Characterization of the *sef14* fimbrial gene cluster and the encoded fimbriae

by

Sharon Carol Clouthier
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

We accept this dissertation as conforming
to the required standard

Dr. W.W. Kay, Supervisor (Department of Biochemistry/Microbiology)

Dr. T.J. Trust, Departmental Member (Department of Biochemistry/Microbiology)

Dr. P.J. Romaniuk, Departmental Member (Department of Biochemistry/Microbiology)

Dr. F. Nano, Departmental Member (Department of Biochemistry/Microbiology)

Dr. B. Glickman, Outside Member (Department of Biology)

Dr. S. Moseley, External Examiner (Department of Microbiology, University of Washington)

Supervisor: W.W. Kay

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_r 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 labeiled 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 *Kpn*I fragment identified SefD and SefE as 18K and 30K M_r 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.

Examiners:

Dr. W.W. Kay, Supervisor (Department of Biochemistry/Microbiology)

Dr. T.J. Trust, Departmental Member (Department of Biochemistry/Microbiology)

Dr. P.J. Romaniuk, Departmental Member (Department of Biochemistry/Microbiology)

Dr. F. Nano, Departmental Member (Department of Biochemistry/Microbiology)

Dr. B. Glickman, Outside Member (Department of Biology)

Dr. S. Moseley, External Examiner (Department of Microbiology, University of Washington)

TABLE OF CONTENTS

Title page i

Abstract ii

Table of Contents v

List of Tables xiii

List of Figures or Illustrations xiv

List of Abbreviations xvii

Acknowledgments xxi

Dedication xxiii

CHAPTER I. INTRODUCTION

1. Fimbriae

A. Terminology 1

B. Classification 1

2. Fimbriae Structure

A. Composition 4

B. Primary structure of the fimbrins 5

C. 2° and 3° structure of the fimbriae 10

3. Components involved in fimbrial biosynthesis and assembly

A. Chaperone 12

B. Usher 15

C. Minor subunits 17

D. Models for fimbriae assembly 18

4. Genetic organization of fimbrial gene clusters 23

5. Regulation

A. Transcriptional regulation

a. Transcriptional regulatory proteins

i. Lrp 28

ii. Members of the AraC family of transcriptional regulators 29

iii. PapB-like and PapI-like proteins 30

iv. cAMP-CRP 31

b. Phase variation

i. Inversion-dependent phase variation 32

ii. Methylation-dependent phase variation 34

c. Environmental control of fimbriae expression 36

d. Two component regulatory systems 38

B. Post-transcriptional regulation 40

6. *Salmonella* bacteria and their fimbriae 41

CHAPTER II. CHARACTERIZATION OF THE *sef14* GENE CLUSTER

1. MATERIALS AND METHODS

A. Bacterial strains 45

a. Production of rifampicin resistant 3b mutant 45

b. Production of Tn*phoA* mutants 46

B. Media and growth conditions 47

C. Plasmids and plasmid construction

a. Construction of pSC1 47

b. Construction of pSC2 50

c. Construction of pSC3, 4, 5 and 8 50

d. Construction of pSC6 52

D. DNA ligation 53

E. Production of competent cells 53

F. Transformation of competent cells 54

G. Purification of chromosomal DNA

a. Proteinase method 54

b. Method of Alm *et al.* (1993) 55

H. Polymerase chain reaction assays

a. PCR amplification of 5' end of *sefA* and Tn*phoA* 56

b. PCR amplification of *sefC* 57

c. PCR amplification of *sefD* 58

I. DNA sequencing and computer analysis

a. DNA sequence analysis of *sefABC* 59

b. DNA sequence analysis of the IS3 element and *sefDE*$_1$*E*$_2$ 61

J. Protein purification

a. Purification of SEF14 fimbriae 61

b. Partial purification of SEF18 fimbriae 62

c. Overexpression and purification of SefD 63

K. Preparation of immune serum

a. Antiserum to SEF14 fimbriae 64

b. Antiserum to SefD 64

L. SDS-PAGE and Western blot analysis 65

M. *In vitro* transcription-translation 66

N. Electron microscopy 67

O. Dot blot hybridization

a. Hybridization with *sefD* probe 68

b. Hybridization with *rns* probe 69

P. Pulsed field gel electrophoresis 70

Q. Southern blot hybridization 70

2. RESULTS

A. Nucleotide sequence and protein determination 71

B. *In vitro* expression of *sefA*, -*B*, -*C* and *sefD* 92

C. Identification of SEF14 and SEF18 fimbriae 96

D. Characterization of Tn*phoA* mutants 106

E. Distribution of *sefD* and SefD 108

F. Mapping *sefA* and *sefD* 113

3. DISCUSSION

A. Nucleotide sequence and protein determination 117

B. Distribution of *sefD* and SefD 125

CHAPTER III. ANALYSIS OF *sefA* TRANSCRIPTION

1. MATERIALS AND METHODS

A. Bacterial strains, plasmids, media and growth conditions 127

B. Primer extension of RNA transcripts 127

C. DNA sequencing and computer analysis 129

D. mRNA stability 129

E. Expression of SEF14 fimbriae 129

F. RNA extraction 130

G. Electrophoresis and Northern transfer of RNA 131

H. Northern blot hybridization 132

I. Purification of SEF14 fimbriae 132

J. Preparation of immune serum 133

K. SDS-PAGE and Western blot analysis 133

2. RESULTS

A. Mapping the 5' end of the *sefA* transcript 133

B. Analysis of *sefA* mRNA stability 134

C. Characterizing how temperature effects fimbriae expression

a. Thermo-regulated expression of SEF14 fimbriae 135

b. Thermo-regulation of *sefA* transcription 139

3. DISCUSSION 139

CHAPTER IV. CHARACTERIZATION OF SEFA AND SEFB

1. MATERIALS AND METHODS

A. Bacterial strains and plasmids 146

B. DNA ligation 146

C. PCR amplification of *sefB* 147

D. Cloning PCR-amplified *sefB* 147

E. Media and growth conditions 148

F. Purification of SefA and SefB from DH5α/pSC10 150

G. Depolymerization of SEF14 152

H. Isoelectric focusing 152

I. Sedimentation equilibrium 153

J. Cross-linking of proteins 154

K. GluC digestion, reverse phase HPLC and peptide sequence analysis of SefA 155

L. Purification of SEF14 fimbriae 156

M. Preparation of immune serum

a. Antiserum to SEF14 fimbriae 156

b. Antiserum to SefB 156

N. SDS-PAGE and Western blot analysis 158

2. RESULTS

A. Purification of SefA and SefB 158

B. Characterization of SefA and SefB

a. Isoelectric focusing 160

b. Gel filtration 163

c. Depolymerization of SEF14 168

d. Sedimentation equilibrium 168

e. Cross-linking of SefA 174

f. Amino acid sequence analysis 178

3. DISCUSSION 179

General Discussion 188

References 192

LIST OF TABLES

Table 1: Table of bacterial strains 45

Table 2: Table of plasmids 49

Table 3: Table of PCR and hybridization primers 56

Table 4: Table of primers used for DNA sequencing 59

Table 5: Comparison of the predicted amino acid sequence for SefB with those of three fimbrial chaperone proteins 78

Table 6: Comparison of the predicted amino acid sequence for SefC with those of eight fimbrial outer membrane proteins 80

Table 7: Comparison of the predicted amino acid sequence for OrfB to putative IS3 family transposases 84

Table 8. Comparison of the predicted amino acid sequences for $SefE_1$ and $SefE_2$ to transcriptional regulators of the AraC family 94

Table 9: Summary of fimbrins and fimbriae produced by the Tn*phoA* and Tn*10* mutants of *S. enteritidis* 107

Table 10: The distribution of *sefD* and SefD among *Salmonella* isolates and other eubacteria 109

Table 11: Sequence analysis of the N-terminal amino acids from $SefA_L$ and $SefA_H$ 177

LIST OF FIGURES

Figure 1: Schematic diagram of the PapD molecule, illustrating the arrangements of the beta strands in the two domains 14

Figure 2: Model of Pap fimbriae biosynthesis. 19

Figure 3: A. Model for type 1 fimbriae biogenesis
B. Model for K99 fimbriae biogenesis 21

Figure 4: Model for type IV fimbriae biogenesis 23

Figure 5: Genetic organization of fimbrial operons 24

Figure 6: Genetic organization of type IV fimbrial operons 26

Figure 7: Schematic diagram summarizing the components that influence transcription of the *pap* operon either positively or negatively 36

Figure 8: Cloning strategy 48

Figure 9: Plasmid maps of pSC4 and pSC8 51

Figure 10: Open reading frame map of *sefABCDE* and the IS3 element.. 73

Figure 11: Nucleotide sequence of *sefABC* 74

Figure 12: Secondary-structure analysis of SefB and local alignment of SefB and three fimbrial periplasmic chaperone proteins 79

Figure 13: Nucleotide sequence of the IS3 element 83

Figure 14: Nucleotide sequence of the intergenic region between *sefA* and the IS3 element 87

Figure 15: Nucleotide sequence of *sefD* 89

Figure 16: Southern blot hybridization of *Kpn*I digested *S. enteritidis* DNA with *sefA* and *sefD* probes 90

Figure 17: Nucleotide sequence of $sefE_1$ and $sefE_2$ 93

Figure 18: Local alignment of $SefE_1$ and $SefE_2$ and two AraC-like transcriptional regulators 95

Figure 19: Expression of the *sefA, -B* and *-C* genes in an *E. coli in vitro* transcription-translation system 97

Figure 20: Expression of the *sefD* gene in an *E. coli in vitro* transcription-translation system 98

Figure 21: Immunoelectron microscopy of negatively stained cells for SEF14 production 100

Figure 22: Analysis of SEF18 production by immunogold electron microscopy of negatively stained cells 101

Figure 23: Cellular localization of SEF18 103

Figure 24: Analysis of the serological cross-reactivity between the four fimbriae of *S. enteritidis* 3b and their antisera 105

Figure 25: PCR amplification of DNA fragments from *S. enteritidis* 3b Tn*phoA* mutants harboring Tn*phoA* in *sefA* 107

Figure 26: Analysis of SEF18 production by enterobacteria 112

Figure 27: Identification of chromosomal fragments containing *sefA* or *sefD* by Southern blot hybridization 114

Figure 28: Position of the *sef14* gene cluster on the *Xba*I-*Bln*I genomic cleavage map of *S. enteritidis* 116

Figure 29: Mapping of the 5' end of the *sefA* transcript using primer extension 136

Figure 30: Determination of *sefA* mRNA stability 137

Figure 31: Schematic representation of the proposed secondary structures within the *sefABC* mRNA 138

Figure 32: Western blot analysis of SEF14 production at different growth temperatures 140

Figure 33: Analysis of *sefA* transcription by Northern blot

hybridization 141

Figure 34: A. Plasmid map of pSC7. B. Construction of pSC9 and pSC10 149

Figure 35: Affinity chromatography of SefA and SefB isolated from the periplasm of *E. coli* 159

Figure 36: Determination of the pI for SefA 161

Figure 37: Determination of the pI for SefB 162

Figure 38: Gel filtration chromatography of SefA isolated from the periplasm of *E. coli* 164

Figure 39: Gel filtration chromatography of SefB isolated from the periplasm of *E. coli* 165

Figure 40: SDS polyacrylamide gel analysis of the three forms of SefA... 166

Figure 41: Sedimentation equilibrium analysis of $SefA_L$ 169

Figure 42: Sedimentation equilibrium analysis of $SefA_H$ 172

Figure 43: Cross-linking of $SefA_L$ with low concentrations of BS^3..... 175

Figure 44: Cross-linking of $SefA_L$ with higher concentrations of BS^3 176

LIST OF ABBREVIATIONS

Å	angstrom
bp	base pair(s)
BS^3	bis(sulfosuccinimidyl)-suberate
BSA	bovine serum albumin
CD	circular dichroism
CFA	colonization factor antigen
cm	centimeter(s)
CM	carboxy methyl
CS	centisome(s)
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
EDTA	(ethylene diamine)tetraacetic acid
EM	electron microscopy
g	gram(s)
h	hour(s)
IEF	isoelectric focusing
IHF	integration host factor
IM	intramuscular
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	inverted repeat

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	millimeter(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 KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl pH 7.4)
PCR	polymerase chain reaction

PFGE	pulsed-field gel electrophoresis
pI	isoelectric point
pmol	picomole(s)
R	radial distance from axis of rotation
$R_{meniscus}$	radial position of the meniscus
rif	rifampicin
RNA	ribonucleic acid
rpm	rotations per minute
s	second(s)
SC	subcutaneous
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEF	*Salmonella enteritidis* fimbriae
$SefA_H$	higher molecular weight form of periplasmic SefA in SDS polyacrylamide gels
$SefA_L$	lower molecular weight form of periplasmic SefA in SDS polyacrylamide gels
SOC	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
TBS	tris buffered saline
TAE	40 mM Tris HCl, 20 mM sodium acetate, 1 mM EDTA pH 8.3
TE	10 mM Tris pH 8, 1 mM EDTA
TFA	trifluoroacetic acid
µg	microgram(s)

µl	microlitre(s)
µM	micromolar
UV	ultraviolet

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 Washinton, 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 pINIII^{113} 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 rtoary 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 (Lintermans *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* (Tønjum *et al.*, 1993), *Moraxella bovis* (Marrs *et al.*, 1985), *Moraxella nonliquefaciens* (Tønjum *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 corrodens* (Rao and Progulske-Fox, 1993; Tønjum *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* (LngA) (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 varible 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 *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 *E. coli* (Arnqvist *et al.*, 1992) and *S. enteritidis* (Collinson *et al.*, 1991), respectively (Low *et al.*, 1995). The *agfA* fimbrin gene is present in other *Salmonella* isolates as well as *E. coli, Citrobacter* spp., *Shigella sonnei* and *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 fimbrin protein target to prevent nonproductive aggregation or polymerization of fimbrins in the periplasm (Bakker *et al.*, 1991; Kuehn *et al.*, 1991). When the chaperone is bound to the fimbrin, 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 fimbrin 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).

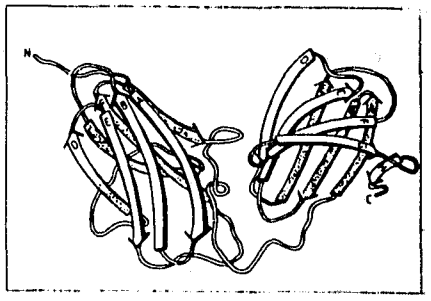


Fig. 1. Schematic diagram of the PapD molecule, illustrating the arrangements of the beta strands in the two domains. Strands A B E and strands C F G form the two beta sheets which are packed together to form each domain (Holmgren and Bränden, 1989).

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*

al., 1992).

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

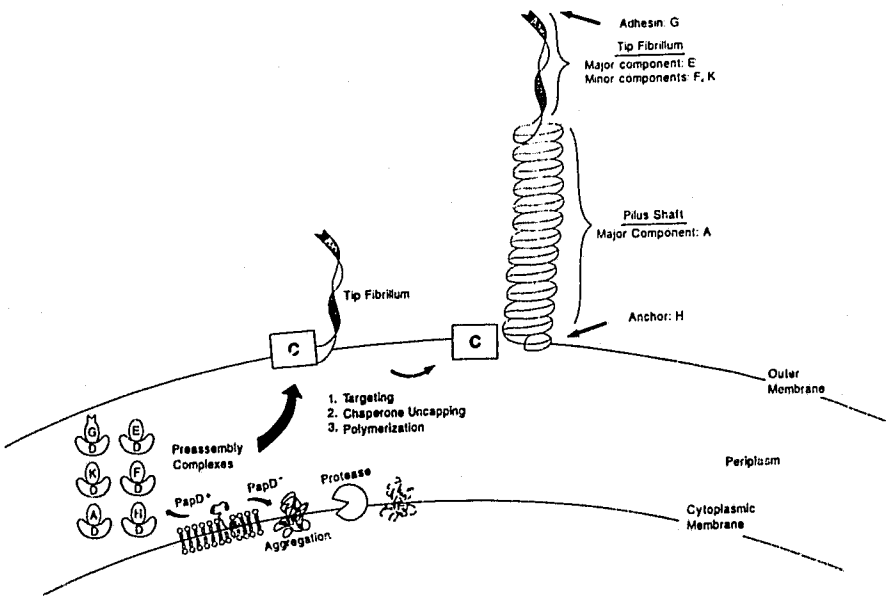


Fig. 2. A model of Pap fimbriae biosynthesis. The assembly details are given in the text (Kuehn *et al.*, 1994).

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

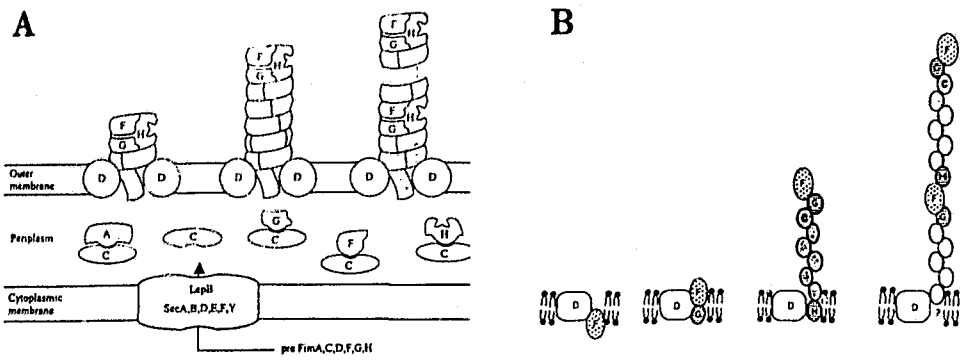


Fig. 3. A. Model for type 1 fimbriae biogenesis. The structural and transport/assembly components encoded by the type 1 fimbriae operon are translocated across the cytoplasmic membrane via the normal Sec export pathway. Fimbrial subunits complex with the chaperone, FimC, in the periplasm and are transported 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 encorporated 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).

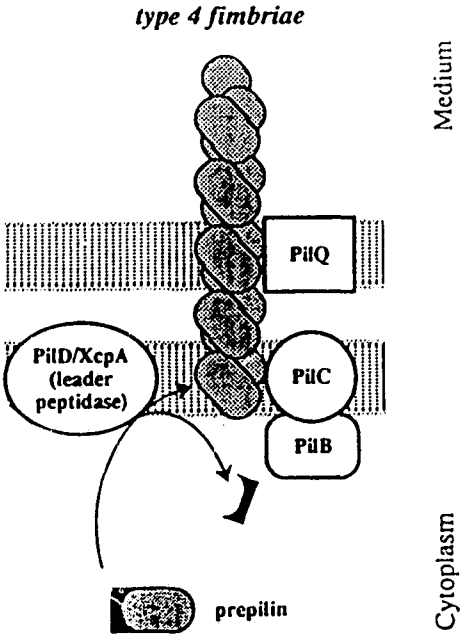


Fig. 4. Model for type IV fimbriae biogenesis. Details are given in the text (Hobbs and Mattick, 1993).

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 *fae* and

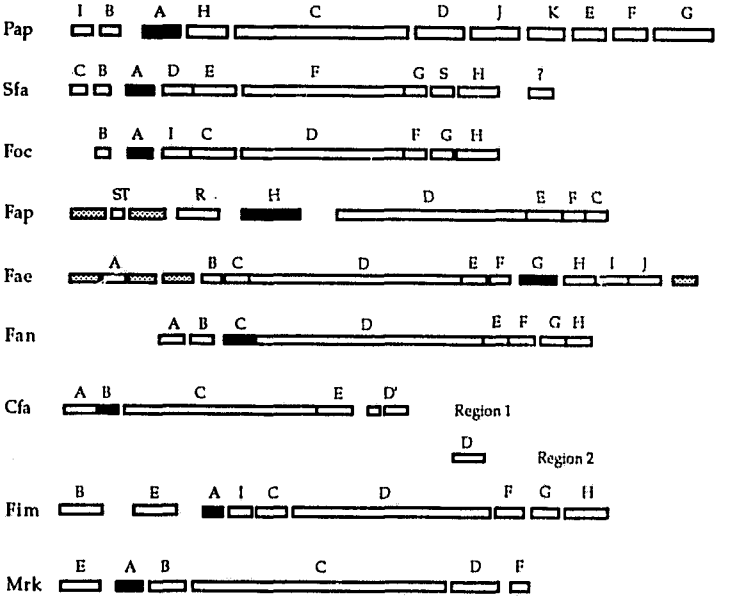


Fig. 5. Genetic organization of fimbrial operons. Shaded boxes represent the major subunit; arrows or cross-hatched boxes indicate IS elements. Chaperone genes are *papD, sfaE, focC, faeE, fanE, fapE, fimC* and *mrkB*. Usher genes are *papC, sfaF, focD, fapD, faeD, fanD, cfaC, fimD* and *mrkC*. Regulation genes are *papBI, sfaBC, focB, fapR, faeAB, fanAB, cfaD, fimBE* and *mrkE* (Allen *et al.*, 1991; De Graaf and Gaastra, 1994; Hacker and Morschhäuser, 1994; Klaasen and De Graaf, 1990; Klemm and Krogfelt, 1994; Kuehn *et al.*, 1994).

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)

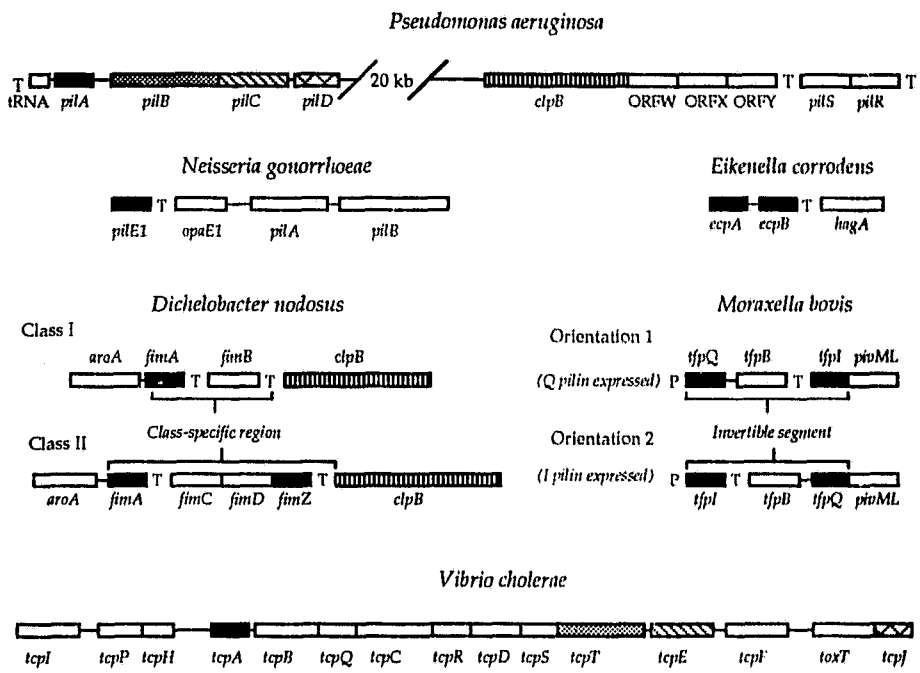


Fig. 6. Genetic organization of type IV fimbrial operons. Arrows indicate the extent and direction of the coding regions. Fimbrial subunit genes are filled with black; other homologue sets are filled with matching patterns. Unfilled arrows indicate genes with no other homologues in the figure. Details 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 *fan* transcription and K99 fimbriae expression (Braaten *et al.*, 1992). Unlike the other Lrp regulated fimbrial operons, the *fan* operon lacks both a *papI*-like regulatory gene as well as GATC box regions. Instead, the *fanA* and *fanB* regulatory genes both share DNA sequence similarities with *papB* (Roosendaal *et al.*, 1987) and encode proteins that stimulate *fan* transcription by a Lrp-independent antitermination mechanism (Braaten *et al.*, 1992). Thus, although *fan* transcription, like *pap, sfa* and *daa* transcription, is positively regulated by Lrp, Lrp regulates the *fan* operon and the *sfa, daa* and *pap* operons by different mechanisms.

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 *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 *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 *papB* and *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 *papI* and *papB*, there are two major transcription starts sites denoted p_I and p_{BA}. PapB binds to two different sites within the *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 *pap* transcription indirectly by stimulating transcription initiation at p_I (Fig. 7). The subsequent expression of PapI helps initiate *pap* transcription by interacting with the Lrp, PapB and CRP regulatory proteins and RNA polymerase all bound to the *pap* regulatory region (Fig. 7) (Van Der Woude *et al.*, 1992).

The *sfa* (Schmoll *et al.*, 1990a), *daa* (Bilge *et al.*, 1993b) and *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 IS*1* 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_1 and T_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 (Båga *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 (Dam). The operons encoding the Pap (Blyn *et al.*, 1990), S (Morschhäuer *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 *pap* transcription (Blyn *et al.*, 1990; Braaten *et al.*, 1994).

The methylation-dependent phase variation in the *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 *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 *pap* phase variation, the histone-like protein does exert a negative effect on the transcription of the *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 comformation and displace H-NS (Forsman *et al.*, 1992; Van Der Woude *et al.*, 1992). The effects of the various proteins on *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

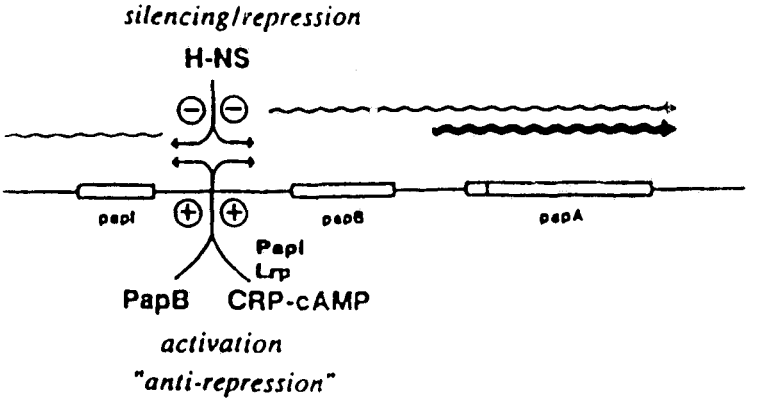


Fig. 7. Schematic diagram summarizing the components that influence transcription of the *pap* operon either positively or negatively (Uhlin, 1994). H-NS represses while PapB, PapI, Lrp and CRP-cAMP activate *pap* expression.

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.

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 26°C (Arnqvist *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 (Fe^{2+}) 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 (σ^{54}) 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 high-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 suseptibility 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 D_1 (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* (Arnqvist *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.

Bacterial strain	Description	Source or Reference
S. enteritidis		
27655-3b	wild type	T. Wadstrom
27655-3b-122	Tn*10* insertion mutant of 3b	Feutrier *et al.*, 1988
27655-3b1b	spontaneous rifampicin resistant mutant of 3b	Collinson *et al.*, 1992
Tn*phoA* 1-2	Tn*phoA* mutant of 3b1b	This study
Tn*phoA* 1-11	Tn*phoA* mutant of 3b1b	This study
Tn*phoA* 1-16	Tn*phoA* mutant of 3b1b	This study
Tn*phoA* 2-3	Tn*phoA* mutant of 3b1b	This study
E. coli		
HB101	F-*hsdS20* (r_B- m_B-) *leu supE44 ara14 galK2 lacY1 proA2 rpsL20* (Str^r) *xyl-5 mtl-1 recA13 mcrB*	Sambrook *et al.*, 1989
XL-1 Blue	F'::Tn*10 proA+B+lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (r_K- m_K+) *supE44 relA1 lac*	Stratagene, La Jolla, Calif
DH5α	F'/*endA1 hsdR17* (r_K-m_K+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1 Δ(lacZYA-argF) U169 (ø80dlacΔ(lacZ)M15)*	Sambrook *et al.*, 1989
TB1	F-*ara Δ(lac-proAB) rpsL* (Str^r)*(ø80dΔ(lacZ)M15) hsdR* (r_K-m_K+)	New England Biolabs, Inc.
SM10 pRT733	*λpir oriR6K tra- mob+* amp^r Km^r	Manoil and Beckwith, 1985
Salmonella serovars	wildtype	see Table 9

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 (rif^R) colonies were streaked onto solid LB media containing rifampicin (250 μg/ml) in two consecutive passes. Screening the rif^R mutants for Congo red binding and the presence of SEF14, SEF17 and SEF21 confirmed that the bacteria were *S. enteritidis*.

b. Production of Tn*phoA* mutants. Tn*phoA* 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_{650}, and then grown statically to 1.0 OD_{650}. The recipient strain, *S. enteritidis* 3b1b was grown in LB and rifampicin (100 μg/ml) shaking to 0.8 to 1.0 OD_{650}. 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

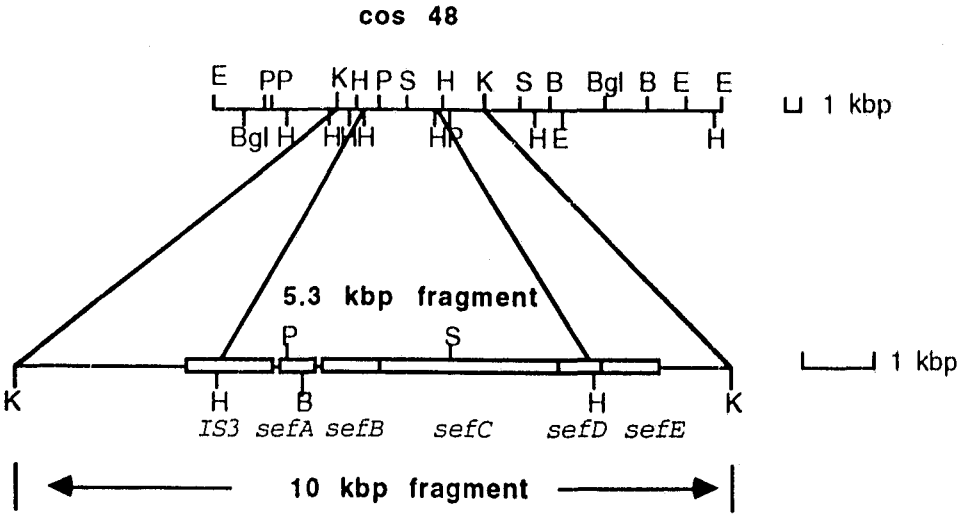


Fig. 8. Cloning strategy. The IS3 element and *sefABCDE* are encoded on the 10 kbp*Kpn* I fragment subcloned from cos48 (44 kb *Eco*R I fragment). The 5.3 kb *Hin*d III fragment originally used to sequence *sefABC* and part of *sefD* is also shown. Restriction-site abbreviations are as follows: B, *Bam*H I; Bgl, *Bgl* II; *Eco*R I; H, *Hin*d III; K, *Kpn* I; P, *Pst* I; and S, *Sma* I.

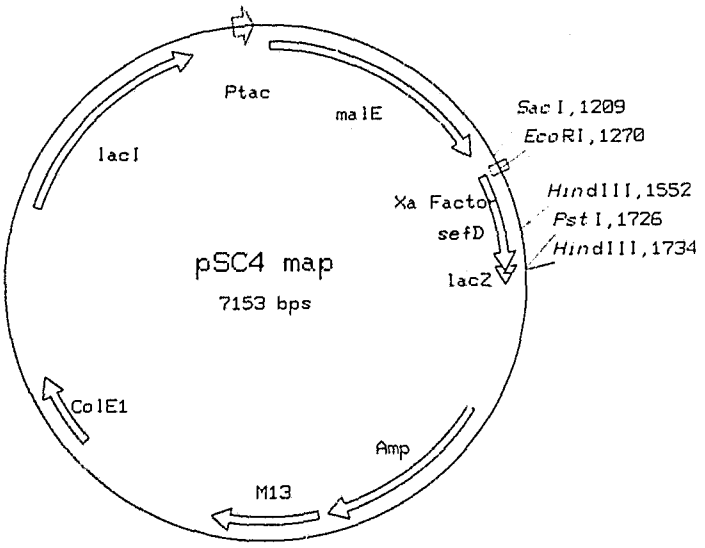
Sma I insert, was ligated together with T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD) to produce the construct designated pSC1. The ligations were transformed into *E. coli* HB101 (Sambrook *et al.*, 1989).

