Characterization of the sef14 fimbrial gene cluster and the encoded fimbriae

by

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B.Sc., University of Victoria, 1990

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry

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ABSTRACT

Salmonella enteritidis produces thin, filamentous fimbriae designated SEF14. A 7.1 kb fragment encoding genes responsible for SEF14 biosynthesis was sequenced and found to contain an IS3 element and five genes, sefABCDE. sefA encoded the structural subunit of SEF14 fimbriae. sefB and sefC encoded proteins homologous to fimbrial chaperones and ushers, respectively. In vitro expression directed by a 5.3 kb fragment identified SefA, SefB and SefC as approximately 14K, 28K and 90K M, proteins, respectively, which correlated with their predicted amino acid sequences. E. coli carrying the same 5.3 kb fragment were unable to assemble SEF14 fimbriae; however, immunogold labelled SEF14 fimbriae were displayed on E. coli clones containing a 44 kb fragment which encompassed the 5.3 kb region. Therefore, sefABC comprised only part of the sef14 operon responsible for the expression and assembly of SEF14 fimbriae.

Further DNA sequence analysis revealed two open reading frames, designated sefD and sefE immediately downstream of sefABC. sefD had the same translational polarity whereas sefE had the opposite polarity as sefABC. In vitro expression of a 10 kb KpnI fragment identified SefD and SefE as 18K and 30K M, proteins, respectively, which correlated with their predicted amino acid sequences. sefE encoded a protein homologous to AraC family transcriptional regulators, whereas the translated protein sequence of sefD was unique. SefD was produced in abundance by wild type S. enteritidis.
Furthermore, unusually long, thin, fimbriae were evident on *S. enteritidis* and *Escherichia coli* by immunoelectron microscopy. Thus, SefD was designated the structural subunit of fimbriae which were shown to be serologically distinct from the three known *S. enteritidis* fimbriae SEF14, SEF17 and SEF21 and were given the name SEF18 fimbriae. DNA hybridization and Western blot analyses showed that SefD was widely distributed among *Enterobacteriaceae*. In addition, *sefD* as well as *sefA* were mapped to the 90 centisome position on the *S. enteritidis* chromosome.

DNA sequence analysis of the region upstream of *sefA*, revealed three open reading frames, *orfABC*, whose genetic organization and sequence was characteristic of IS3 elements. Furthermore, the 289 bp region between the IS3 element and *sefA* contained three putative deoxyadenosine methylase sites and two consensus integration host factor binding sites.

Production of SEF14 fimbriae was thermoregulated since these fimbriae were not expressed by *S. enteritidis* grown below 30°C. Northern blot analysis of RNA isolated from *S. enteritidis* grown at different temperatures indicated that growth temperature regulated *sefA* transcription. Transcription of *sefA* was initiated at two major start sites located upstream of *sefA* and produced an unusually stable *sefA* transcript with a half life of 28 min. Secondary structure analysis of the mRNA transcript for *sefABC* predicted the formation of two stable stem-loop structures in the intercistronic region between *sefA* and *sefB* which may protect the 3' terminus against exonucleolytic attack resulting in the slow *sefA* mRNA decay rate.
SEF14 fimbriae are polymers of the protein SefA. In SDS polyacrylamide gels, SefA isolated from the periplasm of an E. coli clone separated into two forms that differed by only 1-2 kDa. Solution analysis revealed that the lower molecular weight form (SefA_L) was a monomer whereas the higher form (SefA_H) was a dimer. The monomer could be cross-linked to form a dimer but only after SefA_L shifted 1-2 kDa higher in the gel. Thus, the cross-linker was substituting for something in SefA_L that was missing but required for dimerization. Sequence analysis revealed that SefA_L lacked the first 24 N-terminal amino acids which accounted for the lower molecular weight and indicated that these 24 amino acids were required for dimerization. The dimer could be the basic building unit of SEF14 fimbriae.

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<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BS³</td>
<td>bis(sulfosuccinimidyl)-suberate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CFA</td>
<td>colonization factor antigen</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter(s)</td>
</tr>
<tr>
<td>CM</td>
<td>carboxy methyl</td>
</tr>
<tr>
<td>CS</td>
<td>centisome(s)</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>(ethylene diamine)tetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IHF</td>
<td>integration host factor</td>
</tr>
<tr>
<td>IM</td>
<td>intramuscular</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IR</td>
<td>inverted repeat</td>
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</table>
IRL  left inverted repeat
IRR  right inverted repeat
IS   insertion sequence
kb   kilobase(s)
kDa  kilodalton
kV   kilovolt(s)
L    litre(s)
LB   Luria broth
mAmp milliamp
mg   milligram(s)
min  minute(s)
ml   millilitre(s)
mM  millimolar
MOPS 4-morpholinepropanesulfonic acid
MW  molecular weight
ng   nanogram(s)
nm  nanometer(s)
OD   optical density
ORF  open reading frame(s)
PBS phosphate buffered saline (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl pH 7.4)
PCR  polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>pmol</td>
<td>picomole(s)</td>
</tr>
<tr>
<td>R</td>
<td>radial distance from axis of rotation</td>
</tr>
<tr>
<td>R_{meniscus}</td>
<td>radial position of the meniscus</td>
</tr>
<tr>
<td>rif</td>
<td>rifampicin</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>s</td>
<td>second(s)</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEF</td>
<td><em>Salmonella enteritidis</em> fimbriae</td>
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<tr>
<td>SefA_{H}</td>
<td>higher molecular weight form of periplasmic SefA in SDS polyacrylamide gels</td>
</tr>
<tr>
<td>SefA_{L}</td>
<td>lower molecular weight form of periplasmic SefA in SDS polyacrylamide gels</td>
</tr>
<tr>
<td>SOC</td>
<td>2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM D-glucose</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TAE</td>
<td>40 mM Tris HCl, 20 mM sodium acetate, 1 mM EDTA pH 8.3</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris pH 8, 1 mM EDTA</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>\mu g</td>
<td>microgram(s)</td>
</tr>
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<td>Description</td>
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<td>--------------------</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre(s)</td>
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<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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</table>
ACKNOWLEDGMENTS

I would like to thank Sandy Kielland (University of Victoria, Protein Sequencing Center) for sequencing my proteins, Dr. Ausio (University of Victoria, Canada) for the sedimentation equilibrium analysis of SefA, C. Furlong (University of Washington, WA) for the fermentation media recipe, J.R. Scott (Emory University Health Sciences Center, GA) for the gift of the plasmid pEU2030, T. Wadstrom (University of Lund, Sweden) for S. enteritidis 27655-3b, K.E. Sanderson (University of Calgary, Canada) for the blots of chromosomal DNA separated by PFGE, F. Nano (University of Victoria) for the gift of pINIII113 B1 designed by M. Inouye, the people in Animal Care who took such good care of the rabbits, Alistair for his help during the fermentation runs, Christina Kay and Blair MacDonald for their help during the purification of periplasmic SefA and SefB, Bill Eaton and Jim Baxter for allowing me to work at Malaspina University-College, Holly Blackburn, Barbara Folkins and Bev Morrison for making room for me at Malaspina University-College, Al Vaisius for giving me the opportunity to teach, Dr. Trust for use of the Superdex 75 HR 10/30 column, Darrel Hardy for advice and assistance with the HPLC, Kirsten Sheffield for drawing the HPLC profiles, Glenn Pryhitka and Kathy Cliff for their time and equipment and Scott Schutz and Albert Labossiere who generally helped me, often when I was in a panic.
I would also like to thank Jan Burian who synthesized many of my oligos and drew the plasmid maps as well as Pam Banser who gave me the DNA panels that were screened for the presence sefD.

I would like to give special thanks to Jamie Doran and Karen Collinson for their advice, patience, generosity, and support in and out of the lab as well as their work on the patent and the diagnostic aspects of SEF14 fimbriae and for teaching me how to write a scientific paper. Without their help, my papers and thesis would not be the same.

And then there was Val Funk, a great friend. Thanks for the talks, the walks and the suppers. Thanks also for helping me set up and run the first set of samples on the Superdex 75 column and for helping me with the rotary IEF apparatus.

Finally, there's Bill, a supervisor like no other. Thanks for having such faith in my ability to do science, for being excited about my results, for not pressuring me when the results were slow in coming, for seeing the bright side of things when all I saw was failure and for sticking by me even when I was working in Nanaimo or doing the daily Nanaimo-Victoria-Nanaimo commutes.
DEDICATION

This thesis is dedicated to my husband, Wayne, whose love, friendship and humor gave me the strength to keep going.
CHAPTER I

Introduction

1. Fimbriae

A. Terminology

Fimbriae are proteinaceous, filamentous structures produced on the surface of a range of bacteria (Duguid et al., 1955). These appendages have also been referred to as threads, filaments, bristles, cilia, fuzz, colonization factor antigen and adhesins (Paranchych and Frost, 1988). In addition, the term "fibrillae" has been used to describe the flexible, thin fimbriae that are only 2 to 3 nm in diameter (Levine et al., 1984; Stirm et al., 1967). Currently, the term "pili" is used interchangeably with fimbriae even though Ottow (1975) suggests that the term "pili" be used for conjugative filaments involved in the transfer of DNA between bacterial cells (e.g. F pili) (Brinton, 1959; 1965). For purposes of simplicity, the term "fimbriae" is used throughout this thesis to describe all non-flagellar, non-conjugative surface appendages.

B. Classification

Although many classification schemes have been presented, one specific scheme has not yet been widely accepted for the classification of fimbriae. Historically, fimbriae have been classified on the basis of their morphology, their adhesive properties, on biochemical grounds or by the receptors to which they adhere.
Classification of fimbriae on the basis of their morphology has been dependent on recent advances in electron microscopy. Using this technique, fimbriae have been divided into three morphological classes: thin, rigid rods with diameters of about 7 nm (e.g. Type 1, CFA I, 987P, CS1, CS2, Pap and S fimbriae) (Gaastra and De Graaf, 1982; Hacker et al., 1985; Klemm, 1985; Levine et al., 1984; Paranchych and Frost, 1988); thin flexible rods with diameters of about 6 nm (e.g. fimbriae from Pseudomonas aeruginosa, Moraxella spp., Neisseria spp. and Dichelobacter (formerly Bacteroides) nodosus (Paranchych and Frost, 1988; Strom and Lory, 1993); and flexible but very thin rods with diameters of only 2-4 nm (e.g. K88, F41, CS3, SEF14, SEF17 and SEF18) (Klemm, 1985; Low et al., 1995; Paranchych and Frost, 1988).

The adhesive properties of fimbriae have also been used for classification. One of the ways to characterize the adherence properties of fimbriae is by hemagglutination reactions in which bacterial strains expressing specific fimbriae show different patterns of activity with red blood cells of different animal species (Duguid et al., 1955). Since the hemagglutination activity of fimbriae is either sensitive or resistant to inhibition by D-mannose, fimbriae have been divided into two categories: those mediating mannose-sensitive (MS) hemagglutination (e.g. Type 1) and those mediating mannose-resistant hemagglutination (MR) (Duguid and Old, 1980). Unfortunately, not all fimbriae cause hemagglutination and thus are excluded from this classification scheme.

Fimbriae have been also classified on biochemical grounds for example
by their amino acid sequence. Bacteria including *Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella nonliquefaciens, Moraxella bovis, Dichelobacter nodosus, Vibrio cholerae,* and *Pseudomonas aeruginosa* produce fimbrins whose sequence begins with a modified N-terminal amino acid (Strom and Lory, 1993). Other fimbrins have a free N-terminus and have a tyrosine as the penultimate amino acid (Kusters and Gaastra, 1994). However, some fimbrins do not fall into either category and thus are excluded from this classification scheme.

Finally, fimbriae have also been classified by the receptors to which they adhere. For example, P fimbriae from uropathogenic *E. coli* bind to glycolipids which contain the disaccharide α-Gal-(1-4)-β-Gal and are present on human erythrocytes and on epithelial cells of the urinary tract (Bock et al., 1985; Källenius et al., 1980; Leffler and Svanborg-Edén, 1981). S fimbriae adhere to glycoproteins terminating with α-sialic acid-(2, 3)-β-Gal (Korhonen et al., 1984; Parkkinen et al., 1986) whereas K99 fimbriae interact with the carbohydrate portion of the glycolipid hematoside found specifically on horse erythrocytes (De Graaf and Gaastra, 1994). However, the usefulness of this classification scheme is limited by a number of factors including the fact that fimbriae composed of the same major subunit can have different receptor binding specificities (Lund et al., 1988b; Marklund et al., 1992; Strömberg et al., 1990).

Without a classification scheme to keep the large and growing number
of fimbriae categorized, significant relationships between these fimbriae can be missed and/or forgotten. To prevent this confusion, the reader is referred to several extensive reviews that have tabulated the important characteristics of various fimbriae (De Graaf and Gaastra, 1994; Duguid and Old, 1980; Hacker and Morschhäuser, 1994; Kuehn et al., 1994; Strom and Lory, 1993).

2. Fimbrial Structure

A. Composition

Fimbriae are usually composed of a major fimbrial subunit (fimbrin) and several types of minor subunits. Minor subunits are proteins closely related in amino acid sequence to the fimbrins but they are low in abundance in the fimbrial structure. One fimbrial component that has a specialized function is the adhesin (Hanson and Brinton, 1988; Kuehn et al., 1992; Lindberg et al., 1986; Lund et al., 1987; 1988a; Minion et al., 1986; Moch et al., 1987). In a few cases, the adhesin is actually the major subunit, forming the bulk of the fimbrial fiber (Bakker et al., 1992; Bühler et al., 1991; Schifferli et al., 1991a; Willensen and De Graaf, 1993). More often, the adhesins are minor subunits associated with the tips of the fimbriae (Hanson and Brinton, 1988; Kuehn et al., 1992; Lindberg et al., 1986; 1987; Lund et al., 1987; 1988a; Minion et al., 1986; Moch et al., 1987). Other minor proteins are essential for fimbrial biogenesis and will be discussed later.
B. Primary structure of the fimbrins

Features common to all fimbrins include low cysteine and methionine content and a low percentage of basic and aromatic residues (Collinson et al., 1991). In addition, all fimbrins have an N-terminal signal sequence composed of polar uncharged or hydrophobic residues, although the specific sequence and the number of residues vary considerably. The primary amino acid sequence of the major fimbrial subunits are the basis for a recently proposed classification scheme which divides fimbriae from *E. coli* and *Salmonella* into seven classes (Low et al., 1995). Fimbrins from other bacteria also fall into one of these seven classes.

Fimbrins of class 1 [P (Båga et al., 1984; Rhen et al., 1985; Van Die and Bergmans, 1984; Van Die et al., 1986), S (Schmoll et al., 1987), Type 1 (Klemm, 1984; Orndorff and Falkow, 1985), F17 (Lintemans et al., 1988), K99 (Roosendaal et al., 1984), F107 (Imberechts et al., 1992), 987P (Isaacson and Richter, 1981) and Type 3 (Gerlach et al., 1988)] and class 2 [F1845 (Bilge et al., 1989) and 075X (Swanson et al., 1991)] have two cysteine residues (Low et al., 1995) which form a cys-cys bridge in the native molecules (Jann et al., 1981). The difference between the two classes of fimbrins is the spacing between the two cysteine residues: 38-43 amino acids in class 1 and 31 amino acids in class 2. Other residues conserved in the two classes of fimbrins include a phenylalanine residue located between the two cysteines and a tyrosine residue located 2 or 4 amino acids from the C-terminus in class 1 and 2, respectively (Low et al., 1995). The role of the conserved phe is unknown but
the penultimate tyrosine is essential for the expression of subunits that are conformationally stable and capable of interacting with the periplasmic carrier protein (Simons et al., 1990a). Furthermore, the C-terminus of PapG, the tip adhesin of Pap pili, is essential in forming a preassembly complex consisting of PapG and PapD, the periplasmic chaperone (Hultgren et al., 1989). This interaction, which is necessary for the proper assembly of the fimbriae, prevents proteolytic degradation (Hultgren et al., 1989) and nonproductive collisions of interactive subunits (Kuehn et al., 1991). The different positions of the tyrosine may be due to differences in the interaction between the subunit and the respective periplasmic chaperones.

The third class of fimbrins [K88 (Dykes et al., 1985; Gaastra et al., 1981), CS31A (Korth et al., 1991) and F41 (Anderson and Moseley, 1988)] lack the central cysteine residues although they do have the penultimate tyrosine at their C-termini (Girardeau et al., 1991; Low et al., 1995). In addition, these fimbrins have in common 15 amino acids in their leader sequences and 4 proline residues in the mature fimbrin sequence. The conserved prolines are located within or immediately adjacent to hydrophobic domains that are supposed to form a common hydrophobic core in each of the three fimbrins (Girardeau et al., 1991). These hydrophobic amino acid clusters associated with proline may have a similar function to that of disulfide bridges for they can maintain the local folding and the structural integrity of the molecules (Girardeau et al., 1991).

The fourth class are fimbrins of type IV fimbriae, also collectively called
N-methylphenylalanine (NMePhe) fimbriae. Members of this class have highly conserved N-termini and N-methylated amino acids (phenylalanine, methionine, leucine or serine) as the first amino acid of the mature fimbrin (Kaufman and Taylor, 1994; Strom and Lory, 1993; Tennent and Mattick, 1994). The type IV class can be further subdivided into two groups. Group A consists of fimbrins from Pseudomonas aeruginosa (Finlay et al., 1986; Johnson et al., 1986; Strom and Lory, 1986), Neisseria gonorrhoeae (Meyer et al., 1984), Neisseria meningitidis (Tonjum et al., 1993), Moraxella bovis (Marrs et al., 1985), Moraxella nonliquefaciens (Tonjum et al., 1991), Moraxella lacunata (Marrs et al., 1990), Dichelobacter nodosus (Elleman and Hoyne, 1984; McKern et al., 1983), Branhamella catarrhalis (Marrs and Weir, 1990) and Eikonella correndens (Rao and Progulske-Fox, 1993; Tonjum et al., 1993). The fimbrin precursor has a short positively charged leader sequence, either 6-8 amino acids long, that is cleaved between an invariant glycine and phenylalanine prior to assembly into fimbriae (Nunn and Lory, 1991). Substitution of serine for glycine at position -1 of the profimbrin abolishes proteolytic processing and results in a nonfimbriated phenotype (Koomey et al., 1991). Site-directed mutagenesis has established that a variety of hydrophobic amino acids can be tolerated at the N-terminal position occupied by the methylated phenylalanine (Strom and Lory, 1991; 1992).

The mature fimbrin is divided into three regions. The highly conserved N-terminal region is hydrophobic and contains an invariant glutamic acid located five amino acids from the N-terminus. The glutamate
at +5 is essential for assembly and efficient methylation but dispensible for cleavage (Koomey et al., 1991; MacDonald et al., 1993; Paslocke and Paranchych, 1988; Strom and Lory, 1991; 1992). In addition, two tyrosine residues in the hydrophobic N-terminus are conserved in all type IV fimbrins of group A and are at the subunit/subunit interface in both native fimbriae and in reassembled fimbriae filaments (Watts and Kay, 1982; Watts et al., 1983). The central region is variable whereas the C-terminal region contains a pair of conserved cysteines that form a disulfide loop (Sastry et al., 1985; Schoolnik et al., 1984). The C-terminal region of the Pseudomonas fimbrin is exposed at the tip of the fimbrial strand and is associated with the binding of Pseudomonas fimbriae to glycosphingolipid receptors (Lee et al., 1994; Sheth et al., 1994). Thus, the C-terminal region is the receptor binding domain.

Group B of this class consists of fimbrins from Vibrio cholerae (TcpA) (Faast et al., 1989), enteropathogenic E. coli (BfpA) (Donnenberg et al., 1992; Girón et al., 1991; Sohel et al., 1993) and enterotoxigenic E. coli (LmgA) (Girón et al., 1994). The fimbrin precursors of this class have longer leader peptides than those in group A: 25 and 13 amino acids for preTcpA and preBfpA, respectively (Donnenberg et al., 1992; Faast et al., 1989; Sohel et al., 1993; Strom and Lory, 1993). Since the lngA gene has not yet been sequenced, the length of the leader peptide is not known. As in the case with group A members, the signal peptides of both TcpA (Faast et al., 1989) and BfpA (Donnenberg et al., 1992; Sohel et al., 1993) end in glycine. However, unlike the invariant NMePhe in group A, the first amino acid of the mature fimbrins in group B is
N-methyl methionine (TcpA), a modified leucine (BfpA) or a modified serine (LngA). The modification of the leucine and serine are unknown. Members of group B have a conserved N-terminal region, a variable central domain and a C-terminal region that contains a pair of cysteines that may form an intrachain disulfide bond.

The fifth class are fimbrins from CFA I and CS1 fimbriae of enterotoxigenic \textit{E. coli} (Low et al., 1995). At the amino acid level, there is 92% similarity and 55% identity between the predicted sequences of these two proteins (Perez-Casal et al., 1990). Unlike fimbrins of class 1, 2 and 3, class 5 fimbrins lack the cysteines and the C-terminal tyrosine residues (Perez-Casal et al., 1990).

Class 6 fimbrins are from curli and SEF17 fimbriae from \textit{E. coli} (Arnqvist et al., 1992) and \textit{S. enteritidis} (Collinson et al., 1991), respectively (Low et al., 1995). The \textit{agfA} fimbrin gene is present in other \textit{Salmonella} isolates as well as \textit{E. coli}, \textit{Citrobacter} spp., \textit{Shigella sonnei} and \textit{Enterobacter cloacae} (Doran et al., 1993a). These fimbrins have similar total amino acid compositions in that the percentages of basic, potentially acidic, hydrophobic, aromatic and polar uncharged amino acidss are comparable (Collinson et al., 1992). These fimbrins also have an unusual abundance (36-47%) of the small amino acids serine, glycine and alanine. In addition, the fimbrins that have been sequenced have highly conserved N-terminal amino acid sequences that start with GVVPQ (Collinson et al., 1992). Thus, the class 6 fimbrins are also known as the GVVPQ class.
The sole members of the class 7 and class 8 fimbrins come from the SEF14 (Clouthier et al., 1993) and SEF18 (Clouthier et al., 1994) fimbriae, respectively, of *S. enteritidis*. These fimbrins have no homology to any other known fimbrins, thereby forming two separate classes.

C. **2° and 3° structure of the fimbriae**

Two general types of fimbrial structures have been reported: thick, rigid fibres and thin, flexible fibres. A combination of X-ray fiber diffraction and electron microscopy has shown that the helical symmetry of the rigid type 1 fimbriae from *E. coli* is 3.125 subunits per turn of a 23.2 Å pitch helix (Brinton, 1965). These results compare well with the 3.3 subunits per turn of a 24.45 Å pitch helix obtained for Pap fimbriae which are also thick fibres (Gong and Makowski, 1992). Further analysis of the fiber diffraction data shows that the type 1 and Pap fimbrial subunits are tightly packed in a right-handed α-helix and separated by an 8 Å and a 7.42 Å axial rise, respectively (Brinton, 1965; Gong and Makowski, 1992). STEM (scanning transmission electron microscopy) indicates that both type 1 and Pap fimbriae are about 65 Å in diameter with a small central cavity 15 Å across (Gong and Makowski, 1992). Freeze etch EM and STEM images of Pap fimbriae reveal a thin, open-helical fiber extending from the end of each fimbria (Gong and Makowski, 1992; Kuehn et al., 1992). These tip structures are composed of four minor fimbrial proteins (PapK-PapE-PapF-PapG). PapE forms the linear polymer that ends
with PapG, the adhesin (Kuehn et al., 1992). Thus, Pap fimbriae are composites of thin and thick fimbriae.

NMePhe or type IV fimbriae are also thick fibers whose structure has been examined by X-ray diffraction. These studies have shown that fimbriae from P. aeruginosa strains PAK and PAO consist of 5.06-5.08 subunits per 41-Å turn of helix (Watts et al., 1983). The crystallization of N. gonorrhoeae fimbriae and the subsequent X-ray diffraction analysis has led to the proposal that each fimbrin folds into an antiparallel 4-α-helix bundle (Parge et al., 1987; 1990). Like type 1 and Pap fimbriae, both the P. aeruginosa and N. gonorrhoeae fimbriae have the overall appearance of a cylinder with a central channel.

The three dimensional structure of thin, flexible fimbriae such as K88, K99, CS3, SEF14, SEF17 and SEF18 has not yet been reported. However, EM studies show that these fimbriae are extended and that they lack a central channel (Clouthier et al., 1993; 1994; Collinson et al., 1991; Isaacson, 1977; Levine et al., 1984; Stirm et al., 1967). Their structure resembles that of the tip fiber located at the ends of Pap fimbriae.

Further biochemical analysis of all fimbriae indicate that the structural components of fimbriae are not covalently linked but are held together by hydrophilic and hydrophobic bonds to form a very stable structure. These structures are so stable that in some cases boiling at low pH (McMicheal and Ou, 1979) or treatment with 90% formic acid (Collinson et al., 1991) is required to promote depolymerization.
3. Components involved in fimbrial biosynthesis and assembly

The biosynthesis of fimbriae requires the transport of fimbrial subunits across the cytoplasmic membrane, the periplasm and the outer membrane; the polymerization of fimbrial subunits at the cellular surface; and the anchoring of fimbriae to the cell envelope. Transport of the fimbrial subunits across the cytoplasmic membrane to the periplasm occurs in a Sec-dependent manner (Dodd et al., 1984). In this pathway, the cytosolic molecular chaperone SecB maintains the translocation competence of preproteins in the cytosol and targets them to the cytoplasmic membrane (Kumamoto, 1991) where a complex consisting of SecA (Oliver, 1993), SecD (Matsuyama et al., 1993), SecE (Tokuda et al., 1991), SecF (Sagara et al., 1994) and SecY (Nishiyama et al., 1991) assists in preprotein insertion and translocation across the inner membrane. SecA, the central protein in this pathway, is conserved among eubacteria suggesting that a SecA-dependent export system is common to all prokaryotes (Sadaie et al., 1991; Takamatsu et al., 1992).

A. Chaperone

Transport of K88, K99 and Pap fimbrial subunits across the periplasm is accomplished by a periplasmic chaperone protein (Bakker et al., 1991; Kuehn et al., 1991; Lindberg et al., 1989). Furthermore, all well characterized fimbrial operons include a gene encoding a protein which is a member of the fimbrial periplasmic chaperone family (Holmgren et al., 1992; Van Rosmalen and Saier, 1994). The role of this protein is to bind to interactive assembly surfaces
on its fimbirin protein target to prevent nonproductive aggregation or polymerization of fimbirins in the periplasm (Bakker et al., 1991; Kuehn et al., 1991). When the chaperone is bound to the fimbirin, aggregation is prevented whereas its release results in polymerization of the fimbrial rod (Kuehn et al., 1991). The chaperone also functions to stabilize the fimbrial subunits, for, in the absence of a chaperone, the fimbrial subunits are rapidly degraded in the periplasm (Bakker et al., 1991; De Graaf and Klaasen, 1986; De Graaf et al., 1984; Klemm et al., 1985; Lindberg et al., 1989; Orndorff and Falkow, 1984a). Unlike the general cytoplasmic chaperones, the periplasmic fimbrial chaperones seem to maintain their substrates in a folded, native-like state (Hultgren et al., 1989; Kuehn et al., 1991). In addition, the binding of the chaperone to the fimbirin is reversible and the release mechanism is seemingly ATP independent (Kuehn et al., 1991).

The X-ray crystallographic structure of PapD, the periplasmic chaperone protein of Pap fimbriae shows that PapD consists of two globular domains oriented in a boomerang shape such that a cleft is formed between the two domains (Fig. 1) (Holmgren et al., 1988; Holmgren and Bränden, 1989). Each domain is a β-barrel structure formed by two antiparallel β-sheets connected by a flexible loop region giving this protein a topology similar to an immunoglobulin fold (Fig. 1) (Holmgren and Bränden, 1989). If the periplasmic chaperone proteins are evolutionarily related to the immunoglobulins, then the antigen binding fold of the immunoglobulins may correspond to the cleft in the chaperone proteins (Holmgren et al., 1992).
An analysis of site-directed mutations in solvent-exposed cleft residues reveals that the cleft region between the two domains forms the fimbrin binding pocket (Slonim et al., 1992). In addition, the crystal structure of PapD complexed with a C-terminal peptide of PapG shows that the peptide is anchored within the cleft by hydrogen bonds (Hultgren et al., 1993). Point mutations that abolish the PapD-peptide interactions also abolish the ability of PapD to bind subunits in vitro further demonstrating the function of the cleft in subunit binding (Hultgren et al., 1993). PapD can interact with PapA, PapH, PapK, PapE, PapF and PapG, fimbrin proteins which share a similar C-terminal sequence. The chaperone may differentially accommodate the fimbrin subunit side chains in its cleft, resulting in different affinities between PapD and the related fimbrial proteins. These differences in affinity may assist in the ordered biogenesis of the composite Pap fimbriae (Slonim et
Detailed sequence comparison of eleven putative periplasmic chaperone proteins reveals that all of these proteins possess the overall topology of an immunoglobulin fold (Holmgren et al., 1992; Van Rosmalen and Saier, 1994). Most of the conserved residues are within the β-strands and are critical to maintaining the structural integrity of the protein. One group of invariant residues contributes to the hydrophobic core whereas another group of conserved residues form an internal salt bridge necessary to orient the two domains toward each other to form the binding cleft. A third group of invariant residues are critical in positioning the orienting loop structures which link the β-strands (Holmgren et al., 1992). The variable regions occur primarily in the loops connecting the β-strands as well as in the flexible linker which connects the two domains (Holmgren et al., 1992; Van Rosmalen and Saier, 1994).

