mM NaCl). The pellet was resuspended in 2 ml of 25% (wt/vol) sucrose, 50 mM Tris-HCl (pH 8.0) and 1 ml of lysozyme (10 mg/ml in 250 mM EDTA [pH 8.0]) was added and the mixture placed on ice for 20 min. TE buffer (750 μl) and 250 μl of lysis solution consisting of 5% [wt/vol] sarkosyl, 50 mM Tris-HCl (pH 8.0) and 62.5 mM EDTA (pH 8.0) were added, together with 10 mg of solid pronase. The mixture was gently vortexed, transferred to a 50 ml Ehrlenmeyer flask and incubated at 56°C for 1 h. This was followed by three extractions with TE-saturated phenol and two extractions with diethyl-ether. The genomic DNA was precipitated with four volumes of 100% ethanol and resuspended in 1 ml of 1× TE buffer.

2.14 Analysis and manipulation of DNA

2.14.1 DNA quantitation

The concentration of DNA in solutions was determined by measurement of absorption at 260 nm using a Pharmacia LKB Ultrospec Plus spectrophotometer and assuming that an A_{260} of 1.0 is equivalent to 50 μg DNA per ml (Miller, 1972; Maniatis *et al.,* 1982).

2.14.2 Restriction endonuclease digestion of DNA

DNA samples were digested using the restriction enzyme buffers recommended by the manufacturers. For double restriction digests, 10× SPK buffer consisting of 200mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 500 mM KCl, and 50% (vol/vol) glycerol was sometimes used for incompatible restriction enzyme buffer conditions. About 100 to 500 ng of plasmid DNA or purified restriction fragments were incubated with 2 units of each restriction enzyme in a final volume of 20 μl at 37°C for 1-2 h, with the exception of *Sma*I digests, which were carried out at 25°C. The reactions were terminated by heating at 65°C for 10 min. Prior to electrophoresis on an agarose gel, a one-tenth volume of tracking dye consisting of 15% Ficoll (wt/vol), 0.1% (wt/vol), bromophenol blue, and 100 ng per ml RNase A, was added. For the digestion of 4 μg of chromosomal DNA, the same method was used, however, about 4-6 units of each enzyme was used for digestion for 4h.

2.14.3 Calculation of sizes of restriction fragments

The sizes of restriction enzyme fragments were calculated by comparing their relative mobility with that of *Eco*RI-digested *Bacillus subtilis* bacteriophage SPP1 DNA, and/or with that of *Bgl*II-digested phage λ DNA. The calculated molecular sizes in

kilobase (kb) pairs of the SPP1 *Eco*RI standard fragments (Ratcliffe *et al.*, 1979) obtained from Bresatec (Adelaide, South Australia) were: 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, and 0.36. The molecular sizes of the *Bgl*II-digested phage λ DNA were: 22.01, 13.29, 9.68, 2.39, and 0.65 kb, as derived from the New England Biolabs catalogue.

2.14.4 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at room temperature on horizontal, 0.8% or 1% (wt/vol) agarose gels (Seakem HGT), 13 cm long, 10 cm wide and 0.7 cm thick. Gels were electrophoresed at 100 V for 4-5 h in either 1× TBE buffer (67 mM Tris base, 22 mM boric acid and 2 mM EDTA at pH 8.0), or 1× TAE buffer (40 mM Tris acetate and 2 mM EDTA at pH 8.0). After electrophoresis the gels were stained in deionised water containing 2 μg/ml ethidium bromide. DNA bands were visualized by trans-illumination with UV light and photographed using either Polaroid 667 positive film or using Mitsubishi thermal paper (K65HM) on a Mitsubishi video copy processor fitted with a Tracktel camera.

For preparative gels, Sea Plaque (Seakem) low-gelling-temperature agarose at a concentration of 0.6% (wt/vol) was used for separation of restriction fragments, which were recovered by the following methods:

Method 1: DNA bands were excised and the agarose melted at 65°C. The DNA fragment was then purified using the QIAEX DNA gel extraction protocol as detailed in the QIAEX handbook (QIAGEN).

Method 2: After electrophoresis the required DNA bands were excised and then placed inside dialysis tubing with about 300 μl of 1× TAE buffer. This was then positioned in an electrophoretic tank filled with 1× TAE buffer. A 200 mA current was

applied for 30 min, causing the DNA to move out of the gel into the buffer contained in the dialysis tubing. The current was then reversed for about 30 sec. The buffer containing the DNA was removed from the dialysis tubing, and the DNA was extracted with an equal volume of TE-saturated phenol, precipitated by adding two volumes of absolute ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2) and pelleted at 15,000 rpm in a microfuge (Biofuge 15; Heraeus). The pellet was washed once in 1 ml 70% (vol/vol) ethanol, dried *in vacuo* in a Speedivac, and resuspended in 1× TE buffer.

2.14.5 Dephosphorylation of DNA with alkaline phosphatase

Plasmid DNA was dephosphorylated using CIAP according to Maniatis *et al.* (1982). $ZnCl_2$ was added to 100 ng to 2 µg of digested DNA in restriction buffer to a final concentration of 100 nM. For restriction digests that generated 5' overhanging ends, 1 unit of CIAP was added and the mixture was incubated at 37°C for 30 min. For restriction digests that generated 3' overhanging, or blunt ends, the incubation period was reduced to 15 min at 37°C. Then, another unit of CIAP was added to each sample and the mixtures were incubated at 55°C for a further 45 min. In all cases, the reaction was terminated by the addition of EDTA (pH 8.0) to a final concentration of 3 mM, followed by heating at 65°C for 10 min. The volume of each sample was made up to 100 µl with Milli-Q water before extracting with Tris-saturated phenol (pH 7.5), chloroform, isoamyl alcohol (25:24:1). 20 µg glycogen were added to each sample as a carrier before precipitating the DNA with 3 volumes of 100% ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2) and incubation at -20°C for 30 min. DNA was recovered by centrifugation (15 min, microfuge), pellets were washed with 70% (vol/vol) ethanol and dried *in vacuo*. Each DNA sample was resuspended in an appropriate volume of Milli-Q water or 1× TE to 1 µg/µl and stored at -20°C.

2.14.6 End-filling with Klenow fragment

Protruding ends created by cleavage with restriction endonucleases were filled in using the Klenow fragment of *E. coli* DNA polymerase I. About 1 μg of digested DNA or purified DNA fragments, 2 μl of 10× Klenow buffer (100 mM Tris-HCl [pH 8.0] and 100 mM $MgCl_2$), 1 μl of each dNTP (2 mM) and 1 unit of Klenow fragment were combined in a final volume of 20 μl and incubated for 15 min at 37°C. DNA was then extracted twice in phenol/chloroform in a total volume of 100 μl and precipitated in 2 volumes of 100% ethanol and one-tenth volume of 3M sodium acetate for 30 min in a dry ice/ethanol bath. Pellets were washed once in 1 ml 70% (vol/vol) ethanol, dried *in vacuo* and resuspended in a total volume of 20 μl of 1× TE buffer.

2.14.7 *In vitro* cloning

The DNA fragment to be subcloned (about 200 ng) was cleaved in either single or double restriction enzyme digests. This was combined with 20 ng of similarly cleaved vector DNA, then ligated with 1 unit of T4 DNA ligase in a volume of 20 μl in a buffer containing a final concentration of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 600 nM ATP at 4°C for 16 h. For blunt-end ligation, a two-step method described by Damak and Bullock (1993) was used. In the first step, about 60 ng per μl of the insert DNA was combined with 60 ng per μl of vector DNA, before adding 1 unit of T4 DNA ligase and the mixture kept at room temperature initially for 1 h in a 10 μl volume. In the second step, the ligation mix was diluted to 20 times original volume with water; T4 DNA ligase and buffer were adjusted to their original concentration, and the ligation reaction was allowed to proceed further for another 16 h at 4°C. The ligated DNA was then used directly for transformation of *E. coli* strains. Wherever possible,

transformants were screened for insertional inactivation of the appropriate drug resistance gene (eg. Ap or Tc) prior to plasmid DNA isolation.

2.14.7.1 Cosmid cloning

High molecular weight genomic DNA prepared from strain 11RX was partially digested with *Sau*3AI. Samples were taken at specific time intervals and aliquots were electrophoresed on a 0.8% (wt/vol) agarose gel in Tris-acetic acid-EDTA (TAE) buffer. DNA fragments of approximately 35-45kb were excised from the gel, electro-eluted and extracted using Tris-EDTA (TE)-saturated phenol/chloroform. The purified DNA was ligated to pPM2101 which had previously been completely digested with *Bam*HI and dephosphorylated with CIAP. Ligation was allowed to proceed initially at room temperature for 10 h, and then overnight at 4°C. The ligation mix was packaged into bacteriophage λ (Collins and Hohn, 1978) using the *in vitro* Packagene System (Promega) and then transduced into *E. coli* AAEC189.

500 *E. coli* transductants obtained from cosmid cloning were screened by colony blot DNA hybridisation with oligonucleotide #571 previously end-labelled with DIG-dUTP and terminal transferase (Boehringer-Mannheim), and by colony immunoblotting with one of the SEF14-reactive MAbs, KAP14-1. One of the cosmid clones, pPM3901, reacted strongly in colony immunoblotting and was subsequently used throughout this study.

2.14.7.2 Construction of plasmid pYZ100

Plasmid pYZ100, a pT7-7 derivative coding for SefA Ag, was constructed by Dr Yan Zheng-Xin (Max-Planck Institute for Biology, Tübingen, Germany), as follows. A 2.2 kb *Hin*dIII and *Eco*RI T7 terminator fragment from pEP12 was first inserted into *Hin*dIII and

*Eco*RI sites of polylinker region of pBA and subsequently cleaved out with *Hin*dIII and *Cla*I and ligated into the *Hin*dIII and *Cla*I sites of pT7-7, generating pYZ57. The *sefA* fragment was amplified by PCR using pPM3911 as a template with introduction of a *Nde*I site at the start codon ATG by employing the primer pair designated YZ020 and YZ021 (see Table 2.2). The PCR product was digested with *Nde*I and *Sal*I, and inserted into pYZ57 cleaved with *Nde*I and *Sal*I, yielding pYZ57-*sefA*. Finally, a 200 bp *Xho*I and *Sal*I T1 terminator fragment from pDS3 was first filled-in with Klenow and then ligated into the blunt-ended *Bgl*II site of pYZ57-*sefA*. The resulting plasmid with the T1 terminator fragment in the right orientation upstream of T7 promoter was designated pYZ100.

2.14.7.3 Cloning of *sefA* into pEV41b

An in-frame *sefA* construct was generated in pEV41b as follows. The N-terminal 21 amino acid signal sequence coding region of the protein was removed by digesting the region encompassing *sefA* in pPM3911 with *Pvu*II (see Fig. 4.4B, C, Chapter 4). Another blunt end was created at the C-terminal coding region by cleaving the same DNA with *Sna*BI. Several fragments were generated, but the 580 bp *Pvu*II-*Sna*BI fragment of interest was gel-extracted and cloned by blunt-end ligation into the *Sma*I site in the polylinker of pEV41b. The ligation mix was transformed into *E. coli* 2136 (Earhart *et al.,* 1979; Remaut *et al.*, 1983) and incubated overnight at 28°C. Clones of the appropriate size and orientation were selected by restriction analysis of DNA from transformants, and in-frame fusion was confirmed by dye-deoxy terminator sequencing of the recombinant DNA with oligonucleotide #816 derived from bases 441-460 of pEV41b. The *sefA* construct thus generated (pPM3913) consists of an additional 30 N-terminal coding residues contributed by the pEV41b vector, and was used to transform *E. coli* UT5600[p*cI857*].

2.14.8 Exonuclease deletion

Carboxyl-terminal deletions of *fanC* and *sefA* were carried out with exonuclease III [Exo III] (Henikoff, 1984) using the Erase-a-Base kit (Promega) according to the manufacturer's recommendations. For C-terminal deletion of *fanC*, a CsCl DNA preparation of *fanC::phoA* clone (pPM3918) was digested with *Hin*dIII and the ends protected from Exo III digestion by filling with α-phosphorothioate nucleotides. An Exo III-sensitive site was created by digesting the linearised DNA with *Eco*RV and deletion was carried out at 30°C with reactions being terminated at 30 sec intervals, generating approximately 100 bp deletions during each time interval. Ligated deletion products were used to transform *E. coli* CC118 (a *phoA*-negative strain) and plated on nutrient agar plates containing Ap and X-pho to select in-frame *fanC::phoA'* fusions. Exact sizes of *fanC* nested products was determined by dye-terminator sequencing of nested DNA fragments with oligonucleotide #565.

For *sefA* deletion, a CsCl DNA preparation of the *sefA* clone (pPM3913) was initially digested with *Hin*dIII and the site was protected against Exo III digestion by filling with α-phosphorothioate nucleotides. An Exo III-sensitive site was created by digesting the clone with *Cla*I and deletion carried out at 22°C with reactions being terminated at 20 sec intervals, generating deletions of approximately 20-25 bp during each time interval. Plasmids from each time point were ligated with T4 DNA ligase and transformed into *E. coli* 2136 λ *cI857*, grown at 28°C, and transformants selected for appropriate nested products by restriction analysis of plasmid mini-preps. Exact sizes of nested products were determined by dye-deoxy terminator sequencing of their carboxy-terminal coding regions with oligonucleotide #926 complementary to bases 601-620 of pEV41b.

For digestion with Exo III, 5 μg of DNA was dissolved in 60 μl of a solution containing 66 mM Tris-HCl (pH 8.0),and 0.66 mM $MgCl_2$ before adding 400 units Exo III. 2.5 μl aliquots were removed at intervals and added to 7.5 μl of a solution containing 40.5 mM potassium acetate (pH 4.6), 338 mM NaCl, 1.35 mM $ZnSO_4$, and 6.75% (vol/vol) glycerol containing 2.25 units of S1 nuclease. The mixture was incubated at room temperature for 30 min before the addition of 1 μl of a solution of 300 mM Tris base and 50 mM EDTA and incubation at 65°C for 10 min. Samples were then ligated using the T4 DNA ligase mix.

2.14.9 *In vitro* transcription/translation

The *E. coli* S30 extract system for circular DNA (Promega, catalogue number L1020) was used for *in vitro* transcription/translation of nested DNA sequences generated in *sefA* in pPM3913, following the procedure outlined in the technical bulletin. [^{35}S]-methionine was not used in any of the experiments because of the absence of internal methionine residues in SefA protein. Generated products were then detected by Western blotting with either an antiserum specific for SEF14, or with a panel of SEF14-positive MAbs.

2.14.10 Polymerase Chain Reaction (PCR) Protocol

The procedure used was a modification of Saiki and Gelfand (1989). PCR amplification was performed in a 50 μl reaction volume containing PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris (pH 8.4), 50 mM KCl, and 100 μg per ml of gelatin), 1.5 U of Taq polymerase (Perkin-Elmer Cetus), 1 μM of each primer, 100 ng of DNA template, and 200 mM of each of the four dNTPs. The reaction mixture was resuspended thoroughly by vortexing, centrifuged briefly, and a small overlay of paraffin was added to prevent

evaporation during cycling. The thermal cycler (Perkin-Elmer Cetus, Model 480) was programmed to incubate samples at 95°C for 5 min and then to carry out 25 cycles consisting of 95°C for 30 sec (denaturation), 50°C for 30 sec (annealing), 72°C for 1 min, 30 sec (extension), followed by a final extension at 54°C for 5 min. 5 µl of this reaction product was analysed on a 0.8% agarose gel and the remaining product was purified and used for cloning or labelling for Southern hybridisation. Before cloning, PCR products were digested with the appropriate enzyme(s) and treated with proteinase K as described by Crowe *et al.* (1991) to improve the efficiency of cloning.

2.14.11 Labelling of DNA probes

2.14.11.1 Labelling of oligonucleotide #571

The 3'-end of oligonucleotide 571 was labelled by adding 100 ng of the DNA to 2.5 µl of 10× tailing buffer (1.4 M Potassium cacodylate, 300 mM Tris [pH 7.2], and 1 mM DTT), 2.5 µl DIG-dUTP, 1 µl terminal transferase, and 1 µl 400 mM $CoCl_2$, in a total volume of 25 µl. The reaction mixture was incubated at 37°C for 1 h. The DNA was spot-checked for labelling as detailed in Section 2.14.12 and stored at -20°C until required.

2.14.11.2 Labelling the 168 bp PCR product of *sefA*

The N-terminal region of sefA was amplified by PCR using oligonucleotide #571 and #572 to generate a 168 bp *sefA* PCR product. Reactions incorporating DIG-dUTP were performed using random-primed labelling: About 50-100 ng of the fragment to be labelled was resuspended in 15 µl Milli-Q water, boiled for 5 min and then chilled on ice for 2 min before adding 2 µl of a solution containing 1 mM of each dNTP, 650 nM DIG-dUTP, 142.5 mM Tris-HCl (pH 8.1), 5.7 mM DTT, 14.25 mM $MgCl_2$, 114 mM KCl, 2 µl of 400 µg/ml random primer (hexamer) and 2 units Klenow enzyme. The mixture was

incubated at 37°C overnight and the reaction was stopped by the addition of EDTA (pH 8.0) to a concentration of 20 mM. Unincorporated label was removed by precipitating the DNA with a one-tenth volume of 4 M LiCl and three volumes of 100% ethanol at -20°C for 30 min. Glycogen (20 μg) was added as a carrier. DNA was recovered by centrifugation (15 min, microfuge) the pellet washed with 70% (vol/vol) ethanol, and dried *in vacuo*. The DNA was resuspended in 100 μl Milli-Q water and stored at -20°C. Percentage incorporation of label was not determined, but labelling was checked by blotting one-tenth of the probe onto a nitrocellulose (NC) disc and detecting bound DIG-dUTP (see Section 2.14.12).

2.14.12 DNA dot blotting with oligonucleotide probes

This procedure was used to screen transductants obtained from the cosmid cloning of the 11RX genomic DNA (see Section 2.14.7.1). The transductants were grown up overnight on agar plates and the colonies were transferred to NC discs (Schleicher and Schuell, BA 85, 0.45 μm) and lysed by placing the NC discs with colony side up onto Whatman 3 MM paper soaked in 500 mM NaOH for 5 to 10 minutes. The discs were subjected to 5 min treatments by placing them sequentially on Whatman 3 MM paper soaked in the following solutions: (a), 100 mM NaOH and 1.5 M NaCl; (b), 1 M Tris-HCl (pH 7.5, twice); (c), 500 mM Tris-HCl (pH 7.5); and (d), 1.5 M NaCl. The discs were then air dried, and heated under vacuum between Whatman 3 MM sheets for 2 h at 80°C. All bacterial debris was removed by a 2 h wash at 65°C in 3× SSC (20× SSC is a solution containing 340 mM NaCl, 75 mM sodium citrate [pH 7.0] and 0.2% (wt/vol) SDS.

Prior to hybridisation, NC discs were incubated for 2 h at 42°C in 15 ml of prehybridisation solution consisting of 56.25 ml of 4 M NaCl, 22.5 ml of 1 M Tris-HCl (pH 7.6), 9 ml of 250 mM EDTA, 25 ml of 50× Denhardt's reagent [0.1% (wt/vol) Ficoll,

0.1% (wt/vol) polyvinylpyrrolidone, 0.1% (wt/vol) fraction V BSA], 2.5 ml of denatured herring sperm DNA made up to a final volume of 250 ml and a final concentration of 0.2% (wt/vol) SDS. The prehybridisation fluid was discarded and replaced with fresh hybridisation buffer (same as for prehybridisation solution but without herring sperm DNA). Labelled oligonucleotide #571 was added and hybridisation allowed to proceed for 16-24 hr at 42°C. After hybridisation, NC discs were washed extensively at room temperature. The washing procedure consisted of three washes in 5× SSC without SDS for 10 min each wash. The discs were then washed briefly in a solution containing 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl, and then incubated for at least 30 min in 5% (w/v) skim milk in the same buffer to block non-specific binding sites on the discs. The discs were then washed briefly in the same buffer and incubated in buffer containing a 1/5,000 dilution of anti-DIG Ab-horse radish peroxidase (POD) conjugate (Boehringer-Mannheim) for 1 h and washed again four times for 5 min in a buffer consisting of 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl, followed by two 5 min washes in PBS. Finally, the discs were soaked in 2 ml of ECL detection reagent (Boehringer-Mannheim) for 1 min in a transparent plastic bag after all bubbles had been excluded, before exposing them to X-ray film at room temperature. All washes were carried out at room temperature.

2.14.13 DNA hybridisation by Southern blotting

Single and/or double digests of DNA were carried out using restriction endonucleases and the restriction fragments were separated electrophoretically on a 1% agarose slab gel. Unidirectional transfer of DNA from agarose gels to NC paper (Schleicher and Schuell, BA 85, 0.45 μm) was performed as described by Southern (1975) and modified by Maniatis *et al.* (1982).

Prior to hybridisation with labelled probe (the DIG-dUTP-labelled 168 bp *sefA* PCR product) filters were incubated for 2 hr at 42°C in prehybridisation solution consisting of 50% (vol/vol) formamide, 50 mM sodium phosphate buffer (pH 6.4), 5× SSC (pH 7.0), 5× Denhardt's reagent and 100 µg/ml of single stranded herring sperm DNA (Sigma) (Maniatis *et al.,* 1982). Prehybridisation fluid was discarded and replaced with fresh hybridisation buffer (same solution as prehybridisation solution, but without herring sperm DNA). The 168 bp labelled *sefA* fragment was denatured by boiling for 10 min, added to the filter in the hybridisation solution, and hybridisation was allowed to proceed for 16-24 hr at 42°C. After hybridisation, stringency washing of the filter was carried out twice for 5 min with a solution of 2× SSC and 0.1% (wt/vol) SDS at 42°C, and then twice for 15 min with a solution of 0.2× SSC and 0.1% (wt/vol) SDS at 42°C. Detection of hybridised DNA was by ECL using anti-digoxigenin-POD Fab fragments as described in Section 2.14.12.

2.14.14 Sequencing using dye-labelled primers

Sequencing reactions were carried out using 1 µg of double stranded plasmid DNA with the Applied Biosystems Prism dye-primer cycle sequencing ready reaction kit as detailed below.

Reagent	A	C	G	T
Ready Reaction premix (µl)	4	4	8	8
DNA template (µl)	1	1	2	2
Total volume (µl)	5	5	10	10

About 20 µl of light mineral oil was added to each reaction mixture which was centrifuged briefly. Tubes were placed in a Perkin-Elmer Cetus thermal cycler (Model 480) preheated to 95°C and cycling was allowed to proceed as follows:

Rapid thermal ramp to 95°C

95°C for 30 sec

Rapid thermal ramp to 55°C

55°C for 30 sec

Rapid thermal ramp to 70°C

70°C for 60 sec

(repeated for 15 cycles)

This was followed by:

Rapid thermal ramp to 95°C

95°C for 30 sec

Rapid thermal ramp to 70°C

70°C for 60 sec

(for a further 15 cycles)

After the cycles were completed, samples were subjected to a rapid thermal ramp to 4°C and were combined in 80 µl of 95% (vol/vol) ethanol, mixed thoroughly, and kept on ice for 15 min to precipitate the DNA. The DNA was pelleted at 15,000 rpm for 30 min in a microcentrifuge (Biofuge 15, Heraeus), washed in 250 µl of 70% (vol/vol) ethanol, centrifuged for another 5 min, and dried *in vacuo* for 3 min and stored at -20°C until needed. Prior to loading onto the sequencing gel the DNA preparations were resuspended in 5 µl deionized formamide/50 mM EDTA (pH 8.0) at a 5:1 (vol/vol) ratio, and heated to 95°C for 2 min.

2.14.15 Sequencing with dye-labelled terminators

Sequencing reactions were carried out using 1 µg of double-stranded plasmid DNA on a Perkin-Elmer Cetus Model 480 thermal cycler following the protocol provided by Applied Biosystems. To 5.0 µl (1.0 µg) of double-stranded DNA template, 9.5 µl of

terminator premix and 3.2 pmol of primer were added and the volume was made up to 20 µl with deionised water. Each mixture was overlaid with 40 µl mineral oil prior to cycling. The following cycle protocol was used:

Rapid thermal ramp to 96°C

96°C for 30 sec

Rapid thermal ramp to 50°C

50°C for 15 sec

Rapid thermal ramp to 60°C

60°C for 4 min

(for a total of 25 cycles)

After the cycles were completed, samples were subjected to a rapid thermal ramp to 4°C and each reaction mixture (20 µl) was precipitated with a mixture 50 µl of 95% (vol/vol) ethanol and 2 µl of 3 M sodium acetate, pH 5.2 and held for 10 min on ice. DNA was pelleted at 15,000 rpm for 30 min (Biofuge 15, Heraeus) at 4°C and the pellet was washed in 250 µl of 70% (vol/vol) ice-cold ethanol. Samples were dried *in vacuo* and stored at -20°C until needed. Prior to loading onto the sequencing gel the samples were resuspended in 5 µl of a mixture of deionized formamide/50 mM EDTA (pH 8.0) at a 5:1 (vol/vol) ratio and heated to 95°C for 2 min.

2.14.16 Analysis of DNA sequences

Preliminary sequencing data obtained from the 373A automated sequencer were analysed using the Applied Biosystems Analysis version 1.2.1, and Seq Ed program version 1.0. Sequencing data were analysed using the following computer programs: LKB DNA and protein analysis programs, DNASIS and PROSIS (Hitachi Software). A search

for homology of unknown DNA sequence data with any known sequences was made using BLASTN and BLASTX at NCBI (Altschul *et al.,* 1990; Gish and States, 1993)

2.15 Protein analysis

2.15.1 Preparation and purification of SEF14 protein

The method used for large scale preparation/purification of the SEF14 protein of 11RX was a modification of the methods of Feutrier *et al.* (1986) and Müller *et al.* (1991) and consisted of subculturing 1 ml aliquots of an overnight CBT broth culture of 11RX grown at 37°C with shaking, onto each of 10 glass trays (290 mm^2) containing 250 ml CBT agar. Trays were incubated overnight at 37°C and the bacteria from each tray were harvested with 25 ml physiological saline to which 10 mM Mg^{2+} and 5 mM Ca^{2+} had been added. By this process, approximately 200 ml of bacterial suspension was obtained which was centrifuged at 7,000 × *g* for 5 min, the pellet of bacteria was washed once in the saline-Mg^{2+}-Ca^{2+} mixture used above before being resuspended in the same solution and heated at 65°C for 30 min. After cooling to room temperature, the suspension was centrifuged at 7,000 × *g* for 20 min. The resulting supernatant was stored overnight at 4°C after adding 200 mg sodium acetate and 0.001% sodium azide (NaN_3). A precipitate, mainly LPS, was formed during this time. This was collected by ultracentrifugation at 200,000 × *g* at 4°C for 90 min. The pellet was analysed for protein and LPS content to determine the amount of protein pelleted along with the LPS. To the supernatant (150 ml) was added ammonium sulphate [$(NH_4)_2SO_4$] to 50% saturation and the solution kept at 4°C for 2 h. The aggregate that formed (established to be SEF14) was harvested by centrifugation at 20,000 × *g* for 20 min at 4°C, resuspended in 10 ml physiological saline and dialysed twice against 500 ml saline in a dialysis tubing with an exclusion limit of 10 kDa. The supernatant obtained from the last centrifugation was also analysed for residual

protein and LPS. The purified protein was stored at -20°C in aliquots of 100 μg/ml and was used in most assays at 1 μg per ml.

2.15.2 Preparation of SEF14 for N-terminal sequencing

A large amount of 11RX suspension was prepared essentially as described above for the purification of the 14 kDa protein up to the heat elution step, with the exception that 11RX was grown on nutrient agar and cells were washed and resuspended in PBS. After the heat elution step, the bacterial suspension was brought to room temperature and centrifuged at 12,000 × *g* for 10 min. The supernatant was precipitated with $(NH_4)_2SO_4$ to 50% saturation at 4°C for 2 h. The aggregate formed was collected by centrifugation at 12,000 × *g* for 10 min, resuspended in PBS and dialysed for 2 h at 4°C against sterile deionised water to which 0.0002% NaN_3 had been added. A 2 ml aliquot of this preparation was electrophoresed in SDS on a 15% polyacrylamide gel and lightly stained before transfer to a polyvinylidene difluoride membrane (Bio-Rad) at 200 mA for 2 h in a Trans-Blot Cell (Bio-Rad). The transfer buffer used contained 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 10% (vol/vol) methanol. The protein bands transferred were stained for 10 min with Comassie Brilliant Blue using standard procedures and the membrane was sent to Dr. A. Gooley (Macquarie University, Sydney, Australia) for analysis on an Applied Biosystems 470A protein sequencer.

2.15.3 Preparation of whole cell lysates of bacteria

Bacterial suspensions were prepared for Western blotting by resuspending the pellets of 1 ml aliquots of overnight or mid-exponential phase cultures in 50 μl PBS, and mixing them with an equal volume of 2× sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, and

0.05% (wt/vol) bromophenol blue (Lugtenberg *et al.*, 1975). The samples were sonicated briefly with a Branson Model B15 Sonifier and heated to 100°C for 3 min before storing at -20°C.