Table 2. Table of plasmids.

Plasmids	Description	Source
cos48	44 kbp of *S. enteritidis* DNA in pHC79	Feutrier *et al.*, 1988
pKX1	5.3-kb *Hind* III fragment of *S. enteritidis* DNA from cos48 subcloned into pTZ19R	Müller *et al.*, 1989
del A10, -B15, -B23, -C1, -D5, -D8, -D9, -D16, -D19, -E1, -E2 and -E21	pTZ18R derivatives containing sequential deletions of the 5.3-kb *Hind* III pKX1 fragment	Müller *et al.*, 1989
pSC1	1907 bp *Sma* I deletion in the 5.3-kb fragment from pKX1	This study
pSC2	10-kbp *Kpn* I fragment from cos48 subcloned into pUC19	This study
pSC3	447 bp PCR-amplified *sefD* gene from *S. enteritidis* cloned into pMal-c2	This study
pSC4	447 bp PCR-amplified *sefD* gene from*S. enteritidis* cloned into pMal-p2	This study
pSC5	447 bp PCR-amplified *sefD* gene from *S. enteritidis* cloned into pUC18	This study
pSC6	1046 bp *Hind* III/*Pst* I fragment from cos48 cloned into pTZ18R	This study
pSC7	741 bp PCR-amplified *sefB* gene from *S. enteritidis* cloned into pMal-p2	This study (Ch. IV)
pSC8	2445 bp PCR-amplified *sefC* gene from *S. enteritidis* cloned into pMal-p2	This study
pSC9	1573-bp *Bsm* I fragment from pKX1 subcloned into pUC19	This study (Ch. IV)
pSC10	1624 bp *Eco*R I/*Hind* III fragment from pSC9 subcloned into pINIII^{113}-B1	This study (Ch. IV)
pEU2030	1200 bp *Hha* I fragment encompassing the *rns* gene cloned into pUC18	J.R. Scott
pSFA	584-bp *Dra* I fragment cloned into pUC19	J.L. Doran

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 *Eco*R 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 *Eco*R 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



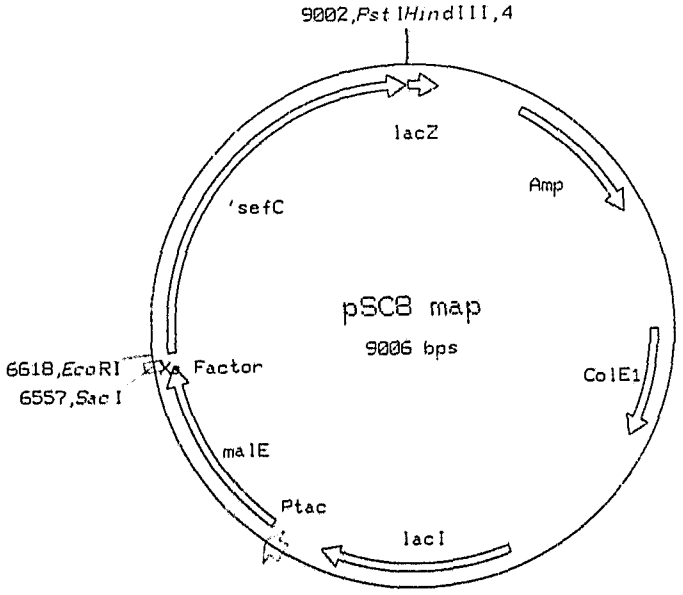


Fig. 9. Plasmid maps: pSC4 (A), and pSC8 (B). *sefC* and *sefD* were PCR amplified, purified, digested with *Eco*R I and *Pst* I, purified and ligated into the *Eco*R I/*Pst* I sites of pMal-p2. The map of pSC3 is identical to that of pSC4 except that the *malE* gene in pSC3 has a deletion of the signal 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 *Hin*d III and *Pst* I (New England Biolabs Inc.), and rerun on a 1% agarose gel in TAE buffer. The appropriate *Hin*d III/*Pst* I band was purified from the gel using an electroeluter (Pharmacia-LKB) and then ligated to *Hin*d 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 $MgCl_2$, 5 mM ATP, 5 mM DTT and 25% (w/v) polyethylene glycol-8000], vector and insert DNA and sterile dH_20. 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 $CaCl_2$ 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 OD_{660} 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 $MnCl_2$, 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 $CaCl_2$, 5 mM $MnCl_2$). 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.

b. Method of Alm *et al.*, (1993). Cells from 20 ml overnight 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.

PCR primers	Primer sequence (5' to 3')	Source
ST2	GGAGATTTTGGAATTCGCGTAAATCAGCATCTGCA GTAGC	U of Victoria
sefB-T (Ch. IV)	GCTACCTTCCAGAATTCTCTTCGGAAAGTAAAAAAA	U of Calgary
sefB-B (Ch. IV)	TTGTTTACACCTGCAGCTAATAATCTCTTATAATT	U of Calgary
sefC-T	TGGAAATGTTGAATTCAGACAATATAATTTCGACT	U of Calgary
sefC -B	GACGAATTATCTGCAGTCATTTGCACACTCCATTT	U of Calgary
sefD-T2	GCTTTTTAAATGGAGTGAATTCATGAATCAGTAT AATTCG	U of Victoria
sefD-B2	CGCCAATTTGCGCTGCAGTTATTATAATTCAATTTC	U of Victoria
Tn*phoA*	AAAACGGGAAAGGTTCCG	U of Calgary
Hybridization probes	**Probe sequence (5' to 3')**	**Source**
*sefA*1	CAGCCAACTGGAGTCAGG	U of Calgary

a. PCR amplification of 5' end of *sefA* and Tn*phoA*.

Chromosomal DNA used for the PCR analysis was prepared from *S. enteritidis* 3b and the four Tn*phoA* 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 (Tn*phoA*) was derived from the $IS50_L$ sequence in Tn*phoA*. 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 $MgCl_2$ 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 amplications 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 *Eco*R 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 $MgCl_2$ 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 *Eco*R 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 $MgCl_2$ 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,

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

Sequencing primers	Primer sequence (5' to 3')	Source
D16b	TACTGCTGAACGTAGAA	Ultra Diagnostics
B15a	GTTACAATAAATGGAGC	Ultra Diagnostics
B15b	AATTGAGCATATCCAAA	Ultra Diagnostics
B15c	ATAATCTCTTATAATTT	Ultra Diagnostics
Oligo B15	GGTTGTGTGTAAAAGGAGT	Ultra Diagnostics
E2	TGATTCAAATTTTTTTT	Ultra Diagnostics
E21	TCTACCAATAGTTACAC	Ultra Diagnostics
A10	CATAATTGTAGCTTAAG	Ultra Diagnostics
E1	TAGTTTGCTCCGATCTC	Ultra Diagnostics
C1	CAAGAGCTAGTGGCTTT	Ultra Diagnostics
D10	TGAACTAATGAGGATTT	Ultra Diagnostics
sefU1	GCCAAGCTTTCGCCCGCAGC	U of Victoria
sefU2	GACATACTTCATTTCAGGC	U of Victoria
sefU3	CGTCGCGAAATTCAGGCG	U of Victoria
sefU4	GTGGATATTAGATATTCC	U of Victoria
sefU5	GCAGGTGCTGCGGGCGAAAG	U of Victoria
sefU6	GTGGATGATCCATTAATAC	U of Victoria
sefU7	CCACCAAAAAGCCATGCAAG	U of Victoria
sefU8	CGCGACATACTTCGCGAAAC	U of Victoria
sefU9	CTGTGGCGGCGTAAAAGAC	U of Victoria
sefU10	GCCACAGTGCCTGTGCGTC	U of Victoria
sefU11	CCACGGCCAGATAAAGCC	U of Victoria
sefU12	CCTCGCGGATGACGGCAC	U of Victoria
sefD	GCGACAGAAATTGAATTA	U of Victoria
sefE	TAGTATTTTCATTCTCAA	U of Victoria
sefE2	CAGTTAATTCCATTGTCGG	U of Victoria
sefE3	CTTAATGGTAACAATACAG	U of Victoria
sefE4	CCTCTAGAGTCGACCTGCAG	U of Victoria
sefE5	CTTTATTAGATATGATTATCCG	U of Victoria
sefE6	CACTTATAACAACCACACATGG	U of Victoria
sefE7-2	CTAGAGGCAACATAGCC	U of Victoria
sefE8	CGCTACAGAACATTTAGCG	U of Victoria
sefE9	GACATTTCAACGAAATGGA	U of Victoria
sefE10	TTACAGAATCCTTCTGGATG	U of Victoria
sefE11	CACATGGCTTACACTTGC	U of Victoria
sefE12	GAGTTTTATGCTTATTTGAG	U of Victoria
sefE13	GTCATTATGCATATTCAC	U of Victoria
sefE14	CCAAACGCGCTGCTTTTTGC	U of Victoria
Universal reverse	TGCGTGGCCACTGCCAC	U of Calgary

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, 1988) 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 *sefDE$_1$E$_2$*

The open reading frames *sefD*, *sefE$_1$* and *sefE$_2$* 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_2O 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_2O, dialyzed against sterile distilled H_2O (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 centrifigation (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 encorporated 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 antisera. 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, Broma, Sweden) at 0.8 mA/cm^2 for 90 min. After staining proteins with Amido Black, the 14,000 M_r 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/cm^2 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 [^{35}S]-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 [^{35}S]-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 H_2O 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 [α-^{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 [α-^{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 *Eco*R I and *Hin*d 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 *Eco*R I and *Hin*d 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-^{32}P]-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 *Hin*d 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 M_r, respectively. The predicted M_r (14,436) and amino acid composition of SefA confirmed the M_r 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). 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 GENPEPT data bases 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

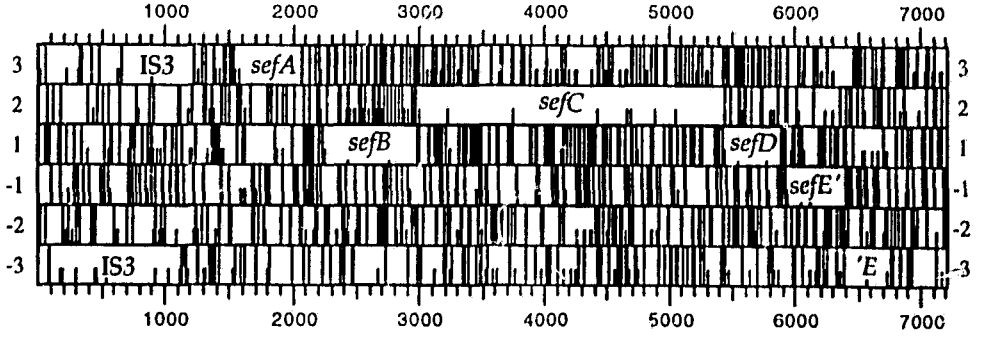


Fig. 10. Open reading frame map of *sefABCDE*$_1$*E*$_2$ and the IS3 element. The DNA sequence was analyzed in all six reading frames which are labelled from 1 to 3 and -1 to -3 on each side of the diagram. Full lines represent putative translation stop sites whereas the half lines represent putative 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.

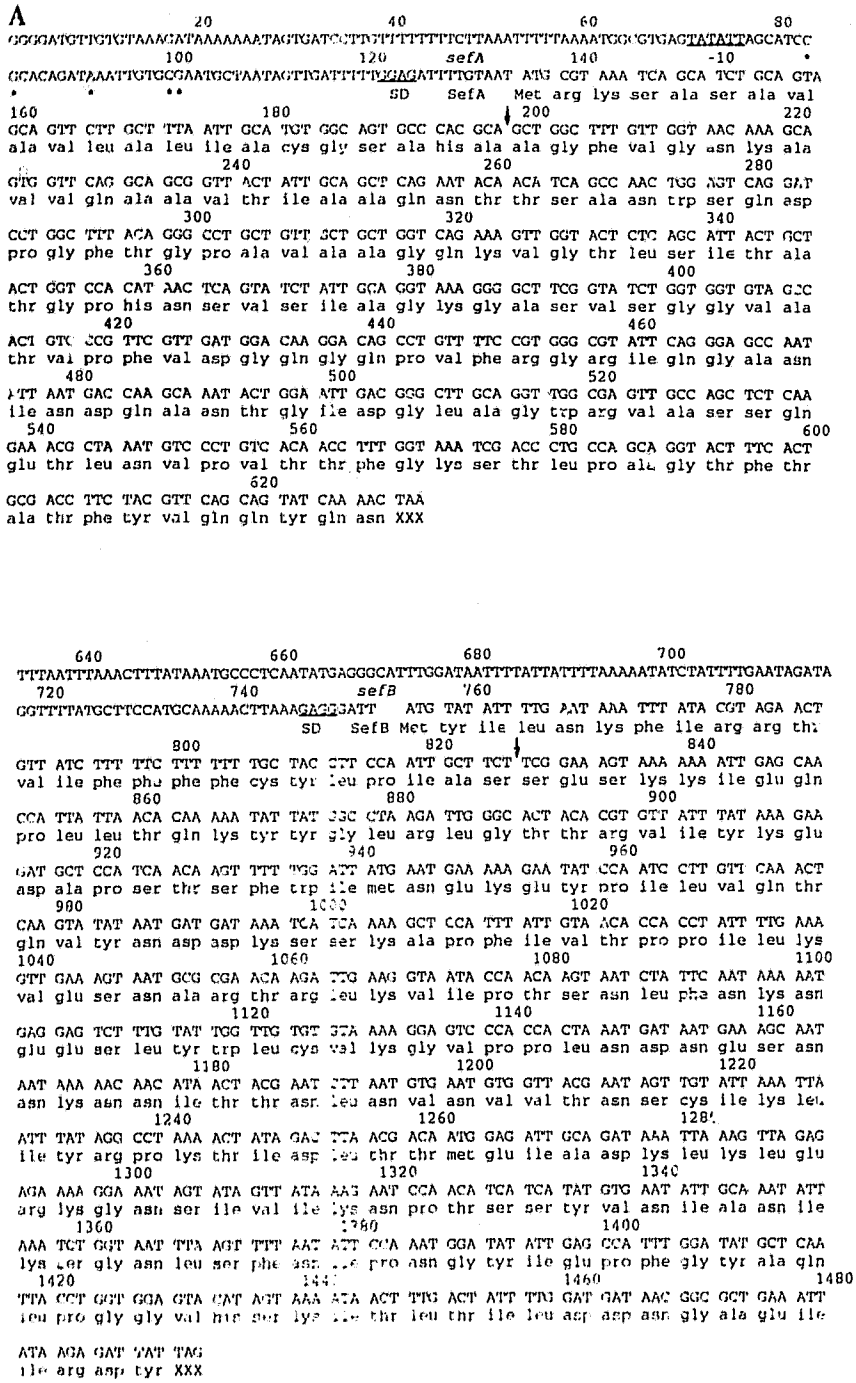
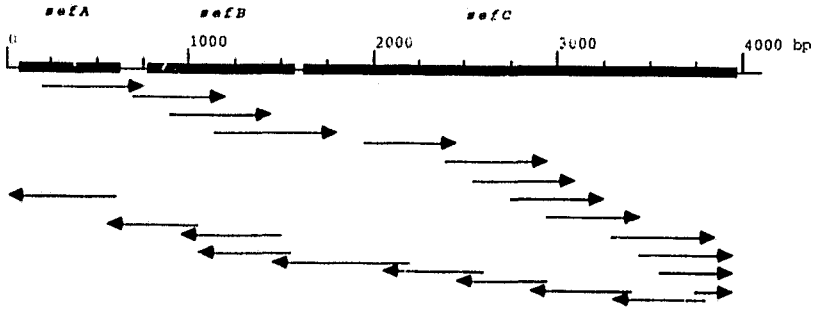


Fig. 11. Nucleotide sequence of *sefABC*. (A) Consensus Shine-Dalgarno (SD) sequences, located 8 to 14 bp upstream of the translational start site of each gene, and the -10 putative promoter region are underlined. The termination codons are indicated as XXX, the transcription start sites are indicated 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.

```
    1500  selC              1520                  1540                  1560
TTTAAGCTTTAAACAAATG AAG AAA ACC ACA ATT ACT CTA TTT GTT TTA ACC AGT GTA TTT CAC TCT
      SD  SelC  Met lys lys thr thr ile thr leu phe val leu thr ser val phe his ser
                      1580                  1600                  1620
GGA AAT GTT TTC TCC AGA CAA TAT AAT TTC GAC TAT GGA AGT TTG AGT CTT CCT CCC GGT GAG
gly asn val phe ser arg gln tyr asn phe asp tyr gly ser leu ser leu pro pro gly glu
                  1640                  1660                  1680
AAT GCA TCT TTT CTA AGT GTT GAA ACG CTT CCT GGT AAT TAT GTT GTT GAT GTA TAT TTG AAT
asn ala ser phe leu ser val glu thr leu pro gly asn tyr val val asp val tyr leu asn
              1700                  1720                  1740
AAT CAG TTA AAA GAA ACT ACT GAG TTG TAT TTC AAA TCA ATG ACT CAG ACT CTA GAA CCA TGC
asn gln leu lys glu thr thr glu leu tyr phe lys ser met thr gln thr leu glu pro cys
          1760                  1780                  1800
TTA ACA AAA GAA AAA CTT ATA AAG TAT GGG ATC GCC ATC CAG GAG CTT CAT GGG TTG CAG TTT
leu thr lys glu lys leu ile lys tyr gly ile ala ile gln glu leu his gly leu gln phe
      1820                  1840                  1860
GAT AAT GAA CAA TGC GTT CTC TTA GAG CAT TCT CCT CTT AAA TAT ACT TAT AAC GCG GCT AAC
asp asn glu gln cys val leu leu glu his ser pro leu lys tyr thr tyr asn ala ala asn
  1880                  1900                  1920                  1940
CAA AGT TTG CTT TTA AAT GCA CCA TCT AAA ATT CTA TCT CCA ATA GAC AGT GAA ATT GCT GAT
gln ser leu leu leu asn ala pro ser lys ile leu ser pro ile asp ser glu ile ala asp
                  1960                  1980                  2000
GAA AAT ATC TGG GAT GAT GGC ATT AAC GCT TTT CTT TTA AAT TAC AGA GCT AAT TAT TTG CAT
glu asn ile trp asp asp gly ile asn ala phe leu leu asn tyr arg ala asn tyr leu his
              2020                  2040                  2060
TCT AAG GTT GGA GGA GAA GAT TCA TAC TTT GGT CAA ATT CAA CCT GGT TTT AAT TTT GGT CCC
ser lys val gly gly glu asp ser tyr phe gly gln ile gln pro gly phe asn phe gly pro
          2080                  2100                  2120
TGG CGG CTA AGG AAT CTA TCA TCT TGG CAA AAC TTG TCA AGC GAA AAA AAA TTT GAA TCA GCA
trp arg leu arg asn leu ser ser trp gln asn leu ser ser glu lys lys phe glu ser ala
      2140                  2160                  2180
TAT ATT TAT GCT GAG CGA GGT TTA AAA AAA ATA AAC AGC AAA CTA ACA GTT GGG GAC AAA TAT
tyr ile tyr ala glu arg gly leu lys lys ile lys ser lys leu thr val gly asp lys tyr
          2200                  2220                  2240
ACC AGT GCA GAT TTA TTC GAT AGC GTA CCA TTT AGA GGC TTT TCT TTA AAT AAA GAT GAA AGT
thr ser ala asp leu phe asp ser val pro phe arg gly phe ser leu asn lys asp glu ser
      2260                  2280                  2300
ATG ATA CCT TTC TCA CAG AGA ACA TAT TAT CCA ACA ATA CGT GGT ATT GCG AAA ACC AAT GCG
met ile pro phe ser gln arg thr tyr tyr pro thr ile arg gly ile ala lys thr asn ala
  2320                  2340                  2360                  2380
ACT GTA GAA GTA AGA CAA AAT GGA TAC TTG ATA TAT TCT ACT TCA GTC CCC CCC GGG CAA TTC
thr val glu val arg gln asn gly tyr leu ile tyr ser thr ser val pro pro gly gln phe
                  2400                  2420                  2440
GAG ATA GGT AGA GAA CAA ATT GCT GAT CTT GGT GTT GGG GTT GGG CTT GAT GTT AGC ATT
glu ile gly arg glu gln ile ala asp leu gly val gly val gly val leu asp val ser ile
              2460                  2480                  2500
TAT GAA AAA AAT GGG CAG GTC CAA AAC TAT ACA GTG CCA TAT TCA ACT CCT GTA TTA TCT TTG
tyr glu lys asn gly gln val gln asn tyr thr val pro tyr ser thr pro val leu ser leu
          2520                  2540                  2560
CCT GAT GGA TAT TCT AAA TAT AGT GTA ACT ATT GGT AGA TAC AGG GAG GTT AAC AAT GAT TAT
pro asp gly tyr ser lys tyr ser val thr ile gly arg tyr arg glu val asn asn asp tyr
      2580                  2600                  2620
ATC GAT CCT GTT TTT TTT GAA GGG ACT TAT ATA TAT GGT CTG CCT TAT GGG TTT ACT TTA TTT
ile asp pro val phe phe glu gly thr tyr ile tyr gly leu pro tyr gly phe thr leu phe
  2640                  2660                  2680
GGT GGA GTG CAA TGG GTA AAT ATT TAT AAT TCA TAT GCC ATA GGC GCA AGT AAA GAT ATT GGT
gly gly val gln trp val asn ile tyr asn ser tyr ala ile gly ala ser lys asp ile gly
    2700                  2720                  2740
GAG TAT GGT GCT CTG TCT TTT GAC TGG AAA ACA TCT GTT TCG AAG ACT GAT ACA TCC AAT GAA
glu tyr gly ala leu ser phe asp trp lys thr ser val ser lys thr asp thr ser asn glu
2760                  2780                  2800                  2820
AAT GGT CAT GCA TAT GGG ATT AGA TAC AAT AAA AAT ATC GCT CAG ACA AAC ACC GAA GTA TCT
asn gly his ala tyr gly ile arg tyr asn lys asn ile ala gln thr asn thr glu val ser
                  2840                  2860                  2880
TTG GCT AGT CAT TAC TAT TAT TCG AAA AAT TAT AGA AGT TTT TCT GAA GCA ATT CAT AGT AGC
leu ala ser his tyr tyr tyr ser lys asn tyr arg ser phe ser glu ala ile his ser ser
              2900                  2920                  2940
GAG CAT GAT GAA TTT TAC GAT AGA AAT AAG AAA TCA ACA ACC TCT ATG TTA TTA AGT CAG GCA
glu his asp glu phe tyr asp arg asn lys lys ser thr thr ser met leu leu ser gln ala
          2960                  2980                  3000
TTA GGA TCT CTA GGT TCT GCT GAC TTA AGC TAC AAT TAT GAT AAA TAT TGG AAA GAT GAA GGT
leu gly ser leu gly ser val asp leu ser tyr asn tyr asp lys tyr trp lys his glu gly
      3020                  3040                  3060
AAA AAA TCA ATA ATT GCT AAT GAC GGG AAG AAT TTA AAT GGT GTT TGG TTA TGG CTT TCA TAT
lys lys ser ile ile ala asn asp gly lys asn leu asn gly val trp leu trp leu ser tyr
```

```
      3080                        3100                        3120
ACG AAA AGT ACA TCA AAG ATT AGT GAA GAA AAT GAA GAT TTA TTC AGT TTT CTA CTC AGT GTA
thr lys ser thr ser lys ile ser glu glu asn glu asp leu phe ser phe leu leu ser val
  3140                        3160                        3180                        3200
CCT TTG CAA AAA CTT ACA AAT CAT GAA ATG TAT GCT ACA TAT CAA AAC TCA TCC TCT TCA AAG
pro leu gln lys leu thr asn his glu met tyr ala thr tyr gln asn ser ser ser ser lys
                    3220                        3240                        3260
CAT GAT ATG AAT CAT GAT TTA GGT ATT ACT GGT GTG GCA TTT AAT AGC CAA TTG ACA TGG CAA
his asp met asn his asp leu gly ile thr gly val ala phe asn ser gln leu thr trp gln
                3280                        3300                        3320
GCA AGA GGG CAA ATA GAA GAT AAA TCG AAA AAT CAA AAG GCT ACA TTT TTA AAT GCT TCT TGG
ala arg gly gln ile glu asp lys ser lys asn gln lys ala thr phe leu asn ala ser trp
              3340                        3360                        3380
CGA GGT ACT TAT GGG GAG ATC GGA GCA AAC TAT AGT CAT AAT GAA ATA AAT CGT GAT ATT GGG
arg gly thr tyr gly glu ile gly ala asn tyr ser his asn glu ile asn arg asp ile gly
            3400                        3420                        3440
ATG AAT GTT TCT GGT GGG GTG ATT GCT CAT TCA TCA GGA ATT ACG TTT GGT CAG AGT ATA TCG
met asn val ser gly gly val ile ala his ser ser gly ile thr phe gly gln ser ile ser
          3460                        3480                        3500
GAT ACT GCT GCA CTG GTA GAG GCT AAA GGT GTA AGT GGG GCA AAA GTT CTG GGC CTA CCA GGT
asp thr ala ala leu val glu ala lys gly val ser gly ala lys val leu gly leu pro gly
      3520                        3540                        3560
GTT AGA ACC GAT TTT AGA GGC TAT ACA ATA TCC AGT TAT CTT ACT CCA TAT ATG AAT AAC TTC
val arg thr asp phe arg gly tyr thr ile ser ser tyr leu thr pro tyr met asn asn phe
 3580                        3600                        3620                        3640
ATA TCT ATA GAT CCA ACA ACG TTA CCA ATA AAT ACG GAT ATT AGG CAA ACT GAT ATT CAA GTA
ile ser ile asp pro thr thr leu pro ile asn thr asp ile arg gln thr asp ile gln val
                    3660                        3680                        3700
GTT CCT ACC GAA GGT GCT ATT GTA AAA GCT GTA TAT AAA ACA AGC GTG GGT ACT AAT GCA TTA
val pro thr glu gly ala ile val lys ala val tyr lys thr ser val gly thr asn ala leu
                3720                        3740                        3760
ATT AGA ATT ACA AGA ACA AAT GGA AAG CCA CTA GCT CTT GGC ACA GTT CTT TCA CTT AAG AAT
ile arg ile thr arg thr asn gly lys pro leu ala leu gly thr val leu ser leu lys asn
              3780                        3800                        3820
AAT GAT GGA GTA ATC CAA TCA ACA TCT ATT GTT GGC GAA GAT GGT CAG GCA TAT GTA TCT CGA
asn asp gly val ile gln ser thr ser ile val gly glu asp gly gln ala tyr val ser gly
            3840                        3860                        3880
TTG TCA GGA GTG CAA AAA TTA ATC GCT TCG TGG GGG AAT AAG CCC TCC GAT ACT TGT ACA GTT
leu ser gly val gln lys leu ile ala ser trp gly asn lys pro ser asp thr cys thr val
      3900                        3920                        3940
TTT TAC TCT CTT CCC GAT AAA AAC AAA GGT CAG ATT AGC TTT TTA AAT GGA GTG TGC AAA TGA
phe tyr ser leu pro asp lys asn lys gly gln ile ser phe leu asn gly val cys lys XXX
```

B



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 (Gierasch, 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

Chaperone Protein	Bacterial Strain	Percent Identity[a]	Percent Similarity[a]	Combined Percent	Amino acid Overlap	Total number of amino acids	Molecular Weight
PapD	*E. coli*	28	47	75	163	239	28,500[b]
CS3-1	*E. coli*	31	44	75	224	241	27,000[c]
MrkB	*K. pneumoniae*	27	49	76	171	232	25,000[d]

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 *et al.*, 1989

c. Jalajakumari *et al.*, 1989

d. Allen *et al.*, 1991

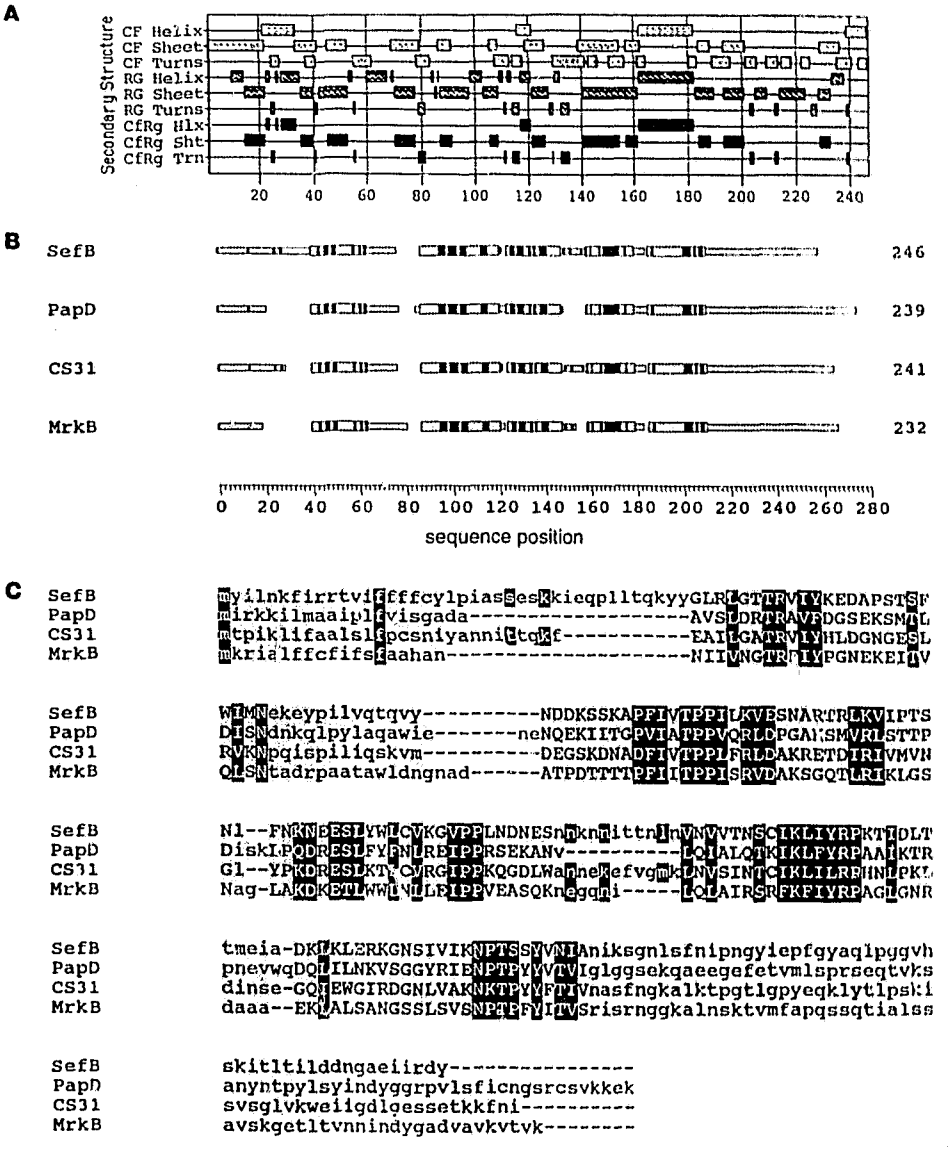


Fig. 12. Secondary-structure analysis of SefB and local alignment of SefB and three fimbrial periplasmic chaperone proteins. (A) Secondary-structure analysis of the predicted amino acid sequence for SefB. (B) Schematic representation and aligned sequences (C) of fimbrial chaperone proteins analyzed for 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