**B. Usher**

Transport of the fimbrial subunits across the outer membrane is accomplished by a large outer membrane protein that is also encoded by all well characterized fimbrial operons (Allen et al., 1991; Dodson et al., 1993; Klemm and Christiansen, 1990; Mooi et al., 1986; Roosendaal and De Graaf, 1989; Schmoll et al., 1990a; Van Rosmalen and Saier, 1994). Expression of this outer membrane protein is required for fimbriae production on the cell
surface. Mutations in these proteins have no effect on the amount of the fimbrial subunit present in cell extracts but do result in a bald phenotype (De Graaf et al., 1984; De Graaf and Klaasen, 1986; Klemm et al., 1985; Klemm and Christiansen, 1990; Mooi et al., 1982; 1983; Norgren et al., 1987; Orndorff and Falkow, 1984a). In addition, overproduction of these outer membrane proteins affects cellular permeability to exogenous substances and leads to cell death suggesting that these proteins form pores through which fimbrial subunits are able to pass (Klemm and Christiansen, 1990; Norgren et al., 1987).

In vitro studies with partially purified PapC from the Pap fimbrial operon have shown that this outer membrane protein not only assists in the transport of protein across the outer membrane but also facilitates the polymerization and assembly of the fimbrial subunits into mature fimbriae (Dodson et al., 1993). Thus, the outer membrane protein appears to be a passive channel that also has an active role in determining the order of the fimbrial subunit passage. This ordering function has been referred to as that of an usher (Dodson et al., 1993).

Detailed sequence comparison of eleven putative outer membrane usher proteins show that the N-terminal third of these proteins exhibit the largest degree of sequence similarity. The central third of these proteins are the least conserved (Van Rosmalen and Saier, 1994). These findings suggest that the N-terminal third of the usher proteins are more important to structure and function than are the central and C-terminal domains (Van Rosmalen and Saier, 1994).
The usher proteins are relatively hydrophilic consisting mainly of amphipathic β-strands, β-turns and loops (Van Rosmalen and Saier, 1994). In addition, the membrane spanning domains appear to adopt a β-barrel structure, with joining surface-exposed β-turns or loops of various lengths (Schifferli and Alrutz, 1994). The proposed β-barrel structure is expected to be essential for structural stability in the membrane. In support of this model, β-turn-inducing linker insertions which target the predicted β-sheets are nonpermissive whereas linker insertions at the predicted turns or at the junctions of the predicted β-strands and turns are permissive (Schifferli and Alrutz, 1994).

C. Minor subunits

All fimbrial gene clusters characterized to date encode proteins similar in size and sequence to chaperones and ushers. Thus, all fimbriae seem to be exported by an identical pathway. However, some fimbrial gene clusters encode minor fimbrial proteins that also seem to be involved in fimbrial biogenesis. In the case of Pap fimbriae, PapF and PapK are minor components of the tip fiber and are essential as initiators of polymerization and as adaptor proteins (Jacob-Dubuisson et al., 1993). PapF is required to initiate tip fiber assembly and to correctly present the PapG adhesin so that it can mediate receptor binding. In turn PapK terminates tip fiber growth and initiates the formation of the fimbrial shaft (Jacob-Dubuisson et al., 1993). Another minor, fimbrin-like protein, PapH, is required to anchor each fimbria to the cell and
to modulate fimbrial length (Båga et al., 1987). Finally, PapJ, which contains a potential nucleotide-binding site, is a periplasmic protein required to maintain fimbriae integrity (Tennent et al., 1990). It may act as a chaperone-like protein that ensures the proper assembly of heteropolymeric Pap fimbriae perhaps by energizing components of the fimbrial biogenesis pathway (Tennent et al., 1990). All of these proteins, with the exception of PapJ, are minor components of the Pap fimbriae. Similar proteins have been identified in other systems. For instance, FaeC initiates assembly of K88 fimbriae (Oudega et al., 1989). FanF, FanG and FanH control the length, the initiation and the elongation, respectively, of K99 fimbriae (Simons et al., 1990b; 1991) while FimF and FimG initiate and terminate, respectively, type 1 fimbrial assembly (Russell and Orndorff, 1992). Thus, in spite of variations in fimbrial structure, fimbriae assembly in gram-negative bacteria seems to require proteins with similar functions and structures.

D. Models for fimbriae assembly

Fimbrial subunits are initially synthesized as signal sequence-containing precursors which are processed into mature proteins. The mature subunits are added to the base of the growing fimbriae close to the surface of the outer membrane (Lowe et al., 1987).

Using the Pap system, a general model for fimbrial assembly has been proposed in which the differential affinities of the various fimbrial proteins for PapC and PapD, the relative abundance of each of the subunit proteins and
the complementary surfaces on each subunit type are all factors that influence the ordered assembly of these fimbriae (Fig. 2) (Hultgren et al., 1993). Initially, the complexes that PapD forms with each of the three most distal tip subunits (PapG, PapF and PapE) bind PapC specifically in vitro. However, the complexes between PapD and the most proximal tip subunit (PapK) and the major rod subunit (PapA) do not bind PapC (Dodson et al., 1993). This binding specificity ensures that the tip fiber is assembled before the fimbrial rod (Fig. 2) (Dodson et al., 1993; Hultgren et al., 1993). Thus, PapD-PapG, which has the highest affinity for PapC, binds to PapC first thereby ensuring PapG's localization at the distal end of the pilus tip. The subsequent binding
of PapD-PapF to PapC initiates tip growth and provides the complementary surfaces capable of linking PapG to PapE (Jacob-Dubuisson et al., 1993). PapE subunits then polymerize into the tip fiber upon multiple rounds of PapD-PapE binding and PapE encorporation. Since PapD-PapK and PapD-PapA are unable to bind to empty PapC sites, the fimbrial rods cannot be made in the absence of the tip fiber. The binding site for PapD-PapK seems to be the polymerized tip in the context of PapC. The incorporation of PapK terminates the growth of the tip and seems to create a binding site for PapD-PapA (Fig. 2) (Dodson et al., 1993). The targeting of PapD-PapA complexes to PapC allows polymerization of the fimbrial rods (Dodson et al., 1993). Due to differences in fimbrial structure, variations on this general assembly scheme have been worked out for other fimbrial systems (Fig. 3) (Klemm and Krogfelt, 1994; Simons et al., 1991).

Fimbriae of the type IV group A class are assembled by a mechanism different from that just described. This is exemplified by a study showing that expression of *D. nodosus* fimbrin in *E. coli* results in the association of the fimbrin with the inner membrane of *E. coli* but no surface fimbriae (Elleman et al., 1986a). However, expression of *D. nodosus*, *M. bovis* and *N. gonorrhoeae* fimbrin genes in *P. aeruginosa* results in the formation of fimbriae in the heterologous host, suggesting that the basic machinery involved in the biogenesis of the type IV group A fimbriae is conserved (Beard et al., 1990; Elleman et al., 1986b; 1990; Hoyne et al., 1992). Similar heterologous expression of type IV group B fimbriae (Tcp, Bfp or Lng) by those
Fig. 3. A. Model for type 1 fimbriae biogenesis. The structural and transport/assembly components encoded by the type 1 fimbriae operon are translocated across the cytoplasmic membrane via the normal Sec export pathway. Fimbrial subunits complex with the chaperone, FimC, in the periplasm and are transported to FimD, the assembly platform. FimF, FimG and FimH are inserted first followed by the major subunit FimA whose incorporation is interspersed with complexes of FimF, FimG and FimH (Klemm and Krogfelt, 1994). B. Model for K99 fimbriae biogenesis. FanF initially recognizes FanD, the assembly platform, followed by FanG, a minor subunit and FanC, the major fimbrial subunit. This process is repeated with FanH being incorporated to form a link between FanC and FanF. Although not shown, translocation across the periplasm requires the chaperone FanE which protects FanC, F, G and H against proteolytic degradation (Simons et al., 1991). These fimbriae (K99 and type 1) do not have the distinct adhesive tip fibers observed on the Pap fimbriae. Instead, adhesion occurs both at the tip and laterally.

bacteria that express type IV group A fimbriae has not been reported. However, products of the tcp gene cluster show sequence similarity with the biogenesis proteins of P. aeruginosa suggesting that the overall mechanism of type IV biogenesis may be conserved (Kaufman et al., 1993) For this reason, only the genes involved in biogenesis of P. aeruginosa fimbriae will be discussed further.

Four gene products are involved in the biogenesis of P. aeruginosa fimbriae: PilB, PilC, PilD (Koga et al., 1993; Nunn et al., 1990) and PilQ (Martin
et al., 1993). Although mutations in any one of the three genes results in the absence of fimbriae on the cell surface, each mutant synthesizes the fimbrial subunit, PilA, at a level comparable with that produced in the wild-type bacteria (Nunn et al., 1990). PilB contains a consensus nucleotide binding sequence (Whitchurch et al., 1991), GlyXXXXGlyLys(Thr), common to many prokaryotic nucleotide-binding proteins (Walker et al., 1982). Thus, PilB may be a cytoplasmic nucleotide binding protein that supplies energy for subunit translocation or assembly (Koga et al., 1993; Whitchurch et al., 1991). PilC appears to be an integral inner membrane protein, the function of which is not known but one possibility is that it provides an assembly platform for the fimbrial strand (Fig. 4) (Hobbs and Mattick, 1993; Nunn et al., 1990). Similarly, PilQ is an outer membrane protein whose function is unknown but necessary for type IV fimbrial biogenesis (Martin et al., 1993). Finally, PilD is an inner membrane protein which cleaves the leader sequence (Nunn and Lory, 1991) and catalyzes the N-methylation of the N-terminal residue in the P. aeruginosa fimbrin (Fig. 4) (Strom et al., 1993). Unlike PilB and PilC whose function is restricted to fimbrial biogenesis, PilD has an additional role in the secretion of proteins that are released from P. aeruginosa into the surrounding media (Nunn and Lory, 1992; Strom et al., 1991). A tentative model for type IV fimbrial biogenesis has been proposed (Fig. 4) (Tennent and Mattick, 1994). The secretion of the type IV fimbrin across the cytoplasmic membrane is highly specific and involves PilD, the signal peptidase and possibly the inner membrane protein PilC. Post-secretional folding may be
achieved by an unidentified chaperone. Fimbrin assembly and translocation across the outer membrane likely involves PilQ, the large outer membrane protein (Fig. 4). Whether type IV fimbriae are homopolymers or heteropolymers with associated minor subunit proteins is not yet resolved (Tennent and Mattick, 1994).

Fig. 4. Model for type IV fimbriae biogenesis. Details are given in the text (Hobbs and Mattick, 1993).

4. Genetic organization of fimbrial gene clusters

The structure, biosynthesis, assembly and regulation of a given fimbrial type requires several different proteins. All the fimbrial operons of Enterobacteriaceae members encode proteins that make up the bulk of the fimbrial structure (major and minor subunits) as well as proteins that
determine the binding specificity of the fimbriae (adhesins). Furthermore, they contain proteins that are essential for the assembly of the fimbriae (chaperone and usher). Finally, they contain proteins that modulate expression of the fimbrial system (Fig. 5). The fimbrial genes which encode these proteins are generally clustered in large, 7-9 kbp gene clusters as exemplified by the *pap* operon (9 kb) in uropathogenic *E. coli* (165), the *fue* and *fan* operons which encode the K88ab (8 kb) and K99 (7 kb) fimbriae from porcine and bovine enterotoxigenic *E. coli*, respectively, the *fim* operon (9 kb) which encodes the type 1 fimbriae of *E. coli*, the *sfa* operon (8 kb) which
encodes the S fimbriae of E. coli (Schmoll et al., 1990a), the fap operon which encodes the 987P fimbriae of enterotoxigenic E. coli (De Graaf and Klaasen, 1986; Schifferli et al., 1991b) and the mrk operon (7 kb) which encodes the type 3 fimbriae of Klebsiella pneumoniae (Fig. 5) (Allen et al., 1991). However, in some cases such as the coo (CS1), cfa (CFA/I) and agg (AAF/I) gene clusters (Nataro et al., 1993; Smith et al., 1982; Willshaw et al., 1983), the genes involved in fimbrial biosynthesis and regulation are physically separated. For the cfa and agg systems, two regions of a single plasmid are required: region 1 contains the structural gene for the major fimbrin (Nataro et al., 1993; Willshaw et al., 1985); region 2 contains the regulatory gene, cfaR/cfaD (CFA/I) (Caron and Scott, 1990; Savelkoul et al., 1990) or aggR (AAF/I) (Fig. 5) (Nataro et al., 1994). In the case of the coo system, the structural gene for the major CS1 antigen, CooA, is located on a plasmid different from the one encoding the positive regulatory protein Rns (Caron et al., 1989; Perez-Casal et al., 1990)

Although the genes responsible for the biogenesis of E. coli fimbriae are clustered near the structural subunit genes, the type IV structural subunit genes are often surrounded by genes that do not encode biogenesis functions with the exception of the genes required for Tcp assembly. Even among themselves, these systems lack organizational similarity (Fig. 6). In P. aeruginosa, the fimbrial subunit gene, pilA, is located upstream and in the opposite transcriptional orientation of pilB, C and D which are required for fimbrial biogenesis (Hobbs et al., 1988; Nunn et al., 1990). About 25 kbp
downstream of \( pilA-D \) are located the genes \( pilS \) and \( pilR \) which encode the two-component sensor-regulator system that controls transcription of \( pilA \) (Fig. 6) (Hobbs et al., 1993). PilQ is encoded by a gene located elsewhere on the chromosome (Martin et al., 1993)

**Fig. 6.** Genetic organization of type IV fimbrial operons. Arrows indicate the extent and direction of the coding regions. Fimbrial subunit genes are filled with black; other homologue sets are filled with matching patterns. Unfilled arrows indicate genes with no other homologues in the figure. Details are given in the text (Tennent and Mattick, 1994).

In \( D. \ nodosus \), there are two classes of genomic arrangement associated with the two classes of fimbrial subunit (Fig. 6) (Hobbs et al., 1991; Mattick et al., 1991). In both classes, the fimbrial subunit gene, \( fimA \), is preceded by \( aroA \)
and followed by a termination signal (Hobbs et al., 1991). In class I strains, the remainder of the operon contains one gene, *fimB*, whereas in class II strains there are three genes, *fimC*, *D* and *Z* (Fig. 6) (Hobbs et al., 1991). *FimZ* is a duplicate fimbrial subunit that is homologous to *FimA* whereas *FimB*, *C* and *D* are membrane proteins of unknown function (Hobbs et al., 1991).

The genetic organizations for other type IV fimbriae are different again. In *M. bovis*, two partial fimbrial subunit genes, *tfpQ* and *tfpI*, are located on an invertible segment of DNA, which alternates orientation relative to an external promoter and translation initiation/N-terminal coding sequences (Marrs et al., 1988). *TfpQ* and *tfpI* are separated by the gene *tfpB* and the invertible segment is followed on the promoter side by the gene *pivML* which encodes the invertase (Fig. 6) (Fulks et al., 1990; Lenich and Glasgow, 1994). In *E. corrodens*, two tandemly arranged fimbrial subunit genes, *ecpA* and *ecpB*, are followed by a transcription termination signal (Fig. 6) (Rao and Progulske-Fox, 1993; Tønjum et al., 1993). Finally, in *N. gonorrhoeae*, a number of partial fimbrial subunit genes designated *pilS* represent silent loci which function as reservoirs of structural and antigenic variants. These loci can be exchanged into the expression locus, *pilE*, by nonreciprocal recombination (Haas and Meyer, 1986; Segal et al., 1985; Seifert et al., 1988). The genes, *pilA* and *pilB*, found downstream of *pilE*, encode proteins which modulate the level of *pilE* expression (Fig. 6) (Taha et al., 1988; 1991). Because the ancillary genes encoding the proteins required for type IV fimbrial biogenesis are scattered in different parts of the chromosome, many of them
have not yet been found. Consequently, the actual mechanism of type IV fimbrial biogenesis remains unclear.

5. Regulation

A. Transcriptional regulation

a. Transcriptional regulatory proteins

i. Lrp

Lrp is a global regulatory protein that positively regulates operons necessary in famine conditions but negatively regulates those which are necessary in rich environments (Calvo and Matthews, 1994; Newman et al., 1992). In some cases, the activity of Lrp is either positively or negatively affected by leucine whereas in other cases, leucine has no effect on Lrp activity. The Lrp-regulated fimbrial operons include the fae, sfa, daa and pap gene clusters which are leucine-non-responsive (Braaten et al., 1992; Hulsman et al., 1994; Van Der Woude and Low, 1994) as well as the fim and fan operons which are leucine-responsive (Braaten et al., 1992; Gally et al., 1993). Lrp activates transcription from fan, pap, daa, fim and sfa genes but represses transcription from fae genes. The negative effect of Lrp on fae transcription may be due to an additional GATC box near the RNA polymerase binding site. The binding of Lrp and the fae regulatory protein, FaeA, to this region prevents transcription initiation and/or Dam methylation of the GATC sites (Hulsman et al., 1994).

Exogenous leucine stimulates fim inversion and type 1 fimbriae
expression (Gally et al., 1993) but reduces \textit{fan} transcription and K99 fimbriae expression (Braaten et al., 1992). Unlike the other Lrp regulated fimbrial operons, the \textit{fan} operon lacks both a \textit{papI}-like regulatory gene as well as GATC box regions. Instead, the \textit{fanA} and \textit{fanB} regulatory genes both share DNA sequence similarities with \textit{papB} (Roosendaal et al., 1987) and encode proteins that stimulate \textit{fan} transcription by a Lrp-independent antitermination mechanism (Braaten et al., 1992). Thus, although \textit{fan} transcription, like \textit{pap}, \textit{sfa} and \textit{daa} transcription, is positively regulated by Lrp, Lrp regulates the \textit{fan} operon and the \textit{sfa}, \textit{daa} and \textit{pap} operons by different mechanisms.

\textit{ii. Members of the AraC family of transcriptional regulators}

Expression of the CFA/I and CFA/II fimbrial colonization factor antigens as well as the 987P and AAF/I fimbriae is regulated by the proteins CfaD/CfaR (Caron and Scott, 1990; Savelkoul et al., 1990), Rns (Caron et al., 1989), FapR (Klaasen and De Graaf, 1990) and AggR (Nataro et al., 1994), respectively. These proteins share amino acid sequence similarity with each other as well as with the DNA binding domain of AraC, the transcriptional regulator of the \textit{E. coli} arabinose operon (Gallegos et al., 1993). Consequently, these proteins belong to the AraC family of transcriptional regulators. In each case, expression of the fimbrial operon is activated by its regulatory protein (Caron and Scott, 1990; Klaasen and De Graaf, 1990; Nataro et al., 1994; Savelkoul et al., 1990). The mechanism of \textit{cfa} activation by CfaD/CfaR
involves removing the repressive effect mediated by H-NS. Whether CfaD binds to the DNA to diminish the effect of H-NS is unknown (Jordi et al., 1992). However, if DNA binding occurs, then the fimbrial activator could cause alterations in the local DNA conformation thereby counteracting the silencing by H-NS. The other positive regulators, homologous with CfaD, may have the same mode of action.

iii. PapB-like and PapI-like proteins

The \textit{papB} and \textit{papI} genes encode trans-acting proteins that stimulate transcription of the Pap fimbrial operon (Båga et al., 1985). In the 400 bp intergenic region between \textit{papI} and \textit{papB}, there are two major transcription starts sites denoted \( p_I \) and \( p_{BA} \). PapB binds to two different sites within the \textit{papI-papB} intergenic region. The highest-affinity binding site is 60 bp upstream of the \( p_I \) transcription start site and the lower-affinity site overlaps the -10 site of the \( p_{BA} \) promoter (Forsman et al., 1989). The binding of PapB to the high-affinity site enhances \textit{pap} transcription indirectly by stimulating transcription initiation at \( p_I \) (Fig. 7). The subsequent expression of PapI helps initiate \textit{pap} transcription by interacting with the Lrp, PapB and CRP regulatory proteins and RNA polymerase all bound to the \textit{pap} regulatory region (Fig. 7) (Van Der Woude et al., 1992).

The \textit{sfa} (Schmoll et al., 1990a), \textit{daa} (Bilge et al., 1993b) and \textit{fae} (Hulsman et al., 1994) operons express regulatory proteins with amino acid sequence
similarities to PapI and PapB. Expression of the K99 determinant also involves two promoters and two trans-acting products FanA and FanB which share sequence similarity with each other and with PapB (Roosendaal et al., 1987). The expression of the sfa and daa operons are regulated by a mechanism which is similar to the mechanism controlling expression of the pap operon (Van Der Woude and Low, 1994). In contrast, expression of the fae and fan operons seem to be controlled by more complex mechanisms. In the fae operon, the presence of two IS1 elements in the regulatory region affects the activity of the FaeA (PapI-like) and FaeB (PapB-like) regulatory proteins (Hulsman et al., 1994). FaeA has a negative effect and FaeB has a positive effect on transcription of the fae operon (Hulsman et al., 1994). In contrast, FanA and FanB both exert a positive effect on the fan operon expression possibly by acting in trans as anti-terminators at the two terminators T\textsubscript{1} and T\textsubscript{2} (De Graaf, 1988; Roosendaal et al., 1987; 1989).

iv. cAMP-CRP

The cAMP receptor protein (CRP) complex (cAMP-CRP) is a global regulator that influences the transcription of several fimbriae operons in response to the level of glucose. High levels of glucose repress the synthesis of K99 (Isaacson, 1980), CFA/I (Karjalainen et al., 1991a), CFA/II (Evans et al., 1991) and Pap (Bága et al., 1985) fimbriae. The effect of catabolite repression on K99 fimbriae production is dependent on the host strain and is restricted to
the synthesis of fimbrial subunits. The assembly of these fimbriae is not affected (Girardeau et al., 1982; Isaacson, 1983). In the case of the pap operon, cAMP-CRP has been shown to bind adjacent to the high-affinity PapB-binding site and activate transcription from the $p_I$ and $p_{BA}$ promoters (Båga et al., 1985; Göransson et al., 1989a). The binding of the cAMP-CRP complex may alter the local DNA conformation which alleviates the transcriptional silencing mediated by H-NS (Forsman et al., 1992). Thus, cAMP-CRP acts as an anti-repressor of pap operon transcription (Fig. 7).

b. Phase variation
   
   i. Inversion-dependent phase variation

Expression of the E. coli type 1 fimbriae is transcriptionally regulated by a phase variable mechanism (Brinton, 1959; Eisenstein, 1981) involving inversion of a 314-bp DNA segment that carries the promoter for fimA the gene coding for the major subunit of type 1 fimbriae (Abraham et al., 1985). Inversion of this DNA segment by site-specific recombination is controlled, in part, by the products of the fimB and fimE (hyp) genes which encode proteins with sequence similarity to site-specific recombinases (Eisenstein et al., 1987; Klemm, 1986; Orndorff and Falkow, 1984b). FimE promotes inversion of the 314-bp segment to the 'OFF' orientation whereas FimB promotes inversion in either direction (Klemm, 1986; McClain et al., 1991; 1993; Pallesen et al., 1989).

In addition to FimB and FimE, four other elements, not encoded by the
fim gene cluster, affect the phase variation of type 1 fimbriae. The first element is the integration host factor (IHF), a histone-like protein which induces a conformational change in the 314-bp DNA fragment to make recombination more favorable (Dorman and Higgins, 1987; Eisenstein et al., 1987). The second element, H-NS, is another histone-like protein which is encoded by pilG (Kawula and Orndorff, 1991; Spears et al., 1986)/osmZ (Higgins et al., 1988; Hulton et al., 1990). In contrast to IHF, H-NS seems to repress the fim switch. The effect of these two histone-like proteins on fim expression suggests that DNA topology is important in regulating the expression of type 1 fimbriae.

A third element that affects type 1 phase variation is the site-specific DNA binding protein Lrp (leucine responsive regulatory protein) (Blomfield et al., 1993). This protein stimulates phase variation of type 1 fimbriation by binding in and adjacent to the fim switch to form a nucleoprotein complex that is required for recombination (Gally et al., 1994).

The fourth regulator of type 1 phase variation is a minor leucine tRNA species specific for the codon UUG (Burghoff et al., 1993). Because the fimB gene contains six TTG codons and fimE contains only two such codons, the tRNA specific for UUG favors production of FimB over FimE resulting in the 'ON' orientation of the switch. Thus, the level of this leucine tRNA is important for regulation of type 1 phase variation (Burghoff et al., 1993). In summary, regulation of type 1 fimbriation involves a number of factors that influence the switching ability endowed by the FimB and FimE proteins.
These factors allow that bacteria to control type 1 fimbriation in response to various environmental signals.

ii. Methylation-dependent phase variation

Expression of other fimbriae is transcriptionally regulated by a phase variation mechanism that involves differential methylation of two GATC sites by deoxyadenosine methylase (Dâm). The operons encoding the Pap (Blyn et al., 1990), S (Morschhäuser et al., 1993; Van Der Woude and Low, 1994) and F1845 (Bilge et al., 1993b; Van Der Woude and Low, 1994) fimbriae all contain conserved DNA sequences designated GATC box I and GATC box II, which share 11 and 8 bps of sequence identity, respectively (Van Der Woude et al., 1992). With respect to the pap operon, phase-OFF cells contain a fully methylated GATC I site and an unmethylated GATC II site. Conversely, phase-ON cells contain an unmethylated GATC I site and a fully methylated GATC II site (Blyn et al., 1990; Braaten et al., 1991). The two GATC sites are located within the pap regulatory region and overlap binding sites for the global regulatory protein Lrp (Blyn et al., 1990; Nou et al., 1993). PapI, a regulatory protein of the pap operon, plays an essential role in modulating the switch between pap phase states. PapI does not bind to DNA by itself but modulates the binding of Lrp (Nou et al., 1993). In the absence of PapI, Lrp binds to sites near GATC II and inhibits methylation of this GATC site, forming the phase-OFF methylation pattern. Methylation of the unoccupied GATC I site inhibits the binding of Lrp-PapI to this site thereby maintaining the OFF phenotype until DNA replication occurs. Immediately following
DNA replication, a hemimethylated GATC I site is generated. Lrp-PapI binds and inhibits Dam methylation of this site resulting in Pap fimbriae expression (Braaten et al., 1994). Because Dam and Lrp-PapI essentially compete for binding to the same GATC sites, the level of Dam in the cell also regulates Pap phase variation. Increased levels of Dam result in increased methylation of the GATC I site which then inhibits binding of Lrp-PapI as well as formation of the ON phase. Reduced levels of Dam decreases the methylation of the GATC II site which is required for \textit{pap} transcription (Blyn et al., 1990; Braaten et al., 1994).

The methylation-dependent phase variation in the \textit{pap} operon is also regulated by the catabolite repressor protein (CRP) in response to carbon sources and by the regulatory protein, PapB (Van Der Woude et al., 1992). Both CRP and PapB stimulate transcription of \textit{papI} (Fig. 7) (Båga et al., 1985; Göransson et al., 1989a). Thus, these proteins control the level of PapI which in turn regulates the frequency of the switch between the ON and OFF states.

Although H-NS does not effect \textit{pap} phase variation, the histone-like protein does exert a negative effect on the transcription of the \textit{pap} genes (Fig. 7) (Göransson et al., 1990; White-Ziegler et al., 1990). The repression of H-NS may be alleviated by the antirepressor activity of the CRP and PapB activator proteins. One possible mechanism may be the formation of a nucleoprotein complex with cAMP-CRP and other Pap regulatory proteins which alter the local DNA conformation and displace H-NS (Forsman et al., 1992; Van Der Woude et al., 1992). The effects of the various proteins on \textit{pap} expression is
summarized in Fig. 7.

The sfa and daa operons contain genes with sequence identity to papI and papB (Bilge et al., 1993b; Schmoll et al., 1990a), are positively regulated by Lrp and are under OFF-ON phase variation control (Van Der Woude and Low, 1994). Thus these operons have regulatory properties similar to that of the pap operon (Van Der Woude and Low, 1994). Unlike the pap operon, the fae operon is not under phase variation control even though this operon contains the two GATC boxes (Van Der Woude et al., 1992), is negatively regulated by Lrp and contains two genes encoding proteins which are homologous to PapB and PapI (Hulsman et al., 1994). Regulation of the fae operon is not well understood.

Fig. 7. Schematic diagram summarizing the components that influence transcription of the pap operon either positively or negatively (Uhlin, 1994). H-NS represses while PapB, PapI, Lrp and CRP-cAMP activate pap expression.
c. Environmental control of fimbriae expression

Production of fimbriae is affected by a variety of environmental factors including temperature, iron, growth rate, osmolarity and as already discussed carbon sources and aliphatic amino acids (leucine). In general, fimbrial expression is repressed by temperatures less than 26-28 °C. For example, Pap (Göransson and Uhlin, 1984), S (Schmoll et al., 1990b), K88 (Ørskov et al., 1961), K99 (De Graaf et al., 1980; Isaacson, 1983; Roosendaal et al., 1986; Van Der Woude et al., 1990b), CS1 and CFA/I (Evans et al., 1975) fimbriae are expressed only at temperatures above 25°C. The thermoregulation occurs at the level of transcriptional initiation and is mediated by the histone-like protein H-NS (Göransson et al., 1990; Jordi et al., 1992; Morschhäuser et al., 1993; White-Ziegler et al., 1990). Fimbrial expression at the higher temperatures depends upon the alleviation of the repressive effect of H-NS by antirepressors of the fimbrial gene systems (Göransson et al., 1989b; Jordi et al., 1992; Morschhäuser et al., 1993; Van Der Woude et al., 1992). In contrast, Curli fimbriae is not expressed at temperatures above 76°C (Arnvist et al., 1992). This regulation occurs at the transcriptional level but the molecular mechanism is unknown.

Synthesis of CFA/I fimbriae is also transcriptionally regulated by iron availability (Karjalainen et al., 1991b). Fur (ferric uptake regulator), the protein that mediates this regulation, binds iron (Fe²⁺) and represses CFA/I synthesis by binding to DNA in the subunit promoter region (Bagg and
Neilands, 1987; Karjalainen et al., 1991b).