2.15.4 Cell fractionation

The procedure used for cell fractionation of 11RX cells and *E. coli* AAEC189 cells carrying the 11RX cosmid clone pPM3901 was a modification of that described by Osborn *et al.* (1972). In each case, cells were grown to mid-exponential phase in 50 ml CBT broth and then pelleted at 10,000 × *g* for 10 min. The supernatant was kept on ice and the pellet was resuspended in 1 ml 20% sucrose in 30 mM Tris-HCl (pH 8.1). The bacterial cells were converted to sphaeroplasts by adding 100 µl of 1 mg/ml lysozyme in 100 mM EDTA (pH 7.3) and placing the suspensions on ice for 30 min. The suspension was then centrifuged at 10,000 × *g* for 10 min and the supernatant was kept as the periplasmic fraction. The pellet was freeze-thawed three times by placing on dry ice for 30 min and at 37°C 5 min each time. The freeze-thawed pellet was resuspended in 3 ml 3 mM EDTA (pH 7.3) and sonicated by pulsing in a B15 Branson Sonifier cell disruptor. Unlysed cells were removed by centrifugation at 8,000 × *g* at 4°C for 5 min and the resulting supernatant was centrifuged at 200,000 × *g* for 1 h. The supernatant obtained from the last centrifugation was established as the cytoplasmic fraction, and the pellet was established as the membrane fraction. This was resuspended in 1 ml of a solution of 2% Triton X-100 in 0.25 M Tris-HCl (pH 7.5) and kept at room temperature for 15 min before centrifugation at 200,000 × *g* for 1 h. The Triton X-100-soluble fraction was established as the inner (cytoplasmic) membrane fraction, while the insoluble fraction (the pellet) was established as the outer membrane fraction. The pellet was resuspended in 500 µl of 1× sample loading buffer for SDS-PAGE analysis. The supernatant from the first centrifugation was

concentrated 10-fold with 50% trichloroacetic acid, resuspended in 50 µl saline with a drop of 500 mM NaOH, and added to an equal volume of 2× sample loading buffer. All other samples were also resuspended accordingly and loaded for SDS-PAGE analysis and Western blotting.

2.15.5 Protein overproduction

2.15.5.1 Overproduction of FanC::PhoA fusions

The plasmid pGP1-2 carries the T7 RNA polymerase gene under the control of the bacteriophage λ p_L promoter, and the λ *cI857* (temperature sensitive) repressor gene (Tabor and Richardson, 1985). When a plasmid containing a T7 RNA polymerase dependent promoter is introduced into cells containing pGP1-2, transcription from this promoter can be induced by shifting the growth temperature from 30°C to 42°C. As the T7 RNA polymerase is resistant to the antibiotic Rif, transcription that is dependent on the host RNA polymerase can be selectively inhibited with this antibiotic, allowing specific labelling or large scale production of proteins encoded by genes cloned behind a T7 RNA polymerase dependent promoter (Tabor and Richardson, 1985). This system was used for overproduction of FanC::PhoA fusions using Minca broth supplemented with antibiotics as required.

2.15.5.2 Overproduction of SefA protein

The recombinant plasmid pPM3913 (Section 2.14.7.3) was used for high level expression of SefA protein *in vitro* in *E. coli* K-12 strains ECC219 and UT5600[p*cI857*] after temperature induction as described by Strebel *et al.* (1986) and Pohlner *et al.* (1993). This system employs the λ p_L promoter for temperature-regulated transcription, and the translation initiation region of the bacteriophage MS2 polymerase gene, to direct an

efficient transcription and translation of *sefA*. The medium used for SefA overproduction was Terrific broth supplemented with antibiotics as required.

2.15.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on 5% stacking and 15% separating polyacrylamide gels using a modification of the protocol of Lugtenberg *et al.* (1975) as described previously by Achtman *et al.* (1978). Gels were 15 cm or 20 cm long, 11 cm wide, and 1.5 mm thick. Samples were heated at 100°C for 3 min in SDS sample buffer consisting of 25 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS; 10% (vol/vol) glycerol; 5% (vol/vol) β-mercaptoethanol, and 15% (wt/vol) bromophenol blue prior to loading. Gels were generally electrophoresed at 100 V for 5-8 hr. Proteins were stained with gentle agitation overnight at room temperature in 0.06% (wt/vol) Coomassie Brilliant Blue G250 dissolved in 5% (vol/vol) perchloric acid. Destaining was accomplished with several changes of 5% (vol/vol) acetic acid, with gentle agitation for 24 hr. Size markers (Pharmacia) were 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).

2.15.7 Colony (dot) blotting and Western blotting

Bacteria for colony (dot) blotting were grown to mid-exponential phase in CBT or LB broth. They were washed once in physiological saline and then resuspended to 4×10^9 cells per ml in saline. About 10 µl of each bacterial suspension was spotted onto a nitrocellulose disc, allowed to air-dry for 5 min and then lysed with 500 mM HCl for 30 min. Unlysed cells were removed with a jet stream of saline, and the disc was blocked with 5% (wt/vol) skim milk powder in 0.05% Tween 20 plus 20 mM Tris-buffered saline. Primary Ab in colony blotting was either a 1:5,000 dilution of the specific mouse anti-

11RX or anti-K99 serum, or, a 1:500 dilution of the SEF14- or K99-positive MAbs, while the secondary Ab was goat anti-mouse IgG conjugated to HRP. Detection of the bound Ab was by ECL.

Western blotting of SDS-PAGE fractionated proteins were performed by a modification of the procedure of Towbin *et al.* (1979). Fractionated proteins were transferred onto NC in a Trans-Blot Cell apparatus (Bio-Rad). Blotting was done at 200 mA constant current for 2 h in 2.8 litres of transfer buffer consisting of 200 mM glycine, 20 mM Tris and 5% (vol/vol) methanol. Transferred proteins were blotted with primary and secondary Abs as described above for colony blotting.

2.15.8 Electron microscopy

2.15.8.1 Preparation of protein A-gold

Colloidal gold (10 nm-15 nm diameter) was treated with dextran, activated with glutaraldehyde and then coupled to protein A (Pharmacia), according to the method of Hicks and Molday (1984).

2.15.8.2 Immuno-electron microscopy

Bacteria for immuno-electron microscopy were grown statically in CBT broth for 48 h at 37°C. They were resuspended in PBS to 10^{11} cells per ml and 20 µl of each suspension were spotted onto a piece of parafilm. Nickel grids (200 mesh, Graticules) coated with collodion were treated with 100 µg/ml poly-L-lysine for 5 min before being placed on the 20 µl samples of bacterial suspension for 5 min. After attachment of bacteria, the grids were blocked with 3% BSA-PBS for 3 min and incubated with a 1:10 dilution of the SEF14 reactive MAb KAP14-1 in 3% BSA-PBS for 15 min. The grids were washed twice with PBS, incubated with a 1:80 dilution of protein A-gold for 10 min,

and washed twice with deionised water. Finally, the grids were negatively stained with 1% uranyl acetate for 30 sec, blotted dry and examined under a Philips CM 100 transmission electron microscope.

2.15.9 Determination of protein concentration

Protein concentration of solutions was estimated at A_{280} using an Ultrospec Plus spectrophotometer (Pharmacia, LKB Biochrom, Milton Keynes, England), with BSA as a standard. An A_{280} of 1.4 was taken as equivalent to 1 mg per ml of protein.

2.16 LPS (silver) staining

SDS-PAGE of samples was performed on a 15% acrylamide gel after digestion of protein with proteinase K (Hitchcock and Brown, 1983). The gel was silver-stained according to the method described by Tsai and Frasch (1982) as follows:

i) fixation overnight in 40% (vol/vol) ethanol and 10% (vol/vol) acetic acid in water;

ii) oxidation for 5 min with 0.7% (wt/vol) periodic acid in 40% (vol/vol) ethanol, 10% (vol/vol) acetic acid in water;

iii) 4 washes with water for 30 min each wash;

iv) staining for 10 min in a solution containing 28 ml of 0.1 N NaOH, 2 ml of concentrated NH_4OH and 5 ml of 20% (wt/vol) $AgNO_3$ in a total volume of 150 ml;

v) developing in a solution of 50 mg citric acid and 500 µl formaldehyde in 1 litre of water. The citric acid was dissolved in water and heated to 37°C and formaldehyde was added just before use. Milli-Q water was used to rinse all glassware and to prepare the solutions used.

2.17 *In vivo* studies

2.17.1 Colonisation and competition studies.

For colonisation studies, 11RX and its derivatives (PE908 and PE909) and 7314 and its *sefA::aphA3* mutant derivative (PE910) were used, and two repeat experiments were performed. In each experiment, mice were divided into 3 groups of 25 mice per cage. After withholding feed for 4 h, the mice were fed with 35 µl of a 10% (wt/vol) solution of sodium bicarbonate by means of a sterile micropipette followed by infection with 30 µl of 1-2 × 10^9 CFU of either 11RX, PE908 or PE909 strains in phosphate-buffered saline (PBS, pH 7.4). For strains 7314 and PE910, 30 µl of 1 × 10^4 CFU of the bacterial suspensions were used. At days 1, 4, 8, 14 and 21 post-infection, 5 mice were sacrificed from each group by cervical dislocation and the spleen and Peyer's patches of all five mice from each group were homogenised separately in sterile normal saline, diluted as necessary, and viable counts determined in triplicate by culturing samples on XLD agar with or without Km overnight at 37°C.

In the first competition experiment, 15 mice were infected orally with a mixture of 11RX and PE908 at 1 × 10^8 and 8.5 × 10^7 CFU, respectively (1.2:1 ratio). In a later experiment, 15 mice were fed with a mixture of 5 × 10^4 organisms of strain 7314 and 5 × 10^4 organisms of strain PE910. At days 1, 4 and 8, five mice were sacrificed, and viable counts of bacteria in their spleens and Peyer's patches determined as above, using XLD agar with and without Km to determine the relative proportions of the two types of bacteria present in both tissues.

2.17.2 LD_{50} measurements

The LD_{50} of *S.* Enteritidis strain 7314 and that of its isogenic *sefA::aphA-3* mutant strain (PE910) was determined using standard methodology. Groups of 5 mice were

infected orally with either strain 7314 or strain PE910 at 1×10^8, 5×10^7, 1×10^7, 5×10^6, 1×10^6, 5×10^5, or 1×10^5 CFU, respectively. Mice were observed over 3 weeks and any deaths recorded on a daily basis; mice that displayed roughened hair on their coat, a reluctance to move and severe weight loss were also sacrificed and recorded as deaths due to infection. The LD_{50} values were calculated according to the method of Reed and Muench (1938).

2.17.3 Protection studies

For protection studies mice were set up into seven groups of 10 mice per cage. The mice were deprived of feed and water for 4 h before being fed with 30 µl of a 10% (wt/vol) solution of sodium bicarbonate followed by an oral dose of a suspension of 1-2 $\times 10^9$ live bacteria of strains 11RX, PE908, PE914, PE915 PE916 or SL3261 in phosphate buffered saline (PBS, pH 7.4). As a control, one group of mice were fed with PBS only. After one week, mice received a "booster" oral dose of the same bacteria. Thirty five days later, they were anaesthetised and bled from the retro-orbital plexus. They were challenged orally a week later with an approximately 200 LD_{50} (5×10^7) dose of the virulent *S.* Enteritidis strain 7314. The mice were observed for 30 days after challenge. In a separate experiment, mice were grouped as described above, but were infected only once with the same dose of bacteria, and challenged 42 days later with the same virulent strain.

2.18 *In vitro* adherence and invasion studies

These assays were carried out essentially as described by Hale and Bonventre (1979) and Lee *et al.* (1992), but using HeLa cells, and with some modifications. HeLa cells were dispensed onto cover slips (13 mm diameter) in 24-well tissue culture trays (Corning 25820) at 4×10^4 cells per well to form non-confluent monolayers, and incubated

at 37°C overnight in 5% CO_2. Approximately 3 h before infection, culture medium was aspirated from the monolayers and replaced with antibiotic-free medium. Monolayers for adhesion assays were treated with cytochalasin D at 5 μg/ml for about 30 min before infection to prevent bacterial uptake or invasion (Sory and Cornelis, 1994). They were then cultured in triplicate with 4×10^5 CFU of exponential-phase cultures of 11RX, PE908, 7314, or PE910 grown in CBT broth. After incubation for 1 h, the monolayers were washed six times in D-PBS to remove unattached bacteria, and then disrupted with 200 μl of 0.5% Triton X-100 in PBS for 10 min. The lysates were then diluted in PBS, and plated on LB agar with or without Km overnight at 37°C to enumerate the number of viable bacteria. Alternatively, cultured HeLa cells were washed six times in D-PBS, fixed in cold methanol for 10 min, and stained with Giemsa solution. The number of adherent bacteria and number of infected Hela cells per 100 HeLa cells was determined by counting duplicate fields using a 100× (oil immersion) objective.

For invasion studies, HeLa cell monolayers were cultured in triplicate with a multiplicity of infection of 10 of bacteria per cell. After 2 h incubation, monolayers were washed twice in D-PBS and growth of extracellular bacteria was inhibited by the addition of Gm (50 μg/ml) in culture medium with 5% foetal bovine serum for a further 2 h. The monolayers were then washed twice in D-PBS, disrupted with 0.5% Triton X-100, and viable counts were determined as described above. Alternatively, the monolayers were washed twice in D-PBS after culture, fixed to the cover slips by immersing them in cold methanol for 10 min, and stained with Giemsa solution. The percentage of HeLa cells infected was calculated as: (number of HeLa cells infected/total number of HeLa cells counted × 100). In each assay, 200 HeLa cells were examined in duplicate fields and those with one or more bacteria associated with them were recorded as infected. In addition,

wherever it was possible to do so, the number of bacteria per HeLa cell were counted and recorded.

To determine the relative number of internalised organisms in an *in vitro* competition experiment using a mixture of 11RX and PE908, and a mixture of 7314 and PE910, monolayers were set up in triplicate as above and cultured with a mixture of either 11RX strain and PE908, or a mixture of 7314 strain and PE910. After incubation for 2 h, monolayers were washed in D-PBS five times after incubation with Gm. The HeLa cells were disrupted with 200 µl of 0.5% Triton X-100 in PBS for 10 min, aliquots were diluted and plated onto LB agar with and without Km, and incubated overnight at 37°C.

2.19 Measurement of bacterial growth in HeLa cells

HeLa cell monolayers were cultured with bacteria at a multiplicity of infection of between 5 and 10 bacteria per cell. After 1 h of culture, the monolayers were treated with 50 µg/ml of Gm. After 2 h incubation with Gm, the concentration was reduced to 10 µg/ml. At that time and at 2, 4, 8, 12, and 24 h thereafter, triplicate wells from each bacterial infection were disrupted with 0.5% Triton X-100 for 10 min and internalised bacteria were enumerated as described in Section 2.18.

2.20 Double immunofluorescence microscopy

HeLa cells were infected with bacteria as described for the invasion assay. After incubation with Gm for 2 h, monolayers were fixed onto the cover slips with 3.7% formalin in PBS overnight at 4°C. The cells were then permeabilised with 500 µl of 0.1% Triton X-100 in PBS for 1 min at room temperature. Intracellular bacteria were labelled with a rabbit anti-*S.* Enteritidis 11RX serum (primary Ab), and goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (goat anti-rabbit FITC; Sigma) as the secondary

Ab. The host cell cytoskeleton was labelled with a phalloidin-rhodamine conjugate (Sigma), and the preparations were examined under an Olympus BH-2 fluorescence microscope with a 100× (oil immersion) objective.

CHAPTER 3

Preliminary characterisation of SefA

3.1 Introduction

Previous work by Kotlarski *et al.* (1989) had demonstrated that T cells from *S.* Enteritidis 11RX (11RX)-immunised animals can be induced to proliferate *in vitro* and to release cytokines when cultured in the presence of antigen presenting cells and formalin-killed 11RX organisms or soluble 11RX antigens. In a subsequent experiment, Vordermeier and Kotlarski (1990a) used the "T cell Western blot" technique described by Abou-Zeid *et al.* (1987) and Lamb *et al.* (1988) to establish that T-cell stimulating antigens of 11RX localised in the region containing proteins of approximately 14-18 kDa, but were also present with sizes of 24, 34 and 50-60 kDa.

The existence of two different pathways of antigen processing could explain why some fragmented peptides associate with Class I MHC products whereas others complex with Class II molecules and elicit $CD8^+$ and $CD4^+$ T-cell responses, respectively (Falo *et al.,* 1985; Germain, 1986; Morrison *et al.*, 1986; Braciale *et al.*, 1987). It has been shown that when spleen cells are cultured with appropriate peptide fragments of ovalbumin (OVA), strong peptide-specific responses by Lyt 2^+ ($CD8^+$) T cells are induced (Carbone *et al.*, 1988; Moore *et al.*, 1988). Fragments of OVA cleaved by trypsin and cyanogen bromide were both effective, whereas native OVA did not provoke any detectable response (Carbone *et al.*, 1988). The introduction of OVA directly into the cytoplasm of EL4 tumour cells resulted in presentation of this protein as OVA peptide-Class I MHC molecule complexes (Moore *et al.*, 1988). It is believed that this approach could be employed in the presentation of well defined antigens of IBPs in the context of Class I as well as Class II MHC molecules of antigen presenting cells (Unanue, 1984; von Boehmer *et al.*, 1989), and would enable a detailed evaluation of the relative importance of the two types of complexes in the induction of resistance to IBPs and an understanding of the

biology of interaction of IBP antigens with antigen presenting cells. To achieve this aim, Vordermeier and Kotlarski (1990b) suggested that it is essential to use purified antigens, preferably of relatively low molecular weight, thereby reducing the number of different antigenic determinants being studied. Preliminary work with the partially purified low molecular weight 16 kDa protein of 11RX by Vordermeier and Kotlarski (1990b) indicated that this protein might be a suitable candidate antigen for such studies. The authors also observed that formalin-killed suspensions of other organisms within the family *Enterobacteriaceae* , such as *E. coli*, were able to induce proliferation of, and IL-2 release from 11RX-primed T cells. This finding led the authors to suggest that an 11RX Ag(s) localised in the 16-18 kDa region could be the "common" Ag(s) expressed by some members of the family.

As a continuation of the work of Vordermeier and Kotlarski (1990a, b), this chapter describes the purification of the 16 kDa antigen, its characterisation and an evaluation of its immunogenic properties. Data are presented which establish that the 16 kDa protein referred to as AP16 by Vordermeier and Kotlarski (1990b) is actually a precursor form of the 14 kDa structural subunit protein (SefA) of *S.* Enteritidis fimbriae (SEF14) described in the literature (Feutrier *et al.*, 1986, 1988; Thorns *et al.*, 1990), and characterised by others during the course of this study (Clouthier *et al.*, 1993; Turcotte and Woodward, 1993). Evidence is also presented which establish that the protein is not a "common" Ag expressed by a number of organisms belonging to the family *Enterobacteriaceae*.

3.2 Results

3.2.1 N-terminal amino acid sequence of the immunogenic protein

The first step in the detailed characterisation of the 16 kDa protein described by Vordermeier and Kotlarski (1990a, b) was to prepare the protein for sequencing to ascertain its identity. The procedure adopted is detailed in Section 2.15.2 in Chapter 2. Analysis of the amino acid sequence of the SDS-PAGE-purified protein established its homology to the published sequence FM$SALEN (Swiss Protein Identification number)

of the 14 kDa fimbrial protein (SEF14) of *S.* Enteritidis (Table 3.1). The sequence blot gave 30 clear cycles with only two differences to the published sequence. These were the Glu/Val difference in position 9, and the Asn/Ser difference in position 27. The sequence data of Turcotte and Woodward (1993) are similar to those reported here except for the Asn/Ser difference in position 27. As a consequence of this finding, the immunogenic fimbrial protein will be referred to as SEF14 here and in subsequent chapters of this thesis.

Table 3.1 N-terminal amino acid sequences of SEF14 fimbrial proteins of *S.* Enteritidis strains 11RX, 27655-3b and 1246/89.

Strain	Amino acid sequence[a]	Reference(s)
11RX	AGFVGNKAEVQAAVTIAAQNTTSANWNQDP	This study
27655-3b	AGFVGNKAVVQAAVTIAAQNTTSANWSQDP	Clouthier *et al.*, 1993
1246/89	AGFVGNKAEVQAAVTIAAQNTTSANWSQDP	Turcotte and Woodward, 1993

[a] For strain 11RX, the amino acid sequence was deduced chemically from SDS-PAGE-purified protein by A. Gooley, Macquarie University, Sydney, Australia; for strains 27655-3b and 1246/89, the amino acid sequences were deduced by DNA sequencing. Underlining indicates amino acids that are not identical in all three sequences.

3.2.2 Purification of SEF14.

An extension of the various approaches employed in defining and characterising the T-cell stimulatory antigens of 11RX was the purification of its highly immunogenic 14 kDa fimbrial protein. This was accomplished by differential ultracentrifugation and ammonium sulphate precipitation as described in Chapter 2 (Section 2.15.1). As a consequence of identifying the 14 kDa protein of 11RX as a fimbrial protein, the organisms were grown on CBT agar with the expectation that this medium would increase the yield of the protein based on our findings with the K99 fimbrial Ag of enterotoxigenic *E. coli* expressed in 11RX (unpublished observations), and indeed this was found to be the case. The ultracentrifugation step was introduced to remove LPS from the SEF14 preparation, however, SDS-PAGE analysis (Fig. 3.1A) shows that a substantial amount of the starting material, including some fimbrial protein, was also removed by this process. This loss was, however, compensated for by the purity of the final SEF14 preparation

which was confirmed by silver staining which detected no LPS contamination (Fig. 3.1B). The total protein yield from this procedure was estimated to be 1.5 mg/g dry weight of bacteria.

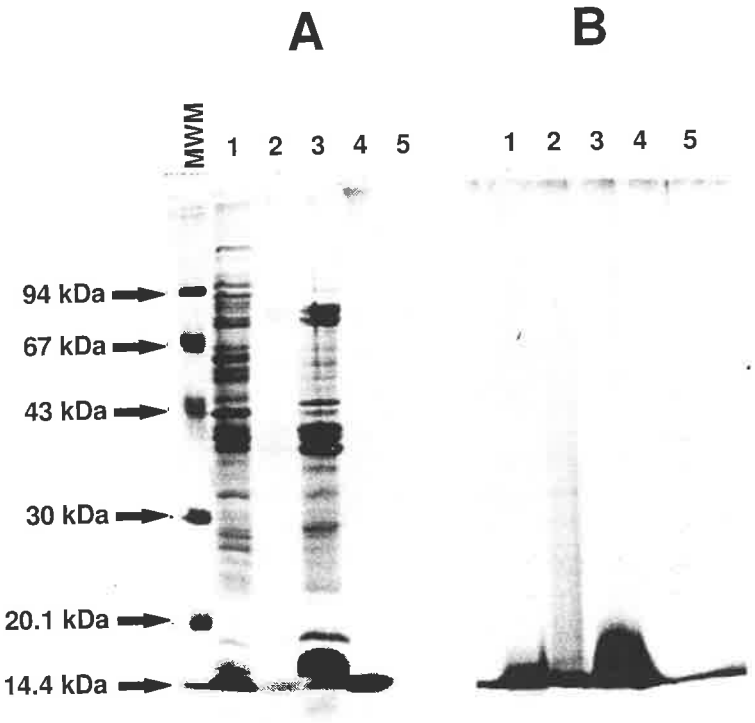


Figure 3.1 (A) SDS-PAGE analysis of fractions obtained during the purification of SEF14. The proteins were visualised by staining with Coomassie blue. Lanes: MWM= low-molecular-weight markers (Pharmacia) in kilodaltons; 1, whole cell lysate of 11RX (1 mg of protein); 2, heat-eluted fraction (200 μg of protein); 3, LPS pellet (500 μg of protein); 4, SEF14 (100 μg); 5, residual supernatant. (B) Same samples in panel A but silver stained. Samples in panel B were treated with proteinase K before electrophoresis.

3.2.3 *In vitro* response of normal and 11RX-primed T cells to SEF14.

LPS is mitogenic for B cells and is known to be a contaminant of proteins which localise in the 14-18 kDa region of SDS-PAGE fractionated preparations of salmonellae unless other procedures are included to remove the LPS. Because it is often difficult to prepare T-cell populations completely free of B cells, it was important to establish that the biological activity of the purified SEF14 preparation was not partly due to LPS contamination. To rule out this possibility, a comparison of the *in vitro* ability of SEF14, formalin-fixed 11RX, soluble extracts of 11RX and 11RX-LPS to stimulate 11RX-primed and normal T cells was made. Control cultures containing either normal or 11RX-primed

T cells in the presence or absence of the T-cell mitogen ConA were included for comparison. The purified SEF14 preparation induced 11RX-primed T cells to proliferate to a level similiar to that observed with either formalin-fixed 11RX or soluble extracts of 11RX, whereas 11RX-LPS had no stimulatory effect (Table 3.2); none of these antigens could stimulate purified T cells from normal animals (Table 3.3). As expected, both types of T cells were equally responsive to ConA (Tables 3.2 and 3.3).

Table 3.2. *In vitro* proliferation of T cells from 11RX-immunised mice.

Stimulant[a]	[^{3}H]-TdR incorporation (cpm, mean ± SEM) after 3 days of culture[b]		
	T cells	T cells + NPC	NPC
SEF14 (1 µg/ml)	5,023 ± 496	43,522 ± 2,803	79 ± 22
F11RX (1 µg/ml)	5,798 ± 531	42,103 ± 3,301	101 ± 16
S11RX (10 µg/ml)	ND[c]	35,659 ± 980	89 ± 18
LPS (10 µg/ml)	697 ± 335	1,063 ± 489	153 ± 88
ConA (1 µg/ml)	23,217 ± 3,320	66,778 ± 3,885	106 ± 34
Medium only	66 ± 14	863 ± 232	52 ± 21

[a] F11RX, formalin-killed 11RX; S11RX, soluble extracts of 11RX; ConA, concanavalin A; NPC, normal peritoneal cells.
[b] 1×10^5 T cells, and 2×10^4 NPC were used per well. Cultures were pulsed for 4 h with [^{3}H]-TdR before harvest. Data presented are for quadruplicate cultures.
[c] ND, not determined.

Table 3.3. *In vitro* proliferation of T cells from nonimmunised mice.

Stimulant[a]	[^{3}H]TdR incorporation (cpm, mean ± SEM) after 3 days of culture[b]		
	T cells	T cells + NPC	NPC
SEF14 (1 µg/ml)	1,563 ± 58	6,283 ± 593	79 ± 22
F11RX (1 µg/ml)	2,365 ± 389	10,120 ± 1,662	101 ± 16
S11RX (10 µg/ml)	ND[c]	3,222 ± 453	89 ± 18
LPS (10 µg/ml)	2,069 ± 653	5,344 ± 688	153 ± 88
ConA (1 µg/ml)	51,023 ± 2,998	60,038 ± 3,220	106 ± 34
Medium only	80 ± 16	ND	52 ± 21

[a, b, c] See footnotes for Table 3.2.

Purified SEF14 (at 1 μg/ml) was also able to induce secretion of IL-2 into the supernatants of 11RX-primed T-cell cultures, to an extent comparable to soluble extracts of 11RX (at 10 μg/ml; Table 3.4), corroborating the results obtained earlier by Vordermeier and Kotlarski (1990b) with the partially purified protein. However, 11RX-LPS did not induce significant levels of IL-2 secretion (data not shown). These, and the observation that no LPS was detected in the purified SEF14 preparation by LPS staining of gels following SDS-PAGE fractionation (Fig. 3.1B), makes it unlikely that LPS might have contributed to the stimulatory activity of SEF14 when added to cultures of T cells and NPC.

There was some variability in the relatively high levels of IL-2 released from 11RX-primed T cells cultured with purified SEF14 or soluble extracts of 11RX when no NPC were added, ranging from almost undetectable levels to more than 50% of those released when NPC were also present. This was interpreted to indicate that the 11RX-primed T cell suspensions used in these studies differed in the number of contaminating antigen presenting cells (APC) that remained after purification. Since significant proliferation of all the T-cell preparations used in these duplicate experiments was not obtained unless NPC were added, it was inferred that induction of cytokine release from these T cells required smaller numbers of antigen-presenting cells than induction of proliferative responses.

Table 3.4. Ability of 11RX antigens to induce IL-2 production and elicit DTH reaction.

Stimulant[a]	IL-2 released (U/ml)[b]			% Swelling at the following time after challenge[c]	
	T cells	T cells + NPC	NPC	24 h	48 h
SEF14 (1 μg/ml)	43	81	<2	23.5 ± 5.3	31 ± 7.7
S11RX (10 μg/ml)	35	67	<2	ND[d]	ND
F11RX (1 μg/ml)	ND	ND	ND	34.6 ± 2.2	55 ± 7.8
Medium only	<2	<2	<2	1.7 ± 1.0	0.6 ± 0.5

[a] See Table 3.2, footnote *a*.
[b] Samples tested were culture supernatants harvested after 24 h of incubation of 11RX-primed T cells, NPC and antigen and assayed by using the CTLL cell line.
[c] Results are means ± SEM for groups of three mice. The percent footpad swelling was calculated on the basis of measurements of right (control) and left (test) hind footpad thicknesses.
[d] ND, not determined.

3.2.4 DTH responses.

It is generally accepted that *in vitro* proliferative responses of T cells on their own are insufficient to determine whether cell-mediated immunity to *Salmonella* had been mounted by the animals providing the T cells. This is because the proliferation assay does not distinguish between T cells with effector functions and helper T cells involved in facilitating either a humoral or a CMI response. The purified SEF14 preparation was therefore used as an eliciting Ag in DTH assays to demonstrate its antigenic activity *in vivo* as detailed in Section 2.10.9 (Chapter 2). A local DTH reaction was elicited when SEF14 was added to fractionated IPCs and then injected into the hind footpads of non-immunised mice, with maximum swelling occuring 24 h after injection (data not shown). Similarly, when SEF14 was injected into the left hind footpads of 11RX-immunised mice, a significant DTH response was elicited, with maximum swelling occuring 48 h after injection (Table 3.4). Little or no swelling was observed at 24 h and the swelling was significantly reduced at 72 h (data not shown). The percentage swelling was calculated using the footpad thickness of the uninoculated (right) hind footpad of each test mouse as the baseline measurement. Comparable results were obtained with the partially purified protein (Vordermeier and Kotlarski, 1990b).