OM Protein	Bacterial Strain	Percent Identity[a]	Percent Similarity[a]	Combined Percent	Amino Acid Overlap	Molecular Weight
PapC	*E. coli*	23.1	44.1	67.2	745	81,000[b]
FaeD	*E. coli*	24.0	45.0	69.0	741	82,200[c]
FanD	*E. coli*	22.4	48.1	70.5	701	84,500[d]
MrkC	*K. pneumoniae*	26.9	47.3	74.2	714	91,000[e]
CS3-3	*E. coli*	28.9	46.7	75.6	373	48,000[f]
CS3-4	*E. coli*	29.3	48.4	77.7	264	33,000[f]
CS3-5	*E. coli*	34.0	46.7	80.7	145	20,000[f]
CS3-2	*E. coli*	30.7	63.2	93.9	547	63,000[f]

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 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)

b. Norgren *et al.*, 1987

c. Mooi *et al.*, 1986

d. Rossendaal and DeGraaf, 1989

e. Allen *et al.*, 1991

f. Jalajakumari *et al.*, 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 M_r and 12,300 M_r, 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 B_1, B_2 or B_3 (Fig. 13A). Depending on where translation was initiated, a protein of 41,000, 35,000 or 33,000 M_r 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 IS*3* from *E. coli*. Thus, an IS*3* 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 data bases 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* IS*3*. 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 IS*3* element did not correspond to the position of *orfIII* in the IS*3* 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 IS*3* 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 IS*3* from *E. coli* (Table 7). The alignment of the

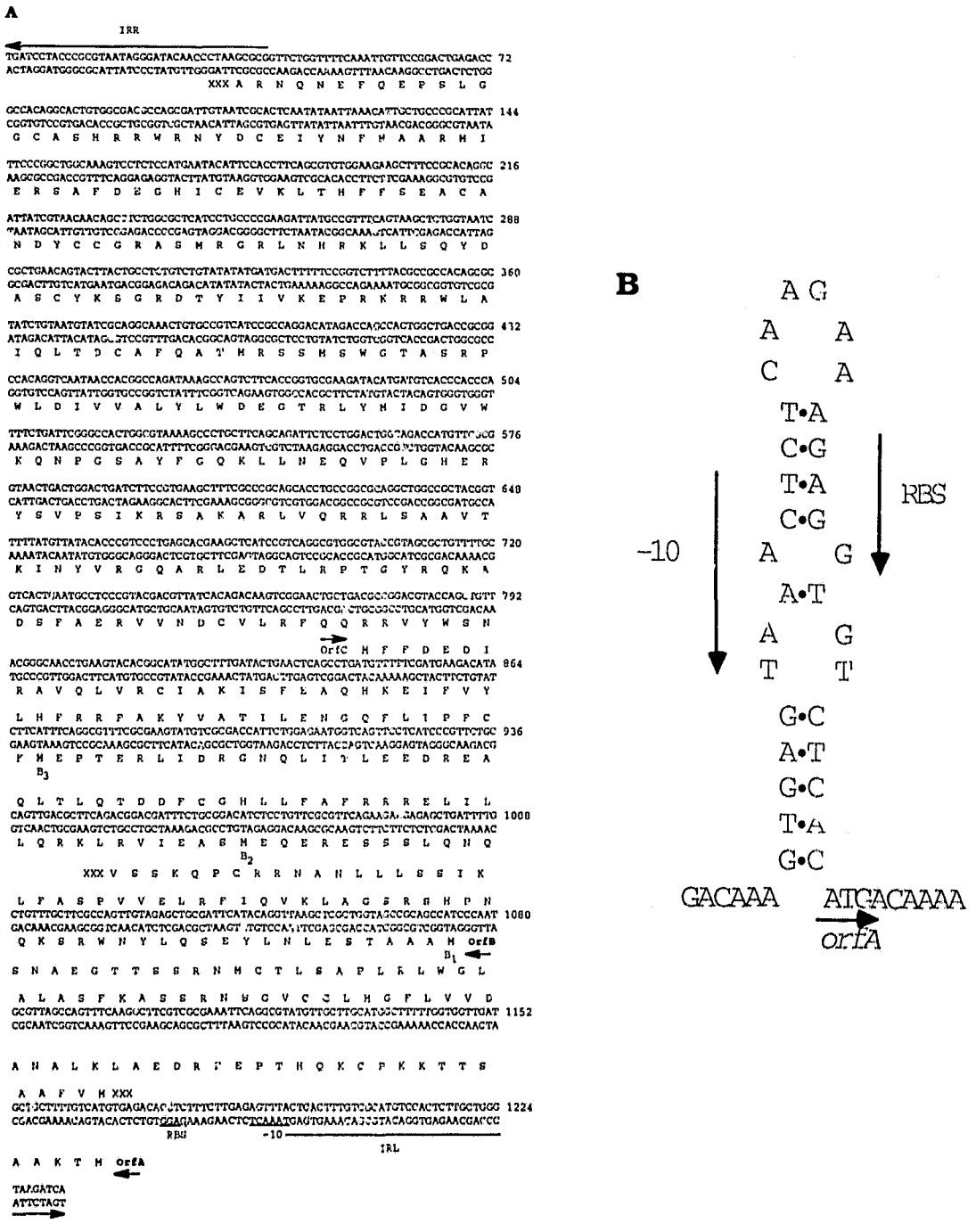


Fig. 13. A. Nucleotide sequence of the IS3 element. Consensus Shine-Dalgarno (RBS) sequences and the -10 region are underlined and the termination codons are indicated as XXX. The direction of each ORF is indicated by the short arrows whereas the two long arrows represent the 39 bp inverted repeats. The numbering is only for reference and does not correspond to the position of the IS3 element relative to *sefA*. B. Schematic representation of the proposed secondary structure within the left IR of the IS3 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.

Putative Transposase	Bacterial Strain	IS element	Percent Identity[a]	Percent Similarity[a]	Combined Percent	Amino acid Overlap	Total Amino acids
OrfI	*E.coli*	IS3	80.5	6.9	87.4	277	288
OrfB	*Erwinia amylovora*	Tn*5393*	40.3	16	56.3	268	285
	Acinetobacter calcoaceticus		41	17	58	235	272
Protein 1	*Lactococcus lactis*		31.8	13.8	45.6	305	384
	Lactococcus lactis	IS*1069*	37.6	17.2	54.8	274	299
OrfB	*Leptospira interrogans*		38	25.6	63.6	234	282
Protein B	*Shigella sonnei*	IS*600*	36.3	18.1	54.4	256	272
Orf1	*Bacillus stearothermophilus*		30.2	22.4	52.6	255	287
Protein B	*E. coli*	IS*150*	34.4	20.8	55.2	221	283
	E. coli	IS*1203*	29.5	21.3	50.8	258	296
	Corynebacterium glutamicum		33.2	17	50.2	253	298
	Pseudomonas cepacia	IS*401*	34.4	18.8	53.2	160	229
Protein 4	*Agrobacterium tumefaciens*		31	21.6	52.3	213	301
Protein B	*Shigella sonnei*	IS*629*	29.3	20	49.3	229	296
C3	*Mycoplasma hyorhinis*	IS*1221*	31.2	18.3	49.5	186	474
	Mycobacterium tuberculosis	IS*986*	34.5	20.4	54	142	278
	Mycobacterium bovis	IS*987*	34.5	20.4	54.9	142	346
	Lactobacillus casei	IS*L1*	31.2	15.6	46.8	186	274
Orf2	*Streptococcus agalactiae*	IS*861*	32.4	19	51.4	179	277
	E. coli	IS*3411*	28.3	21	49.3	152	240
	Pediococcus pentosaceus		42.9	21.4	64.3	98	195
	Lactobacillus sake		30.4	21.1	51.5	171	302
	Mycoplasma hyopneumoniae		27.1	21.5	48.6	181	275
	Lactobacillus johnsonii	IS1223	32	28.2	60.2	103	313
	Mycoplasma pulmonas	IS*1138*	32	24.3	56.3	103	400

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 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 B_3 resulting in the production of a 33,000 M_r protein. However, frameshift analysis suggested that translation of *orfB* was initiated at B_2. In the IS3 element from *E. coli*, the start and stop codons for *orfI* and *orfII*, respectively, overlap and a frameshift event fuses the products of the two genes. In the IS3 element from *S. enteritidis*, the B_2 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 IS*1203* of *E. coli* 0111:H- (296 aa), IS*629* of *Shigella sonnei* (296 aa) and IS*3411* of *E. coli* (240 aa) suggested that translation of *orfB* was initiated at B_1 to produce a 41,000 M_r 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 B_1 and B_3 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 *sefA* revealed several elements that were also present in the regulatory regions of other fimbrial operons. Alignment of the -10 region of the *sefA* promoter (see Chapter III) to the -10 region of the promoters in the *pap, sfa, daa* and *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 *sef14* regulatory region at a comparable position (Fig. 14). Two more GATC sites were also present in the *sef14* regulatory region but these did not overlap the known GATC sites in the other operons. The GATC site located farthest from the *sefA* promoter was actually located within the left inverted repeat of the IS3 element (Fig. 14). All three *sef14* GATC sites were situated on inverted repeats, just like in the *pap, fae, daa* and *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 *sef14* regulatory region, one of which was located at the same position as the second GATC site of the *pap, sfa, daa* and *fae* operons (Fig. 14).

The nucleotide sequence of DNA immediately downstream of *sefABC* revealed a fourth open reading frame (ORF) designated *sefD*. This gene had the same translational polarity as *sefABC* (Fig. 10). In fact, the AUG start codon for *sefD* overlapped the UGA stop codon of *sefC* (Fig. 15). To eliminate the possibility that DNA rearrangement had occurred during cloning, the

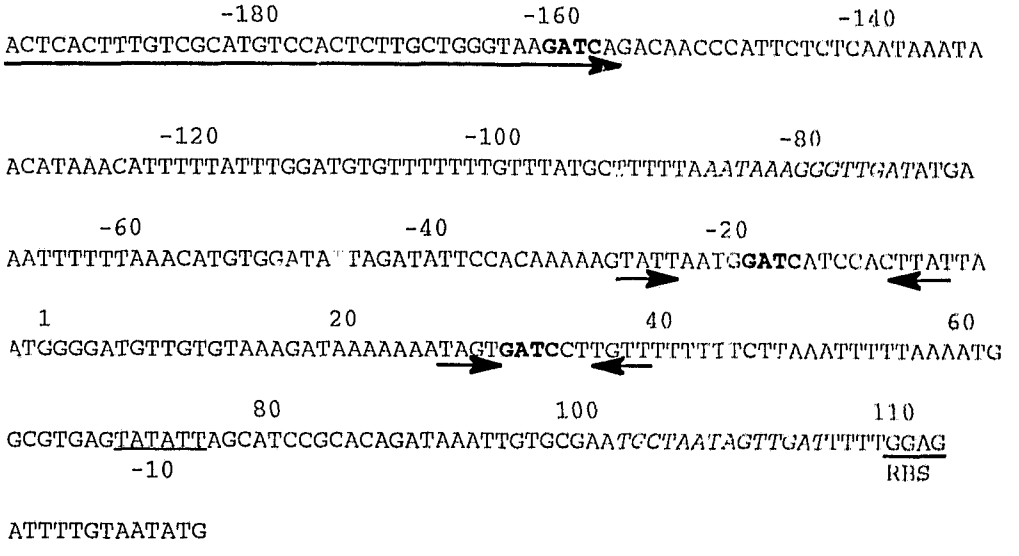


Fig. 14. Nucleotide sequence of the intergenic region between *sefA* and the IS3 element. The numbers above the sequence refer to the position of the nucleotides relative to *sefA*. Each GATC site is in bold, the corresponding inverted repeats are indicated by the short arrows, the consensus IHF sites 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_r (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
TTTTTACTCTCTTCCCGATAAAAACAAAGGTCAGATTAGCTTTTTAAATGGAGTGTGCAA
```

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
```

