JOURNAL OF BACTERIOLOGY, May 1993, p. 2523–2533
0021-9193/93/092523-11$02.00/0
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Vol. 175, No. 9

Characterization of Three Fimbrial Genes, *sefABC*, of *Salmonella enteritidis*

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Received 3 December 1992/Accepted 12 February 1993

Salmonella enteritidis **produces thin, filamentous fimbriae designated SEF14. A 3.9-kb region of a 5.3-kb fragment encoding genes responsible for SEF14 biosynthesis was sequenced and found to contain three genes,** ***sefABC. sefA*** **encoded a novel fimbrin, the structural subunit of SEF14 fimbriae.** ***sefB*** **and** ***sefC*** **encoded proteins homologous to** ***Escherichia coli*** **and** ***Klebsiella pneumoniae*** **fimbrial periplasmic chaperone proteins and fimbrial outer membrane proteins, respectively, and are the first such genes to be characterized from** ***Salmonella*** **spp. In vitro expression directed by the 5.3-kb DNA fragment identified SefA, SefB, and SefC as approximately 14,000-, 28,000-, and 90,000-M_r proteins, respectively, which correlated with their predicted amino acid sequences.** ***sefB*** **and** ***sefC*** **were not expressed in the absence of** ***sefA*****. Primer extension analysis of** ***sefABC*** **revealed two major transcription start sites located upstream of** ***sefA*****. Transcription of** ***sefBC*** **also initiated from the** ***sefA*** **promotor region. 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*** **indicative of differential regulation of SefA, SefB, and SefC translation.** ***E. coli*** **cells carrying the 5.3-kb DNA fragment of** ***S. enteritidis*** **DNA were unable to assemble distinguishable SEF14 fimbriae; however, immunogold-labelled SEF14 fimbriae were displayed on** ***E. coli*** **clones containing a 44-kb DNA fragment which encompassed the 5.3-kb region. Therefore,** ***sefABC*** **genes make up part of a complex** ***sef*** **operon responsible for the expression and assembly of SEF14 fimbriae.**

Many pathogenic bacteria are capable of invading host cells and subsequently residing as intracellular pathogens (9). This complex process can be viewed as a temporal series of precisely controlled biochemical events, the first of which entails attachment to the host cell surface (15). Various bacterial host cell attachment mechanisms exist; one such strategy found with certain bacteria that infect epithelial cell surfaces is mediated by fimbriae (17).

Fimbriae, also called pili, are proteinaceous, filamentous surface structures primarily composed of helically arranged, identical, protein subunits called fimbrins (30). In some cases, fimbriae also contain additional minor components with specialized functions, such as adhesins (18, 29). Several proteins appear to be involved in the structure, biosynthesis, and assembly of a given fimbrial type (19). Typically, the fimbrial genes which encode these proteins are organized into operons as exemplified by molecular studies of the genes encoding K88ab and K99 fimbriae from porcine and bovine enterotoxigenic *Escherichia coli*, respectively (5), Pap pili from uropathogenic *E. coli* (14), and type 1 fimbriae from *E. coli* (28). Several of these fimbrial operons share fundamental similarities in genetic organization (14, 29). Much less is known about the genetic organization or the functions of the various gene products involved in the biosynthesis of fimbriae from other members of the family *Enterobacteriaceae*, including the important invasive enteropathogens of the genus *Salmonella*.

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The rising pathogen *Salmonella enteritidis* has been recognized as the leading cause of salmonellosis in humans in North America (41) and Europe (11, 33–35). This current pandemic is largely attributed to contamination of poultry and the transovarion infection of eggs (4, 34). One highly virulent, human isolate of *S. enteritidis* produces at least three distinct fimbriae: SEF14 (7, 8), SEF17 (2), and SEF21 (24). Fimbriae analogous to SEF17 have also been found in clinical isolates of *E. coli* (3).

The harboring of a multiplicity of fimbrial types by *S. enteritidis* raises important questions as to their respective function(s), biosynthesis, genetic regulation, and possible role(s) in pathogenesis. Answers to these fundamental questions will lead to a better understanding of the pathogenesis of this organism. In this study, we determined the DNA sequence of three genes from the SEF14 operon of *S. enteritidis* and found that *sefA* encodes a unique fimbrin whereas *sefB* and *sefC* encode genes whose translated proteins share homology with *E. coli* fimbrial chaperone and outer membrane proteins. Moreover, *sefABC* expression was characterized at the level of transcription and translation.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *S. enteritidis* 27655-3b was provided by T. Wadstrom (University of Lund, Lund, Sweden). The *S. enteritidis sefABC* gene cluster present on a 44-kb insert in cos48 (8) was subcloned as a 5.3-kb *Hin*dIII fragment into pTZ19R to create plasmid pKX1 (25). pSC1 is pKX1 with a *Sma*I fragment deleted. *E. coli* HB101 (38) was the host for the

