Cloning, DNA nucleotide sequence and distribution of the gene encoding the SEF14 fimbrial antigen of *Salmonella enteritidis*

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(Received 13 October 1992; revised 21 January 1993; accepted 24 March 1993)

Monoclonal antibody 69/25, specific for the *Salmonella enteritidis* fimbrial antigen (SEF14), was used to screen a pUC-based *S. enteritidis* gene library and a positive clone was identified. Subcloning experiments demonstrated that a 584 bp *Dra*I DNA fragment was the minimal chromosomal segment capable of directing SEF14 antigen expression. Western blotting of *Escherichia coli* recombinants identified a gene product of M_r 16000 as a precursor to the M_r 14300 mature fimbrial subunit protein. The DNA nucleotide sequence of the *Dra*I fragment was determined and was shown to contain a single open reading frame with two potential f-Met start codons and a hydrophobic signal sequence. Downstream of a putative peptidase cleavage site, the deduced amino acid sequence showed considerable homology with the N-terminal amino acid sequence of what was originally described as the type 1 fimbrial subunit of *Salmonella enteritidis* and later redefined as SEF14. The gene encoding SEF14, designated as *sefA*, was shown to be limited in distribution to *Salmonella blegdam*, *S. dublin*, *S. enteritidis*, *S. gallinarum*, *S. moscow*, *S. pullorum*, *S. rostock*, *S. seremban* and *S. typhi*, all belonging to *Salmonella* group D. However, expression of the SEF14 antigen was limited to *S. dublin*, *S. enteritidis*, *S. moscow* and *S. blegdam*. The nucleotide sequence of the *sefA* gene shared no homology with the *Salmonella fimA* gene encoding type 1 fimbriae, and these genes showed distinct patterns of distribution within salmonellae.

Introduction

Fimbriae are surface appendages found on many species of *Enterobacteriaceae* which often display adhesive properties (Duguid & Old, 1980; Parry & Rooke, 1985; De Graaf & Mooi, 1986). Some fimbriae are considered virulence factors because they initiate adherence, which is the first step in the colonization of host mucosal surfaces (Pearce & Buchanan, 1980; Korhonen *et al.*, 1982; Finlay & Falkow, 1989; Johnson, 1991; Krogfelt, 1991). Fimbriae are classified according to their morphological and cell-binding properties (Duguid & Old, 1980; Pearce & Buchanan, 1980; De Graaf & Mooi, 1986). Type 1 fimbriae are rigid organelles of 7 nm diameter which mediate α -D-mannose-sensitive agglutination (Abraham *et al.*, 1988; Clegg & Gerlach, 1987). These fimbriae are considered ubiquitous amongst the

Enterobacteriaceae (Clegg & Gerlach, 1987; Buchanan *et al.*, 1985). DNA sequence analysis of the type 1 fimbrial antigen genes of *S. typhimurium* (Purcell *et al.*, 1987) indicates a monomeric protein unit of M_r approximately 17000–18000. It is assumed that type 1 fimbriae are likely to display antigenic heterogeneity (Nowotarska & Mulczyk, 1977; Salit & Gotschlich, 1977; Buchanan *et al.*, 1985; Old & Adegbola, 1985; Clegg *et al.*, 1987). Indeed, DNA hybridization studies have shown considerable nucleotide sequence divergence amongst type 1 fimbrial subunit sequences of *Enterobacteriaceae* (Buchanan *et al.*, 1985). Other major classes of fimbriae reported in *Salmonella* are type 2, morphologically similar to type 1 but which do not agglutinate erythrocytes (Duguid *et al.*, 1966), and type 3, flexible structures of 3–5 nm diameter which mediate mannose-resistant haemagglutination (Duguid & Old, 1980). These classes of fimbriae have been described on only a few *Salmonella* serotypes but evidence for their role in pathogenesis is growing (Duguid *et al.*, 1976; Hornick *et al.*, 1991; Krogfelt, 1991; Collinson *et al.*, 1992; Tesh & O'Brien, 1992).