Growth rate (μ) affects the synthesis of K99, K88, S and 987P fimbriae (Jacobs and De Graaf, 1985; Schmoll et al., 1990b; Van Der Woude et al., 1989; Van Verseveld et al., 1985). K99 production is arrested at growth rates below 0.25 h⁻¹, but production increases sharply at higher growth rates (Van Verseveld et al., 1985). Similarly, 987P and K88 fimbriae production increases with increasing growth rate, with an optimum at μ=0.4 h⁻¹ (Jacobs and De Graaf, 1985; Schmoll et al., 1990b; Van Der Woude et al., 1989). Growth-rate-dependent regulation of K99 synthesis occurs at the transcriptional and post-transcriptional level and may involve the growth-rate related stability of a mRNA species or mRNA secondary structure (Van Der Woude et al., 1990a). In general, however, the molecular mechanism involved in growth-rate-dependent regulation is unknown.

Osmolarity is another environmental factor that regulates fimbriae production. Pap, S, type 1, CFA/I and Curli fimbriae are optimally expressed under low osmolarity conditions (Kunin et al., 1994; Olsén et al., 1993; Schmoll et al., 1990b). Again, the regulatory mechanism is unknown but operates at the level of transcription (Olsén et al., 1993).

d. Two component regulatory systems

The promoter of the fimbrial subunit genes of P. aeruginosa, D. nodosus and N. gonorrhoeae require the rpoN gene product (σ54) for
transcription (Ishimoto and Lory, 1989). To initiate RNA synthesis, σ^{54} requires a trans-activating factor (Kustu et al., 1989). Generally, this protein is part of a two-component sensor-regulator system that controls expression of a set of genes (Albright et al., 1989; Gross et al., 1989). The trans-activating factor is activated through phosphorylation by the sensor in response to specific environmental signals. PilR and PilS form a two-component regulatory system that controls expression of type IV fimbriae in *P. aeruginosa*. The regulator, PilR, binds to the DNA upstream of the *pilA* promoter region and then, through a DNA-looping mechanism, interacts at the promoter with RNA polymerase containing σ^{54} to allow *pilA* transcription to proceed (Hobbs et al., 1993; Ishimoto and Lory, 1992). This action depends on activation of PilR by PilS, which is probably a histidine protein kinase (Boyd et al., 1994; Hobbs et al., 1993). The nature of the signal that activates PilS is unknown. In *N. gonorrhoeae*, the gene products of *pilA* and *pilB* may also form a similar two-component regulatory system (Taha and Marchal, 1990; Taha et al., 1991).

Expression of the type IV fimbriae of *V. cholerae* (Tcp) is also regulated by signal transduction but the mechanism differs from the standard 'two-component' model. In this case, the system is composed of three components: a sensor (ToxS) (DiRita and Mekalanos, 1991); a membrane spanning transcriptional activator (ToxR) (DiRita and Mekalanos, 1991; Miller et al., 1987); and a member of the AraC family of transcriptional activators (ToxT) (DiRita et al., 1991; DiRita, 1992). Upon activation by the appropriate
environmental stimulus, ToxS interacts with ToxR and initiates the assembly of ToxR dimers in the membrane. The ToxR dimers are active for DNA binding and transcriptionally activate expression of toxT (DiRita and Mekalanos, 1991; DiRita, 1992). In turn, ToxT activates tcp expression (DiRita et al., 1991, DiRita, 1992). Thus, the regulation of the tcp genes by ToxR is indirect, being mediated through ToxT.

B. Post-transcriptional regulation

Fimbrial genes are organized in clusters and are expressed as polycistronic transcriptional units. However, the output of individual gene products is variable resulting in the major subunit being produced in larger amounts than any of the other fimbrial gene products. This differential gene expression is regulated post-transcriptionally in the pap, cfa and daa operons. In the pap operon, the dicistronic papB-papA transcript is endonucleolytically cleaved by RNaseE between the two cistrons (Nilsson and Uhlin, 1991). The resulting papB transcript is highly unstable, with a half-life of only 2.5 min, whereas the resulting papA transcript has a half-life of about 27 min (Båga et al., 1988). The stem-loop structures at the 5' and 3' ends of the papA mRNA may protect this transcript against exonucleolytic degradation. Processed mRNA and differential decay of the processed mRNA transcripts also account for the relatively high level of expression of the major subunit proteins CfaB and DaaE which are assembled into CFA/I and F1845 fimbriae, respectively. Unlike pap, the endonucleolytic processing is RNaseE-independent (Bilge et
al., 1993a; Jordi et al., 1993).

6. *Salmonella* bacteria and their fimbriae

*Salmonella* bacteria are Gram-negative, facultative anaerobic rods that belong to the family Enterobacteriaceae. Subdivision of the genus *Salmonella* into species has traditionally been accomplished by phage typing, serotyping and antimicrobial susceptibility testing. Serotyping is based on the Kaufman-White scheme in which organisms are represented by the numbers and letters given to the different O (somatic), Vi (capsular) and H (flagellar) surface antigens (Le Minor, 1984). The antigenic profile of a particular organism is determined using polyclonal antiserum to the various antigens in haemagglutination assays. Antigenic profiles with particular O antigens in common are collected into an O group (A-Z) and arranged alphabetically by H antigens within the group. For example, all *S. enteritidis* strains belong to the O group labelled D₁ (Le Minor, 1984). However, the O antigenic formulae of some salmonellae can be changed through lysogenization by specific phage. The presence of the alternate O antigen in strains of these groups changes the name of the organism (Le Minor, 1984). These serovars can be further subdivided based on the sensitivity to bacteriophage that recognize specific O or Vi antigens or on the basis of the resistance to antibiotics. However, in recent years, with the advent of molecular techniques, the phylogenetics of *Salmonella* has also been studied using plasmid profile analysis (Mayer, 1988;
Shlaes and Currie-McCumber, 1986; Stanley and Baquar, 1994), DNA restriction fragment polymorphism (RFLP) (Stanley and Baquar, 1994; Wachsmuth, 1986), chromosomal probe fingerprinting (Stanley and Baquar, 1994; Tompkins et al., 1986) and multilocus enzyme electrophoresis (Beltran et al., 1988; Stanley and Baquar, 1994).

Salmonella bacteria are invasive enteropathogens that give rise to gastroenteritis, enteric fever or bacteremia in humans and animals (Groisman and Saier, 1990). Although salmonellosis (food poisoning due to human infection with Salmonella bacteria) is preventable, the world wide incidence of this disease has been increasing steadily during the 1980's and 1990's (Cooke, 1990; Mason, 1994; Pohl et al., 1991; Poppe, 1994; Rampling et al., 1989; Rodrigue et al., 1990; Todd, 1990). Surprisingly, of the over 2000 known Salmonella serotypes, only a few are routinely associated with food poisoning. One strain, S. enteritidis, is now recognized as the leading cause of salmonellosis in humans in North America (Mason, 1994; Poppe, 1994; Todd, 1990) and Europe (Cooke, 1990; Pohl et al., 1991; Rampling et al., 1989; Rivera et al., 1991). In fact, it is considered pandemic in parts of Europe and the third world (Rodrigue et al., 1990). The majority of S. enteritidis infections are associated with the contamination of whole egg shells resulting from vertical transmission of this pathogen from breeder stocks due to transovarian infection (Cowden et al., 1989; Gast, 1994; Hopper and Mawer, 1988; Humphrey, 1994; Rampling et al., 1989).

Most Salmonella enteritidis infections arise from ingestion of these
organisms. Ingested organisms proceed through the intestinal tract where they may interact with the mucosal surface at the Peyer's patches and may adhere to and penetrate into the intestinal epithelial cells (Takeuchi, 1967). One of the first steps towards colonization is stable attachment of the bacteria to each other and/or to the host cell surface (Isberg, 1991). Various bacterial host cell attachment mechanisms exist; one such strategy found with bacteria such as *Salmonella* that infect epithelial cell surfaces is mediated by fimbriae (Finlay and Falkow, 1989).

One highly virulent, human isolate of *S. enteritidis* produces at least four biochemically and serologically distinct fimbriae: SEF14 (Feutrier *et al.*, 1986; 1988), SEF17 (Collinson *et al.*, 1991), SEF18 (Clouthier *et al.*, 1994) and SEF21 (Müller *et al.*, 1991) (*Salmonella enteritidis* fimbriae composed of 14, 17, 18 or 21 kDa subunits, respectively). The nomenclature for *S. enteritidis* fimbriae is summarized as follows: SefA, AgfA, SefD and FimA refer to the fimbrins and *sefA*, *agfA*, *sefD* and *fimA* refer to the genes encoding the fimbrins of the SEF14, SEF17, SEF18 and SEF21 fimbriae, respectively. SEF17 are unusual thin, aggregative fimbriae (Collinson *et al.*, 1991) that mediate binding of *S. enteritidis* to fibronectin and are responsible for a distinctive aggregative colony morphology, autoaggregation, and the ability to bind Congo red (Collinson *et al.*, 1993). Fimbriae analogous to SEF17 have also been found in clinical isolates of *E. coli* (Arnvist *et al.*, 1992; Collinson *et al.*, 1992; Doran *et al.*, 1993a). SEF21 fimbriae are type 1 fimbriae and are responsible for the MS hemagglutination phenotype of *S. enteritidis* (Müller
et al., 1991). Finally, SEF14 and SEF18 are both thin, filamentous fimbriae (Clouthier et al., 1994; Feutrier et al., 1986; 1988) and are the subject of this thesis.

SEF14 fimbriae were discovered by Josiane Feutrier, a former graduate student, who purified the fimbriae from S. enteritidis and then cloned and expressed the fimbrin gene in Escherichia coli (Feutrier et al., 1986, 1988). A 5.3 kbp fragment of her cosmid clone was subcloned and partially characterized by Karl-Heinz Müller, a former research associate (Müller et al., 1989). Initially, both researchers mistakenly identified the SEF14 fimbriae as being type 1-like. However, further investigation by Karl-Heinz Müller revealed the presence of a second fimbriae which had a definite type 1 morphology and was thereafter referred to as SEF21 (Müller et al., 1991). SEF14 and SEF21 were morphologically and biochemically distinct from each other and from the SEF17 fimbriae discovered by Collinson et al. (1991) and the SEF18 fimbriae identified during my Ph.D. program. Initially, the focus of my graduate research was DNA sequence analysis of the 5.3 kb and later a 10 kb fragment of S. enteritidis DNA which had been cloned into E. coli. The following chapters discuss what was found.
CHAPTER II

Characterization of the sef14 gene cluster and its fimbriae

1. MATERIALS AND METHODS

A. Bacterial strains

Table 1. Table of bacterial strains.

<table>
<thead>
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<th>Bacterial strain</th>
<th>Description</th>
<th>Source or Reference</th>
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<td>S. enteritidis</td>
<td>wild type</td>
<td>T. Wadstrom et al., 1988</td>
</tr>
<tr>
<td>27655-3b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27655-3b-122</td>
<td>Tn10 insertion mutant of 3b</td>
<td>Feutrier et al., 1988</td>
</tr>
<tr>
<td>27655-3b-1b</td>
<td>spontaneous rifampicin resistant mutant of 3b</td>
<td>Collinson et al., 1992</td>
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<tr>
<td>TrpA 1-2</td>
<td>TrpA mutant of 3b</td>
<td></td>
</tr>
<tr>
<td>TrpA 1-11</td>
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<td></td>
</tr>
<tr>
<td>TrpA 1-16</td>
<td>TrpA mutant of 3b</td>
<td></td>
</tr>
<tr>
<td>TrpA 2-3</td>
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</tr>
<tr>
<td>E. coli</td>
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<td></td>
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<td>HB101</td>
<td>F-:hsdS20 (rK- mR-) leu supE44 araL4 galK2 lacY1 proA2 rpsL20 (Str') xyl-5 mtl-1 recA13 mcrB</td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>F':Tn10 proA+B+ lacI9 Δ(lacZ)M15/recA1 endA1 gyrA96 (NaI') thi hsdR17 (rK- mK+) supE44 relA1 lac</td>
<td>Stratagene, La Jolla, Calif</td>
</tr>
<tr>
<td>DH5α</td>
<td>F'/:canD1 hsdR17 (rK- mK+) supE44 thi-1 recA1 gyrA (NaI') relA Δ(lacZYA-argF) U169 (a80dΔlacZ)M15</td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td>TBI</td>
<td>F-ara Δ(lac-proAB) rpsL (Str') (a80dΔ(lacZ)M15) hsdR (rK- mK+)</td>
<td>New England Biolabs, Inc.</td>
</tr>
<tr>
<td>SM10 pRT733</td>
<td>apr orIR6K tra-nob+ ampR Kmr</td>
<td>Manoil and Beckwith, 1985</td>
</tr>
<tr>
<td>Salmonella serovars</td>
<td>wildtype</td>
<td>see Table 9</td>
</tr>
</tbody>
</table>

a. Production of the rifampicin resistant 3b mutant. 200 µl of an overnight culture of S. enteritidis 3b was spread onto solid LB media (Rosner, 1972). 25 µl of rifampicin (250 µg/ml) was added to the center of this plate.
which was then incubated for 14 h at 37°C. Four rifampicin resistant (rifR) colonies were streaked onto solid LB media containing rifampicin (250 μg/ml) in two consecutive passes. Screening the rifR mutants for Congo red binding and the presence of SEF14, SEF17 and SEF21 confirmed that the bacteria were *S. enteritidis*.

**b. Production of TnphoA mutants.** *TnphoA* mutants of *S. enteritidis* 3b were generated by mating a spontaneous rifampicin (rif) resistant 3b strain with *E. coli* SM10 carrying the plasmid pRT733 (Manoil and Beckwith, 1985). Briefly, *E. coli/SM10/pRT733*, the donor strain, was grown in LB (Rosner, 1972) and kanamycin (50 μg/ml) shaking to 0.2 OD<sub>650</sub> and then grown statically to 1.0 OD<sub>650</sub>. The recipient strain, *S. enteritidis* 3b1b was grown in LB and rifampicin (100 μg/ml) shaking to 0.8 to 1.0 OD<sub>650</sub>. 3 ml of each culture was pelleted in a microfuge (14,000 x g, 1 min, 22°C), washed twice with 1 ml warm (37°C) LB to remove the antibiotics, and then resuspended in 150 μl warm LB. 37.5 μl of each cell suspension was transferred to a 23 mm filter (Micron Separations Inc., Westboro, MA) placed in the center of warm, solid LB medium and incubated at 37°C for 2 h. Each filter was transferred to a sterile tube containing 2 ml warm LB and vortexed to remove the bacteria. The cells were pelleted in the microfuge (14,000 x g, 1 min, 22°C), resuspended in 300 μl warm LB and plated in 150 μl aliquots onto
solid LB medium containing 5-bromo-4-chloro-3-indolyl phosphate (50 μg/ml) and kanamycin (50 μg/ml) in addition to rifampicin (100 μg/ml). Colonies expressing alkaline phosphatase were isolated, grown in static liquid CFA medium (Evans et al., 1977) and tested for the production of SEF14 fimbriae by Western blot analysis.

B. Media and growth conditions. To promote the production of SEF14 fimbriae, bacteria were incubated aerobically in static broth cultures consisting of CFA medium (Evans et al., 1977) at 37°C for 48-60 h.

To promote SEF18 production and to grow strains of E. coli that served as hosts for recombinant plasmid DNA, bacteria were grown aerobically in shaking (250 rpm) LB cultures (Rosner, 1972) at 37°C for 24 h. To overexpress MalE'-SefD fusion proteins, bacteria were grown aerobically in shaking SOC cultures at 37°C.

Stock cultures were stored in 15% (vol/vol) glycerol broth at -70°C.

C. Plasmids and plasmid construction.

a. Construction of pSC1. pKX1 DNA (Table 2) was purified using a Qiagen column (Qiagen, Chatsworth, CA) and digested with Sma I. One Sma I site was located within sefC and the other Sma I site was located within the vector pTZ19R. After digestion, the Sma I enzyme was inactivated by a 20 min incubation at 65°C and the blunt ended DNA, minus the 1907 bp
Fig. 8. Cloning strategy. The IS3 element and sefABCDE are encoded on the 10 kbp Kpn I fragment subcloned from cos48 (44 kb EcoR I fragment). The 5.3 kb Hind III fragment originally used to sequence sefABC and part of sefD is also shown. Restriction-site abbreviations are as follows: B, BamH I; Bgl, Bgl II; EcoR I; H, Hind III; K, Kpn I; P, Pst I; and S, Sma I.
The *Sma* I insert, was ligated together with T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD) to produce the construct designated pSC1. The ligations were transformed into *E. coli* HB101 (Sambrook *et al.*, 1989).

### Table 2. Table of plasmids.

<table>
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<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
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</thead>
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<tr>
<td>cos48</td>
<td>44 kbp of <em>S. enteritidis</em> DNA in pHCl9</td>
<td>Feutrier <em>et al.</em>, 1988</td>
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<tr>
<td>pKX1</td>
<td>5.3-kb <em>Hind</em> III fragment of <em>S. enteritidis</em> DNA from cos48 subcloned into pTZ19R</td>
<td>Müller <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>del A10, -B15, -B23, -C1, -D5, -D8, -D9, -D16, -D19, -E1, -E2 and -E21</td>
<td>pTZ18R derivatives containing sequential deletions of the 5.3-kb <em>Hind</em> III pKX1 fragment</td>
<td>Müller <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>pSC1</td>
<td>1907 bp <em>Sma</em> I deletion in the 5.3-kb fragment from pKX1</td>
<td>This study</td>
</tr>
<tr>
<td>pSC2</td>
<td>10-kbp <em>Kpn</em> I fragment from cos48 subcloned into pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pSC3</td>
<td>447 bp PCR-amplified <em>seiD</em> gene from <em>S. enteritidis</em> cloned into pMal-c2</td>
<td>This study</td>
</tr>
<tr>
<td>pSC4</td>
<td>447 bp PCR-amplified <em>seiD</em> gene from <em>S. enteritidis</em> cloned into pMal-p2</td>
<td>This study</td>
</tr>
<tr>
<td>pSC5</td>
<td>447 bp PCR-amplified <em>seiD</em> gene from <em>S. enteritidis</em> cloned into pUC18</td>
<td>This study</td>
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<td>pSC6</td>
<td>1046 bp <em>Hind</em> III/<em>Pst</em> I fragment from cos48 cloned into pTZ18R</td>
<td>This study</td>
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<td>pSC7</td>
<td>741 bp PCR-amplified <em>seiB</em> gene from <em>S. enteritidis</em> cloned into pMal-p2</td>
<td>This study (Ch. IV)</td>
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<td>pSC8</td>
<td>2445 bp PCR-amplified <em>seiC</em> gene from <em>S. enteritidis</em> cloned into pMal-p2</td>
<td>This study</td>
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<td>pSC9</td>
<td>1573-bp <em>Bsm</em> I fragment from pKX1 subcloned into pUC19</td>
<td>This study (Ch. IV)</td>
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<td>pSC10</td>
<td>1624 bp <em>EcoR</em> I/<em>Hind</em> III fragment from pSC9 subcloned into pNIII113-B1</td>
<td>This study (Ch. IV)</td>
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<td>pEU2030</td>
<td>1200 bp <em>Hha</em> I fragment encompassing the <em>rns</em> gene cloned into pUC18</td>
<td>J.R. Scott</td>
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<tr>
<td>pSFA</td>
<td>584-bp <em>Dra</em> I fragment cloned into pUC19</td>
<td>J.L. Doran</td>
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</table>
b. Construction of pSC2. cos48 DNA (Fig. 8) and the vector pUC19 were purified using Qiagen columns (Qiagen, Chatsworth, CA), digested with Kpn I, run on a 1% agarose gel in TAE buffer (Sambrook et al., 1989) and the 10 kb Kpn I fragment from cos48 (Fig. 8) as well as the Kpn I digested pUC19 DNA were gel purified using Sephaglas (Pharmacia, Uppsala, Sweden). The vector and the Kpn I fragment were then ligated together with T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD) to produce the plasmid designated pSC2. The ligations were transformed into E. coli DH5α (Sambrook et al., 1989).

c. Construction of pSC3, pSC4, pSC5, and pSC8. sefC- or sefD-PCR-amplified DNA was purified using the "Double GeneClean" procedure recommended by the manufacturer (Bio101 Inc., LaJolla, CA). Briefly, the PCR product was purified using GeneCleanII glassmilk, digested with EcoR I and Pst I (New England Biolabs Inc., Beverly, MA) and then purified a second time using GeneCleanII glassmilk. The vectors, pMal-c2, pMal-p2 (New England Biolabs Inc.) or pUC18 (Yannish-Peron et al., 1985) were purified using a Qiagen column (Qiagen, Chatsworth, CA), digested using EcoR I and Pst I (New England Biolabs Inc.), run on a 1% agarose gel in TAE buffer (Sambrook et al., 1989) and then purified from the gel using Sephaglas (Pharmacia, Uppsala, Sweden). The vector and PCR amplified sefC or sefD were then ligated together with T4 DNA ligase (Bethesda Research
Fig. 9. Plasmid maps: pSC4 (A), and pSC8 (B). sefC and sefD were PCR amplified, purified, digested with EcoR I and Pst I, purified and ligated into the EcoR I/Pst I sites of pMal-p2. The map of pSC3 is identical to that of pSC4 except that the maIE gene in pSC3 has a deletion of the signal sequence leading to cytoplasmic expression of the fusion protein. Maps of pKX1, pSC9 and pSC10 appear in Chapter IV. Because pUC18 and pUC19 are commonly used, maps of constructs made using these vectors are not shown.
Laboratories, Gaithersburg, MD). The ligations were transformed into *E. coli* TB1 (New England Biolabs Inc.) or DH5α (Sambrook *et al.*, 1989). The *in vitro* recombination with *sefD* and pMal-c2 and *sefD* and pMal-p2 created the recombinant plasmids, pSC3 and pSC4, respectively, in which *sefD* was translationally fused with *malE*, the gene encoding the maltose binding protein (Fig. 9A). Similarly, *sefC* was translationally fused with *malE* in pSC8 (Fig. 9B). Expression of these constructs in TB1 cells resulted in a fusion protein consisting of MalE' and SefC (MalE'-SefC) or SefD (MalE'-SefD). The same procedure was used to clone PCR-amplified *sefD* into pUC18. The *sefD*/pUC18 construct was designated pSC5 (Table 2).

d. Construction of pSC6. cos48 DNA was purified using a Qiagen column (Qiagen, Chatsworth, CA), digested with *Sma* I (New England Biolabs Inc., Beverly, MA), and run on a 1% agarose gel in TAE buffer (Sambrook *et al.*, 1989). The *Sma* I fragment (Fig. 8) was purified from the gel using an electroeluter (Pharmacia-LKB, Uppsala, Sweden), digested with *Hind* III and *Pst* I (New England Biolabs Inc.), and rerun on a 1% agarose gel in TAE buffer. The appropriate *Hind* III/*Pst* I band was purified from the gel using an electroeluter (Pharmacia-LKB) and then ligated to *Hind* III/*Pst* I cut pTZ18R (Pharmacia) with T4 DNA ligase. The ligations were transformed into *E. coli* DH5α. The resulting clone was designated pSC6.
D. DNA ligations. Each 20 µl ligation reaction contained T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD), 5 µl 5x DNA ligase reaction buffer [250 mM Tris-HCl pH 7.5, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT and 25% (w/v) polyethylene glycol-8000], vector and insert DNA and sterile dH₂O. To ensure that the appropriate ratio of vector to insert DNA was achieved, each ligation was performed using three different ratios of vector to insert DNA: 0.5-1.0 µl vector DNA with 3-14 µl insert DNA depending on the relative concentrations of vector and insert DNA. Blunt end ligations were performed at 14°C for 24 hr using 1 unit of T4 DNA ligase. Cohesive end ligations were performed at 23-26°C for 1-4 hr using 0.1 units of T4 DNA ligase.

E. Production of competent cells. This procedure is a modification of the CaCl₂ method described by Sambrook et al. (1989). Cells from an overnight culture of E. coli were diluted 1:100 into 40 ml LB and grown aerobically at 37°C to an OD₆₆₀ of 0.5. The cells were chilled on ice, centrifuged (7000 x g, 15 min, 4°C), resuspended in 8 ml CM1 buffer (10 mM sodium acetate pH 5.6, 50 mM MnCl₂, 5 mM NaCl), incubated on ice for 20 min, centrifuged (7000 x g, 10 min, 4°C) and resuspended in 2.5 ml CM2 buffer (10 mM sodium acetate pH 5.6, 5% glycerol, 70 mM CaCl₂, 5 mM MnCl₂). 100 µl aliquots of the competent cells were stored in 1.5 ml eppendorf tubes at -70°C.
F. Transformation of competent cells. For every transformation, 1, 2.5 and 7.5 μl of plasmid DNA (1 mg/ml) were added, respectively, to three 100 μl aliquots of thawed competent cells, which were then incubated on ice for 30-40 min and heat shocked at 42°C for 1 min. 1 ml of SOC was added, the cells were incubated at 37°C for 1 h with gentle shaking, pelleted in the microfuge and resuspended in 300 μl SOC. 100-150 μl aliquots were spread onto solid LB medium containing the appropriate antibiotics.

G. Purification of chromosomal DNA

a. Proteinase method. Cells from 100 ml overnight cultures of a variety of Gram positive and Gram negative bacterial strains were centrifuged (3840 x g, 10 min, 4°C), resuspended in 20 ml 50 mM Tris pH 8, 5 mM EDTA, 25% sucrose, mixed with 5 mg lysozyme, and incubated at 22°C for 5 min followed by a 15 min incubation on ice. The solution was then mixed with 2-10 mg proteinase K (Boehringer Mannheim, Germany) in 1 ml 20% SDS, incubated at 65°C for 30-60 min, mixed with 10 ml phenol and then centrifuged (3020 x g, 10 min, 22°C). The aqueous layer (bottom layer) was mixed with 15 ml phenol:chloroform (1:1) and centrifuged (3020 x g, 10 min, 22°C). The subsequent aqueous layer was mixed with 10 ml phenol:chloroform (1:1) and centrifuged (3020 x g, 10 min, 22°C). The DNA was precipitated from the resulting aqueous layer at -20°C for 20 min with 1/10 volume 3 M sodium acetate and 2 volumes cold 95% ethanol, pelleted by
centrifugation (12,100 x g, 15 min, 4°C), washed with 20 ml 70% ethanol at -
20°C for 2 h, pelleted by centrifugation (12,100 x g, 15 min, 4°C) and dissolved
in 5 ml TE. The DNA solution was incubated with RNaseA (final
concentration of 100 µg/ml) for 30 min at 37°C, extracted with 1 volume of
phenol:chloroform (1:1), and precipitated as described above and then
resuspended in 1 ml TE.

cultures of a variety of Gram positive and Gram negative bacterial strains
were pelleted by centrifugation (3020 x g, 15 min, 4°C), resuspended in 2 ml
25% sucrose, 50 mM Tris HCl pH 8 and 1 ml 10 mg/ml lysozyme in 0.25 M
EDTA pH 8, and left on on ice for 30 min. 0.75 ml TE, 0.25 ml lysis buffer (5%
sarcosyl, 50 mM Tris HCl pH 8, 62.5 mM EDTA) and 10 mg solid pronase
(Boehringer Mannheim, Germany) were added to the solution and incubated
at 60°C for 1 h. The DNA was extracted three times with 4 ml phenol
equilibrated with TE and once with 4 ml ether. Between each extraction, the
organic and aqueous layers were separated by centrifugation (484 x g, 15 min,
4°C). The isolated chromosomal DNA was dialyzed extensively against TE.
H. Polymerase chain reaction assays.

Table 3. Table of PCR and hybridization primers.

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<td>GGACATTTTTGGAATTCGCGTAATCAGCATCTGCA GTAGC</td>
<td>U of Victoria</td>
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<td>sefB-T (Ch. IV)</td>
<td>GCTACCTCCAGAATTCTTTCGGAAGATAGAAAAAA</td>
<td>U of Calgary</td>
</tr>
<tr>
<td>sefB-B (Ch. IV)</td>
<td>TGTGTTACACCTGCAAGTAATAATCTTATAATT</td>
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<tr>
<td>sefC-T</td>
<td>TGGAATGTTGAAATTCAGACAATATAATTTCGACT</td>
<td>U of Calgary</td>
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<td>sefC-B</td>
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<td>sefD-T2</td>
<td>GCCTTTTTAATGGAGTGAATGTAAGTAT</td>
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<td>sefD-B2</td>
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<td>TnphoA</td>
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<td>U of Calgary</td>
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</table>

a. PCR amplification of 5' end of sefA and TnphoA.