SefD

```
  4000                          4020                          4040
TTT CTG ATT GTT ACT GGT TTT TTC AGC TCA ACT ATT AAA GCA CAA
phe leu ile val thr gly phe 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 AGT AAT AAA ATA ATA GGT ACG GGG CAT CTA TTT AAC
arg ile thr ser asn lys ile ile gly thr gly his leu phe asn

        4140                          4160
AGA GAG GGA AAA AAA ATC CTC ATT AGT TCA AGT TTA GAA AAA ATT
arg glu gly lys lys ile leu ile ser ser ser leu glu lys ile

  4180                          4200                          4220
AAA AAT ACC CCA GGG GCA TAT ATT ATT AGA GGT CAG AAT AAC TCA
lys asn thr pro gly ala tyr ile ile arg gly gln asn asn ser

                        4240                          4260
GCC CAT AAG CTT AGG ATA AGA ATA GGT GGA GAA GAC TGG CAA CCA
ala his lys leu arg ile arg ile gly gly glu asp trp gln pro

                4280                          4300
GAT AAT TCA GGT ATT GGT ATG 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 GGG AAT GGA GAC ATT CCT GTT 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 GCG 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.

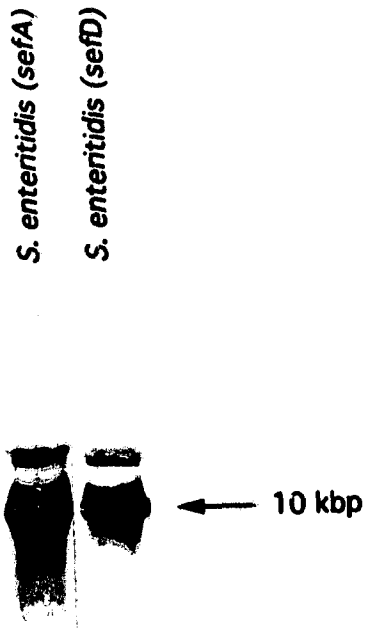


Fig. 16. Southern blot hybridization of *Kpn* I digested *S. enteritidis* DNA with *sefA* and *sefD* probes. The arrow indicates the 10 kb fragment that hybridizes to the radiolabelled *sefA* and *sefD* gene probes.

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, $SefE_2$ 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 $SefE_2$ revealed a helix-turn-helix motif that corresponded to the putative DNA binding domain in CfaD, Rns and AggR. Together, $SefE_1$ and $SefE_2$ 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, $sefE_1$ and $sefE_2$ 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 [^{35}S]-radiolabelled proteins, whereas SefA was identified

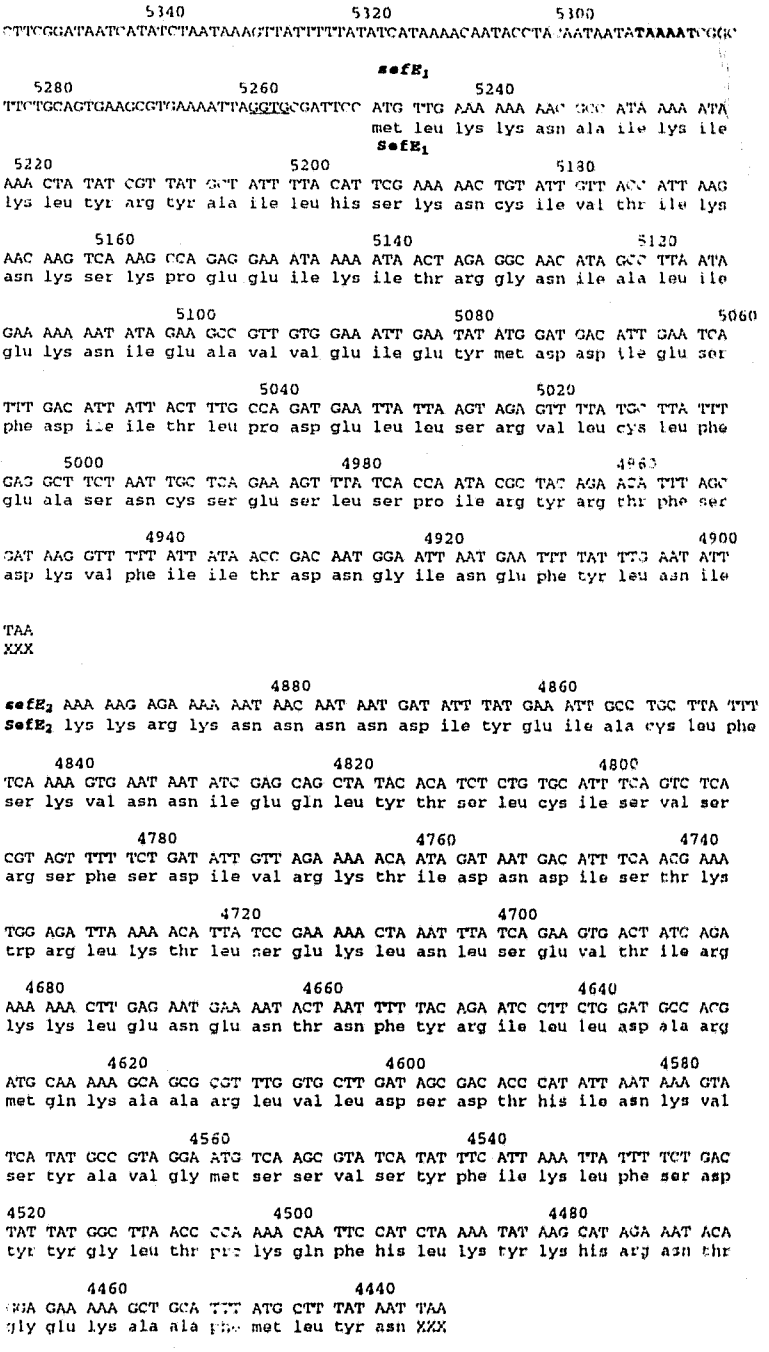


Fig. 17. Nucleotide sequence of *sefE*$_1$ and *sefE*$_2$. The consensus Shine-Dalgarno sequence is underlined and the putative -10 region of the promoter is indicated in bold letters. The numbers above each line refer to the nucleotide position of the two sequences. The predicted amino acid sequences are shown below the DNA sequence.

Table 8. A comparison of the predicted amino acid sequences for $SefE_1$ and $SefE_2$ with those of AraC-like transcriptional regulators.

Transcriptional regulator	Bacterial strain	Regulated system	Percent Identity[a]	Percent similarity[a]	Combined Percent	Amino acid Overlap	Total Amino acids
VirF	*Shigella* sp.	Invasion protein synthesis	40.8	22.7	63.7	238	262
FapR	*E. coli*	Fimbriae biosynthesis	37.3	24.9	62.2	217	260
CfaD	*E. coli*	Fimbriae biosynthesis	46	24	70	130	265
Rns	*E. coli*	Fimbriae biosynthesis	45	23	68	135	265
AggR	*E. coli*	Fimbriae biosynthesis	44	24	68	128	265
CsvR	*E. coli*	Fimbriae biosynthesis	40	29	69	130	301
PerA	*E. coli*	Unknown	36	23	59	137	205
EnvY	*E. coli*	Porin biosynthesis	37	24	61	105	253
AppY	*E. coli*	Acid phosphatase	36	20	56	109	243
AdiY	*E. coli*	Arginine decarboxylase	35	28	63	103	253
UreR	*Proteus mirabilis*	Urease biosynthesis	31	17	50	105	293
UreR	*E. coli*	Urease biosynthesis	30	22	52	97	296
ToxT	*Vibrio cholerae*	Fimbriae biosynthesis	21	29	50	114	276
MsmR	*Streptococcus mutans*	Sucrose utilization	28	27	55	60	278
AdaA	*Bacillus subtilis*	Methyl transferase	29	21	50	55	211

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 $SefE_1$ and $SefE_2$ sequences to each of the other AraC-like transcriptional regulators in the Blast search from March 15, 1995.

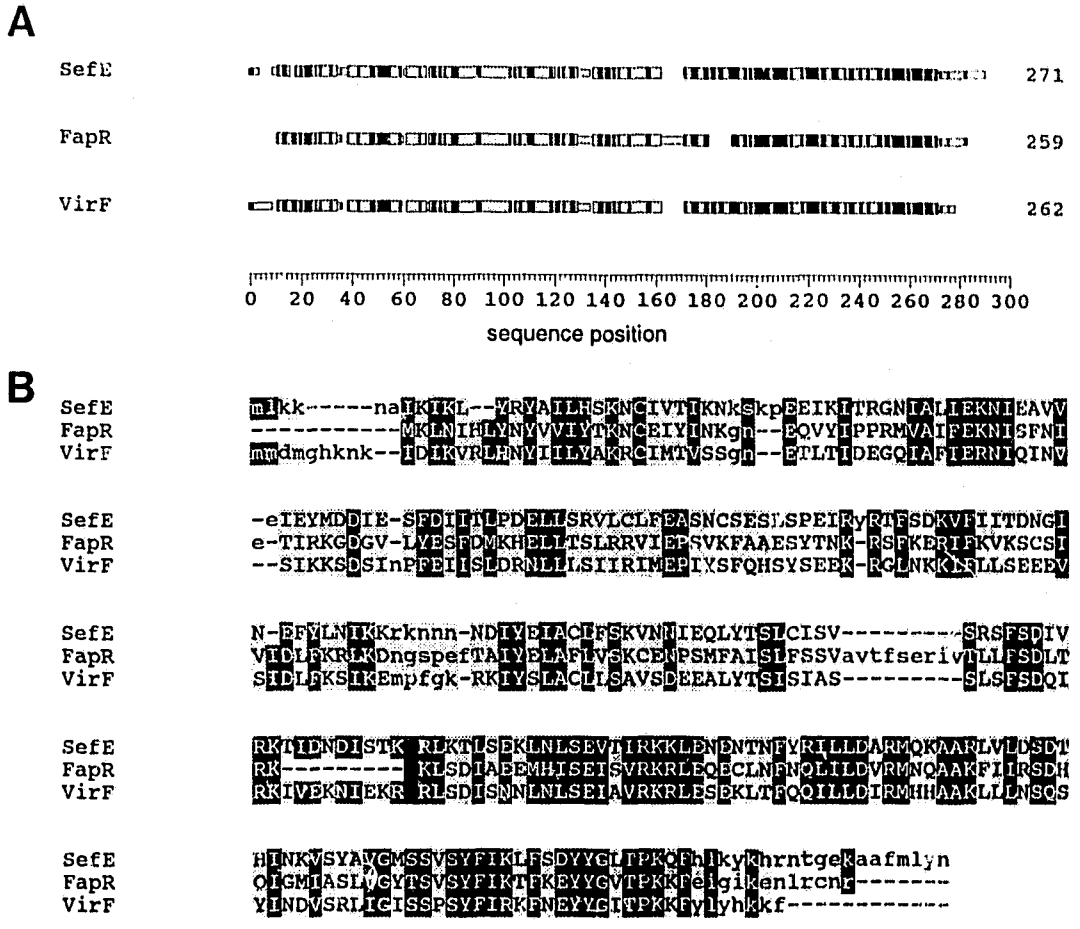


Fig. 18. Local alignment of $SefE_1$ and $SefE_2$ and two AraC-like transcriptional regulators. A. Schematic representation and aligned sequences (B) of fimbrial transcriptional regulators analyzed for statistically significant blocks of similarity. In the schematic (A), the linked sequences are indicated 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 M_r and 18,000 M_r proteins were identified on Western blots as SefA (Fig. 19, lane 7) and SefD (Fig. 20), respectively. The 90,000 M_r protein was identified as SefC while the 70,000, 44,000 and 40,000 M_r 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 M_r protein was identified as SefB. The 16,000 M_r 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 1 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 M_r, 27,000 M_r and 90,000 M_r proteins were expressed from the 5.3 kb DNA fragment and that the 18,000 M_r protein was expressed from the 10 kb *Kpn* I 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

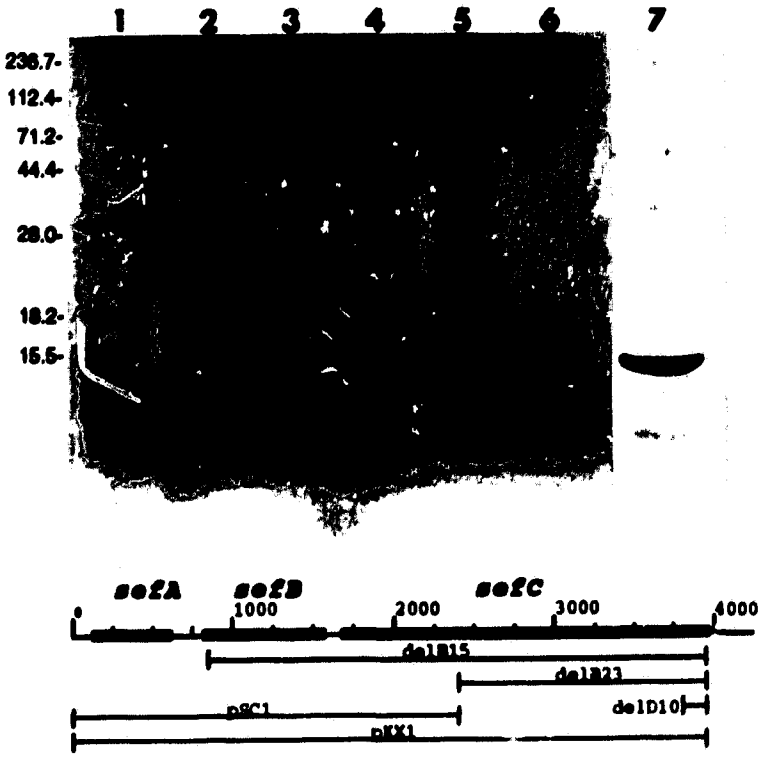


Fig. 19. Expression of the *sefA, -B,* and *-C* genes in an *E. coli in vitro* transcription-translation system; autoradiographs of ^{35}S-methionine-labelled polypeptides, separated on 12.5% acrylamide gels. Lane 1, pTZ19; lane 2, pKX1; lane 3, pSC1; lane 4, delB15; lane 5, delB23; lane 6, delD10; lane 7, Western blot of the *in vitro* transcription-translation of pKX1 developed with antisera generated against denatured SEF14 fimbrin. Below the autoradiograph is a schematic of *sefABC* showing the inserts of various deletion subclones used in the *in vitro* transcription-translation experiments. The size (x10^3) of the molecular weight markers is indicated on the left of the autoradiograph.

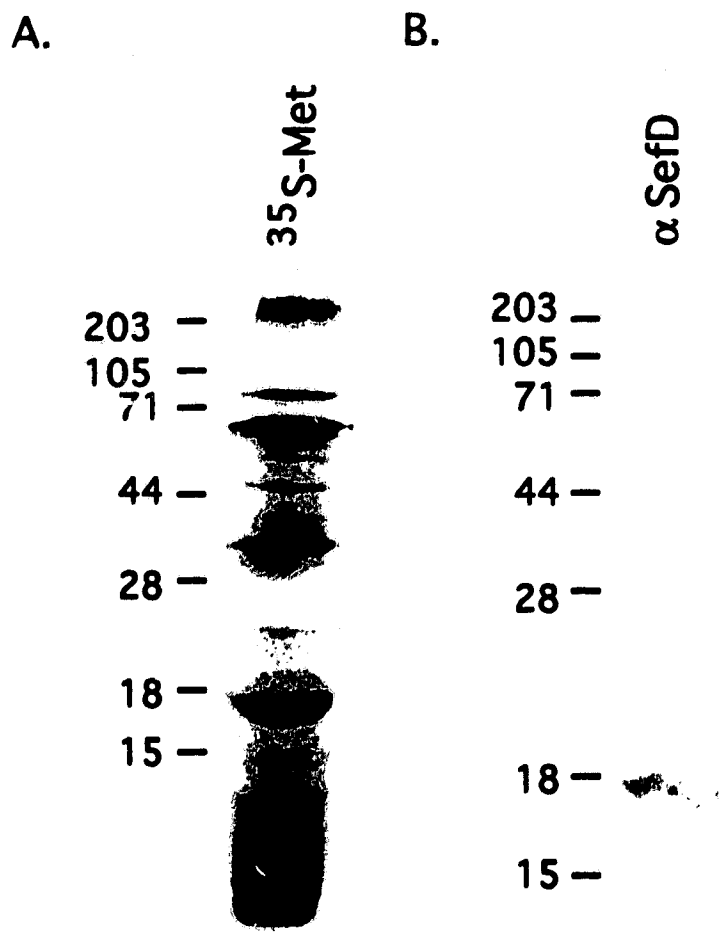


Fig. 20. Expression of the *sefD* gene in an *E. coli in vitro* transcription-translation system. A. Autoradiograph of ^{35}S-methionine-labelled polypeptides separated on a 12.5% acrylamide gel; B. Western blot of the *in vitro* transcription-translation developed with antiserum generated against SefD. 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 microscropy 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_r subunits). Interestingly, SEF18 was often concentrated at the junction of two adjacent cells or found between cells (Fig. 22D).

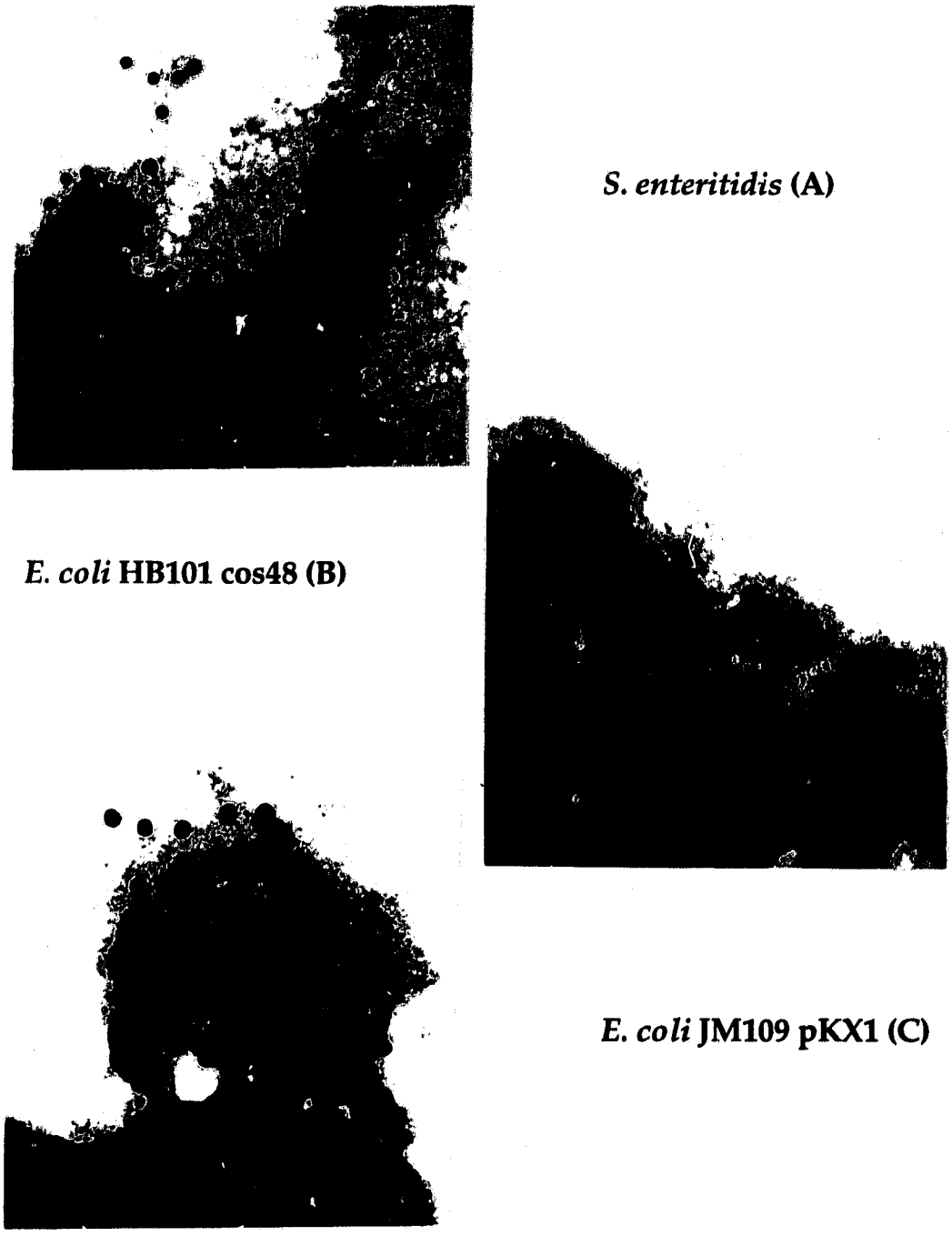
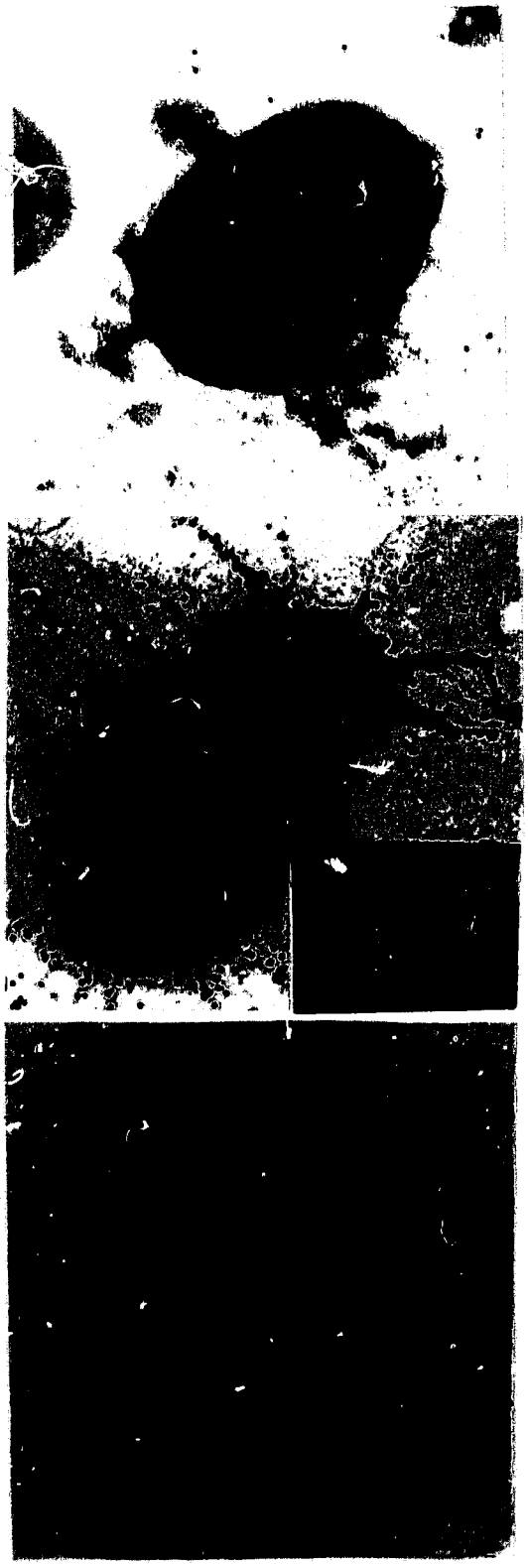


Fig. 21. Immunoelectron microscopy of negatively stained cells for SEF14 production. Cells were grown in 2.5 ml of colonization factor antigen static broth culture for 60 h at 37°C. *S. enteritidis* 27655-3b (A), *E. coli* HB101 containing cos48 (B), and *E. coli* JM109 containing pTZ19 carrying the 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) x94,000; (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 (Appleyard, 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)

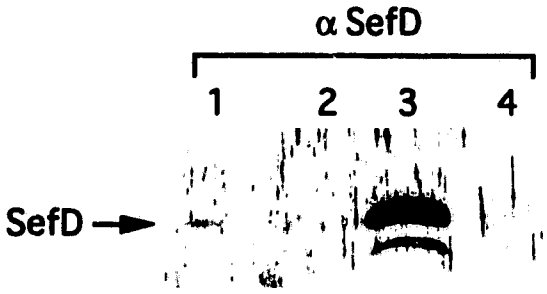


Fig. 23. Cellular localization of SEF18. Western blot of samples taken during the purification of SEF14. Lane 1, supernatant of blended and centrifuged cells; lane 2, supernatant of ultracentrifuged cell membranes; lane 3, pellet of ultracentrifuged cell membranes; lane 4, supernatant of precipitated 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 M_r band. The anti-SefD antisera was generated using the purified 18,000 M_r, 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 M_r) and the uncleaved MalE'-SefD fusion (70,000 M_r). 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 M_r) released by the Factor Xa cleavage and with the MalE' in the

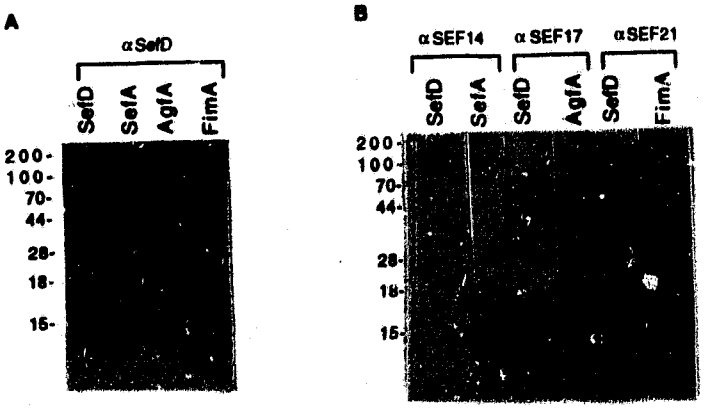


Fig. 24. An analysis of the serological cross-reactivity between the four fimbriae of *S. enteritidis* 3b and their antisera.
A. Western blot analysis of Factor Xa-cut MalE'-SefD fusion (lane 1), purified SEF14 (lane 2), SEF17 (lane 3) and SEF21 (lane 4) with anti-SefD antiserum.
B. Western blot analysis 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 Tn*phoA* mutants. *S. enteritidis* strains defective in the production of SEF14 were generated by Tn*phoA* 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 Tn*phoA* 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 Tn*phoA* located at the junction of Tn*phoA*-generated alkaline phosphatase gene fusions. The PCR amplification resulted in the production of a major DNA fragment of approximately 300 bp for Tn*phoA* 1-2, 350 bp for Tn*phoA* 1-

Table 9. Summary of the fimbrins and fimbriae produced by the Tn*phoA* and Tn*10* mutants of *S. enteritidis*.

Strains	Fimbrin and fimbriae produced			
	SEF14	SEF17	SEF18	SEF21
Tn*phoA* 2-3	-	+	+	+
Tn*phoA* 1-16	-	+	+	+
Tn*phoA* 1-11	-	+	+	+
Tn*phoA* 1-2	-	+	+	+
3b-122	-	+	+	+
3b	+	+	+	+

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.

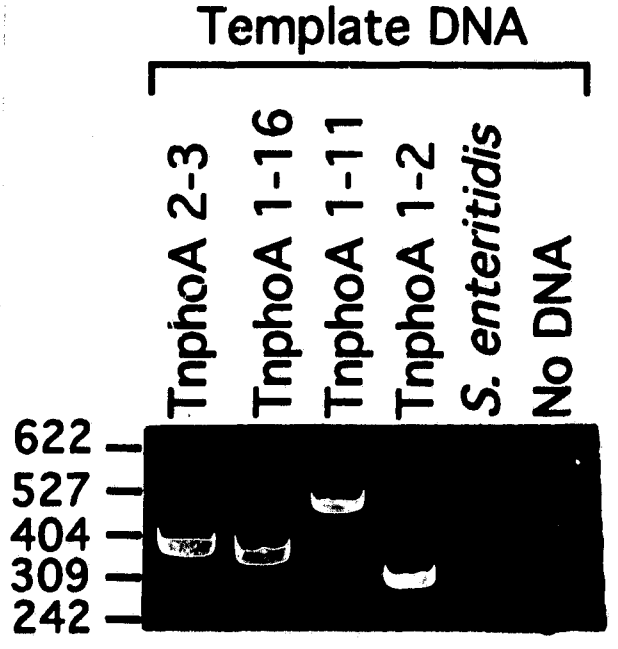


Fig. 25. PCR amplification of DNA fragments from *S. enteritidis* 3b Tn*phoA* mutants harboring Tn*phoA* within *sefA*. The PCR products generated by the primers ST2 and Tn*phoA* are from Tn*phoA* 2-3 (lane 1), Tn*phoA* 1-16 (lane 2), Tn*phoA* 1-11 (lane 3), Tn*phoA* 1-2 (lane 4); *S. enteritidis* 27655-3b (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 Tn*phoA* 2-3 and 500 bp for Tn*phoA* 1-11 (Fig. 25). This data indicates that the four *S. enteritidis* Tn*phoA* 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 M_r 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

Bacterial species and strains	Source[a]	Serogroup	SefD cross-reactive protein[b]	Hybridization[c] to *sefD* probe
***Salmonella* spp.**				
S. albany	TJT	C3	+	++
S. anatum	UVic	E1	nt	++
S. arizonae	TJT		+	++
S. berta				
8392	ATCC	D1	+	+++
89-4065	PVL	D1	nt	+++
90-1271	PVL	D1	nt	+++
S. bovis-morbificans	TJT	C2	+	++
S. cerro	TJT	K	+	++
S. choleraesuis	BBF	C1	+	++
S. dahomey	TJT	X	+	++
S. drypool	UVic	E2	+	++
S. dublin				
15480	ATCC	D1	+	+++
89-3320	PVL	D1	nt	+++
89-3349	PVL	D1	nt	+++
89-4189	PVL	D1	nt	+++
90-243	PVL	D1	nt	+++
90-1176	PVL	D1	nt	+++
S. eastbourne	UVic	D1	+	++
S. enteritidis				
27655-3b	TW	D1	+	+++
27655-3a	TW	D1	nt	++
27036-2II	TW	D1	+	+++
809	LCDC	D1	nt	+++
813	LCDC	D1	nt	+++
907	LCDC	D1	nt	+++
913	LCDC	D1	nt	+++
914	LCDC	D1	nt	+++
930	LCDC	D1	nt	+++
939	LCDC	D1	nt	+++
955	LCDC	D1	nt	+++
972	LCDC	D1	nt	+++
4931	ATCC	D1	+	+++
13076	ATCC	D1	nt	+++
31194	ATCC	D1	nt	+++
89-2749	PVL	D1	nt	+++
JTSe1	JT	D1	nt	+++
S. florida	TJT	H	+	++
S. gallinarum				
9184	ATCC	D1	+	+++
S. gaminara	TJT	I	+	++
S. havana	TJT	G2	+	++

S. infantis				
JTSi1	JT	C1	+	++
S. javiana				
10721	ATCC	D1	+	nt
S. manhatten	TJT	C2	+	nt
S. minnesota	TJT	L	+	++
S. newport	TJT	C2	+	++
S. nienstedten	TJT	C4	+	++
S. oranienburg				
9239	ATCC	C1	+	nt
S. pullorum				
9120	ATCC	D1	+	+++
10398	ATCC	D1	nt	+++
19945	ATCC	D1	nt	+++
89-2331	PVL	D1	nt	+++
90-1175	PVL	D1	nt	+++
S. tennessee	TJT	C1	+	++
S. typhi	UVic	D1	+	nt
S. typhimurium				
962	TJT	B	+	++
Bowmer11	TJT	B	+	++
F18-1	TJT	B	nt	++
F112-2	TJT	B	+	nt
JTSt2	JT	B	nt	++
JTSt3	JT	B	nt	++
S736	TJT	B	+	++
S. worthington	TJT	G2	+	++
Other *Enterobacteriaceae*				
Citrobacter				
freundii				
8090	UVic		+	+
Enterobacter				
aerogenes	UVic		+	+
Erwinia				
caratovora	UVic		+	+
Escherichia				
coli				
11775	ATCC		+	++
C600	ATCC		+	nt
HB101	ATCC		nt	++
E1049a-13	TJT		+	++
438Hf	TJT		+	++
B41M	LE		+	++
HM1475	LE		+	++
Vietman I/1	LE		+	++
Gambia G3	LE		+	++
NG7c	LE		+	++
NG7c1	LE		+	++
VietG	LE		+	++
Hafnia alvei	UVic		+	+
Klebsiella				
pneumoniae				

13883	ATCC	+	++
Proteus vulgaris	UVic	+[d]	+
Providencia rettgeri	UVic	+[d]	+
Serratia marcescens	UVic	-	-
Shigella boydii	UVic	+	nt
Shigella dysenteriae	UVic	+	nt
Shigella flexneri	UVic	+	nt
Shigella sonnei	UVic	+	++
Other eubacteria			
Aeromonas hydrophila	TJT	-	-
Aeromonas salmonicida	WWK	-	-
Bacillus subtilis	UVic	-	-
Pseudomonas aeruginosa	UVic	-	-
Staphylococcus aureus	UVic	-	nt

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; TJT, T.J. Trust, 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.

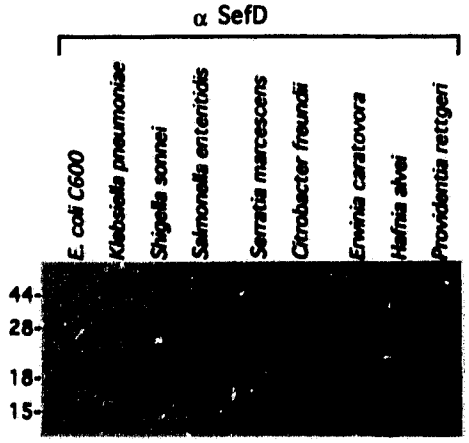


Fig. 26. Analysis of SEF18 production by enterobacteria. Western blot analysis of whole-cell lysates of *E. coli* C600 (lane 1), *K. pneumoniae* 13883 (lane 2), *S. sonnei* (lane 3), *S. enteritidis* 27655-3b (lane 4), *S. marcescens* (lane 5), *Citrobacter freundii* 8090 (lane 6), *Erwinia carotovora* (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 stuctures were also produced on the surface of *E. coli* (Fig. 22D and E), *Klebsiella oxytoca* and *Providencia rettgeri* (Fig. 22Aand 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

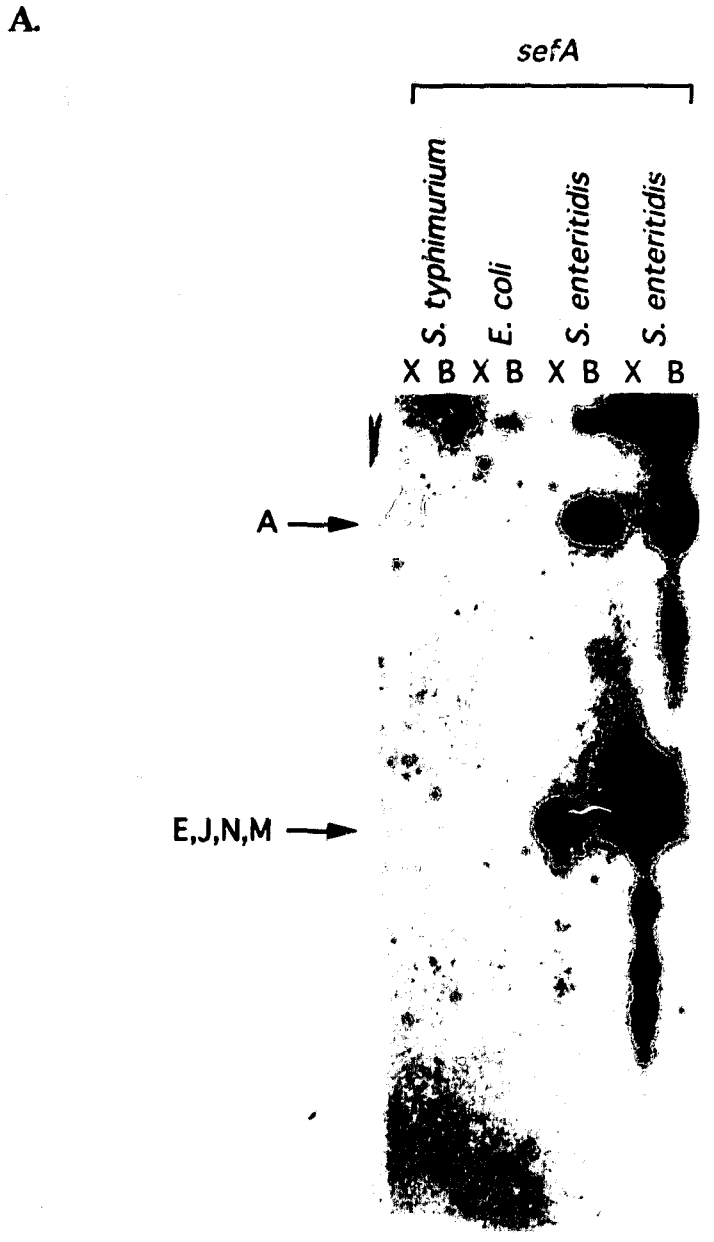
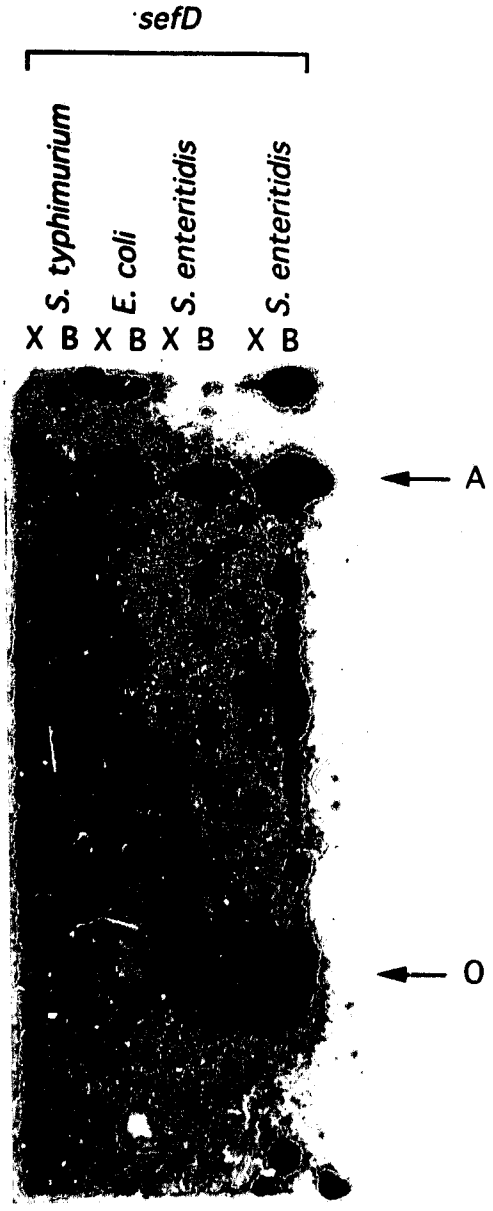


Fig. 27. Identification of chromosomal fragments containing *sefA* (A) or *sefD* (B) by Southern blot hybridization. Autoradiographs of *Xba* I or *Bln* I digests of genomic DNA from *S. typhimurium* LT2 (Lanes 1 and 2), *E. coli* C600 (Lanes 3 and 4), *S. enteritidis* RKS53 (lanes 5 and 6) or *S. enteritidis* 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.

B.


sefD
S. typhimurium
E. coli
S. enteritidis
S. enteritidis
X B X B X B X B
A
O

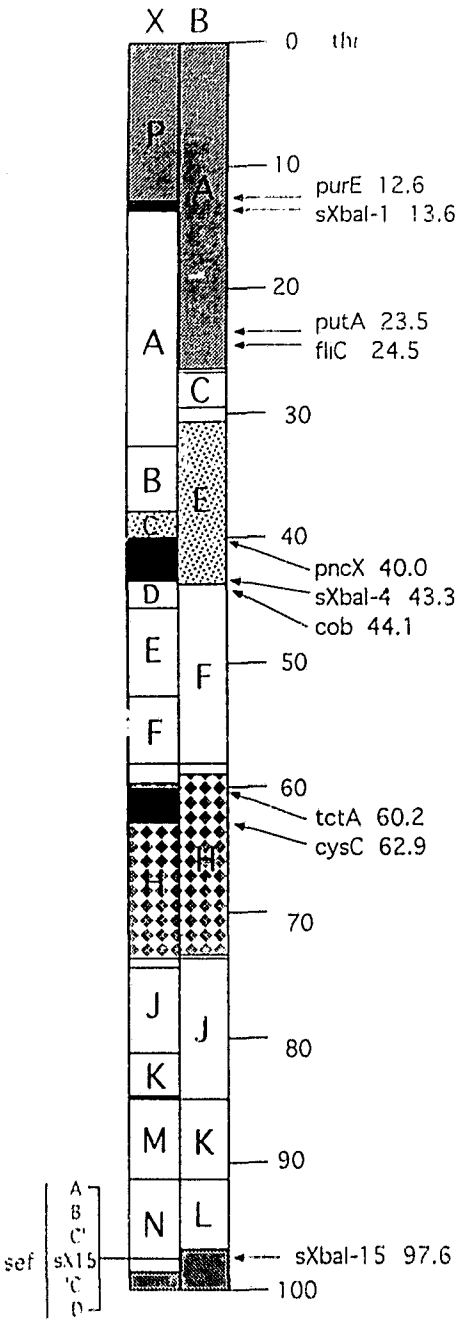


Fig. 28. Position of the *sef14* gene cluster on the *Xba* I-*Bln* I genomic cleavage maps of *S. enteritidis*. The genomic cleavage map previously determined for *S. enteritidis* SSU7998 (Liu *et al.*, 1993b) is shown in linear form, with the physical map divided into 100 centisomes (CS). Each CS of 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 s*Xba* I-1, s*Xba* I-4 or s*Xba* 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, s*Xba* 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, $sefE_1$ and $sefE_2$, and the adjacent IS*5* 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_r 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_r 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.

sefC, the gene immediately downstream of *sefB*, encodes a protein that displays homology to fimbrial outer membrane proteins. Evidence suggesting that SefC is the outer membrane component of the *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 *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, *sefABC* comprises an important part of the *sef14* gene cluster responsible for the expression and assembly of SEF14 fimbriae.

E. coli carrying 44 kb of *S. enteritidis* DNA (cos48) encompassing the *sefABC* operon displays immunogold labelled SEF14 fimbriae. However, immunogold electron microscopy of *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 IS*3* family transposases. The inverted repeats, the overlapping ORFs and the sequence homology are all characteristics shared by IS*3* family elements. Transposable elements often effect the expression of genes adjacent to the insertion site. Some elements such as IS*1163* (Skaugen and Nes, 1994) have a negative effect whereas others such as IS*3* (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 IS*1* 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 IS*1* element

activates 987P fimbriae expression. Although the mechanism is unknown, the IS*1* 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 IS*3* 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 fimbriae produced by this enteric pathogen and have been given the trivial name SEF18 (*S. enteritidis* fimbriae composed of 18,000 M_r 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 Tn*10*

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, $sefE_1$ and $sefE_2$, 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 $SefE_2$ 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 Enterobacterioceae tested with the exception of *Serratia*. The widespread distribution and the antigenic

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 *Dra* 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 *sefA*8 oligonucleotide (TGCGTGGGCACTGCCACA) 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-^{32}P]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 $MgCl_2$ 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 $MgCl_2$, 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°, 30°, 32°, 35° and 37°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°, 32°, 35°, or 37°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 *Eco*R I and *Hin*d 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-^{32}P]-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).

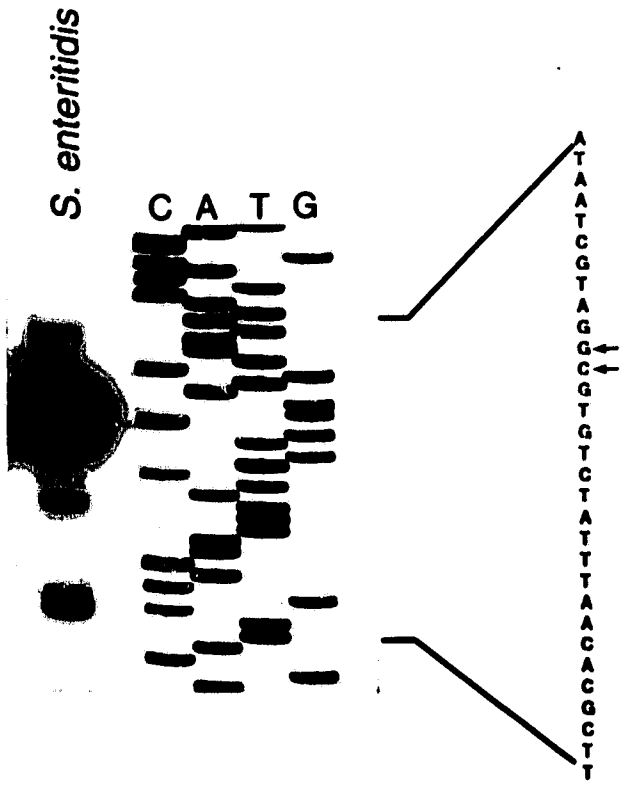
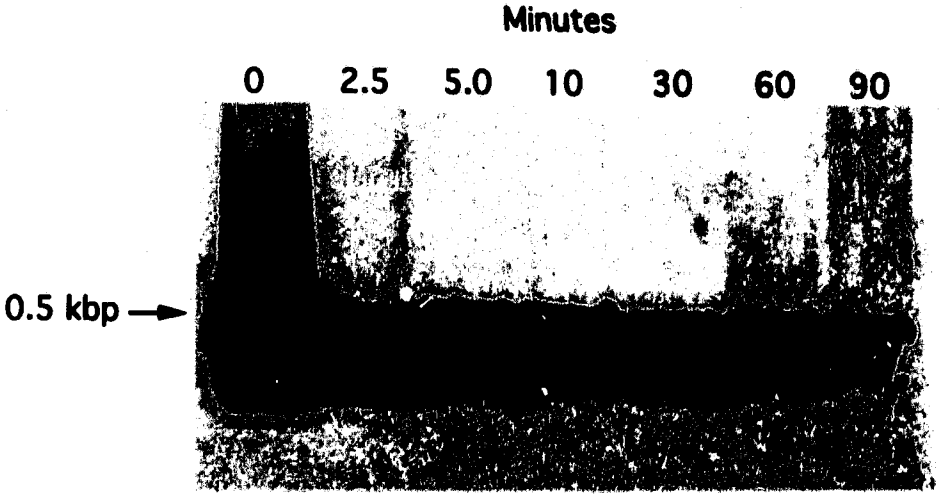


Fig. 29. Mapping of the 5' end of the *sefA* transcript using primer extension. The lane labelled *S. enteritidis* represents the reverse transcriptase products of RNA isolated from this organism grown in colonization factor antigen static broth for 60 h at 37°C. Lanes C, A, T, and G represent the results of dideoxy chain termination sequence reactions in the region encompassing the promoter. The sequence of the γ-^{32}P-labelled primer (TGCGTGGGCACTGCCACA) is complementary to nucleotides 181 to 198 of *sefA*. Arrows indicate the two major transcription initiation sites.

A.



B.

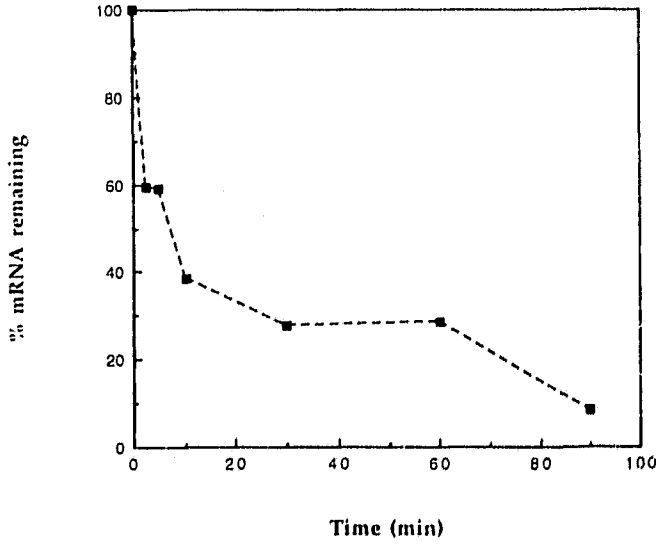


Fig. 30. Determination of *sefA* mRNA stability. (A) After growing for 60 h in CFA broth statically, *S. enteritidis* cells were treated with rifampicin. Northern blot analysis with a ^{32}P-labelled *sefA* probe was performed on the total RNA extracted at 0, 2.5, 5, 10, 30, 60, and 90 min (lanes 1-7, 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.

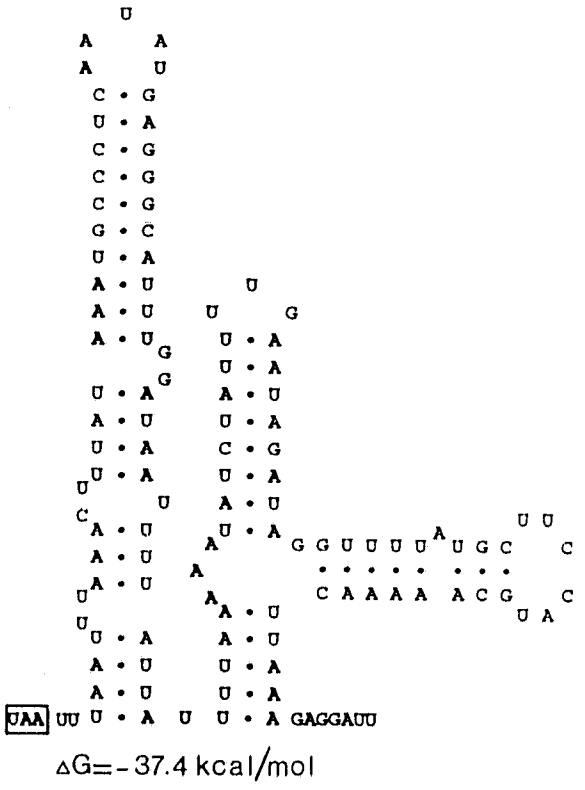


Fig. 31. Schematic representation of the proposed secondary structures within the *sefABC* mRNA. Two stem-loop structures are proposed to form in the intercistronic region of *sefA* and *sefB* mRNA. The *sefA* translation stop codon is boxed. The predicted stability of the stem-loop structures is noted below the schematic.

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 promotors 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

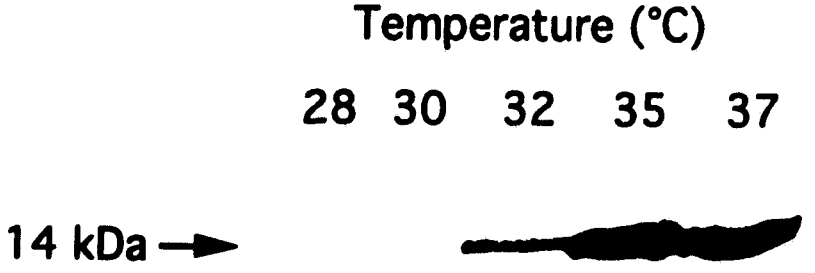


Fig. 32. Western blot analysis of SEF14 production at different growth temperatures. Whole cell extracts of *S. enteritidis* grown in colonization factor antigen broth statically for 60 h at the temperatures indicated were separated on a 12.5% acrylamide gel and transferred to nitrocellulose. The blot was developed using polyclonal antisera generated against SEF14 fimbriae.

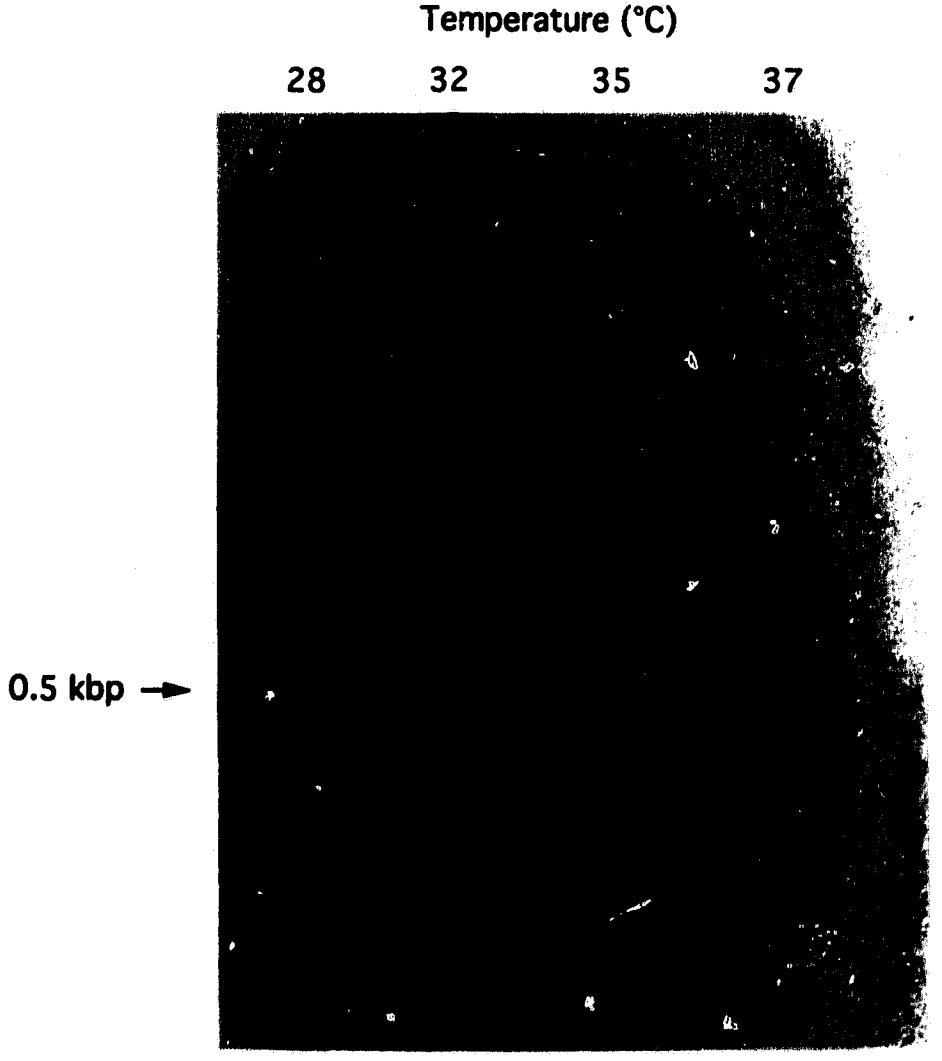


Fig. 33. Analysis of *sefA* transcription by Northern blot hybridization. Total RNA was extracted from *S. enteritidis* cells grown in colonization factor antigen broth statically for 60 h at 28°C (lane 1), 32°C (lane 2), 35°C (lane 3) or 37°C (lane 4). UV illumination of the blot prior to hybridization showed equivalent levels of 16S and 23S in each lane. The RNA samples (10 μg) were hybridized to a ^{32}P-labelled *sefA* probe.

the *sefA*-specific probe. Therefore, transcription of *sefB* and *sefC* is initiated from the *sefA* promotor 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 fimbrin, 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 suseptible 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, $sefE_1$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 *Eco*R I/*Hin*d III fragment encompassing the original *Bsm* I fragment was subcloned from pSC9 into the *Eco*R I/*Hin*d III sites of pINIII^{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^{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 *Eco*R 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 (Boerhringer 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 $MgCl_2$ 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 *Eco*R 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 *Eco*R 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 *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 NH_4Cl, 4.1 g/L KH_2PO_4, 8.9 g/L K_2HPO_4, 40 ml/L 50% glucose, 10 ml/L $MgSO_4$ stock (0.5 g $MgSO_4$ in 10 ml dH_2O), 1 ml/L trace elements (0.05 g $CaCl_2$, 0.018 g $CoCl_2 \cdot 6H_2O$, 0.016 g $CuSO_4 \cdot 5H_2O$, 0.018 g $ZnSO_4 \cdot 7H_2O$, 2 g Na_2EDTA, 2 g $FeCl_2 \cdot 6H_2O$ and 100 µl 1M HCl in 100 ml dH_2O) (C.E. Furlong, University of Washington). Ampicillin was added to a final concentration of 250 µg/ml. 10-30 L of this

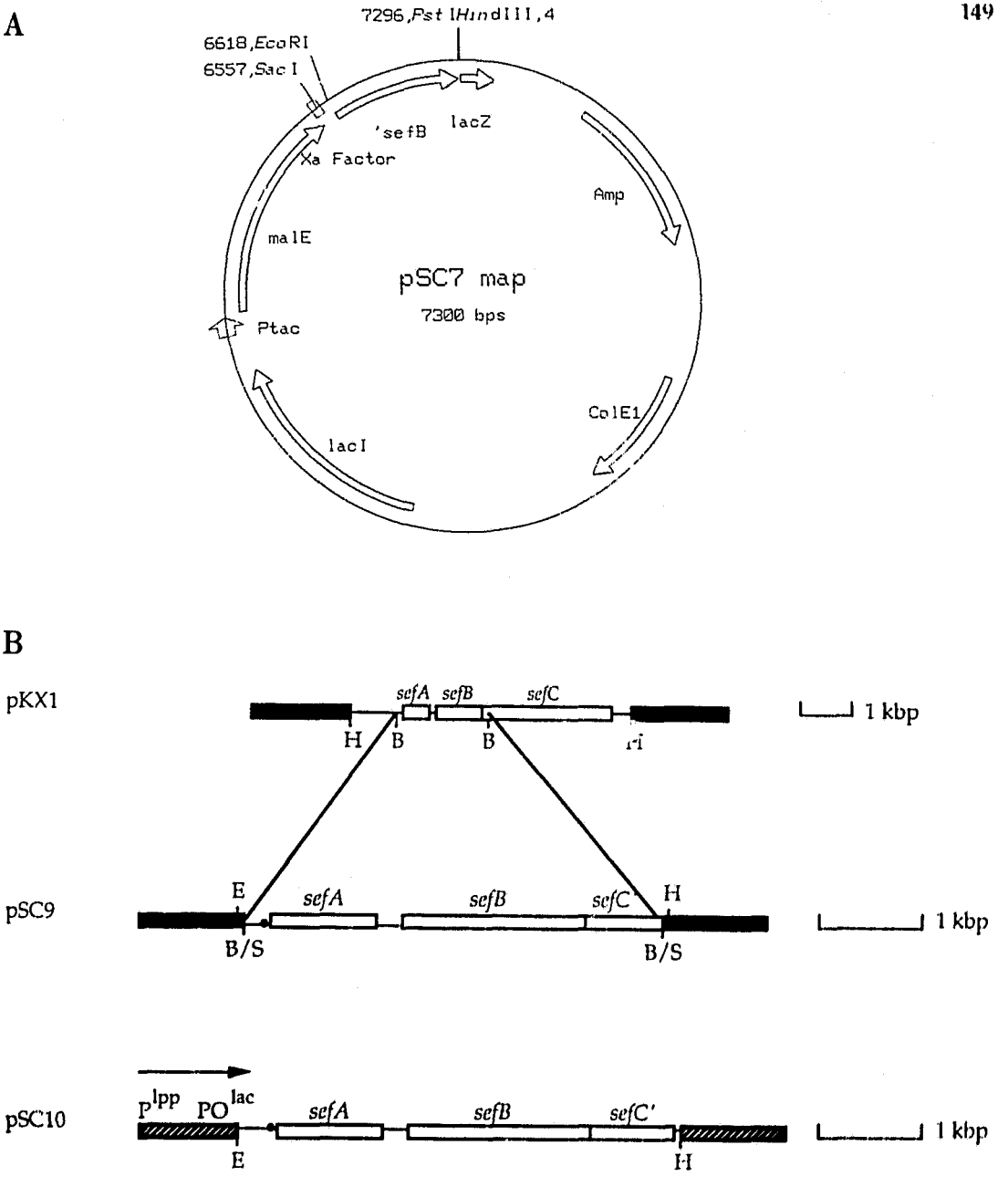


Fig. 34. A. Plasmid map of pSC7. *sefB* was PCR amplified, purified, digested with *Eco*R I and *Pst* I, purified and ligated into the *Eco*R I/*Pst* I sites of pMal-p2. B. Construction of pSC9 and pSC10. The genes are represented by open boxes and the name of the gene is given above each box. Box with small white dots, pTZ19 vector DNA; solid black box, pUC19 vector DNA; box with diagonal lines, pINIII^{113}-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, *Eco*R I.