cloning experiments with cos48 (8). *E. coli* XL-1 Blue (Stratagene, La Jolla, Calif.) was the host for delB15, delB23, and delD10, which are pTZ18R derivatives containing sequential deletions of the cloned 5.3-kb fragment of the *S. enteritidis sef* gene cluster (25). Except when noted, bacteria were grown statically in colonization factor antigen broth (6) at 37°C for 48 h or with shaking (250 rpm) at 37°C in Luria broth (37) supplemented with ampicillin to a final concentration of 250 μg/ml. Stock cultures were stored in 15% (vol/vol) glycerol broth at −70°C.

Purification of SEF14 fimbriae. SEF14 fimbriae were isolated as described previously (24) with the following modifications. SEF14 fimbriae were recovered by centrifugation (15,000 × *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.

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 described previously in Müller et al. (24), except that 500 μg of each protein preparation was used for the initial injections and the subsequent boosts.

SDS-PAGE and Western blot analysis. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (20). Proteins were solubilized in SDS sample buffer, 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 (immunoblot) analysis as described previously (2). Briefly, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, Calif.). The membranes were blocked with skim milk buffer and then incubated with antiserum to denatured SEF14 fimbrin followed by an incubation with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. The immunoreactive proteins were visualized following development with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

DNA sequencing and computer analyses. A series of overlapping deletion subclones were created in the recombinant plasmid pKX1, using DNase I as described previously (25). The deletion subclones were sequenced by the dideoxynucleotide chain termination method (39), using T7 DNA polymerase (Promega, Madison, Wis.). Both strands of the DNA were sequenced completely with the reverse primer for the coding strand and internal oligonucleotide primers purchased from ULTRA Diagnostics Corp. (Seattle, Wash.) for the opposite strand.

The programs contained in MacVector (Intelligenetics, Mountainview, Calif.) were used to determine the order of the overlapping sequenced DNA fragments and to analyze the secondary structures within the predicted amino acid sequence of SefB. DNA Stryder was used to identify open reading frames (ORFs). MACAW (NCBI, Bethesda, Md.) was used to construct regions of local similarity among the four fimbrial chaperone proteins. The RNAFOLD program of PC/GENE (Intelligenetics) was used to predict the secondary structures of the RNA sequence. The predicted amino acid sequence for each ORF was compared with proteins listed in the GenBank, SWISS-PROT, and GENPEPT data bases, using the FASTA program (32).

In vitro transcription-translation. Plasmid-encoded proteins were labelled with ^{35}S-methionine, using a cell-free coupled transcription-translation system (Amersham, Oakville, Ontario, Canada). Plasmids carrying either the 5.3-kb fragment of the *sef* operon or deletions thereof were used as DNA templates. The reactions were incubated at 37°C for 30 min and then dialyzed against distilled H_2O for 10 to 15 min prior to SDS-PAGE analysis and autoradiography with Kodak X-OMAT-AR5 film.

RNA extraction. RNA was prepared from whole cells by a modification of the procedure described by McCormick et al. (22). Briefly, cells from 20 to 50 ml of a 48-h static colonization factor antigen broth culture of *E. coli* HB101 carrying cos48 were lysed with a solution containing 1.5 μl of β-mercaptoethanol, 5 μl of RNasin RNase inhibitor (40,000 U/ml; Promega Corp.), and 200 μl of boiling buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.6], 0.5% Triton X-100, 1 mM 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 plus 2 volumes of 95% ethanol at −70°C for 30 min. DNA was degraded with fast protein liquid chromatography-purified DNase I (Pharmacia, Uppsala, Sweden). Approximate RNA recovery was determined spectrophotometrically at 260 nm.

Primer extension of RNA transcripts. Primer extension reactions were performed by the procedure of Sawers and Bock (40), using RNA prepared as above and an oligonucleotide (18 bp) derived from the *sefA* gene sequence (Regional DNA Synthesis Laboratory, Calgary, Alberta, Canada). Three picomoles of the oligonucleotide (16.5 ng of an 18-mer) was end labelled with 50 μCi of [γ-^{32}P]ATP (38), and then the oligonucleotide was used in the primer extension reactions, which were stopped upon addition of 3 volumes of 95% ethanol–3 M sodium acetate to a final concentration of 0.3 M. RNA was precipitated, recovered by centrifugation (14,000 × *g*, 4°C, 30 min), and washed with 1 ml of ice-cold 70% ethanol. The extension products were dried by vacuum centrifugation (Speedvac; Savant, Farmingdale, N.Y.), resuspended in 5 μl of sequencing dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol

FIG. 1. 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 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.