Thorns *et al.* (1990) produced a panel of monoclonal antibodies (mAbs) to *S. enteritidis* using a mouse

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Abbreviations: IEM, immunoelectron microscopy; mAb, monoclonal antibody.

The nucleotide sequence reported in this paper has been submitted to GenBank and has been assigned the accession number L03833.

immunization protocol designed to elicit an antibody response primarily directed against surface antigens. Immunoelectron microscopy demonstrated that one mAb, designated 69/25, bound specifically to a fimbria-like structure, designated SEFA, but not to type 1 fimbriae, which were also present on the cell surface of *S. enteritidis*. Analysis of the fimbrial antigen indicated a fimbrial subunit of M_r 14300 (Thorns *et al.*, 1990). Three discrete fimbrial antigens have been identified on a human-derived isolate of *S. enteritidis*, strain 27655 (Feutrier *et al.*, 1986; Müller *et al.*, 1991; Collison *et al.*, 1991). The structural subunit of one type of fimbriae, of M_r 21000, designated SEF21, shares significant N-terminal amino acid sequence homology with the structural subunit of type 1 fimbriae of *S. typhimurium* (Müller *et al.*, 1991). The two other fimbriae, with structural subunits of M_r approximately 14000 and 17000, were designated SEF14 (Müller *et al.*, 1991) [mistakenly identified as type 1 when first published (Feutrier *et al.*, 1986)] and SEF17 (Collinson *et al.*, 1991, 1992), respectively. The SEF17 fimbrial subunit shares N-terminal amino acid sequence homology with the structural subunits of curli of *Escherichia coli* (Arnqvist *et al.*, 1992).

As a first step toward establishing the relationship of SEFA with other fimbrial antigens within the *Enterobacteriaceae* and as an essential preliminary to determining its biological role, the gene encoding SEFA was to be identified from within an *S. enteritidis* gene library and its DNA nucleotide sequence determined.

Methods

Bacterial strains, plasmids and media. Wild-type *Salmonella* isolates were from the *Salmonella* reference laboratory, Central Veterinary Laboratory, and were stored on Dorset egg slopes. *Escherichia coli* strain DH5 α [F^- *endA1 hsdR17*(r_k^- m_k^+) *supE44 thi-1 λ^- recA1 gyrA96 relA1 Δ (*argF-lacZYA*)U169 ϕ 80*lacZ* Δ M15)] (Life Technologies; Max Efficiency competent cells) was used as the recipient in transformation experiments, following the manufacturer's recommendations. Luria-Bertani (LB) medium was used for growth of *E. coli* recombinants (Maniatis *et al.*, 1982). Supplements added were ampicillin (100 μ g ml $^{-1}$, Pembritin, Beecham) and X-Gal (40 μ g ml $^{-1}$, Life Technologies). *E. coli* strain DS410 (F^- *minA minB thi ara gal xyl mtl tonA rpsL recA*) was the host for minicell experiments. Plasmid pUC18 (Life Technologies) was used as the standard cloning vector.*

DNA extraction and *S. enteritidis* library preparation. Total genomic DNA was extracted from *Salmonella enteritidis* strain 1246/89 using the method of Goldberg & Ohman (1984) and was further purified by CsCl density-gradient centrifugation following standard procedures (Sambrook *et al.*, 1989). For plasmid isolation, the alkaline extraction procedure of Birnboim & Doly, as described by Maniatis *et al.* (1982), was used for all preparations with the exception of the investigations on plasmid profiles of *Salmonella*, where the method of Kado & Liu (1981) was used. Restriction endonucleases and T4 DNA ligase (Life Technologies) were used in accordance with the manufacturer's recommendations. Calf intestinal phosphatase (CIP, Boehringer Mannheim) was used to remove the 5' phosphate from the linearized