3.2.5 Preparation of anti-11RX serum.

Vordermeier and Kotlarski (1990b) observed that formalin-killed suspensions of other *Enterobacteriaceae* such as *E. coli,* were able to induce proliferation of, and IL-2 release from, 11RX-primed T cells. Given that Ags localised in the 14-18 kDa region were the most effective in inducing IL-2 release from 11RX-primed T cells, this observation led the authors to suggest that 11RX antigen(s) localised in the 14-18 kDa region could be the "common" antigen(s) expressed by a number of *Enterobacteriaceae.* This suggestion is, of course, not compatible with the conclusion that the Ag in the 14-18 kDa region is SEF14, since that would be expressed by other *Enterobacteriaceae*, and required further investigation. Accordingly, extensive absorption of such cross-reacting anti-11RX serum with C5 and several *E. coli* strains before using it in Western blotting

assays of whole cell lysates of these bacteria (Section 2.10.10, Chapter 2) established that SEF14 is unique to *S.* Enteritidis (Fig. 3.2).

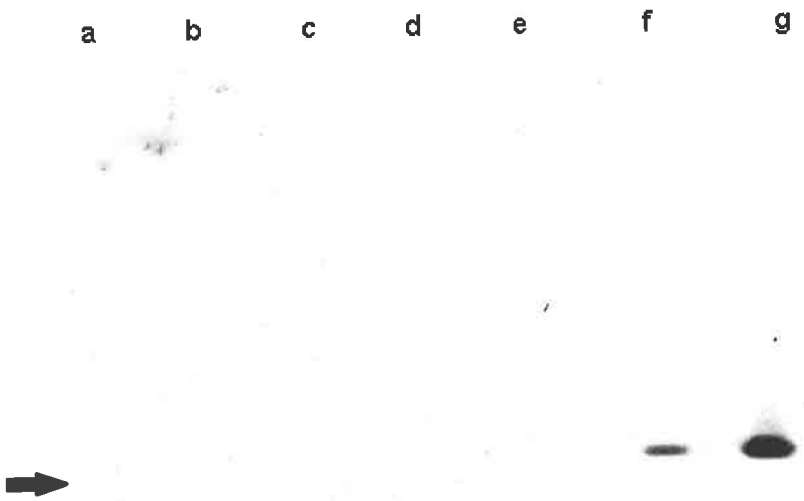


Figure 3.2 Western blotting using an anti-11RX serum extensively absorbed with several species of *Enterobacteriaceae* against whole cell lysates of 11RX, other *Enterobacteriaceae*, and *V. cholerae*. Lanes: a, *S.* Typhimurium C5; b, c, and d, *E. coli* K-12 strains DH1, S17-1, and AAEC189, respectively; e, *V. cholerae* 569B; f, 11RX; g, purified SEF14. Arrow shows the specificity of the antiserum to a band in 11RX corresponding to the SEF14 protein.

3.2.6 Characterisation of monoclonal antibodies to SEF14.

A panel of monoclonal antibodies (MAbs) were raised to SEF14 as described in Chapter 2 (Section 2.10.11). The cell cultures that produced supernatants which reacted specifically with SEF14 in an ELISA were further cloned by limiting dilution and their supernatants were screened by colony and Western blotting. Of the supernatants tested, 11 reacted positively to the whole 11RX preparation by the dot blot method (Section 2.15.7). The specificity of the reactivity of the MAb supernatants to SEF14 was further tested by Western blotting of an SDS-PAGE fractionated whole cell lysate of 11RX and the purified SEF14 preparation under reducing conditions (Section 2.15.7). Using representative blots, it was established that the MAbs bound specifically to one band of

the whole 11RX preparation; this band corresponded to the size of the purified SEF14 preparation (Fig. 3.3).

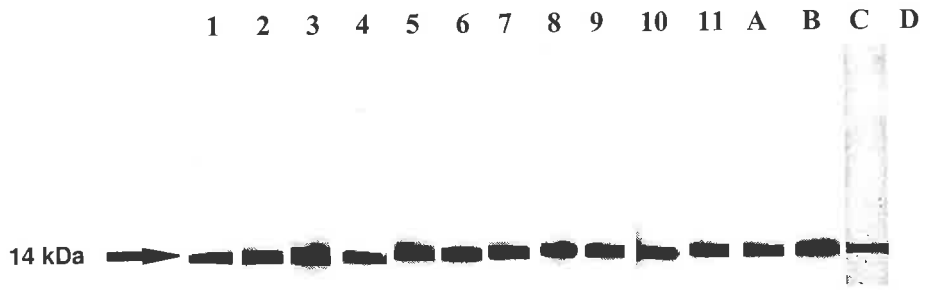


Figure 3.3 Western blotting of SDS-PAGE-fractionated whole-cell lysates of 11RX, showing specificity of the MAbs (KAP14-1 to KAP14-11) raised to the SEF14 protein. Lane A, purified SEF14 blotted with one of the MAbs; lane B, whole 11RX lysate blotted with a SEF14-specific polyclonal serum for comparison; lane C, purified SEF14 stained with 1% amido black; lane D, hybrid supernatant negative for Ab to SEF14.

The abilities of the MAbs produced in this study to detect native and/or denatured SEF14 was examined by (i) immunofluorescence labelling of 11RX bacteria grown for maximal SEF14 production (see Section 2.10.12, Chapter 2), (ii) dot blotting of the native SEF14 preparation from 11RX and (iii) Western blotting of the SEF14 preparation under reducing (denaturing) conditions. Three MAbs to SEF14, designated a-1, a-6 and a-7, were kindly supplied by Dr C. J. Thorns (Central Veterinary Laboratory, Weybridge, Surrey, UK) and were included for comparison. These three MAbs have been characterised previously as belonging to three different epitope clusters as ascertained by competition in a direct-blocking ELISA (Thorns *et al.*, 1992). As shown in Figures 3.4A, and B, all the MAbs examined were able to recognise native SEF14 (by immunofluorescence labelling, and confirmed by dot blotting of native SEF14 preparation), as well as denatured SEF14 (by Western blotting under reducing conditions; in a pattern similar to that of figure 3.3).

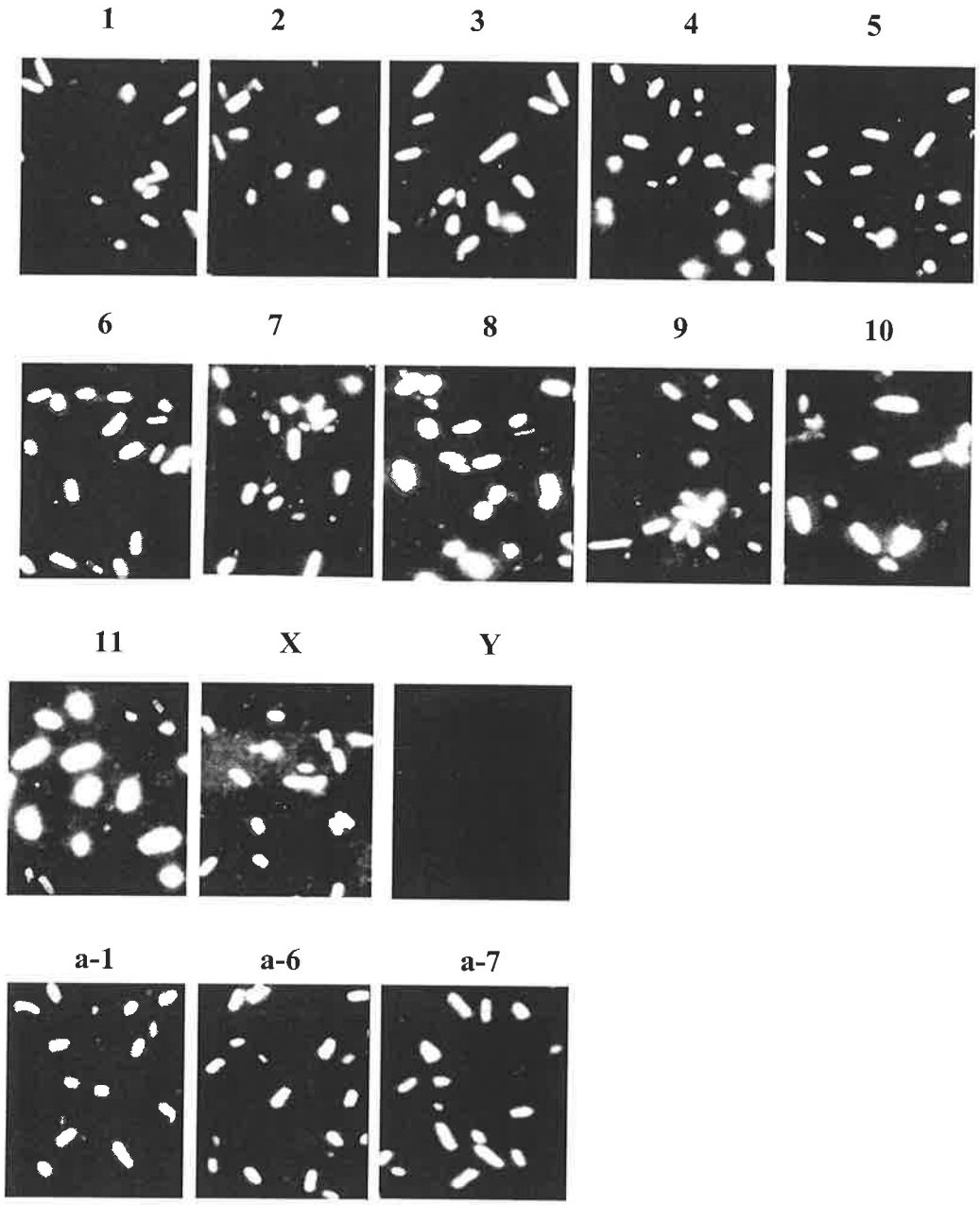


Figure 3.4A Indirect immunofluorescence labelling of 11RX organisms with the various MAbs generated to SEF14 in this study, showing that the MAbs bind to native SEF14 fimbriae. Panels 1 to 11 represent immunofluorescence labelling with MAbs KAP14-1 to KAP14-11 as the primary Abs, and goat anti-mouse IgA, IgM and IgG conjugated to fluorescein isothiocyanate (FITC) as the secondary Ab. Panel X represents immunofluorescence labelling with a SEF14-specific antiserum, panel Y represents immunofluorescence labelling with normal mouse serum (negative control).. Panels a-1, a-6 and a-7 represent immunofluorescence results obtained with the MAbs provided by Dr C. Thorns (CVL, Weybridge, Surrey, England). The Ig isotypes of KAP14-1 to KAP14-11 MAbs are IgG1, IgG1, IgM, IgG1, IgM, IgG2a, IgM, IgG1, (not known), (not known), and IgM, respectively. The Ig isotypes of the MAbs from the CVL are IgG1, IgA, and IgG3, respectively. Magnifications, ×1000.

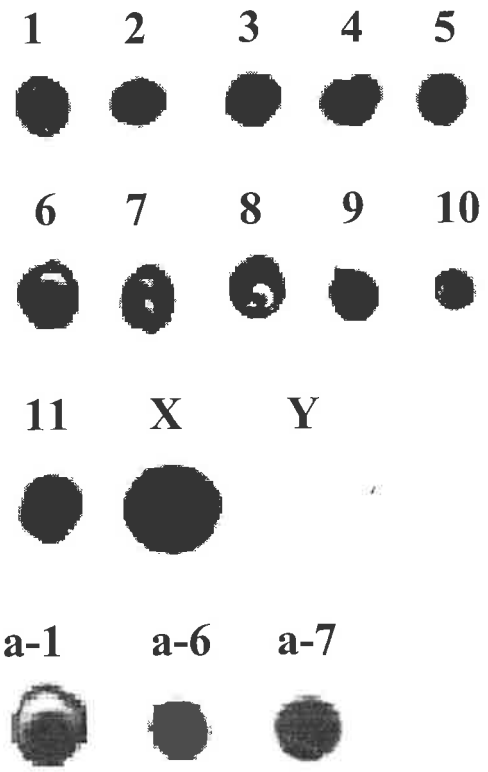


Figure 3.4B Dot blotting of SEF14 preparation from 11RX with the various MAbs (KAP14-1 to KAP14-11) generated to SEF14, and a SEF14-specific antiserum (X). Y represents normal mouse serum (negative control). a-1, a-6 and a-7 represent dot blot results obtained with the MAbs provided by Dr C. Thorns (CVL, Weybridge, Surrey, England).

3.2.7 Immunogold electron microscopy.

11RX organisms were subjected to immunogold electron microscopy using one of the MAbs (KAP14-1) raised to SEF14. Figure 3.5 shows that the antibody binds specifically to fimbrial structures resembling the SEF14 described by Thorns *et al.* (1990) and Clouthier *et al.* (1993).

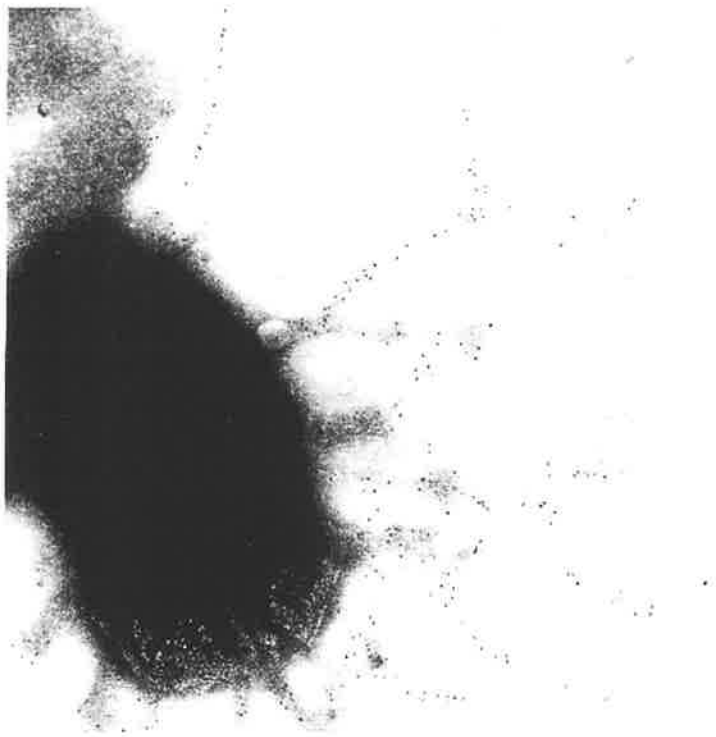


Figure 3.5 Immuno-electron microscopy of uranyl acetate-stained *S.* Enteritidis 11RX strain labelled with protein A-gold after incubation with MAb KAP14-1, which recognises SEF14. Cells were grown in static CBT broth for 48 h at 37°C. Magnification, ×28,500.

3.3 Discussion

One of the requirements for an effective evaluation of the relative importance of class I and class II MHC molecules in the presentation of well-defined antigens of *Salmonella* for induction of T-cell mediated immunity is the availability of purified antigens, preferably of relatively low molecular weight. They are needed not only to reduce the number of different antigenic determinants, but also to abolish non-specific proliferation of lymphoid cells from immune mice due to contaminating LPS in protein antigen preparations of *Salmonella* (Kuusi *et al.,* 1981; Villarreal *et al.*, 1992). This chapter describes the purification and immunogenic characteristics of one such antigen, a 14 kDa fimbrial protein (SEF14) of *Salmonella* Enteritidis 11RX.

Purification of the 14 kDa protein was achieved by differential ultracentrifugation and ammonium sulphate precipitation. The method adopted for purification was

developed by a modification of the protocol published by others (Feutrier *et al.*, 1986; Müller *et al.*, 1991), and was aimed at obtaining reasonable yields of the protein while reducing LPS contamination. The results obtained indicate that the purification procedure was successful and reproducible, and the final product was satisfactory with regards to its homogeneity and suitability for immunological assays.

In previous attempts, nutrient agar was used to grow the 11RX used to prepare this protein. However, when N-terminal amino acid analysis indicated the protein to be a fimbrin, CBT agar was subsequently used because 11RX organisms grown on this medium produce more of SEF14 protein when grown under similar conditions. This was ascertained by comparing yields from both preparations; SDS-PAGE and Western blotting of both preparations with MAb KAP14-1 indicated that both preparations contained SEF14 protein, in different amounts. Moreover, the N-terminal amino acid sequences of the corresponding bands are also the same. As a further step towards obtaining higher yields of the protein, a molecular technique was used to overproduce the fimbrial subunit. This is described in Chapter 6.

The protein appears to be an important immunogen of 11RX because the *in vitro* proliferative response and levels of IL-2 released from 11RX-primed T cells in the presence of purified AP14 are comparable to the response mounted to formalin-killed 11RX, and soluble extracts of 11RX. The levels of proliferation of T-cell lines and clones to the partially purified form of the protein and formalin-fixed 11RX and soluble extracts of 11RX have previously been found to be very similar. These observations confirms earlier evidence provided using the "T cell Western blot" technique employed by Vordermeier and Kotlarski (1990b) in selecting specific AP16-reactive T-cell clones, using mice immunised against whole 11RX and maintaining them with the partially purified protein, that SEF14 is highly immunogenic. In addition, the demonstration that SEF14 was able to elicit a DTH reaction in 11RX-immunised animals confirms the previous suggestion that this protein could be an important immunogenic determinant of 11RX (Vordermeier and Kotlarski, 1990a, b). Standard immunoblot analysis using an *S.* Enteritidis 11RX antiserum extensively absorbed with C5 and several *E coli* strains, as well as using a panel of anti-AP14 monoclonal antibodies suggests that this protein is not

a "common antigen" expressed by a number of organisms belonging to the *Enterobacteriaceae* as was implied in an earlier publication (Vordermeier and Kotlarski, 1990b).

Previously, Vordermeier and Kotlarski (1990a, b) had identified this protein to be 16 kDa in size and thus referred to it as AP16. N-terminal amino acid sequencing has indicated that the 14 kDa protein corresponds to the fimbrial subunit and is homologous to SEF14 previously described by others (Feutrier *et al.*, 1986; Thorns *et al.*, 1990; Clouthier *et al.*, 1993; Turcotte and Woodward, 1993), except for the indicated amino acid differences which do not seem to be potentially significant to the secondary structure or the antigenicity of the protein. Further evidence that the protein corresponds to SEF14 is the demonstration of fimbriae morphologically resembling SEF14 on the surface of 11RX cells by immunoelectron microscopy using a MAb, KAP14-1, raised to the purified protein preparation. This was only achieved, however, when 11RX organisms were grown on CBT medium. Further molecular work with this protein using *E. coli* recombinant clones and Western blot analysis using the MAbs to the 14 kDa form have revealed that the 16 kDa size is the precursor form of the protein, since both bands could be detected with the monoclonal antibodies (see Chapter 4).

It is interesting that the 11RX protein established to be highly immunogenic is a fimbrin, considering the accumulating data being generated regarding the potential importance of immune responses to fimbriae in host immunity to many bacterial species that infect epithelial cell surfaces (Duguid *et al.*, 1966; Duguid and Old, 1980; Pearce and Buchanan, 1980; Tramont and Boslego, 1985; Korhonen *et al.*, 1990; Krogfelt, 1991). This is particularly significant in the context that there has been a worldwide increase in the incidence of nontyphoid salmonellosis in humans caused by *S.* Enteritidis in recent years (Cohen and Gangarosa, 1978; Cooke, 1990; Rodrigue *et al.*, 1990; Todd, 1990; Goodnough and Johnson, 1991; Pohl *et al.*, 1991; Rivera *et al.*, 1991). It was, therefore, anticipated that further work with this protein would allow a more detailed assessment and understanding of the role of this protein in the biology of *S.* Enteritidis, and its significance in induction of resistance to this organism. In addition, T-cell epitope

mapping of the protein should be useful in the design of vaccines to this organism. These propositions are the subject of subsequent chapters.

CHAPTER 4

The role of SefA in the pathogenesis of *S.* Enteritidis

4.1 Introduction

In the previous chapter, data were presented to demonstrate that the purified 14 kDa protein of 11RX is likely to be an important immunogen of this organism. It is able to elicit delayed type hypersensitivity (DTH) in 11RX-primed F1 (BALB/c × C57BL/6) mice and to stimulate *in vitro* proliferation of, and cytokine release from, T lymphocytes obtained from these immunised animals to levels comparable with those induced by the whole organism. This low molecular weight protein was also confirmed to be the structural subunit protein of the 14 kDa *S.* Enteritidis fimbrial antigen (SEF14) previously characterised by others (Feutrier *et al.*, 1986, 1988; Thorns *et al.*, 1990; Clouthier *et al.*, 1993; Turcotte and Woodward, 1993), and will be referred to as SefA here and in subsequent chapters.

The identification of the highly immunogenic 14 kDa protein produced by 11RX as a fimbrin is significant because of the considerable interest fimbriae are generating as potential immunogens against many pathogenic bacteria that colonise epithelial cell surfaces, and also because of the possible role of fimbriae in pathogenesis (Duguid *et al.*, 1966; Duguid and Old, 1980; Pearce and Buchanan, 1980; Tramont and Boslego, 1985; Korhonen *et al.*, 1990; Krogfelt, 1991, Bäumler and Heffron, 1995; Bäumler *et al.*, 1996a, b, c).

Investigations by various workers using tissue culture cell assays as *in vitro* model systems to study *Salmonella*-host cell interactions have indicated that efficient entry of *Salmonella* into cells requires a number of bacterial and host factors (Finlay and Falkow,

1989; Falkow *et al.*, 1992) and several chromosomal loci, most of which map between 58 and 60 min of the *Salmonella* chromosome (Finlay *et al.*,1988; Elsinghorst *et al.*, 1989; Galán and Curtiss, 1989a; Ginocchio *et al.*, 1992; Lee *et al.* 1992; Stone *et al.*, 1992; Altmeyer *et al.*, 1993; Collazo *et al.*, 1995; Mills *et al.*, 1995; Stone and Miller, 1995). In addition, regulatory and structural genes have been described that are necessary for invasion and the survival of salmonellae in macrophages (Fields *et al.*, 1989; Miller *et al.*, 1989; 1992), as demonstrated by observations in murine models of infection (Fields *et al.*, 1986; Finlay *et al.*, 1988; Galán and Curtiss, 1989a, b; Miller *et al.*, 1989, 1992; Stone and Miller, 1995).

This chapter describes the cloning of the region encoding SefA, the characterisation of the protein at the molecular level, and the construction of defined *sefA* mutants which have been used for investigating its role in the pathogenesis of *S.* Enteritidis. Data are presented which implicate the SefA protein as having a role in colonisation of epithelial cell surfaces, but not directly in systemic infection, or in the virulence of *S.* Enteritidis.

4.2 Results

4.2.1 Cosmid cloning

The major aim of this chapter was to assess the role of the SefA protein, the major subunit of SEF14 fimbriae, in the pathogenesis of *S.* Enteritidis. This necessitated cloning the *sefA* gene which was accomplished using a cosmid library (see Section 2.14.7.1). Western blotting of whole cell lysates of the *E. coli* strain AAEC189 carrying cosmid clone pPM3901 using the monoclonal antibody KAP14-1 detected both the precursor (16 kDa) and the mature (14 kDa) forms of the SefA protein (Fig. 4.1). As expected, cell fractionation studies (see chapter 2, Section 2.15.4) reveal that the precursor form of the

protein fractionated to the inner (cytoplasmic) membrane fraction of the recombinant *E. coli* strain carrying the 11RX cosmid, while the mature form fractionated to the outer membrane fraction of the recombinant strain (not shown). Immunogold electron microscopy of this recombinant strain showed the presence of assembled SEF14 fimbriae on the surface of the bacteria (Fig. 4.2).

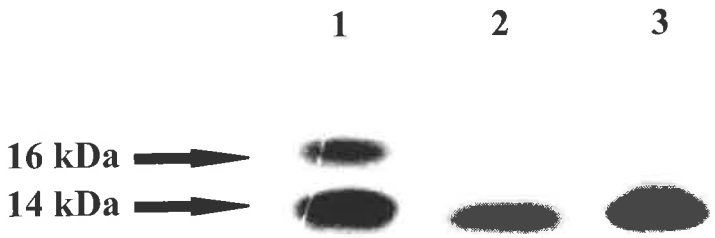


Figure 4.1 Western immunoblot analysis of SDS-PAGE-fractionated whole cell lysates of *S.* Enteritidis 11RX , and the cosmid clone (pPM3901) in *E. coli* AAEC189. Proteins were blotted with the SEF14-reactive MAb KAP14-1. Lanes: **1**, pPM3901 in AAEC189; **2**, 11RX; and **3**, purified SEF14. In the *E. coli* strain carrying the cosmid clone (lane 1), both the precursor (16 kDa) and mature (14 kDa) forms of SEF14 were detected.

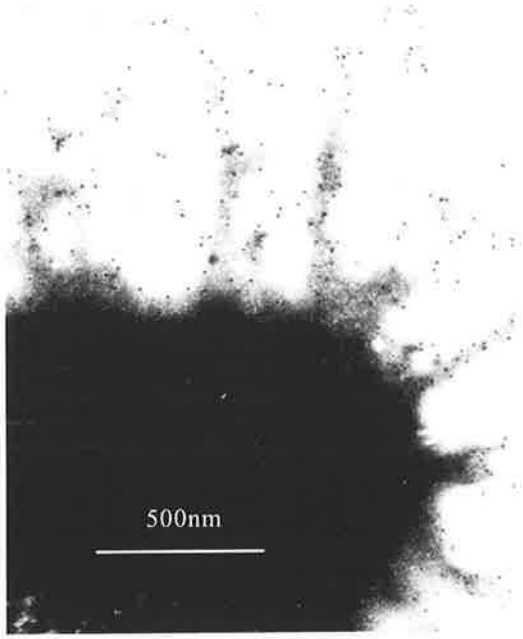


Figure 4.2 Immuno-electron microscopy of uranyl acetate-stained *E. coli* K-12 strain AAEC189 carrying the 11RX cosmid clone pPM3901. The reconbinant strain was labelled with protein A-gold after incubation with MAb KAP14-1, which recognises SEF14. Cells were grown in static CBT broth for 48 h at 37°C. Magnification, ×28,500.

4.2.2 Southern hybridisation and restriction map construction

A limited restriction map in and around *sefA* was constructed by analysing the patterns generated by Southern hybridisation of the restriction fragments of cosmid clone pPM3901 with a DIG-dUTP-labelled 168 bp *sefA* PCR product generated with oligonucleotide #571 and #572 (Section 2.14.13, Chapter 2). A 4.8 kb *Hin*dIII fragment from pPM3901 cosmid DNA (and a 5.3 kb *Hin*dIII fragment from *S.* Enteritidis 11RX chromosomal DNA) gave the smallest single restriction fragments that hybridised with the probe (Fig. 4.3).

Figure 4.3 Southern hybridisation of single and double restriction enzyme fragments generated by complete digestion of the 11RX cosmid clone pPM3901. The restriction fragments were probed with a digoxigenin-dUTP-labelled 168 bp PCR product generated from *sefA*. Lanes: **a,** *Bam*HI/*Eco*RI; **b,** *Bam*HI/*Eco*RV; **c,** *Bam*HI/*Hin*dIII; **d,** *Bam*HI/*Pst*I; **e,** *Bam*HI/*Cla*I; **f,** *Eco*RI/*Eco*RV; **g,** *Eco*RI/*Hin*dIII; **h,** *Eco*RI/*Pst*I; **i,** *Eco*RI/*Cla*I; **j,** *Eco*RV/*Hin*dIII; **k,** *Eco*RV/*Pst*I; **l,** *Eco*RV/*Cla*I; **m,** *Hin*dIII/*Pst*I; **n,** *Hin*dIII/*Cla*I; **o,** *Pst*I/*Cla*I; **p,** *Cla*I; **q,** *Eco*RV; **r,** *Hin*dIII; and **s,** 11RX genomic DNA digested with *Hin*dIII. Molecular size markers (SPP-1) are shown with arrows on the left.

The difference in size between the two *Hin*dIII fragments was because part of the 5.3 kb *Hin*dIII fragment in the chromosome was deleted in the cosmid clone (Fig. 4.4A, B). The 4.8 kb and 5.3 kb *Hin*dIII fragments were cloned into pBluescript KS+, to yield pPM3910 and pPM3911, respectively (Fig. 4.4B, C).