Chromosomal DNA used for the PCR analysis was prepared from S. enteritidis 3b and the four TnphoA mutants of 3b1b (Table 1) as described by Alm et al., (1993) and denatured at 100°C for 2 min just before using. The amplification primer ST2 was designed from the DNA sequence at the 5' end of sefA whereas the primer for the opposite strand (TnphoA) was derived from the IS50L sequence in TnphoA. Amplification was carried out in a 10 µl reaction volume containing 1 µl of the heat denatured chromosomal DNA (approximately 50 ng of DNA), 5 pmoles of each oligonucleotide primer, the nucleoside triphosphates dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Germany) at final concentrations of 0.5 mM each, 1 unit of Taq DNA polymerase (Stratagene, LaJolla, CA) in reaction buffer consisting of 50 mM Tris-HCl pH 8.5, 20 mM KCl, 2.5 mM MgCl₂ and 0.5 mg/ml BSA.
Thermocycling was performed on samples contained in sealed glass capillary tubes inserted into an air driven thermocycler (Idaho Technology, Boise, Idaho) for 30 cycles of denaturation (94°C, 0 s), annealing (55°C, 0 s) and elongation (74°C, 20 s). To determine if the PCR amplifications were successful, the 5 PCR reactions were electrophoresed on 5% polyacrylamide gels in TAE buffer and the DNA was visualized by UV illumination following ethidium bromide staining of the gel (Sambrook et al., 1989).

b. PCR amplification of sefC. sefC amplification was carried out using the primers sefC-T and sefC-B (Table 3) which contained EcoR I and Pst I sites to facilitate cloning of the amplified product. The template, S. enteritidis chromosomal DNA was purified by the method of Alm et al., (1993) and denatured at 100°C for 2 min just before using. Amplification was carried out in a 50 µl reaction volume containing 5 µl of the heat denatured chromosomal DNA (approximately 50 ng of DNA), 5 pmoles of each oligonucleotide primer, the nucleoside triphosphates dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Germany) at final concentrations of 0.5 mM each, 1 unit of Taq DNA polymerase (Stratagene, LaJolla, CA) in reaction buffer consisting of 50 mM Tris HCl pH 8.5, 20 mM KCl, 2.5 mM MgCl₂ and 0.5 mg/ml BSA. Thermocycling was performed on samples contained in sealed glass capillary tubes inserted into an air-driven thermocycler (Idaho Technology, Boise, Idaho) for 30 cycles of denaturation (94°C, 10 s), annealing
(55°C, 10 s) and elongation (74°C, 30 s). To determine if the PCR amplification was successful, the 2445 bp sefC product was electrophoresed in a 1% agarose gel in TAE buffer and visualized by UV illumination following ethidium bromide staining (Sambrook et al., 1989).

c. PCR amplification of sefD. The primers sefD-T2 and sefD-B2 (Table 3), which had been synthesized to encode EcoR 1 and Pst 1 sites to facilitate cloning of the amplified product, were used to amplify sefD. The target DNA, pSC2, was purified using Qiagen columns (Qiagen Inc., Chatsworth, CA). Amplification was carried out in a 50 µl reaction volume containing 5 µl of heat-denatured pSC2 (1 µg/ml), 5 pmol of each primer, the four deoxynucleotide triphosphates (Boehringer Mannheim, Germany) at 0.5 mM each and 0.4 units of Taq DNA polymerase (Stratagene, LaJolla, CA) in reaction buffer consisting of 50 mM Tris-HCl pH 8.5, 20 mM KCl, 2.5 mM MgCl₂ and 0.5 mg/ml BSA. Thermocycling was performed on samples contained in sealed glass capillary tubes inserted in an air-driven thermocycler (Idaho Technology, Boise, Idaho) for 30 cycles of denaturation (94°C, 20 s), annealing (45°C, 10 s) and elongation (74°C, 50 s). The PCR amplified, 447 bp sefD product was electrophoresed on a 1% agarose gel in TAE buffer and visualized by UV illumination following ethidium bromide staining (Sambrook et al., 1989).
I. DNA sequencing and computer analyses.

   a. DNA sequence analysis of *sefABC*. A series of overlapping deletion subclones were created in the recombinant plasmid pKX1 using DNase I as previously described (Table 2) (Müller *et al*., 1989). The deletion subclones were sequenced by the dideoxynucleotide chain termination method (Sanger *et al*., 1977) using T7 DNA polymerase (Promega, Madison, WI).

Table 4. Table of primers used for DNA sequence analysis.

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<th>Sequencing primers</th>
<th>Primer sequence (5' to 3')</th>
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<td>B15a</td>
<td>GCTACAAATATGGAGC</td>
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<td>B15b</td>
<td>AATTGAGCATATCCAAA</td>
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<tr>
<td>B15c</td>
<td>ATAACTCTTATATAATT</td>
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<td>Oligo B15</td>
<td>GCTTGTGTGTAAGCGACT</td>
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</tr>
<tr>
<td>Universal reverse</td>
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<td>U of Calgary</td>
</tr>
</tbody>
</table>
WI). Both strands of the DNA were sequenced completely using the reverse primer for the coding strand and internal oligonucleotide primers (first 11 primers in Table 4: D16b to D10) purchased from ULTRA Diagnostics Corporation (Seattle, WA) for the opposite strand.

The programs contained in MacVector™ (Intelligenetics, Mountainview, CA) were used to determine the order of the overlapping sequenced DNA fragments. DNA Strider™ 1.0 (Marck, 1983) was used to identify open reading frames. MACAW (NCBI, Bethesda, Md.) was used to construct regions of local similarity among the four fimbrial chaperone proteins. The predicted amino acid sequence for each open reading frame was compared to proteins listed in the GenBank, SWISS-PROT and GENPEPT databases, using the FASTA program (Pearson and Lipman, 1988).

b. DNA sequence analysis of the IS3 element and sefDE1E2

The open reading frames sefD, sefE1 and sefE2 and the IS3 DNA were sequenced with custom synthesized oligonucleotide primers (sefU1-U12; sefD; sefE-E14; Table 4) by the dideoxynucleotide chain termination method (Sanger et al., 1977) using Sequenase™ 2.0 version as recommended by the manufacturer (United States Biochemicals, Cleveland, OH). The double stranded DNA template was prepared using a Qiagen column (Qiagen Inc., Chatsworth, CA) and the oligonucleotide primers were synthesized on an Applied Biosystems model 391 PCR-MATE EP DNA synthesizer (Applied
Biosystems Canada Inc., Mississauga, ON, Canada).

The nucleotide sequences obtained were analyzed using DNA Strider™ 1.0 (Marck, 1988). The predicted amino acid sequence for each ORF was compared with proteins listed in the Genbank, SWISS-PROT, and GEN-PEPT data bases using the FASTA program (Pearson and Lipman, 1988).

J. Protein purification.

a. Purification of SEF14 fimbriae. SEF14 fimbriae were isolated as described in Müller et al. (1991). Briefly, S. enteritidis was grown statically in 20 litres of CFA at 37°C for 60 h, harvested by centrifugation (9820 x g, 15 min, 4°C), and resuspended in 1.2 litres of 0.15 M ethanolamine buffer, pH 10.5 (Feutrier et al., 1988). Fimbriae were separated from the cells at room temperature by shearing in a blender (Model 909, biospec Products, Bartlesville, OK.) for three one min intervals, after which cells and cellular debris were removed by centrifugation (12,000 x g, 15 min, 4°C). The resulting supernatant was centrifuged (100,000 x g, 1 h, 4°C) to remove membrane vesicles. This clarified supernatant was dialyzed overnight against 10 mM Tris-HCl pH 7.5 containing 0.2% SDS during which SEF14 precipitated. The following are modifications to the method described in Müller et al. (1991). SEF14 fimbriae were recovered by centrifugation (15,000 x g, 30 min, 4°C) from a crude preparation of SEF14 and SEF21 fimbriae following selective
precipitation of SEF14 during dialysis against 10 mM Tris-HCl, pH 9.5, containing 0.2% SDS (Tris-SDS). The SEF14 fimbrial pellet was washed twice with 400 ml of Tris-SDS and then extensively dialyzed against 0.15 M ethanolamine, pH 10.5, at 4°C for 16 h to dissolve SEF14 fimbriae and residual SEF21 fimbriae. The fimbrial suspension was dialyzed against sterile distilled H$_2$O at 4°C for 16 h to remove the ethanolamine and then dialyzed against Tris-SDS at 4°C for 48 h to selectively reprecipitate SEF14 fimbriae. SEF14 fimbriae were recovered by centrifugation, washed thrice with distilled H$_2$O, dialyzed against sterile distilled H$_2$O (4°C, 24 h), and lyophilized.

b. Partial purification of SEF18 fimbriae. After 24 h of aerobic growth in LB, 500 ml of *E. coli* C600 (Table 9) cells were harvested by centrifugation (10,000 rpm, 4°C, 20 min) and resuspended in 100 ml 10 mM Tris HCl pH 8. The cells were then blended for 4x1 min, recovered by centrifugation (15,300 x g, 4°C, 20 min), and resuspended in 10 ml 10 mM Tris HCl pH 8. The 15 ml suspension was applied to two sucrose gradients consisting of 15, 25, 40, 50, 60 and 75% sucrose in 10 mM Tris HCl pH 8 (5 ml layers) and centrifuged in a Beckman SW28 rotor (80,000 x g, 4°C, 22 h). The cellular debris either settled into a hard pellet at the bottom of the tube, formed a thick band on top of the 75% sucrose layer or became incorporated into the 75% layer. Fractions were collected through a needle inserted into the tube first above the thick band on top of the 75% layer, into this band and
then below this band. SDS-PAGE and Western blots developed using antiserum generated against SefD were used to analyze which fractions contained SEF18.

c. Overproduction and purification of SefD. 1 L SOC and ampicillin (250 µg/ml) was inoculated with 10 ml of an overnight culture of TB1/pSC3, grown at 37°C shaking to 0.5-0.7 OD$_{600}$ and induced with 1 mM IPTG for 2 h at 37°C. The cells were centrifuged (4000 x g, 20 min, 4°C) and resuspended in 50 ml 20 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM EDTA. Lysozyme was then added to a final concentration of 1 mg/ml and the cells were incubated on ice for 30 min, sonicated on ice for 2x2 min (setting 6, 50% duty cycle, continuous cycle time; model W-385, Heat Systems Ultrasonics Inc., Farmingdale, NY) and centrifuged (9000 x g, 30 min, 4°C). MalE'-SefD was purified from the supernatant by affinity chromatography on an amylose resin column (New England Biolabs Inc.). After the supernatant was applied, the column was washed extensively with 20 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM EDTA and MalE'-SefD was eluted with 10 mM maltose in the wash buffer. Column fractions containing the fusion protein were detected by Western blot analysis using anti-MalE antsera. The purified fusion protein was then cleaved with Factor Xa (100 ml column fraction + 2.5 ml Factor Xa at 100 µg/ml). The two proteins MalE' and SefD were separated by SDS-PAGE.
and SefD was eluted from the gel using a protein eluter (BioRad Laboratories, Richmond, CA). The amino acid sequence of the eluted protein was determined by N-terminal amino acid sequence analysis.

K. Preparation of immune serum.

a. Antiserum to SEF14 fimbriae. Purified native SEF14 fimbriae and denatured SEF14 fimbrin protein preparations were used to generate immune sera in female New Zealand white rabbits as previously described in Müller et al. (1991). To prepare the denatured SEF14 fimbrin, SEF14 preparations were subjected to SDS-PAGE after which protein was electrophoretically transferred to nitrocellulose by Western blotting using a LKB Multiphore II electrophoresis unit (LKB-Pharmacia, Bromma, Sweden) at 0.8 mA/cm² for 90 min. After staining proteins with Amido Black, the 14,000 M₀ bands were excised, shredded and emulsified in PBS containing Freund's complete adjuvant. 500 µg of each protein preparation was used for the initial injections and the subsequent boosts that followed 2 and 4 weeks later. Preimmune serum was collected one week prior to the first immunization.

b. Antiserum to SefD. Recovery of SefD from the polyacrylamide gel by elution was always low. To obtain a sufficient quantity of the protein, the Factor Xa cut column fractions were loaded onto 2
preparative gels containing 5 double wide lanes. After electrophoresis, one lane of the gel was stained with Coomassie blue and used to determine the position of SefD in the other gel lanes. The protein was eluted from the subsequent gel slices at 10 mAmp at 4°C for 3 h with SDS and for 1 h without SDS in the elution buffer. The elution membrane had a MW cutoff of 12,000.

500 µl eluted SefD (approximately 500 µg) was mixed with 500 µl complete Freund's adjuvant using two 1 ml syringes connected with a double ended canula. The emulsified solution was injected into a female, New Zealand, white rabbit (52Cr for SefD). Injections were as follows: 1x250 µl intramuscularly (IM); 2x250 µl subcutaneously (SC). For subsequent boosts 2 and 4 weeks later, 1 ml eluted SefD was emulsified with 1 ml incomplete Freund's adjuvant as described above. Injections were as follows: 1x500 µl IM; 2x600 µl SC. In both cases, the prebleed was removed the same day as the initial injection of the antigen and only 2 bleeds were removed after the initial antigen injection prior to euthanizing the rabbit.

L. SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (Laemmli, 1970). Proteins were solubilized in SDS sample buffer [10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% SDS, 0.0625 M Tris-HCl pH 6.8], boiled for 5 min and subjected to electrophoresis through a 5% stacking gel and a 12% separating gel.
Protein bands were visualized by Western blot analysis. Briefly, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (BioRad, Richmond, CA) at 0.8 mAmp/cm² for 1 h using an LKB Multiphor II Electrophoresis System (Pharmacia-LKB, Uppsala, Sweden) and discontinuous buffers (cathode buffer: 40 mM 6-amino-N-hexanoic acid, 20% methanol pH 7.6; anode #1 buffer: 0.3 M Tris, 20% methanol pH 10.4; anode #2 buffer: 25 mM Tris, 20% methanol pH 10.4). The membranes were blocked with skim milk buffer (Tris-buffered saline (TBS) containing 3% skim milk and 0.05% Tween-20) and then incubated with antiserum to denatured SEF14 fimbrin (diluted 1/1000 in TBS Tween-20) followed by an incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase (diluted 1/1000 in TBS Tween-20 containing 1.5% skim milk). The immunoreactive proteins were visualized following development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

M. In vitro transcription-translation. Plasmid-encoded proteins were labelled with [35S]-methionine using a cell-free coupled transcription-translation system (Amersham, Oakville, ON). Plasmids carrying either the 5.3 kb fragment of the sef14 operon (pKX1) or deletions thereof were used as the DNA templates to analyze the production of SefA, SefB and SefC. The DNA template for SefD production was a Kpn I fragment of S. enteritidis.
DNA. This fragment was purified from pSC2 by digesting this plasmid with Kpn I, running the digest on a 1% agarose gel in TAE buffer (Sambrook et al., 1989) and electro-eluting the 10 kb Kpn I band from the gel (Pharmacia-LKB, Uppsala, Sweden).

If necessary, the reactions were made up to 30 µl with dilution buffer and contained 7.5 µl supplement solution (nucleotides, tRNA, inorganic salts, and an energy-generating system), 3 µl amino acids minus methionine, 2 µl [35S]-methionine, 4 µl S-30 extract (from E. coli) and 11 µl Kpn I DNA fragment or 12.5 µl pKX1 plasmid DNA. The reactions were incubated at 37°C for 30 min, chased with 5 µl nonradioactive methionine for 5 min at 37°C and then dialyzed against distilled H2O on 0.025 µM VS filters (Millipore Canada Ltd, Mississauga, ON) for 10-15 min prior to SDS-PAGE analysis and autoradiography using Kodak X-OMAT-AR5 film (Kodak, Rochester, NY).

N. Electron microscopy. SEF14, SEF17, and SEF21 fimbriae and SEF18 fimbriae-like structures on S. enteritidis 3b were immunogold labelled and negatively stained as described previously (Collinson et al., 1991). Briefly, bacterial cells deposited on formvar-coated copper grids were negatively-stained with 1% ammonium molybdate containing 0.1% glycerol. To immunogold label S. enteritidis fimbriae, bacteria-coated grids were incubated on a drop of Tris-NaCl buffer (10 mM Tris, pH 8.0; 0.15 M NaCl) containing
1% skim milk, transferred to a drop of preimmune or immune serum (diluted 1/1000 in Tris-NaCl buffer containing 0.1% skim milk), washed in Tris-NaCl buffer and floated on a drop of protein A gold 15 nm (Auroprobe; Pharmacia, Uppsala, Sweden) (diluted 10 fold in Tris-NaCl buffer containing 0.1% skim milk). The grids were rinsed, negatively-stained as described above air dried and observed with a Hitachi transmission EM operated at 75 kV.

O. Dot blot hybridization.

a. Hybridization with sefD probe. DNA dot blot hybridization was used to screen 58 Salmonella isolates, 10 other members of the family Enterobacteriaceae and 5 other eubacteria. Chromosomal DNA was purified from proteinase K-treated cell lysates by repeated phenol-chloroform extraction and ethanol precipitation (Sambrook et al., 1989) or by the method of Alm et al. (1993). The DNA was quantified by spectroscopy, and 0.5 μg samples were mixed with 0.3 M NaOH (final concentration), incubated at 65°C for 1 h, and mixed with an equal volume of 2 M ammonium acetate. The samples were applied to Hybond-N+ nylon membranes (Amersham Canada Inc., Oakville, ON) using a dot blot manifold. The membrane was rinsed in 2 M ammonium acetate for 5 min and 2x SSC (1x SSC is 0.15 M NaCl, 0.015 M tri-sodium citrate) for 2x5 min and then dried at 80°C for 2 h under vacuum. The sefD gene probe consisted of the amplified 447 bp sefD
PCR fragment labelled with \([\alpha-^{32}P]dATP\) by nick translation (Amersham Canada Inc.). DNA blots were prehybridized at 65°C in prehybridization buffer (5x Denhardt, 20% formamide, 5% SDS; Sambrook et al., 1989) containing 200 μg of herring sperm DNA per ml (Sigma, St. Louis, MO). Following hybridization at 65°C, the membranes were washed at a high stringency (0.2x SSC buffer [1x SSC is 0.15 M NaCl, 0.015 M tri-sodium citrate] 0.1% SDS, 64°C). The results were recorded by autoradiography on X-Omat AR5 film (Kodak, Rochester, N.Y.).

b. Hybridization with \(rns\) probe. DNA dot blot hybridization was used to screen \(S.\ enteritidis\) chromosomal DNA and recombinant plasmids carrying different genes from the \(sef14\) gene cluster. The chromosomal DNA was purified by the method of Alm et al. (1993) whereas the recombinant plasmid DNA was purified using a Qiagen column (Qiagen, Chatsworth, CA). DNA was quantified by spectroscopy and 1 μg samples were applied to Hybond-N+ nylon membranes (Amersham Canada Inc., Oakville, ON) using a dot blot manifold.

The \(rns\) probe consisted of a \(Dra\ I\) fragment isolated from a \(Hind\ III/Sac\) I DNA fragment isolated from pEU2030. The \(Dra\ I\) fragment was labelled with \([\alpha-^{32}P]dATP\) by nick translation (Amersham Canada Inc.). The DNA blots were prehybridized at 65°C in prehybridization buffer (5x Denhardt, 20%...
formamide, 5% SDS; Sambrook et al., 1989) containing 225 µg of herring sperm DNA per ml (Sigma, St. Louis, MO). Following hybridization at 45°C or 65°C, the membranes were washed at a high stringency (0.2x SSC buffer [1x SSC is 0.15 M NaCl, 0.015 M tri-sodium citrate], 0.1% SDS, 45°C). The results were recorded by autoradiography on X-Omat AR5 film (Kodak, Rochester, NY).

P. Pulsed field gel electrophoresis (PFGE). Preparation and digestion of high-molecular-weight genomic DNA, digestion of DNA in agarose blocks and separation of DNA by PFGE were all carried out by Dr. K. Sanderson's group at the University of Calgary using methods reported earlier (Liu et al., 1993a).

Q. Southern blot hybridization. DNA which had been separated by pulsed field gel electrophoresis and then transferred onto Hybond-N+ nylon membranes (Amersham Canada Inc., Oakville, ON) was hybridized to specific oligonucleotides to determine the position of sefA and sefD in the chromosome of S. enteritidis. The blots, generated in Dr. Sanderson's lab, were initially prehybridized for 2 h at 65°C in prehybridization buffer (5x Denhardt, 20% formamide, 5% SDS; Sambrook et al., 1989) containing 200 µg of herring sperm DNA per ml (Sigma, St. Louis, MO). Each blot was then
incubated with one of two separate probes. A 550-bp sefA gene fragment derived from S. enteritidis strain 27655-3b was generated by EcoR I and Hind III restriction enzyme digestion (New England Biolabs Inc., Beverly, MA) of the recombinant plasmid pSFA which contained the 584-bp Dra I fragment encompassing sefA and an additional 60-bp upstream region. A sefD probe was generated by EcoR I and Hind III enzyme digestion (New England Biolabs Inc.) of the recombinant plasmid pSC4 which contained the PCR amplified sefD gene from S. enteritidis 27655-3b. Both sefA and sefD gene fragments were isolated by gel electrophoresis (Sambrook et al., 1989) and purified using the Sephaglas™ Bandprep Kit (Pharmacia, Uppsala, Sweden). These probes were then labelled with [α-32P]-dATP by nick translation (Amersham Canada Inc.) and denatured at 100°C for 3 minutes before being added to the blots. Following a 20 h hybridization at 65°C with one of these two probes, the membranes were washed at high stringency [0.2x SSC buffer (1x SSC is 0.15 M NaCl, 0.015 M tri-sodium citrate) 0.1% SDS]. The results were recorded by autoradiography on X-Omat AR5 film (Kodak, Rochester, NY).

2. RESULTS

A. Nucleotide sequence and protein determination. The DNA fragment from S. enteritidis required for production of SefA fimbrin in E. coli was shown to be a 5.3 kb Hind III fragment isolated from cos48 (Feutrier et al.,
1988), cloned into pTZ19 and designated pKX1 (Müller et al., 1989). Therefore, the DNA sequence of a 3.9 kb region of this fragment was determined by a strategy involving overlapping deletion subclones and internal primers (Fig. 11B). This region was found to contain three open reading frames (ORFs) which were designated sefABC (Fig. 10). All three ORFs demonstrated the same translational polarity. No ORFs were detected in the opposite orientation of the DNA sequence. Each of the three ORFs was preceded by a Shine-Dalgarno consensus sequence for translation initiation (Fig. 11A). The sefABC determinants were 498 bp, 741 bp and 2,445 bp, respectively, and the predicted molecular weights of the encoded proteins were 14,436, 28,012 and 90,268 Mr, respectively. The predicted Mr (14,436) and amino acid composition of SefA confirmed the Mr and amino acid composition of SEF14 fimbrin previously purified by Feutrier et al. (1986). Moreover, the first 60 amino acids of the predicted mature SefA sequence were identical to the N terminal sequence of the purified SEF14 fimbrin (Feutrier et al., 1986). Moreover, the first 60 amino acids of the predicted mature SefA sequence were identical to the N terminal sequence of the purified SEF14 fimbrin (Feutrier et al., 1986). Nucleotide sequences for sefA, -B and -C were submitted to Genbank and given accession numbers L11008, L11009 and L11010, respectively.

Comparison of the predicted amino acid sequences of SefA, SefB and SefC to proteins listed in the GenBank, SWISS-PROT and GENPIEPT databases showed that SefA was a unique fimbrin. Surprisingly, SefB was similar to fimbriae periplasmic chaperone proteins necessary for the assembly of various fimbriae in E. coli and Klebsiella pneumoniae, making sefB the first
Fig. 10. Open reading frame map of $sef^{ABCDEF}_2$ and the IS3 element. The DNA sequence was analyzed in all six reading frames which are labelled from 1 to 3 and -1 to -3 on each side of the diagram. Full lines represent putative translation stop sites whereas the half lines represent putative translation start sites. The ORFs in frames 1 to 3 read from left to right whereas the ORFs in frames -1 to -3 read from right to left. The name of each gene appears in the corresponding ORF. Due to the lack of room, the genes in the IS3 element ($orfA$, $orfB$ and $orfC$) are collectively labelled as IS3 and $sefE_1$ and $sefE_2$ are collectively labelled as $sefE$. The DNA sequence and strategy appear in Fig. 11.
Oligonucleotide primers were synthesized to sequence the opposite strand of DNA. Contiguous sequence. (B) Representation of the strategy used to sequence 3.9 kb of the 5.3-kb region are underlined. The termination codons are indicated as XXX, the transcription start site are indicated with asterisks, and the predicted signal peptidase cleavage sites are indicated with arrows. The numbers above each line refer to the nucleotide position of the sequence.

### Fig. 11. Nucleotide sequence of **sefABC**.

(A) Consensus Shine-Dalgarno (SD) sequences, located 8 to 14 bp upstream of the translational start site of each gene, and the -10 putative promoter region are underlined. The termination codons are indicated as XXX, the transcription start sites are indicated with asterisks, and the predicted signal peptidase cleavage sites are indicated with arrows. The numbers above each line refer to the nucleotide position of the contiguous sequence. (B) Representation of the strategy used to sequence 3.9 kb of the 5.3-kb DNA fragment. Overlapping deletions were generated to sequence the coding strand, and oligonucleotide primers were synthesized to sequence the opposite strand of DNA.
periplasmic chaperone protein gene to be characterized from *Salmonella*. The predicted amino acid sequence of SefB was found to be 28% identical (exact matches) and 47% similar (conservative replacements) to the primary sequence of PapD of Pap pili, 31% identical and 44% similar to the primary sequence of CS3-1 of CS3 fimbriae and 27% identical and 49% similar to the primary sequence of MrkB of Type 3 fimbriae of *Klebsiella pneumoniae* (Table 5). Overall, 18 residues dispersed throughout SefB were identical in all four chaperones and 22 additional residues were identical in three of the four chaperones. An analysis of local sequence similarities indicated that 6 blocks of homology existed among the four fimbrial chaperones (Fig. 12B, C). In SefB, the local similarities corresponded to areas predicted to contain β-sheets and one area predicted to form an α-helix (Fig. 12A). In PapD, the local alignments encompassed the first 10 anti-parallel β-sheets found within the 2 domains of PapD by Holmgren and Bränden (1989) and the hydrophobic linker connecting these two domains. In addition, SefC shared homology to other putative bacterial fimbrial outer membrane proteins, including FanD of K99, FaeD of K88, PapC of Pap fimbriae and the proteins from the CS3 fimbrial operon (Table 6). The predicted amino acid sequences of SefA, SefB and SefC each had a predicted signal sequence of approximately 20 amino acids whose composition was divided into a charged N terminus, a central hydrophobic core and a polar C terminal region typical of prokarytic signal peptides (Giersch, 1989). The most likely cleavage sites for signal peptidase, ala21-
Table 5. A comparison of the predicted amino acid sequence for SefB to three fimbrial chaperone proteins

<table>
<thead>
<tr>
<th>Chaperone Protein</th>
<th>Bacterial Strain</th>
<th>Percent Identity</th>
<th>Percent Similarity</th>
<th>Combined Percent</th>
<th>Amino acid Overlap</th>
<th>Total number of amino acids</th>
<th>Molecular Weight</th>
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<td><em>E. coli</em></td>
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<td>47</td>
<td>75</td>
<td>163</td>
<td>239</td>
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<td><em>E. coli</em></td>
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<td>75</td>
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<td>241</td>
<td>27,000&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>MrkB</td>
<td><em>K. pneumoniae</em></td>
<td>27</td>
<td>49</td>
<td>76</td>
<td>171</td>
<td>232</td>
<td>25,000&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a. Percent identity and similarity refer to the percent of identical and conserved amino acids, respectively, at the same position in pairwise comparisons of the SefB sequence to each of the other three chaperone sequences in Release 16 of SWISS-PROT and Release 64.3 of GenPept (GenBank, IntelliGenetics, Mountain View, CA).
b. Lindberg <i>et al.</i>, 1989
c. Jalajakumari <i>et al.</i>, 1989
d. Allen <i>et al.</i>, 1991
Fig. 12. Secondary-structure analysis of SefB and local alignment of SefB and three fimbrial periplasmic chaperone proteins. (A) Secondary-structure analysis of the predicted amino acid sequence for SefB. (B) Schematic representation and aligned sequences (C) of fimbrial chaperone proteins analyzed for statistically significant blocks of similarity. In the schematic (B), the linked sequences are indicated by the slightly thickened bar, while in the alignment (C), uppercase text is used to indicate residues that have been linked. Linking involves inserting gaps into the alignment to bring the subsequences of the block into alignment. The degree of similarity among the proteins in both panels B and C is indicated by the shading such that the darker the shading, the higher the interrelatedness of the sequences. The length of each predicted amino acid sequence is indicated on the right side of the schematic (B). The amino acid sequences of PapD, CS31, and MrkB were taken from Lindberg et al., 1989, Jalajakumari et al., 1989, and Allen et al., 1991, respectively.
Table 6. A comparison of the predicted amino acid sequence for SefC to eight fimbrial outer membrane proteins

<table>
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<tr>
<th>OM Protein</th>
<th>Bacterial Strain</th>
<th>Percent Identity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent Similarity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Combined Percent</th>
<th>Amino Acid Overlap</th>
<th>Molecular Weight</th>
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<td>745</td>
<td>81,000&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>69.0</td>
<td>741</td>
<td>82,200&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>701</td>
<td>84,500&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>CS3-3</td>
<td><em>E. coli</em></td>
<td>28.9</td>
<td>46.7</td>
<td>75.6</td>
<td>373</td>
<td>48,000&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>CS3-4</td>
<td><em>E. coli</em></td>
<td>29.3</td>
<td>48.4</td>
<td>77.7</td>
<td>264</td>
<td>33,000&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>CS3-5</td>
<td><em>E. coli</em></td>
<td>34.0</td>
<td>46.7</td>
<td>80.7</td>
<td>145</td>
<td>20,000&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>CS3-2</td>
<td><em>E. coli</em></td>
<td>30.7</td>
<td>63.2</td>
<td>93.9</td>
<td>547</td>
<td>63,000&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent identity and similarity refer to the percent of identical and conserved amino acids, respectively, at the same position in pairwise comparisons of the SefC sequence to each of the other 8 proteins listed in Release 16 of SWISS-PROT and Release 64.3 of GenPept (GenBank, Intelligenetics, Mountain View, CA).

<sup>b</sup> Norgren <i>et al.</i>, 1987
<sup>c</sup> Mooi <i>et al.</i>, 1986
<sup>d</sup> Rossendaal and DeGraaf, 1989
<sup>e</sup> Allen <i>et al.</i>, 1991
<sup>f</sup> Jalajakumari <i>et al.</i>, 1989
ala22 (SefA), ser24-ser25 (SefB) and gly30-ser31 (SefC) were determined by the method of von Heijne (1984; Fig. 11A). However, comparison of the primary structure of SefB and SefC with the primary structure of several other periplasmic chaperones (Fig. 12) and fimbrial outer membrane proteins, respectively, suggested that other putative cleavage sites may exist between tyr40-gly41 (SefB) and between ala40-ser41 (SefC).