vector, when appropriate, following the manufacturer's recommendation. For preparation of the gene library, total genomic DNA from *S. enteritidis* strain 1246/89 was partially digested with *Sau3A* following the methods described by Maniatis *et al.* (1982) and resolved by electrophoresis through low-gelling-temperature agarose (Nuseive, FMC, Rockland, ME, USA) in TAE buffer (40 mM-Tris, 20 mM-sodium acetate, 2 mM-EDTA pH 7.7). An agarose block containing DNA fragments of between 2 kb and 5 kb was excised from the gel and the DNA purified using Prep-A-Gene glass milk (Bio-Rad). The prepared DNA was ligated with vector plasmid pUC18 which was cut with *Bam*HI and dephosphorylated. The 1 kb ladder (Life Technologies) was used as DNA size marker. Ligations were done at room temperature for 16 h. *E. coli* strain DH5 α was used as the recipient for transformations.

DNA sequencing. Double-stranded DNA sequencing was done throughout using Sequenase version 2.0 (United States Biochemical Corporation). For each sequencing reaction, 3 μ g plasmid cDNA was denatured at 37 °C for 30 min in 0.2 M-NaOH, 0.2 mM-EDTA in a final volume of 20 μ l. The solution was neutralized by addition of 8 μ l 5 M-ammonium acetate, pH 7.5, and DNA was precipitated by ethanol. The pellet was redissolved in 4 μ l distilled water. Sequencing reactions were done in accordance with the manufacturer's recommendations. Primers for sequencing were synthesized on an ABI PCR-mate following the maker's recommendations and were designed to give overlapping readings on both strands. The nucleotide sequences obtained were compiled and analysed using Microgenie software (Beckman Instruments) and the University of Wisconsin Genetics Computer Group (UWGCG) package at the SERC laboratory, Daresbury, UK.

Polymerase chain reaction (PCR). The methods of Saiki *et al.* (1985) were followed. *Taq* polymerase was supplied by Perkin-Elmer Cetus (Amplitaq) and dNTPs were supplied by Boehringer. Primers were synthesized as described above. For the amplification of the SEF14 structural subunit gene sequence (bases 13–572) the primers were 5'-GGGAATTCGTATATTAGCATCCGAGA-3' (Psefa1) and 5'-GGGAAGCTTTTGATACTGCTGAACGTAG-3' (Psefa3), with *Eco*RI and *Hind*III restriction sites (underlined). For amplification of the type 1 fimbrial subunit gene (*fimA*), the primers were 5'-TTTGAATTCGTGTAATGCAGCCTGTG-3' (Pfima1) and 5'-TTTGGATCCTAAAGGAGGCGTCGGC-3' (Pfima2) (bases 476–896) (Purcell *et al.*, 1987) with *Eco*RI and *Bam*HI sites to facilitate cloning of the amplified products. Target DNA was prepared from cells harvested by centrifuging 1.5 ml of an overnight culture in LB broth. The pellet was resuspended in 200 μ l 10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA, 150 mM-NaCl, vortexed thoroughly in an equal volume of buffered phenol/chloroform (Maniatis *et al.*, 1982) and centrifuged at 13000 *g* for 5 min. The upper aqueous phase was removed and the DNA precipitated by ethanol. The semipurified DNA pellet was resuspended in 200 μ l 10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA and 1 μ l samples were used for PCR. Saiki buffer was adjusted to 5.5 mM-MgCl $_2$. A thermal cycler (Hybaid, Middlesex, UK) was used and the conditions were 94 °C for 1.5 min (denaturing), 50 °C for 1.5 min (annealing), 72 °C for 2.0 min (extension) for 30 cycles.

Cloning of PCR products. Amplified PCR products were subjected to agarose gel electrophoresis through Nusieve low-gelling-temperature agarose in TAE buffer and visualized after ethidium bromide staining (Maniatis *et al.*, 1982) by UV transillumination (Ultraviolet Product Inc., San-Gabriel, CA, USA). Agarose gel blocks containing DNA products were excised and the DNA purified using Prep-A-Gene glass milk. Eluted DNA was digested to completion by relevant restriction endonucleases. The ligation in vector plasmid pUC18, and transformation into strain DH5 α , were as described above.