A

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                    20                      40                      60                      80
GGGGATGTTGTGTAAAGATAAAAAAATAGTGATCCTTGTTTTTTTTCTTAAATTTTTAAAATGGCGTGAGTATATTAGCATCC
                    100                     120     sefA        140          -10    *
GCACAGATAAATTGTGCGAATGCTAATAGTTGATTTTTGGAGATTTTGTAAT ATG CGT AAA TCA GCA TCT GCA GTA
*       *       **                         SD       SefA   Met arg lys ser ala ser ala val
160                 180                                 ↓ 200                           220
GCA GTT CTT GCT TTA ATT GCA TGT GGC AGT GCC CAC GCA GCT GGC TTT GTT GGT AAC AAA GCA
ala val leu ala leu ile ala cys gly ser ala his ala ala gly phe val gly asn lys ala
                    240                         260                             280
GTG GTT CAG GCA GCG GTT ACT ATT GCA GCT CAG AAT ACA ACA TCA GCC AAC TGG AGT CAG GAT
val val gln ala ala val thr ile ala ala gln asn thr thr ser ala asn trp ser gln asp
                300                             320                         340
CCT GGC TTT ACA GGG CCT GCT GTT GCT GCT GGT CAG AAA GTT GGT ACT CTC AGC ATT ACT GCT
pro gly phe thr gly pro ala val ala ala gly gln lys val gly thr leu ser ile thr ala
            360                         380                         400
ACT GGT CCA CAT AAC TCA GTA TCT ATT GCA GGT AAA GGG GCT TCG GTA TCT GGT GGT GTA GCC
thr gly pro his asn ser val ser ile ala gly lys gly ala ser val ser gly gly val ala
        420                         440                         460
ACT GTC CCG TTC GTT GAT GGA CAA GGA CAG CCT GTT TTC CGT GGG CGT ATT CAG GGA GCC AAT
thr val pro phe val asp gly gln gly gln pro val phe arg gly arg ile gln gly ala asn
    480                         500                         520
ATT AAT GAC CAA GCA AAT ACT GGA ATT GAC GGG CTT GCA GGT TGG CGA GTT GCC AGC TCT CAA
ile asn asp gln ala asn thr gly ile asp gly leu ala gly trp arg val ala ser ser gln
 540                        560                         580                             600
GAA ACG CTA AAT GTC CCT GTC ACA ACC TTT GGT AAA TCG ACC CTG CCA GCA GGT ACT TTC ACT
glu thr leu asn val pro val thr thr phe gly lys ser thr leu pro ala gly thr phe thr
                                620
GCG ACC TTC TAC GTT CAG CAG TAT CAA AAC TAA
ala thr phe tyr val gln gln tyr gln asn XXX