The nucleotide sequence of DNA upstream of sefABC revealed three open reading frames designated orfA, orfB, and orfC. orfC had the same translational polarity of sefABC unlike orfA and orfB which were both on the opposite strand and overlapped orfC (Fig. 10). The genes orfA and orfC were 219 bp and 327 bp, respectively, and encoded proteins with the predicted molecular weights of 7,900 M₉ and 12,300 M₉, respectively. Only orfA was preceded by a Shine-Dalgarno consensus sequence for translation initiation (Fig. 13A). A computer search for promoter sequences revealed a putative promoter located partially in the left terminal inverted repeat on the 5' side of orfA. The -10 promoter region and the Shine-Dalgarno sequence were contained in a stem-loop structure (Fig. 13B). The gene, orfB, occurred in frame -1 with respect to orfA and exhibited three possible initiation codons designated B₁, B₂ or B₃ (Fig. 13A). Depending on where translation was initiated, a protein of 41,000, 35,000 or 33,000 M₉ would be synthesized. All three ORFs were contained on a 1232 bp DNA fragment which was flanked by
imperfect inverted repeats (IR) (Fig. 13A). The left IR (IRL) and right IR (IRR) were both 39 bp with 21 bp matching and were 90% and 85% identical to the respective left and right terminal inverted repeats of IS3 from *E. coli*. Thus, an IS3 element was located upstream of *sefA*.

Comparison of the predicted amino acid sequences of OrfA, OrfB and OrfC to protein sequences listed in Genbank, SWISS-PROT and GENPEPT databases showed that OrfA and OrfC were unique. The lack of a protein match for OrfA was surprising considering that of the total 219 bp, 172 bp of *orfA* were identical to the corresponding DNA sequence from *orfII* of *E. coli* IS3. The difference in DNA sequence also accounted for the different lengths of the protein each gene encoded: OrfA (73 aa) and OrfII (99 aa). The lack of a match for OrfC was less surprising since the position of *orfC* within the IS3 element did not correspond to the position of *orfIII* in the IS3 element of *E. coli*.

In contrast to the uniqueness of OrfA and OrfC, OrfB displayed similarity to the putative transposases of insertion sequences from *E. coli*, *Erwinia*, *Acinetobacter*, *Lactococcus*, *Leptospira*, *Shigella*, *Bacillus*, *Corynebacterium*, *Pseudomonas*, *Mycoplasma*, *Mycobacterium*, *Lactobacillus*, *Agrobacterium* and *Pediococcus* whose transposases were members of the IS3 family of transposases. In fact, the predicted amino acid sequence of OrfB was found to be 80.5% identical (exact matches) to the primary sequence of OrfI, the putative transposase for IS3 from *E. coli* (Table 7). The alignment of the
**Fig. 13.** A. Nucleotide sequence of the IS3 element. Consensus Shine-Dalgarno (RBS) sequences and the -10 region are underlined and the termination codons are indicated as XXX. The direction of each ORF is indicated by the short arrows whereas the two long arrows represent the 39 bp inverted repeats. The numbering is only for reference and does not correspond to the position of the IS3 element relative to *orfA*. B. Schematic representation of the proposed secondary structure within the left IR of the IS1 mRNA. The structure encompasses the RBS and the -10 region of the promoter for *orfA*, as indicated by the arrows.
Table 7. A comparison of the predicted amino acid sequence for OrfB with those of IS3 family transposases.

<table>
<thead>
<tr>
<th>Putative Transposase</th>
<th>Bacterial Strain</th>
<th>IS element</th>
<th>Percent Identity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent Similarity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Combined Percent</th>
<th>Amino acid Overlap</th>
<th>Total Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>OrfI</td>
<td>E. coli</td>
<td>IS3</td>
<td>80.5</td>
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<td>277</td>
<td>288</td>
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<tr>
<td>OrfB</td>
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<td>Tn5393</td>
<td>40.3</td>
<td>16</td>
<td>56.3</td>
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<tr>
<td></td>
<td>Acinetobacter calcoaceticus</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Protein 1</td>
<td>Lactococcus lactis</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactococcus lactis</td>
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<td>13.8</td>
<td>45.6</td>
<td>305</td>
<td>384</td>
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<tr>
<td>OrfB</td>
<td>Leptospira interrogans</td>
<td>IS600</td>
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<td>18.1</td>
<td>54.4</td>
<td>256</td>
<td>272</td>
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<tr>
<td>Protein B</td>
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<td></td>
<td></td>
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<td>Orf1</td>
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<td>Orf2</td>
<td>Streptococcus agalactiae</td>
<td>IS861</td>
<td>32.4</td>
<td>19</td>
<td>51.4</td>
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<td></td>
<td>E. coli</td>
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<td>98</td>
<td>195</td>
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<td>Lactobacillus sake</td>
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<td>30.4</td>
<td>21.1</td>
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<td>302</td>
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<td>Mycoplasma hyopneumonia</td>
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<td>21.5</td>
<td>48.6</td>
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<td>56.3</td>
<td>103</td>
<td>400</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent identity and similarity refer to the percent of identical and conserved amino acids, respectively, at the same position in pairwise comparisons of the OrfB sequence to each of the other IS3 family transposases in the Blast search from March 13, 1995.
two proteins suggested that translation of orfB was initiated at B₃ resulting in the production of a 33,000 Mᵣ protein. However, frameshift analysis suggested that translation of orfA was initiated at B₂. In the IS3 element from E. coli, the start and stop codons for orfl and orfll, respectively, overlap and a frameshift event fuses the products of the two genes. In the IS3 element from S. enteritidis, the B₂ start codon of orfB and the stop codon of orfA overlap suggesting that a frameshift could fuse the products of these two genes. In addition, the stop codon for orfA was located in a stem loop structure that could facilitate the frameshift event by causing the elongating ribosome to slow down or pause. However, the alignment of OrfB and the transposases of the insertion sequences from Lactococcus lactis (384 aa), Lactobacillus sake (302 aa), Mycoplasma hypopneumoniae (275 aa) as well as from IS1203 of E. coli 0111:H- (296 aa), IS629 of Shigella sonnei (296 aa) and IS347 of E. coli (240 aa) suggested that translation of orfB was initiated at B₁ to produce a 41,000 Mᵣ protein. Unfortunately, the lack of potential ribosome binding sites and promoters upstream of the three potential translation initiation sites prevented the identification of the true start site. Further protein comparisons revealed that the translated protein sequence between B₁ and B₃ displayed 60% similarity to the M protein from Streptococcus pyogenes. However, the significance of the homology was unknown. In any case, this IS3 element represented the first such element to be found within the S. enteritidis chromosome.
A DNA sequence analysis of the 289 bp intergenic region between the IS3 sequence and \textit{sef} revealed several elements that were also present in the regulatory regions of other fimbrial operons. Alignment of the -10 region of the \textit{sefA} promoter (see Chapter III) to the -10 region of the promoters in the \textit{pap}, \textit{sfa}, \textit{daa} and \textit{fae} operons revealed that one of the two deoxyadenosine methylase (Dam) sites which played an essential role in the control of phase variation in Pap, S and F1845 fimbriae expression, was present in the \textit{sef14} regulatory region at a comparable position (Fig. 14). Two more GATC sites were also present in the \textit{sef14} regulatory region but these did not overlap the known GATC sites in the other operons. The GATC site located farthest from the \textit{sefA} promoter was actually located within the left inverted repeat of the IS3 element (Fig. 14). All three \textit{sef14} GATC sites were situated on inverted repeats, just like in the \textit{pap}, \textit{fae}, \textit{daa} and \textit{sfa} operons. However, the sequence of the inverted repeats was different from those in the other operons. Two consensus IHF binding sites were also present in the \textit{sef14} regulatory region, one of which was located at the same position as the second GATC site of the \textit{pap}, \textit{sfa}, \textit{daa} and \textit{fae} operons (Fig. 14).

The nucleotide sequence of DNA immediately downstream of \textit{sefABC} revealed a fourth open reading frame (ORF) designated \textit{sefD}. This gene had the same translational polarity as \textit{sefABC} (Fig. 10). In fact, the AUG start codon for \textit{sefD} overlapped the UGA stop codon of \textit{sefC} (Fig. 15). To eliminate the possibility that DNA rearrangement had occurred during cloning, the
Fig. 14. Nucleotide sequence of the intergenic region between sefA and the IS3 element. The numbers above the sequence refer to the position of the nucleotides relative to sefA. Each GATC site is in bold, the corresponding inverted repeats are indicated by the short arrows, the consensus IHF sites are represented by italicized letters and the consensus Shine-Dalgarno sequence for sefA is underlined as is the -10 region of the sefA promoter. The left inverted repeat for the IS3 sequence is indicated by the long arrow.
gene organization of pSC2 was confirmed by Southern blot analysis of Kpn I digested S. enteritidis 3b chromosomal DNA hybridized with sefA and sefD specific probes. Both probes hybridized to the same 10 kb Kpn I fragment indicating that the organization of sefABCD was identical in the sequencing template and the chromosome (Fig. 16). Preceding the sefD ORF by 8 bp was a Shine-Dalgarno consensus sequence for translation initiation (GGAG; Fig. 15). The sefD ORF was 447 bp and the predicted molecular weight of the encoded protein, designated SefD, was 16,722 Daltons. However, Western blot analysis of SefD production in S. enteritidis revealed that the protein migrated at approximately 18,000 M, (Fig. 26). The sequence data for sefD appeared in the EMBL/Genbank/DDBJ Nucleotide Sequence Data Libraries under the accession number U07129.

Comparison of the predicted amino acid sequence of SefD to protein sequences listed in the Genbank, SWISS-PROT, and GENPEPT data bases showed that SefD was unique. However, SefD did display 24% identity and 44% similarity to 50 amino acids of Rns, a regulatory protein for the CFA/I fimbrial operon in E. coli (Caron et al., 1989). To determine if this similarity was significant, a radiolabelled rns gene probe was hybridized to genomic S. enteritidis DNA and to plasmid DNA including cos48, pSC6 and pKX1 under high stringency conditions. The probe hybridized to DNA from all four sources. At this point, the evidence suggested that SefD was the positive regulator of the sef14 operon. However, further analysis revealed that the rns
**sefC**

3900 3920 3940
TTTTTACTCTCTTTCCGATAAAAAACAAGGCAGATTAGCTTTTTAAMGGAGTGTGCAA

**sefD**

3960 3980
ATG AAT CAG TAT AAT TCG TCA ATA CCT AAG TTC ATT GTC TCT GTT
met asn gln tyr asn ser ser ile pro lys phe ile val ser val

4000 4020 4040
TTT CTG ATT GTT ACT GGT TTT TTC AGC TCA ACT ATT AAA CCA CAA
phe leu ile val thr gly phe ser ser thr ile lys ala gln

4060 4080
GAA CTT AAA TTA ATG ATT AAA ATA AAT GAG GCT GTT TTT TAT GAC
glu leu lys leu met ile lys ile asn glu ala val phe tyr asp

4100 4120
CGT ATT ACA ACT AAT AAA ATA ATA GGT ACG GGG CAT CTA TTT AAC
arg ile thr ser asn lys ile ile lys ile ser ser leu glu lys ile

4140 4160
AGA GAG GGA AAA AAA ATC CTC ATT AGT AAT AAA ATA ATA GGT ACG GGG CAT CTA TTT AAC
arg glu gly lys leu lys leu ser ser ser leu glu lys ile

4180 4200
AAA AAT ACC CCA GGG GCA TAT ATT ATT GAG GGT CAG AAT AAC TCA
lys asn thr pro gly ala his lys ile ile ser ser leu glu lys ile

4240 4260
GCC CAT AAG CTT AGG ATA AGA ATA GAT CCA GGA GAA GAC ATT CCT GTT GAC
ala his lys leu arg ile arg ile gly gly glu asp trp gln pro

4280 4300
GAT AAT TCA GGT ATT GTG GTA TCT CAT TCT GAT TTT ACT AAT
asp asn ser gly ile gly met val ser his ser asp phe thr asn

4320 4340
GAA TTT AAT ATT TAT TTT TTT GGA AAT GGA GAC ATT CCT GGT GAC
glu phe asn ile tyr phe phe gly asn gly asp ile pro val asp

4360 4380 4400
ACA TAT TTA ATA AGC ATA TAT GCC ACA GAA ATT GAA TTA TAA TAA
thr tyr leu ile ser ile tyr ala thr glu ile glu leu XXX XXX

**Fig. 15.** Nucleotide sequence of *sefD*. The consensus Shine-Dalgarno sequence is underlined as is the *sefC* stop codon which overlaps the ATG start codon for *sefD*. The two consecutive termination codons for *sefD* are indicated as XXX. The numbers above each line refer to the nucleotide position of the contiguous sequence.
Fig. 16. Southern blot hybridization of Kpn I digested S. enteritidis DNA with sefA and sefD probes. The arrow indicates the 10 kb fragment that hybridizes to the radiolabelled sefA and sefD gene probes.
probe was hybridizing to the sefA gene and not to the sefD gene thereby eliminating the hypothesis that sefD encoded a regulatory protein. The hybridization of rns to sefA remains a mystery since Rns and SefA do not display significant amino acid homology [non-specific hybridization has also been seen by Dr. J.R. Scott (Emory University Health Sciences Center, GA); personal communication]. Further evidence that SefD was not the regulator came from the predicted amino acid sequence of SefD which had a putative signal peptidase cleavage site between Ser-24 and Ser-25 as determined by the method of von Heijne (1984) (Fig. 15). The presence of a putative leader sequence suggested that the protein was exported from the cytoplasm to either the periplasmic space or the outer membrane.

Analysis of the DNA sequence downstream of sefD revealed two open reading frames that were designated sefE_1 and sefE_2 (Fig. 17). Further analysis of these ORFs revealed that they had the opposite translational polarity as sefABCD (Fig. 10), were 351 and 462 bp in size and encoded proteins 117 and 154 amino acids in size, respectively. Comparison of these predicted amino acid sequences to protein sequences listed in the Genbank, SWISS-PROT and GENPEPT data bases, showed that SefE_1 and SefE_2 were similar to members of the AraC family of transcriptional regulators including VirF from Shigella sp. and CfaD, Rns, AggR, FapR, CsvR, PerA, EnvY, AppY and AdiY from E. coli, UreR of Proteus mirabilis and TcpN of Vibrio cholerae. The predicted amino acid sequence of both SefE_1 and SefE_2 displayed similarity to VirF of Shigella
sp. and FapR of enterotoxigenic *E. coli* (Table 8). However, **SefE₂** also had 59%, 61% and 58% positive matches (identical residues and conservative replacements) with the C-terminal portions of CfaD, Rns and AggR, respectively, all transcriptional regulators of fimbriae operons in *E. coli* (Table 8). Secondary structure analysis of **SefE₂** revealed a helix-turn-helix motif that corresponded to the putative DNA binding domain in CfaD, Rns and AggR. Together, **SefE₁** and **SefE₂** contained 68% (56% identical and 12% conserved) of the residues in the consensus sequence that define proteins belonging to the AraC family. Thus, on the basis of DNA sequence analysis, **sefE₁** and **sefE₂** were distinct open reading frames. However, on the basis of amino acid sequence homology of the encoded proteins, these ORFs seemed to encode two separate parts of the same protein. Whether these two ORFs were the result of a sequencing error or whether they were vestigial genes or alternatively, whether they encoded two gene products was not clear from the results of this study. Clearly, further studies are required.

**B. In Vitro expression of sefA, -B, -C and sefD.** To confirm that **sefABC** and **sefD** encoded proteins of the predicted sizes, proteins were translated *in vitro* from pKX1 and the *Kpn I* fragment of pSC2. Using a cell free, coupled transcription-translation system, the proteins SefB and SefC were detected as [³⁵S]-radiolabelled proteins, whereas SefA was identified
Fig. 17. Nucleotide sequence of sefE₁ and sefE₂. The consensus Shine-Dalgarno sequence is underlined and the putative -10 region of the promoter is indicated in bold letters. The numbers above each line refer to the nucleotide position of the two sequences. The predicted amino acid sequences are shown below the DNA sequence.
Table 8. A comparison of the predicted amino acid sequences for SefE₁ and SefE₂ with those of AraC-like transcriptional regulators.

<table>
<thead>
<tr>
<th>Transcriptional regulator</th>
<th>Bacterial strain</th>
<th>Regulated system</th>
<th>Percent Identity</th>
<th>Percent similarity</th>
<th>Combined Percent</th>
<th>Amino acid Overlap</th>
<th>Total Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>VirF</td>
<td>Shigella sp.</td>
<td>Invasion protein synthesis</td>
<td>40.8</td>
<td>22.7</td>
<td>63.7</td>
<td>238</td>
<td>262</td>
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<tr>
<td>FapR</td>
<td>E. coli</td>
<td>Fimbriae biosynthesis</td>
<td>37.3</td>
<td>24.9</td>
<td>62.2</td>
<td>217</td>
<td>260</td>
</tr>
<tr>
<td>CfaD</td>
<td>E. coli</td>
<td>Fimbriae biosynthesis</td>
<td>46</td>
<td>24</td>
<td>70</td>
<td>130</td>
<td>265</td>
</tr>
<tr>
<td>Rns</td>
<td>E. coli</td>
<td>Fimbriae biosynthesis</td>
<td>45</td>
<td>23</td>
<td>68</td>
<td>135</td>
<td>265</td>
</tr>
<tr>
<td>AggR</td>
<td>E. coli</td>
<td>Fimbriae biosynthesis</td>
<td>44</td>
<td>24</td>
<td>68</td>
<td>128</td>
<td>265</td>
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<td>CsvR</td>
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<td>EnvY</td>
<td>E. coli</td>
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<td>E. coli</td>
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<td>20</td>
<td>56</td>
<td>109</td>
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<td>E. coli</td>
<td>Arginine decarboxylase</td>
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<td>253</td>
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<tr>
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<td>Proteus mirabilis</td>
<td>Urease biosynthesis</td>
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<td>105</td>
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<td>UreR</td>
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a. Percent identity and similarity refer to the percent of identical and conserved amino acids, respectively, at the same position in pairwise comparisons of the SefE₁ and SefE₂ sequences to each of the other AraC-like transcriptional regulators in the Blast search from March 15, 1995.
Fig. 18. Local alignment of SefE1 and SefE2 and two AraC-like transcriptional regulators. A. Schematic representation and aligned sequences (B) of fimbrial transcriptional regulators analyzed for statistically significant blocks of similarity. In the schematic (A), the linked sequences are indicated by the slightly thickened bar, while in the alignment (B), uppercase text is used to indicate residues that have been linked. Linking involves inserting gaps into the alignment to bring the subsequences of the block into alignment. The degree of similarity among the proteins in both panels A and B is indicated by the shading such that the darker the shading, the higher the interrelatedness of the sequences. The length of each predicted amino acid sequence is indicated on the right side of the schematic (A). The amino acid sequences of FapR and VirF were taken from Klaasen and de Graaf, 1990.
immunologically on a Western blot due to the absence of methionine and SefD was detected by both methods. Several translation products were identified (Fig. 19, lane 2). The 14,000 Mr and 18,000 Mr proteins were identified on Western blots as SefA (Fig. 19, lane 7) and SefD (Fig. 20), respectively. The 90,000 Mr protein was identified as SefC while the 70,000, 44,000 and 40,000 Mr bands were likely minor degradation products of SefC since these bands were absent when pSC1, which contained a deletion in sefC, was used as the template (Fig. 19, lanes 2 and 3). The 27,000 Mr protein was identified as SefB. The 16,000 Mr band seemed to be a minor degradation product of SefB as this band remained when pSC1 was used as a template (Fig. 19, lanes 2, 3). When the three DNase I deletion subclones, delB15, delB23 and delD10, were each used as templates, the bands for SefB, SefC and their minor degradation products were absent (Fig. 19, lanes 4-6) suggesting that sefA and/or its upstream region is necessary for the expression of sefB and sefC. Therefore, the in vitro transcription-translation analysis confirmed that the 14,000 Mr, 27,000 Mr and 90,000 Mr proteins were expressed from the 5.3 kb DNA fragment and that the 18,000 Mr protein was expressed from the 10 kb KpnI fragment as predicted from the DNA sequence analysis (Fig. 20).

C. Identification of SEF14 and SEF18 fimbriae. Immunogold labelling of S. enteritidis cells using polyclonal immune serum raised to the purified SEF14 fimbriae revealed thin, filamentous structures located on the cell
Fig. 19. Expression of the sfa, -B, and -C genes in an E. coli in vitro transcription-translation system; autoradiographs of $^{35}$S-methionine-labelled polypeptides, separated on 12.5% acrylamide gels. Lane 1, pTZ19; lane 2, pXX1; lane 3, pSC1; lane 4, delB15; lane 5, delB23; lane 6, delD10; lane 7, Western blot of the in vitro transcription-translation of pXX1 developed with antisera generated against denatured SEF14 fimbrin. Below the autoradiograph is a schematic of sfaABC showing the inserts of various deletion subclones used in the in vitro transcription-translation experiments. The size ($\times 10^3$) of the molecular weight markers is indicated on the left of the autoradiograph.
Fig. 20. Expression of the sefD gene in an E. coli in vitro transcription-translation system. A. Autoradiograph of 35S-methionine-labelled polypeptides separated on a 12.5% acrylamide gel; B. Western blot of the in vitro transcription-translation developed with antiserum generated against SefD. In both A and B the in vitro transcription/translation template was the 10 kb Kpn I DNA fragment. In the absence of this DNA template, no translation products were present. The size (x10^3) of the molecular weight markers is indicated on the left of the figures.
surface (Fig. 21A). *E. coli* cells hosting pKX1 or cos48 were shown by Western blot analysis to produce SefA (data not shown). Immunoelectron microscopic examination of *E. coli* HB101 containing cos48 revealed the presence of immunogold labelled SEF14 fimbriae on the cell surface (Fig. 21B). Examination of *E. coli* JM109 harboring pKX1 revealed the formation of surface blebs which were labelled with immunogold particles using antiserum generated against SEF14 (Fig. 21C). The latter results confirmed the presence of SefA but indicated that this clone did not encode all of the proteins required to assemble SefA monomers into intact SEF14 fimbriae on the cell surface. *E. coli* JM109 carrying the vector alone did not form these immunogold labelled blebs (data not shown). Therefore, *sefABC* are insufficient for SEF14 production but all genes necessary for SEF14 biosynthesis are encoded on the 44 kb fragment. Moreover, these results demonstrate that production of SEF14 fimbriae was not prohibited in *E. coli.*

SefD was localized to the outer cell surface by immunogold electron microscopy using polyclonal immune sera raised to affinity purified, recombinant SefD. These studies revealed filamentous, immunogold-labelled structures resembling fimbriae on the surface of *S. enteritidis* 3b cells (Fig. 22C). Thus, SefD was concluded to be the major subunit of these fimbriae-like structures designated SEF18 (*S. enteritidis* fimbriae-like proteins composed of 18,000 M, subunits). Interestingly, SEF18 was often concentrated at the junction of two adjacent cells or found between cells (Fig. 22D).
Fig. 21. Immunoelectron microscopy of negatively stained cells for SEF14 production. Cells were grown in 2.5 ml of colonization factor antigen static broth culture for 60 h at 37°C. *S. enteritidis* 27655-3b (A), *E. coli* HB101 containing cos48 (B), and *E. coli* JM109 containing pTZ19 carrying the 5.3-kb *S. enteritidis* fragment (C) were labelled with protein A-gold following incubation with immune serum to native SEF14 fimbriae. SEF14 fimbriae on cells incubated with preimmune serum were not labelled. Ammonium molybdate was used to negatively stain cells. Magnification: (A) x115,000; (B) x54,000; (C) x144,000.
Fig. 22. Analysis of SEF18 production by immunogold electron microscopy of negatively stained cells. *K. oxytoca* (A), *P. rettgeri* (B), *S. enteritidis* 3b (C), *E. coli* C600 (Appieyard, 1954) (D), and *E. coli* SM10 (Simon et al., 1983) (E), were labelled with protein A-gold (15 nm) following incubation with immune serum to denatured SEF18. SEF18 fimbriae on cells incubated with preimmune serum were not labelled. Magnification: A, x14,900; B, x18,000; C, x40,350; D, x23,100; E, x23,800.
S. enteritidis (C)

E. coli C600 (D)

E. coli SM10 (E)
Fig. 23. Cellular localization of SEF18. Western blot of samples taken during the purification of SEF14. Lane 1, supernatant of blended and centrifuged cells; lane 2, supernatant of ultracentrifuged cell membranes; lane 3, pellet of ultracentrifuged cell membranes; lane 4, supernatant of precipitated and centrifuged SEF14 (see SEF14 purification described in Materials and Methods for the details). The band is designated as SefD on the left.
Attempts to purify these fimbriae by the conventional fimbrial purification procedures failed. However, when cells were blended and then spun to equilibrium on a 15-75% sucrose gradient, SEF18 was localized to the outer membrane fraction. Furthermore, an analysis of cell fractions collected during the purification of SEF14 fimbriae revealed that SEF18 was almost exclusively associated with the membrane (Fig. 23).

Since S. enteritidis produces three other known fimbriae, SEF14 (Feutrier et al., 1986), SEF17 (Collinson et al., 1991), and SEF21 (Müller et al., 1991), Western blot studies were performed to determine if SefD was serologically distinct from the fimbrins of these other fimbriae. Western blot analysis revealed that the purified fimbriae SEF14, SEF17, and SEF21 did not react with polyclonal antisera raised to SefD (Fig. 24A). Similarly, purified SefD did not react with immune sera raised against purified SEF14 (SefA), SEF17 (AgfA), or SEF21 (FimA) (Fig. 24B). In lane 1 of Fig. 24A, two high MW bands were detected in addition to the expected 18,000 Mr band. The anti-SefD antisera was generated using the purified 18,000 Mr, Factor Xa cleavage product which consisted of SefD plus 4 amino acid residues from MalE'. Thus, the two bands are present due to cross-reactivity of anti-SefD with MalE' (44,000 Mr) and the uncleaved MalE'-SefD fusion (70,000 Mr). In Fig. 24B, the lanes labelled SefD contained Factor Xa cleaved MalE'-SefD. In lane 5, two bands were present due to cross reactivity of anti-SEF21 with MalE' (44,000 Mr) released by the Factor Xa cleavage and with the MalE' in the
Fig. 24. An analysis of the serological cross-reactivity between the four fimbriae of S. enteritidis 3b and their antisera.
A. Western blot analysis of Factor Xa-cut MalE'-SefD fusion (lane 1), purified SEF14 (lane 2), SEF17 (lane 3) and SEF21 (lane 4) with anti-SefD antiserum.
B. Western blot analysis of SefD with SEF14 (lanes 1 and 2), SEF17 (lanes 3 and 4) or SEF21 (lanes 5 and 6) antisera. SefA, AgfA and FimA are the subunits of SEF14, SEF17 and SEF21 fimbriae, respectively. In both (A) and (B), the sizes (kDa) of the molecular mass markers are indicated on the left.
fusion protein (70,000 M_r) that was not completely cleaved with Factor Xa. Anti-SEF21 did not cross react with SefD since an 18,000 M_r band was not detected. The lack of immunological cross-reactivity between the three fimbriae, SEF14, SEF17, and SEF21 and SEF18 was also demonstrated by immunogold EM (data not shown). Thus, SefD is serologically distinct from SefA, AgfA, and FimA and morphologically distinct from SEF14, SEF17, and SEF21. Furthermore, the SEF14 gene cluster is the first example of an Enterobacteriaceae fimbrial gene cluster to encode two unique fimbrin-like proteins which are assembled into two distinct cell surface structures, SEF14 and SEF18.

D. Characterization of TnphoA mutants. S. enteritidis strains defective in the production of SEF14 were generated by TnphoA mutagenesis. Western blot analysis showed that four mutants did not produce SefA, although they still produced AgfA, SefD and FimA fimbrins of SEF17, SEF18 and SEF21, respectively (Table 9). The mutants were further analyzed to determine the location of the insertion. The sefA gene sequence upstream of TnphoA in all four mutants was amplified by PCR using probes designed from the DNA sequence at the 5' end of sefA and from the IS50_L sequence of TnphoA located at the junction of TnphoA-generated alkaline phosphatase gene fusions. The PCR amplification resulted in the production of a major DNA fragment of approximately 300 bp for TnphoA 1-2, 350 bp for TnphoA 1-
Table 9. Summary of the fimbrins and fimbriae produced by the TnphoA and Tn10 mutants of S. enteritidis.

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The presence of fimbrin subunits SefA, AgfA, SefD and FimA from SEF14, SEF17, SEF18 and SEF21 fimbriae, respectively was determined by Western blot analysis of whole cells. The presence of the fimbriae was determined by immunogold EM analysis of negatively stained whole cells.

Fig. 25. PCR amplification of DNA fragments from S. enteritidis 3b TnphoA mutants harboring TnphoA within sefA. The PCR products generated by the primers ST2 and TnphoA are from TnphoA 2-3 (lane 1), TnphoA 1-16 (lane 2), TnphoA 1-11 (lane 3), TnphoA 1-2 (lane 4); S. enteritidis 27655-3b (lane 5); no DNA control (lane 6). DNA size markers generated by Msp I-digested pBR322 are shown on the left in base pairs.
16 and TnphoA 2-3 and 500 bp for TnphoA 1-11 (Fig. 25). This data indicates that the four S. enteritidis TnphoA mutants unable to produce the SefA fimbrin of SEF14 fimbriae are sefA insertional mutants.