Hybridization. Lysis of bacterial colonies and the capillary transfer of DNA species from agarose gels onto filters were done by the methods

described by Maniatis *et al.* (1982). Preparation of radioisotopically labelled probe, hybridization conditions and post-hybridization washes were as described previously (Woodward *et al.*, 1989).

IPTG induction. Overnight cultures grown in Casamino acid broth were diluted 1:10 in fresh medium and incubated at 37 °C for 30 min, before addition of IPTG (20 µg ml⁻¹). After 2 h incubation, cultures were adjusted to an equivalent OD₆₆₀, centrifuged, and the cell pellets were resuspended in loading buffer for PAGE.

Minicell analysis. *E. coli* strain DS410 harbouring plasmids was grown and minicells were prepared from it following the procedure described by Stocker *et al.* (1984). After resuspension in M9 minimal medium containing 30% (v/v) glycerol to OD₆₀₀ 2.0, which corresponds to approximately 2 × 10¹⁰ cells ml⁻¹, purified minicells were stored at -70 °C. For labelling, 100 µl of prepared minicells were preincubated at 37 °C to eliminate mRNA encoding chromosomal gene products. Minicells were labelled with [³⁵S]methionine and the labelling reaction quenched with unlabelled L-methionine as recommended. Labelled cells were boiled in loading buffer (Laemmli, 1970) and polypeptides were separated by SDS-PAGE. Gels were either dried and autoradiographed for 16 h at room temperature or submitted to Western blotting (Towbin *et al.*, 1979) as described below.

Immunoblotting procedure. Proteins from bacterial cultures grown overnight and from minicell preparations were separated on a denaturing 15% (w/v) polyacrylamide gel as described by Laemmli (1970). Proteins were transferred to nylon-supported nitrocellulose membranes (Hybond-C Extra, Amersham) by the method of Towbin *et al.* (1979). Bacterial colonies grown on nitrocellulose membranes were lysed by exposure to a chloroform-saturated atmosphere. Membranes were blocked for 1 h in a solution of TPBS (0.01 M-PBS, pH 7.2, 0.05% Tween 20) with 3% (w/v) gelatin. Development was by an alkaline phosphatase detection system (Bio-Rad) with mAb 69/25 (Thorns *et al.*, 1990) or a monospecific rabbit polyclonal antiserum (Thorns *et al.*, 1992) as the primary antibody.

Immunoelectron microscopy (IEM). The binding of mAb 69/25 and polyclonal rabbit serum specific for the SEF14 fimbrial antigen was as previously described (Thorns *et al.*, 1990) using gold-labelled anti-Ig for detection.

Latex agglutination. Blue latex particles (0.8 µm Estapor, Rhône-Poulenc) were coated with antibodies following the procedure of Hechemy & Michaelson as reported elsewhere (Thorns *et al.*, 1992).

Results

Cloning the SEF14 antigen gene

Total genomic DNA from *S. enteritidis* strain 1246/89 was digested partially with *Sau*3A. Fragments 2–5 kb in size were ligated with *Bam*HI-digested and dephosphorylated pUC18 DNA. The ligation mixture was used to transform *E. coli* strain DH5α. Recombinant colonies were replica-plated onto fresh agar medium and nylon-supported nitrocellulose membranes for immunoscreening using mAb 69/25 specific for the SEFA fimbrial antigen. Of about 2500 recombinants screened, one strain, designated CT15, expressed the SEFA antigen. The recombinant plasmid, designated pVW400, was extracted from strain CT15 and analysed by restriction endonuclease digestion. The insert was de-

termined to be 2.4 kb in size and convenient restriction sites within the cloned fragment were used to create a series of overlapping subclones by ligation with suitably prepared pUC18. The minimum-sized fragment capable of directing synthesis of the SEFA antigen, as determined by immuno-dot blot analysis of recombinant clones, was a 584 bp *Dra*I fragment cloned into the *Sma*I site of pUC18, giving recombinant plasmid pVW406.