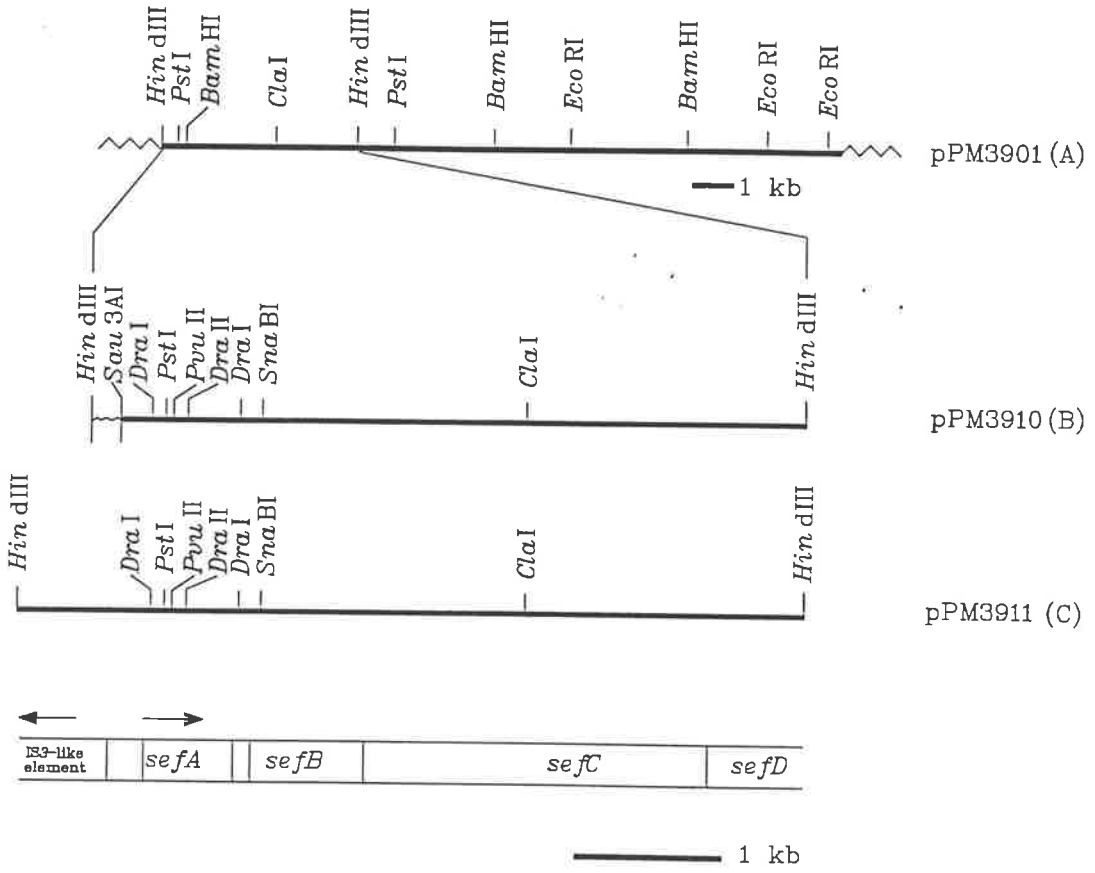


Figure 4.4 (A): Limited restriction map of *S.* Enteritidis 11RX cosmid clone pPM3901, showing the regions in and around *sefA*. This map was derived by single and double digestion with restriction endonucleases, and only the relevant part of the cosmid is shown. **(B):** Linear restriction map of the 4.8 kb *Hin*dIII fragment subcloned from pPM3901 into pBluescript KS$^+$ (pPM3910). The *Hin*dIII site on the left hand side of pPM3910 derives from the cosmid DNA. **(C):** Linear restriction map of the 5.3 kb *Hin*dIII fragment cloned from the 11RX chromosome (pPM3911), showing the position of IS*3*-like element relative to *sefA*. Arrows indicate the direction of transcription of the genes.

Southern blotting was performed on a series of single and double digests of pPM3910 and pPM3911 as described earlier (Section 2.14.13). An 8 kb *Bam*HI fragment from pPM3901 was also cloned into pBluescriptKS+ (pPM3912) and similarly probed

with the DIG-dUTP-labelled 168 bp *sefA* fragment. Based on the results of these blots and the restriction analysis of the DNA sequence of the gene, a restriction map of the fimbrin gene and its exact position and orientation, was generated (Fig. 4.4A, B, C), and was similar to the results of Feutrier *et al.* (1988).

4.2.3 Nucleotide sequence of *sefA*

In order to determine the DNA nucleotide sequence of *sefA*, purified DNA from cosmid clone pPM3901 was partially digested with *Sau*3AI and then ligated to a *Bam*HI-cleaved and CIAP-treated pBluescript KS+ plasmid vector. The ligation mix was used to transform *E. coli* DH5α and selected transformants were screened by DNA hybridisation with an end-labelled oligonucleotide 571 and by immunoblotting with the SEF14-reactive MoAb KAP14-1. The smallest *Sau*3AI subclone (pPM3909) that was positive in these reactions was subjected to dye primer cycle reactions (forward and reverse) and dye-deoxy terminator cycle reactions on a 373A DNA sequencer (Applied Biosystems) to completely sequence the region. Both strands of the DNA were sequenced.

Translation of the DNA sequence revealed a putative ORF of 165 amino acid residues including a 21-amino acid signal sequence. This region was preceded by a good ribosome-binding sequence, and a potential σ^{70} promoter sequence (Fig. 4.5). The molecular weight was in agreement with the M_r of SEF14 described by other workers (Feutrier *et al.*, 1988; Thorns *et al.*, 1990; Clouthier *et al.*, 1993; Turcotte and Woodward, 1993). The deduced amino acid sequence obtained from pPM3909 differed from the N-terminal sequence of the purified fimbrin (Chapter 3, Table 3.1) at position 27, where serine was identified instead of asparagine. The presence of serine at this position is consistent with the sequence data of Turcotte and Woodward (1993) and that of Clouthier *et al.* (1993). The DNA sequence of the *sefA* clone obtained in this study was identical to

that of Turcotte and Woodward (1993). The glutamine/valine difference at position 9 compared with that published by Clouthier *et al.* (1993) was also confirmed.

```
   1  AAG CTT TCG CCC GCA GCA CCT GCC GGC GCA GGC TGG CCG CTA CGG TTT TTA TGT TAT ACA  60
  61  CCC GTC CCT GAG CAC GAA GCT CAT CCG TCA GGC GTG GCG TAC CGT AGC GCT GTT TTG CGT 120
 121  CAC TGA ATG CCT CCC GTA CGA CGT TAT CAC AGA CAA GTC GGA ACT GCT GAC GCC GGA CGT 180
 181  ACC AGC TGT TAC GGG CAA CCT GAA GTA CAC GGC ATA TGG CTT TGA TAC TGA ACT CAG CCT 240
 241  GAT GTT TTT CGA TGA AGA CAT ACT TCA TTT CAG GCG TTT CGC AAA GTA TGT CGC GAC CAT 300
 301  TCT GGA GAA TGG TCA GTT CCT CAT CCC GTT CTG CCA GTT GAC GCT TCA GAC GGA CGA TTT 360
 361  CTG CGG ACA TCT CCT GTT CGC GTT CAG AAG AAG AGA GCT GAT TTT GCT GTT TGC TTC GCC 420
 421  AGT TGT AGA GCT GCG ATT CAT ACA GGT TAA GCT CGC TGG TAG CCG CAG CCA TCC CAA TGC 480
 481  GTT AGC CAG TTT CAA GCT TCG TCG CGA AAT TCA GGC GTA TGT TGC TTG CAT GGC TTT TTG 540
 541  GTG GTT GAT GCT GCT TTT GTC ATG TGA GAC ACC TCT TTC TTG AGA GTT TAC TCA CTT TGT 600
 601  CGC ATG TCC ACT CTT GCT GGG TAA GAT CAG ACA ACC CAT TCT CTC AAT AAA TAA CAT AAA 660
 661  CAT TTT TAT TTG GAT GTG TTT TTT TGT TTA TGC TTT TTA AAT AAA GGG TTG ATA TGA AAT 720
 721  TTT TTA AAC ATG TGG ATA TTA GAT ATT CCA CAA AAA GTA TTA ATG GAT CAT CCA CTT ATT 780
 781  AAT GGG GAT GTT GTG TAA AGA TAA AAA AAT AGT GAT CCT TGT TTT TTT TCT TAA ATT TTT 840
 841  AAA ATG GCG TGA GTA TAT TAG CAT CCG CAC AGA TAA ATT GTG CGA ATG CTA ATA GTT GAT 900
                      -10
 901  TTT TGG AGA TTT TGT AAT ATG CGT AAA TCA GCA TCT GCA GTA GCA GTT CTT GCT TTA ATT 960
   1       RBS                 M   R   K   S   A   S   A   V   A   V   L   A   L   I  14
                                ↓
 961  GCA TGT GGC AGT GCC CAC GCA GCT GGC TTT GTT GGT AAC AAA GCA GAG GTT CAG GCA GCG 1020
  15   A   C   G   S   A   H   A   A   G   F   V   G   N   K   A   E   V   Q   A   A   34

1021  GTT ACT ATT GCA GCT CAG AAT ACA ACA TCA GCC AAC TGG AGT CAG GAT CCT GGC TTT ACA 1080
  35   V   T   I   A   A   Q   N   T   T   S   A   N   W   S   Q   D   P   G   F   T   54

1081  GGG CCT GCT GTT GCT GCT GGT CAG AAA GTT GGT ACT CTC AGC ATT ACT GCT ACT GGT CCA 1140
  55   G   P   A   V   A   A   G   Q   K   V   G   T   L   S   I   T   A   T   G   P   74

1141  CAT AAC TCA GTA TCT ATT GCA GGT AAA GGG GCT TCG GTA TCT GGT GGT GTA GCC ACT GTC 1200
  75   H   N   S   V   S   I   A   G   K   G   A   S   V   S   G   G   V   A   T   V   94

1201  CCG TTC GTT GAT GGA CAA GGA CAG CCT GTT TTC CGT GGG CGT ATT CAG GGA GCC AAT ATT 1260
  95   P   F   V   D   G   Q   G   Q   P   V   F   R   G   R   I   Q   G   A   N   I  114

1261  AAT GAC CAA GCA AAT ACT GGA ATT GAC GGG CTT GCA GGT TGG CGA GTT GCC AGC TCT CAA 1320
 115   N   D   Q   A   N   T   G   I   D   G   L   A   G   W   R   V   A   S   S   Q  134

1321  GAA ACG CTA AAT GTC CCT GTC ACA ACC TTT GGT AAA TCG ACC CTG CCA GCA GGG ACT TTC 1380
 135   E   T   L   N   V   P   V   T   T   F   G   K   S   T   L   P   A   G   T   F  154

1381  ACT GCG ACC TTC TAC GTT CAG CAG TAT CAA AAC TAA
  55   T   A   T   F   Y   V   Q   Q   Y   Q   N   *
```

Figure 4.5 DNA nucleotide sequence of *sefA*, the gene coding for the SefA subunit protein of SEF14 fimbriae of *S.* Enteritidis, with the deduced amino acid sequence of the open reading frame. The consensus ribosome-binding sequence (RBS, underlined) was located 11-14 bp upstream of the translational start site of the gene. A potential σ^{70} promoter sequence (-10 region) is underlined. The signal peptidase cleavage site is indicated by a vertical arrow. The region upstream of *sefA* showing extensive homology to the IS*3*-related element from *E. coli* is boxed.

The DNA coding region upstream of *sefA* was sequenced from pPM3911. Translation of this sequence revealed extensive homology to the insertion sequence IS*3* from *E. coli* (Timmerman and Tu, 1985).

4.2.4 Construction of sefA complementing plasmid

A region flanking *sefA* was amplified using oligonucleotide #929 and #954 with incorporated *Nde*I and *Hin*dIII sites (underlined), respectively (Table 2.2, Chapter 2). The amplified product (617 bp) was digested with these enzymes, cloned into the corresponding sites in pET17-b to generate pPM3914, and used to transform *E. coli* DH5α. Western immunoblot analysis of whole cell lysates of the recombinant strain with the SEF14-reactive MoAb KAP14-1 detected the precursor (16 kDa) and mature (14 kDa) forms of SefA (not shown), in agreement with others (Thorns *et al.*, 1990; Müller *et al.*, 1991).

4.2.5 Construction and analysis of *sefA* mutants

To investigate the role of SefA protein in the pathogenesis of *S.* Enteritidis, the *sefA* gene in 11RX and in a virulent strain, *S.* Enteritidis 7314, was interrupted with a kanamycin resistance (*aphA-3*) cassette using an allelic exchange technique. The procedure employed pCACTUS, a temperature sensitive cloning vector with a chloramphenicol resistance marker and a counter-selectable *sacB* gene constructed by Clark, C. A, R. Doyle, M. Heuzenroeder, and P. A. Manning (manuscript in preparation). In this procedure, cloned *sefA* DNA with the appropriate mutation was exchanged with the corresponding chromosomal copy by homologous recombination, using the selectable markers.

To construct the *sefA* mutant, a 580 bp *Dra*I fragment encompassing *sefA* (Fig.4.4B, C) was cloned into the *Sma*I site in the pCACTUS polylinker to yield pPM3915 (Fig. 4.6). An *aphA-3* cassette with a kanamycin resistance marker from pUC18K vector (Ménard *et al.*, 1993), was used for the nonpolar mutagenesis by cloning it as an 850 bp *Sma*I fragment into an end-filled *Dra*II site in *sefA* in pPM3915, generating pPM3916 (Fig. 4.6).

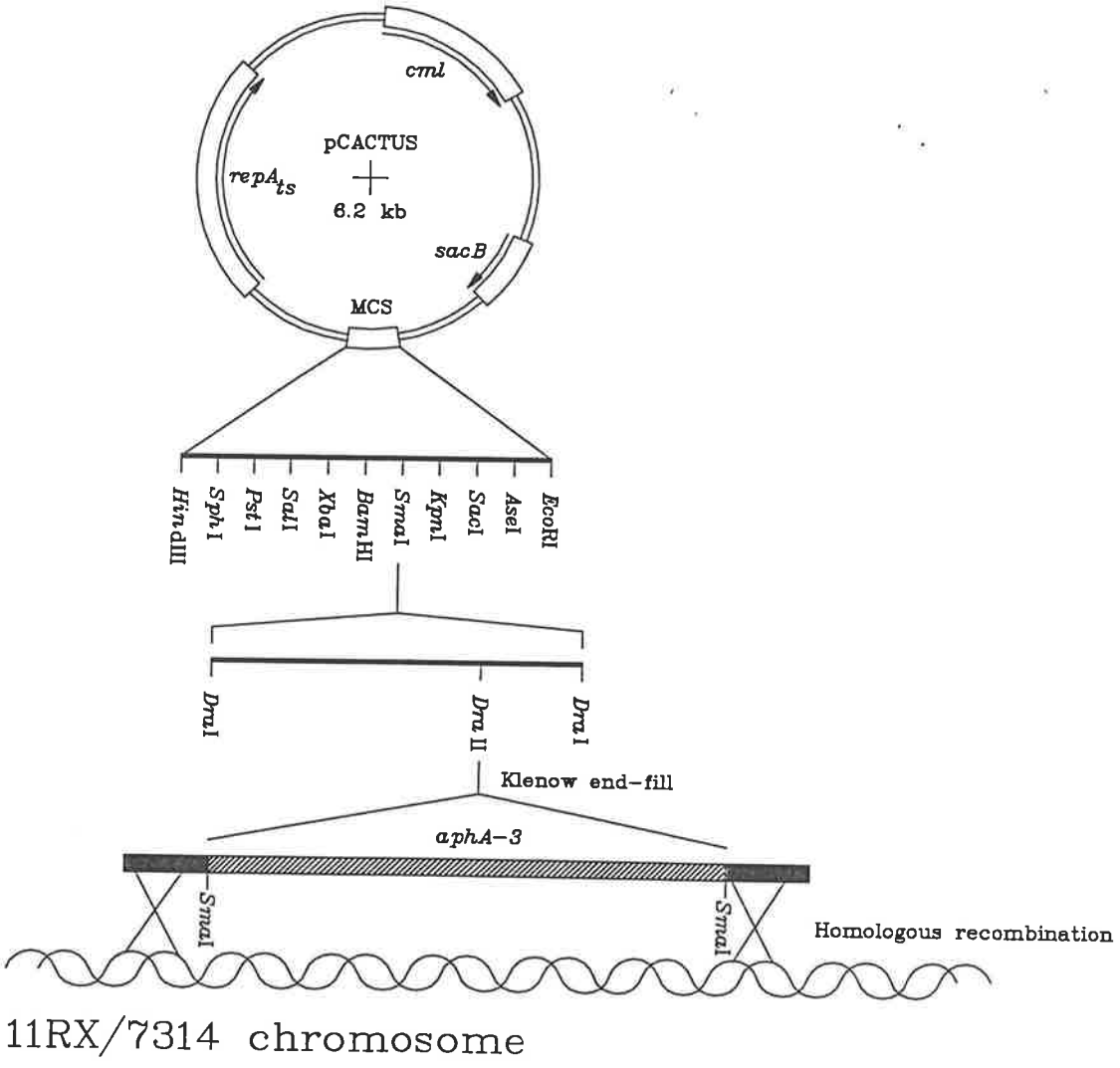


Figure 4.6 Schematic representation of the construction of non-polar mutation in *sefA* with a kanamycin-resistant *aphA-3* cassette with the aid of the suicide vector pCACTUS (see Section 4.2.5 for full description).

About 1 to 2 μg of pPM3916 was electroporated into 1×10^9 cells of electrocompetent 11RX and 7314 strains, using the Gene Pulser apparatus (Bio-Rad). Electrotransformants were selected on LB agar at 30°C in the presence of chloramphenicol (Cm) and were checked for the presence of pPM3916 by plasmid isolation and restriction enzyme digestion. The transformants were then used in the allelic exchange which was allowed to proceed at 30°C in the presence of Cm, and the plasmid integrates were selected at the non-permissive temperature for plasmid replication (42°C) in the presence of Km. Bacteria carrying plasmid cointegrates were resolved by *sacB* negative selection on NaCl-free LB agar plates containing 6% sucrose, the metabolic product of which is toxic to Gram-negative bacteria, to select recombinant bacteria that had both excised and cured the selectable markers, in a manner similar to that described by Blomfield *et al.* (1991b).

Mutation in *sefA* was confirmed by Southern hybridisation, PCR and Western blotting. Southern hybridisation was carried out by digesting chromosomal DNA from both the parent strain and the putative *sefA::aphA-3* mutant strains with *Hin*dIII and probing either with the DIG-dUTP-labelled 168 bp PCR product from *sefA,* or with a similarly labelled 850 bp *aphA-3* cassette. PCR of the parents and the mutants was carried out with oligonucleotide primers #808 and #809. Western blotting of the various strains was carried out using MoAb KAP14-1 specific for SEF14 as primary antibody.

Complementation of mutants was carried out by electroporating pPM3914 into the electrocompetent mutants. Alternatively, complementation of mutants was achieved by backcrossing pPM3915 into the mutants by allelic exchange. The backcross progeny were screened for the loss of Km resistance. Reversion to wild type phenotype was confirmed by immunoelectron microscopy in the complemented mutants, and by PCR, Southern hybridisation, Western blotting and immunoelectron microscopy in the backcross progeny. The 11RX and 7314 mutants were designated PE908 and PE910 respectively, while the 11RX and 7314 backcross progeny were designated PE909 and PE911 respectively.

Southern analysis of the *Hin*dIII-digested chromosomal DNA of 11RX, 7314, and their *sefA* mutant derivatives, PE908 and PE910, respectively, using the DIG-dUTP-labelled *sefA* probe, showed that the *aphA-3* cassette was inserted into *sefA* in the chromosome as indicated by an increase in size of the *Hin*dIII fragment in the PE908 and PE910 (Fig. 4.7A). When the chromosomal digests were probed with a DIG-dUTP labelled *aphA-3* cassette, the larger fragment was detected only in PE908 and PE910 (Fig. 4.7B).

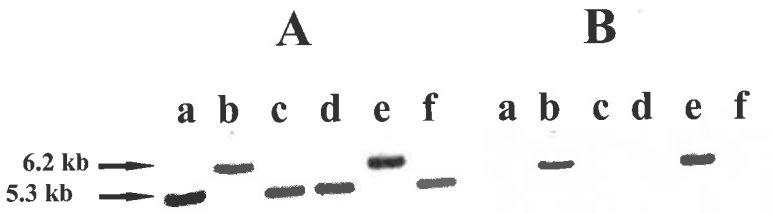


Figure 4.7 Southern hybridization of *Hin*dIII-digested genomic DNA of *S.* Enteritidis strains 11RX, 7314 and their derivatives. Panel **A** was probed with a digoxigenin-dUTP-labelled 168 bp *sefA* PCR product, panel **B** was probed with a similarly labelled 850 bp *Sma*I fragment encompassing the *aphA-3* cassette. Lanes (both panels): **a**, strain 11RX; **b**, *sefA::aphA-3* mutant strain of 11RX (PE908); **c**, 11RX *sefA* backcross strain (PE909); **d**, strain 7314; **e**, *sefA::aphA-3* mutant strain of 7314 (PE910); and **f**, 7314 *sefA* backcross strain (PE911).

In a PCR to amplify *sefA* in the chromosomal preparations of these strains using oligonucleotide #808 and #809, a 1.4 kb product, 850 bp larger than the expected *sefA* PCR product, was detected in all the putative mutants tested (not shown), further confirming the insertion of the *aphA-3* cassette into *sefA* in the chromosome.

Western blot analysis of whole cell lysates of the parent and mutant strains with the KAP14-1 MoAb showed that SefA protein was detected only in the parent and backcross

strains, demonstrating that the insertion of the *aphA-3* cassette into *sefA* results in the abolition of the production of SefA protein in the mutants (Fig. 4.8).

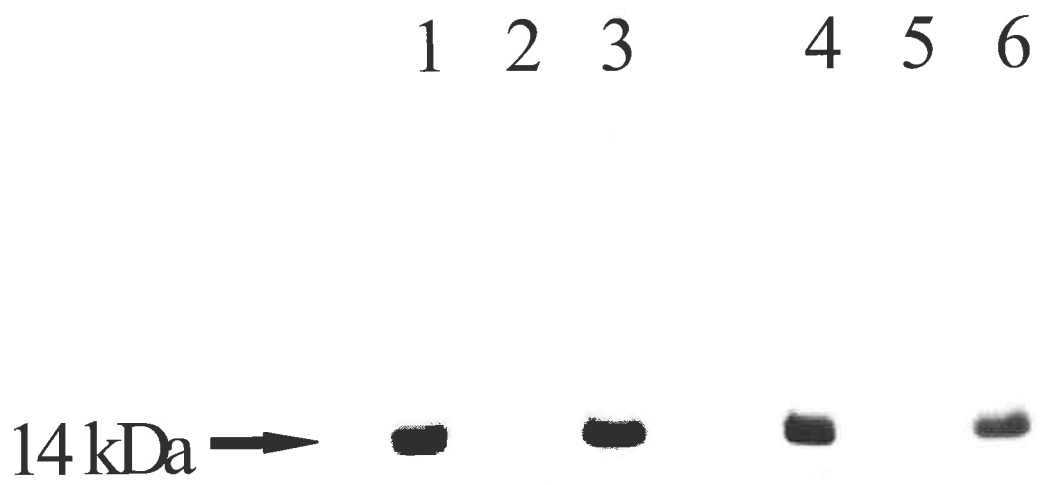


Figure 4.8 Western blotting of whole cell lysates of *S.* Enteritidis strains 11RX and 7314, and their derivatives using a SEF14-reactive MAb, KAP14-1. Samples were heated at 100°C for 3 min in SDS sample loading buffer before electrophoresis. Lanes:1, strain 11RX; 2, *sefA::aphA-3* mutant strain of 11RX (PE908); 3, backcross strain of PE908 (PE909) 4, strain 7314; 5, *sefA::aphA-3* mutant strain of 7314 (PE910); and 6, backcross strain of PE910 (PE911).

To restore the production of SEF14 fimbriae in strains PE908 and PE910, the *sefA* defect was complemented by electroporating pPM3914 into the *sefA* mutants. The strains were then subjected to immunogold electron microscopy. SEF14 fimbriae production was restored on the surface of the complemented mutants (Fig. 4.9), further demonstrating that the *aphA-3* cassette was not polar on the downstream genes *sefB* and *sefC*, which encode for the chaperone and the outer membrane protein (usher), respectively (Clouthier *et al.*, 1993).

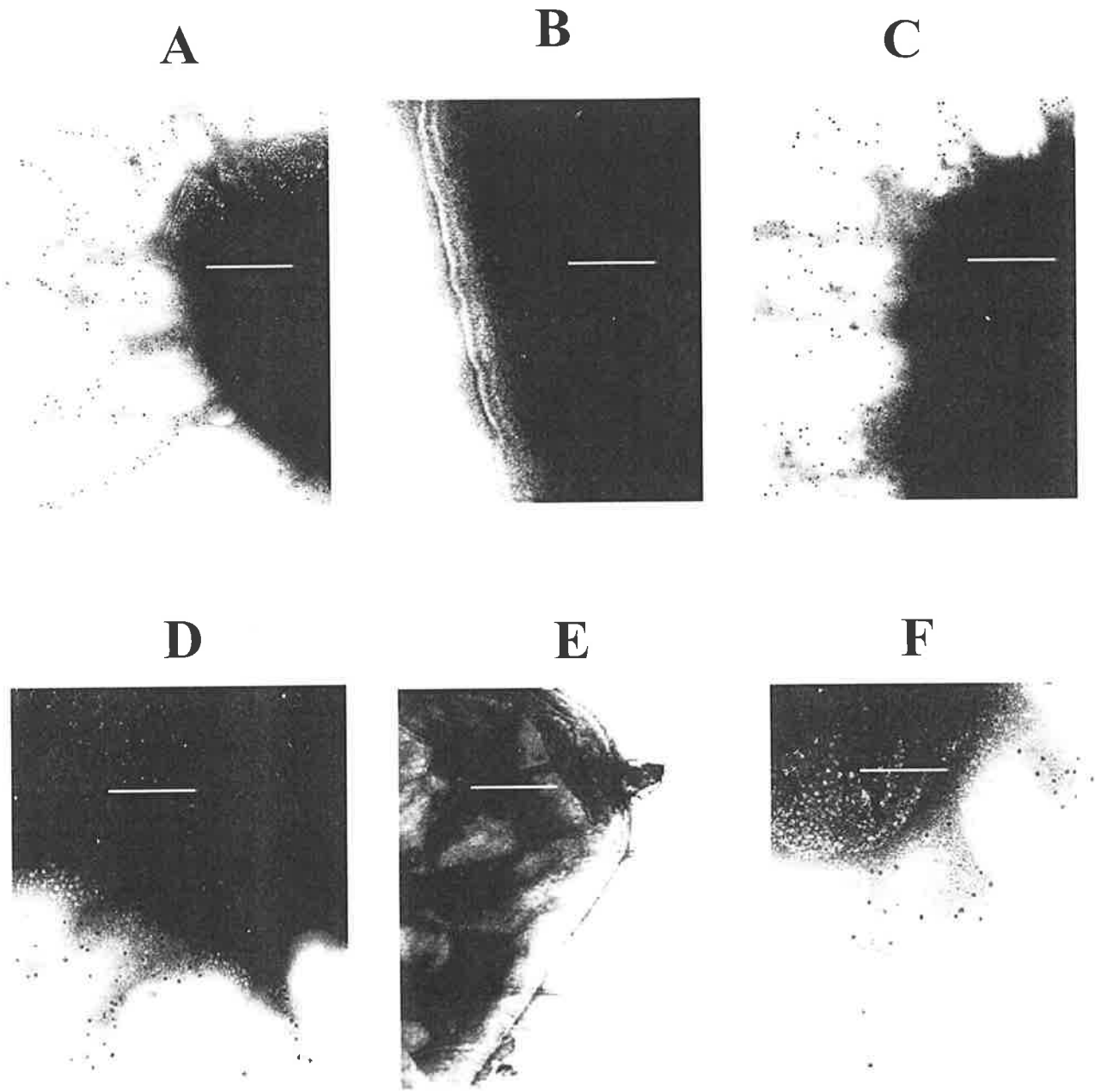


Figure 4.9 Immuno-electron microscopy of uranyl acetate-stained *S.* Enteritidis strains 11RX and 7314, their *sefA::aphA-3* mutant counterparts, and the complemented derivatives of the mutants. Bacteria were labelled with protein A-gold after incubation with the SEF14-reactive MAb KAP14-1. SEF14 fimbriae production was restored in the mutants by *in trans* complementation of SefA defect with pPM3914, a plasmid carrying a functional *sefA* gene. Panels: **A**, strain 11RX; **B**, *sefA::aphA-3* mutant strain of 11RX (PE908); **C**, strain PE908 complemented with pPM3914; **D**, strain 7314; **E**, *sefA::aphA-3* mutant strain of 7314 (PE910); and **F**, strain PE910 complemented with pPM3914. Bars, 200nm.

4.2.6 Colonisation studies

To assess the role of SefA in the pathogenesis of *S.* Enteritidis, the abilities of 11RX, 7314 and their isogenic *sefA* mutants, PE908 and PE910, to colonise the Peyer's patches and invade the spleens of BALB/c mice were compared (Section 2.17.1). To define the role of the SefA protein in colonisation and invasion, the backcross strains PE909 and PE911 were used as additional controls. They were derived from strains PE908 and PE910, respectively, by replacing the disrupted *sefA* gene with the wild type copy.