E. Distribution of sefD and SefD. The PCR amplified sefD gene was hybridized to DNA from a total of 73 bacterial strains represented as DNA dot blots on nylon membranes under high stringency conditions (Table 10). The sefD gene probe hybridized to all members of the family Enterobacteriaceae tested with the exception of Serratia marcescens. Strong hybridization signals were obtained with DNA isolated from S. enteritidis, S. dublin, S. pullorum, S. gallinarum, and S. berta as well as with DNA from Klebsiella pneumoniae and Shigella sonnei. DNA isolated from various E. coli strains and the other Salmonella species hybridized to the sefD probe with moderate intensity whereas DNA isolated from Enterobacter, Citrobacter, Erwinia, Hafnia, Providencia, Proteus and Shigella sonnei hybridized weakly to the sefD probe (Table 10). DNA preparations from strains of other eubacteria did not hybridize to the sefD probe. Western blot analysis of these strains confirmed the expression of an 18,000 Mr protein that was strongly cross-reactive with SefD (Table 10 and Fig. 26). Proteus vulgaris and Providencia rettgeri produced an immunologically cross-reactive band that migrated with an apparent molecular weight of 17,000. Those strains negative for sefD by DNA dot blot analysis were also negative for SefD production as determined by
Table 10. The Distribution of sefD and SefD among *Salmonella* isolates and other eubacteria

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<th>SefD cross-reactive proteinb</th>
<th>Hybridizationc to sefD probe</th>
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**Other Enterobacteriaceae**

**Citrobacter**

freundii

8090

**Enterobacter aerogenes**

UVic + +

**Erwinia carotovora**

UVic + +

**Escherichia coli**

11775

ATCC + ++

C600

ATCC + nt

HB101

ATCC nt ++

E1049a-13

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438Hf

TTT + ++

B41M

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HM1475

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Vietman I/1

LE + ++

Gambia G3

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NG7c

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NG7c1

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**Haemophilus alvei**

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**Klebsiella pneumoniae**
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**Other eubacteria**

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a. ATCC, American Type Culture Collection; BBF, B.B. Finlay, Biotechnology Laboratory and Departments of Biochemistry and Microbiology, University of British Columbia, Vancouver, British Columbia, Canada; JT, J. Tomas, Departement Microbiologie, Universitat de Barcelona, Barcelona, Spain; LCDC, H. Lior, Laboratory Center for Disease Control, Ottawa, Ontario, Canada; LE, L. Emödy, University Medical School, Institute of Microbiology, Pécs, Hungary; PVL, G. Thiele, Provincial Veterinary Laboratory of British Columbia, Abbotsford, British Columbia, Canada; TT, T. J. Tomas, Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada; TW, T. Wadström, University of Lund, Lund, Sweden; UVic, Culture Collection of the Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada; WWK, W.W. Kay, Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada.

b. Strains grown aerobically overnight in LB were screened for immunoreactive SefD proteins by Western blot analysis as described in the text. Production of SefD cross-reactive proteins was recorded as positive (+), negative (-) or not tested (nt).

c. Hybridization of membrane-bound bacterial DNA to the 442-bp *sefD* probe. The results of the hybridization are reported as weakly (+), moderately (++), or strongly (+++) positive or negative (-).

d. The SefD immunologically cross-reactive band migrated with an apparent MW of 17,000 rather than with an apparent MW of 18,000 as observed with S. *enteritidis* 27655-3b SefD fimbrin.
Fig. 26. Analysis of SEF18 production by enterobacteria. Western blot analysis of whole-cell lysates of *E. coli* C600 (lane 1), *K. pneumoniae* 13883 (lane 2), *S. sonnei* (lane 3), *S. enteritidis* 27655-26 (lane 4), *S. marcescens* (lane 5), *Citrobacter freundii* 8090 (lane 6), *Erwinia carotovora* (lane 7), *Hafnia alvei* (lane 8), and *P. rettgeri* (lane 9) with anti-SefD antiserum. The source of each strain can be found in Table 7. The sizes of the molecular mass markers are indicated on the left in kDa.
Western blot analysis (Table 10 and Fig. 26). Subsequent immunogold EM studies indicated that SEF18 fimbriae-like structures were also produced on the surface of *E. coli* (Fig. 22D and E), *Klebsiella oxytoca* and *Providencia rettgeri* (Fig. 22A and B). The apparent morphology and distribution of the immunogold labelled structures on these strains varied from filamentous structures of variable length radiating from the cell to amorphous structures (Fig. 22).

**F. Mapping sefA and sefD.** The *sefA* and *sefD* radiolabelled probes were hybridized to DNA isolated from *S. enteritidis* RKS53, *S. enteritidis* 27655-3b, *S. typhimurium* LT2 and *E. coli* C600, digested with either *Xba* I or *Bln* I and separated by pulsed field gel electrophoresis. The 16 *Xba* I and 12 *Bln* I fragments had been given a letter and arranged in alphabetical order around a circular genomic map of the chromosome of *S. enteritidis* by Liu et al. (1993b). To determine which digestion fragments hybridized to the probes, the Southern blots were initially hybridized to the radiolabelled *sefA* or *sefD* probes, exposed to X-ray film and then hybridized to radiolabelled, whole *S. enteritidis* DNA. By overlaying the autoradiogram obtained from the *sefA*-or *sefD* probed blot on the autoradiogram obtained by probing the same blot with the radiolabelled *S. enteritidis* DNA and comparing the results to ethidium bromide stained gels of digested genomic *S. enteritidis* DNA whose bands had been assigned a letter, the *sefA*- and *sefD*-positive bands
Fig. 27. Identification of chromosomal fragments containing sefA (A) or sefD (B) by Southern blot hybridization. Autoradiographs of Xba I or Bln I digests of genomic DNA from S. typhimurium LT2 (Lanes 1 and 2), E. coli C600 (Lanes 3 and 4), S. enteritidis RKS53 (lanes 5 and 6) or S. enteritidis 27655-3b (lanes 7 and 8), separated by PFGE. The identity of the sefA or sefD positive fragments are indicated by the arrows and letters on the right and left sides of each autoradiograph.
S. typhimurium
E. coli
S. enteritidis

X B X B X B
X B
S. enteritidis
Fig. 28. Position of the sefA gene cluster on the Xba I-Bln I genomic cleavage maps of S. enteritidis. The genomic cleavage map previously determined for S. enteritidis SSU7998 (Liu et al., 1993b) is shown in linear form, with the physical map divided into 100 centisomes (CS). Each CS of the S. enteritidis chromosome is 46 kb (Liu et al., 1993b). The thr gene is located at 0 CS and the previously determined position of relevant genes or Xba I sites (designated sXba I-1, sXba I-4 or sXba I-15 for sites 1, 4 or 15, respectively, located clockwise around the chromosome from thr) are noted on the right of the S. enteritidis map (Liu et al., 1993b). The newly mapped genes are indicated on the left of the linear map. The sefA and sefD genes are located on Xba I fragments N and O, respectively, of S. enteritidis on either side of the Xba I site, sXba I-15.
corresponded to the Bln I fragment designated A. The Xba I-N fragment overlapped the Bln I-A fragment in the genomic cleavage map of S. enteritidis (Fig. 28). The sefA probe did not hybridize to the S. typhimurium or E. coli DNA. In contrast, the sefD probe hybridized to DNA from the two strains of S. enteritidis as well as to DNA from E. coli C600 but not to DNA from S. typhimurium LT2 (Fig. 27B). The sefD probe hybridized to one Xba I band designated O and to one Bln I band designated A from the two S. enteritidis strains. These two DNA fragments overlapped in the genomic cleavage map of S. enteritidis (Fig. 28). The hybridization of the sefA and sefD probes to different Xba I restriction fragments was not surprising since an Xba I cleavage site was found in sefC through restriction enzyme cleavage site analysis of the DNA sequence for sefABCD. An analysis of where the fragments overlapped revealed that the sefA and sefD genes were localized on the genomic map to between the twelfth Bln I and sixteenth Xba I cleavage sites which corresponded to approximately 4500 kb or 90 centisomes (CS) of the circular chromosome (Fig. 28). In addition, these results showed that the SEF14 gene cluster ran clockwise from sefA to sefD in the genomic map of S. enteritidis.

3. DISCUSSION

A. Nucleotide sequence and protein determination. The nucleotide sequence of four genes, sefABCD, two ORFs, sefE₁ and sefE₂, and the adjacent IS₅ element represents the first sequence of a fimbrial operon from the
important invasive enteropathogen *S. enteritidis*. The gene *sefA* encodes a novel protein whose predicted *M*<sub>r</sub> and amino acid composition matches that previously reported for the SEF14 fimbrin (Feutrier *et al.*, 1986). Furthermore, the first 60 predicted amino acids are identical to the N terminal amino acid sequence reported for the SEF14 fimbrin (Feutrier *et al.*, 1986). These results demonstrate that *sefA* encodes the structural subunit of SEF14 fimbriae, SefA.

The adjacent, downstream gene, *sefB*, encodes a fimbrial periplasmic chaperone protein and represents the first fimbrial periplasmic chaperone gene characterized from *Salmonella*. The amino acid sequence and *M*<sub>r</sub> of SefB are very similar to that of three fimbriae periplasmic chaperone proteins from *E. coli* and *K. pneumoniae*. Furthermore, the presence of invariant residues in SefB and the three previously described chaperone proteins suggests that these proteins are functionally similar. According to the well-characterized P fimbrin biogenesis system in *E. coli* (Kuehn *et al.*, 1992), the chaperone binds to pilus subunit proteins in the periplasmic space and modulates the assembly of fimbrin monomers into fimbriae without becoming a component of the final structure. Preliminary analysis suggests that SefB shares the same secondary structure characteristics of PapD including two anti-parallel β-sheet-containing domains separated by a linker consisting of a number of hydrophobic amino acids. Interestingly, the blocks of similarity between SefB and PapD correspond to areas predicted to contain either a β-sheet or an α-helix in both proteins suggesting that the interaction
between fimbrial chaperone proteins and their respective fimbrial subunit(s) is similar even though the fimbrin amino acid sequences are unique. Perhaps, the C terminal region of each chaperone protein is responsible for unique fimbrin recognition since this area displays a minimal amount of mutual similarity among the four previously mentioned chaperone proteins.

\textit{sefC}, the gene immediately downstream of \textit{sefB}, encodes a protein that displays homology to fimbrial outer membrane proteins. Evidence suggesting that SefC is the outer membrane component of the \textit{sef14} operon includes the fact that SefC has nine putative membrane spanning domains (data not shown), a predicted signal sequence, and a high \( M_r \), comparable to those of analogous outer membrane proteins from other fimbrial operons. All fimbrial systems characterized to date encode a high molecular weight outer membrane protein, presumably essential for the assembly and surface localization of fimbriae (Low \textit{et al}., 1995; Van Rosmalen and Saier, 1994). The current hypothesis suggests that these outer membrane proteins play an active role in the dissociation of the chaperone from fimbrin and fimbrin accessory proteins as they are assembled into fimbriae (Hultgren and Normark, 1992). Thus, \textit{sefABC} comprises an important part of the \textit{sef14} gene cluster responsible for the expression and assembly of SEF14 fimbriae.

\textit{E. coli} carrying 44 kb of \textit{S. enteritidis} DNA (cos48) encompassing the \textit{sefABC} operon displays immunogold labelled SEF14 fimbriae. However, immunogold electron microscopy of \textit{E. coli} carrying the 5.3 kb DNA fragment
subcloned from cos48 shows that distinguishable SEF14 filamentous fimbriae are not assembled. Thus, accessory proteins must exist which are not encoded by the 5.3 kb fragment encoding sefABC. By analogy to other fimbrial systems, accessory proteins involved in the assembly of SEF14 fimbriae could include a protein(s) determining fimbriae length, an adhesin(s), an adaptor, proteins that initiate the assembly of the fimbriae filaments, and regulatory proteins (Low et al., 1995).

The genes, orfA, orfB and orfC, form overlapping ORFs which are flanked by imperfect inverted repeats of 39 bp. orfA and orfC encode unique proteins whereas orfB encodes a protein that displays amino acid sequence similarities to IS3 family transposases. The inverted repeats, the overlapping ORFs and the sequence homology are all characteristics shared by IS3 family elements. Transposable elements often effect the expression of genes adjacent to the insertion site. Some elements such as IS1163 (Skaugen and Nes, 1994) have a negative effect whereas others such as IS3 (Aronson et al., 1989; Charlier et al., 1982) stimulate transcription of adjacent genes using promoters that mediate outwardly directed transcription or by the formation of hybrid promoters during the insertion process. In two cases, IS insertions are biologically significant for the control of fimbriae production. The presence of the two nontandem, inverted IS1 elements in the fae regulatory region enhances K88 fimbriae production and may explain the absence of phase variation in K88 expression (Hulsman et al., 1994). Similarly, an IS1 element
activates 987P fimbriae expression. Although the mechanism is unknown, the IS1 element activates expression of fapR, the gene encoding a regulatory protein that activates the silent promoter of the 987P fimbrial gene cluster (Klaasen and De Graaf, 1990; Klaasen et al., 1990). Unfortunately, the effect, if any, of the IS3 element on sef14 expression is unknown.

Little is known about the mechanisms that regulate the expression of the sef14 gene cluster. DNA sequence analysis of the intergenic region between the IS3 sequence reveals several regulatory elements that may be involved in controlling sef14 expression. Because GATC sites occur only once every 256 bp in E. coli, the presence of three GATC sites within 193 bp of sef14 DNA is unusual. A cluster of deoxyadenosine methylase (Dam) sites, GATC I and GATC II, is also present in the pap (Blyn et al., 1990), sfa (Schmoll et al., 1990a), daa (Bilge et al., 1993b) and fae (Hulsman et al., 1994) operons. Differential methylation of these sites regulates the expression of these fimbriae operons. In the pap operon, if Lrp and PapI bind to GATC I, their binding inhibits methylation of this GATC site by Dam and facilitates the formation of an active transcriptional complex. However, if this GATC site is first methylated by Dam, binding of Lrp and PapI is inhibited (Van Der Woude et al., 1992). The presence of these conserved GATC box domains within sef14 suggests that this gene cluster may be subject to a similar regulatory control mechanism involving Lrp and DNA methylation. Furthermore, the presence of IHF binding sites suggests that IHF may also be
involved in regulating sef14 transcription, possibly by facilitating the bending of DNA to form a transcriptionally active complex with Lrp and RNA polymerase. However, further studies on the methylation state of the GATC sites and the effect of Lrp and IHF on sef transcription will be required to determine if and how these proteins affect transcription of the sef14 operon.

The gene, sefD, is found downstream of sefABC. The ORF demonstrates the same translational polarity as sefABC. Furthermore, the start codon for sefD overlaps the translational stop codon for sefC. Consequently, sefD is characterized as a member of the sef14 gene cluster. SefD, the protein encoded by this gene, is unique and is assembled into thin, fimbriae on the cell surface of S. enteritidis 27655-3b. These newly characterized fimbriae are biochemically and serologically distinct from SEF14 (Feutrier et al., 1986), SEF17 (Collinson et al., 1991) and SEF21 (Müller et al., 1991), the three known fimbrial types produced by S. enteritidis 27655-3b. Thus, these filaments represent the fourth fimbrial produced by this enteric pathogen and have been given the trivial name SEF18 (S. enteritidis fimbriae composed of 18,000 M, subunits). Furthermore, the SEF14 cluster is the first example in the Enterobacteriaceae of a fimbrial gene cluster that encodes two fimbrins which are assembled into two distinct cell surface structures, SEF14 and SEF18.

The presence of two fimbrins encoded in one fimbrial operon is
unusual for the fimbrial operons of the Enterobacteriaceae. However, this genetic organization is common in fimbrial operons of non-enteric bacteria such as *Moraxella bovis* (Fulks et al., 1990), *Bacteroides nodosus* (Hobbs et al., 1991), and *Neisseria gonorrhoeae* (Segal et al., 1986). *Bacteroides nodosus* produces two classes of type 4 fimbriae. Class II strains carry a fimbrial operon encoding two subunit genes *fimA* and *fimZ* having the same translational polarity. The purpose of the second subunit gene is unknown, but Hobbs et al (1991) suggest FimZ may be an aberrant subunit that disrupts the structure or assembly of the fimbrial strand during assembly-disassembly cycles. This situation seems unlikely for SefA and SefD as SefD assembles into fimbriae distinct from SEF14.

Similarly, *Moraxella bovis* produces two antigenically distinct fimbriae called Q and I (Fulks et al., 1990). The I fimbrial gene represents a reserve cassette of variant sequences to increase the scope for structural variation as was initially characterized in *N. gonorrhoeae* (Segal et al., 1986). SEF14 and SEF18 may be involved in antigenic variation but how *sefD* might be preferentially expressed over *sefA* is unknown.

Although it is not known whether the assembly of SEF14 and SEF18 is mediated by SefB and SefC or whether SEF18 has its own assembly-specific proteins, the unique genetic organization raises interesting questions regarding the relative regulation and expression of each protein. Western blot and immunogold EM analysis of chromosomal Tn*phoA* and Tn10
mutants of *S. enteritidis* unable to produce SEF14 indicate that SEF18 is still expressed and assembled on the cell surface. Similarly, SEF14 fimbriae are not expressed at temperatures below 28°C whereas SEF18 is produced independent of growth temperature. Furthermore, *sefBC*, the genes encoding the two proteins involved in transport and assembly, are found only in those *Salmonella* species that express *sefA*. Thus the preliminary data suggests that SEF14 and SEF18 are independently expressed and assembled.

The function of SEF18 is unknown, however the apparent localized concentration of these fimbriae at the junction between adjacent cells suggests that they may play a role in cell-cell adherence. Furthermore, attempts to purify SEF18 using the conventional fimbriae purification procedures of blending or heat treatment (Korhonen *et al.*, 1983) were not successful. Thus, these structures appear to be anchored more firmly in the outer membrane, a characteristic that may enhance the stability of cell-cell interactions. Fimbriae, unrelated to SEF18, have also been implicated in the role of cell aggregation (Collinson *et al.*, 1993; Girón *et al.*, 1991). Perhaps the SEF18 fimbriae maintain *S. enteritidis* cell aggregates while other fimbriae engage specific receptors on the intestinal epithelial cell surface or other relevant surfaces. More detailed genetic analysis will be required to determine if SEF18 is involved in adhesion.

The ORFs, *sefE₁* and *sefE₂*, are found downstream of *sefD* but demonstrate the opposite translational polarity as *sefABCD*. The translated
protein sequences are similar to several proteins including VirF and FapR. VirF of *Shigella flexneri* positively regulates transcription of the genes involved in invasion of epithelial cells by shigellae (Adler et al., 1989) whereas FapR of enterotoxigenic *E. coli*, is the positive regulator of expression of the 987P fimbriae operon (Klassen and DeGraaf, 1990). Both proteins are members of the AraC family of transcriptional regulators which are characterized by the following features: 1) all members are positive transcriptional factors; 2) the C-terminal DNA binding domain is organized as a helix-turn-helix motif; and 3) transcription of most of the regulatory genes is divergent from the gene or operon they regulate (Gallegos et al., 1993). Conserved features of the putative DNA binding domain are found within the predicted sequences of SefE2 and putative transcription of the two open reading frames is divergent from *sefABCD*. However, further work is required to characterize these open reading frames before any comment can be made regarding the role, if any, of the putative translated products in the transcriptional regulation of the *sef14* gene cluster.

**B. Distribution of *sefD* and SefD.** SefD is assembled into thin, fimbriae on the cell surface of *S. enteritidis* 27655-3b. Phenotypic screening by Western blotting and genotypic screening by DNA hybridization show that SefD homologues are found in all members of the Enterobacteriaceae tested with the exception of *Serratia*. The widespread distribution and the antigenic
The conservation of SEF18 among the various members of the Enterobacteriaceae is remarkable. Type 1 fimbriae have also been observed on many different species of enterobacteria, however, DNA hybridization results have indicated that sequences encoding Type 1 fimbriae within these bacteria are not conserved (Clegg and Gerlach, 1987; Doran et al., 1994). Similarly, agglutination studies have demonstrated distinct serological groups which display little or no antigenic cross-reactivity (Clegg and Gerlach, 1987). Other fimbriae such as SEF17 and its E. coli homologue, curli, are even less widely distributed (Doran et al., 1993a). The conservation of the DNA sequence of the SEF18 subunit and the epitopes recognized by antibodies generated against SefD throughout the Enterobacteriaceae make SEF18 the most widely distributed and antigenically conserved fimbriae known thus far (Clouthier et al., 1994).
CHAPTER III

Analysis of sefA transcription

1. MATERIALS AND METHODS

A. Bacterial strains, plasmids, media and growth conditions.

Salmonella enteritidis 27655-3b was provided by T. Wadstrom (University of Lund, Sweden). cos48 consists of a 44 kb insert of S. enteritidis DNA cloned into pHC79 (Feutrier et al., 1988). pSFA was a gift from J.L. Doran (Microtek International Ltd.) and contains the 584-bp Dr I fragment of S. enteritidis 27655-3b DNA encompassing sefA and an additional 60-bp upstream region cloned into the Sma I site of pUC18.

Bacteria were grown statically in colonization factor antigen (CFA) broth (Evans et al., 1977) at various temperatures for 48-60 h. Cultures of cells containing cos48 were supplemented with ampicillin to a final concentration of 250 μg/ml. Stock cultures were stored in 15% glycerol broth at -70°C.

B. Primer extension of RNA transcripts. Primer extension reactions were performed according to the procedure of Sawers and Bock (1989) using RNA prepared as below and the sefA8 oligonucleotide (TGCGTGCCACTGCCACA) derived from the sefA gene sequence (Regional DNA Synthesis Laboratory, Calgary, AB). Three pmol of the oligonucleotide
(16.5 ng of an 18-mer) was end-labelled using 50 μCi of [γ-32P]ATP (Sambrook et al., 1989) and then incubated for 1 h at -70°C to 10 ng S. enteritidis RNA in the presence of 0.3 M sodium acetate, 10 mM MgCl₂ and 3 volumes 95% ethanol. The precipitated RNA was pelleted in the microfuge (15,000 x g, 30 min, 4°C), resuspended in 4.5 μl 100 mM KCl, 50 mM Tris HCl pH 8.3, denatured at 95°C for 30 s and annealed at 55°C for 20 min. 3.25 μl of the annealing reaction was added to 3.25 μl 250 mM Tris HCl pH 8.3, 250 mM KCl, 50 mM MgCl₂, 1.6 μl 0.1 M 1,4-dithiothreitol, 1.4 μl distilled water, 1.5 μl of each dNTP from 2 mM stocks (Boehringer Mannheim, Germany) and extended for 1 h at 42°C in the presence of 1 μl AMV reverse transcriptase (6.5U) (Amersham Canada Inc., Oakville, ON). The primer extension reactions were stopped upon addition of three volumes of 95% ethanol and 3 M sodium acetate to a final concentration of 0.3 M. Nucleic acids were precipitated, recovered by centrifugation (14,000 x g, 4°C, 30 min) and washed with 1 ml ice cold 70% ethanol. The extension products were dried by vacuum centrifugation (Speed Vac, Savant Instruments Inc., Farmingdale, NY), resuspended in 5 μl sequencing dye (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF), and applied to a 6% sequencing gel with sefA sequencing reaction mixes in adjacent lanes. The primer used in the sequencing reactions corresponded to that used in the primer extension reaction. The gels were dried for 2 h at 80°C and the results
were recorded by autoradiography on X-Omat AR5 film (Kodak, Rochester, NY).

C. DNA sequencing and computer analysis. The DNA in the region encompassing the sefA promoter was sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977), using T7 DNA polymerase (Promega, Madison, Wis.). The primer used in the sequencing reactions corresponded to that used in the primer extension reactions.

The RNAFOLD program of PC/GENE (Intelligenetics) was used to predict the secondary structures of the RNA sequence.

D. mRNA stability. Rifampicin was added to the S. enteritidis culture grown at 37°C for 48 h to a final concentration of 200 μg/ml. Cell growth was arrested at different times (0-90 min) by quickly mixing the cell culture samples with an equal volume of crushed frozen killer solution (10 mM sodium azide, 200 μg/ml chloramphenicol in minimal medium). RNA was then extracted, run on formaldehyde/agarose gels, transferred to nylon membranes and analyzed by Northern blot hybridization as described below.

E. Expression of SEF14 fimbriae. To investigate if there was a correlation between the growth temperature and phenotypic expression of SEF14 fimbriae, S. enteritidis 27655-3b cells were grown statically in CFA broth.
at $28^\circ$, $30^\circ$, $32^\circ$, $35^\circ$ and $37^\circ$C for 60 h. 1 ml samples of each culture were centrifuged (14,000 x g, 2 min, 20°C), resuspended in 200 μl 1x SDS sample buffer, boiled for 10 min and subjected to electrophoresis as described below. SEF14 synthesis was detected by Western blotting with rabbit immune serum raised to purified SEF14 fimbriae as described below.

F. RNA extraction. RNA was prepared from whole cells by a modification of the procedure described by McCormick et al. (1991). Briefly, cells from 20 to 50 ml of a 48 h static CFA broth culture of S. enteritidis 27655-3b grown at $28^\circ$, $32^\circ$, $35^\circ$, or $37^\circ$C were lysed with a solution containing 1.5 μl β-mercaptoethanol, 5 μl RNasin® ribonuclease inhibitor (40,000 U/ml) (Promega Corp., Madison, WI) and 200 μl boiling buffer (140 mM NaCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl pH 8.6, 0.5% Triton X-100, 1 mM 1,4-dithiothreitol, 1% SDS). Protein was separated from the nucleic acid by three phenol:chloroform extractions. The nucleic acids were precipitated in 2.5 M ammonium acetate and two volumes of 95% ethanol at -70°C for 30 min. DNA was degraded with FPLC purified DNase I (Pharmacia, Uppsala, Sweden). Approximate RNA recovery was determined spectrophotometrically at 260 nm.
G. Electrophoresis and Northern transfer of RNA. The electrophoretic separation of extracted RNA and the subsequent Northern transfer to Hybond N+ (Amersham Canada Inc., Oakville, ON) membranes were performed as described in Fourney et al. (1992). RNA samples were concentrated in a Speed Vac concentrator (Savant Instruments Inc., Farmingdale, NY), adjusted to 5 μl with autoclaved water, added to 25 μl electrophoresis sample buffer (0.1 ml deionized formamide, 0.15 ml 10x 4-morpholinepropanesulfonic acid [MOPS], 0.24 ml formaldehyde, 0.1 ml deionized RNase-free water, 0.1 ml glycerol, 0.08 ml 10% (w/v) bromophenol blue), and heated at 65°C for 15 min. 1 μl of ethidium bromide solution (1 mg/ml) was introduced into each sample prior to electrophoresis.

The RNA was electrophoresed on a 1% agarose gel containing 1x MOPS and 0.66 M formaldehyde at 30V for 12 h. After electrophoresis, the gel was soaked for 20 min in 0.05 M NaOH made up in 1x SSC (0.15 M NaCl, 0.015 M tri-sodium citrate) and two 20 min periods in 10x SSC at room temperature with gentle shaking. The Hybond N+ nylon membrane (Amersham Canada Inc.) was prewet in distilled water and then soaked in 10x SSC for 5 min. The RNA was transferred to the membrane in 10x SSC by capillary action using a small pore sponge to enhance capillary action (from the bottom, the layers include sponge, 3 pieces of 3 MM paper, gel, nylon membrane, 3 pieces of 3 MM paper, 3 inches of paper towels and a weight of about 0.5 kg). The RNA was fixed to the membrane by baking for 2 h at 80°C under vacuum.
H. Northern blot hybridization. The DNA probe used to detect *sefA* mRNA was the 550-bp *sefA* gene fragment generated by *EcoR I* and *Hind III* restriction enzyme digestion of the recombinant plasmid pSFA. The *sefA* gene fragment was isolated by agarose gel electrophoresis (Sambrook *et al.*, 1989) and purified using the Sephaglas™ Bandprep Kit (Pharmacia, Uppsala, Sweden). This probe was then labelled with [α-32P]-dATP by nick translation (Pharmacia, Uppsala, Sweden) and denatured at 100°C for 3 minutes before being added to the blots.

After the RNA was transferred onto the nylon membrane, the filter was baked at 80°C for 2 h, pre-hybridized for 2 h at 65°C in a hybridization buffer containing 6x SSC (1x SSC is 0.15 M NaOH, 0.015 M tri-sodium citrate), 5x Denhardt, 20% formamide, 5% SDS and 15 mg/ml herring sperm DNA, and then hybridized at 65°C for 18 h in the same buffer containing the denatured, radiolabelled *sefA* probe. The hybridized filters were washed at 65°C in 2x SSC for 10 min, 0.2x SSC for 10 min followed by a 7 min wash in 0.2x SSC. The results were recorded by autoradiography on X-Omat AR5 film (Kodak, Rochester, NY).

I. Purification of SEF14 fimbriae. SEF14 fimbriae were isolated as previously described in the methods section of Chapter II.
J. Preparation of immune serum. Purified native SEF14 fimbriae and denatured SEF14 fimbrin protein preparations were used to generate immune sera in female New Zealand white rabbits as previously described in the methods section of Chapter II.

K. SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) as previously described in Chapter II.

2. RESULTS
   
   A. Mapping the 5' end of the sefA transcript. In vitro expression of sefA, -B and -C suggested that translation of SefB and SefC was dependent on the presence of sefA and/or the region upstream of sefA. To confirm this, the transcription start sites for sefA, sefB and sefC were determined. Primer extension studies using RNA isolated from S. enteritidis grown statically at 37°C consistently revealed transcription start sites immediately upstream of sefA. These included two major extension products as well as several minor ones (Fig. 29). When the primer extension reaction was performed at 50°C, a temperature expected to destabilize secondary structures, reverse transcriptase still stopped at all the sites with the same frequency suggesting that stem-loop structures were not blocking the migration of reverse transcriptase. No
transcription start sites could be found immediately upstream of *sefB* or *sefC* (data not shown). These results indicated that the 5' end of the mRNA transcript of *sefABC* was initiated upstream of *sefA*.

**B. Analysis of sefA mRNA stability.** The stability of the *sefA* transcript found in RNA isolated from *S. enteritidis* grown statically at 37°C was examined by Northern blot analysis with total RNA prepared at different time points after the addition of rifampicin (Fig. 30A). Hybridization with a *sefA*-specific probe revealed the presence of a major 0.5 kb transcript corresponding in size to that expected for a *sefA* (0.646 kb) mRNA species. 1.4 kb and 4.0 kb transcripts corresponding in size to the *sefAB* (1.395 kb) and *sefABC* (3.859 kb) transcripts, respectively, were also detected using the *sefA*-specific probe. Autoradiograms of the Northern blot revealed that the 0.5 kb *sefA* transcript could still be detected 90 min after the inhibition of transcription initiation. The kinetics of *sefA* mRNA decay were analyzed by plotting the decay rate, determined from densitometric tracings of the autoradiograms, against time (Fig. 30B). Due to the nonlinear distribution of points, a line that encompassed all the points could not be drawn. However, if the line was drawn through the first four time points, the half life of the *sefA* transcript, determined from the slope of this line, was approximately 24 min. The larger transcripts were only visible at t = 0.