Nucleotide sequence analysis of SEF14 antigen gene

The DNA nucleotide sequence of the *Dra*I fragment is given, along with deduced amino acid sequence, in Fig. 1(a). An open reading frame was identified with two potential f-Met start codons (bases 46 and 79, Fig. 1a), giving putative protein products of 176 and 165 amino acid residues, respectively, with respective *M_r* values of 18000 and 16500. A Shine-Dalgarno ribosome-binding consensus sequence, GGAGA, was located at position 65–69, exactly 9 bp upstream of the second ATG start codon (position 79). The first ATG start codon (position 46) was located within a region showing the potential to form a significant hairpin loop secondary structure (positions 16–54) (Fig. 1b). The presence of a potential -10 promoter sequence, TATATTA, at positions 13–19 suggests that this region might be involved in promoting expression of the gene encoding the SEFA antigen, hereafter designated as *sefA*. The DNA sequence was compared with those in the GenBank and EMBL databases but showed no significant homology with any described sequences. However, the deduced amino acid sequence shared significant homology with the N-terminal amino acid sequence of a fimbrial antigen from *S. enteritidis* (Fig. 1a) originally described as type 1 by Feutrier *et al.* (1986) but renamed SEF14 by Müller *et al.* (1991).

Minicell and Western blot analysis of recombinant plasmids

Plasmids pVW406 and pUC18, into which the *sefA* gene was cloned, were transformed in separate experiments into the *E. coli* minicell-producing strain DS410. Polypeptides encoded by these plasmids were labelled with [³⁵S]methionine and compared by SDS-PAGE and autoradiography. No polypeptide with an *M_r* (16500) compatible with that of a deduced *sefA* gene product was observed, although the β-galactosidase major band was absent from the pVW406 experiment but present in the pUC18 control experiment (data not shown). To confirm expression of the SEF14 antigen, minicells containing pVW406 were subjected to Western blot analysis using the SEFA antigen-specific mAb 69/25 as probe. Poly-

Fig. 1. (a) Nucleotide sequence of the 584 bp *Dra*I DNA fragment encoding the SEF14 antigen, with the deduced amino acid sequence of the large open reading frame. The N-terminal amino acid sequence determined chemically by Feutrier *et al.* (1986) is aligned. The two possible ATG f-Met start codons are double-underlined and a region of potential secondary folding is single-underlined. (b) Potential secondary structure of the putative promoter region (1–81) is given along with potential –10 regions (p1 and p2, boxed), ribosome-binding site (rbs) and the two ATG f-Met start codons (double-underlined).

Fig. 2. Western blots of polypeptides expressed in *E. coli* recipients harbouring recombinant plasmids, using mAb 69/25 as a probe to detect SEFA antigen. Lanes: 1, M_r markers (Rainbow, Amersham); 2, *E. coli* DH5 α ; 3, *E. coli* DH5 α (pUC18); 4, *E. coli* DS410 minicells harbouring pVW406; 5, *E. coli* DH5 α (pVW406) induced with IPTG; 6, *E. coli* DH5 α (pVW406), uninduced; 7, *E. coli* DH5 α (pVW400) induced with IPTG; 8, *E. coli* DH5 α (pVW400), uninduced; 9, *S. enteritidis* 1246/89; 10, purified SEF14 fimbriae.