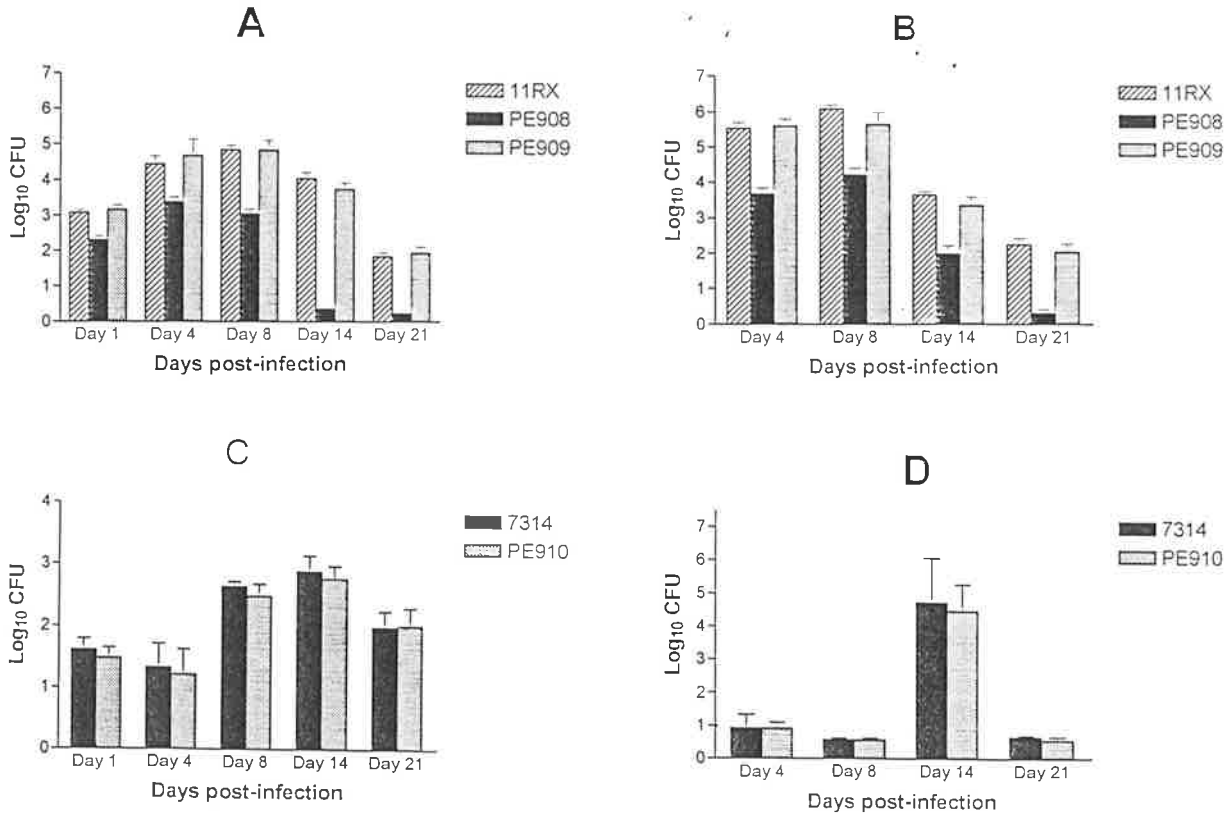


Figure 4.10 Log_{10} CFU of *S.* Enteritidis strains 11RX, 7314 and their derivatives in the Peyer's patches and spleens of BALB/c mice dosed orally with 1-2 × 10^9 organisms of strain 11RX, PE908, or PE909 (A and B), and 1 × 10^4 organisms of strain 7314 or PE910 (C and D) and then killed at intervals. Values are mean (± standard deviation) of five mice per time point for each bacterial strain.

As shown in Figure 4.10A, strain 11RX and PE909 colonised the Peyer's patches better and reached about 100-fold higher numbers by day 8 than PE908 when mice were infected orally with approximately the same dose of bacteria. Strain 11RX and PE909

were also able to persist longer in the Peyer's patches. Appreciable numbers of these bacteria could still be recovered from the Peyer's patches up to 3 weeks after infection, whereas PE908 could no longer be recovered by day 14. These differences could not be attributed to alteration in the growth rate of the bacteria as ascertained by *in vitro* measurements in CBT broth (data not shown). However, the invasion of the spleen did not seem to be appreciably affected by the mutation as indicated by the observation that there was a similar ratio recovered from the spleen in comparison to the numbers recovered from Peyer's patches (Fig. 4.10B). Nevertheless, the outcome was a large number of organisms in the spleens of mice infected with the strain without a mutation in *sefA* (11RX). One would therefore expect a difference in LD_{50} between the two strains.

The results obtained in the colonisation studies with 11RX and its isogenic mutant led to a similar experiment with the virulent 7314 strain and its isogenic mutant, PE910. Because strain 7314 is highly virulent for mice (LD_{50} of ca 2.5×10^5), they were separately fed with 1×10^4 organisms of strains 7314 or PE910 and monitored over a 21-day period. Quite interestingly, there was no obvious difference between the two strains in the pattern of colonisation of Peyer's patches and spleens of the mice (Fig. 4.10C, D) over the period, indicating that the *sefA* mutation did not have a detectable effect on the colonisation of the host epithelium by the SefA-deficient strain. It was also noted that unlike strains 11RX and PE908, virtually all the 7314 and PE910 organisms had been cleared from the spleens of the infected mice by day 21, although a few organisms could still be recovered from the Peyer's patches. This was likely due to the lower dose of the virulent strain and its SefA mutant counterpart used, to ensure that infection of the mice could be followed up to day 21.

To verify these results further, competition for colonisation of Peyer's patches between 11RX and PE908 was evaluated in two separate experiments. In both

experiments, 11RX was able to outcompete PE908 in Peyer's patches over an 8-day period, as shown in Table 4.1A, where the input ratio of 11RX and PE908 was 1.2:1 (1×10^8 and 8.5×10^7 organisms, respectively). It also appears that due to the effect(s) of competition, PE908 could not persist in Peyer's patches as long as it did in the absence of competition. However, an appreciable number of the PE908 strain was able to invade and persist in the spleen.

Table 4.1A *In vivo* competition between strain 11RX and its *sefA::aphA-3* mutant (PE908) after mixed oral infections.

Days after oral infection*	11RX vs PE908 numbers in:[a]			
	Peyer's patches		Spleen	
	11RX	PE908	11RX	PE908
1	3.0 ± 0.3	2.1 ± 0.2	ND[b]	ND
4	4.4 ± 0.5	2.8 ± 0.7	3.8 ± 0.4	2.0 ± 0.4
8	5.1 ± 0.3	2.6 ± 0.9	5.1 ± 0.3	2.5 ± 0.3

* Mice were infected orally with a mixture of strain 11RX and PE908 at 1×10^8 and 8.5×10^7 organisms, respectively (1.2:1 input ratio).
[a] Values are mean CFU ± standard deviation of organisms recovered from Peyer's patches and spleens of 5 mice. CFU are expressed in $\log_{10}$ values.
[b] ND = not determined.

Subsequently, competition between the virulent *S.* Enteritidis strain 7314 and its *sefA* mutant counterpart, PE910, was examined over an 8-day period, using 5×10^4 organisms of each strain. Again, the relative ratios of the two strains recovered were not markedly different in the Peyer's patches and spleens of mice over the period (Table 4.1B). A similar result was obtained in a repeat experiment.

Table 4.1B *In vivo* competition between strain 7314 and its *sefA::aphA-3* mutant (PE910) after mixed oral infections.

Days after oral infection*	7314 vs PE910 numbers in:[a]			
	Peyer's patches		Spleen	
	7314	PE910	7314	PE910
1	ND[b]	ND	ND	ND
4	3.8 ± 0.7	3.8 ± 0.8	2.4 ± 0.3	2.2 ± 0.6
8	2.9 ± 0.5	2.8 ± 0.4	5.1 ± 0.3	4.8 ± 0.5

* Mice were infected orally with a mixture of strain 7314 and PE910 at 5×10^4 organisms each (1:1 input ratio).
[a] Values are mean CFU ± standard deviation of organisms recovered from Peyer's patches and spleens of 5 mice. CFU are expressed in $\log_{10}$ values.
[b] ND = not determined.

The virulent *S.* Enteritidis strain 7314 and its s*efA* mutant derivative (PE910) were used to determine the role, if any, of SEF14 in the virulence of this organism in orally infected BALB/c mice (Section 2.17.2). In this assay, it was observed that the LD_{50} of PE910 was very similar (ca 2.5×10^5 CFU) to that of the parent 7314 strain, suggesting that strain 7314 may have additional factors which could compensate for SefA loss *in vivo,* or that SefA has no role in virulence.

4.2.7 *In vitro* adherence and invasion of HeLa cells

These assays were carried out to complement the results obtained from the mouse colonisation studies. The methods used in these assays are outlined in Section 2.18 of Chapter 2. In the Triton X-100 treated monolayers, 0.7% of adherent 11RX organisms, and 0.3% of PE908 adherent organisms respectively, were recovered from the original inoculum, compared to 1.0% and 1.1% of adherent 7314 and PE910 organisms, respectively (Table 4.2A). In the Giemsa-stained preparations of the adhesion assay, only 3% of the PE908-infected HeLa cells displayed adherent bacteria, compared to about 8% in the monolayers which had been exposed to 11RX organisms. The number of bacteria

adhering to the HeLa cells also varied, averaging about 5 adherent bacteria for PE908 organisms, to about 12 for 11RX organisms. For HeLa cells exposed to 7314 and PE910 organisms, the proportion and the number of Giemsa-stained adherent bacteria were comparable, with about 20% of the HeLa cells displaying adherent bacteria, and the number of adherent bacteria per HeLa cell averaging between 15-18 organisms (Table 4.2B).

Table 4.2A Comparison of *in vitro* adherence and invasion of HeLa cell monolayers by 11RX, PE908, 7314 and PE910.

Strain	(%) Adherence[a]	(%) Invasion[b]
11RX	0.7 ± 0.2	3.0 ± 0.9
PE908	0.3 ± 0.1	1.8 ± 0.6
7314	1.0 ± 0.4	5.2 ±1.1
PE910	1.1 ± 0.3	4.9 ± 1.0

[a] For adherence assays, HeLa cells were cultured with bacteria at 4×10^5 organisms (a multiplicity of infection of 10) for 1 h. Values represent the means ± standard deviations of triplicate samples from one of three similar experiments. Percent adherence represent the percentage of bacteria recovered relative to the initial inoculum after Triton X-100 lysis of HeLa cells.

[b] For invasion assays, HeLa cells were cultured with bacteria at 4×10^5 organisms each and incubated for 2 h. Values represent the means ± standard deviations of triplicate samples from one of three similar experiments. Percent invasion represent the percentage of gentamicin-resistant bacteria recovered relative to the initial inoculum after Triton X-lysis of HeLa cells.

Table 4.2B Comparison of *in vitro* adherence and invasion of HeLa cell monolayers by 11RX, PE908, 7314 and PE910 in Giemsa-stained preparations.

Strain	(%) Adherence[a]	(%) Invasion[b]
11RX	8 1 ± 2.1(12.2 ± 3.8)	12.1 ± 4.2 (20.3 ± 4.7)
PE908	3.4 ± 1.5 (5.8± 2.2)	5.2 ± 2.3 (8.0 ± 3.5)
7314	20.4 ± 4.5 (18.7 ± 3.6)	20 ± 3.9 (≥30)
PE910	20.2 ± 5.1 (15.6 ± 3.2)	18 ± 3.5(≥30)

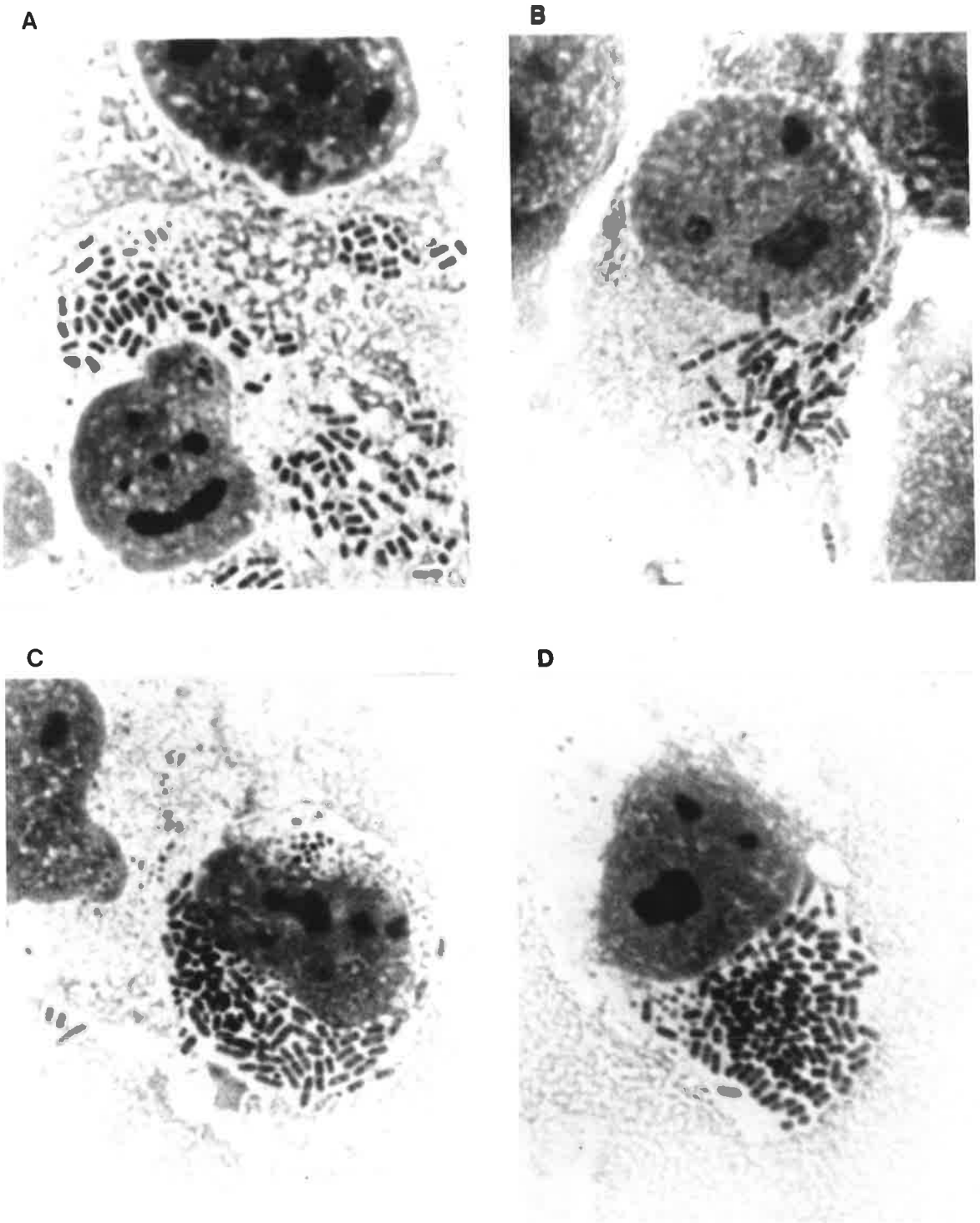
[a] For adherence assays, HeLa cells were cultured with bacteria at 4×10^5 organisms (a multiplicity of infection of 10) for 1 h. Values represent the mean number of cells (± standard deviation) displaying adherent bacteria per 100 HeLa cells; values in brackets represent average number of adherent bacteria (± standard deviation) per infected HeLa cell in Giemsa-stained preparations.

[b] For invasion assays, HeLa cells were cultured with bacteria at 4×10^5 organisms each and incubated for 2 h. Values represent the average number of cells (± standard deviation) with intracellular organisms per 100 HeLa cells; values in brackets represent average number of bacteria counted (± standard deviation) per invaded HeLa cell in Giemsa-stained preparations.

In the invasion assay, about 3% of the 11RX organisms cultured with the HeLa cells were recovered by Triton X-100 lysis of the monolayers after Gm treatment, compared to about 2% of PE908 organisms. The virulent strain 7314 and its isogenic SefA mutant were recovered in comparably higher, but similar numbers, where about 5% of the initial inoculum were recovered (Table 4.2A). In the Giemsa-stained preparations, 12% of the HeLa cells were invaded by one or more 11RX organisms, whereas about 5% of HeLa cells were invaded by PE908 (Table 4.2B). The HeLa cells appeared normal in these assays, and a high proportion (about 60%) of those invaded by 11RX had between 15-25 intracellular bacteria per cell. In the case of PE908-invaded cells, about half of the HeLa cells invaded had between 5-15 intracellular PE908 organisms (Fig. 4.11A, B). A few of the HeLa cells invaded by bacteria (approximately 5-6%) harboured more than 50 intracellular 11RX or PE908 organisms. In contrast, it was noted that a better invasion of the HeLa cells was established by 7314 and PE910 after 2 h of incubation, where about 20% of intact HeLa cells were invaded by both strains (Table 4.2B), and a relatively high proportion (20%) of the invaded cells had more than 50 intracellular bacteria (Fig. 4.11C, D). These results were confirmed using immunofluorescence microscopy (not shown).

In a competition experiment designed to examine the invasion of HeLa cells by 11RX and PE908 at an input ratio of 1:1, the "output" ratio recovered from the Triton X-100 lysates of the invaded HeLa cells was 29:1 in favour of 11RX (not shown), indicating that 11RX organisms were able to outcompete their isogenic *sefA* mutant. This observation is significant because when HeLa cells were infected separately with doses of 11RX or PE908 organisms which were similar to those used in the competition assay, approximately similar numbers of 11RX organisms were recovered from the Triton X-100 lysates of the HeLa cells as those recovered from the competition experiment, whereas the number of PE908 organisms recovered was 6 times higher than the number recovered in the competition experiment (not shown). These data imply that PE908 was at a

Figure 4.11 Invasion of HeLa cells by **A**, 11RX; **B**, *sefA::aphA-3* mutant strain of 11RX (PE908); **C**, 7314; and **D**, *sefA::aphA-3* mutant strain of 7314 (PE910). HeLa cells were invaded with the bacterial strains at 4×10^5 organisms each for 2 h. HeLa cells were stained with Giemsa 2 h after gentamicin treatment of invaded cells. Magnifications, ×1000.


A
B
C
D

competitive disadvantage for entry into HeLa cells compared to 11RX, and supports the results of the *in vivo* competition experiments. Competition between 7314 and PE910 for invasion of HeLa cells was not observed (not shown), which was also consistent with the results obtained from the *in vivo* work.

The ability of *S.* Enteritidis to survive and grow within HeLa cells was investigated by monitoring the rate of intracellular growth of 11RX, 7314, PE908, and PE910 strains over a 24 h period. The results obtained indicated that the organisms multiplied very slowly within the HeLa cells, with PE908, the *sefA* delete strain of 11RX, showing the slowest rate of intracellular multiplication (Fig. 4.12).

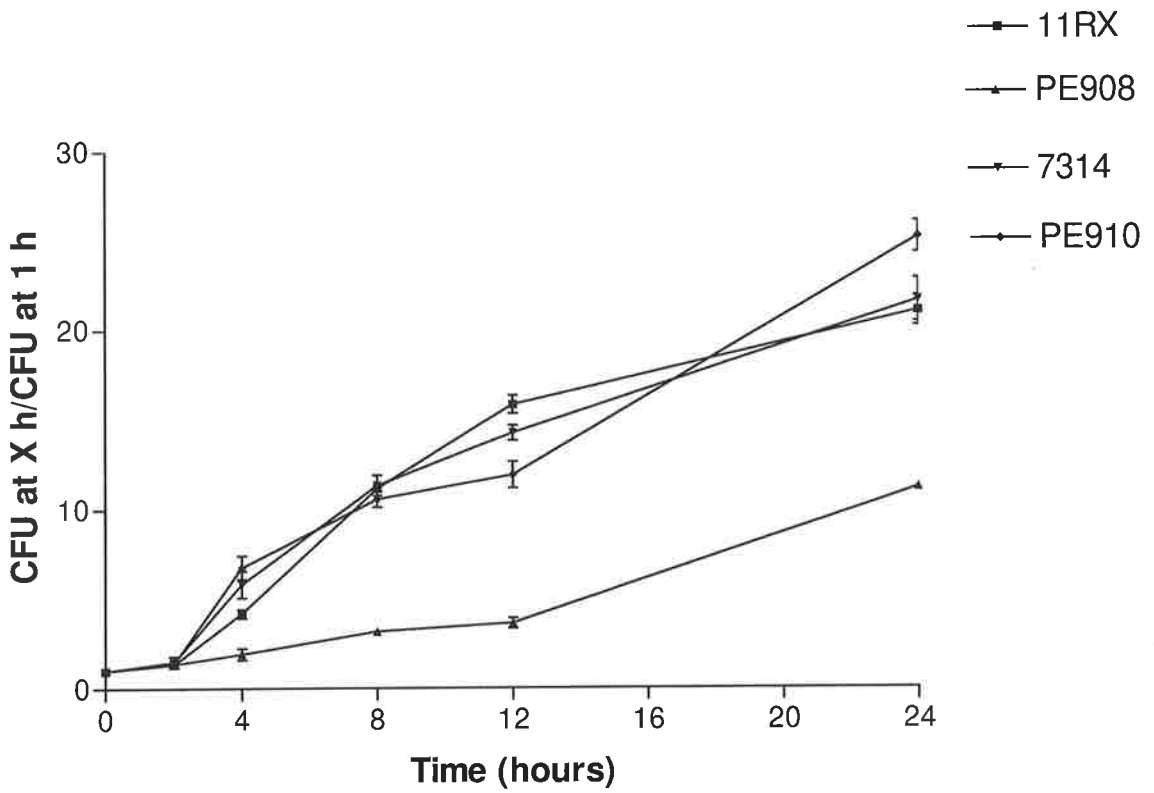


Figure 4.12 Measurement of intracellular growth of *S.* Enteritidis strains 11RX, 7314 and their *sefA::aphA-3* mutant derivatives in HeLa cells over a 24 h period. Intracellular growth was determined by dividing the number of intracellular bacteria at the indicated time periods with the number present after 1 h of infection. Each data point represents the mean (± SEM) of three samples from one of three separate experiments.

These results are similar to the findings of Sansonetti *et al.* (1986) with *S.* Typhimurium C5 and of Mroczenski-Wildey *et al.* (1989) with *S.* Typhi, using HeLa cells, and also comparable to the results of Finlay *et al.* (1988) with *S.* Choleraesuis using polarised MDCK epithelial cells. Quite recently, Mills and Finlay (1994) demonstrated similar findings in HeLa cells using *S.* Typhi Ty2 and *S.* Typhimurium SL1344.

4.3 Discussion

The development of safe and effective vaccines against diseases caused by salmonellae requires a clear definition and detailed understanding of their determinants of pathogenicity as well as an elucidation of the host-pathogen relationships. In this respect, direct evidence for the role of surface appendages produced by salmonellae in the disease process is emerging, and this study relates directly to this issue. Using *in vivo* and *in vitro* approaches, the role of the structural subunit protein, SefA, of the 14 kDa fimbrial antigen (SEF14) of *Salmonella* Enteritidis in the pathogenesis of this organism was investigated.

In Chapter 3, it was demonstrated that SEF14 could induce strong T-lymphocyte responses in BALB/c mice. As an extension of that work, the structural subunit gene, *sefA*, was cloned into *E. coli*, and *sefA* was mutated in the chromosome of *S.* Enteritidis by allelic exchange with a copy that has been inactivated by interruption with a nonpolar kanamycin resistance (*aphA-3*) cassette. The effect of this mutation on the ability of *S.* Enteritidis strains to colonise the Peyer's patches and to invade the spleens of BALB/c mice was investigated.

The attenuated *S.* Enteritidis 11RX strain was clearly shown to be able to colonise better and persist longer in the Peyer's patches than its SefA-deficient counterpart, PE908. This difference was even more pronounced in a competition experiment between the two

bacterial strains, where it was demonstrated that PE908 was markedly outcompeted by strain 11RX. These findings were supported by *in vitro* adherence studies and also by *in vitro* competition experiments using HeLa cells where similar observations were made. From these results it was concluded that SEF14 may be important in the attachment of *S.* Enteritidis 11RX to the host epithelium in the early stages of infection by either targeting the organism to a specific receptor, or by bringing the organism into close proximity of the eucaryotic cell in order to trigger its uptake. However, *in vivo* and *in vitro* competition experiments between the highly virulent *S.* Enteritidis strain 7314 and its SEF14-deficient counterpart, PE910, provided no evidence for any pronounced difference between the strains.

These contradictory observations are likely to be related to the nature of the two strains used in this study. For instance, *S.* Enteritidis 11RX is a rough strain derived from the virulent *S.* Enteritidis strain 11, and lacks the O-and the H-antigens of the parent. It is therefore likely that fimbriae, especially SEF14, become more important in strain 11RX for colonisation and infection of the host because it is attenuated. In contrast, it appears that *S.* Enteritidis 7314, being highly virulent, relies less on SEF14, and probably employs alternative means to colonise host epithelium during infection. The production of other fimbriae, namely SEF17 (Collinson *et al.*, 1991), SEF18 (Clouthier *et al.*, 1993), and SEF21 (Müller *et al.*, 1991) has not been assessed in 11RX or 7314, but it would be interesting to evaluate the effect of a sequential knockout of these in addition to SEF14, on the colonisation of epithelial cell surfaces by these *S.* Enteritidis strains. In order to draw any general conclusions, however, it would be necessary to extend these observations to other host species than mice, because they may possess different or additional receptors for bacterial adherence. Tissue tropism could also be different in other host species.

The significance of SEF14 in the virulence of *S.* Enteritidis was also investigated in mice by comparing the LD_{50} of the virulent *S.* Enteritidis 7314 strain with that of its

SEF14-deficient counterpart, PE910. No difference was detected, implying that SEF14 does not play a significant role in the virulence of *S.* Enteritidis. Again, it would be necessary to investigate the effect of SEF14 mutation in other hosts, as well as evaluating the role that other fimbriae of this organism may play in its pathogenesis and virulence.

From observations made in this study, SefA does not seem to have a direct role in systemic infection as assessed by the invasion of spleens of orally-infected mice. This was reflected by the observation that a similar ratio of bacteria - 11RX, PE908, 7314 or PE910 - that colonised and persisted in the Peyer's patches, was observed in the spleens of these mice. To support this notion, it was observed that there was no noticeable difference in the number of bacteria recovered from spleens of mice 2-3 weeks after intraperitoneal immunisation with either the parent or the SEF14-deficient strains (data not shown), further indicating that the expression of these fimbriae is probably not critical once the bacteria have crossed the intestinal barrier. These findings were corroborated by the *in vitro* invasion of HeLa cells by these bacteria, and are not totally unexpected since a cluster of invasion loci, regulatory genes and other bacterial components have been identified and characterised as having a direct role in invasion and survival in eucaryotic cells; in some cases, the presence of these genes has been correlated with virulence in salmonellae (Fields *et al.*, 1986; Finlay *et al.*, 1988; Galán and Curtiss, 1989a, b; Miller *et al.*, 1989, 1992; Stone and Miller, 1995).

The observation that the *S.* Enteritidis strains used in this study multiplied slowly within HeLa cells in the first few hours after infection is consistent with the findings of others (Sansonetti *et al.*, 1986; Finlay *et al.*, 1988; Mroczenski-Wildey *et al.*, 1989), using different strains of *Salmonella* and probably reflects the fact that an initial lag of several hours is needed for these organisms to adjust to the harsh environment within a membrane-bound vacuole, before they reach maximal intracellular division rates. This is in contrast

to *Shigella* species that escape the endosomic vacuole soon after entry and multiply extensively within the cytoplasm (Sansonetti *et al.*, 1986; Finlay and Falkow, 1989).

The sequence upstream of *sefA* showed extensive homology to the insertion sequence IS*3* of *E. coli* (Timmerman and Tu, 1985), and to our knowledge, this finding is a second report of this insertion element in *Salmonella*, the first being that reported downstream of *invH* by Altmeyer *et al.* (1993). The significance of this finding is not immediately known, however, proximal movable elements have been shown in certain instances to participate in the regulation of expression of virulence factors or other genes (Reynolds *et al.*, 1981; Ou *et al.*, 1988), and this may be the case with the SEF14 fimbrial operon since no regulatory genes have been identified in this operon up to date.

This chapter clearly demonstrates that SEF14 is needed for *S.* Enteritidis strain 11RX, but not for strain 7314, to colonise host epithelium. Further investigations, for example receptor identification and characterisation, identification and characterisation of other colonisation factors, invasion and virulence genes, are still needed to gain a detailed understanding of the pathogenesis of *S.* Enteritidis and its interaction with the host.

CHAPTER 5

SefA protein is a protective antigen of *S.* Enteritidis

5.1 Introduction

Various models have been studied to analyse the use of live attenuated mutants of *Salmonella* as protective and immunogenic carriers in different animals (Hoiseth and Stocker, 1981; Levine and Hornick, 1981; Curtiss *et al.*, 1987; Curtiss and Kelly, 1987; Stocker, 1988; Gálan and Curtiss, 1989b; Cooper *et al.*, 1990; Hormaeche *et al.*, 1991; Cooper *et al.*, 1993; Gherardi *et al.*, 1993a, b; McGhee and Kiyono, 1993; Cooper *et al.*, 1994a; Nishikawa *et al.* 1994), and as Ag delivery vectors for oral vaccines in experimental animals (Stevenson and Manning, 1985; Brown *et al.*, 1987; Cárdenas and Clements, 1992, 1993; McGhee and Kiyono, 1993; Paton *et al.*, 1993; Cooper *et al.*, 1994a, b; Jackson *et al.*, 1994; Redman *et al.*, 1994). It is generally accepted that live attenuated *Salmonella* strains provide better antimicrobial protection than killed vaccines (Collins and Carter, 1974; Germanier and Fürer, 1975; Levine *et al.*, 1983; George *et al.*, 1987; Levine *et al.*, 1987), and are capable of eliciting humoral responses as well as cell-mediated immunity (Collins, 1974). Furthermore, fully defined genetic deletion mutants are more desirable for use as live vaccines for purposes of quality control (Dougan *et al.*, 1993).