        640                     660                     680                     700
TTTAATTTAAACTTTATAAATGCCCTCAATATGAGGGCATTTGGATAATTTTATTATTTTAAAAATATCTATTTTGAATAGATA
  720                   740          sefB        760                             780
GGTTTTATGCTTCCATGCAAAAACTTAAAGAGGGATT    ATG TAT ATT TTG AAT AAA TTT ATA CGT AGA ACT
                                 SD     SefB Met tyr ile leu asn lys phe ile arg arg thr
                    800                         820     ↓                       840
GTT ATC TTT TTC TTT TTT TGC TAC CTT CCA ATT GCT TCT TCG GAA AGT AAA AAA ATT GAG CAA
val ile phe phe phe phe cys tyr leu pro ile ala ser ser glu ser lys lys ile glu gln
            860             ↓           880                         900
CCA TTA TTA ACA CAA AAA TAT TAT GGC CTA AGA TTG GGC ACT ACA CGT GTT ATT TAT AAA GAA
pro leu leu thr gln lys tyr tyr gly leu arg leu gly thr thr arg val ile tyr lys glu
        920                     940                     960
GAT GCT CCA TCA ACA AGT TTT TGG ATT ATG AAT GAA AAA GAA TAT CCA ATC CTT GTT CAA ACT
asp ala pro ser thr ser phe trp ile met asn glu lys glu tyr pro ile leu val gln thr
    980                     1000                            1020
CAA GTA TAT AAT GAT GAT AAA TCA TCA AAA GCT CCA TTT ATT GTA ACA CCA CCT ATT TTG AAA
gln val tyr asn asp asp lys ser ser lys ala pro phe ile val thr pro pro ile leu lys
1040                    1060                            1080                            1100
GTT GAA AGT AAT GCG CGA ACA AGA TTG AAG GTA ATA CCA ACA AGT AAT CTA TTC AAT AAA AAT
val glu ser asn ala arg thr arg leu lys val ile pro thr ser asn leu phe asn lys asn
                        1120                            1140                        1160
GAG GAG TCT TTG TAT TGG TTG TGT GTA AAA GGA GTC CCA CCA CTA AAT GAT AAT GAA AGC AAT
glu glu ser leu tyr trp leu cys val lys gly val pro pro leu asn asp asn glu ser asn
                1180                            1200                        1220
AAT AAA AAC AAC ATA ACT ACG AAT CTT AAT GTG AAT GTG GTT ACG AAT AGT TGT ATT AAA TTA
asn lys asn asn ile thr thr asn leu asn val asn val val thr asn ser cys ile lys leu
            1240                        1260                        1280
ATT TAT AGG CCT AAA ACT ATA GAC TTA ACG ACA ATG GAG ATT GCA GAT AAA TTA AAG TTA GAG
ile tyr arg pro lys thr ile asp leu thr thr met glu ile ala asp lys leu lys leu glu
        1300                            1320                        1340
AGA AAA GGA AAT AGT ATA GTT ATA AAG AAT CCA ACA TCA TCA TAT GTG AAT ATT GCA AAT ATT
arg lys gly asn ser ile val ile lys asn pro thr ser ser tyr val asn ile ala asn ile
    1360                        1380                        1400
AAA TCT GGT AAT TTA AGT TTT AAT ATT CCA AAT GGA TAT ATT GAG CCA TTT GGA TAT GCT CAA
lys ser gly asn leu ser phe asn ile pro asn gly tyr ile glu pro phe gly tyr ala gln
 1420                       1440                            1460                            1480
TTA CCT GGT GGA GTA CAT AGT AAA ATA ACT TTG ACT ATT TTG GAT GAT AAC GGC GCT GAA ATT
leu pro gly gly val his ser lys ile thr leu thr ile leu asp asp asn gly ala glu ile

ATA AGA GAT TAT TAG
ile arg asp tyr XXX
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    1500  sefC              1520                                    1540                       1560
TTTAAGGTGTAAACAAATG AAG AAA ACC ACA ATT ACT CTA TTT GTT TTA ACC AGT GTA TTT CAC TCT
    SD  SefC  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 AAG 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 GTT 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 ACT TTT TCT GAA GCA ATT CAT AGT AGC
leu ala ser his tyr tyr tyr ser lys asn tyr arg thr phe ser glu ala ile his ser ser
                  2900                          2920                    2940
GAG CAT GAT GAA TTT TAC GAT AAA AAT AAG AAA TCA ACA ACC TCT ATG TTA TTA AGT CAG GCA
glu his asp glu phe tyr asp lys asn lys lys ser thr thr ser met leu leu ser gln ala
              2960                         2980                         3000
TTA GGA TCT CTG GGT TCT GTT AAC TTA AGC TAC AAT TAT GAT AAA TAT TGG AAA CAT GAA GGT
leu gly ser leu gly ser val asn leu ser tyr asn tyr asp lys tyr trp lys his glu gly
          3020                         3040                         3060
AAA AAA TCA ATA ATT GCT AGT TAT GGG AAG AAT TTA AAT GGT GTT TCG TTA TCG CTT TCA TAT
lys lys ser ile ile ala ser tyr gly lys asn leu asn gly val ser leu ser leu ser tyr
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FIG. 1—*Continued.*

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       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 GGA
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
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B

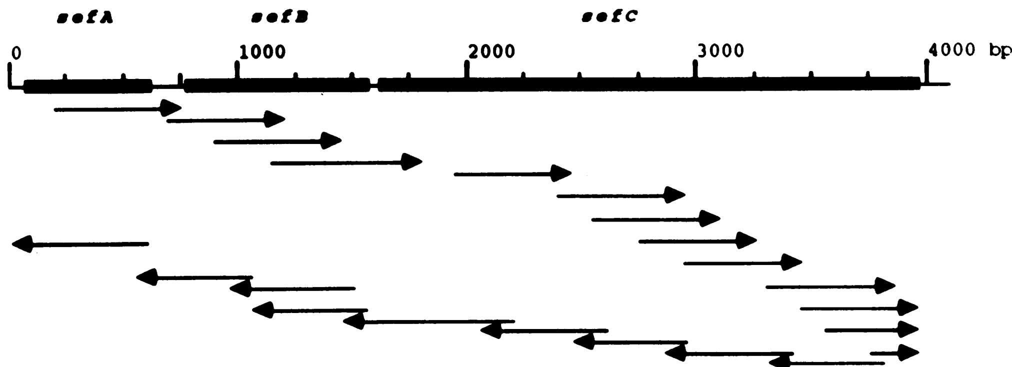

FIG. 1—*Continued.*

FF), and applied to a 6% sequencing gel with *sefA* sequencing reaction mixes in adjacent lanes.