peptides with apparent M_r values of 14300 and 16000 were observed (Fig. 2, lane 4), suggesting the presence of both a mature subunit protein and its precursor in the minicells bearing pVW406. *E. coli* cells harbouring recombinant plasmids pVW400 and pVW406 were grown separately in Casamino acid broth with and without IPTG. Equivalent protein loadings from each culture were analysed by SDS-PAGE. Polypeptides were immunoblotted using mAb 69/25 (Fig. 2). No discernible differences in band intensity were observed. Sequence analysis of plasmids pVW400 and pVW406 (data not shown) demonstrated that the directions of transcription of the *sefA* gene and of the *lacZ* gene of plasmid pUC18 were opposite in plasmid pVW400 but were the same in plasmid pVW406. Sequence analysis of three other derivatives of pUC18 with the *sefA*-bearing *Dra*I fragment cloned into the *Sma*I site, which had been selected on the basis of *lacZ* inactivation and antigen-reactive immuno-dotblots with mAb 69/25, had the *lacZ* and *sefA* genes in the same transcriptional orientation. IEM studies were done to test for the presence of the SEFA antigen on the cell surface of *E. coli* recombinants harbouring pVW400 and pVW406. No labelling was observed in these strains. However, the positive control *S. enteritidis* strain 1246/89 was shown by IEM to produce both the SEFA fimbrial structure described by Thorns *et al.* (1990) and Müller *et al.* (1991), and

fimbriae which did not label with mAb 69/25 which had a typical type 1 morphology.

S. enteritidis encodes type 1 fimbriae homologous with those of *S. typhimurium*

The presence of type 1-like fimbriae on *S. enteritidis* strain 1246/89 raised the question whether the gene encoding the structural subunit of those type 1-like fimbriae was homologous with that of *S. typhimurium*. Furthermore, it was essential to discriminate between the two fimbrial structures at the genetic level. To do this, PCR primers were synthesized using the published *fimA* sequence of the type 1 fimbrial structural subunit gene of *S. typhimurium* strain S850 (Purcell *et al.*, 1987). PCR was used to amplify a 420 bp intragenic fragment from *fimA*. The amplified product was cloned into pUC18 and the DNA nucleotide sequence confirmed as identical with the published sequence. The same primers were used in PCR to amplify the internal fragment from *S. enteritidis* strain 1246/89. The PCR products from two separate experiments were cloned into pUC18 and four individual recombinant plasmids sequenced. Only five base differences were observed when the nucleotide sequences were compared with that of the *S. typhimurium* *fimA* fragment sequence (M. J. Woodward, A. M. Warner & C. Turcotte, unpublished data).

SEFA and type 1 fimbriae genes show distinct distribution patterns in salmonellae

Individual isolates of 74 *Salmonella* serotypes representing 17 serogroups were tested by colony hybridization for the presence of sequences homologous with the *sefA* and *fimA* genes (Table 1). The *Dra*I fragment isolated from pVW400 was radiolabelled and used as a probe in these experiments. Of the 1185 isolates tested, only *S. blegdam*, *S. dublin*, *S. enteritidis*, *S. gallinarum*, *S. moscow*, *S. pullorum*, *S. rostock*, *S. seremban* and *S. typhi*, all belonging to serogroup D, hybridized with the *sefA* probe (Table 1). To probe for type 1 fimbriae, the cloned 420 bp PCR product from *S. typhimurium* was excised from the pUC18 vector, radiolabelled and used under stringent conditions as a gene probe to screen 273 salmonellae representative of 74 serotypes after colony lysis. The probe hybridized with all serotypes tested, supporting the generally held view that the *fimA* gene is ubiquitous among salmonellae.

The interesting distribution of the *sefA* gene could have been related to the presence of a plasmid. Therefore, to test the location of the *sefA* gene, Southern hybridization experiments were done using the *sefA* probe and, as a positive plasmid control, a probe derived from the virulence region of the *S. dublin* 75 kbp plasmid