media were inoculated with 0.3-1.5 L, respectively, of an overnight culture of DH5α/pSC10, respectively. At 1.0 OD_{650}, IPTG was added to the culture to a final concentration of 0.5 mM. NH_4OH and 50% glucose were added automatically to maintain the culture at pH 7. Supplements of $MgSO_4$, trace elements, 50% glucose and 10x LB were added at 5-7 OD_{650} to maintain logarithmic growth. At 8.0 OD_{650}, the cells were concentrated and washed with 10-25 L PBS (Sambrook *et al.*, 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 *et al.* (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 $MgCl_2$ 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, Uppsula, 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 $SefA_H$ whereas the lower form was designated $SefA_L$. 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_3PO_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_2O 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.

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

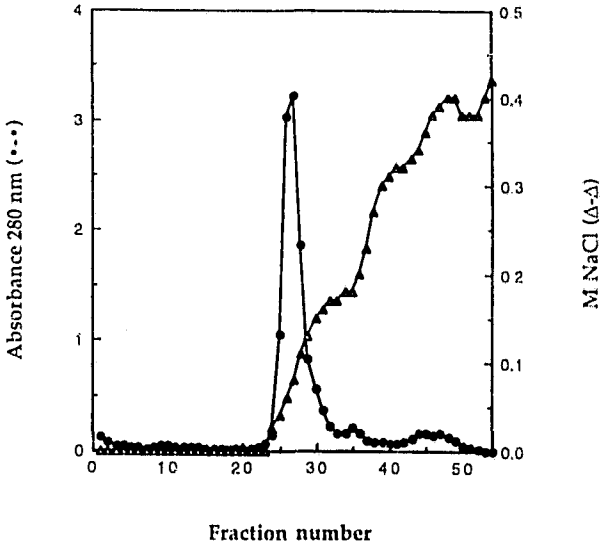
intramuscularly (IM); 2x250 µl subcutaneously (SC). For subsequent boosts 2 and 4 weeks later, 1 ml eluted SefB was emulsified with 1 ml incomplete Freund's adjuvant as described above. Injections were as follows: 1x500 µl IM; 2x600 µl SC. In both cases, the prebleed was removed the same day as the initial 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 pINIII^{113}-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

A.



B.

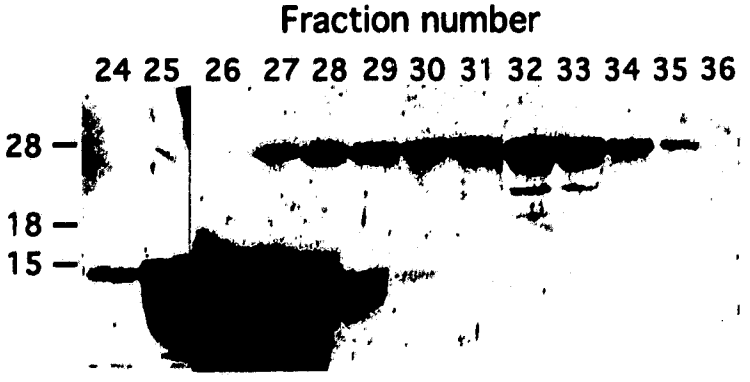


Fig. 35. Affinity chromatography of SefA and SefB isolated from the periplasm of *E. coli*. (A) SefA- or SefB-positive fractions from the DEAE Sepharose column were pooled and applied to a CM Sepharose column for affinity chromatography in 20 mM Tris HCl pH 6.5. Upon application of a linear NaCl gradient, 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

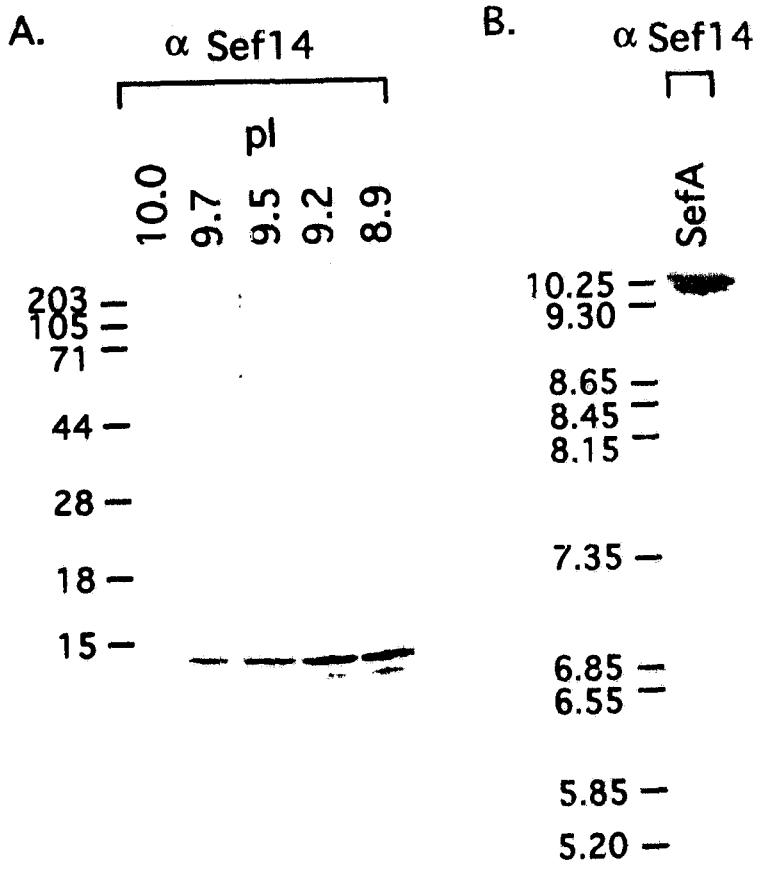


Fig. 36. Determination of the pI for SefA. (A) Western blot analysis of rotary IEF fractions run on a 12.5% SDS polyacrylamide gel which was blotted and developed using anti-SEF14 antiserum. Fractions with pI 10.0 (lane 1), 9.7 (lane 2), 9.5 (lane 3), 9.2 (lane 4) and 8.9 (lane 5) contained SefA. Molecular 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.

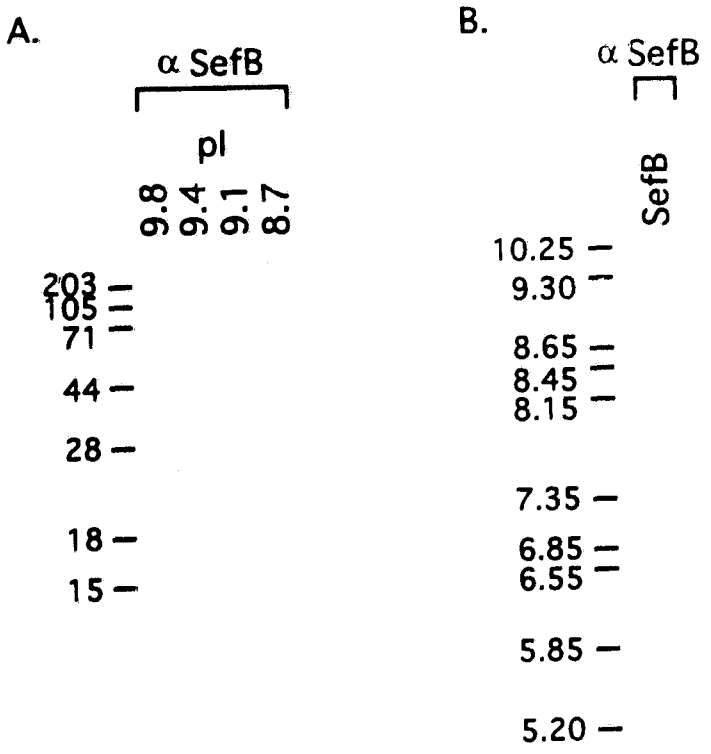


Fig. 37. Determination of the pI for SefB. (A) Western blot analysis of rotary IEF fractions run on a 12.5% SDS polyacrylamide gel which was blotted and developed using anti-SefB antiserum. Fractions with pI 9.8 (lane 1), 9.4 (lane 2), 9.1 (lane 3) and 8.7 (lane 4) contained SefB. Molecular weight standards 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 pI markers are indicated on the left.

which was similar to the predicted pI of 9.96.

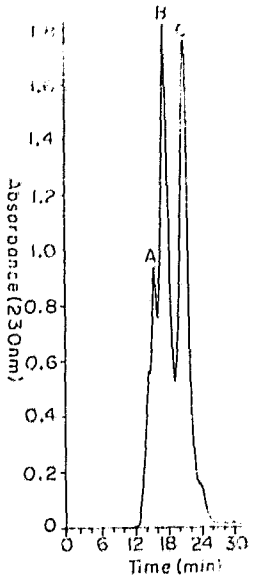
Native polyacrylamide gels of SefB showed multiple, closely spaced bands between pH 8.65 and 9.3 (Fig. 37B). For preparative IEF, the focusing fractions with pH 8.7 to 9.8 contained SefB (Fig. 37A). The average pI for SefB, calculated from the results of similar experiments, was 9.5 which is similar 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 ($SefA_H$) that migrated slightly slower (1,000-2,000 M_r) in SDS-PAGE than the form of SefA found in Peak C ($SefA_L$) (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).

A



B

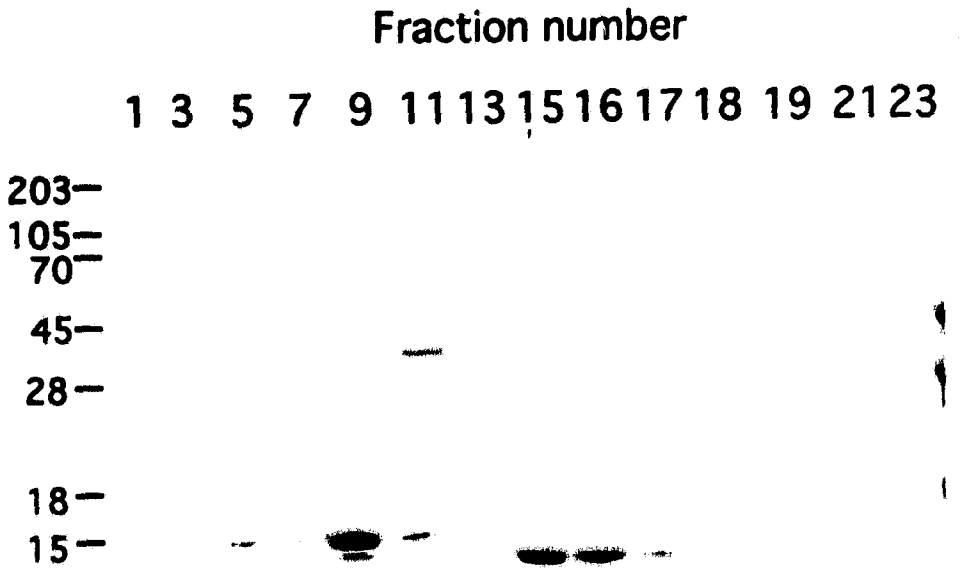


Fig. 38. Gel filtration chromatography of SefA isolated from the periplasm of *E. coli*. (A) SefA-positive fractions from the CM Sepharose column were applied to a Superdex 75 HR 10/30 column for gel filtration in 0.1 M Tris HCl, 0.1 M NaCl pH 7.5. Samples were taken at 30 second intervals. The column 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 8, 9 and 10, contains $SefA_H$ and peak C, which corresponds to fractions 14, 15 and 16, contains $SefA_L$.

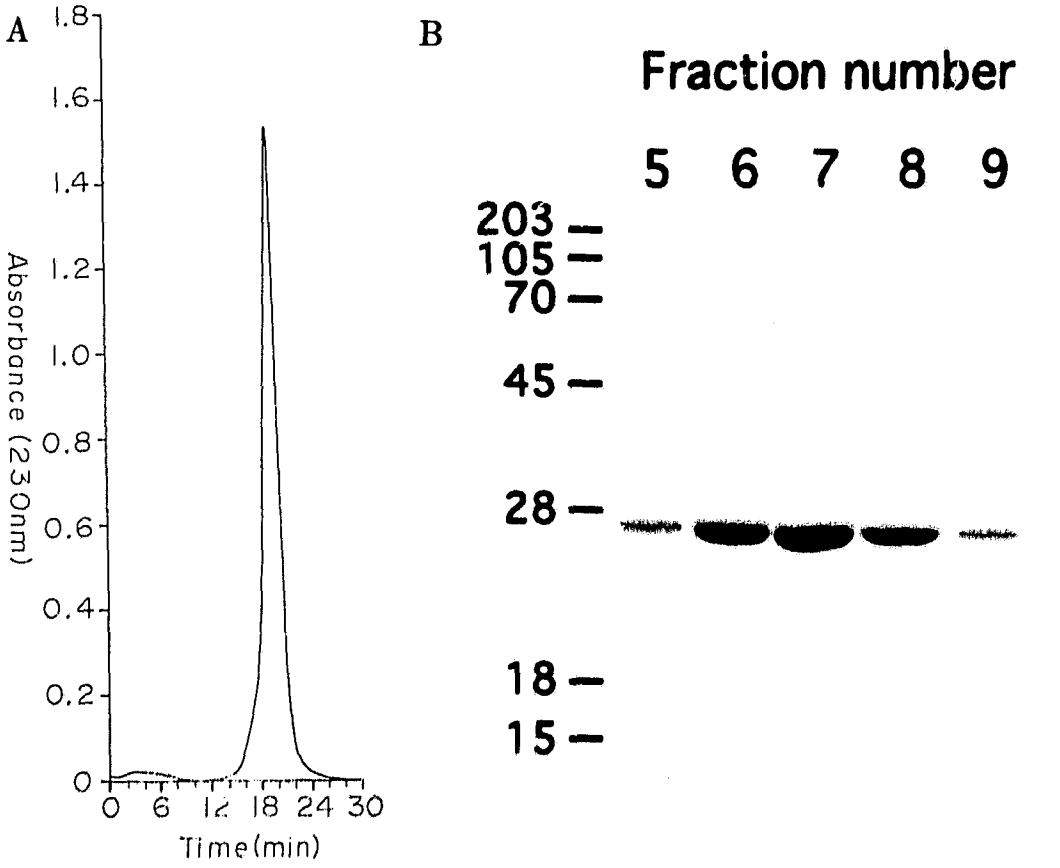


Fig. 39. Gel filtration of SefB isolated from the periplasm of *E. coli*. (A) SefB-positive fractions from the CM Sepharose column were applied to a Superdex 75 HR 10/30 column for gel filtration in 0.1 M Tris HCl, 0.1 M NaCl pH 7.5. Samples were taken at 30 second intervals. The column was calibrated 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.

A.

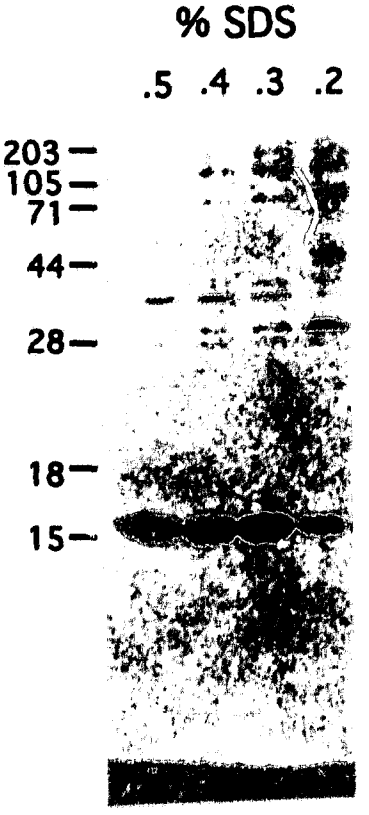
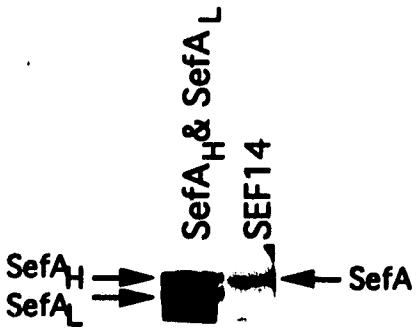
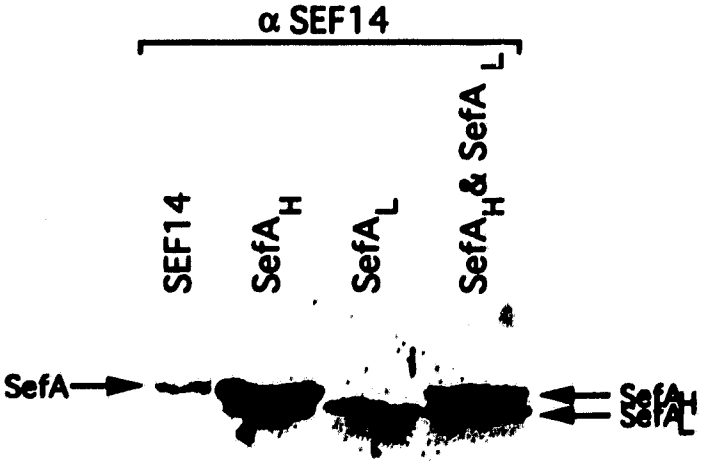


Fig. 40. SDS polyacrylamide gel analysis of the three forms of SefA. (A) Purified SEF14 was incubated with 0.5% (lane 1), 0.4% (lane 2), 0.3% (lane 3), and 0.2% (lane 4) SDS at 37°C for 30 min, mixed with an equal volume of native 2x sample buffer (only 0.2% SDS and no β-mercaptoethanol) and separated on a 12.5% polyacrylamide gel containing only 0.10% 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 $SefA_H/SefA_L$ (lane 1) and purified SEF14 (lane 2). (C) Western blot analysis of purified SEF14 (lane 1), $SefA_H$ (lane 2), $SefA_L$ (lane 3) and $SefA_H/SefA_L$ (lane 4) using antiserum to SEF14 fimbriae.

B.


SefA$_H$& SefA$_L$
SEF14
SefA$_H$
SefA$_L$
SefA

C.


α SEF14
SEF14
SefA$_H$
SefA$_L$
SefA$_H$& SefA$_L$
SefA
SefA$_H$
SefA$_L$

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 $SefA_{H}$ in SDS polyacrylamide gels suggesting that $SefA_{H}$ 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

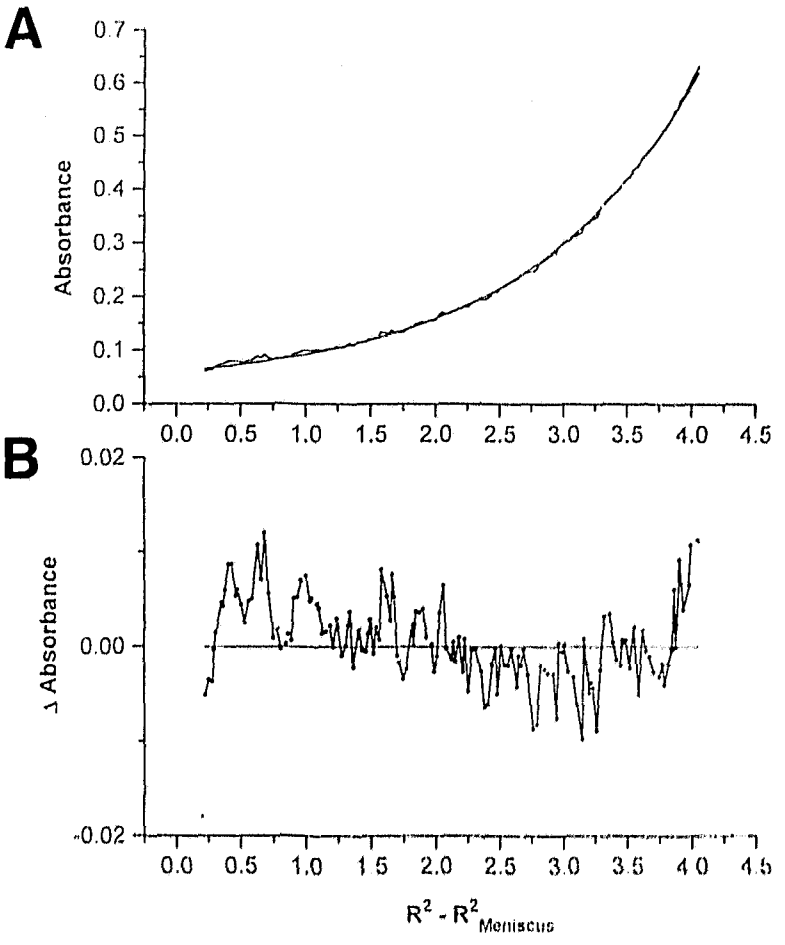
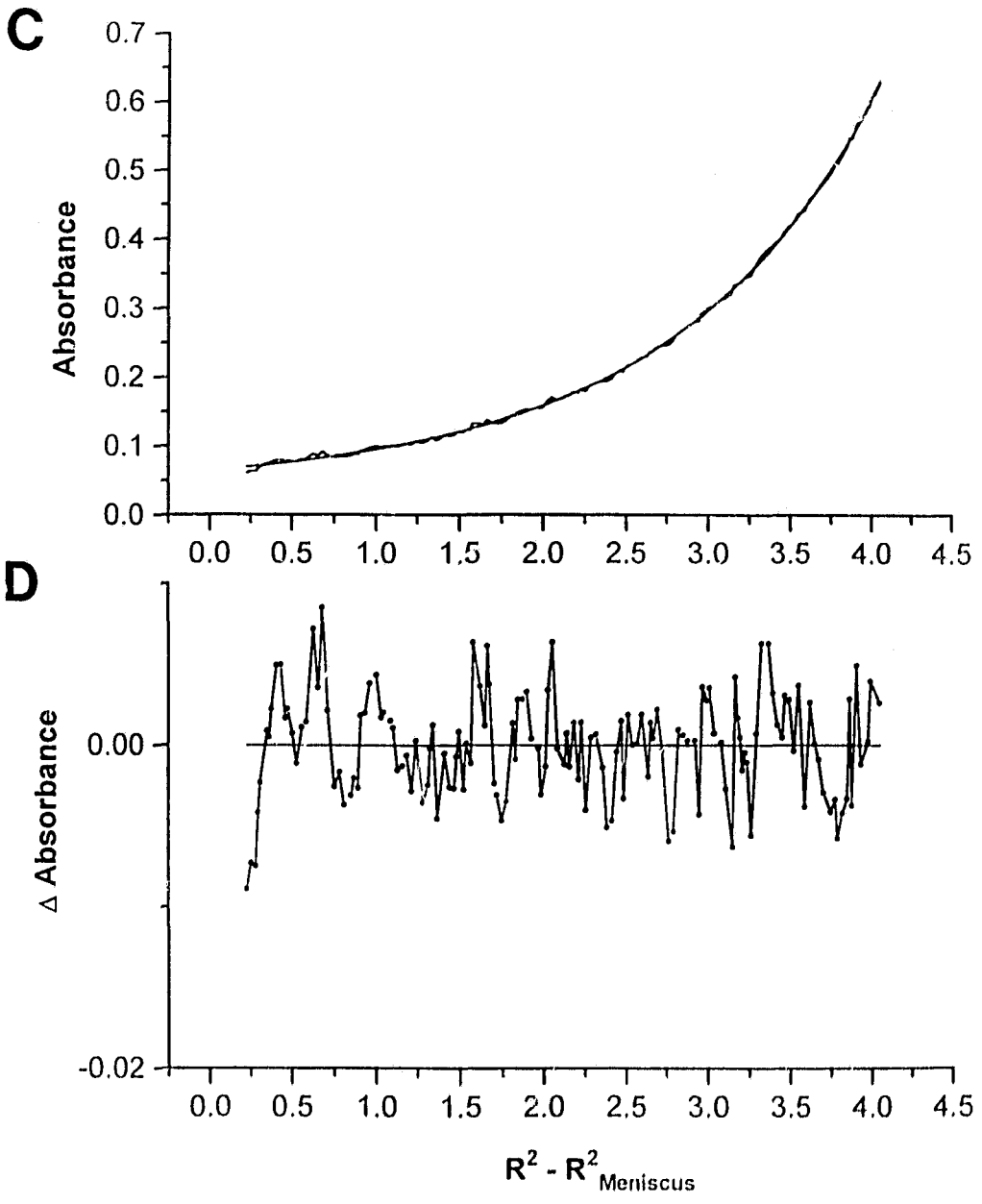
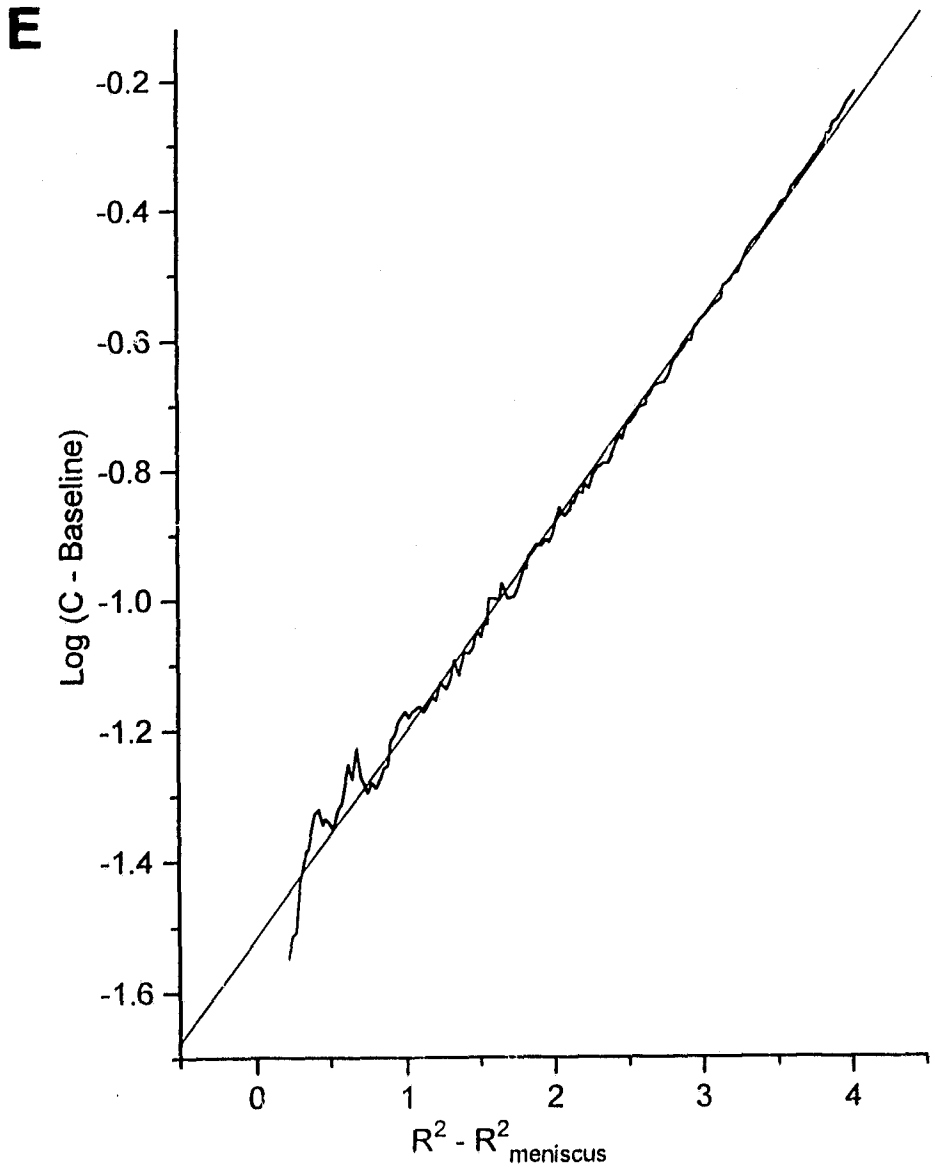


Fig. 41. Sedimentation equilibrium analysis of $SefA_L$. $SefA_L$ was dialyzed in 0.1M Tris HCl, 0.1M NaCl pH 7.5 buffer, concentrated to 0.245 OD_{280nm} and analyzed using the partial specific volume of 0.7293. The 120 µl sample was loaded into a standard 12-mm double-sector cell and equilibrated using 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^2-$R^2_{meniscus}$ where R is the radial distance from the axis of rotation and $R_{meniscus}$ is the radial position of the meniscus. The absorbance deviation of the $SefA_L$ equilibrium curve from the monomer or a dimer model curves is shown in B and D, respectively. The acceptable level of error is ±0.02 absorbance units at 280 nm. (E) The $SefA_L$ equilibrium curve was linearized in the linear regression plot of log(C-Baseline) versus R^2-$R^2_{meniscus}$ where C is the concentration of the solute and baseline refers to the initial concentration of the solute.


C
Absorbance
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
D
Δ Absorbance
0.00
-0.02
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
R2 - R2 Meniscus


E
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
Log (C - Baseline)
0
1
2
3
4
R2 - R2meniscus

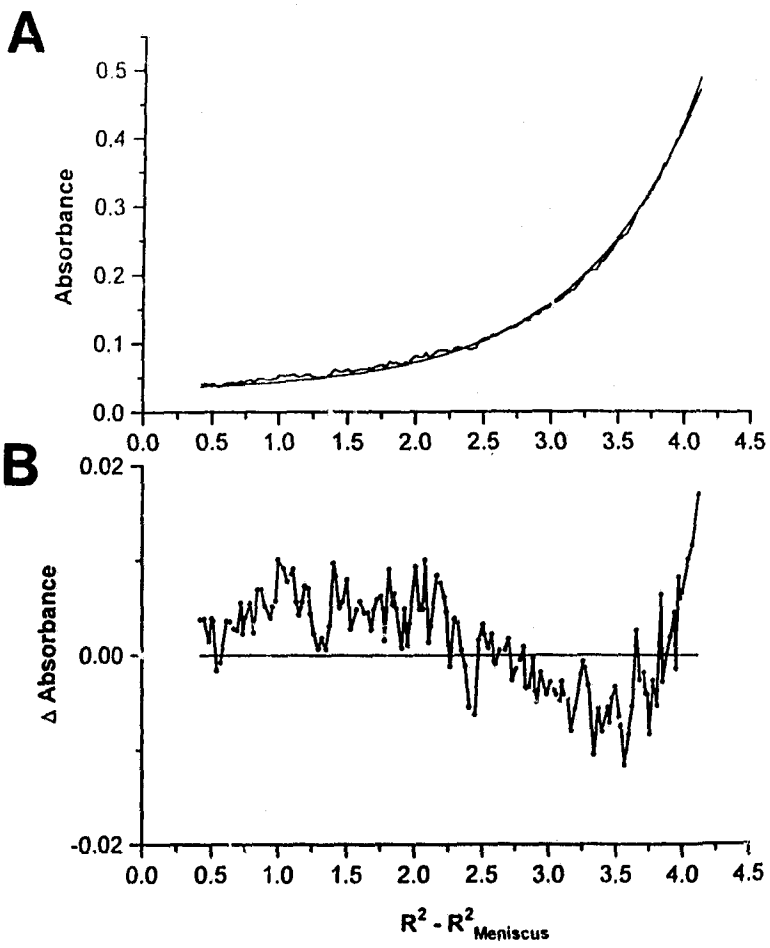
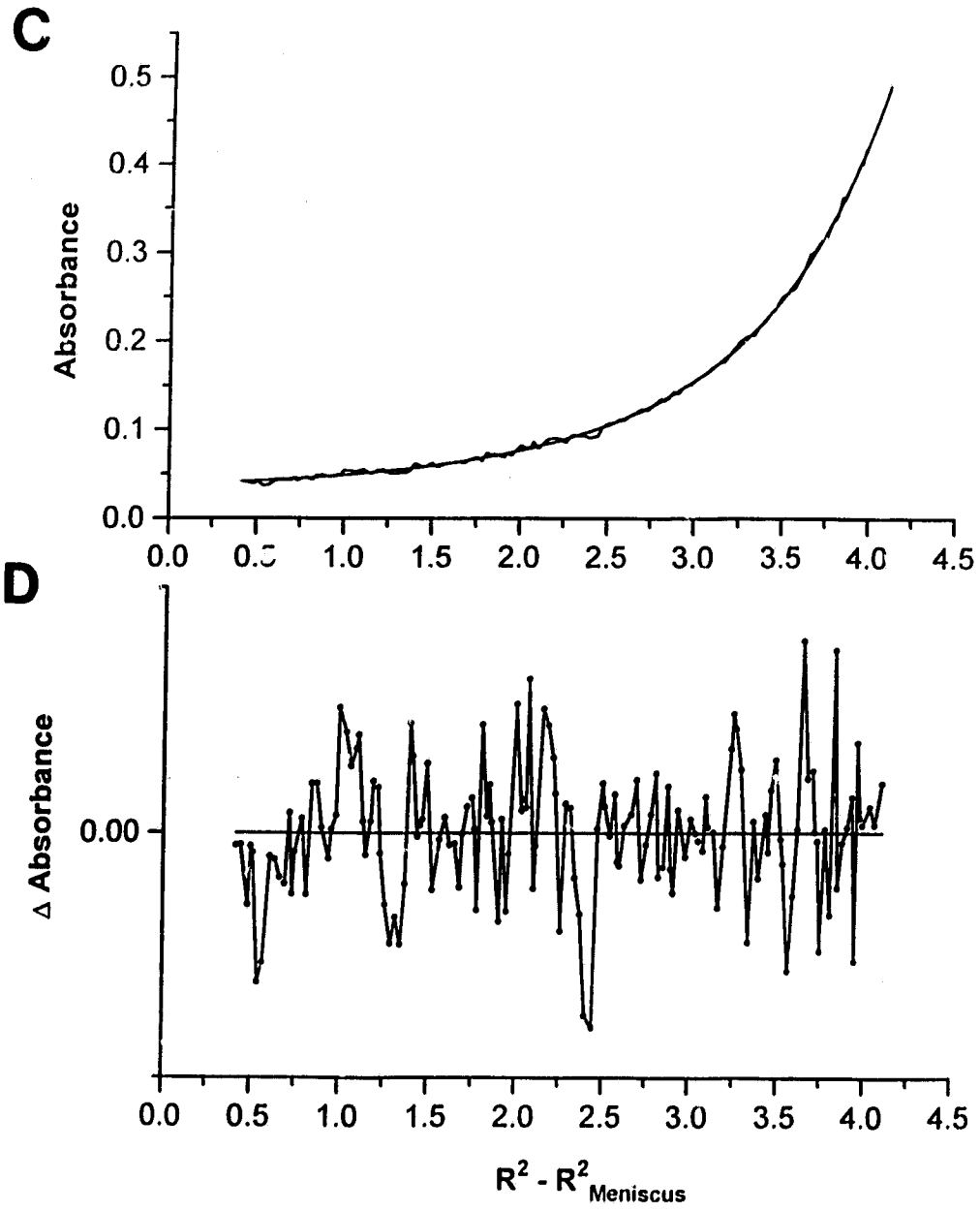


Fig. 42. Sedimentation equilibrium analysis of $SefA_H$. $SefA_H$ was dialyzed in 0.1M Tris HCl, 0.1 M NaCl pH 7.5 buffer, concentrated to 0.245 OD_{280} and analyzed using the partial specific volume of 0.7293. The 120 µl sample was loaded into a standard 12-mm double-sector cell and equilibrated using 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^2-$R^2_{meniscus}$ where R is the radial distance from the axis of ratation and $R_{meniscus}$ is the radial position of the meniscus. The level of deviation of the $SefA_H$ 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.


C
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
D
Δ Absorbance
0.00
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
R2 - R2 Meniscus

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 $SefA_L$ existed in a monomeric form, dimers of $SefA_L$ were also present in solution. In contrast, $SefA_H$ had a 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 $SefA_L$, $SefA_H$ existed predominately as a dimer in solution. However, analysis of $SefA_H$ 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)-suberate], a water-soluble and non-cleavable cross-linker with a spacer arm of 11.4 Å that forms cross-links between two closely

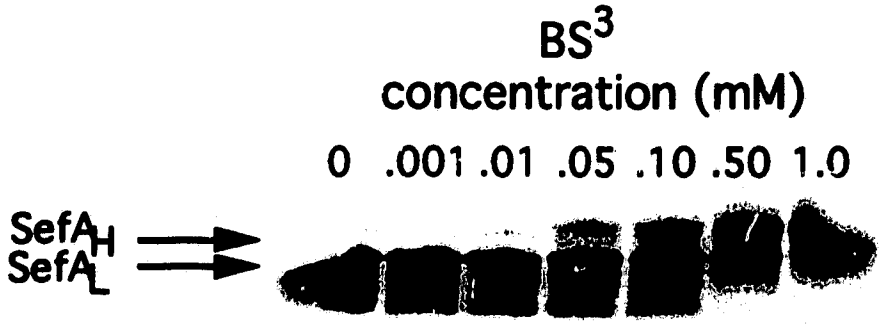


Fig. 43. Cross-linking of $SefA_L$ with low concentrations of BS^3. $SefA_L$ was cross-linked with BS^3 at concentrations of 0 mM (lane 1), 0.001 mM (lane 2), 0.01 mM (lane 3), 0.05 mM (lane 4), 0.10 mM (lane 5), 0.50 mM (lane 6) and 1.0 mM (lane 7), run on a 12.5% SDS-polyacrylamide gel, and stained with Coomassie Blue. The position of the $SefA_L$ and $SefA_H$ bands are indicated on the left.

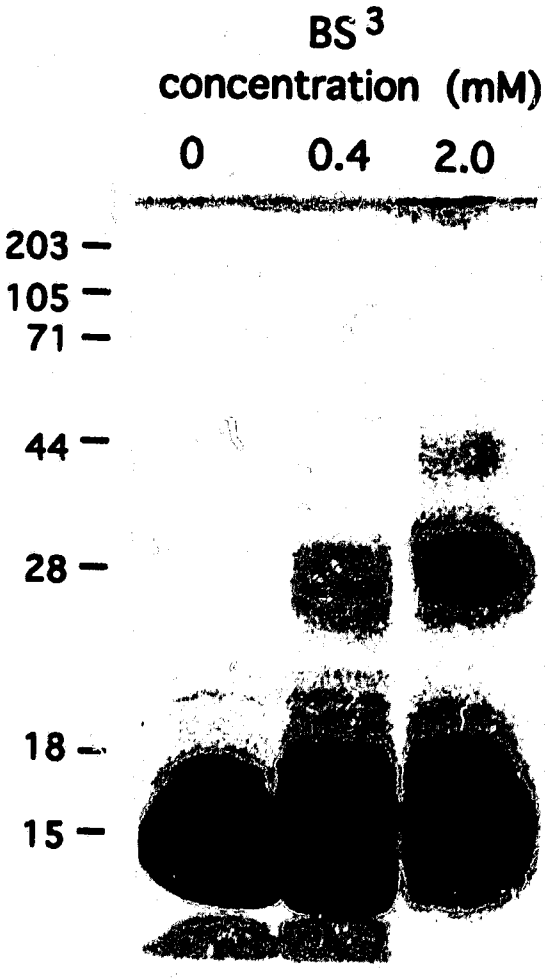


Fig. 44. Cross-linking $SefA_L$ with higher concentrations of BS^3. $SefA_L$ was cross-linked with BS^3 at concentrations of 0 mM (lane 1), 0.4 mM (lane 2) and 2.0 mM (lane 3), run on a 12.5% SDS polyacrylamide gel and stained with Coomassie Blue. The sizes of the molecular weight markers are indicated on the left in kDa.

Table 11. Summary of the N-terminal sequence analysis of $SefA_H$ and $SefA_L$

$SefA_H$			$SefA_L$
Sequence 1	**Sequence 2**	**Sequence 3**	**Sequence 1**
ala	ala	asn	asn
gly	val	trp	trp
phe	thr	ser	ser
val	ile	gln	gln
gly	ala	asp	asp
asn	ala	pro	pro
lys	gln	gly	gly
ala	asn	phe	phe
val	thr	thr	thr
val	thr	gly	gly
gln	ser	pro	pro
ala	ala	ala	ala
ala	asn	val	val
val	trp	ala	ala
thr	ser	ala	ala
ile	gln	gly	gly
ala	asp	gln	gln
ala	pro	lys	lys
gln	gly		val
	asn		gly
	thr		thr
	thr		leu
			ser
			ile
			thr
			ala
			thr
			gly
			pro

associated polypeptides. SDS-PAGE analysis of BS^3-treated $SefA_L$ revealed that with increasing concentrations of BS^3 (0.001-1 mM) the migration rate of $SefA_L$ in the gel decreased to that of $SefA_H$ (Fig. 43). Furthermore, gel filtration analysis revealed that cross-linked $SefA_L$ now migrated through the column with the same retention time as native $SefA_H$ (data not shown). At higher concentrations of BS^3 (1-2 mM), dimers and trimers of $SefA_L$ were formed (Fig. 44). SDS-PAGE analysis of BS^3-treated $SefA_H$ 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 $SefA_L$ and $SefA_H$ but that an intrapeptide cross-link had to form within $SefA_L$ 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 $SefA_L$ and $SefA_H$ was used to determine if the sequences of the two proteins were the same. This analysis revealed that the N-terminal amino acid of $SefA_L$ 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 $SefA_H$ was determined to be ala^1. The sequence data

also revealed that several truncated forms of SefA co-migrated with $SefA_H$ 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 *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 *et al.*, 1993) that prevent nonproductive aggregation of fimbrial subunits imported into the periplasm (Kuehn *et al.*, 1991), protect the fimbrial subunit from proteolytic cleavage and enhance the processing of the subunit's signal peptide (Bakker *et al.*, 1991; Hultgren *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 *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 *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 dH_2O suggests that charged groups are exposed on the surface of the protein. In contrast, the high insolubility of SEF14 fimbriae in dH_2O 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 65Å 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 15Å 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 α-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 *E. coli* has permitted the first such study. Gel filtration and equilibrium ultracentrifugation analysis of SefA isolated from the periplasm of *E. coli* reveal that SefA exists as both a monomer ($SefA_L$) and a dimer ($SefA_H$). 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, $SefA_L$ and $SefA_H$ as well as SefA derived from SEF14 fimbriae have different migration rates through SDS-PAGE such that $SefA_L$ migrates slightly faster than $SefA_H$ and SefA from fimbriae. N-terminal amino acid sequence analysis of $SefA_L$ and $SefA_H$

reveals that the N-terminal amino acid in $SefA_H$ is ala^1 but is asn^{25} in $SefA_L$. The loss of 24 amino acids from the N-terminus of SefA would create a 12,200 M_r protein which explains the faster migration of $SefA_L$ through SDS-PAGE. Since $SefA_L$ 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 $SefA_L$ also form in solution. N-terminal amino acid sequence analysis of a band cut out of a stained blot of $SefA_H$ run on a 12.5% polyacrylamide gel containing SDS reveals that truncated forms of SefA also co-migrate with $SefA_H$ even though they are missing the first 12, 17 or 24 N-terminal amino acids. Why these truncated forms of $SefA_H$ are not migrating as distinct bands in SDS-PAGE and how $SefA_L$ differs from the truncated form of SefA that is missing 24 N-terminal amino acids but yet co-migrates with $SefA_H$ 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 $SefA_L$ in SDS-PAGE is converted to that of $SefA_H$ in the presence of low concentrations of BS[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 $SefA_L$. 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 $SefA_H$ 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, *sefABCDE*$_1$*E*$_2$, 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 *sefE*$_1$/*E*$_2$ encode proteins homologous to other fimbrial periplasmic chaperones, ushers and transcriptional regulators, respectively. Together, the genes *sefABCDE*$_1$*E*$_2$ 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 $SefE_1$ and $SefE_2$ 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 $sefE_1$ and $sefE_2$ 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.

References

Abraham, J.M., Freitag, C.S., Clements, J.R. and Eisenstein, B.I. (1985) An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli.Proceedings of the National Academy of Sciences of the United States of America* **82**:5724-5727.

Adler, B., Sasakawa, C., Tobe, T., Makino, S., Komatsu, K. and Yoshikawa, M. (1989) A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri. Molecular Microbiology* **13**:627-635.

Albright, L.M., Huala, E. and Ausubel, F.M. (1989) Prokaryotic signal transduction mediated by sensor and regulator protein pairs. *Annual Review of Genetics* **23**:311-336.

Allen, B. L., Gerlach, G.-F. and Clegg, S. (1991) Nucleotide sequence and functions of *mrk* determinants necessary for expression of Type 3 fimbriae in *Klebsiella pneumoniae. Journal of Bacteriology* **173**:916-920.

Alm, R. A., Guerry, P., and Trust, T. J. (1993) Distribution and polymorphism of the flagellin genes among isolates of *Campylobacter coli* and *Campylobacter jejuni. Journal of Bacteriology* **175**:3051-3057.

Anderson, D.G. and Moseley, S.L. (1988) *Escherichia coli* F41 adhesin: Genetic organization, nucleotide sequence and homology with the K88 determinant. *Journal of Bacteriology* **170**:4890-4896.

Arnqvist, A., Olsén, A., Pfeifer, J., Russell, D.G. and Normark, S. (1992) The Crl protein activates cryptic genes for curli formation and fibronectin binding in *Escherichia coli* HB101. *Molecular Microbiology* **6**:2443-2452.

Aronson, B.D., Levinthal, M. and Somerville, R.L. (1989) Activation of a cryptic pathway for threonine metabolism via specific IS3-mediated alteration of promoter structure in *Escherichia coli. Journal of Bacteriology* **171**:5503-5511.

Båga, M., Normark, S., Hardy, J., O'Hanley, P., Lark, D., Olsson, O., Schoolnik, G. and Falkow, S. (1984) Nucleotide sequence of the *papA* gene encoding the Pap pilus subunit of human uropathogenic *Escherichia coli. Journal of Bacteriology* **157**:330-333.

Båga, M., Göransson, M., Normark, S. and Uhlin, B.E. (1985) Transcriptional

activation of a Pap pilus virulence operon from uropathogenic *Escherichia coli*. *EMBO Journal* **4**:3887-3893.

Båga, M., Norgren, M. and Normark, S. (1987) Biogenesis of *E. coli* Pap pili: PapH, a minor pilin subunit involved in cell anchoring and length modulation. *Cell* **49**:241-251.

Båga, M., Göransson, M., Normark, S. and Uhlin, B.E. (1988) Processed mRNA with differential stability in the regulation of *E. coli* pilin gene expression. *Cell* **52**:197-206.

Bagg, A. and Neilands, J.B. (1987) Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiological Reviews* **51**:509-518.

Bakker, D., Vader, C.E.M., Roosendaal, B., Mooi, F.R., Oudega, B. and De Graaf, F.K. (1991) Structure and function of periplasmic chaperone-like proteins involved in the biosynthesis of K88 and K99 fimbriae in enterotoxigenic *Escherichia coli*. *Molecular Microbiology* **5**:875-886.

Bakker, D., Willensen, P.T.J., Simons, L.H., Van Zijderveld, F.G. and De Graaf, F.K. (1992) Characterization of the antigenic and adhesive properties of FaeG, the major subunit of K88 fimbriae. *Molecular Microbiology* **6**:247-255.

Beard, M.K.M., Mattick, J.S., Moore, L.J., Mott, M.R., Marrs, C.F. and Egerton, J.R. (1990) Morphogenetic expression of *Moraxella bovis* fimbriae (pili) in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **172**:2601-2607.

Beltran, P., Musser, J.M., Helmuth, R., Farmer III, J.J., frenchs, W.M., Wachsmuth, I.K., Ferris, K., McWhorter, A.C., Wells, J.G., Cavioto, A. and Selander, R.K. (1988) Toward a population genetic analysis of *Salmonella*: Genetic diversity and relationships among strains of serotypes *S. choleraesuis*, *S. derby*, *S. dublin*, *S. enteritidis*, *S. heidelberg*, *S. infantis*, *S. newport* and *S. typhimurium*. *Proceedings of the National Academy of Sciences of the United States of America* **85**:7753-7757.

Bilge, S.S., Clausen, C.R., Lau, W. and Moseley, S.L. (1989) Molecular characterization of a fimbrial adhesin, F1845, mediating diffuse adherence of diarrhea-associated *Escherichia coli* to HEp-2 cells. *Journal of Bacteriology* **171**:4281-4289.

Bilge, S.S., Apostol, Jr., J.M., Aldape, M.A. and Moseley, S.L. (1993a) mRNA processing independent of RNaseIII and RnaseE in the expression of the F1845 fimbrial adhesin of *Escherichia coli*. *Proceddings of the National Academy of Sciences of the United States of America* **90**:1455-1459.

Bilge, S.S., Apostol, Jr., J.M., Fullner, K.J. and Moseley, S.L. (1993b)

Transcriptional organization of the F1845 fimbrial adhesin determinant of *Escherichia coli*. *Molecular Microbiology* **7**:993-1006.

Blomfield, I.C., Calie, P.J., Eberhardt, K.J., McClain, M.S. and Eisenstein, B.I. (1993) Lrp stimulates phase variation of type 1 fimbriation in *Escherichia coli* K-12. *Journal of Bacteriology* **175**:27-36.

Blyn, L.B., Braaten, B.A. and Low, D.A. (1990) Regulation of *pap* pilin phase variation by a mechanism involving differential Dam methylation states. *EMBO Journal* **9**:4045-4054.

Bock, K., Breimer, M.E., Bringde, A., Hansson, G.G., Karlsson, K.-A., Larson, G., Leffler, H., Samuelsson, B.-E., Strömberg, N., Svanborg-Edén, C. and Thurin, J. (1985) Specificity of binding of a strain of uropathogenic *Escherichia coli* to Galα1-4Gal-containing glycosphingolipids. *Journal of Biological Chemistry* **260**:8545-8551.

Boyd, J.M., Koga, T. and Lory, S. (1994) Identification and characterization of PilS, an essential regulator of pilin expression in *Pseudomonas aeruginosa*. *Molecular and General Genetics* **243**:565-574.

Braaten, B.A., Blyn, L.B., Skinner, B.S. and Low, D.A. (1991) Evidence for a methylation-blocking factor (*mbf*) locus involved in *pap* pilus expression and phase variation in *Escherichia coli*. *Journal of Bacteriology* **173**:1789-1800.

Braaten, B.A., Platko, J.V., Van Der Woude, M.W., Simons, B.H., De Graaf, F.K., Calvo, J.M. and Low, D.A. (1992) Leucine-responsive regulatory protein controls the expression of both the *pap* and *fan* pili operons in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **89**:4250-4254.

Braaten, B.A., Nou, X., Kaltenbach, L.S. and Low, D.A. (1994) Methylation patterns in *pap* regulatory DNA control pyelonephritis-associated pili phase variation in *E. coli*. *Cell* **76**:577-588.

Brinton, C.C. (1959) Non-flagellar appendages of bacteria. *Nature* (London) **183**:782-786.

Brinton, C.C. (1965) The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram negative bacteria. *Transactions of the New York Academy of Science* **27**:1003

Bühler, T., Hoschützky, H. and Jann, K. (1991) Analysis of colonization factor antigen 1, an adhesin of enterotoxigenic *Escherichia coli* 078:H11: Fimbrial morphology and location of the receptor-binding site. *Infection and Immunity* **59**:3876-3882.

Burghoff, R.L., Pallesen, L., Krogfelt, K.A., Newman, N.V., Richardson, M., Bliss, J.L., Laux, D.C. and Cohen, P.S. (1993) Utilization of the mouse large intestine to select an *Escherichia coli* F-18 DNA sequence that enhances colonization ability and stimulates type 1 fimbriae synthesis. *Infection and Immunity* **61**:1293-1300.

Calvo, J.M. and Matthews, R.G. (1994) The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. *Microbiological Reviews* **58**:466-490.

Caron, J., Coffield, L.M. and Scott, J.R. (1989) A plasmid-encoded regulatory gene, *rns*, required for expression of the CS1 and CS2 adhesins of enterotoxigenic *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **86**:963-967.