Electron microscopy. SEF14 fimbriae on *S. enteritidis*, *E. coli* JM109 containing pKX1, or *E. coli* HB101 containing cos48 were immunogold labelled and negatively stained as described previously (2).

Nucleotide sequence accession number. The nucleotide sequences reported herein for *sefA*, *-B*, and *-C* have been submitted to GenBank and given accession numbers L11008, L11009, and L11010, respectively.

RESULTS

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*dIII fragment

TABLE 1. Comparison of the predicted amino acid sequence for SefB with those of three fimbrial chaperone proteins

Chaperone protein	Bacterial strain	% Identity[a]	% Similarity[a]	Combined %	Amino acid overlap	Total no. of amino acids	Mol wt (reference)
PapD	*E. coli*	28	47	75	163	239	28,500 (21)
CS3-1	*E. coli*	31	44	75	224	241	27,000 (16)
MrkB	*K. pneumoniae*	27	49	76	171	232	25,000 (1)

[a] Percent identity and similarity refer to the percentage 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).

FIG. 2. 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. (21), Jalajakumari et al. (16), and Allen et al. (1), respectively.

TABLE 2. Comparison of the predicted amino acid sequence for SefC with those of eight fimbrial outer membrane proteins

Protein	Bacterial strain	% Identity[a]	% Similarity[a]	Combined %	Amino acid overlap	Mol wt (reference)
PapC	*E. coli*	23.1	44.1	67.2	745	81,000 (27)
FaeD	*E. coli*	24.0	45.0	69.0	741	82,200 (23)
FanD	*E. coli*	22.4	48.1	70.5	701	84,500 (36)
MrkC	*K. pneumoniae*	26.9	47.3	74.2	714	91,000 (1)
CS3-3	*E. coli*	28.9	46.7	75.6	373	48,000 (16)
CS3-4	*E. coli*	29.3	48.4	77.7	264	33,000 (16)
CS3-5	*E. coli*	34.0	46.7	80.7	145	20,000 (16)
CS3-2	*E. coli*	30.7	63.2	93.9	547	63,000 (16)

[a] Percent identity and similarity refer to the percentage of identical and conserved amino acids, respectively, at the same position in pairwise comparisons of the SefC sequence to each of the other eight proteins listed in release 16 of SWISS-PROT and release 64.3 of GenPept (GenBank, Intelligenetics).

isolated from cos48 (8), cloned into pTZ19, and designated pKX1 (25). 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. 1B). This region was found to contain three ORFs which were designated *sefABC* (Fig. 1A). 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. The *sefABC* determinants were 498, 741, 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 purified previously by Feutrier et al. (7). Moreover, the first 60 amino acids of the predicted SefA sequence were identical to the N-terminal sequence of the purified SEF14 fimbrin (7).

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 fimbrial periplasmic chaperone proteins necessary for the assembly of various fimbriae in *E. coli* and *Klebsiella pneumoniae*, making *sefB* the first chaperone protein gene to be characterized from *Salmonella* species. 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 *K. pneumoniae* (Table 1). 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 six blocks of homology existed among the four fimbrial chaperones (Fig. 2B and C). In SefB, the local similarities corresponded to areas predicted to contain β-sheets and one area predicted to form an α-helix (Fig. 2A). In PapD, the local alignments encompassed the first 10 antiparallel β-sheets found within the two domains of PapD by Holmgren and Bränden (13) and the hydrophobic linker connecting these two domains. In addition, SefC shared homology with 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 2). 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 (10). The most likely cleavage sites for signal peptidase, Ala-21 to Ala-22 (SefA), Ser-24 to Ser-25 (SefB), and Gly-30 to Ser-31 (SefC), were determined by the method of von Heijne (42) (Fig. 1A). However, comparison of the primary structure of SefB and SefC with the primary structure of several other periplasmic chaperones (Fig. 2) and fimbrial outer membrane proteins, respectively, suggests that other putative cleavage sites may exist between Tyr-40 and Gly-41 (SefB) and between Ala-40 and Ser-41 (SefC).