In considering the relative importance of the surface or intracellular expression of an Ag in eliciting an immune response, Brown *et al.* (1987) demonstrated that the attenuated *aroA S.* Typhimurium vaccine strain was able to elicit both humoral and cellular immune responses to a cloned intracellular Ag, β-galactosidase, in mice. This finding

accentuates the ability of attenuated salmonellae to elicit both humoral and cell-mediated immune responses to cloned, especially protective, heterologous intracellular Ags.

The nature of the protective Ags produced by salmonellae is a subject of some controversy. For example, some investigators have reported an increased protection in mice immunised with outer membrane proteins, while others find porin preparations from *S.* Typhimurium to be adequately effective (Udhayakumar and Muthukkaruppan, 1987a, b; Isibasi *et al.*, 1988; Matsui and Arai, 1989, 1990). These proteins were recognised by T cells obtained from previously immunised donors (Young *et al.*, 1988b; Vordermeier and Kotlarski, 1990a). In addition, considerable interest is being generated in fimbriae as potential immunogens in animals against many bacteria which infect epithelial cell surfaces (Duguid *et al.*, 1966; Duguid and Old, 1980; Pearce and Buchanan, 1980; Tramont and Boslego, 1985; Korhonen *et al.*, 1990; Sun *et al.*, 1990; Krogfelt, 1991; Sun *et al.,* 1991). Recently, it was reported that orally administered hen egg-yolk antibodies specific for the purified SEF14 fimbrin of *S.* Enteritidis provided passive immunisation against experimental salmonellosis in mice (Peralta *et al.*, 1994). The results obtained during the preliminary characterisation of SEF14 in this study (Chapter 3) indicated that the purified SEF14 fimbrial Ag of *S.* Enteritidis 11RX is likely to be an important immunogen of because of its ability to elicit delayed-type hypersensitivity reactions in 11RX-primed mice, and its ability to stimulate strong *in vitro* proliferation of, and cytokine release from, T cells obtained from these animals. As an extension of that work, an attempt was made to evaluate the importance of the structural subunit protein (SefA) of SEF14 in the biology of *S.* Enteritidis by investigating its role in protection in mice challenged with a virulent *S.* Enteritidis strain, using a variety of Ag delivery systems and various immunological assays. Part of the work presented here was carried out by Dr Yan Zheng-Xin (Max-Planck Institute for Biology, Tübingen, Germany). Dr Yan constructed plasmids pYZ84 and pYZ100 and carried out the *in vitro* and *in vivo* stability tests on the

recombinant vaccine strains used. Data are presented which indicate that SefA is likely to be one of the Ags of *S.* Enteritidis which can elicit protection against virulent challenge in mice.

5.2 Results

The LD_{50} of *S.* Enteritidis strain 7314 was determined as ca 2.5×10^5 by the oral route, and <10 organisms by the intraperitoneal and the intravenous routes in BALB/c mice. The LD_{50} of *S.* Enteritidis 11RX in F1 mice has been previously established as ca 2×10^6 organisms by both the intravenous and intraperitoneal routes (Davies, 1975; Ashley, 1976).

5.2.1 Production of SefA by *Salmonella* vaccine strains.

The *aroA* mutant of *S.* Typhimurium, strain SL3261, and the SL3261::pYZ84 strain (PE914) were electro-transformed with plasmids (pYZ100 and pPM3911) encoding the *sefA* gene (see Chapter 2, Section 2.14.7.4, and Chapter 4, Section 4.2.2), to yield strains PE915 and PE916, respectively. These strains were tested for SefA production after growth at 28°C and 37°C with or without ampicillin selection, using Western blot analysis of whole bacterial lysate with a MAb specific for SefA. The bands detected correspond to the 14 kDa SefA protein and its premature form (16 kDa), similar to the findings of Thorns *et al.,* (1990) and Müller *et al.,* (1991) who have both analysed *E. coli* recombinant strains expressing the SefA protein. As expected, strain PE915 (SL3261::pYZ84[pYZ100]) produced more SefA at 37°C owing to derepression of the $\lambda\, p_L$ promoter. (Fig. 5.1).

The cellular location of SefA produced by the vaccine strains *in vitro* was examined by immuno-electron microscopy with the SEF14 MAb as described in Chapter 2, Section 2.15.8.2. SefA produced by strain SL3261 carrying pPM3911 (PE916) was detected as a

fimbrial protein, whereas strain SL3261::pYZ84[pYZ100] (PE915) produced large amounts of fimbrin which formed long bundles of aggregates outside the cell and left the SefA-producing cells as empty ghosts (not shown).

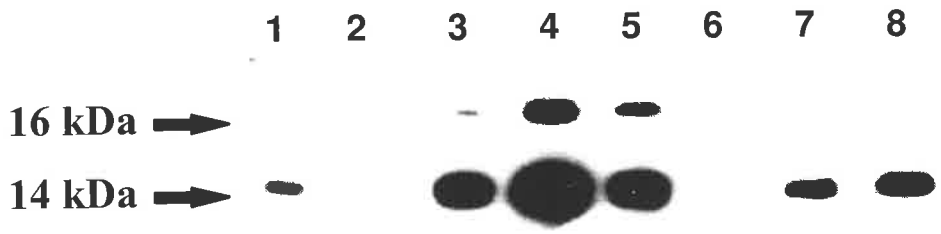


Figure 5.1 Western blotting of whole cell lysates of *Salmonella* vaccine strains using a SEF14-reactive MAb KAP14-1. Samples were heated at 100°C for 3 min in SDS sample loading buffer before electrophoresis. Lanes:1, strain 11RX; 2, PE908; 3, PE915 grown at 28°C; 4, PE915 grown at 37°C, 5, PE916; 6, PE914; 7, strain 7314; and 8, purified SEF14.

The *in vitro* stability of the recombinant vaccine strains was determined by growing bacteria in LB medium in the absence of antibiotics and transferring a fraction of the culture (1/1000) into fresh LB every 12 hours. Equivalent dilutions of each culture were spread onto LB and LB+Amp plates and incubated for 16 hours at 37°C. The colonies which grew on both plates were counted and the data obtained was used to estimate the percentage of Ap^R colonies. It was found that the PE916 strain completely lost its plasmid after overnight growth in non-selective medium, whereas a large proportion of strain PE915 organisms still contained the plasmid when grown under the same condition (Table 5.1).

Table 5.1 *In vitro* and *in vivo* stability of strains used in protection studies.

Antibiotic(s)	CFU of bacteria recovered from:[a]				
	in vitro culture (from LB broth)[b]		*in vivo* growth (from spleens)[c]		
	PE915	PE916	PE914	PE915	PE916
Ap	36 ± 4.2	0	231 ± 83.8	11.3 ± 2.5	0
Km	36 ± 0.7	ND[d]	ND	ND	ND
Ap+Sm	ND	ND	225 ± 58.9	9 ± 3.5*	11.7 ± 21
None	32 ± 14.8	240 ± 14.8	ND	ND	ND

[a] The CFU values shown are total number of viable bacteria recovered multiplied by 10^7.
[b] Values shown are means ± standard deviation of CFU of bacteria counted from duplicate LB agar plates.
[c] Values represent means ± standard deviation of CFU of bacteria recovered from spleens of three mice after 3 days of oral immunisation.
[d] ND, Not determined.
* All the bacteria growing on the Ap+Sm plates were Ap^R when transferred to LB agar containing Ap.

5.2.2 Oral immunisation and protection

In order to test the *in vivo* stability of the recombinant vaccine strains, groups of three mice were sacrificed three days after oral immunisation and their spleens were removed and homogenised separately. One half of each spleen homogenate was plated onto LB/Sm agar and the other onto LB/Ap+Sm. The colonies were counted and the percentage of Ap^R colonies calculated as before. Three days after oral immunisation, small numbers of recombinant bacteria were found in the spleen. The two strains producing SefA (PE915 and PE916) were present in lower numbers than the strain carrying the T7 promoter plasmid used as a control (PE914). Consistent with the results reported above, strain PE916 had lost the plasmid coding for SefA completely, but by contrast, 100% of strain PE915 was still carrying the plasmid.

5.2.3 Antibody responses to SefA

Mice infected orally with approximately 1×10^9 organisms of *S.* Enteritidis 11RX and *S.* Typhimurium *aroA* strains expressing SefA protein (PE915 and PE916) elicited significant IgG responses to SEF14 as measured by ELISA; the highest titre was obtained

from mice orally infected with PE915 (Table 5.2), reflecting the level of expression of the Ag produced by the strain used for immunisation. The response was specific for SefA, as strains SL3261, PE914, and PE908 did not elicit any detectable response. When LPS was the sensitising Ag in the ELISA, sera from mice immunised with the various strains showed high and similar anti-LPS IgG reactivity (Table 5.2), reflecting their common LPS structure. Similarly, an anti-SefA IgA response was also detected in the serum of mice immunised with strain PE915; this response was weaker in mice immunised with 11RX or PE916 and undetectable in mice immunised with strain PE908, SL3261, or PE914. Except for strain PE915, none of the strains induced a significant anti-LPS IgA response in mice infected with the various strains (Table 5.2). Similarly, significant IgG and serum IgA responses were induced against SefA when mice were immunised intraperitoneally (not shown), indicating that SefA is a strong immunogen of *S.* Enteritidis.

Table 5.2 Antibody responses induced by various bacterial strains.

Sera from mice immunised with:[a]	SefA status	Total IgG titre against:[b]		IgA titre against:[b]	
		SEF14	LPS	SEF14	LPS
11RX	chromosome	2.7 ± 0.5	4.4 ± 0.4	1.7 ± 0.6	1.7 ± 0.2
PE908	absent	0	5.2 ± 1.0	0	1.7 ± 0.4
SL3261	absent	0	3.9 ± 0.3	0	1.6 ± 0.4
PE914	absent	0	3.9 ± 0.4	0	1.6 ± 0.6
PE915	plasmid	4.8 ± 0.8	4.7 ± 1.0	3.0 ± 0.4	2.9 ± 0.8
PE916	plasmid	2.0 ± 0.3	3.7 ± 0.4	1.7 ± 0.2	1.6 ± 0.5

[a] Mice were immunised orally with 1-2 × 10^9 CFU of each bacterial strain. They were bled 35 days after secondary immunisation with the same dose of the bacterial preparations.

[b] Immunoglobulin (IgG, IgA) titres were expressed as the reciprocal of the $\log_{10}$ dilution of pooled sera giving an absorbance of 0.4 above the background at A_{405}. Values are mean ± standard deviation of triplicate ELISA titres.

5.2.4 *In vitro* responses of primed T cells to various Ags

The T-cell responses of mice to the various strains expressing the SefA Ag was investigated in a standard T-cell proliferation assay (Section 2.10.4, Chapter 2). The NW-fractionated intraperitoneal cells prepared from the immune mice were fairly homogeneous suspensions of T cells with little variation from preparation to preparation, ranging between 95 to 98%. In addition to the T cells, NPC were added to provide Ag-presenting cells, and the T-cell mitogen, concanavalin A, was included as a control. A high proportion of each responding T-cell population (70 to 75%) was of the $L3T4^+$ phenotype as determined by fluorescence activated cell sorting using various T cell markers.

Table 5.3 *In vitro* proliferation of T-cells from mice immunised with various bacterial preparations.

Stimulant [a]	[^{3}H]-TdR incorporation (cpm, mean ± SEM) of T-cells primed with[b]:		
	11RX	PE915	PE916
11RX (1 μg/ml)	57,220 ± 3, 640	25,578 ± 1, 484	28,617 ± 2, 276
PE908 (1 μg/ml)	31,266 ± 1, 955	14,836 ± 1, 033	16,329 ± 2, 154
SL3261 (1 μg/ml)	14,983 ± 1, 093	10,423 ± 1, 210	8,246 ± 1, 661
PE914 (1 μg/ml)	14,365 ± 998	8,252 ± 1, 101	6,572 ± 954
PE915 (1 μg/ml)	31,280 ± 1, 863	49,504 ± 3, 683	32,860 ± 3, 310
PE916 (1 μg/ml)	28,165 ± 1, 906	45,614 ± 3, 055	42,500 ± 3, 022
SEF14 (1 μg/ml)	45,513 ± 2, 066	48,177 ± 4, 075	33,464 ± 782
ConA (1 μg/ml)	60,435 ± 4, 041	65,720 ± 3, 461	62,079 ± 5, 043
LPS (10 μg/ml)	1,093 ± 434	ND[c]	ND
NPCs only	989 ± 142	1,896 ± 288	1,126 ± 236
Medium only	672 ± 80	587 ± 62	750 ± 45

[a] Formalin-killed preparations of 11RX, PE908, SL3261, PE914, PE915, and PE916 were used as stimulants in this assay. ConA, concanavalin A; NPC, normal peritoneal cells.
[b] 1×10^5 primed T cells, and 2×10^4 NPC were used per well in each assay. Cultures were pulsed for 4 h with [^{3}H]-TdR prior to harvest. Data presented are for quadruplicate cultures, and a typical result from one of three experiments is presented.
[c] ND, not determined.

As shown in Table 5.3, purified SEF14, formalin killed 11RX, formalin-killed PE915, and formalin-killed PE916 organisms all induced strong proliferation of T cells

harvested from mice immunised with 11RX, PE915, or PE916. In contrast, formalin-killed SL3261 alone or formalin-killed PE914 were not as stimulatory. In addition, T cells from SL3261-immune mice responded well to ConA, but showed little proliferation against the various stimulating Ags (data not shown). It was observed that formalin-killed PE908 organisms induced significant proliferation of T cells harvested from 11RX-immune mice, although the response was lower than that induced by 11RX. Presumably this was due to the presence of T-cell stimulatory Ags other than SEF14 in the PE908 strain. The result obtained when T cells of 11RX-immune mice was cultured with formalin-fixed 11RX or purified SEF14 is in agreement with earlier reports (Vordermeier and Kotlarski, 1990a, b) and further supports the suggestion made in Chapter 3 that SEF14 is likely to be an important immunogen of *S.* Enteritidis.

5.2.5 Cytokine assays

The abilities of the various antigenic preparations to stimulate T cells from immunised mice was further analysed by assaying the profiles of IL-2 and IFN-γ production in supernatants from cultures of unfractionated peritoneal cells and Ags as described in Sections 2.10.6 and 2.10.7 in Chapter 2. Table 5.4 shows the results of a typical experiment. Formalin-killed 11RX stimulated the highest IL-2 and IFN-γ release, while formalin-killed SL3261 induced the lowest release of these cytokines. The formalin-killed PE915 and PE916 strains also induced significant levels of these cytokines.

When purified SEF14 was added to cultures of 11RX-primed T cells, significant release of cytokines was obtained. However, the IL-2 release and IFN-γ production levels induced by adding strain PE908 to 11RX-primed T cells were similar to those induced by the

isogenic parent, indicating that Ags other than SefA protein were able to stimulate a subset of the T cells primed against *S.* Enteritidis.

Table 5.4 Cytokine production by T-cells stimulated with various bacterial preparations.

Supernatants from primed peritoneal cells stimulated with:[a]	IL-2 release (units/ml)	IFN-γ production (units/ml)
11RX	96	45
PE908[b]	78	45
SL3261	43	10
PE915	70	30
PE916	64	22
SEF14[b]	58	20

[a] Mice were immunised intraperitoneally with live organisms and the primed peritoneal cells were stimulated with the corresponding formalin-killed preparations.
[b] Purified SEF14 and formalin-killed preparation of PE908 were used to stimulate 11RX-primed T-cells.

Although this has not been demonstrated directly in the present study, it is quite possible that these cytokines, particularly IFN-γ, may play an active role in the protection of mice against virulent challenge by augmenting the bactericidal activity of phagocytic cells, as reported by Matsumura *et al.* (1990).

5.2.6 Protection after oral challenge

The abilities of *S.* Enteritidis 11RX and the SefA protein of *S.* Enteritidis to confer immunity to BALB/c mice against challenge with the virulent *S.* Enteritidis 7314 strain was also assessed in two sets of experiments. Table 5.5 shows a typical result obtained from one set of the challenge experiments.

When two oral doses of bacteria were used to immunise the mice, strain 11RX gave the best level of protection (80%), followed by PE908 strain (70%), PE915 (70%), and PE916 with 60% level of protection. In contrast, SL3261 and PE914 gave very little protection (20% each) against challenge by *S.* Enteritidis 7314. In general (and not surprisingly), a greater level of protection was observed in mice given a booster (secondary)

dose of organisms than in those induced in mice given a single immunising dose. Obviously, the high level of protection in mice immunised with the PE908 strain indicates that *S.* Enteritidis produces other protective Ags, and supports the interpretation of the results obtained from the T-cell proliferation assay results.

Table 5.5 Protection profiles of mice after virulent challenge.

Immunising bacteria[a]	% survival of mice with:[b, c]	
	One immunisation	Two immunisations
11RX	60	80
PE908	60	70
SL3261	0	20
PE914	0	20
PE915	60	70
PE916	40	60
PBS (control)	0	ND

[a] Mice were immunised orally with 1-2 × 10^9 CFU of each bacterial preparation.
[b, c] Challenge dose was 200 LD_{50} (ca 5 × 10^7 CFU) of virulent *Salmonella* Enteritidis 7314; 10 mice were used per group.

5.3 Discussion

In this Chapter, the role of SefA, the structural subunit of the 14 kDa fimbrial protein (SEF14) of this organism in protection against virulent challenge was investigated using BALB/c mice as the experimental model. Impetus for this study arose from the finding that SEF14 induces strong T-cell responses, and also from reports from various workers that fimbriae are immunogenic and the demonstration that fimbrial Ags can act as effective vaccines in various animals, inducing effective immunity to many bacteria that infect epithelial cell surfaces (Duguid and Old, 1980; Pearce and Buchanan, 1980; Tramont and Boslego, 1985; Korhonen *et al.*, 1990; Krogfelt, 1991).

It was observed that the SefA fimbrial Ag of *S.* Enteritidis 11RX induced strong and specific antibody responses in mice immunised either by the oral or intraperitoneal route. This result is consistent with the work of Dr Thorns and co-workers (Central Veterinary Laboratory, Weybridge, Surrey, England, personal communication) who also observed that chickens are capable of mounting a significant antibody response to this Ag. Furthermore, the demonstration of strong proliferation of, and cytokine release from, T cells obtained from intraperitoneally-immunised mice cultured with the various strains expressing the SefA Ag corroborates earlier reports (Vordermeier and Kotlarski, 1990a, b) and confirms the capability of this fimbrial Ag to elicit both humoral and cell-mediated responses in mice.

To evaluate the relative importance of the surface or intracellular expression of the Ag in eliciting a T-cell-mediated immune response, a comparison of the proliferative responses of *S.* Enteritidis 11RX-primed T cells to either the formalinised bacterial preparations, or their lysed counterparts, was made. In these assays, the proliferative responses of the responding T cells to the two sets of antigenic preparations were similar (data not shown). This result suggests that the intracellularly localised Ag is also accessible to T cells and implies that surface expression or secretion from the cell is not essential for immunogenicity of a cloned Ag expressed in attenuated salmonellae, as previously suggested by Brown *et al* (1987). They demonstrated humoral and cellular immunity in mice to a cloned intracellular protein, β-galactosidase, expressed in an *aroA S.* Typhimurium vaccine strain. A notable observation in the present study was that the SefA-deficient *S.* Enteritidis 11RX strain (strain PE908) was also able to induce a significant, albeit reduced, *in vitro* T-cell response. Presumably this indicates that the responding T cells (mainly of the $L3T4^+$ phenotype) represent a mixed population of T cells with a range of different Antigenic specificities which can respond to any of the stimulatory Ags present in the vaccine preparations used.

The ability of SefA to protect mice against challenge with virulent *S.* Enteritidis 7314 was investigated in this study. The results obtained show that *S.* Enteritidis 11RX provided the highest level of protection (80%). This was not surprising because this strain has been demonstrated previously to be protective, and also provides a high level of resistance against antigenically-unrelated bacteria (Ushiba *et al.*, 1959; Saito *et al.*, 1960; Rowley *et al.*, 1968). It is somewhat surprising, however, that 100% protection could not be induced. A variation in Ag expression may account for this. However, *S.* Enteritidis 11RX is a rough strain and its flagella Ag does undergo phase switching whereas the challenge strain is smooth and is always flagellated; it is therefore likely that the absence or poor expression of both LPS and flagellar Ags, particularly the LPS O-Ag, may have contributed to the reduced ability of the 11RX strain to induce a 100% protective immune response. This is corroborated by the recent work of Hormaeche *et al.* (1996), and is further supported by the observation that a high anti-LPS Ab titre was generated prior to challenge in the immunised mice used in the present study. The result also strongly suggests that humoral response, in addition to CMI response is needed for effective protection against challenge with the virulent strain. This conclusion is consistent with the recent report by Peralta *et al.* (1994) that a survival rate of 77.8% was generated in mice against experimental salmonellosis by orally administered hen-egg yolk Abs raised to SEF14 of *S.* Enteritidis, compared to 32% survival in control mice fed with a preparation of normal egg-yolk Abs.

The SL3261 *aroA* strain carrying the recombinant plasmid with *sefA* (PE915) gave 70% protection, compared to 20% obtained with either the same strain alone or the same strain carrying the T7 promoter vector alone (PE914). The *aroA* strain carrying pBluescriptKS+ with *sefA* (PE916) was somewhat less protective (60%), attesting to the utility of the high level Ag expression system of PE915. Nonetheless, these results indicate that protective immunity against *S.* Enteritidis can be achieved using both an *S.* Typhimurium vaccine strain presenting SefA Ag in its native form (fimbrin) as produced by

PE916, and the one producing large amounts recombinant SefA as cell-free aggregates as found in PE915, and suggests that the decisive factor for inducing an optimal T cell response by live recombinant bacterial vaccine strains appears to be the dosage of the recombinant Ags produced. The form in which the heterologous Ag is presented may play only a secondary role. The lower level of protection obtained with PE916 compared to PE915 is probably because of the relative instability of PE916 *in vivo*, although the work of Cárdenas and Clements (1993) suggests that the most important event for the development of an immune response against a foreign Ag may be the initial amount of Ag that primes the immune system, rather than the persistence of the vector in host tissues.

Altogether, the results obtained in this study indicate that SefA is protective and are consistent with the results of T cell assays and antibody responses. In general, mice receiving secondary immunisation were better protected, confirming the importance of multiple immunisation in raising the level of protection. However, the SefA deficient strain of 11RX (PE908) was also protective, indicating that protective Ags other than SefA are produced by *S.* Enteritidis. The results are clearly consistent with those obtained from the T-cell proliferation assays and humoral responses, and are not at all surprising, given the large variety of molecules produced by this organism. Indeed it would have been much more surprising to find that SefA was the only molecule produced by *S.* Enteritidis that was able to induce effective protection against challenge with virulent *S.* Enteritidis organisms.

From the aforegoing, it seems reasonable to conclude that SefA is necessary, but not sufficient for protection of mice against virulent challenge by *S.* Enteritidis, or that SefA may be protective on its own, but that there are other Ags in *S.* Enteritidis which can also induce effective immunity. It is likely that a sequential knockout of other fimbriae produced by *S.* Enteritidis (such as SEF17, SEF18, and SEF21) may provide a better and a more integrated approach to the understanding of the relative importance of these fimbriae in the biology of the organism and in conferring resistance to infection.

CHAPTER 6

Epitope analysis of FanC and SefA

6.1 Introduction

The work carried out in this chapter was an investigation of an earlier proposition by Vordermeier and Kotlarski (1990b) that the low molecular weight Ag of *S.* Enteritidis, which has been identified as the SEF14 fimbrial protein, might be a suitable candidate Ag to study the interactions between class I and class II MHC molecules in the pathways of Ag processing in the induction of a T cell response.

It is generally agreed that the identification of epitopes within protein molecules could be valuable in the design of "subunit" vaccines and for diagnostic purposes, and several predictive methods have been described for the identification of potential B-cell epitopes (Hopp and Woods, 1981; Kyte and Doolittle, 1982; Tainer *et al.*, 1984; Westhof *et al.*, 1984; Barlow *et al.*, 1986; Parker *et al.*, 1986, von Heijne, 1992). It is believed that B-cell epitopes are surface-exposed regions of a protein and as such are probably hydrophilic and more mobile than interior regions. Consequently, algorithms for hydrophilicity and accessibility have been used to predict antigenicity (Janin, 1979; Parker *et al.*, 1986). In contrast, T-cell epitopes generally consist of short, linear peptides and predictive theories for T-cell epitopes based on analysis of amino acid sequences have indicated that these sites tend to be amphipathic structures (DeLisi and Berzofsky, 1985; Lamb *et al.*, 1987; Rothbard *et al.,* 1987; Rothbard and Taylor, 1988).

A variety of practical approaches have been used either ~~singly~~ singularly or in combination, in the identification of potential B and T cell epitopes within protein Ags. These include:

(i) the construction and expression of a sub-library containing randomly generated fragments of the gene of interest in phage or plasmid expression vectors (Young and Davis, 1985; Mehra *et al.*, 1986; Lamb *et al.*, 1987; Warren *et al.*, 1990; Phalipon *et al.*, 1992; Barzu *et al.*, 1993);

(ii) the chemical synthesis of overlapping peptides of a known protein (Geysen *et al.*, 1987; Bray *et al.*, 1990; Seabrook *et al.*, 1990; Ashbridge *et al.*, 1992; Petersen *et al.*, 1992; Joys and Street, 1993; Robinson *et al.*, 1993; Vordermeier *et al.*, 1993);

(iii) the use of N- and/or C-terminal deletion mutagenesis of the gene and expression of the truncated protein as a fusion protein in a suitable vector (Gross and Rohrmann, 1990; Hickling *et al.*, 1992; Wunderlich *et al.*, 1992; Skakoon and Dunn, 1993; Oettinger and Andersen, 1994);

(iv) the use of cyanogen bromide-cleavage of proteins (Kahlert *et al.*, 1992; Bertram *et al.*, 1994) or proteolytic digestion of proteins to generate peptide fragments (Bertram 1991; Mercier *et al.,* 1993; Ramanathan *et al.,* 1993);

(v) the use of site-directed mutagenesis (Ramanathan *et al.*, 1993; Schönherr *et al.*, 1993; Simon *et al.*, 1993); and

(vi) the use of MAbs raised against synthetic peptides derived from the predicted epitopes of a protein in epitope mapping of the original protein (Krogfelt *et al.*, 1987; Mercier *et al.*, 1993; Simon *et al.*, 1993; Tahara *et al.*, 1993).

At the commencement of this study, neither the identity nor the nucleotide sequence of the SefA Ag was not known. In order to establish systems to define the B- and T-cell epitopes of the protein, an analogous Ag, the K99 fimbrial protein of enterotoxigenic *E. coli* (ETEC), was employed as a model. The K99 fimbrial Ag was an attractive model because it is a fimbrin, it was well characterised, and the complete genetic organisation of its operon had been determined (De Graaf *et al.*, 1984; Roosendaal *et al.*, 1984; Roosendaal *et al.*, 1987a, b; Oudega and de Graaf, 1988; Roosendaal and de Graaf,

1989; Simons *et al.*, 1990b; Bakker *et al.*, 1991; Simons *et al.*, 1991). In addition, the major subunit protein of K99, FanC, is of relatively low molecular weight, composed of 159 amino acid residues preceded by a N-terminal signal sequence of 22 amino acid residues (Roosendaal *et al.*, 1984). Furthermore, it had been demonstrated (Bertram, 1991; Bertram *et al.*, 1994) that K99 fimbriae were stably expressed on the surfaces of *Salmonella* strains and could generate specific anti-K99 T-cell responses *in vitro* and *in vivo* as measured by *in vitro* proliferation and cytokine release from lymphoid cells of immunised animals, and by DTH responses in immunised mice when challenged with K99 in the footpad. The findings of Bertram *et al.* (1994) also indicated that the FanC protein might contain one or a few T-cell epitopes, and also provided an indirect insight into the potential immunogenicity of surface Ags produced by salmonellae.

As an extension of the work of Bertram *et al.* (1994), an attempt was made to characterise the B and T cell epitopes of the FanC polypeptide corresponding to the major subunit of K99 fimbriae. This was carried out using a recombinant approach involving the fusion of FanC and its truncated derivatives to a reporter, the *E. coli* alkaline phosphatase (PhoA), to generate stable recombinant fusion proteins. The data obtained from the epitope mapping studies of FanC was applied to evaluate the potential B and T cell epitope(s) of SefA, but using a slightly different approach to ensure easy purification of SefA peptides from the fusion partner. This was carried out by stepwise deletions of *sefA* (in pPM3913) from the C-terminal end. Due to the failure of this approach, a series of overlapping synthetic peptides were used to characterise the T cell epitopes of SefA. The results obtained and the interpretation of the results, are presented.

6.2 Results and Discussion

6.2.1 Determination of C-terminal deletion end-points in *fanC*

Figure 6.1A shows a schematic representation of the construction of *fanC::phoA* fusions in pPM3918. Essentially, a 1.7 kb *Hpa*I fragment encompassing *fanC* and part of *fanD* was cloned from pFK99 into the *Hin*cII-*Hin*cII site of pUC1318, a hybrid vector with a symmetric polylinker, to generate pPM3917. The recombinant vector was then digested with *Bam*HI which cuts on both sides outside the insert, to generate a fragment which was subsequently cloned into the *Bam*HI site upstream of *phoA'* in the polylinker of pPM3500, to yield pPM3918.