Caron, J. and Scott, J.R. (1990) A *rns*-like regulatory gene for colonization factor antigen 1 (CFA/1) that controls expression of CFA/1 pilin. *Infection and Immunity* **58**:874-878.

Charlier, D., Piette, J. and Glansdorff, N. (1982) IS3 can function as a mobile promoter in *E. coli*. *Nucleic Acids Research* **10**:5935-5948.

Clegg, S., and Gerlach, G. F. (1987) Enterobacterial fimbriae. *Journal of Bacteriology* **169**:934-938.

Clouthier, S. C., Müller, K.-H., Doran, J. L., Collinson, S. K., and Kay, W. W. (1993) Characterization of three fimbrial genes, *sefABC*, of *Salmonella enteritidis*. *Journal of Bacteriology* **175**:2523-2533.

Clouthier, S.C., Collinson, S.K. and Kay, W.W. (1994) Unique fimbriae-like structures encoded by *sefD* of the SEF14 fimbrial gene cluster of *Salmonella enteritidis*. *Molecular Microbiology* **12**:

Collinson, S. K., Emödy, L., Müller, K.-H., Trust, T. J., and Kay, W. W. (1991) Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. *Journal of Bacteriology* **173**:4773-4781.

Collinson, S. K., Emödy, L., Trust, T.J. and Kay, W.W. (1992) Thin aggregative fimbriae from diarrheagenic *Escherichia coli*. *Journal of Bacteriology* **174**:4490-4495.

Collinson, S. K., Doig, P. C., Doran, J. L., Clouthier, S. C., Trust, T. J., and Kay, W. W. (1993) Thin aggregative fimbriae mediate binding of *Salmonella enteritidis* to fibronectin. *Journal of Bacteriology* **175**:12-18.

Cooke, M. (1990) Epidemiology of foodborne illness: UK. *Lancet* **336**:790-793.

Cowden, J.M., Chisholm, D., O'Mahony, M., Lynch, D., Mawer, S.L., Spain, G.E., Ward, L. and Rowe, B. (1989) Two outbreaks of *Salmonella enteritidis* phage type 4 infection associated with the consumption of fresh shell-egg products. *Epidemiology and Infection* **103**:47-52.

De Graaf, F.K., Wientjes, F.B. and Klaaasen-Boor, P. (1980) Production of K99 antigen by enterotoxigenic *Escherichia coli* strains of antigen groups 08, 09, 020 and 0101 grown at different conditions. *Infection and Immunity* **27**:216-221.

De Graaf, F.K., Krenn, B.E. and Klaasen, P. (1984) Organization and expression of genes involved in the biosynthesis of K99 fimbriae. *Infection and Immunity* **43**:508-514.

De Graaf, F.K. and Klaasen, P. (1986) Organization and expression of genes involved in the biosynthesis of 987P fimbriae. *Molecular and General Genetics* **204**:75-81.

De Graaf, F. K. (1988) Fimbrial structures of enterotoxigenic *E. coli*. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* **54**:395-404.

De Graaf, F.K. and Gaastra, W. (1994) Fimbriae of enterotoxigenic *E. coli*. In *Fimbriae: Adhesion, genetics, biogenesis and vaccines*. London: CRC Press, pp. 53-83.

DiRita, V.J. and Mekalanos, J.J. (1991) Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. *Cell* **64**:29-37.

DiRita, V.J., Parsot, C., Jander, H. and Mekalanos, J.J. (1991) Regulatory cascade controls virulence in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America* **88**:5403-5407.

DiRita, V.J. (1992) Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. *Molecular Microbiology* **6**:451-458.

Dodd, D.C., Bassford, Jr., P.J. and Eisenstein, B.I. (1984) Dependence of secretion and assembly of type 1 fimbrial subunits of *Escherichia coli* on normal protein export. *Journal of Bacteriology* **159**:1077-1079.

Dodson, K.W., Jacob-Dubuisson, F., Striker, R.T. and Gultgren, S.J. (1993) Outer membrane PapC usher discriminately recognizes periplasmic chaperone-pilus subunit complexes. *Proceedings of the National Academy of Sciences of the United States of America* **90**:3670-3674.

Donnenberg, M.S., Girón, J.A., Nataro, J.P. and Kaper, J.B. (1992) A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. *Molecular Microbiology* **6**:3427-3437.

Doran, J. L., Collinson, S. K., Burian, J., Sarlós, G., Todd, E. C. D., Munro, C. K., Kay, C. M., Banser, P. A., Peterkin, P. I., and Kay, W. W. (1993a) DNA-based diagnostic tests for *Salmonella* species targeting *agfA*, the structural gene for thin, aggregative fimbriae. *Journal of Clinical Microbiology* **31**:2263-2273.

Doran, J.L., Collinson, S.K., Kay, C.M., Banser, Burian, J., Munro, C.K., Lee, S.H., Somers, J.M., Todd, E.C.D., and Kay, W.W. (1993b) *fimA* and *tctC* and SEF21 fimbriae based diagnostics for *Salmonella*. *Molecular and Cellular Probes* **8**:291-310.

Doran, J.L., Collinson, S.K., Clouthier, S.C., Cebula, T.A., Koch, W.H., Burian, J. and Kay, W.W. (1995) *sefA* genes of *Salmonella*. Submitted.

Dorman, C.J. and Higgins, C.F. (1987) Fimbrial phase variation in *Escherichia coli* : Dependence on integration host factor and homologies with other site-specific recombinases. *Journal of Bacteriology* **169**:3840-3843.

Drlica, K. (1992) Control of bacterial DNA supercoiling. *Molecular Microbiology* **6**:425-433.

Duguid, J.P., Smith, I.W., Dempster, G. and Edmunds, P.N. (1955) Non-flagellar filamentous apendages ("fimbriae') and haemagglutinating activity in *Bacterium coli*. *Journal of Pathology and Bacteriology***70**:335-348.

Duguid, J. P. and Old, D. C. (1980) Adhesive Properties of Enterobacteriaceae in *Bacterial Adherence: Receptors and Recognition*, Series B, Vol. 6, Beachey, E. H. (ed). London: Chapman and Hall.pp. 186-217.

Dykes, C.W., Halliday, I.H., Read, M.J., Hobden, A.N. and Harford, S. (1985) Nucleotide sequences of four variants of the K88 gene of porcine enterotoxigenic *Escherichia coli*. *Infection and Immunity* **50**:279-283.

Eisenstein, B.I. (1981) Phase variation of type 1 fimbriae in *Escherichia coli* is under transcriptional control. *Science* **214**:336-339.

Eisenstein, B.I., Sweet, D.S., Vaughn, V. and Friedman, D.I. (1987) Integration host factor is required for the DNA inversion that controls phase variation in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **84**:6506-6510.

Elleman, T.C. and Hoyne, P.A. (1984) Nucleotide sequence of the gene

encoding pilin of *Bacteroides nodosus*, the causal organism of ovine footrot. *Journal of Bacteriology* **160**:1184-1187.

Elleman, T.C., Hoyne, P.A., Emery, D.L., Stewart, D.J. and Clark, B.L. (1986a) Expression of the pilin gene from *Bacteroides nodosus* in *Escherichia coli*. *Infection and Immunity* **51**:187-192.

Elleman, T.C., Hoyne, P.S., Stewart, D.J., McKern, N.M. and Peterson, J.E. (1986b) Expression of pili from *Bacteroides nodosus* in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **168**:574-580.

Evans, D.G., Silver, R.P., Evans, D.J., Chase, D.G. and Gorbach, S.L. (1975) Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. *Infection and Immunity* **12**:656-667.

Evans, D. G., Evans jr., D.J. and Tjoa, W. (1977) Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: Correlation with colonization factor. *Infection and Immunity* **18**:330-337.

Evans, D.G., Evans, Jr., D.J., Karjalainen, T.K. and Lee, C.-H. (1991) Production of colonization factor antigen II of enterotoxigenic *Escherichia coli* is subject to catabolite repression. *Current Microbiology* **23**:71-74

Faast, R., Ogierman, M.A., Stroeger, U.J. and Manning, P.A. (1989) Nucleotide sequence of the structural gene, *tcpA*, for a major pilin subunit of *Vibrio cholerae*. *Gene* **85**:227-231.

Feutrier, J., Kay, W. W., and Trust, T. J. (1986) Purification and characterization of fimbriae from *Salmonella enteritidis*. *Journal of Bacteriology* **168**:221-227.

Feutrier, J., Kay, W. W., and Trust, T. J. (1988) Cloning and expression of a *Salmonella enteritidis* fimbrin gene in *Escherichia coli*. *Journal of Bacteriology* **170**:4216-4222.

Finlay, B.B., Pasloske, B.L. and Paranchych, W. (1986) Expression of the *Pseudomonas aeruginosa* PAK pilin gene in *Escherichia coli*. *Journal of Bacteriology* **165**:625-630.

Finlay, B. B., and Falkow, S. (1989) Common themes in microbial pathogenicity. *Microbiological Reviews* **53**:210-230.

Forsman, K., Göransson, M. and Uhlin, B.E. (1989) Autoregulation and multiple DNA interactions by a transcriptional regulatory protein in *E. coli*

pili biogenesis. *EMBO Journal* **8**:1271-1277.

Forsman, K., Sondén, B., Göransson, M. and Uhlin, B.-E. (1992) Antirepression function in *Escherichia coli* for the cAMP-CRP receptor protein transcriptional activator. *Proceedings of the National Academy of Sciences of the United States of America* **89**:9880-9884.

Fourney, R.M., Miyakoshi, J., Day III, R.S. and Paterson, M.C. (1992) Northern blotting: Efficient RNA staining and transfer. *Focus* **10**:5-7.

Fulks, K. A., Maris, C. F., Stevens, S. P., and Green, M. R. (1990) Sequence analysis of the inversion region containing the pilin genes of *Moraxella bovis*. *Journal of Bacteriology* **172**:310-316.

Gaastra, W., Mooi, F.R., Stuitje, A.R. and De Graaf, F.K. (1981) The nucleotide sequence of the gene encoding the K88ab protein subunit of porcine enterotoxigenic *Escherichia coli*. *Federation of European Microbiological Societies Microbiology Letters* **12**:41-46.

Gaastra, W. and DeGraaf, F.K. (1982) Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. *Microbiological Reviews* **46**:129-161.

Gallegos, M.-T., Michán, C. and Ramos, J.L. (1993) The XylS/AraC family of regulators. *Nucleic Acids Research* **21**:807-810.

Gally, D.L., Bogan, J.A., Eisenstein, B.I. and Blomfield, I.C. (1993) Environmental regulation of the *fim* switch controlling type 1 fimbrial phase variation in *Escherichia coli* K-12: Effects of temperature and media. *Journal of Bacteriology* **175**:6186-6193.

Gally, D.L., Rucker, T.J. and Blomfield, I.C. (1994) The leucine-responsive regulatory protein binds to the *fim* switch to control phase variation of type 1 fimbrial expression in *Escherichia coli* K-12. *Journal of Bacteriology* **176**:5665-5672.

Gast, R.K. (1994) Understanding *Salmonella enteritidis* in laying chickens: The contributions of experimental infections. *International Journal of Food Microbiology* **21**:107-116.

Gerlach, G.-F., Allen, B.L. and Clegg, S. (1988) Molecular characterization of the type 3 (MR/K) fimbriae of *Klebsiella pneumoniae*. *Journal of Bacteriology* **170**:3547-3553.

Gierasch, L. M. (1989) Signal sequences. *Biochemistry* **28**:923-930.

Girardeau, J.P., Dubourguier, H.C. and Gouet, P.H. (1982) Effect of glucose and amino acids on expression of K99 antigen in *Escherichia coli*. *Journal of General Microbiology* **128**:2243-2249

Girardeau, J.-P., Bertin, Y., Martin, C., Vartanian, M.D. and Boeuf, C. (1991) Sequence analysis of the *clpG* gene, which codes for surface antigen CS31A subunit: Evidence of an evolutionary relationship between CS31A, K88 and F41 subunit genes. *Journal of Bacteriology* **173**:7673-7683.

Girón, J.A., Ho, A.S.Y., and Schoolnik, G.K. (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science* **254**:710-713.

Girón, J.A., Levine, M.M. and Kaper, J.B. (1994) Longus: A long pilus ultrastructure produced by human enterotoxigenic *Escherichia coli*. *Molecular Microbiology* **12**:71-82.

Goldblum, K. and Apirion, D. (1981) Inactivation of the ribonucleic acid-processing enzyme ribonuclease E blocks cell division. *Journal of Bacteriology* **146**:128-132.

Goldstein, E. and Drlica, K. (1984) Regulation of bacterial DNA supercoiling: Plasmid linking numbers vary with growth temperature. *Proceedings of the National Academy of Sciences of the United States of America***81**:4046-4050.

Gong, M. and Makowski, L. (1992) Helical structure of P pili from *Escherichia coli*. *Journal of Molecular Biology* **228**:735-742.

Göransson, M. and Uhlin, B.E. (1984) Environmental temperature regulates transcription of a virulence pili operon in *E. coli*. *EMBO Journal* **33**:2885-2888.

Göransson, M., Forman, K., Nilsson, P. and Uhlin, B.E. (1989a) Upstream activating sequences that are shared by two divergently transcribed operons mediate cAMP-CRP regulation of pilus-adhesin in *Escherichia coli*. *Molecular Microbiology* **3**:1557-1565.

Göransson, M., Forsman, K. and Uhlin, B.E. (1989b) Regulatory genes in the thermoregulation of *Escherichia coli* pili gene transcription. *Genes and Development* **3**:123-130.

Göransson, M., Sondén, B., Nilsson, P., Dagberg, B., Forsman, K., Emanuelsson, K. and Uhlin, B.E. (1990) Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli*. *Nature* (London) **344**:682-685.

Groisman, E.A. and Saier, Jr., M.H. (1990) *Salmonella* virulence: New clues to intramacrophage survival. *Trends in Biochemistry* **15**:30-33.

Gross, R., Aricó, B. and Rappuoli, R. (1989) Families of bacterial signal-transducing proteins. *Molecular Microbiology* 3:1661-1667.

Haas, R. and Meyer, T.F. (1986) The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: Evidence for gene conversion. *Cell* **44**:107-115.

Hacker, J., Schmidt, G., Hughes, C., Knapp, S., Marget, M. and Goebel, W. (1985) Cloning and characterization of genes involved in production of mannose-resistant, neuraminidase-suseptible (X) fimbriae from a uropathogenic 06:K15:H31 *Escherichia coli* strain. *Infection and Immunity* **47**:434-440.

Hacker, J. and Morschhäuer, J. (1994) S and F1C fimbriae. In *Fimbriae: Adhesion, genetics, biogenesis and vaccines.* London: CRC Press, pp. 27-36.

Hanson, M.S. and Brinton, Jr., C.C. (1988) Identification and characterization of *E. coli* type-1 pilus tip adhesion protein. *Nature* (London) **332**:265-268.

Higgins, C. F., Ames, G.F.-L., Barnes, W.M., Clement, J.M. and Hofnung, M. (1982) A novel intercistronic regulatory element of prokaryotic operons. *Nature* (London) **298**:760-762.

Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G. and Bremer, E. (1988) A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli. Cell* **52**:569-584.

Hobbs, M., Dalrymple, B., Delaney, S.F. and Mattick, J.S. (1988) Transcription of the fimbrial subunit gene and an associated transfer RNA gene of *Pseudomonas aeruginosa. Gene* **62**:219-227.

Hobbs, M., Dalrymple, B. P., Cox, P. T., Livingstone, S. P., Delaney, S. F., and Mattick, J. S. (1991) Organization of the fimbrial gene region of *Bacteroides nodosus*: Class I and class II strains. *Molecular Microbiology* **5**:543-560.

Hobbs, M., Collie, E.S.R., Free, P.D., Livingston, S.P. and Mattick, J.S. (1993) PilS and PilR, a two component transcriptional regulatory system controlling expression of type 4 fimbriae of *Pseudomonas aeruginosa. Molecular Microbiology* **7**:669-682.

Hobbs, M. and Mattick, J.S. (1993) Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: A general system for the formation of surface-associated protein complexes. *Molecular Microbiology* **10**:233-243.

Holmgren, A., Bränden, C.-I., Lindberg, F. and Tennent, J.M. (1988) Preliminary X-ray study of PapD crystals from uropathogenic *Escherichia coli*. *Journal of Molecular Biology* **203**:279-280.

Holmgren, A., and Bränden, C.-I. (1989) Crystal structure of chaperone protein PapD reveals an immunoglobulin fold. *Nature* (London) **342**:248-251.

Holmgren, A., Kuehn, M.J., Bränden, C.-I. and Hultgren, S.J. (1992) Conserved immunoglobulin-like features in a family of periplasmic pilus chaperones in bacteria. *EMBO Journal* **11**:1617-1622.

Hopper, S.A. and Mawer, S. (1988) *Salmonella enteritidis* in a commercial layer flock. *Veterinary Records* **123**:351.

Hoyne, P.A., Haas, R., Meyer, T.F., Davies, J.K. and Elleman, T.C. (1992) Production of *Neisseria gonorrhoeae* pili (fimbriae) in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **174**:7321-7327.

Hulsman, T.T., Bakker, D., Klaasen, P. and De Graaf, F.K. (1994) Leucine-responsive regulatory protein, IS*1* insertions, and the negative regulator FaeA control the expression of the *fae* (K88) operon in *Escherichia coli*. *Molecular Microbiology* **11**:525-536.

Hultgren, S.J., Lindberg, F., Magnusson, G., Kihlberg, J., Tennent, J.M. and Normark, S. (1989) The PapG adhesin of uropathogenic *Escherichia coli* contains separate regions for receptor binding and for the incorporation into the pilus. *Proceedings of the National Academy of Science of the United States of America* **86**:4357-4361.

Hultgren, S. J., and Normark, S. (1992) Chaperone-assisted assembly and molecular architecture of adhesive pili. *Annual Review of Microbiology* **45**:383-415.

Hultgren, S.J., Abraham, S., Caparon, M., Falk, P., Geme, J.W. and Normark, S. (1993) Pilus and nonpilus bacterial adhesins: Assembly and function in cell recognition. *Cell* **73**:887-901.

Hulton, C.S., Seirafi, A., Hinton, J.C.D., Sidebotham, J.M., Waddell, L., Pavitt, G.D., Owen-Hughes, T., Spassky, A., Buc, H. and Higgins, C.F. (1990) Histone-like protein H1 (H-NS), DNA supercoiling, and gene expression in bacteria. *Cell* **63**:631-642.

Humphrey, T.J. (1994) Contamination of egg shell and contents with *Salmonella enteritidis*: A review. *International Journal of Food Microbiology* **21**:31-40.

Imberechts, H., De Grove, H., Schlicker, C., Bouchet, H., Pohl, P., Charlier, G., Bertschinger, H., Wild, P., Vandekerckhove, J., Van Damme, J., Van Montagu, M. and Lintermans, P. (1992) Characterization of F107 fimbriae of *Escherichia coli* 107/86, which causes edema disease in pigs, and nucleotide sequence of the F107 major fimbriae subunit gene, *fedA*. *Infection and Immunity* **60**:1963-1971.

Isaacson, R.E. (1977) K99 surface antigen of *Escherichia coli*: Purification and partial characterization. *Infection and Immunity* **15**:272-279.

Isaacson, R.E. (1980) Factors regulating expression of the *Escherichia coli* pilus K99. *Infection and Immunity* **28**:190-194

Isaacson, R.E. and Richter, P. (1981) *Escherichia coli* 987P pilus: Purification and partial characterization. *Journal of Bacteriology* **146**:784-789.

Isaacson, R.E. (1983) Regulation of expression of *Escherichia coli* pilus K99. *Infection and Immunity* **40**:633-639.

Isberg, R. R. (1991) Discrimination between intracellular uptake and surface adhesion of bacterial pathogens. *Science* **252**:934-938.

Ishimoto, K.S. and Lory, S. (1989) Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (RpoN) of RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* **86**:1954-1957.

Ishimoto, K.S. and Lory, S. (1992) Identification of *pilR*, which encodes a transcriptional activator of the *Pseudomonas aeruginosa* pilin gene. *Journal of Bacteriology* **174**:3514-3521.

Jacob-Dubuisson, F., Heuser, J., Dodson, K., Normark, S. and Hultgren, S. (1993) Initiation of assembly and association of the structural elements of a bacterial pilus depend on two specialized tip proteins. *EMBO Journal* **12**: 837-847.

Jacobs, A.A.C. and De Graaf, F.K. (1985) Production of K88, K99 and F41 fibrillae in relation to growth phase, and a rapid procedure for adhesin purification. *Federation of European Microbiological Societies Microbiology Letters* **26**:15-19.

Jalajakumari, M. B., Thomas, C.J., Halter, R. and Manning, P.A. (1989) Genes for biosynthesis and assembly of CS3 pili of CFA/II enterotoxigenic *Escherichia coli*: Novel regulation of pilus production by bypassing an amber codon. *Molecular Microbiology* **3**:1685-1695.

Jann, K., Jann, B. and Schmidt, B. (1981) SDS polyacrylamide gel electrophoresis and serological analysis of pili from *Escherichia coli* from different pathogenic origin. *Federation of European Microbiological Societies Microbiology Letters* **11**:21-25.

Johnson, K., Parker, M.L. and Lory, S. (1986) Nucleotide sequence and transcriptional initiation site of two *Pseudomonas aeruginosa* pilin genes. *Journal of Biological Chemistry* **261**:15703-15708.

Jones, C.H., Jacob-Dubuisson, F., Dodson, K., Kuehn, M., Slonim, L., Striker, R. and Hultgren, S.J. (1992) Adhesin presentation in bacteria requires molecular chaperones and ushers. *Infection and Immunity* **60**:4445-4451.

Jordi, B.J.A.M., Dagberg, B., De Haan, L.A.M., Hamers, A.M., Van Der Zeijst, B.A.M., Gaastra, W. and Uhlin, B.E. (1992) The positive regulator CfaD overcomes the repression mediated by histone-like protein H-NS (H1) in the CFA/1 fimbrial operon of *Escherichia coli*. *EMBO Journal* **11**:2627-2632.

Jordi, B.J.A.M., op den Camp, I.E.L., De Haan, L.A.M., Van Der Zeijst, B.A.M. and gaastra, W. (1993) Differential decay of RNA of the CFA/1 fimbrial operon and control of the relative gene expression. *Journal of Bacteriology* **175**:7976-7981.

Kallenius, G. Möllby, R., Svenson, S.B., Winberg, J., Lundblad, A., Svensson, S. and Cedergren, B. (1980) The P^k antigen as receptor for the haemagglutinin of pyelonephritic *Escherichia coli*. *Federation of European Microbiological Societies Microbiology Letters* **7**:297-302.