A stable secondary structure formation was predicted to exist on the 3'
end of the sefA mRNA. Bases 636-689 (ΔG= -24.6 kcal/mol) and bases 691-746 (ΔG= -12.8 kcal/mol) were predicted to form a pair of stem-loop structures in the intercistronic region between sefA and sefB (Fig. 31). Following these putative stem-loop structures was a stretch of 10 uridine residues interrupted by two adenine residues (Fig. 11A). Regions unusually rich in adenine and uracil residues existed within these stem-loop structures (bases 679-709) reminiscent of RNase E cleavage sites.

C. Characterizing how temperature affects fimbriae expression.

a. Thermo-regulated expression of SEF14 fimbriae. Since environmental conditions influence the expression of many genes, the affect of temperature on the expression of SEF14 fimbriae was examined. To investigate the correlation between the growth temperature and phenotypic expression of SEF14 fimbriae, S. enteritidis was grown at 28°, 30°, 32°, 35° and 37°C. SEF14 synthesis was analyzed by Western blots using polyclonal immune serum raised to purified SEF14 fimbriae (Fig. 32). Western blot analysis revealed that the production of SEF14 was strongly dependent on growth temperature. SEF14 fimbriae were absent during growth at 28°C and 30°C but were produced in increasing quantities by cells grown at temperatures over 30°C (Fig. 32).
Fig. 29. Mapping of the 5' end of the sefA transcript using primer extension. The lane labelled S. enteritidis represents the reverse transcriptase products of RNA isolated from this organism grown in colonization factor antigen static broth for 60 h at 37°C. Lanes C, A, T, and G represent the results of dideoxy chain termination sequence reactions in the region encompassing the promoter. The sequence of the $\gamma^{32}$P-labelled primer (TGCGTGGGCACTGCCACA) is complementary to nucleotides 181 to 198 of sefA. Arrows indicate the two major transcription initiation sites.
Fig. 30. Determination of sefA mRNA stability. (A) After growing for 60 h in CFA broth statically, *S. enteritidis* cells were treated with rifampicin. Northern blot analysis with a $^{32}$P-labelled sefA probe was performed on the total RNA extracted at 0, 2.5, 5, 10, 30, 60, and 90 min (lanes 1-7, respectively) after the addition of rifampicin. (B) Kinetics of mRNA decay in rifampicin-treated cells. The amount of the sefA transcript at different times after rifampicin addition were determined by densitometric scanning of autoradiograms after the experiment described in (A). The half-life of the sefA transcript was estimated from the slope of a line drawn through the first four time points.
Fig. 31. Schematic representation of the proposed secondary structures within the *sefABC* mRNA. Two stem-loop structures are proposed to form in the intercistronic region of *sefA* and *sefB* mRNA. The *sefA* translation stop codon is boxed. The predicted stability of the stem-loop structures is noted below the schematic.

$\Delta G = -37.4 \text{ kcal/mol}$
b. Thermo-regulation of sefA transcription. To determine whether temperature affected SEF14 expression at the transcriptional or translational level, production of the sefA mRNA transcript at various temperatures was investigated by Northern blot hybridization. To ensure that each lane contained equivalent levels of RNA, the amounts of 16S and 23S rRNA in each lane on the blot were analyzed by UV illumination prior to hybridization and were found to be similar. Hybridization of the sefA probe to RNA isolated from S. enteritidis cells grown at 28°, 32°, 35°, and 37°C revealed that the 0.5 kb transcript was produced only in cells grown from 32°C to 37°C (Fig. 33). No transcripts were detected at 28°C suggesting that temperature regulated the expression of SEF14 fimbriae at the transcriptional level.

3. DISCUSSION

The sefABC genes are transcribed as a single mRNA transcript. In vitro expression of sefABC in Chapter 2 indicates that sefB and sefC are not expressed in the absence of sefA. Primer extension analysis in this section reveals that no transcription start sites or promoters are located immediately upstream of sefB or sefC (data not shown). Furthermore, the 5' ends of several mRNA transcripts map to the region upstream of sefA and transcripts corresponding in size to sefAB and sefABC mRNA species are detected with
Fig. 32. Western blot analysis of SEF14 production at different growth temperatures. Whole cell extracts of *S. enteritidis* grown in colonization factor antigen broth statically for 60 h at the temperatures indicated were separated on a 12.5% acrylamide gel and transferred to nitrocellulose. The blot was developed using polyclonal antisera generated against SEF14 fimbriae.
Fig. 33. Analysis of sefA transcription by Northern blot hybridization. Total RNA was extracted from S. enteritidis cells grown in colonization factor antigen broth statically for 60 h at 28°C (lane 1), 32°C (lane 2), 35°C (lane 3) or 37°C (lane 4). UV illumination of the blot prior to hybridization showed equivalent levels of 16S and 23S in each lane. The RNA samples (10 μg) were hybridized to a 32P-labelled sefA probe.
the sefA-specific probe. Therefore, transcription of sefB and sefC is initiated from the sefA promoter region. The significance of multiple minor transcription start sites upstream of sefA is not clear. The multiple bands may be the result of pausing near the end of the duplex or they could represent variable 5' ends clustered within a few nucleotides.

Transcription initiation from the sefA promoter region produces a stable sefA transcript. Northern blot analysis with total RNA prepared at different time points after transcription termination indicates that the 0.5 kb transcript is detectable 90 min after the termination of transcription. The half-life of the sefA transcript, roughly estimated to be 24 min, was significantly longer than the common bacterial mRNA half-life of only a few minutes (Koraimann and Hogenauer, 1989). Thus, the sefA transcript is unusually stable. Since transcription of sefABC has been shown to initiate from the sefA promoter region, the absence of the 1.4 kb sefAB and the 4.0 kb sefABC transcripts after time zero suggests that the larger transcripts are much less stable than the 0.5 kb transcript.

Given that the sef14 operon is transcribed as a single mRNA transcript and that the sefA transcript is unusually stable, a post-transcriptional mechanism must exist which regulates the relative production of the fimbriin, the chaperone protein and the outer membrane protein. One potential mechanism involves post-transcriptional, endonucleolytic cleavage of the primary mRNA transcript at adenine-uracil rich regions (Nilsson and
Uhlin, 1991). The extensive adenine-uracil rich sequences located in the regions flanking the mRNA transcript for sefA is consistent with this mechanism. Cleavage at these adenine-uracil rich sites would result in discrete mRNA transcripts displaying differential stability to exonucleolytic degradation. Specifically, the sefA transcript could be stabilized by the secondary structures predicted to form in the intercistronic region between sefA and sefB. The stem-loop structures resemble rho-independent transcriptional terminators which have been shown to protect mRNA against 3'-5' exonucleolytic attack (Newbury et al., 1987; Petersen, 1992) and have also been implicated in the lowered expression of distally located genes in multicistronic operons (Higgins et al., 1982). The 3' end of the sefAB and sefABC transcripts, lacking such stabilizing structures, would be more susceptible to degradation. Alternatively, the lower levels of the sefAB and sefABC transcripts may be a result of inefficient transcription termination at these structures. A direct consequence of either mechanism would be differential regulation of SefA, SefB, and SefC translation which would account for the experimentally observed high levels of SefA relative to SefB and SefC (data not shown).

Processed mRNA and differential decay of the processed mRNA transcripts account for the relatively high levels of expression of the major subunit proteins CfaB, DaaE and PapA which are assembled into CFA/I, F1845 and Pap fimbriae, respectively (Nilsson and Uhlin, 1991; Bilge et al., 1993b,
Jordi et al., 1993). Unlike the cfa and daa operons, the endonucleolytic processing of the pap mRNA is dependent on RNaseE, an endonuclease that usually cleaves in adenine-uracil rich regions (Nilsson and Uhlin, 1991). Unfortunately, the effect of RNaseE, RNaseP, or RNaseIII, if any, on sefA mRNA is unknown.

Expression of SEF14 fimbriae is subject to thermoregulation. SEF14 is present during growth at 37°C but is not produced by S. enteritidis grown at 30°C or below. Temperature control of fimbriation is also observed for the pap (Göransson and Uhlin, 1984), fan (Van Der Woude et al., 1990b), sfa (Schmoll et al., 1990b), fae (Ørskov et al., 1961) and cfa (Evans et al., 1975) operons which are expressed only at temperatures above 25°C. In each case, including the sef14 operon, the thermoregulation occurs at the level of transcription, and in some cases, is mediated by H-NS, a histone-like protein that binds to double stranded DNA. In the pap and cfa operons, H-NS promotes gene silencing by binding in the promoter region and repressing transcription (Göransson et al., 1990; Jordi et al., 1992; White-Ziegler et al., 1990). The effect, if any, of H-NS on the temperature dependent control of SEF14 production is unknown.

The silencing effect of H-NS in the pap and cfa operons is overcome by the positive regulatory proteins cAMP-CRP and CfaD, respectively (Forsman et al., 1992; Jordi et al., 1992). The mechanism by which the silencing effect of H-NS is overcome by CfaD is not known (Jordi et al., 1992). However, cAMP-
CRP interaction with the _papI-papB_ intercistronic DNA induces bending which may alter the DNA conformation and play a role in the antirepressor effect of cAMP-CRP on _pap_ operon expression (Forsman _et al._, 1992). In the _sef14_ gene cluster, DNA sequence analysis of the region downstream of _sefD_ has revealed two ORFs, _sefE_ and _sefE_ 2, whose predicted amino acid sequences display significant homology to CfaD and other members of the AraC family of transcriptional activators (see Chapter II). However, it is unknown whether these ORFs are contiguous, whether they are expressed and/or whether the putative protein product(s) have any effect on the thermoregulation of _sefA_ transcription.

Alternatively or in addition to H-NS, the thermoregulation of _sef14_ expression may involve changes in the negative superhelicity of _S. enteritidis_ chromosomal DNA induced by temperature (Drlica, 1992; Goldstein and Drlica, 1984). The degree of DNA supercoiling in prokaryotes is determined in part by the coordinate action of two enzymes, DNA gyrase and DNA topoisomerase I (Goldstein and Drlica, 1984; Menzel and Gellert, 1983). Gyrase introduces negative supercoils while topoisomerase I relaxes the DNA. Thus, inhibitors of DNA gyrase such as novobiocin, relax the DNA and effect expression of genes requiring an increase in DNA supercoiling. Unfortunately, the effect of DNA supercoiling, if any, on the temperature regulation of SEF14 production is unknown. Thus, the mechanism controlling the thermoregulation of _sefA_ transcription remains to be determined.
CHAPTER IV
Characterization of SefA and SefB

1. MATERIALS AND METHODS.

A. Bacterial strains and plasmids. *Salmonella enteritidis* 27655-3b was provided by T. Wadstrom (University of Lund, Lund, Sweden). *E. coli* DH5α (New England Biolabs Inc.) was the host for pSC7, pSC9 and pSC10, recombinant plasmids carrying sefA and/or sefB. PCR-generated sefB was subcloned into pMAL-p2 (New England Biolabs Inc.) to create plasmid pSC7 (Fig. 34A). To construct pSC9, the 1573 bp *Bsm*I fragment from pKX1 was treated with T4 DNA polymerase (New England Biolabs) to enable subcloning the fragment into the *Sma*I site of pUC19 (Fig. 3B). The *EcoR*I/*Hind*III fragment encompassing the original *Bsm*I fragment was subcloned from pSC9 into the *EcoR*I/*Hind*III sites of pINIII\textsuperscript{113}-B1 (Masui et al., 1984) to create pSC10 (Fig. 34B). The original *Bsm*I fragment carried sefA, minus its promoter but with its own ribosome binding site, and sefB. In pSC10, these two genes were cloned downstream of the IPTG-inducible *lpp/lac* promoter/operator system of pINIII\textsuperscript{113}-B1.

B. DNA ligation. DNA ligations were performed as previously described in the methods section of Chapter II.
C. PCR amplification of *sefB*. Genomic *S. enteritidis* DNA used for the PCR reaction was prepared by the method of Alm *et al.* (1993) and heat denatured at 100°C for 5 min immediately before use. The primers *sefB*-T and *sefB*-B (Table 3) which had been synthesized to encode EcoR I and Pst I sites to facilitate cloning of the amplified product, were used to amplify *sefB*. Amplification was carried out in a 50 μl reaction volume containing 5 μl of heat denatured, genomic, *S. enteritidis* DNA (1 μg/ml), 5 pmol of each primer, the four deoxynucleotide triphosphates (Boehringer Mannheim, Germany) at 0.5 mM each and 0.4 units of Taq DNA polymerase (Stratagene, LaJolla, CA) in reaction buffer consisting of 50 mM Tris HCl pH 8.5, 20 mM KCl, 2.5 mM MgCl₂ and 0.5 mg/ml BSA. Thermocycling was performed on samples contained in sealed glass capillary tubes inserted in an air-driven thermocycler (Idaho Technology, Boise, Idaho) for 30 cycles of denaturation (94°C, 10 s), annealing (55°C, 10 s) and elongation (74°C, 30 s). The 640 bp *sefB* product was electrophoresed in a 1% agarose gel in TAE buffer and visualized by UV illumination following ethidium bromide staining (Sambrook *et al.*, 1989).

D. Cloning PCR-amplified *sefB*. *sefB*-PCR-amplified DNA was purified using the "Double GeneClean" procedure recommended by the manufacturer (Bio101 Inc., LaJolla, CA). Briefly, the PCR product was purified using GeneCleanII glassmilk, digested with EcoR I and Pst I (New England Biolabs
Inc., Beverly, MA) and then purified a second time using GeneCleanII
glassmilk. The vector, pMal-p2 (New England Biolabs Inc.), was purified
using a Qiagen column (Qiagen, Chatsworth, CA), digested using EcoRI and
PstI (New England Biolabs Inc.), run on a 1% agarose gel in TAE buffer
(Sambrook et al., 1989) and then purified from the gel using Sephaglas
(Pharmacia, Uppsala, Sweden). The vector and PCR amplified sefB were then
ligated together with T4 DNA ligase (Bethesda Research Laboratories,
Gaithersburg, MD) in accordance with the manufacturer's instructions. The
ligations were transformed into E. coli DH5α (Sambrook et al., 1989). The
manipulations created the recombinant plasmid pSC7 in which sefB was
translationally fused with malE, the gene encoding the maltose binding
protein (Fig. 34A).

E. Media and growth conditions. Cells used for the isolation of SefA
and SefB were grown at 37°C in a complex media composed of the following
ingredients: LB supplemented with 5.3 g/L NH₄Cl, 4.1 g/L KH₂PO₄, 8.9 g/L
K₂HPO₄, 40 ml/L 50% glucose, 10 ml/L MgSO₄ stock (0.5 g MgSO₄ in 10 ml
dH₂O), 1 ml/L trace elements (0.05 g CaCl₂, 0.018 g CoCl₂•6H₂O, 0.016 g
CuSO₄•5H₂O, 0.018 g ZnSO₄•7H₂O, 2 g Na₂EDTA, 2 g FeCl₂•6H₂O and 100 μl
1M HCl in 100 ml dH₂O) (C.E. Furlong, University of Washington).
Ampicillin was added to a final concentration of 250 μg/ml. 10-30 L of this
Fig. 34. A. Plasmid map of pSC7. *sefB* was PCR amplified, purified, digested with *EcoR I* and *Pst I*, purified and ligated into the *EcoR I/Pst I* sites of pMal-p2. B. Construction of pSC9 and pSC10. The genes are represented by open boxes and the name of the gene is given above each box. Box with small white dots, pTZ19 vector DNA; solid black box, pUC19 vector DNA; box with diagonal lines, pINIII113-B1 vector DNA. The arrow indicates the direction of transcription. P, promoter; PLpp, lipoprotein promoter; POlac, lac promoter/operator; *, original ribosome binding site of *sefA*. Only the relevant restriction sites are indicated and the abbreviations are as follows: H, *Hind III*; B, *Bsm I*; S, *Sma I*; E, *EcoR I*. 
media were inoculated with 0.3-1.5 L, respectively, of an overnight culture of DH5α/pSC10, respectively. At 1.0 OD<sub>650</sub>, IPTG was added to the culture to a final concentration of 0.5 mM. NH₄OH and 50% glucose were added automatically to maintain the culture at pH 7. Supplements of MgSO₄, trace elements, 50% glucose and 10x LB were added at 5-7 OD<sub>650</sub> to maintain logarithmic growth. At 8.0 OD<sub>650</sub>, the cells were concentrated and washed with 10-25 L PBS (Sambrook <i>et al.</i>, 1989) using the Millipore filtering system (Millipore Canada Ltd., Mississauga, ON).

F. Purification of SefA and SefB from DH5α/pSC10. The washed and concentrated cell suspension was pelleted by centrifugation (18,600 x g, 4°C, 20 min). The cell pellet with a wet weight of 73 g was quickly resuspended in 341 ml of 20% sucrose, 20 mM Tris HCl pH 8 using a high torque mixer (Cole Parmer, Chicago). EDTA was added to a final concentration of 1 mM and the cell suspension was gently stirred for 10-15 min until the solution was homogenous. As described by Lindström <i>et al.</i> (1970), the outer membrane was disrupted upon the addition of EDTA and lysozyme to a final concentration of 5 mM and 50 µg/ml, respectively. After 5 min of gentle stirring at room temperature, the spheroplasts were stabilized by the addition of MgCl₂ to a final concentration of 20 mM and pelleted by centrifugation (28,100 x g, 4°C, 20 min). The supernatant (400 ml), containing the periplasmic proteins, was dialyzed (3 x 5 L 10 mM Tris HCl pH 7.3, 4°C),
centrifuged (28,100 x g, 4°C, 15 min), and applied to a 20 x 2.5 cm column of DEAE sepharose CL-6B (Pharmacia, Uppsala, Sweden) equilibrated at 4°C with 10 mM Tris HCl pH 7.3. The flow rate was adjusted to 0.5 ml/min. 4 ml fractions were collected and analyzed by SDS-PAGE and Western blot for the presence of SefA and/or SefB. Fractions containing SefA and SefB were pooled, dialyzed (3 x 5 L 20 mM Tris HCl pH 6.5, 4°C) and centrifuged (28,100 x g, 4°C, 15 min). The supernatant was applied to a 20 x 2.5 cm column of CM sepharose CL-6B (Pharmacia) at a flow rate of 0.6 ml/min. After the supernatant was loaded, the column was washed with 200 ml 20 mM Tris HCl pH 6.5. SefA and SefB were eluted into 5 ml fractions using a linear gradient of 0.04-0.14 M NaCl followed by a wash of 100 ml 0.14 M NaCl in 20 mM Tris HCl pH 6.5 and then a second linear gradient of 0.14-0.5 M NaCl in 20 mM Tris HCl pH 6.5. The SefA positive fractions were pooled, concentrated, dialyzed and then loaded onto a 70 x 1.5 cm Superdex 75 (Pharmacia) column equilibrated with 100 mM Tris HCl, 100 mM NaCl pH 7.5. Using a flow rate of 0.4 ml/min, fractions of 1.5 ml were collected. The SefA positive fractions from the preparative Superdex 75 column were concentrated and loaded onto an analytical 30 x 1 cm Superdex 75 HR column. SDS-PAGE analysis of the gel filtration fractions revealed that SefA from the early fractions ran 1-2 kDa higher than SefA from the later fractions. The higher form of SefA was designated Sef\(A_H\) whereas the lower form was designated Sef\(A_L\). SefB positive fractions eluted from the CM sepharose column were also applied to
the 30 x 1 cm Superdex 75 column to achieve greater resolution from contaminating proteins.

G. Depolymerization of SEF14. 10 µl of a SEF14 fimbriae solution (2 mg/ml) was mixed with an equal volume of different concentrations of SDS, incubated at 37°C for 1 h and then added to 20 µl 2x SDS-PAGE sample buffer containing only 0.2% SDS and lacking β-mercaptoethanol. These samples, which were not boiled, were then loaded onto SDS polyacrylamide gels containing only 0.1% or 0.05% SDS rather than the usual 2% SDS. The proteins were electrophoretically transferred onto nitrocellulose (BioRad Laboratories, Richmond, CA) using the LKB Multiphor II System (Pharmacia-LKB, Uppsala, Sweden). Immunoblots of these gels were then developed with antisera generated against SEF14 fimbriae.

H. Isoelectric focusing (IEF). Purified SefA and SefB were dialyzed against 10 mM Tris HCl pH 7.3, diluted to 50 ml in the same buffer containing 1 ml ampholytes (pH 3-10) and then subjected to separate rotary preparative isoelectric focusing (IEF) (BioRad Laboratories, Richmond, CA) carried out at 12 watts until the volts stabilized (usually 3-4 h). Following the run, the focused proteins were transferred into 20 tubes, the pH of each fraction was determined and SDS-PAGE and immunoblotting were used to determine which fractions contained the protein of interest.
Purified SefA and SefB were also analyzed by flatbed IEF (Pharmacia-LKB, Uppsala, Sweden) using precast, native, 5% polyacrylamide gels pH 3.5-9.5 (Pharmacia). Mineral oil was applied to the top of the flatbed apparatus, the gel was placed on top of the oil layer, electrode strips soaked in NaOH (cathode) or H$_3$PO$_4$ (anode) were placed approximately 1 cm from the top and bottom of the gel. Sample strips were placed in the middle of the gel and 15-20 µl of sample or 15 µl of high pI standards (pH 5-10; Pharmacia) were loaded onto each strip. Power was applied (30 watt, 50 mAmp, 1500 volt) for 45 min, the sample strips were removed, and the run was continued for 45 more minutes. The gel was cooled by a continuous flow of cold water through the lower chamber of the flatbed IEF apparatus. The gel was either silver stained, stained with Coomassie Blue, or transferred onto nitrocellulose (BioRad Laboratories, Richmond, CA) using an LKB Multiphor II Electrophoresis System (Pharmacia-LKB) and discontinuous buffers (anode 1: 3 M Tris pH 8; anode 2: 0.1 M Tris pH 8; cathode: 0.1 M arginine, 0.01% (w/v) SDS pH 8) at pH 8 or by capillary action for 24 h in 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, 20% methanol. The blots were developed using antisera generated against SEF14 or SefB as described previously in Chapter II.

I. Sedimentation equilibrium. The samples used in the sedimentation runs were dialyzed extensively against 100 mM Tris, 100 mM NaCl pH 7.5 and
adjusted so that the final OD$_{280}$ reading of the sample was 0.245. Sedimentation runs were performed at 20°C on a Beckman Analytical XL-A ultracentrifuge equipped with a photoelectric scanner accessory in accordance with the methods described by Beckman. Experiments were reported as photoelectric scans at OD$_{280}$. The partial specific volume of SefA and SefB, determined from their predicted amino acid sequences, were 0.7293 ml/g and 0.7434 ml/g, respectively.

J. Cross-linking of proteins. Proteins samples were dialyzed against and then diluted in PBS (Sambrook et al., 1989) to a final concentration of 2-4 mg/ml. For cross-linking, 10 μl of the dialyzed protein sample was added to 2 μl PBS, 3 μl dH$_2$O and 5 μl 0.2, 0.4, 0.6, 0.8, 1.0 or 2.0 mM BS$_3$ [Bis(sulfosuccinimidyl)-suberate; Pierce Chemical Co., USA] and then incubated at 22°C for 30 min. The cross-linking reactions were stopped by the addition of 4 μl 50 mM ethanolamine in PBS pH 7.5. An equal volume of 2x SDS-PAGE sample buffer was added to each cross-linking reaction and analyzed by SDS-PAGE, the gels of which were either stained with Coomassie Blue or immunoblotted and developed with antisera generated against SEF14 or SefB.
K. GluC (Protease V8) digestion, reverse phase HPLC and peptide sequence analysis of SefA. GluC is a Staphylococcal serine protease that hydrolyzes peptide bonds specifically at the carboxylic side of Glu and Asp in the buffer conditions used below. 500 μl of a solution of SefA_L at 1.885 OD_{280} and 500 μl of a solution of SefA_H at 0.83 OD_{280} were each added to 300 μl GluC (0.1 mg) (Boehringer Manneheim, Germany) in KPO_4 buffer pH 7.8 (Sambrook et al., 1989) and incubated at 37°C for 6 h. 200 μl of each reaction was injected onto a 4.6 mm x 25 cm RP300 column (10 μm, C8 reverse phase column with a 300 Å pore; Brownlee Labs, Applied Biosystems Inc., Foster City, CA) equilibrated with 0.1% TFA. The peptides were eluted with a gradient of 0-60% acetonitrile. The fractions containing peptides were concentrated using a Speed Vac concentrator (Savant Instruments Inc., Farmingdale, NY) and then sequenced using an Applied Biosystems model 470A gas-phase sequencer or a model 473 liquid-phase sequencer with on-line phenylthiohydantoin analysis.

To sequence the N-terminus of SefA_H and SefA_L, the purified proteins were run on a 12.5% polyacrylamide gel containing SDS, blotted onto ProBlott nylon membrane (Applied Biosystems Inc.), and stained with 0.1% Coomassie Blue R-250 in 40% methanol/1% acetic acid. The protein bands were cut out of the membrane and the N-terminal amino acid sequence of each protein was sequenced using an Applied Biosystems model 470A gas-phase sequencer or a model 473 liquid-phase sequencer with on-line phenylthiohydantoin analysis.
analysis.

L. Purification of SEF14 fimbriae. SEF14 fimbriae were isolated as previously described in the methods section of Chapter II.

M. Preparation of immune serum.

a. Antiserum to SEF14. Purified native SEF14 fimbriae and denatured SEF14 fimbrin protein preparations were used to generate immune sera in female New Zealand white rabbits as previously described in the methods section of Chapter II.

b. Antiserum to SefB. The MalE'-SefB fusion protein, the source of SefB used to generate anti-SefB serum, was over-expressed and affinity purified from the periplasm of TB1/pSC7 as follows. 1 L SOC and ampicillin (250 μg/ml) was inoculated with 10 ml of an overnight culture of TB1/pSC7, grown at 37°C shaking to 0.4-0.5 OD 600 and induced with 1 mM IPTG for 2 h at 37°C. The cells were centrifuged (4000 x g, 20 min, 4°C) and resuspended in 400 ml 30 mM Tris, 20% sucrose pH 8. After 800 μl 0.5 M EDTA pH 8 was added, the cells were gently stirred at room temperature for 10 min, centrifuged (8000 x g, 20 min, 4°C), resuspended in 400 ml ice cold 5 mM MgSO 4 , stirred on ice for 10 min and centrifuged (8000 x g, 20 min, 4°C). The MalE'-SefB fusion protein was affinity purified from the supernatant on
an amylose resin column (New England Biolabs Inc., Beverly, MA) and eluted from the column with 10 mM maltose in 20 mM Tris HCl pH 7.4, 100 mM NaCl, 1 mM EDTA. Column fractions containing the fusion protein were detected by Western blot analysis using anti-MalE antisera. The purified fusion protein was then cleaved with Factor Xa (100 µl column fraction + 2 to 5 µl Factor Xa at 100 µg/ml) (New England Biolabs Inc.). The two proteins, MalE' and SefB, were separated by SDS-PAGE and SefB was eluted from the gel using a protein eluter (BioRad Laboratories, Richmond, CA). The sequence of the eluted protein was confirmed to be that of SefB by N-terminal amino acid sequence analysis.

Recovery of SefB from the polyacrylamide gel by elution was always low. To obtain a sufficient quantity of the protein, the Factor Xa cut column fractions were loaded onto 2 preparative gels containing 5 double wide lanes. After electrophoresis, one lane of the gel was stained with Coomassie blue and used to determine the position of SefB in the other gel lanes. The protein was eluted from the subsequent gel slices at 10 mAmp at 4°C for 3 h with SDS and for 1 h without SDS in the elution buffer. The elution membrane had a MW cutoff of 12,000.

500 µl eluted SefB (approximately 500 µg) was mixed with 500 µl complete Freund's adjuvant using two 1 ml syringes connected with a double ended canula. The emulsified solution was injected into a female, New Zealand, white rabbit (59Cr for SefB). Injections were as follows: 1x250 µl
intramuscularly (IM); 2x250 μl subcutaneously (SC). For subsequent boosts 2 and 4 weeks later, 1 ml eluted SefB was emulsified with 1 ml incomplete Freund’s adjuvant as described above. Injections were as follows: 1x500 μl IM; 2x600 μl SC. In both cases, the prebleed was removed the same day as the initial injection of the antigen and only 2 bleeds were removed after the initial antigen injection prior to euthanizing the rabbit.

N. SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis was carried out as previously described in Chapter II.

2. RESULTS

A. Purification of SefA and SefB. To obtain further information on the structure and assembly of SEF14 fimbriae, sefA and sefB were cloned downstream of the lpp/lac promoter/operator system of pINIII13-B1. sefA encodes the major subunit of SEF14 fimbriae while sefB encodes a putative periplasmic chaperone protein. Expression of sefAB was induced by the addition of IPTG and both proteins were localized to the periplasmic space from which they were subsequently purified. One of the more crucial steps in the purification procedure was the production of the spheroplasts. A balance between the rupture of the outer membrane and protection of the inner
Fig. 35. Affinity chromatography of SefA and SefB isolated from the periplasm of *E. coli*. (A) SefA- or SefB-positive fractions from the DEAE Sepharose column were pooled and applied to a CM Sepharose column for affinity chromatography in 20 mM Tris HCl pH 6.5. Upon application of a linear NaCl gradient, 5 ml fractions were collected. The absorbance at 280 nm and the conductivity of each fraction were determined and plotted against the fraction number. (B) 10 μl aliquots from the column fractions were run on a 12.5% SDS polyacrylamide gel and were screened for the presence of SefA or SefB by Western blot analysis using polyclonal antiserum raised to SEF14 or SefB, respectively. The sizes of the molecular weight standards (kDa) are shown on the left. SefA eluted at 0.08 M NaCl whereas SefB eluted at 0.16 M NaCl.
membrane had to be maintained to maximize the release of periplasmic proteins and to minimize contamination from cytoplasmic proteins. The resulting osmotic shock was then applied to a DEAE Sepharose column on which neither SefA nor SefB were retained. However, the column did remove some of the contaminating proteins. The CM Sepharose column retained both SefA and SefB which were eluted at 0.08 M and 0.16 M NaCl, respectively (Fig. 35). The preparative and analytical gel filtration columns were used to further resolve SefA and SefB from contaminating proteins. The use of denaturing agents was avoided throughout the purification scheme and yet evidence of a complex of these two proteins was not found. The yield of the purification procedure was disappointingly low with only 15 mg of SefA and 5 mg of SefB recovered from 40 L of cells.