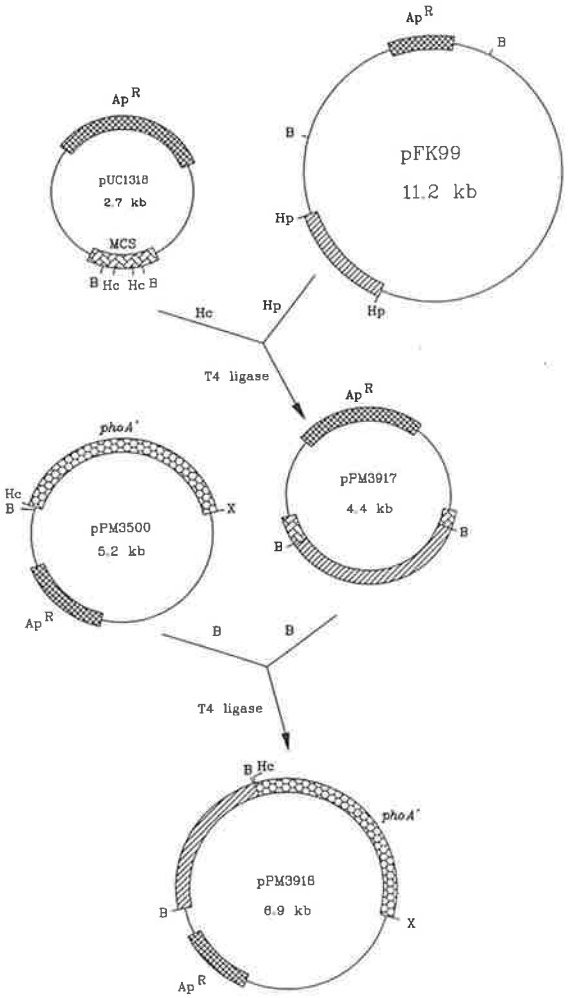


Figure 6.1A Schematic representation of the construction of *fanC::phoA'* fusions and the generation of pPM3918. Abbreviations : B, *Bam*HI; Hc, *Hin*cII; Hp, *Hpa*I; X, *Xho*I; Ap^R, ampicillin-resistance gene, MCS, multiple cloning site (see Section 6.2.1 for full description).

The vector pPM3500 was constructed by C. A. Clark in our laboratory by cloning the *phoA* gene lacking a promoter and a signal sequence (*phoA'*) into the *Hin*cII-*Xho*I site of the polylinker of pBluescriptSK+. The generation of a series of C-terminal deletions of *fanC* in pPM3918 is described in Section 2.14.8, Chapter 2. Nutrient agar plates containing Ap and X-pho were used to screen for in-frame *fanC::phoA'* fusions in *E. coli* K-12 strain CC118 (a *phoA*-negative strain). Since PhoA is only active in the periplasm (Manoil and Beckwith, 1985), blue colonies on X-pho plates indicate those carrying in-frame fusions as a result of *phoA'* being transported into the periplasm since *phoA'* itself lacks a signal sequence. The exact positions of the C-terminal deletion end-points in *fanC* fused to *phoA'* was determined by dye-terminator sequencing reactions with oligonucleotide #565 complementary to the N-terminal coding region of the *phoA'* gene. The complete nucleotide sequence of *fanC*, with the deduced amino acid sequence of the open reading frame is shown in Fig. 6.1B.

6.2.2 Overproduction of FanC::PhoA fusions.

Initial attempts to overproduce the FanC::PhoA fusion proteins and peptides derived from exonuclease deletions of *fanC* in pPM3918 using the T3 RNA polymerase/promoter system, and by IPTG induction, were unsuccessful, and the exact reason for the failure is unclear. Consequently, the deletion derivatives of *fanC* were amplified by PCR using oligonucleotides #746 and #779, the PCR products were digested with *Nde*I and *Kpn*I and cloned into the corresponding sites in the expression vector pET17b. High level expression of the FanC::PhoA fusion proteins and peptides in pET17b was achieved using the T7 RNA polymerase/promoter system of Tabor and Richardson (1985). After induction, the FanC::PhoA fusions were the predominant proteins in the cell (Fig. 6.2, lanes 1-12). Fusion to PhoA was further confirmed by Western blotting of the fusion proteins with anti-bacterial alkaline phosphatase (Fig. 6.3).

```
  1  ATG AAA AAA ACA CTG CTA GCT ATT ATC TTA GGT GGT ATG GCT TTT GCG 48
  1  M   K   K   T   L   L   A   I   I   L   G   G   M   A   F   A  16

 49  ACT ACC AAT GCT TCT GCG AAT ACA GGT ACT ATT AAC TTC AAT GGC AAA 96
 17  T   T   N   A   S   A   N   T   G   T   I   N   F   N   G   K  32
                                     ↑d

 97  ATA ACG AGT GCT ACT TGT ACA ATT GAC CCT GAG GTC AAT GGT AAT CGT 144
 33  I   T   S   A   T   C   T   I   D   P   E   V   N   G   N   R  48
             ↑c                                          ↑b

145  ACA TCA ACT ATA GAT CTT GGG CAG GCT GCT ATT AGT GGT CAT GGC ACT 192
 49  T   S   T   I   D   L   G   Q   A   A   I   S   G   H   G   T  64
                                                     ↑a

193  GTA GTG GAT TTT AAA CTA AAA CCA GCG CCC GGC AGT AAT GAC TGC CTA 240
 65  V   V   D   F   K   L   K   P   A   P   G   S   N   D   C   L  80

241  GCG AAA ACA AAT GCT CGT ATT GAC TGG TCT GGT TCT ATG AAC AGT TTA 288
 81  A   K   T   N   A   R   I   D   W   S   G   S   M   N   S   L  96

289  GGT TTT AAT AAT ACA GCT TCA GGA AAT ACT GCT GCT AAA GGA TAC CAT 336
 97  G   F   N   N   T   A   S   G   N   T   A   A   K   G   Y   H 112
     ↑12                         ↑11                 ↑10

337  ATG ACT TTG CGC GCA ACA AAC GTT GGA AAT GGG TCT GGT GGT GCT AAT 384
113  M   T   L   R   A   T   N   V   G   N   G   S   G   G   A   N 128
                         ↑9                  ↑8

385  ATT AAT ACT TCA TTC ACT ACG GCT GAA TAC ACT CAC ACT TCT GCA ATT 432
129  I   N   T   S   F   T   T   A   E   Y   T   H   T   S   A   I 144
                         ↑7              ↑6

433  CAG TCA TTT AAC TAT TCA GCC CAG CTG AAA AAA GAT GAC CGC GCT CCG 480
145  Q   S   F   N   Y   S   A   Q   L   K   K   D   D   R   A   P 160
       ↑5          ↑4                              ↑3

481  TCT AAT GGT GGA TAT AAA GCT GGC GTA TTT ACT ACT TCA GCA TCC TTC 528
161  S   N   G   G   Y   K   A   G   V   F   T   T   S   A   S   F 176
                             ↑2

529  TTA GTC ACT TAT ATG TAA TAT TTA AAG TAT TTT ACA TTG CGG GCA TAT 576
177  L   V   T   Y   M   *                                       182

577  CTA TGA TTG CCC GCA ATA TTA CTG ATG GAT ATT ATA TGA ATA GAA AAA 624
                                     ↑1

625  AAC ATC AGA TTT TAA AAA TTT TAT TGT TGT GTC TAA TAA GCA GTA A   670
```

Figure 6.1B DNA nucleotide sequence of *fanC*, the gene coding for the FanC subunit protein of K99 fimbriae of enterotoxigenic *E. coli*, with the deduced amino acid sequence of the open reading frame. Vertical arrows (numbered) indicate the positions of the end points of the C-terminal deletions generated for epitope analysis in *fanC::phoA'* fusions. The last base of each truncated **fanC** gene is shown in boldface typeset. Deletions a, b, c, and d (arrowed) were also fused to *phoA'*, but the fusion products were not detected by SDS-PAGE analysis or by Western blotting with anti-alkaline phosphatase serum (not shown). The underlined sequence corresponds to a region in FanC that fits into the motif common to T cell epitopes as predicted by DeLisi and Berzofsky (1985) and Rothbard and Taylor (1988).

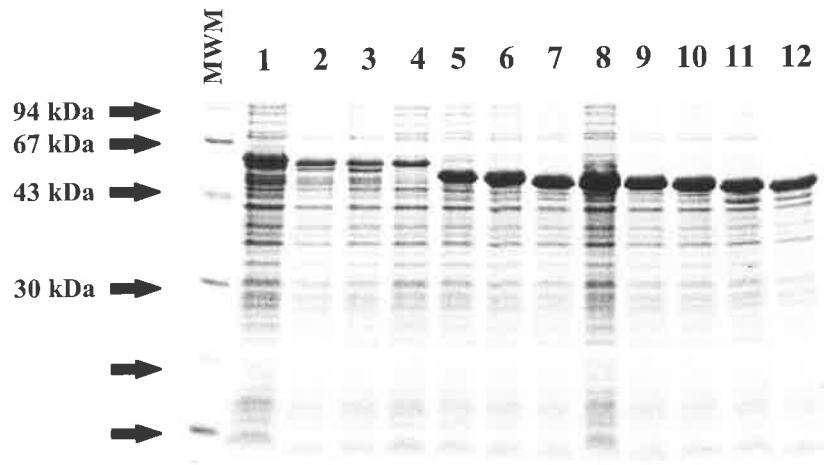


Figure 6.2 SDS-PAGE analysis of overexpression of FanC::PhoA fusion polypeptides in *E. coli* K-12 strain DH5 cells harbouring the T7 RNA polymerase plasmid pGP1-2. The polypeptides were visualised by staining with Coomassie blue. Lanes: MWM= low molecular-weight markers (Pharmacia); 1 to 12 represents various polypeptides derived by carboxyl-terminal truncations of *fanC* fused to *phoA'* as numbered in Figure 6.1B. Samples were heated at 100°C for 3 min in SDS sample loading buffer before electrophoresis.

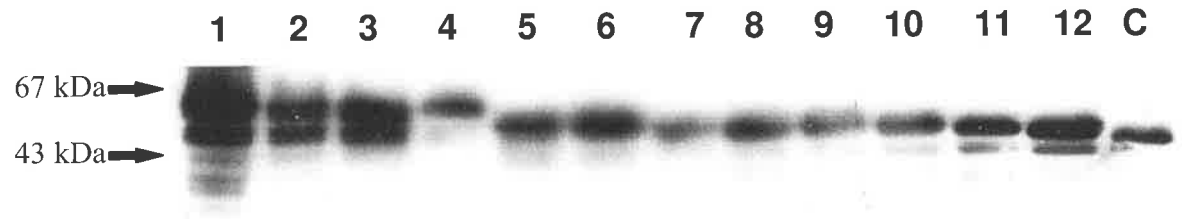


Figure 6.3 Western blotting of SDS-PAGE-fractionated whole-cell lysates of samples loaded in Figure 6.2 with an Ab to bacterial alkaline phosphatase. The full length fusion is 67 kDa in size (lane 1). A gradual reduction in the sizes of the fusions could be seen. Lane C is a control representing an unfused alkaline phosphatase, 43 kDa in size.

6.2.3 Epitope analysis of FanC

6.2.3.1 B cell epitope mapping of FanC

An attempt was made to characterise the B-cell epitopes of FanC by Western blotting of the various fusion proteins with an antiserum specific for the K99 fimbrial Ag, and with a panel of MAbs generated to the K99 Ag. The specific anti-K99 serum could only recognise the full-length FanC::PhoA polypeptide and fusion peptides with not less than 47 amino acid deletions from the C-terminus of FanC (Figs. 6.1B and 6.4A). The intensity of the Ab reaction with the recognised FanC peptides also appears to reduce progressively with increasing deletion, although the corresponding protein gel (Fig. 6.2, lanes 1-12) did not indicate a bias in the relative amount of protein loaded. Since a polyclonal Ab was used, the observation suggests that there are several epitopes being sequentially deleted from the C-terminus of the FanC polypeptide. It seems unlikely that the truncated peptides may have been degraded by proteases because similar results were obtained when these proteins were expressed in *lon* (χ2875), *degP* (KS474 and ECC219) and *ompT* (BL21) strains of *E. coli*. Furthermore, the addition of 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (Sigma P-7626) to the fusion proteins immediately after induction did not result in a pronounced difference in the results (not shown). In addition, Western blotting of unboiled preparations of the fusion proteins with the specific anti-K99 serum under non-reducing conditions was also carried out to verify whether the non-recognition was as a result of denaturation of the peptides. Similar results were obtained, except that the intensity of the signal from the resultant autoradiogram was higher than that obtained with boiled preparations. It is possible that once fused to alkaline phosphatase, the conformation of the peptides could have changed so that they were no longer recognised by Abs specific for the native form as readily. This suggestion is consistent with the results of Simons *et al.* (1990a), who ascribed a normal expression and

binding capacity of K99 fibrillae to the presence of the penultimate and conserved tyrosine residue of FanC.

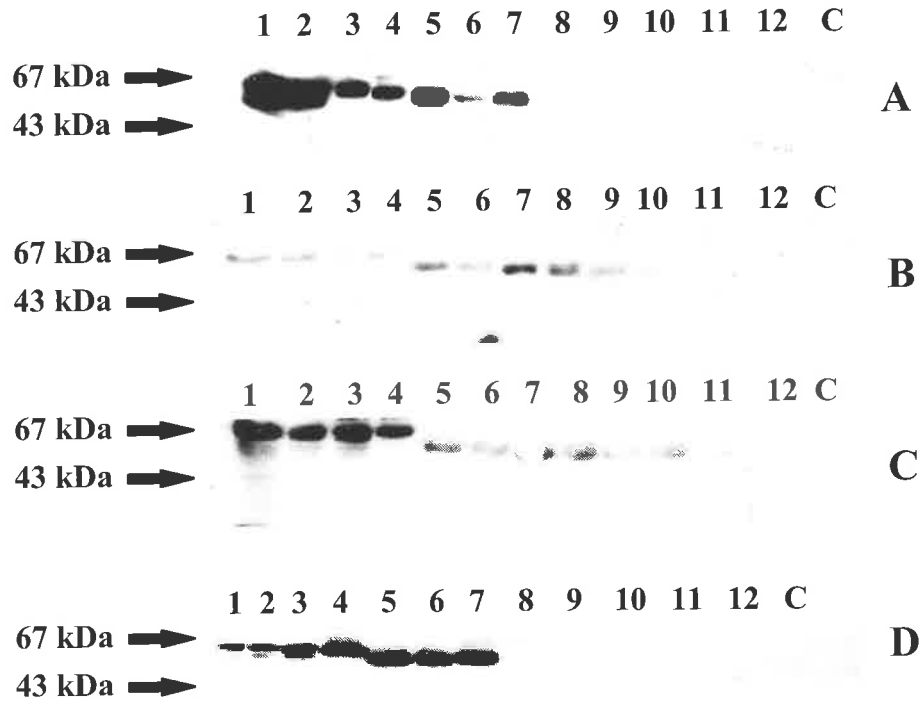


Figure 6.4 Western immunoblot analysis of samples loaded in Figure 6.2, with **A,** a K99 specific antiserum; **B,** MAb KK99-1 raised to K99; **C**, MAb KK99-2; and **D**, MAb KK99-4. A similar blotting pattern as with MAb KK99-2 (blot **C**) was obtained with MAb KK99-3 (not shown). Similar results were obtained in two repeat experiments. For full description, see text (Section 6.2.3.1).

The MAbs raised to K99 were also examined for binding to the fusion proteins by Western blotting. Figures 6.4B to 6.4D shows the results obtained with the fusion proteins reacted with MAbs KK99-1, KK99-2, KK99-3, and KK99-4. Quite interestingly, the MAbs seem to be recognising more fusion proteins than the K99-specific antiserum. A plausible explanation would be that the range of specificity of Abs in the antiserum are not all present at a high enough concentration, and therefore could not bind the peptides at the dilution of the antiserum used. Alternatively, it is likely that the repertoire of the K99 specific antiserum is biased towards recognition of the carboxyl terminal portion of FanC.

Taken together, the results indicate that the B-cell epitopes of the FanC protein are highly likely to be continuous, although it appears that there is an immunodominant region at the C-terminus of the protein as judged by the decrease in intensity of binding of the smaller fusion peptides. A summary of the results of the B-cell epitope mapping studies is presented in Table 6.1.

Table 6.1 Summary of B cell epitope mapping of FanC with various antibodies.

FanC::PhoA fusion protein/ peptide[a]	number of amino acids deleted[b]	Reactivity obtained with:[c]				
		K99-specific antiserum	KK99-1	KK99-2	KK99-3	KK99-4
1	none	+	+	+	+	+
2	14	+	+	+	+	+
3	24	+	+	+	+	+
4	33	+	+	+	+	+
5	36	+	+	+	+	+
6	43	+	+	+	+	+
7	48	+	+	+	+	+
8	57	-	+	+	+	-
9	63	-	+	+	+	-
10	71	-	+	+	+	-
11	77	-	-	+	+	-
12	85	-	-	-	-	-

[a] FanC protein and C-terminal deleted peptides are numbered as they are shown in Figure 6.2.
[b] Values represent the number of amino acids deleted from the C-terminus of FanC in the respective clones.
[c] The results shown were based on reactivity of the various antibodies against the FanC::PhoA fusions in a Western blot as shown in Figure 6.4.

6.2.3.2 T cell epitope mapping of FanC

The various fusion proteins were also employed to identify the potential T-cell epitopes of the FanC protein by analysing their ability to stimulate SA9-primed T cells in a standard *in vitro* proliferation assay (Section 2.10.4), using the "T cell Western blot" technique described by Abou-Zeid *et al.* (1987) and Lamb *et al.* (1988). The relevant characteristics of SA9, SA5 and 11RX are described in Table 2.3 in Chapter 2. Figure 6.5 shows the results of the T-cell epitope mapping of FanC. As shown in the figure, the full

length FanC::PhoA fusion potein and all the FanC::PhoA peptides (lanes 1-12, in the order shown in figure 6.2) induced the proliferation of SA9-primed fractionated peritoneal cells (FIPCs) to a similar extent as judged by ^{3}H-[TdR] incorporation after 72 h of culture. The overproduced alkaline phosphatase protein (PhoA) on its own is not as stimulatory. As expected, the primed peritoneal cells proliferated in response to stimulation by purified K99 Ag and formalin-killed preparation of SA9 organisms. Not surprisingly, formalin-killed preparations of SA5 and 11RX organisms were also stimulatory, but to a lesser extent, since SA5 and 11RX share the same Ags as SA9, but they do not carry the gene for the expression of the K99 Ag.

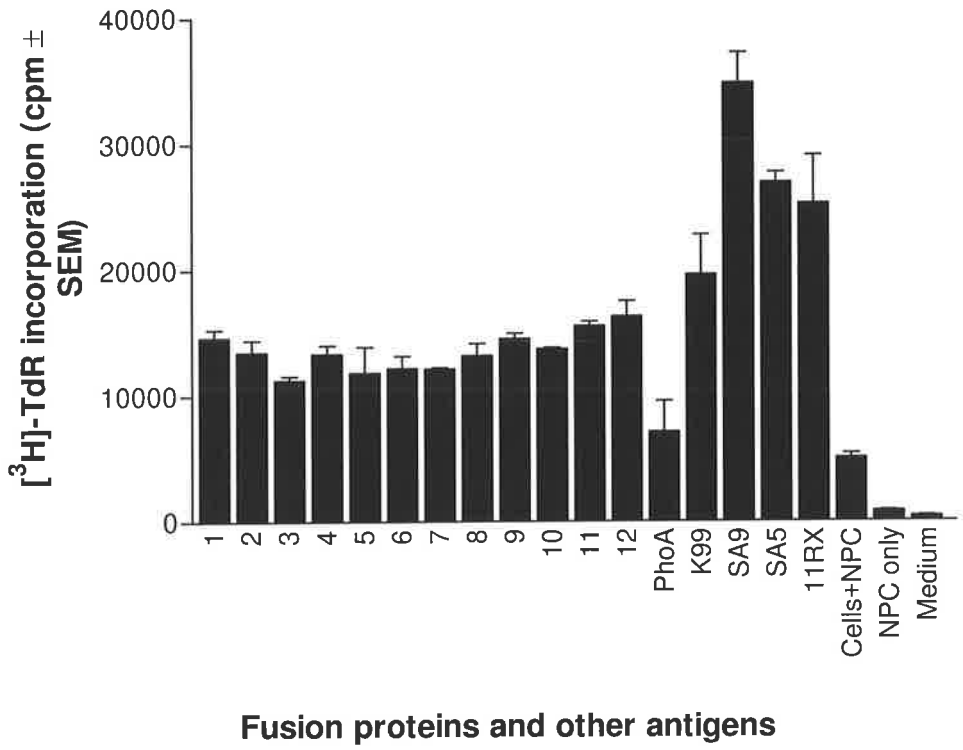


Figure 6.5 T cell stimulation profiles of FanC::PhoA fusions. Fusion proteins and unfused alkaline phosphatase (PhoA) were used at 5 mg/ml to stimulate peritoneal cells (FIPCs) harvested from mice 14 days after i.p. immunisation with live SA9 organisms. Purified K99, concanavalin A (ConA), and formalin-killed preparations of SA9, SA5 and 11RX were used at 1 μg/ml each in these assays. Proliferation was measured by [^{3}H]-TdR uptake after 72 h of culture. [^{3}H]-TdR uptake from ConA-stimulated T cells in this assay was 113,572 ± 9,052 counts. Cells+NPC=fractionated peritoneal cells cultured with normal peritoneal cells (NPC) only, as control.

The overall proliferation levels of the primed peritoneal cells were lower than expected, except with stimulation by ConA. This probably indicates that a relatively low population of T cells primed to the K99 Ag were present in the peritoneal preparations. However, similar results were obtained in a repeat experiment, and the proliferation profiles of fractionated spleen cells harvested from the i.p. immunised animals to these Ags were comparable to those shown in figure 6.5 (not shown).

It was somewhat disappointing that none of the FanC::PhoA fusion peptides was immunodominant, as anticipated based on previous work on K99 by Bertram *et al.* (1994). The reason may be that the FanC peptides produced by fusion to alkaline phosphatase are folded such that they could not be processed properly by the APCs, making the relevant epitope regions unavailable for recognition by the T cells. A search was made in the FanC coding region for the presence of motifs common to T cell epitopes as predicted by DeLisi and Berzofsky (1985) and Rothbard and Taylor (1988). The only region in FanC that correlates with the motif is the Arg-Ala-Pro-Ser amino acid sequence at positions 158-161 of the FanC polypeptide (underlined in figure 6.1B). It would be interesting to examine the immunogenic characteristics of a synthetic peptide spanning this region in future work.

6.2.4 Overproduction of SefA

In order to generate C-terminal truncated peptides of SefA for B and T cell epitope analysis, a *sefA* construct was generated in pEV41b (Section 2.14.3). The resulting construct, pPM3913, was used for overproduction of SefA in *E. coli* K-12 strains ECC219 and UT5600[p*cI857*] (Section 2.15.5.2). After induction, SefA was the predominant protein in the cell (Fig. 6.6A, lane 1). The increase in size of the overproduced protein from the usual 14 kDa to 17 kDa was due to the fusion of SefA to a 30-amino acid leader peptide contributed by the pEV41b vector. Western blotting with the SEF14-reactive MAb KAP14-1 was used to confirm that the overproduced protein was SefA (Fig. 6.6B).

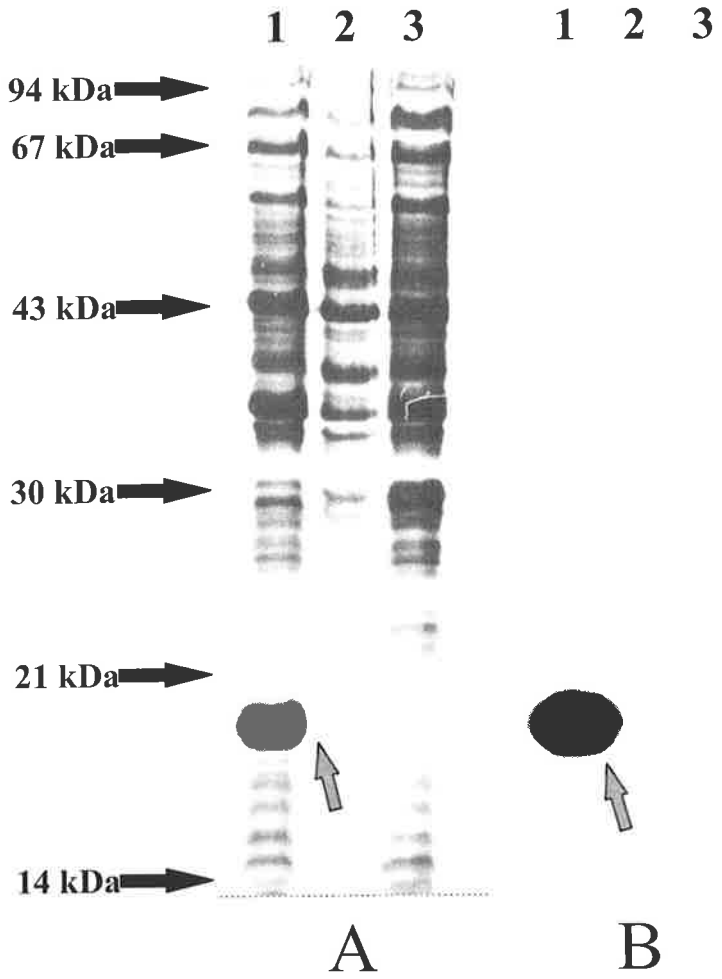


Figure 6.6 (**A**) SDS-PAGE analysis of overproduction of SefA in *E. coli* UT5600[p*c1857*]. Samples were heated at 100°C for 3 min in SDS sample loading buffer before electrophoresis. Lanes: 1, Cells induced for SefA production at 42°C; 2, Uninduced cells; 3, Cells carrying vector alone , as control. (**B**) Western immunoblot analysis of samples in panel **A** using the SEF14-reactive MAb KAP14-1. Arrows point to the overproduced SefA protein.

6.2.5 Epitope analysis of SefA

6.2.5.1 SefA peptides are not produced by C-terminal deletions of *sefA*

Having successfully overproduced SefA protein from pPM3913, a series of C-terminal deletions in the *sefA* clone in pEV41b (pPM3913) were generated [see Section 2.14.8] in order to yield peptides for B- and T-cell epitope mapping of the protein, by exploiting the vector's additional features which included an upstream His_6 tag to facilitate

a single step affinity purification of the protein and its peptides, and an IgA protease cleavage site for *in vitro* processing of fusions.

Disappointingly, unlike the full-length fusion protein, the peptide products of the generated nested deletions could not be detected by Coomassie blue staining of their SDS-PAGE-fractionated preparations (not shown). This was analysed further by Western blotting of SDS-PAGE-fractionated whole cell lysates of these clones with the specific SEF14 antiserum and the SEF14-reactive MAbs KAP14-1, KAP14-4 and KAP14-8; none of the deletions in SefA could be detected with any of these antibodies. It was presumed that the peptides might have been degraded by proteases, so an attempt was made to express the peptides in *lon*, *degP* and *ompT* strains of *E. coli* as was done for the FanC::PhoA clones. Again, the peptides could not be detected on protein gels or by Western blotting in any of these attempts. Figure 6.7A shows a typical result obtained by Western blotting of the fusions with either the specific SEF14 antiserum or the SEF14-reactive MAbs in these *E. coli* strains. To rule out the possibility of degradation of the peptides or/and mRNA instability, I attempted to express these fusions in a cell-free system by *in vitro* transcription/translation, using the *E. coli* S30 extract kit (Section 2.14.9). The fusion peptides could not be detected by this system either (Fig. 6.7B), although the full length fusion protein could be detected, indicating that the negative result obtained was not due to a technical failure. In fact, this problem was discussed with our collaborators at the Central Veterinary Laboratory, Weybridge, Surrey, England, who were interested in defining the B cell epitopes of the SefA Ag. Interestingly, they had tried a similar procedure, also with no success.

Figure 6.7 Western immunoblot analyses of SDS-PAGE-fractionated translated carboxyl-terminal deletions of *sefA* in pPM3913. (**A**), in *E. coli* UT5600[pc*1857*] (similarly in *lon*, *degP* and *ompT* strains of *E. coli*), and (**B**), by *in vitro* transcription/translation using the *E. coli* S30 extract. Lanes (both panels): **1**, *Pvu*II-*Sna*BI fragment encompassing full length *sefA* and part of *sefB*, cloned into pEV41b (pPM3913); **2**, a C-terminal deletion in pPM3913 down to, and including the TAA stop codon of *sefA*; and **3-12**, progressive internal deletions in *sefA*. Similar results were obtained in several repetitions using the specific SEF14 antiserum and various SEF14-reactive MAbs in all blots, so only the typical blots are shown.