Karjalainen, T.K., Evans, D.G., Evans, Jr., D.J., Graham, D.Y. and Lee, C.-H. (1991a) Catabolite repression of the colonization factor antigen I (CFA/I) operon of *Escherichia coli*. *Current Microbiology* **23**:307-313.

Karjalainen, T.K., Evans, D.G., Evans, Jr., D.J., Graham, D.Y. and Lee, C.-H. (1991b) Iron represses the expression of CFA/1 fimbriae of enterotoxigenic *E. coli*. *Microbial Pathogenesis* **11**:317-323.

Kaufman, M.R., Shaw, C.E., Jones, I.D. and Taylor, R.K. (1993) Biogenesis and regulation of the *Vibrio cholerae* toxin-coregulated pilus: Analogies to other virulence factor secretory systems. *Gene* **126**:43-49.

Kaufman, M.R. and Taylor, R.K. (1994) Fimbriae of *Vibrio cholerae*. In *Fimbriae: Adhesion, genetics, biogenesis and vaccines*. London: CRC Press, pp. 157-170.

Kawula, T.H. and Orndorff, P.E. (1991) Rapid site-specific DNA inversion in *Escherichia coli* mutants lacking the histonelike protein H-NS. *Journal of*

Bacteriology **173**:4116-4123.

Klaasen, P. and De Graaf, F.K. (1990) Characterization of FapR, a positive regulator of expression of the 987P operon in enterotoxigenic *Escherichia coli*. *Molecular Microbiology* **4**:1779-1783.

Klaasen, P., Woodward, M.J., Van Zijderveld, F.G. and De Graaf, F.K. (1990) The 987P gene cluster in enterotoxigenic *Escherichia coli* contains an ST_{pa} transposon that activates 987P expression. *Infection and Immunity* **58**:801-807.

Klemm, P. (1984) The *fimA* gene encoding the type-1 fimbrial subunit of *Escherichia coli*: Nucleotide sequence and primary structure of the protein. *European Journal of Biochemistry* **143**:395-399.

Klemm, P. (1985) Fimbrial adhesins of *Escherichia coli*. *Review of Infectious Diseases* **7**:321-340.

Klemm, P., Jorgensen, B.J., Van Die, I., De Ree, H. and Bergmans, H. (1985) The *fim* genes responsible for synthesis of type 1 fimbriae in *Escherichia coli*, cloning and genetic organization. *Molecular and General Genetics* **199**:410-414.

Klemm, P. (1986) Two regulatory *fim* genes, *fimB* and *fimE*, control the phase variation of type 1 fimbriae in *Escherichia coli*. *EMBO Journal* **5**:1389-1393.

Klemm, P. and Christiansen, G. (1990) The *fimD* gene required for cell surface localization of *Escherichia coli* type 1 fimbriae. *Molecular and General Genetics* **220**:334-338.

Klemm, P. and Krogfelt, K.A. (1994) Type 1 fimbriae of *Escherichia coli*. In *Fimbriae: Adhesion, genetics, biogenesis and vaccines*. London: CRC Press, pp. 9-26.

Koga, T., Ishimoto, K. and Lory, S. (1993) Genetic and functional characterization of the gene cluster specifying expression of *Pseudomonas aeruginosa* pili. *Infection and Immunity* **61**:1371-1377.

Koomey, M., Bergstrom, S., Blake, M. and Swanson, J. (1991) Pilin expression and processing in pilus mutants of *Neisseria gonorrhoeae*: Critical role of Gly_1 in assembly. *Molecular Microbiology* **5**:279-287.

Koraimann, G. and Hogenauer, G. (1989) A stable core region of the *tra* operon mRNA of plasmid R1-19. *Nucleic Acids Research* **17**:1283-1298.

Korhonen, T.K., Tarkka, E., Tanta, H. and Haahtelu, K. (1983) Type 3 fimbriae

of *Klebsiella* sp.: Molecular characterization and role in bacterial adhesion to plant roots. *Journal of Bacteriology* **155**:860-865.

Korhonen, T.K., Väisänen-Then, V., Rhen, M., Pere, A., Parkkinen, J. and Finne, J. (1984) *Escherichia coli* fimbriae recognizing sialyl galactosides. *Journal of Bacteriology* **159**:762-766.

Korth, M.J., Schneider, R.A. and Moseley, S.L. (1991) An F41-K88-related genetic determinant of bovine septicemic *Escherichia coli* mediates expression of CS31A fimbriae and adherence to epithelial cells. *Infection and Immunity* **59**:2333-2340.

Kuehn, M. J., Normark, S. and Hultgren, S.J. (1991) Immunoglobulin-like PapD chaperone caps and uncaps interactive surfaces of nascently translocated pilus subunits. *Proceedings of the National Academy of Sciences of the United States of America* **88**:10586-10590.

Kuehn, M. J., Heuser, J., Normark, S. and Hultgren, S.J. (1992) P pili in uropathogenic *E. coli* are composite fibres with distinct fibrillar adhesive tips. *Nature* (London) **356**:252-255.

Kuehn, M.J., Haslam, D., Normark, S. and Hultgren, S.J. (1994) Structure, function and biogenesis of Escherichia coli P pili. In *Fimbriae: Adhesion, genetics, Biogenesis and vaccines.* London: CRC Press, pp. 37-51.

Kumamoto, C.A. (1991) Molecular chaperones and protein translocation across the *Escherichia coli* inner membrane. *Molecular Microbiology* **5**:19-22.

Kunin, C.M., Hua, T.H., Guerrant, R.L. and Bakaletz, L.O. (1994) Effect of salicylate, bismuth, osmolytes and tetracycline resistance on expression of fimbriae by *Escherichia coli. Infection and Immunity* **62**:2178-2186.

Kusters, J.G. and Gaastra, W. (1994) Fimbrial operons and evolution in fimbriae. In *Fimbriae: Adhesion, Genetics, Biogenesis and Vaccines.* London: CRC Press, pp. 179-196.

Kustu, S., Santero, E., Keener, J., Popham, D. and Weiss, D. (1989) Expression of σ^{54} (*ntrA*)-dependent genes is probably united by a common mechanism. *Microbiology Reviews* **53**:367-376.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London)**227**:680-685.

Lee, K.K., Sheth, H.B., Wong, W.Y., Sherburne, R., Paranchych, W., Hodges, R.S., Lingwood, C.A., Krivan, H. and Irvin, R.T. (1994) The binding of

Pseudomonas aeruginosa pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. *Molecular Microbiology* **11**:705-713.

Leffler, H. and Svanborg-Edén , C. (1981) Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. *Federation of European Microbiological Societies Microbiology Letters* **8**:127-134.

Le Minor, L. (1984) Genus III: *Salmonella*. In *Bergies Manual of Systematic Bacteriology. Vol. 1.* Baltimore: The Williams and Wilkins Co., pp. 427-458.

Lenich, A.G. and Glasgow, A.C. (1994) Amino acid sequence homology between Piv, an essential protein in site-specific DNA inversion in *Moraxella lacunata*, and transposases of an unusual family of insertion elements. *Journal of Bacteriology* **176**:4160-4164.

Levine, M. M., Ristaino, P., Marley, G., Smyth, C., Knutton, S., Boedeker, E., Black, R., Young, C., Clements, M. L., Cheney, C., and Patnaik, R. (1984) Coli surface antigens 1 and 3 of colonization factor antigen II-positive enterotoxigenic *Escherichia coli*: Morphology, purification, and immune responses in humans. *Infection and Immunity* **44**:409-420.

Lindberg, F., Lund, B. and Normark, S. (1986) Genes products specifying adhesion of uropathogenic *Escherichia coli* are minor components of pili. *Proceedings of the National Academy of Sciences of the United States of America* **83**:1891-1895.

Lindberg, F., Lund, B., Johansson, L. and Normark, S. (1987) Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. *Nature* (London) **328**:84-87.

Lindberg, F., Tennent, J.M., Hultgren, S.J., Lund, B. and Normark, S. (1989) PapD, a periplasmic transport protein in P-pilus biogenesis. *Journal of Bacteriology* **171**:6052-6058.

Lindstrom, E.B., Boman, H.G. and Steele, B.B. (1970) Resistance of *Escherichia coli* to penicillins. VI. Purification and characterization of the chromosomally mediated penicillinase present in *ampA*-containing strains. *Journal of Bacteriology* **101**:218-231.

Lintermans, P., Pohl, P., Deboeck, F., Bertels, A., Schlicker, C., Vandekerckhove, J., Van Damme, J., Van Montagu, M. and De Grove, H. (1988) Isolation and nucleotide sequence of the F17A gene encoding the structural protein of the F17 fimbriae in bovine enterotoxigenic *Escherichia coli*. *Infection and Immunity* **56**:1475-1484.

Liu, S.-L., Hessel, A. and Sanderson, K.E. (1993a) The *Xba*I-*Bln*I-*Ceu*I genomic cleavage map of *Salmonella typhimurium* LT2, determined by double digestion, end-labelling, and pulsed-field gel electrophoresis. *Journal of Bacteriology* **175**:4104-4120.

Liu, S.-L., Hessel, A. and Sanderson, K.E. (1993b) The *Xba*I-*Bln*I-*Ceu*I genomic cleavage map of *Salmonella enteritidis* shows an inversion relative to *Salmonella typhimurium* LT2. *Molecular Microbiology* **10**:655-664.

Low, D., Braaten, B., van der Woude, M. (1995) Fimbriae. In Press.

Lowe, M.A., Holt, S.C. and Eisenstein, B.I. (1987) Immunoelectron microscopic analysis of elongation of type 1 fimbriae in *Escherichia coli*. *Journal of Bacteriology* **169**:157-163.

Lund, B., Lindberg, F., Marklund, B.-I. and Normark, S. (1987) The PapG protein is the α-D-galactopyranosyl-(1-4)-β-D-galactopyranose-binding adhesin of uropathogenic *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **84**:5898-5902.

Lund, B., Lindberg, F. and Normark, S. (1988a) Structure and antigenic properties of the tip-located P pilus proteins of uropathogenic *Escherichia coli*. *Journal of Bacteriology* **170**:1887-1894.

Lund, B., Marklund, B.-I., Strömberg, N., Lindberg, F., Karlsson, K.A. and Normark, S. (1988b) Uropathogenic *Escherichia coli* can express serologically identical pili of different receptor binding specificities. *Molecular Microbiology* **2**:255-263.

MacDonald, D.L., Paslocke, B.L. and Paranchych, W. (1993) Mutations in the fifth position glutamate in *Pseudomonas aeruginosa* pilin affect the transmethylation of the N-terminal phenylalanine. *Canadian Journal of Microbiology* **39**:500-510.

Manoil, C. and Beckwith, J. (1985) Tn*phoA*: A transposon probe for protein export signals. *Proceedings of the National Academy of Sciences of the United States of America* **82**:8129-8134.

Marck, C. (1988) DNA Strider: A 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic Acids Research* **16**:1829-1836.

Marklund, B.-I., Tennent, J.M., Garcia, E., Hamers, A., Båga, M., Lindberg, F., Gaastra, W. and Normark, S. (1992) Horizontal gene transfer of the *Escherichia coli pap* and *prs* operons as a mechanism for the development of

tissue-specific adhesive properties. *Molecular Microbiology* **6**:2225-2242.

Marmur, J. (1961) A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *Journal of Molecular Biology* **3**:208-218.

Marrs, C.F., Schoolnik, G., Koomey, J.M., Hardy, J., Rothbard, J. and Falkow, S. (1985) Cloning and sequencing of a *Moraxella bovis* pilin gene. *Journal of Bacteriology* **163**:132-139.

Marrs, C.F., Ruehl, W.M., Schoolnik, G.K. and Falkow, S. (1988) Pilin gene phase variation of *Moraxella bovis* is caused by an inversion of the pilin genes. *Journal of Bacteriology* **170**:3032-3039.

Marrs, C.F., Rozsa, F.W., Hackel, J., Stevens, S.P. and Glasgow, A.C. (1990) Identification, cloning and sequencing of *piv*, a new gene involved in inverting the pilin genes of *Moraxella lacunata*. *Journal of Bacteriology* **172**:4370-4377.

Marrs, C.F. and Weir, S. (1990) Pili (fimbriae) of *Branhamella* species. *American Journal of Medicine* **88**:36s-40s.

Martin, P.R., Hobbs, M., Free, P.D., Jeske, Y. and Mattick, J.S. (1993) Characterization of *pilQ*, a new gene required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. *Molecular Microbiology* **9**:857-868.

Mason, J. (1994) *Salmonella enteritidis* control programs in the United States. *International Journal of Food Microbiology* **21**:155-169.

Masui, Y., Mizuno, T. and Inouye, M. (1984) Novel high-level expression cloning vehicles: 10^4-fold amplification of *Escherichia coli* minor protein. *Bio-Technology* **2**:81-85.

Matsuyama, S., Fujita, Y. and Mizushima, S. (1993) SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. *EMBO Journal* **12**:265-270.

Mattick, J.S., Anderson, B.J., Cox, P.T., Dalrymple, B.P., Bills, M.M., Hobbs, M. and Egerton, J.R. (1991) Gene sequences and comparison of the fimbrial subunits representative of *Bacteroides nodosus* serotypes A to I: Class I and class II strains. *Molecular Microbiology* **5**:561-573.

Mayer, L.W. (1988) Use of plasmid profiles in epidemiologic surveillance of disease outbreaks and in tracing the transmission of antibiotic resistance. *Clinical Microbiology Reviews* **1**:228-243.

McClain, M.S., Blomfield, I.C. and Eisenstein, B.I. (1991) Roles of *fimB* and

fimE in site specific DNA inversion associated with phase variation of type 1 fimbriae in *Escherichia coli*. *Journal of Bacteriology* **173**:5308-5314.

McCormick, J. R., Zengel, J.M. and Lindahl, L. (1991) Intermediates in the degradation of mRNA from the lactose operon of *Escherichia coli*. *Nucleic Acids Research* **19**:2767-2776.

McKern, N.M., O'Donnell, I.J., Inglis, A.S., Stewart, D.J. and Clark, B.L. (1983) Amino acid sequence of pilin from *Bacteroides nodosus* (strain 198), the causative organism of ovine footrot. *Federation of European Biochemical Societies Letters* **164**:149-153.

McMicheal, J.C. and Ou, J.R. (1979) Structure of common pili from *Escherichia coli*. *Journal of Bacteriology* **138**:969-975.

Menzel, R. and Gellert, M. (1983) Regulation of the genes for *E. coli* DNA gyrase: Homeostatic control of DNA supercoiling. *Cell* **34**:105-113.

Meyer, T.F., Billyard, E., Haas, R., Storzbach, S. and So, M. (1984) Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. *Proceedings of the National Academy of Sciences of the United States of America* **81**:6110-6114.

Miller, V.L., Taylor, R.K. and Mekalanos, J.J. (1987) Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. *Cell* **48**:271-279.

Minion, F.C., Abraham, S.N., Beachey, E.H. and Goguen, J.D. (1986) The genetic determinant of adhesive function in type 1 fimbriae of *Escherichia coli* is distinct from the gene encoding the fimbrial subunit. *Journal of Bacteriology* **165**:1033-1036.

Moch, T., Hoschützky, J., Hacker, J., Kröncke, K.-D. and Jann, K. (1987) Isolation and characterization of the α-sialyl-β-2,3-galactosyl-specific adhesin from fimbriated *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **84**:3462-3466.

Mooi, F.R., Wouters, C., Wijfjes, A. and De Graaf, F.K. (1982) Construction and characterization of mutants impaired in the biosynthesis of the K88ab antigen. *Journal of Bacteriology* **150**:512-521.

Mooi, F.R., Wijfjes, A. and De Graaf, F.K. (1983) Identification and characterization of precursors in the biosynthesis of the K88ab fimbria of *Escherichia coli*. *Journal of Bacteriology* **154**:41-49.

Mooi, F. R., Claassen, I., Bakker, D., Kuipers, H. and De Graaf, F.K. (1986)

Regulation and structure of an *Escherichia coli* gene coding for an outer membrane protein involved in the export of K88ab fimbrial subunits. *Nucleic Acids Research* **14**:2443-2457.

Morschhäuser, J., Uhlin, B.-E. and Hacker, J. (1993) Transcriptional analysis and regulation of the *sfa* determinant coding for S fimbriae of pathogenic *Escherichia coli* strains. *Molecular and General Genetics* **238**:97-105.

Müller, K. - H., Trust, T.J. and Kay, W.W. (1989) Fimbriation genes of *Salmonella enteritidis. Journal of Bacteriology* **171**:4648-4654.

Müller, K.-H., Collinson, S. K., Trust, T. J., and Kay, W. W. (1991) Type 1 fimbriae of *Salmonella enteritidis. Journal of Bacteriology* **173**:4765-4772.

Nataro, J.P., Yikang, D., Giron, J.A., Savarino, S.J., Kothary, M.H. and Hall, R. (1993) Aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli* requires two unlinked plasmid regions. *Infection and Immunity* **61**:1126-1131.

Nataro, J.P., Yikang, D., Yingkang, D. and Walker, K. (1994) AggR, a transcriptional activator of aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli. Journal of Bacteriology* **176**:4691-4699.

Newbury, S.F., Smith, N.H. and Higgins, C.F. (1987) Differential mRNA stability controls relative gene expression within a polycistronic operon. *Cell* **51**:1131-1143.

Newman, E.B., D'Ari, R. and Lin, R.T. (1992) The leucine-Lrp regulon in *E. coli*: A global response in search of a Raison d'Etre. *Cell* **68**:617-619.

Nilsson, P., and Uhlin, B.E. (1991) Differential decay of a polycistronic*Escherichia coli* transcript is initiated by RNase E-dependent endonucleolytic processing. *Molecular Microbiology* **5**:1791-1799.

Nishiyama, K., Kabuyama, Y., Akimaru, J., Matsuyama, S., Tokuda, H. and Mizushima, S. (1991) SecY is an indispensible component of the protein secretory machinery of *Escherichia coli. Biochimica et Biophysica Acta* **1065**:89-97.

Norgren, M., Baga, M., Tennent, J.M. and Normark, S. (1987) Nucleotide sequence, regulation and functional analysis of the *papC* gene required for cell surface localization of Pap pili of uropathogenic *Escherichia coli. Molecular Microbiology* **1**:169-178.

Nou, X., Skinner, B., Braaten, B., Blyn, L., Hirsch, D. and Low, D. (1993) Regulation of pyelonephritis-associated pili phase-variation in *Escherichia*

coli: Binding of the PapI and the Lrp regulatory proteins is controlled by DNA methylation. *Molecular Microbiology* **7**:545-553.

Nunn, D., Bergman, S. and Lory, S. (1990) Products of three accessory genes, *pilB, pilC and pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. *Journal of Bacteriology* **172**:2911-2919.

Nunn, D.N. and Lory, S. (1991) Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. *Proceedings of the National Academy of Sciences of the United States of America* **88**:3281-3285.

Nunn, D.N and Lory, S. (1992) Components of the protein-excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. *Proceedings of the National Academy of Sciences of the United States of America* **89**:47-51.

Oliver, D.B. (1993) SecA protein autoregulated ATPase catalyzing preprotein insertion and translocation across the *Escherichia coli* inner membrane. *Molecular Microbiology* **7**:159-165.

Olsén, A., Arnqvist, A., Hammar, M., Sukupolvi, S. and Normark, S. (1993) The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. *Molecular Microbiology* **7**:523-536.

Orndorff, P. E., and Falkow, S. (1984a) Organization and expression of genes responsible for type 1 piliation in *Escherichia coli*. *Journal of Bacteriology* **159**:736-744.

Orndorff, P.E. and Falkow, S. (1984b) Identification and characterization of a gene product that regulates type 1 piliation in *Escherichia coli*. *Journal of Bacteriology* **160**:61-66.

Orndorff, P.E. and Falkow, S. (1985) Nucleotide sequence of *pilA*, the gene encoding the structural component of type 1 pili in *Escherichia coli*. *Journal of Bacteriology* **162**:454-457.

Ørskov, I., Ørskov, F., Sojka, W.J. and Leach, J.M. (1961) Simultanious occurrence of *E. coli* B and L antigens in strains from diseased swine. *Acta Pathologica et Microbiologica Scandinavica* **53**:404-422.

Ottow, J.C.G. (1975) Ecology, physiology and genetics of fimbriae and pili. *Annual Review of Microbiology* **29**:79-108.

Oudega, B., De Graaf, M., De Boer, L., Bakker, D., Vader, C.E.M., Mooi, F.R. and De Graaf, F.K. (1989) Detection and identification of FaeC as a minor

component of K88 fibrillae of *Escherichia coli. Molecular Microbiology* **3**:645-652.

Pallesen, L., Madsen, O. and Klemm, P. (1989) Regulation of the phase switch controlling expression of type 1 fimbriae in *Escherichia coli. Molecular Microbiology* **3**:925-931.

Paranchych, W., and Frost, L. S. (1988) The physiology and biochemistry of pili. *Advances in Microbial Physiology* **29**:53-114.

Parge, H.E., McRee, D.E., Capozza, M.A., Bernstein, S.L., Getzoff, E.D. and Tainer, J.A. (1987) Three dimensional structure of bacterial pili. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* **53**:447-453.

Parge, H.E., Bernstein, S.L., Deal, C.D., McRee, D.E., Christensen, D., Capozza, M.A., Kays, B.W., Fieser, T.M., Draper, D., So, M., Getzoff, E.D. and Tainer, J.A. (1990) Biochemical purification and crystallographic characterization of the fiber-forming protein pilin from *Neisseria gonorrhoeae. Journal of Biological Chemistry* **265**:2278-2285.

Parkkinen, J., Rogers, G.N., Korhoren, T.K., Dahr, W. and Finne, J. (1986) Identification of the O-linked sialyloligosaccharides of glycophorin A as the erythrocyte receptors for S-fimbriated *Escherichia coli. Infection and Immunity* **54**:37-42.

Paslocke, B.L. and Paranchych, W. (1988) The expression of mutant pilins in *Pseudomonas aeruginosa*: Fifth position glutamate affects pilin methylation. *Molecular Microbiology* **2**:489-496.

Pearson, W. R., and Lipman, D. J. (1988) Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences of the United States of America* **85**:2444-2448.

Perez-Casal, J., Swartley, J.S. and Scott, J.R. (1990) Gene encoding the major subunit of CS1 pili of human enterotoxigenic *Escherichia coli. Infection and Immunity* **58**:3594-3600.

Petersen, C. (1992) Control of functional mRNA stability in bacteria: Multiple mechanisms of nucleolytic and non-nucleolytic inactivation. *Molecular Microbiology* **6**:277-282.