In vitro expression of *sefA*, *-B*, and *-C*. To confirm that *sefABC* encoded proteins of the predicted sizes, proteins were translated in vitro from pKX1. By using a cell-free, coupled transcription-translation system, proteins SefB and SefC were detected as ^{35}S-radiolabelled proteins, whereas

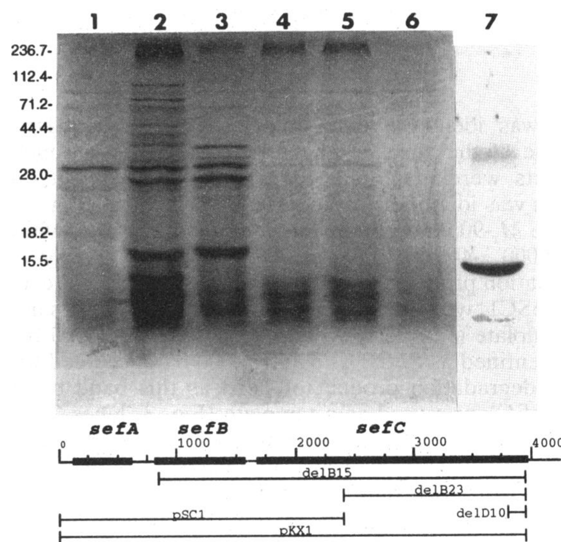

FIG. 3. Expression of the *sefA*, *-B*, and *-C* genes in an *E. coli* in vitro transcription-translation system: autoradiograph of ^{35}S-methionine-labelled polypeptides, separated on a 12.5% acrylamide gel. Lane 1, pTZ19; lane 2, pKX1; lane 3, pSC1; lane 4, delB15; lane 5, delB23; lane 6, delD10; lane 7, Western blot of the in vitro transcription-translation of pKX1 developed with antisera generated against denatured SEF14 fimbrin. The size (10^3) of the molecular weight markers is indicated on the left. Below the autoradiograph is a schematic of the *sef* gene cluster showing the inserts of various deletion subclones used in the in vitro transcription-translation experiments.

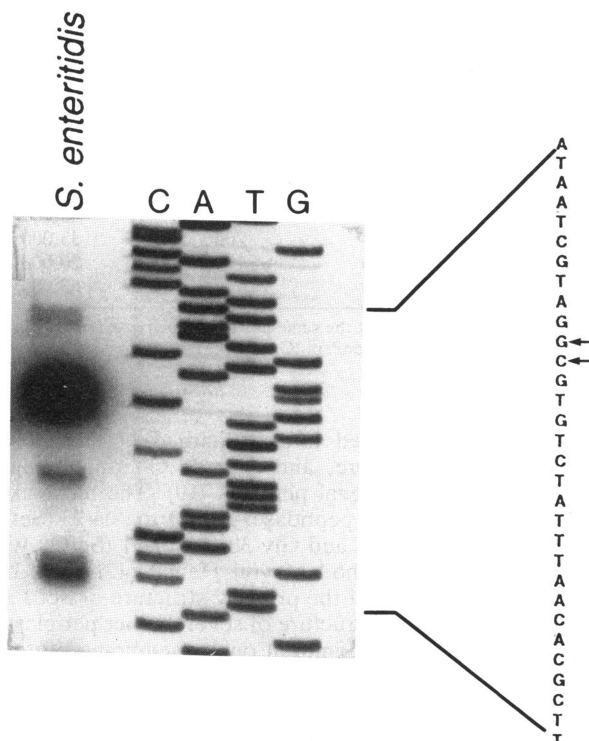

FIG. 4. Mapping of the 5′ end of the *sefA* transcript, using primer extension. The lane labelled *S. enteritidis* represents the reverse transcriptase products of RNA isolated from this organism grown in colonization factor antigen static broth for 60 h at 37°C. Lanes C, A, T, and G represent the results of dideoxy chain termination sequence reactions in the region encompassing the promoter. The sequence of the γ-^{32}P-labelled primer (TGCGTGGGCACTGCC ACA) is complementary to nucleotides 181 to 198 of *sefA*. Arrows indicate the two major transcription initiation sites.

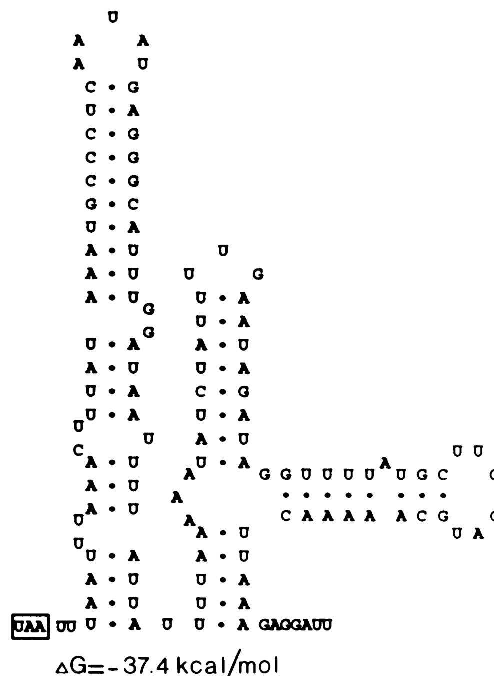

FIG. 5. Schematic representation of the proposed secondary structures within the *sefABC* mRNA. Two stem-loop structures are proposed to form in the intercistronic region of *sefA* and *sefB* mRNA. The *sefA* translation stop codon is boxed. The predicted stability of the stem-loop structures is noted below the schematic.