B. Characterization of SefA and SefB

a. Isoelectric focusing. Isoelectric focusing of the crude osmotic shock from DH5α/pSC10 or of CM Sepharose fractions containing either SefA or SefB revealed the presence of multiple bands that cross reacted with antisera generated against either the SEF14 fimbriae or SefB, respectively. Native polyacrylamide IEF gels of SefA showed multiple closely spaced bands between pH 9.3 and 10.25 (Fig. 36B). For preparative IEF, the focusing fractions contained predominately SefA_H (Fig. 36A). The average pI for SefA, calculated from the results of two separate preparative focusing runs, was 9.6
Fig. 36. Determination of the pi for SefA. (A) Western blot analysis of rotary IEF fractions run on a 12.5% SDS polyacrylamide gel which was blotted and developed using anti-SEP14 antiserum. Fractions with pi 10.0 (lane 1), 9.7 (lane 2), 9.5 (lane 3), 9.2 (lane 4) and 8.9 (lane 5) contained SefA. Molecular weight standards in kDa are indicated on the left. (B) Western blot analysis of SefA separated according to pi on a 5% polyacrylamide, native IEF gel. The blot of the gel was developed using antiserum raised to SEF14. The pH of the pi markers are indicated on the left.
Fig. 37. Determination of the pl for SefB. (A) Western blot analysis of rotary IEF fractions run on a 12.5% SDS polyacrylamide gel which was blotted and developed using anti-SefB antiserum. Fractions with pl 9.8 (lane 1), 9.4 (lane 2), 9.1 (lane 3) and 8.7 (lane 4) contained SefB. Molecular weight standards in kDa are indicated on the left. (B) Western blot analysis of SefB focused on a 5% polyacrylamide, native IEF gel. The blot of the gel was developed using antiserum raised to SefB. The pH of the pl markers are indicated on the left.
which was similar to the predicted pI of 9.96.

Native polyacrylamide gels of SefB showed multiple, closely spaced bands between pH 8.65 and 9.3 (Fig. 37B). For preparative IEF, the focusing fractions with pH 8.7 to 9.8 contained SefB (Fig. 37A). The average pI for SefB, calculated from the results of similar experiments, was 9.5 which is similar to the predicted pI of 9.11.

b. Gel filtration. Gel filtration chromatography revealed that SefA migrated in two partially overlapping peaks centered at 35 kDa and 15 kDa (Fig. 38A). Peak B contained a form of SefA (SefAH) that migrated slightly slower (1,000-2,000 M,) in SDS-PAGE than the form of SefA found in Peak C (SefAL) (Fig. 38B).

The possibility that these were two different proteins was eliminated by showing that both proteins reacted with antisera raised to purified SEF14 fimbriae (Fig. 40C). Both forms of SefA were stable such that conversion from one form to another did not occur. For instance, when SefA from peak B was reapplied to the Superdex 75 column, the protein migrated with the same retention time obtained the first time through the column. This was also true for the form of SefA from peak C.

Gel filtration analysis of SefB purified from the periplasm of E. coli suggested that the protein was a 28 kDa monomer (Fig. 39).
Fig. 38. Gel filtration chromatography of SefA isolated from the periplasm of *E. coli*. (A) SefA-positive fractions from the CM Sepharose column were applied to a Superdex 75 HR 10/30 column for gel filtration in 0.1 M Tris HCl, 0.1 M NaCl pH 7.5. Samples were taken at 30 second intervals. The column was calibrated using cytochrome C (12 kDa), β-lactoglobulin (37 kDa), bovine serum albumin (66 kDa), and human transferrin (90 kDa) as molecular weight marker proteins. The molecular weight of the proteins in peaks A, B and C were estimated to be 70, 35, and 15 kDa, respectively. (B) 10 μl aliquots from the column fractions collected every 30 seconds were run on a 12.5% SDS polyacrylamide gel and stained with Coomassie Blue. The sizes of the molecular weight standards (kDa) are shown on the left. Peak B, which corresponds to fractions 3, 9 and 10, contains SefA_H and peak C, which corresponds to fractions 14, 15 and 16, contains SefA_L.
Fig. 39. Gel filtration of SefB isolated from the periplasm of E. coli. (A) SefB-positive fractions from the CM Sepharose column were applied to a Superdex 75 HR 10/30 column for gel filtration in 0.1 M Tris HCl, 0.1 M NaCl pH 7.5. Samples were taken at 30 second intervals. The column was calibrated using cytochrome C (12 kDa), β-lactoglobulin (37 kDa), bovine serum albumin (66 kDa), and human transferrin (90 kDa) as molecular weight marker proteins. The single peak corresponds to an estimated molecular weight of 30 kDa. (B) 10 μl aliquots from the column fractions collected every 30 seconds were run on a 12.5% SDS polyacrylamide gel and stained with Coomassie Blue. The sizes of the molecular weight standards (kDa) are shown on the left. The peak corresponds to fractions 6, 7, and 8.
Fig. 40. SDS polyacrylamide gel analysis of the three forms of SefA. (A) Purified SEF14 was incubated with 0.5% (lane 1), 0.4% (lane 2), 0.3% (lane 3), and 0.2% (lane 4) SDS at 37°C for 30 min, mixed with an equal volume of native 2x sample buffer (only 0.2% SDS and no β-mercaptoethanol) and separated on a 12.5% polyacrylamide gel containing only 0.1% SDS, blotted onto nitrocellulose and developed using anti-SEF14 antiserum. The sizes of the molecular weight standards (kDa) are shown on the left. (B) Coomassie blue stained SDS polyacrylamide gel of SefA_L/SefA_H (lane 1) and purified SEF14 (lane 2). (C) Western blot analysis of purified SEF14 (lane 1), SefA_L (lane 2), SefA_H (lane 3) and SefA_L/SefA_H (lane 4) using antiserum to SEF14 fimbrin.
c. Depolymerization of SEF14. To determine if one of the forms of SefA was preferentially assembled into SEF14 fimbriae, depolymerized SEF14 fimbriae were analyzed by SDS-PAGE. The depolymerization of SEF14 fimbriae required at least 0.2% SDS when the sample was not heated to 100°C (Fig. 40A). If the concentration of SDS in either the gel or the sample buffer was not high enough, multimers of SefA formed, the predominate multimer being the dimer (Fig. 40A). Other detergents (ie. NP-40, Triton X-100, Tween-20 and CHAPS) or chaotropic agents (ie. urea) seemed to have little effect on the solubility of these fimbriae (data not shown). Further analysis showed that the fimbrin from the depolymerized SEF14 fimbriae co-migrated with SefA$_{11}$ in SDS polyacrylamide gels suggesting that SefA$_{11}$ was preferentially assembled into the fimbriae (Fig. 40B and C).

d. Sedimentation equilibrium. To examine why SefA was migrating through the gel filtration column in two different peaks, the approximate molecular weight of SefA from each peak was determined by equilibrium ultracentrifugation. In 100 mM Tris, 100 mM NaCl pH 7.3, the same buffer used for the gel filtration columns, SefA$_L$ had an average molecular weight of 14,960 and fit the single component model (Fig. 41B). The slight deviations were well within the ±0.02 absorbance unit boundaries and were not considered significant. Furthermore, the molecular weight of SefA$_L$ predicted using the single component model (14,960 Da) matched more
Fig. 41. Sedimentation equilibrium analysis of SefA<sub>L</sub>. SefA<sub>L</sub> was dialyzed in 0.1M Tris HCl, 0.1M NaCl pH 7.5 buffer, concentrated to 0.245 OD<sub>280nm</sub> and analyzed using the partial specific volume of 0.7293. The 120 µl sample was loaded into a standard 12-mm double-sector cell and equilibrated using a 3-mm column length at a radial velocity of 30,000 rpm for 18 h at 20°C. The data was fitted to monomer (A) and dimer (C) model curves of absorbance at 280 nm versus R<sup>2</sup>-R<sub>2meniscus</sub>, where R is the radial distance from the axis of rotation and R<sub>2meniscus</sub> is the radial position of the meniscus. The absorbance deviation of the SefA<sub>L</sub> equilibrium curve from the monomer or dimer model curves is shown in B and D, respectively. The acceptable level of error is ±0.02 absorbance units at 280 nm. (B) The SefA<sub>L</sub> equilibrium curve was linearized in the linear regression plot of log(C-Baseline) versus R<sup>2</sup>-R<sub>2meniscus</sub>, where C is the concentration of the solute and baseline refers to the initial concentration of the solute.
Fig. 42. Sedimentation equilibrium analysis of SefA<sub>H</sub>. SefA<sub>H</sub> was dialyzed in 0.1 M Tris HCl, 0.1 M NaCl pH 7.5 buffer, concentrated to 0.245 OD<sub>280</sub> and analyzed using the partial specific volume of 0.7293. The 120 µl sample was loaded into a standard 12-mm double-sector cell and equilibrated using a 3-mm column length at a radial velocity of 30,000 rpm for 18 h at 20°C. The data was fitted to monomer (A) and dimer (C) model curves of absorbance at 280 nm versus R<sup>2</sup> - R<sup>2</sup><sub>meniscus</sub> where R is the radial distance from the axis of rotation and R<sub>meniscus</sub> is the radial position of the meniscus. The level of deviation of the SefA<sub>H</sub> curve from the model monomer and dimer curves is shown in (B) and (D), respectively. The Δ Absorbance scale in D is the same as that in B. The acceptable level of error is ±0.02 absorbance units at 280 nm.
closely the molecular weight of the monomer (12,169 Da) compared to that of
the dimer (20,637 Da) predicted from the monomer-dimer model. However,
the wave-like distribution of points in the single component model suggested
that even though the majority of SefAL existed in a monomeric form, dimers
of SefAL were also present in solution. In contrast, SefAH had an average
molecular weight of 34,104 Da and fit the monomer-dimer model (Fig. 42D).
Although the estimated molecular weight of 34,000 Da was higher than the
expected value of 29,000 Da, the 34,000 Da value was within the ±5% error
expected from equilibrium centrifugation when this technique is used to
determine molecular weight. The relative stoichiometry of the reaction
indicated that, unlike SefAL, SefAH existed predominately as a dimer in
solution. However, analysis of SefAH using the single component model
showed that monomers were also present. Thus, in solution, SefA isolated
from the periplasm of *E. coli* existed in two native states, a monomer and a
dimer.

e. Cross-linking of SefA. Further proof that SefA formed a
dimer came from cross-linking experiments. In separate studies, both forms
of SefA were incubated with increasing concentrations of BS\(^3\)
[Bis(sulfosuccinimidyl)-suverate], a water-soluble and non-cleavable cross-
linker with a spacer arm of 11.4 Å that forms cross-links between two closely
Fig. 43. Cross-linking of SefA\textsubscript{L} with low concentrations of BS\textsuperscript{3}. SefA\textsubscript{L} was cross-linked with BS\textsuperscript{3} at concentrations of 0 mM (lane 1), 0.001 mM (lane 2), 0.01 mM (lane 3), 0.05 mM (lane 4), 0.10 mM (lane 5), 0.50 mM (lane 6) and 1.0 mM (lane 7), run on a 12.5% SDS-polyacrylamide gel, and stained with Coomassie Blue. The position of the SefA\textsubscript{L} and SefA\textsubscript{H} bands are indicated on the left.
Fig. 44. Cross-linking SefA_L with higher concentrations of BS\textsuperscript{3}. SefA_L was cross-linked with BS\textsuperscript{3} at concentrations of 0 mM (lane 1), 0.4 mM (lane 2) and 2.0 mM (lane 3), run on a 12.5% SDS polyacrylamide gel and stained with Coomassie Blue. The sizes of the molecular weight markers are indicated on the left in kDa.
Table 11. Summary of the N-terminal sequence analysis of $\text{SefA}_H$ and $\text{SefA}_L$

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associated polypeptides. SDS-PAGE analysis of BS$^3$-treated Sef$\text{A}_L$ revealed that with increasing concentrations of BS$^3$ (0.001-1 mM) the migration rate of Sef$\text{A}_L$ in the gel decreased to that of Sef$\text{A}_H$ (Fig. 43). Furthermore, gel filtration analysis revealed that cross-linked Sef$\text{A}_L$ now migrated through the column with the same retention time as native Sef$\text{A}_H$ (data not shown). At higher concentrations of BS$^3$ (1-2 mM), dimers and trimers of Sef$\text{A}_L$ were formed (Fig. 44). SDS-PAGE analysis of BS$^3$-treated Sef$\text{A}_H$ indicated only the formation of dimers and trimers with concentrations of BS$^3$ ranging from 0.1 mM to 1.0 mM (data not shown). Together, these results showed that BS$^3$ could cross-link the polypeptide chains of Sef$\text{A}_L$ and Sef$\text{A}_H$ but that an intrapeptide cross-link had to form within Sef$\text{A}_L$ before dimerization occurred.

f. Amino acid sequence analysis. N-terminal amino acid sequence analysis of the intact proteins as well as the peptides generated by GluC digestion of Sef$\text{A}_L$ and Sef$\text{A}_H$ was used to determine if the sequences of the two proteins were the same. This analysis revealed that the N-terminal amino acid of Sef$\text{A}_L$ was asn$^{25}$ rather than ala$^1$, the first amino acid determined from N-terminal amino acid sequence analysis performed on SEF14 fimbriae purified from the surface of S. enteritidis (Table 11). The N-terminal amino acid of Sef$\text{A}_H$ was determined to be ala$^1$. The sequence data
also revealed that several truncated forms of SefA co-migrated with SefA\textsubscript{H} on SDS-PAGE even though 12, 17, or 24 N-terminal amino acids were missing (Table 11).

3. DISCUSSION

SefA and SefB have been purified from the periplasm of an \textit{E. coli} clone carrying a plasmid that contains the genes encoding these two proteins. SefA is the major fimbrin of SEF14 fimbriae whereas SefB, on the basis of protein sequence similarity, is a member of the superfamily of periplasmic chaperones (Clouthier \textit{et al.}, 1993) that prevent nonproductive aggregation of fimbrial subunits imported into the periplasm (Kuehn \textit{et al.}, 1991), protect the fimbrial subunit from proteolytic cleavage and enhance the processing of the subunit's signal peptide (Bakker \textit{et al.}, 1991; Hultgren \textit{et al.}, 1989). Although subunit/chaperone complexes have been isolated from other fimbrial systems, a stable SefA-SefB complex was not found in the fractions collected from the ion exchange or gel filtration columns in this study. Lindberg \textit{et al.} (1989) also found it difficult to maintain a subunit-chaperone complex throughout their entire purification scheme. They were, however, able to recover evidence of a periplasmic complex. Similarly, Bakker \textit{et al.} (1991) showed that FaeG, the subunit of K88 fimbriae, formed a complex with the
chaperone FaeE. Although a stable chaperone-subunit complex was not found in this study, it does not mean that SefA and SefB do not associate. If they do associate, however, the affinity between the two proteins is so low that the complex is easily dissociated.

Further analysis of periplasmic SefA reveals that the solubility of this protein is significantly different from SefA that has been assembled into fimbriae. SefA isolated from the periplasm of an *E. coli* clone is very soluble since this protein can be lyophilized and then easily resuspended in water. In contrast, SEF14 fimbriae are extremely insoluble and only go into solution at high pH (pH 10.5) or in the presence of greater than 0.2% SDS. The high solubility of periplasmic SefA in dH2O suggests that charged groups are exposed on the surface of the protein. In contrast, the high insolubility of SEF14 fimbriae in dH2O suggests that hydrophobic regions of SefA are exposed on the external surface of each fimbria. The exposure of these hydrophobic regions would explain why the presence of SEF14 fimbriae increases the hydrophobicity of the cell surface of *S. enteritidis* (Feutrier *et al.*, 1986).

The structural components of fimbriae are not covalently linked but seem to be held together in an extremely stable form by hydrophobic and hydrophilic interactions (Watts *et al.*, 1982; 1983). Preliminary analyses show that SEF14 fimbriae are also very stable. CD and EM analyses of SEF14 fimbriae in 0.15 M ethanolamine pH 10.5 reveal that high pH has little effect
on secondary structure and that the fimbriae are still intact [Dr. K. Collinson (University of Victoria); Dr. C. Kay (University of Alberta); personal communication]. Type IV fimbriae from *P. aeruginosa* are also resistant to alkali denaturation until pH 11. Even then, neutralization of Type IV fimbriae which have been treated at pH 13 results in regeneration of the original CD spectrum indicating that alkali denaturation is reversible (Watts *et al.*, 1983). The addition of 0.2% SDS to SEF14 fimbriae results in the formation of aggregates. SDS-PAGE of these aggregates, using less than the recommended amount of SDS in the gel and sample buffer, reveals that the major aggregative form of fimbrial SefA is a dimer. Addition of higher concentrations of SDS (0.3 to 0.5% SDS) substantially decreases dimer formation leaving mainly SefA monomers. Even boiling SEF14 fimbriae in 1% SDS for 10 min does not completely eliminate dimer formation. 1% SDS also dissociates Type IV fimbriae from *P. aeruginosa* into monomers (Watts *et al.* 1982). However, the mild, nonionic detergent, octyl glucoside, dissociates these fimbriae into a mixture of monomers and dimers, an effect similar to that of 0.2% SDS on SEF14 fimbriae. Since higher concentrations of SDS are required to disrupt SefA dimers, the interaction between two SefA monomers within the dimer must be stronger than the interaction between two dimers within a single SEF14 fimbrial strand. SDS is both a strongly anionic and hydrophobic detergent. Thus, the hydrophobic hydrocarbon end could disrupt hydrophobic interactions while the charged sulfate end could disrupt
hydrogen bonds or salt linkages within SEF14 fimbriae. Since urea and nonionic or zwitterionic detergents do not depolymerize SEF14 fimbriae, a combination of hydrophobic and hydrophilic interactions must maintain the integrity of these fimbriae.

Secondary structure predictions indicate that SefA is composed of 14% α-helix, 35% β-structure, 27% β-turn and 24% random coils. The two α-helical regions are contained within the first 41 N-terminal amino acids whereas the β-structure is found within the central and C-terminal regions of SefA. Unfortunately, the details of the predicted secondary structure of SefA cannot be resolved into a model for fimbrial morphology. Data from X-ray diffraction and hydrodynamic studies had to be obtained before a model for fimbrial structure could be developed for the Type IV fimbriae of *Pseudomonas aeruginosa*. Similarly, X-ray diffraction data has led to more information on the structure of the Type 1 and Pap fimbriae of *E. coli*. In all three cases, the fimbrial subunits are tightly packed into a right-handed α-helix giving rise to a thick fiber ranging in diameter from 52 Å for Type IV fimbriae to 65 Å for Type 1 and Pap fimbriae. Type IV fimbriae have a central channel of 12 Å whereas Type 1 and Pap fimbriae have a central cavity of 15 Å in diameter (Brinton, 1965; Gong and Makowski, 1992; Watts et al., 1983). Unfortunately, X-ray diffraction does not produce interpretable diffraction of the thin fibrillae located at the tip of Pap fimbriae. However, STEM analysis reveals that these structures are about 20 Å in diameter (Gong and Makowski,
1992) which corresponds to the diameter of SEF14 fimbriae. Thus, SEF14 fimbriae, like these tip fibrillae, may also be highly curved filaments that have a helical twist. In accordance with this, preliminary CD analysis of SEF14 fimbriae in the presence and absence of SDS shows some \( \alpha \)-helical character with measured values that closely reflect those predicted from the amino acid sequence. However, more structural information will have to wait for X-ray diffraction analysis and hydrodynamical studies with SEF14 fimbriae.

Because the major fimbrial subunits are generally susceptible to degradation and aggregation in the periplasm, essentially nothing is known about their morphology in this cell compartment prior to assembly into fimbriae. The stability of SefA isolated from the periplasm of \textit{E. coli} has permitted the first such study. Gel filtration and equilibrium ultracentrifugation analysis of SefA isolated from the periplasm of \textit{E. coli} reveal that SefA exists as both a monomer (SefA\textsubscript{L}) and a dimer (SefA\textsubscript{H}). The SefA monomer and the SefA subunit from the dimer both cross-react with SEF14 fimbriae antisera and have the same very basic pI of 9.6 indicating that they are essentially the same protein. However, SefA\textsubscript{L} and SefA\textsubscript{H} as well as SefA derived from SEF14 fimbriae have different migration rates through SDS-PAGE such that SefA\textsubscript{L} migrates slightly faster than SefA\textsubscript{H} and SefA from fimbriae. N-terminal amino acid sequence analysis of SefA\textsubscript{L} and SefA\textsubscript{H}
reveals that the N-terminal amino acid in SefAH is ala\textsuperscript{1} but is asn\textsuperscript{25} in SefAL.
The loss of 24 amino acids from the N-terminus of SefA would create a 12,200 M\textsubscript{r} protein which explains the faster migration of SefAL through SDS-PAGE. Since SefAL exists primarily as a monomer in solution and is missing 24 N-terminal amino acids, the N-terminus of SefA must be involved in dimerization. However, other parts of the molecule must also contribute to dimerization since the sedimentation equilibrium data indicates that dimers of SefAL also form in solution. N-terminal amino acid sequence analysis of a band cut out of a stained blot of SefAH run on a 12.5% polyacrylamide gel containing SDS reveals that truncated forms of SefA also co-migrate with Sef\textsubscript{A\textsubscript{H}} even though they are missing the first 12, 17 or 24 N-terminal amino acids. Why these truncated forms of SefAH are not migrating as distinct bands in SDS-PAGE and how SefAL differs from the truncated form of SefA that is missing 24 N-terminal amino acids but yet co-migrates with SefAH is unknown.

Cross-linking studies provide further support that N-terminal residues are involved in the dimerization of SefA. These studies show that the migration of SefAL in SDS-PAGE is converted to that of SefAH in the presence of low concentrations of BS\textsuperscript{3}. Since this cross-linker reacts with amino groups such as those found on lysine, glutamine and asparagine residues, perhaps the cross-linker initially reacts with asn at the N-terminus of SefAL. Even
though BS$^3$ only has a MW of 572.43 Da, multiple interactions between the cross-linker and SefA_L could account for the decrease in migration rate of BS$^3$ cross-linked SefA_L through SDS polyacrylamide gels. However, the cross-linking studies also show that higher concentrations of BS$^3$ promote the dimerization of SefA_L monomers that are missing the first 24 N-terminal amino acids. Thus, the cross-linker is substituting for one or more of these residues. Perhaps the amino groups on one or more of the five basic residues, asn$^6$, lys$^8$, gln$^{11}$, gln$^{19}$ and asn$^{20}$, that are missing from the N-terminus of SefA_L, are involved in the dimerization of SefA.

The amino terminal domain of FaeG, the subunit of K88 fimbriae, has also been putatively identified as the region of the subunit involved in subunit-subunit interaction (Bakker et al., 1991). Furthermore, the N-terminus of the Type IV fimbrial subunit is involved in subunit-subunit interactions in the native fimbriae. However, Watts et al. (1983) propose that the N-terminus is involved not in making the dimer but rather in making dimer-dimer contact. Although the N-terminus of SefA seems to be involved in dimerization, it is not known whether the two subunits interact through their N-termini or whether the N-terminus of one subunit interacts with an alternate region of the other subunit.

This study provides the first evidence that dimers of fimbrial subunits can exist in the periplasm in the presence of a chaperone. Multimeric
complexes of FaeG have been detected in degP mutants in the absence of FaeE. However, Bakker et al. (1991) show that these multimers are not exported to the cellular surface indicating that only FaeG monomers delivered by FaeE can be accepted by the fimbrial growth points at the outer membrane. Similar results have been obtained with PapG, the adhesin for Pap fimbriae (Kuehn et al., 1991). These studies lead to the proposal that the role of the periplasmic chaperone is to bind to interactive assembly surfaces on the fimbrin to prevent nonproductive aggregation of subunits in the periplasm (Bakker et al., 1991; Kuehn et al., 1991). However, the observation in this study that SefA exists as a dimer in the periplasm suggests that SEF14 fimbrial subunits are assembled in the periplasm as dimers after which they are translocated to the outer membrane for addition to the growing organelle. Whether or not the SefA dimer is actually the basic building unit for SEF14 fimbriae remains to be investigated.

Unfortunately, this study was unable to determine the role of SefB in SEF14 biogenesis. Perhaps SefB minimizes the cleavage of N-terminal amino acids from SefA in the periplasm thereby increasing the efficiency of SEF14 assembly. Such a role would explain the SEF14 positive phenotype observed by Müller et al. (1989) upon insertional inactivation of sefB by mini Mu transposons. Alternatively, SefB may present the SefA dimer to the outer membrane usher for assembly into fimbriae. In any case, further characterization of SefB will require a modification to the overexpression
system so that the production of SefB is enhanced. Once sufficient quantities of SefA and SefB have been purified from the periplasm, the proteins can be characterized through biophysical analyses in conjunction with cross-linking studies. The results may help determine whether or not these two proteins form specific complexes with each other.
General Discussion

This research has characterized six genes, sefABCDE1E2, an IS3 element, the fimbrins of SEF14 and SEF18 fimbriae, as well as sefA transcription. The function of the IS3 element, if any, is unknown. sefA and sefD encode the fimbrins of SEF14 and SEF18 fimbriae, respectively, whereas sefB, sefC and sefE1/E2 encode proteins homologous to other fimbrial periplasmic chaperones, ushers and transcriptional regulators, respectively. Together, the genes sefABCDE1E2 form the sef14 gene cluster encoding two fimbriae that are assembled and expressed on the surface of S. enteritidis.

SEF14 and SEF18 are also expressed on the surface of other bacteria. However, unlike SEF18 which is ubiquitous among Enterobacteriaceae, SEF14 is only present in five serogroup D1 serovars all of which are pathogens of humans and/or commercially reared animals: S. berta (humans), S. dublin (cattle), S. enteritidis (humans), S. gallinarum (poultry) and S. pullorum (poultry) (Doran et al., submitted). The sequences of the fimbrin gene in each of the five group D1 serovars expressing SEF14 are highly conserved. Unfortunately, the similarity precludes the development of both a monoclonal antibody probe specific for each serovar and a S. enteritidis-specific DNA probe-based assay (Doran et al., submitted). However, sefA can be used in a PCR-based assay to detect five significant Salmonella pathogens.
Such an assay would be useful in rapid screening of clinical and veterinary samples, natural and domestic water supplies, estuarine and coastal shellfish growing waters, sewage effluents and waste from food processing plants (Doran et al., submitted). In addition, characterization of the two fimbrin genes, sefA and sefD, has laid the foundation for research directed toward the development of heterologous vaccines in S. enteritidis for the prevention of disease caused by human and animal pathogens.

Pathogenic bacteria are exposed to a variety of environments and differentially express virulence factors in response to these changes. Understanding the factors that control the expression of SEF14 fimbriae may provide some insight into the environment surrounding the bacteria when these fimbriae are expressed. The expression of this putative attachment mechanism is initiated at two transcription start sites located upstream of sefA and is regulated by temperature. Transcription initiation may be regulated by SefE₁ and SefE₂ which share homology with VirF and FapR, members of the AraC family of transcriptional regulators involved in the transcriptional activation of thermally regulated virulence regulons in enteric pathogens. However, further characterization of the sefE₁ and sefE₂ genes and their putative protein products is required to determine if they play a role in the thermoregulation of sefA transcription. Regardless of how subunit production is regulated, SEF14 fimbriae are preferentially expressed at 37°C suggesting that they play a role in attachment within the chicken or
human host, possibly along the gastrointestinal tract. Determining the fimbrial receptor as well as the specific cell type carrying that receptor may localize where SEF14 fimbriae start playing a role in the pathogenesis of *S. enteritidis*.

The inherent stability of fimbriae has provoked an interest into how these structures are synthesized and the features that make the final structure so stable. Characterization of the SEF14 fimbrin, SefA, shows that SefA can dimerize and that the N-terminal amino acids of SefA are required for dimerization. The presence of SefB in the periplasm may minimize the cleavage of these N-terminal amino acids from SefA and maximize the efficiency of SEF14 assembly. Biochemical analysis of SEF14 fimbriae indicates that these dimers are not covalently linked but are held together by hydrophilic and hydrophobic bonds. The interactions between two SefA monomers within the dimer seem to be stronger than the interactions between two dimers within a single SEF14 fimbrial strand. These structural details become important when the fimbriae are used to express heterologous antigens for the purpose of vaccine development.

In conclusion, the *sef14* gene cluster displays various characteristics some of which are unique and some that are similar to other fimbrial systems. Characterizing the DNA has led to a better understanding of the organization, expression and regulation of the genes required for SEF14 fimbrial biosynthesis. Researching the encoded proteins has resulted in a
better understanding of the assembly and structure of SEF14 fimbriae. Together, the studies have provided the foundation for the development of novel diagnostics and vaccines for pathogenic *Salmonella* bacteria. Hopefully, this knowledge will provide some advantage to researchers trying to determine the biological significance of these fimbriae.
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Title of Dissertation:

CHARACTERIZATION OF THE sef14 GENE CLUSTER AND THE ENCODED FIMBRIAE

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