6.2.5.2 Use of synthetic peptides to define T-cell epitopes of SefA

In a further approach to define the T cell epitopes of SefA, aliquots of nineteen overlapping synthetic peptides of SefA (generously donated by Dr C. Thorns, Central Veterinary Laboratory, Weybridge, Surrey, England), were used during the last few stages of thesis preparation. Each of these peptides is 16 amino acids in length, overlapping by 8 amino acids and covering the entire length of mature SefA (Fig. 6.8). The peptides were synthesised using a solid phase Synergy Fmoc peptide synthesiser (Applied Biosystems), and were dissolved in 60% DMSO in sterile double deionised water at 10 mg/ml. To characterise the T cell epitope(s) of SefA, each peptide was used in an *in vitro* T-cell proliferation assay (as described in Section 2.10.4) at 50, 5, and 0.5 μg/ml. In this assay, primed T cells were obtained from fractionated intraperitoneal cells and fractionated spleen cells of BALB/c mice that had been immunised i.p. 14 days earlier with live 11RX

organisms. Figure 6.9 shows the results obtained from one of two experiments, with similar results, using the peptides at 50 μg/ml which gave the most consistent results.

```
1 MRKSASAVAV 10
         11 LALIACGSAHAAGFVG 26   (P45)
                 19 AHAAGFVGNKAEVQAA 34 (P46)
                         27 NKAEVQAAVTIAAQNT 42       (P48)
                                 35 VTIAAQNTTSANWSQD 50      (P53)
                                         43 TSANWSQDPGFTGPAV 58    (P54)
                                                 51 PGFTGPAVAAGQKVGT 66   (P55)
51 PGFTGPAVAAGQKVGT 66      (P55)
        59 AAGQKVGTLSITATGP 74    (P73)
                67 LSITATGPHNSVSIAG 82  (P49)
                        75 HNSVSIAGKGASVSGG 90 (P50)
                                83 KGASVSGGVATVPFVD 98      (P51)
                                        91 VATVPFVDGQGQPVFR 106    (P57)
                                                99 GQGQPVFRGRIQGANI 114   (P58)
99 GQGQPVFRGRIQGANI 114    (P55)
       107 GRIQGANINDQANTGI 122   (P60)
               115 NDQANTGIDGLAGWRV 130 (P61)
                       123 DGLAGWRVASSQETLN 138       (P62)
                               131 ASSQETLNVPVTTFGK 146     (P64)
                                       139 VPVTTFGKSTLPAGTF 154    (P65)
                                               147 STLPAGTFTATFYVQQ 162   (P66)
                                                       155 TATFYVQQYQN 165 (P63)
```

Figure 6.8 Amino acid sequences of 19 synthetic peptides spanning the entire region of the 14 kDa SefA protein of *S.* Enteritidis. Each peptide is 16 amino acids in length and overlaps with the adjacent peptide by 8 amino acids. Peptide numbers are shown in brackets; amino acid positions are indicated bold face typeset. Peptides are a kind gift from Dr C. Thorns, Central Veterinary Laboratory, Weybridge, Surrey, England.

As seen in the figure, peptide P55 was the most stimulatory to primed peritoneal and spleen T cells. In addition, peptides P46 and P51, and two other overlapping peptides, P61 and P62, were also stimulatory to the T cells. As expected, purified SefA Ag and formalin-killed preparation of 11RX organisms induced strong proliferative responses from the immune T cells. It should be noted that other peptides also appeared to be somewhat stimulatory, although to a lesser extent, and there was a high background proliferative response from the immune T cell population cultured with APCs alone

(labelled cells + NPCs; Fig 6.9). In the former case, this may indicate that SefA has an array of T cell epitopes. As a general observation, however, the proliferation levels of the primed peritoneal and spleen cells were lower than expected as observed with the primed T cells in the K99 assays, except with stimulation by ConA. The likely reason for this is the relatively poor priming of T cells to SefA antigen. This could probably be addressed in future experiments by immunising the mice with a secondary dose of the live 11RX organism, or boosting with the purified SefA preparation.

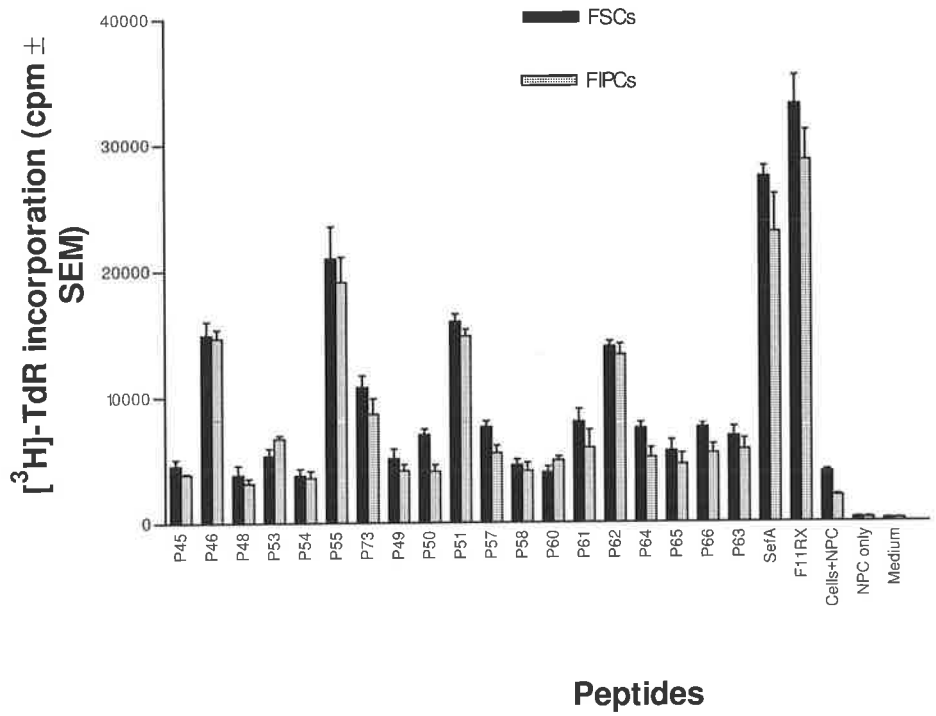


Figure 6.9 T cell stimulation profiles of SefA synthetic peptides. Peptides were used at 50 mg/ml to stimulate spleen cells (FSCs) and peritoneal cells (FIPCs) harvested from mice 14 days after i.p. immunisation with live 11RX organisms. Purified SefA, formalin-killed preaparation of 11RX (F11RX), and concanavalin A (ConA) were used at 1 μg/ml each in these assays. Proliferation was measured by [^{3}H]-TdR uptake after 72 h of culture. [^{3}H]-TdR uptake from ConA-stimulated T cells in this assay was 108,674 ± 9,845 counts. Cells+NPC=FSCs or FIPCs cultured with normal peritoneal cells (NPC) as control.

It seems very likely that the amino acid sequence Gly-Pro-Ala-Val-Ala-Ala-Gly corresponding to positions 55-61 of the protein (spanned by peptide P55) is the immunodominant T cell epitope region of the SefA Ag. This reasoning is based not only

on the T cell proliferation results, but also because the amino acid sequence forms a pattern similar to the motif for T cell epitopes predicted by DeLisi and Berzofski (1985) and Rothbard and Taylor (1988). Four other potential T cell epitope regions in the protein based on the prediction are (1), the Gly-Phe-Val-Gly sequence corresponding to amino acid positions 23-26 of the protein (spanned by peptide P46); (2), the Gly-Val-Ala-Thr sequence corresponding to positions 90-93 (spanned by peptide P51); (3), the Gly-Leu-Ala-Gly sequence corresponding to positions 124-127 (spanned by peptides P61 and P62); and (4), the Arg-Val-Ala-Ser sequence corresponding to positions 129-132 of the polypeptide (spanned by peptide P62). All of these predicted regions, except for the one spanned by peptide P61, were most stimulatory to the primed T cell used in this study. It would be important to work with these peptides further to confirm the present results.

6.3 Summary

The studies reported in this chapter were designed to characterise the B- and T-cell epitopes of FanC and SefA, the structural subunit proteins of K99 and SEF14 fimbriae, respectively, using a recombinant fusion technique and synthetic peptides. The recombinant approach was adopted for a number of reasons. First, production of proteins and peptides by recombinant DNA techniques is believed to be cheaper, easily renewable and an efficient alternative to chemical synthesis of peptides (Gross and Rohrmann, 1990; Wunderlich *et al.*, 1992; Skakoon and Dunn, 1993; Oettinger and Andersen, 1994). Second, fusion of truncated proteins to a larger protein such as alkaline phosphatase (PhoA) could lead to stabilisation of the peptides which otherwise would be rapidly degraded within the *E. coli* cell (Hoffman and Wright, 1985). Finally, the fusion products could easily be purified from other cellular proteins by affinity chromatography with

antibody to alkaline phosphatase. In this chapter, it was clearly demonstrated that fusion of the C-terminal truncations of FanC to PhoA led to the stabilisation of the FanC peptides.

The carboxyl-terminal residues of many fimbrial subunit proteins and subunit-like proteins which function as minor components is highly conserved, and in most cases, the penultimate residue is a tyrosine (Båga *et al.*, 1984; Mooi *et al.*, 1984; Roosendaal *et al.*, 1984; Orndorff and Falkow, 1985; Lindberg *et al.*, 1986; Båga *et al.*, 1987; Klemm and Christiansen, 1987; Roosendaal *et al.,* 1987b; Hultgren *et al.*, 1989). It is believed that that this tyrosine residue is critical for the conformational stability of these proteins and for their stable interaction with the periplasmic chaperone protein. This has been well established in the case of Pap pili and K99 fimbriae (Hultgren *et al*, 1989; Simons *et al.*, 1990a). Recently in our laboratory, studies involving epitope analysis of the CstH major subunit protein of CS3 fibrillae (Jalajakumari *et al.,* 1989), were performed. In these experiments, various amino- and carboxyl-terminal deletions of *cstH* fused to *phoA'* were evaluated using a panel of MAbs to CS3 fibrillae and a CS3-specific antiserum, and produced similar findings (B. Yakhchali, 1996). It seems conceivable that this may also be the case with the SefA subunit in this study, although the C-terminal tyrosine residue in SefA is the third from last residue. It is quite possible that fusion of the SefA peptides to a larger protein such as PhoA could lead to a stabilisation of the peptides, as was achieved with the K99 peptides in this study. It is tempting to speculate from these results that this is a common phenomenon across many fimbrial types.

The results obtained from T cell proliferation assays with FanC::PhoA fusions did not indicate the presence of any immunodominant T cell epitope in this protein, contrary to what was anticipated. In addition, the fusion partner, alkaline phoshatase, was found, to an extent, to be stimulatory to T cells in this study. These findings (and those from the B-cell epitope mapping) highlight the potential problems and drawbacks of the use of recombinant protein fusion technique to characterise B- and T-cell epitopes of certain

proteins, although the technique has been used successfully in other systems (see Gross and Rohrmann, 1990; Hickling *et al.*, 1992; Wunderlich *et al.*, 1992; Skakoon and Dunn, 1993; Oettinger and Andersen, 1994).

A series of overlapping synthetic peptides were used to characterise the T cell epitopes of the SefA antigen. Unfortunately, these peptides were only available at the concluding stages of this work and as such only a limited set of experiments were performed. However, the results obtained indicate the presence of a potential immunodominant epitope of this antigen at amino acid positions 55-61 of the polypeptide; other potential immunogenic regions of the protein were also indicated. Further investigations are needed to get a better understanding of the present results. These would include investigating if the peptides are MHC class I- or MHC class II-restricted using relevant T cell clones and mice of different haplotypes. It would also be interesting to see if these peptides are able to generate protective immunity against challenge by virulent *S.* Enteritidis. The results from such investigations would add to current knowledge on the generation of immunity during the disease process, and could aid in the design of vaccines to *S.* Enteritidis.

CHAPTER 7

Summary and conclusions

7.1 Previous studies

This work was commenced as a continuation of the many studies in our laboratory over the years in trying to understand the nature of cell-mediated immunity to *Salmonella* infections, using an attenuated strain, *S.* Enteritidis 11RX, as a model, and employing various *in vitro* and *in vivo* systems as tools. Earlier studies had established that mice infected with the attenuated 11RX strain were protected against challenge by the isogenic virulent strain 11, as well as against challenge by the highly virulent *S.* Typhimurium C5 strain. In fact, mice immunised with 11RX were also shown to be resistant to infections by antigenically unrelated bacteria, such as *Listeria monocytogenes* (Rowley *et al.*, 1968).

Subsequently, the ability of the 11RX strain to protect against lethal infection was ascribed to an enhanced bactericidal activity of macrophages in the presence of antibacterial Abs (Rowley *et al.*, 1968), and more importantly, to the ability of the 11RX organisms to induce T cell-mediated immunity, comprising mainly T cells of the MHC class II-restricted $L3T4^+$ phenotype, but also of the MHC class I-restricted $Lyt2^+$ phenotype, especially after secondary infection (Davies and Kotlarski 1974, 1976; Attridge and Kotlarski 1985a, b; Kotlarski *et al.*, 1989; Pope *et al.*, 1994; Pope and Kotlarski, 1994). These findings led Vordermeier and Kotlarski (1990a, b) to characterise the Ags of the attenuated 11RX strain that were capable of stimulating T cells of 11RX-immunised mice. Their study identified a highly immunogenic low-molecular weight 16 kDa protein of 11RX as capable of eliciting delayed-type hypersensitivity, and able to stimulate *in vitro* proliferation of, and cytokine release from, T cells harvested from 11RX primed F_1 mice to

levels comparable to those induced by the whole organism. The observations in the study conducted by Vordermeier and Kotlarski (1990b) that formalin-killed suspensions of other organisms within the family *Enterobacteriaceae,* such as *E. coli*, were able to induce proliferation of, and IL-2 release from 11RX-primed T cells, led the authors to suggest that an 11RX Ag(s) localised in the 16-18 kDa region could be the "common" Ag(s) expressed by some members of the family. The 16 kDa protein was partially purified, but was, however, associated with LPS.

7.2 Current work and perspectives

As an extension of the work of Vordermeier and Kotlarski (1990b), the initial aim of this thesis was to make a homogeneous preparation of the immunogenic 16 kDa protein of 11RX free of LPS, to aid in its characterisation. This was necessary to abolish non-specific proliferation of lymphoid cells due to LPS contamination because it is often difficult to prepare T cell populations completely free of B cells (Kuusi *et al.*, 1981; Villarreal *et al.,* 1992). Furthermore, Vordermeier and Kotlarski (1990b) had proposed that to gain a better understanding of the pathways of Ag processing in the induction of cell-mediated immunity to intracellular bacterial pathogens, it is essential to use purified Ags, preferably of a relatively low molecular weight, thereby reducing the number of antigenic determinants being studied. It was suggested in that study that the immunogenic 16 kDa low molecular weight protein of 11RX might be a suitable candidate Ag for such studies.

The first step in the characterisation of the immunogenic 16 kDa protein was its purification. This was accomplished by differential ultracentrifugation and ammonium sulphate precipitation, a protocol developed by a modification of methods published by Feutrier *et al.* (1986), and Müller *et al.* (1991). The purity of the protein was ascertained

by SDS-PAGE analysis and by silver staining of the SDS-PAGE-fractionated preparations, which confirmed that the protein was free of LPS contamination. The slightly faster mobility of the purified protein on SDS-PAGE was the first indication that this protein might be smaller than the 16 kDa size previously reported. The purified protein was shown to be highly immunogenic as determined by standard *in vitro* T cell proliferation assays, induction of cytokines from T cells harvested from 11RX-immunised mice, and by its ability to elicit delayed-type hypersensitivity reactions in mice, to levels similar to that elicited by a formalin-killed preparation of the whole 11RX organism. Using standard immunoblot analyses with a highly specific 11RX antiserum and a panel of MAbs raised to the protein, it was also demonstrated that the purified protein is not the "common" Ag expressed by some members of the family *Enterobacteriaceae*, as previously suggested by Vordermeier and Kotlarski (1990b).

Preliminary characterisation of the highly immunogenic low molecular weight protein by N-terminal sequencing established its identity with the 14 kDa fimbrial Ag of *S.* Enteritidis (SEF14), previously described by Feutrier *et al.* (1986) and Thorns *et al.* (1990), and later characterised by Turcotte and Woodward (1993) and Clouthier *et al.* (1993). The demonstration of fimbriae morphologically resembling SEF14 fimbriae on the surface of 11RX bacteria by immuno-electron microscopy with one of the MAbs raised to this protein confirmed the identity of this protein. Further evidence on the size of the protein was obtained later by Western blotting of an *E. coli* recombinant strain carrying an 11RX cosmid with the MAbs raised to the protein, which showed that both the 14 and the 16 kDa bands were detected by the MAbs. The two bands were later confirmed by sequence analysis of the gene coding for the subunit protein, *sefA,* as the precursor (16 kDa) and mature (14 kDa) forms of the protein.

The identification of the highly immunogenic low molecular weight Ag of 11RX as the SEF14 fimbrin of *S.* Enteritidis opened new perspectives for studying the 11RX

organism, and evaluating the role of the protein in the biology of the organism, considering the emerging evidence on the role of fimbriae in the pathogenesis of many bacteria that infect epithelial cell surfaces, and also because of the role of fimbriae in the generation of immunity in hosts to bacteria that infect epithelial cell surfaces. To address these questions, a molecular approach was adopted, and this initially involved cloning the gene coding for the protein which was achieved by making a cosmid library of the 11RX chromosome in *E. coli*. The gene (*sefA*) coding for the structural subunit protein, SefA, was identified and characterised by restriction mapping and sequencing.

To define the role of SefA in the biology of *S.* Enteritidis, defined *sefA* mutants were constructed in strain 11RX and in a virulent *S.* Enteritidis strain 7314, by interrupting *sefA* with a nonpolar kanamycin-resistance *aphA-3* cassette (Ménard *et al.*, 1993) with the aid of a suicide vector pCACTUS (Clark *et al.*, manuscript in preparation). The mutation in both strains resulted in the abolition of SefA production and consequently, a loss of SEF14 fimbriae assembly. The abilities of the *sefA::aphA-3* mutant derivatives of strains 11RX and 7314 to colonise the Peyer's patches and establish a systemic infection in the spleens of BALB/c mice was compared with their isogenic parents. The results obtained indicated that the mutation of *sefA* in the 11RX strain resulted in a pronounced reduction in the ability of the *sefA::aphA-3* mutant to colonise (and persist in) the Peyer's patches of BALB/c mice, implying a role for SEF14 fimbriae in this process. The invasion of the spleens of these mice was not appreciably affected, indicating that the expression of these fimbriae is probably not critical once the bacteria have crossed the epithelial cell barrier. Evidence to support this notion was provided by the observation that there was no noticeable difference in the number of bacteria recovered from spleens of mice 2-3 weeks after intraperitoneal infection with either the parent or the SEF14-deficient strains of 11RX (data not shown). In contrast, there were no differences in the abilities of the virulent strain 7314 and its *sefA::aphA-3* isogenic mutant to either colonise or persist in the Peyer's

patches, or invade the spleens of the mice. These results were correlated with *in vitro* adherence and invasion of HeLa cells where similar observations were made. It is likely that the contradictory observations reported here were due to the nature of the two strains used in the study. Strain 11RX lacks the LPS O-(somatic) Ag and has a variable flagellar Ag phase, whereas strain 7314 possesses a smooth LPS phenotype and is always flagellated. It therefore seems likely that 11RX, being rough, relies more on SEF14 fimbriae for colonisation of the host epithelium because it is attenuated, while strain 7314 may possess other (fimbrial?) structures that could compensate for the SEF14 loss.

The concurrent production of other known fimbriae of *S.* Enteritidis, namely SEF17 (Collinson *et al.*, 1991), SEF18 (Clouthier *et al.*, 1993), and SEF21 (Müller *et al.*, 1991) in 11RX or 7314 was not assessed in this study and has not been reported elsewhere in the literature. It is envisaged that this would be a challenging task, since distinct fimbriae are produced under different growth conditions and at different growth stages of bacteria, both *in vitro* and *in vivo*. However, it is known that in some organisms, alterations in the LPS phenotype leads to altered export or production of certain fimbriae (Pilipcinec *et al.* 1994; Iredell, J. R., and Manning, P. A., manuscript submitted). An attempt to complement the O-Ag defect in strain 11RX with a cosmid carrying the *rfb* and *rfc* genes of *S.* Typhimurium (Neal *et al.,* 1993) was unsuccessful, indicating that the rough phenotype of 11RX was not due to an *rfb* defect. Nonetheless, it would be interesting to evaluate the effect of a sequential knockout of the other fimbriae in addition to SEF14, on the colonisation of epithelial cell surfaces by these *S.* Enteritidis strains. To draw any general conclusions, however, it would be necessary to extend these observations to host species other than mice, because they may possess different or additional receptors for bacterial adherence, and tissue tropism could also be different in other host species. The recent evidence that distinct fimbrial types contribute to the tissue tropism of *S.* Typhimurium for the murine villous small intestine (Bäumler *et al.*, 1996a) and murine Peyer's patches

(Bäumler *et al.*, 1996b), and to attachment to, and invasion of, epithelial cell lines (Bäumler and Heffron, 1995; Bäumler *et al.*, 1996c), has provided good support for this view. The identification and characterisation of additional host cell receptors, signalling molecules that trigger the host cell response leading to bacterial uptake, and other factors which are yet to be defined, would also aid in a better understanding of *Salmonella*-host cell interactions.

The role of SefA in the virulence of *S.* Enteritidis was assessed by LD_{50} measurements in BALB/c mice orally infected with the virulent *S.* Enteritidis strain 7314 and its *sefA::aphA-3* isogenic mutant counterpart. The results show that there was no difference between the LD_{50} of both strains (ca 2.5×10^5), implying that SefA does not have a measurable role in the virulence of *S.* Enteritidis. Taken together, these findings are not totally unexpected since a cluster of invasion loci, regulatory genes and other bacterial components have been identified and characterised as having a direct role in invasion and survival of salmonellae in eucaryotic cells, and in some cases, the presence of these genes has been correlated with virulence in salmonellae (Fields *et al.*, 1986; Finlay *et al.*, 1988; Galán and Curtiss, 1989a, b; Miller *et al.*, 1989, 1992; Mills *et al.*, 1995; Stone and Miller, 1995). Recently, it was shown that a 35-40 kb of DNA encompassing a contiguous region of *Salmonella* chromosome at centisome 63 encodes determinants for entry into mammalian cells (Mills *et al.*, 1995). Nucleotide sequence analysis of this region which has been carried out so far has revealed the presence of at least 28 genes, some of which have been genetically and functionally characterised (see review by Galán, 1996). However, it is not known whether additional invasion determinants are encoded downstream of these regions and this is a potential area of further research.

The sequence upstream of *sefA* showed extensive homology to the insertion sequence IS*3* of *E. coli* (Timmerman and Tu, 1985). This observation is the second report of this insertion element in *Salmonella*, the first being downstream of *invH* as reported by

Altmeyer *et al.* (1993). The significance of this finding is yet to be investigated, however, virulence factors have been shown to be encoded within or near sequences resembling transposons (So *et al.*, 1979; Mekalanos, 1983; Knapp *et al.*, 1986; Miller *et al.,* 1990), and Falkow *et al.* (1987) proposed that this topological relationship may be related to the acquisition or spread of such traits. Furthermore, proximal movable elements have been shown in certain instances to participate in the regulation of expression of virulence factors or other genes (Reynolds *et al.*, 1981; Ou *et al.*, 1988). It is therefore likely that the presence of the IS*3*-like element may be related to the regulation of the SEF14 fimbrial operon especially since there are no data regarding the characterisation of regulatory genes in this operon.

The genetic strategies hitherto used in the identification of virulence factors in bacteria (Isberg and Falkow, 1985; Fields *et al.*, 1986; Taylor *et al.*, 1987; Fields *et al.*, 1989; Miller *et al.*, 1989) have been limited by their inability to reproduce accurately host environmental factors *in vitro*. In this study, for example, SefA was demonstrated to be an important Ag *in vitro*, at least as demonstrated by its ability to induce strong, specific T cell and humoral responses in mice. However, the expected correlation of the *in vitro* data was not observed *in vivo*, as assessed by LD_{50} measurements. The reasons for these inconsistencies are unclear. However, Mahan *et al.* (1993) devised a genetic system that positively selects for bacterial genes that are specifically induced in host tissues. Using the *in vivo* expression technology (IVET), the authors were able to identify *in vivo* induced genes (*ivi* genes) in *S.* Typhimurium. These *ivi* genes were poorly expressed on laboratory media, but they were expressed at elevated levels by bacteria persisting in the tissues of BALB/c mice. Mutations in the *ivi* genes conferred virulence defects on *S.* Typhimurium, implying that genes selected by the IVET technique play an important role in infection and/or pathogenesis. This genetic system and related systems are already in general use in a wide variety of bacterial-host systems and are potentially applicable to vaccine

development for protection against *Salmonella* infections and to a wide variety of other microorganisms.

The observations that the purified SefA protein was able to induce *in vitro* proliferation of and cytokine release from T cells obtained from 11RX-immunised mice, and its capability to elicit delayed-type hypersensitivity reactions in these animals, stimulated the investigation of its potential as an Ag able to induce a protective immune response against challenge with a virulent strain of *S.* Enteritidis. Furthermore, the fact that SefA is a fimbrial subunit is significant in itself because of the potential of fimbriae as immunogens in animals against many bacteria which infect epithelial cell surfaces (Duguid *et al.*, 1966; Duguid and Old, 1980; Pearce and Buchanan, 1980; Tramont and Boslego, 1985; Korhonen *et al.*, 1990; Sun *et al.*, 1990; Krogfelt, 1991; Sun *et al.,* 1991). In this study, the ability of SefA to protect mice against challenge by the virulent *S.* Enteritidis strain 7314 was investigated in orally infected BALB/c mice, using a variety of Ag delivery systems. The results obtained showed that an immune response to SefA is protective, consistent with recent work of Peralta *et al.* (1994), who demonstrated that orally administered hen egg-yolk Abs specific for the purified SEF14 fimbrin provided passive immunisation against experimental salmonellosis in mice. However, because SEF14 is unique to *S.* Enteritidis and other group D salmonellae, it is not expected that it would be cross-protective against infections by other salmonellae. The data obtained in this study also indicated the presence of other protective Ags of *S.* Enteritidis. At present, the mechanisms of protection and the nature and identity of the Ags able to induce protective immune responses against *Salmonella* infections are yet to be fully clarified, and future work in this direction is needed. However, it is expected that protection would involve an interplay between humoral and cell-mediated immune responses, and evidence for these are continually emerging (Udhayakumar and Muthukkaruppan, 1987a, b; Nauciel, 1990; Muthukkumar and Muthukkaruppan, 1993; Pope and Kotlarski, 1994).

In an earlier study, Vordermeier and Kotlarski (1990b) proposed that the 16 kDa protein of *S.* Enteritidis 11RX (identified in this study as SEF14) might be a suitable candidate Ag to study the interactions between class I and class II MHC molecules in the pathways of Ag processing in the induction of a T cell response. Part of the work reported in this thesis was an evaluation of this proposition. In addition, the identification and characterisation of SefA as protective Ag of *S.* Enteritidis has also stimulated the interest in characterising the reactive B- and T-cell epitopes of the SefA Ag. However, at the commencement of this study, neither the identity nor the nucleotide sequence of the SefA Ag was known. A well-characterised analogous Ag, the FanC subunit protein of the K99 fimbriae of enterotoxigenic *E. coli*, was therefore employed as a model to establish systems to define the B- and T-cell epitopes of SefA. The approach adopted was a recombinant technique, involving the fusion of FanC and its carboxyl-terminal truncated derivatives to a reporter, the *E. coli* alkaline phosphatase, to generate stable recombinant fusion proteins.

The results obtained from the B cell epitope mapping studies of FanC by immunoblotting of the recombinant fusions with a K99-specific antiserum and a panel of MAbs suggests that the B cell epitopes of FanC are likely to be continuous, the use of more MAbs for mapping and inhibition ELISA with these MAbs is likely to improve and confirm this data. The various fusion proteins were also employed to identify the potential T-cell epitopes of the FanC protein by their abilities to stimulate SA9-primed T cells in a standard *in vitro* proliferation assay using the "T cell Western blot" technique described by Abou-Zeid *et al.* (1987) and Lamb *et al.* (1988). Contrary to the suggestion by Bertram *et al.* (1994) that the K99 fimbrial antigen should contain one or a few T cell epitopes, none of the FanC::PhoA fusion peptides was immunodominant. It is hypothesised that fusion of these peptides to alkaline phosphatase had resulted into altered folding such that they could not be processed properly by APCs, making the relevant epitope regions unavailable for

recognition by T cells. This finding highlights the potential problems and drawbacks of the recombinant fusion technique, and points to the fact that care should be taken when interpreting results from such studies. At the very least, results from such assays should be complemented with assays using synthetic peptides.

As a consequence of the potential drawbacks of the recombinant fusion technique, a slightly different approach was initially adopted to produce SefA peptides for the characterisation of the potential B and T cell epitopes of SefA. The approach involved C-terminal deletions of *sefA* fused at its N-terminus to a His_6 tag and an IgA protease cleavage site in plasmid pEV41b to allow for easy purification and *in vitro* processing of fusions. Unfortunately, this approach failed because carboxyl-terminal deleted SefA peptides were extremely unstable, and could not be detected by any of the several methods used. In a further approach to define the T cell epitopes of SefA, a series of overlapping synthetic peptides of SefA were examined for their abilities to stimulate the proliferation of T cells harvested from 11RX-immunised BALB/c mice. The results obtained indicated the presence of a potential immunodominant T cell epitope of SefA at amino acid positions 55-61 of the polypeptide, judged by a high level of proliferation from T cells stimulated by peptides spanning this region. It is worth pointing out that the results obtained from the T cell epitope mapping studies are not conclusive, because the synthetic peptides were only available during the last stages of this work. Clearly, further investigations, including MHC class restriction analysis of the peptides using appropriate T cell clones and mice of different haplotypes, and generation of protective immunity by these peptides against virulent challenge, are needed to provide a better understanding of the results reported in this thesis. It is also anticipated that the results from such investigations could have an important implication for vaccine design against *S.* Enteritidis, and in the design of vaccine strategies for other salmonellae.

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