SefA was identified immunologically on a Western blot because of the absence of methionine. Several translation products were identified (Fig. 3, lane 2). The M_r-14,000 protein was identified on Western blots as SefA (Fig. 3, lane 7). The M_r-90,000 protein was identified as SefC, while the M_r-70,000, -44,000, and -40,000 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. 3, lanes 2 and 3). The M_r-27,000 protein was identified as SefB. The M_r-16,000 band seemed to be a minor degradation product of SefB as this band remained when pSC1 was used as a template (Fig. 3, lanes 2 and 3). When the three DNase I deletion subclones, delB15, delB23, and delD10, were each used as templates, the bands for SefB and SefC and their minor degradation products were absent (Fig. 3, lanes 4 to 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 M_r-14,000, -27,000, and -90,000 proteins were expressed from the 5.3-kb DNA fragment as predicted from the DNA sequence analysis (Fig. 1).

Transcriptional analysis of *sefABC*. To confirm that translation of SefB and SefC was dependent on the presence of *sefA* and/or the region upstream of *sefA*, the transcription start sites for *sefA*, *sefB*, and *sefC* were determined. Primer extension studies consistently revealed transcription start sites immediately upstream of *sefA*. These included two major extension products as well as several minor ones (Fig. 4). When the primer extension reaction was performed at 50°C, a temperature expected to destabilize secondary structures, reverse transcriptase still stopped at all of 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*.

A stable secondary-structure formation was predicted to exist on the 3′ end of the *sefA* mRNA. Bases 636 to 689 (ΔG = −24.6 kcal [ca. −102.9 kJ]/mol) and bases 691 to 746 (ΔG = −12.8 kcal [ca. −53.5 kJ]/mol) were predicted to form a pair of stem-loop structures in the intercistronic region between *sefA* and *sefB* (Fig. 5). Following these putative stem-loop structures was a stretch of 10 uridine residues interrupted by 2 adenine residues (Fig. 1A). Regions unusually rich in adenine and uracil residues existed within these stem-loop structures (bases 679 to 709), reminiscent of RNase E cleavage sites.

Identification of SEF14 fimbriae. Immunogold labelling of *S. enteritidis* cells with polyclonal immune serum raised to the purified SEF14 fimbriae revealed thin, filamentous organelles located on the cell surface (Fig. 6A). *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. 6B). 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. 6C). This result con-