Pohl, P., Lintermans, P., Marin, M. and Couturier, M. (1991) Epidemiological study of *Salmonella enteritidis* strains of animal origin in Belgium. *Epidemiology and Infection* **106**:11-16.

Polard, P. and Chandler, M (1995) Bacterial transposases and retroviral integrases. *Molecular Microbiology* **15**:13-23.

Poppe, C. (1994) *Salmonella enteritidis* in Canada. *International Journal of Food Microbiology* **21**:1-5.

Rampling, A., Upson, R., Ward, L., Anderson, F.R., Peters, E. and Rowe, B. (1989) *Salmonella enteritidis* phage type 4 infection of broiler chickens: A hazard to public health. *Lancet* **ii**:436-438.

Rao, V.K. and Progulske-Fox, A. (1993) Identification of a novel type 4 pilin gene arrangement: Cloning and sequence of two N-methyl-phenylalanine pilin genes from *Eikenella corrodens*. *Journal of General Microbiology* **139**:651-660.

Rhen, M., van Die, I., Rhen, V. and Bergmans, H. (1983) Comparison of the nucleotide sequences of the genes encoding the KS71A and F7 fimbrial antigens of uropathogenic *Escherichia coli*. *European Journal of Biochemistry* **151**:573-577.

Rivera, M. J., Rivera, N., Castillo, J., Rubio,M.C. and Gómez-Lus, R. (1991) Molecular and epidemiological study of *Salmonella* clinical isolates. *Journal of Clinical Microbiology* **29**:927-932.

Rodrigue, D.C., Tauxe, R.V. and Rowe, B. (1990) International increase in *Salmonella enteritidis*: A new pandemic? *Epidemiology and Infection* **105**:21-27.

Roosendaal, B., Gastra, W. and De Graaf, F.K. (1984) The nucleotide sequence of the gene encoding the K99 subunit of enterotoxigenic *Escherichia coli*. *Federation of European Microbiological Societies Microbiology Letters* **22**:253-258.

Roosendaal, B., Van Bergen en Henegouwen, P.M.P. and De Graaf, F.K. (1986) Subcellular localization of K99 fimbrial subunits and effect of temperature on subunit synthesis and assembly. *Journal of Bacteriology* **165**:1029-1032.

Roosendaal, B., Boots, M. and De Graaf, F.K. (1987) Two novel genes, *fanA* and *fanB*, involved in the biogenesis of K99 fimbriae. *Nucleic Acids Research* **15**:5973-5984.

Roosendaal, B., Damoiseaux, J., Jordi, W. and de Graaf, F.K. (1989) Transcriptional organization of the DNA region controlling expression of the K99 gene cluster. *Molecular and General Genetics* **215**:250-256.

Roosendaal, B., and De Graaf, F.K. (1989) The nucleotide sequence of the *fanD* gene encoding the large outer membrane protein involved in the biosynthesis of K99 fimbriae. *Nucleic Acids Research* **17**:1263.

Rosner, J. L. (1972) Formation, induction and curing of bacteriophage P1 lysogens. *Virology* **48**:679.

Russell, P.W. and Orndorff, P.E. (1992) Lesions in two *Escherichia coli* type 1 pilus genes alter pilus number and length without affecting receptor binding. *Journal of Bacteriology* **174**:5923-5935.

Sadaie, Y., Takamatsu, H., Nakamura, K. and Yamane, K. (1991) Sequencing reveals similarity of the wild-type *div*$^+$ gene of *Bacillus subtilis* to the *Escherichia coli secA* gene. *Gene* **98**:101-105.

Sagara, K., Matsuyama, S. and Mizushima, S. (1994) SecF stabilizes SecD and SecY, components of the protein translocation machinery of the *Escherichia coli* cytoplasmic membrane. *Journal of Bacteriology* **176**:4111-4116.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: A laboratory manual*. Second Edition. cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**:5463-5467.

Sastry, P.A., Finlay, B.B., Paslocke, B.L., Paranchych, W., Pearlstone, T.R. and Smillie, L.B. (1985) Comparative studies of the amino acid and nucleotide sequences of pilin derived from *Pseudomonas aeruginosa* PAK and PAO. *Journal of Bacteriology* **164**:571-585.

Savelkoul, P.H.M., Willshaw, G.A., McConnell, M.M., Smith, H.R., Hamers, A.M., Van Der Zeijst, B.A.M. and Gaastra, W. (1990) Expression of CFA/1 fimbriae is positively regulated. *Microbial Pathogenesis* **8**:91-99.

Sawers, G., and Bock, A. (1989) Novel transcriptional control of the pyruvate formate-lyase gene: Upstream regulatory sequences and multiple promotors regulate anaerobic expression. *Journal of Bacteriology* **171**:2485-2498.

Schifferli, D.M., Beachey, E.H. and Taylor, R.K. (1991a) Genetic analysis of 987P adhesion and fimbriation of *Escherichia coli*: The *fas* genes link both phenotypes. *Journal of Bacteriology* **173**:1230-1240.

Schifferli, D.M., Beachey, E.H. and Taylor, R.K. (1991b) 987P fimbrial gene

identification and protein characterization by T7 RNA polymerase-induced transcription and Tn*phoA* mutagenesis. *Molecular Microbiology* **5**:61-70.

Schifferli, D.M. and Alrutz, M.A. (1994) Permissive linker insertion sites in the outer membrane protein of 987P fimbriae of *Escherichia coli*. *Journal of Bacteriology* **176**:1099-1110.

Schmoll, T., Hacker, J. and Goebel, W. (1987) Nucleotide sequence of the *sfaA* gene coding for the S-fimbrial protein subunit of *Escherichia coli*. *Federation of European Microbiological Societies Microbiology Letters* **41**:229-235.

Schmoll, T., Morschbäuer, J., Ott, M., Ludwig, B., Van Die, I. and Hacker, J. (1990a) Complete genetic organization and functional aspects of the *Escherichia coli* S fimbrial adhesin determinant: Nucleotide sequence of the genes *sfaB,C,D,E,F*. *Microbial Pathogenesis* **9**:331-343.

Schmoll, T., Ott, M., Oudega, B. and Hacker, J. (1990b) Use of a wild-type gene fusion to determine the influence of environmental conditions on expression of the S fimbrial adhesin in an *Escherichia coli* pathogen. *Journal of Bacteriology* **172**:5103-5111.

Schoolnik, G.K., Fernandez, R., Tai, J., Rothbard, J. and Gotschlich, E. C, (1984) Gonococcal pili: Primary structure and receptor binding domain. *Journal of Experimental Medicine* **159**:1351-1370.

Segal, E., Billyard, E., So, M., Storzbach, S. and Meyer, T.F. (1985) Role of chromosomal rearrangement in *Neisseria gonorrhoeae* pilus phase variation. *Cell* 40:293

Segal, E., Hagblom, P., Seifert, H. S., and So, M. (1986) Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. *Proceedings of the National Academy of Sciences of the United States of America* **83**:2177-2181.

Seifert, H.S., Ajioka, R.S., Marchal, C., Sparling, P.F. and So, M. (1988) DNA transformation leads to pilin antigenic variation in *Neisseria gonorrhoeae*. *Nature* (London) **336**:392-395.

Sheth, H.B., Lee, K.K., Wong, W.Y., Srivastava, G., Hindsgaul, O., Hodges, R.S., Paranchych, W. and Irvin, R.T. (1994) The pili of *Pseudomonas aeruginosa* strains PAK and PAO bind specifically to the carbohydrate sequence βGalNAc(1-4)βGal found in glycosphingolipids asialo-GM_1 and asialo-GM_2. *Molecular Microbiology* **11**:715-723.

Shlaes, D. and Currie-McCumber, C.A. (1986) Plasmid analysis in molecular

epidemiology: A summary and future directions. *Review of Infectious Diseases* **8**:738-746.

Simons, B.L., Rathman, P., Malij, C.R., Oudega, B. and De Graaf, F.K. (1990a) The penultimate tyrosine residue of the K99 fibrillar subunit is essential for stability of the protein and its interaction with the periplasmic carrier protein. *Federation of European Microbiological Microbiology Letters* **67**:107-112.

Simons, B.L., Willemsen, P.T.J., Bakker, D., Roosendaal, B., De Graaf, F.K. and Oudega, B. (1990b) Structure, localization, and function of FanF, a minor component of K99 fibrillae of enterotoxigenic *Escherichia coli*. *Molecular Microbiology* **4**:2041-2050.

Simons, L.H., Willensen, P.T.J., Bakker, D., De Graaf, F.K. and Oudega, B. (1991) Localization and function of FanH and FanG, minor components of K99 fimbriae of enterotoxigenic *Escherichia coli*. *Microbial Pathogenesis* **11**:325-336.

Skaugen, M. and Nes, I.F. (1994) Transposition in *Lactobacillus sake* and its abolition of lactocin S production by insertion of IS*1163*, a new member of the IS*3* family. *Applied and Environmental Microbiology* **60**:2818-2825.

Slonim. L.N., Pinkner, J.S., Bränden, C.-I. and Hultgren, S.J. (1992) Interactive surface in the PapD chaperone cleft is conserved in pilus chaperone superfamily and essential in subunit recognition and assembly. *EMBO Journal* **11**:4747-4756.

Smith, H.R., Willshaw, G.A. and Rowe, B. (1982) Mapping of a plasmid coding for colonization factor antigen 1 and heat-stable enterotoxin production, isolated from an enterotoxigenic strain of *Escherichia coli*. *Journal of Bacteriology* **149**:264-275.

Sohel, I., Puente, J.L., Murray, W.J., Vuopio-Varkila, J. and Schoolnik, G.K. (1993) Cloning and characterization of the bundle-forming pilin gene of enteropathogenic *Escherichia coli* and its distribution in *Salmonella* serotypes. *Molecular Microbiology* **7**:563-575.

Spears, P.A., Schauer, D. and Orndorff, P.E. (1986) Metastable regulation of type 1 piliation in *Escherichia coli* and isolation and characterization of an phenotypically stable mutant. *Journal of Bacteriology* **168**:179-185.

Stanley, J. and Baquar, N. (1994) Phylogenetics of *Salmonella enteritidis*. *International Journal of Food Microbiology* **21**:79-87.

Stirm, S., Orskov, F., Orskov, I. and Birch-Andersen, A. (1967) Episome-carried surface antigen K88 of *Escherichia coli*. III. Morphology. *Journal of*

Bacteriology **93**:740-748.

Strom, M.S. and Lory, S. (1986) Cloning and expression of the pilin gene of *Pseudomonas aeruginosa* PAK in *Escherichia coli*. *Journal of Bacteriology* **165**:367-372.

Strom, M.S. and Lory, S. (1991) Amino acid substitutions in pilin of *Pseudomonas aeruginosa*: Effect on leader peptide cleavage, amino-terminal methylation, and pilus assembly. *Journal of Biological Chemistry* **266**:1656-1664.

Strom, M.S., Nunn, D. and Lory, S. (1991) Multiple roles of the pilus biogenesis protein PilD: Involvement of PilD in excretion of enzymes from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **173**:1175-1180.

Strom, M.S. and Lory, S. (1992) Kinetics and sequence specificity of processing of prepilin by PilD, the type IV leader peptidase of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **174**:7345-7351.

Strom, M.S. and Lory, S. (1993) Structure-function and biogenesis of the type IV pili. *Annual Review of Microbiology* **47**:565-596.

Strom, M.S., Nunn, D.N and Lory, S. (1993) A single bifunctional enzyem, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. *Proceedings of the National Academy of Sciences of the United States of America* **90**:2404-2408.

Strömberg, N., Marklund, B.-I., Lund, B., Ilver, D., Hamers, A., Gaastra, W., Karlsson, K.-A. and Normark, S. (1990) Host-specificity of uropathogenic *Escherichia coli* depends on differences in binding specificity to Galα1-4Gal-containing isoreceptors. *EMBO Journal* **9**:2001-2010.

Swanson, T.N., Bilge, S.S., Nowicki, B. and Moseley, S.L. (1991) Molecular structure of the Dr adhesin: Nucleotide sequence and mapping of receptor-binding domain by use of fusion constructs. *Infection and Immunity* **59**:261-268.

Taha, M.K., So, M., Seifert, H.S., Billyard, E. and Marchal, C. (1988) Pilin expression in *Neisseria gonorrhoeae* is under both positive and negative transcriptional control. *EMBO Journal* **7**:4367-4378.

Taha, M.K. and Marchal, C. (1990) Conservation of *Neisseria gonorrhoeae* pilus expression regulatory genes *pilA* and *pilB* in the genus *Neisseria*. *Infection and Immunity* **58**:4145-4148.

Taha, M.K., Dupuy, B., Saurin, W., So, M. and Marchal, C. (1991) Control of

pilus expression in *Neisseria gonorrhoeae* as an original system in the family of two-component regulators. *Molecular Microbiology* **5**:137-148.

Takamatsu, H., Fuma, S.-I., Nakamura, K., Sadaie, Y., Shinkai, A., Matsuyama, S.I., Mizushima, S. and Yamane, K. (1992) In vivo and in vitro characterization of the *secA* gene product of *Bacillus subtilis*. *Journal of Bacteriology* **174**:4308-4316.

Takeuchi, A. (1967) Electron microscopic studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. *American Journal of Pathology* **50**:109-136.

Tennent, J.M., Lindberg, F. and Normark, S. (1990) Integrity of *Escherichia coli* P pili during biogenesis: Properties and role of PapJ. *Molecular Microbiology* **4**:747-758.

Tennent, J.M. and Mattick, J.S. (1994) Type 4 fimbriae. In *Fimbriae: Adhesion, genetics, biogenesis and vaccines*. London: CRC Press, pp.

Todd, E. (1990) Epidemiology of foodborne illness: North America. *Lancet* **336**:788-790.

Tokuda, H., Akimaru, J., Matsuyama, S., Nishiyama, K., and Mizushima, S. (1991) Purification of SecE and reconstitution of SecE-dependent protein translocation activity. *Federation of European Biochemical Societies Letters* **279**:233-236.

Tompkins, L.S., Troup, N., Lebigne-Rousell, A. and Cohen, M.L. (1986) Cloned, random chromosomal sequences as probes to identify *Salmonella* species. *Journal of Infectious Diseases* **154**:156-162.

Tønjum, T., Marrs, C.F., Razsa, F., Bovre, K. (1991) The type 4 pilin of *Moraxella nonliquefaciens* exhibits unique similarities with the pilins of *Neisseria gonorrhoeae* and *Dichelobacter (Bacteroides) nodosus*. *Journal of General Microbiology* **137**:2483-2490.

Tønjum, T., Weir, S., Bovre, K., Progulske-Fox, A. and Marrs, C.F. (1993) Sequence divergence in two tandemly located pilin gnees of *Eikenella corrodens*. *Infection and Immunity* **61**:1909-1916.

Uhlin, B.E. (1994) Regulation of *E. coli* fimbrial expression. In *Fimbriae: Adhesion, genetics, biogenesis and vaccines*. London: CRC Press, pp.

Van Der Woude, M.W., De Graaf, F.K. and Van Verseveld, H.W. (1989) Production of the fimbrial adhesin 987P by enterotoxigenic *Escherichia coli* during growth under controlled conditions in a chemostat. *Journal of*

General Microbiology **135**:3421-3429.

Van Der Woude, M.W., Arts, P.A., Bakker, D., Van Verseveld, H.W. and De Graaf, F.K. (1990a) Growth-rate-dependent synthesis of K99 fimbrial subunits is regulated at the level of transcription. *Journal of General Microbiology* **136**:897-903.

Van Der Woude, M.W., Braster, M., Van Verseveld, H.W. and De Graaf, F.K. (1990b) Control of temperature-dependent synthesis of K99 fimbriae. *Federation of European Microbiological Societies Microbiology Letters* **68**:183-188.

Van Der Woude, M.W., Braaten, B.A. and Low, D.A. (1992) Evidence for global regulatory control of pilus expression in *Escherichia coli* by Lrp and DNA methylation: Model building based on analysis of *pap*. *Molecular Microbiology* **6**:2429-2435.

Van Der Woude, M.W. and Low, D.A. (1994) Leucine-responsive regulatory protein and deoxyadenosine methylase control the phase variation and expression of the *sfa* and *daa* pili operons in *Escherichia coli*. *Molecular Microbiology* **11**:605-618.

Van Die, I. and Bergmans, H. (1984) Nucleotide sequence of the gene encoding the $F7_2$ fimbrial subunit of a uropathogenic *Escherichia coli* strain. *Gene* **32**:83-90.

Van Die, I., van E., M., Zuidweg, E., Hoekstra, W., de Tee, H., van den Bosch, H. and Bergmans, H. (1986) Functional relationship among the gene clusters encoding F71, F72, F9 and F11 fimbriae of human uropathogenic *Escherichia coli*. *Journal of Bacteriology* **167**:407-410.

Van Rosmalen, M. and Saier, Jr., M.H. (1994) Structural and evolutionary relationships among two families of bacterial periplasmic chaperone proteins which function cooperatively in fimbrial assembly. Submitted.

Van Verseveld, H.W., Bakker, P., Van Der Woude, T., Terleth, C. and De Graaf, F.K. (1985) Production of fimbrial adhesins K99 and F41 by enterotoxigenic *Escherichia coli* as a function of growth-rate domain. *Infection and Immunity* **49**:159-163.

von Heijne, G. (1984) How signal sequences maintain cleavage specificity. *Journal of Molecular Biology* **173**:243-251.

Wachamuth, K. (1986) Molecular epidemiology of bacterial infections: Examples of methodology and of investigations of outbreaks. *Review of Infectious Diseases* **8**:682-692.

Walker, J.E., Saraste, M., Runswick, J.J., Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO Journal* **1**:945-951.

Watts, T.H. and Kay, C.M. (1982) Dissociation and characterization of pilin isolated from *Pseudomonas aeruginosa* strains PAK and PAO. *Canadian Journal of Biochemistry* **60**:867-872.

Watts, T.H., Kay, C.M. and Paranchych, W. (1983) Spectral properties of three quaternary arrangements of *Pseudomonas* pilin. *Biochemistry* **22**:3640-3646.

Whitchurch, C.B., Hobbs, M., Livingston, S.P., Krishnapillai, V. and Mattick, J.S. (1991) Characterization of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialized protein export system widestread in eubacteria. *Gene* **101**:33-44.

White-Ziegler, C.A., Blyn, L.B., Braaten, B.A. and Low, D.A. (1990) Identification of an *Escherichia coli* genetic locus involved in thermoregulation of the *pap* operon. *Journal of Bacteriology* **172**:1775-1782.

Willensen, P.T.J. and De Graaf, F.K. (1993) Multivalent binding of K99 fimbriae to the N-glycolyl-GM_3 ganglioside receptor. *Infection and Immunity* **61**:4518-4522.

Willshaw, G.A., Smith, H.R. and Rowe, B. (1983) Cloning of regions encoding colonization factor antigen 1 and heat-stable enterotoxin in *Escherichia coli*. *Federation of European Microbiological Societies Microbiology Letters* **16**:101-106.

Willshaw, G.A., Smith, H.R., McConnell, M.M. and Rowe, B. (1985) Expression of cloned plasmid regions encoding colonization factor antigen 1 (CFA/1) in *Escherichia coli*. *Plasmid* **13**:8-16.

Yannish-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-119.

VITA

Surname: Clouthier **Given Names:** Sharon Carol

Place of Birth: Brandon, Manitoba, Canada

Educational Institutions Attended:

University of Victoria	1982-1985, 1989-1990, 1991-1995
Malaspina University-College	1986-1988

Degrees Awarded:

B.Sc.	University of Victoria	1990

Honors and Awards:

University of Victoria Fellowship	1991
B.C. Science Council G.R.E.A.T. Grant	1992-1995
William Armstrong Memorial Prize	1994

Publications:

Clouthier, S.C., Müller, K.-H., Doran, J.L., Collinson, S.K. and Kay, W.W. (1993) Characterization of three genes, *sefA, B* and C of *Salmonella enteritidis*. *Journal of Bacteriology* **175**:2523-2533.

Collinson, S.K., Doig, P.C., Doran, J.L., Clouthier, S.C., Trust, T.J. and Kay, W.W. (1993) Thin aggregative fimbriae mediate binding of *Salmonella enteritidis* to fibronectin. *Journal of Bacteriology* **175**:12-18.

Clouthier, S.C., Collinson, S.K. and Kay, W.W. (1994) Unique fimbriae-like structures encoded by *sefD* of the SEF14 fimbrial gene cluster of *Salmonella enteritidis*. *Molecular Microbiology* **12**:893-903.

Doran, J.L. Kay, W.W., Collinson, S.K. and Clouthier, S.C. (1994) Methods and compositions for detection of *Salmonella*. Filed with the US patent office on April 26, 1994. PCT filings in Europe, Japan, Canada, Australia and New Zealand. Form filings in Taiwan, Mexica and Korea.

Kay, W.W., Collinson, S.K., Clouthier, S.C. and Doran, J.L. (1994) Methods and compositions for *Salmonella*-based vaccines. Filed with the US patent office on April 26, 1994. PCT filings in Europe, Japan, Canada, Australia and New Zealand. Form filings in Taiwan, Mexica and Korea.

Collinson, S.K., Doran, J.L., Clouthier, S.C., Sanderson, K.E., Liu, S.-L., Banser, P.A. and Kay, W.W. (1995) Mapping of four fimbrin genes, *agfA*, *fimA*, *sefA* and *sefD*, on the *Salmonella enteritidis* chromosome. *Gene*. Submitted.

Doran, J.L., Collinson, S.K., Clouthier, S.C., Cebulo, T., Koch, W.H., Burian, J., Todd, E.C.D. and Kay, W.W. (1995) *sefA*-based detection of *Salmonella*. *Molecular and Cellular Probes*. Submitted.

Clouthier, S.C. and Kay, W.W. (1995) Characterization of SefA, the subunit of SEF14 fimbriae. In preparation.

Clouthier, S.C. and Kay, W.W. (1995) Analysis of *sefA* transcription in *Salmonella enteritidis*. In preparation.

Collinson, S.K., Clouthier, S.C., Banser, P.A. Doran, J.L. and Kay, W.W. (1995) Genetic analysis of the *S. enteritidis agfBA* operon encoding proteins involved in SEF17 production. In preparation.

PARTIAL COPYRIGHT LICENSE

I hereby grant the right to lend my dissertation to users of the University of Victoria Library, and to make single copies only for such users or in response to a request from the Library of any other university, or similar institution, on its behalf or for one of its users. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by me or a member of the University designated by me. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Title of Dissertation:

CHARACTERIZATION OF THE *sef14* GENE CLUSTER AND THE ENCODED FIMBRIAE

Author:

_____

(Signature)

Sharon C. Clouthier

(Name)

Sept 25, 1996

(Date)