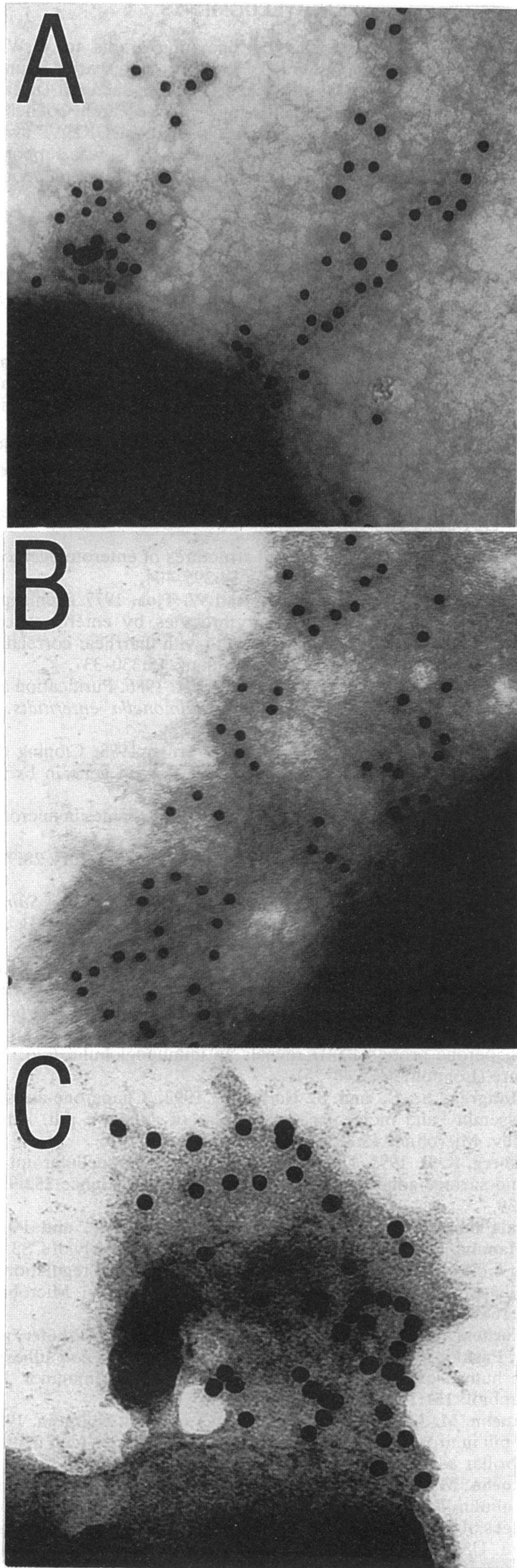

FIG. 6. Immunoelectron microscopy of negatively stained cells for SEF14 production. Cells were grown in 2.5 ml of colonization factor antigen static broth culture for 60 h at 37°C. *S. enteritidis* 27655-3b (A), *E. coli* HB101 containing cos48 (B), and *E. coli* JM109 containing pTZ19 carrying the 5.3-kb *S. enteritidis* fragment (C) were labelled with protein A-gold following incubation with immune serum to native SEF14 fimbriae (2). Ammonium molybdate was used to negatively stain cells (2). Magnification: (A) ×115,000; (B) ×94,000; (C) ×144,000.

firmed 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* genes 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.*

DISCUSSION

The nucleotide sequence of three genes, *sefABC*, from *S. enteritidis* 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 match those reported previously for the SEF14 fimbrin (7). Furthermore, the first 60 predicted amino acids are identical to the N-terminal amino acid sequence reported for the SEF14 fimbrin (7). 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* species. The amino acid sequence and M_r of SefB are very similar to those of three fimbrial 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* (18), 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 antiparallel 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 *sef* 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 (29). 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 (14). Thus, *sefABC* genes make up an important part of the *sef* operon responsible for the expression and assembly of SEF14 fimbriae.

The *sefABC* genes are transcribed as part of a single mRNA transcript. *sefB* and *sefC* were not expressed in the absence of *sefA*, and no transcription start sites or promotors were found immediately upstream of *sefB* or *sefC*. Furthermore, the 5′ ends of several mRNA transcripts were mapped to the region upstream of *sefA*. 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 breathing near the end of the duplex or they could represent variable 5′ ends all clustered within a few nucleotides.

Given that the *sef* operon is transcribed as a single mRNA transcript, a posttranscriptional mechanism must exist which regulates the relative production of the fimbrin, the chaperone protein, and the outer membrane protein. One potential mechanism involves posttranscriptional, RNase E-dependent endonucleolytic cleavage of the primary mRNA transcript at adenine-uracil-rich regions (26). Consistent with this, extensive adenine-uracil-rich sequences are located in the regions flanking the mRNA transcript for *sefA*. Cleavage at these sites would result in discrete mRNA transcripts displaying differential stability to exonucleolytic degradation. Specifically, the *sefA* transcript would 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 (32) and have also been implicated in the lowered expression of distally located genes in multicistronic operons (12). Conversely, the transcripts for *sefB* and *sefC* lacking such stabilizing structures would be more susceptible to degradation. A direct consequence of this posttranscriptional processing would be differential regulation of SefA, SefB, and SefC translation. This would account for the experimentally observed high levels of SefA (data not shown). The relationship between this processing and regulation of the *sef* operon is currently under investigation.

The genetic organization of the *sef* operon is similar to the Fae (23), Fan (5), CS3 (16), and Pap (14) fimbrial operons which are approximately 10 kb in length and consist of 8 to 11 genes. *E. coli* carrying 44 kb of *S. enteritidis* DNA (cos48) encompassing the *sefABC* operon displayed immunogold-labelled SEF14 fimbriae. However, immunogold electron microscopy of *E. coli* carrying the 5.3-kb DNA fragment subcloned from cos48 showed that distinguishable SEF14 filamentous fimbriae were 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 fimbrial length, an adhesin(s), an adaptor, proteins that initiate the assembly of the fimbrial filaments, and regulatory proteins (14).

ACKNOWLEDGMENTS

This project was supported by operating grants to W.W.K. provided by the British Columbia Health Care Research Foundation, the Networks of Centers of Excellence in Bacterial Disease, and The Natural Sciences and Engineering Research Council of Canada (NSERC). S.C.C. was the recipient of a GREAT award from the British Columbia Science Council. S.K.C. was supported by an NSERC Postdoctoral Fellowship, and J.L.D. was supported by an NSERC Industrial Fellowship.

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