(Woodward *et al.*, 1989). In separate experiments, each probe was hybridized with Southern transfers of plasmid profiles and of *Eco*RI and *Bgl*II digests of genomic DNA from various *Salmonella* serotypes which hybridized with the *sefA* probe and from *S. typhimurium* as a SEFA fimbriae-negative control. The *sefA* gene probe hybridized only with the genomic DNA and the virulence region DNA probe hybridized only with the virulence plasmids. Interestingly, some restriction fragment length polymorphism was observed for the *sefA* region in the probed chromosomal digests. For example, *Bgl*II digests of genomic DNA from *S. berta*, *S. blegdam*, *S. enteritidis*, *S. gallinarum* and *S. pullorum* gave 18 kb fragments which hybridized with the *sefA* probe, whereas *S. dublin* and *S. rostock* on the one hand and *S. seremban* and *S. typhi* on the other gave hybridizing fragments of 16 kb and 24 kb, respectively.

Not all *sefA*⁺ isolates express the SEFA antigen

Thorns *et al.* (1990) reported that all isolates of *S. enteritidis* and about 90% of *S. dublin* isolates expressed the SEFA antigen at the cell surface. However, the hybridization experiments (Table 1) indicated that other group D serotypes possessed the *sefA*⁺ gene. Agglutination experiments using latex particles coated with mAb 69/25 were done on 311 isolates of group D salmonellae which hybridized with the *sefA* probe. Whilst all *S. blegdam*, *S. enteritidis* and *S. moscow* isolates and 93% of *S. dublin* isolates agglutinated, indicating surface presentation of the antigen, *S. berta*, *S. gallinarum*, *S. pullorum*, *S. rostock*, *S. seremban* and *S. typhi* did not (Table 1). Furthermore, Western blotting with mAb 69/25 and SEFA-monospecific polyclonal antisera of whole-cell lysates of single isolates of *S. berta*, *S. gallinarum*, *S. pullorum*, *S. rostock*, *S. seremban* and *S. typhi* did not give evidence of expression of the SEFA antigen either as a surface or cytoplasmic antigen. IEM confirmed the presence of the SEFA antigen as a fimbria-like structure on single isolates of *S. blegdam* and *S. moscow*, four isolates of *S. enteritidis* and four isolates of *S. dublin* which agglutinated latex particles coated with mAb 69/25.

The *sefA* gene is conserved

It was possible that the SEFA antigen displayed antigenic heterogeneity which might explain the inability to detect antigen expression using the available immunological reagents on some of those isolates which hybridized with the *sefA* gene probe. To test this, PCR was used to amplify the *sefA* gene from single isolates of *S. gallinarum* and *S. pullorum* which were negative in the latex agglutination and Western blotting experiments. For

Table 1. *Salmonella* strains examined by hybridization with *sefA* and *fimA* gene probes and by latex agglutination for surface expression of *sefA* gene product

The results are shown as the number of probe-positive isolates over the number of isolates tested. For *sefA*⁺, the values in parentheses are the number of latex-positive isolates over the number of isolates tested. ND, Not done.

Serogroup	Serotype	<i>sefA</i> [†]	<i>fimA</i> [†]
B	<i>S. agama</i>	0/1	1/1
	<i>S. agona</i>	0/10	1/1
	<i>S. bredeney</i>	0/10	1/1
	<i>S. derby</i>	0/9	1/1
	<i>S. heidelberg</i>	0/11	1/1
	<i>S. indiana</i>	0/1	1/1
	<i>S. reading</i>	0/2	1/1
	<i>S. schwarzengrund</i>	0/1	1/1
	<i>S. stanley</i>	0/5	1/1
	<i>S. typhimurium</i>	0/264	64/64
C1	<i>S. bareilly</i>	0/1	1/1
	<i>S. infantis</i>	0/12	1/1
	<i>S. lille</i>	0/1	1/1
	<i>S. livingstone</i>	0/9	1/1
	<i>S. mbandaka</i>	0/9	1/1
	<i>S. montevidео</i>	0/8	1/1
	<i>S. ohio</i>	0/6	1/1
	<i>S. oranienburg</i>	0/1	1/1
	<i>S. oslo</i>	0/1	1/1
	<i>S. tennessee</i>	0/6	1/1
	<i>S. thompson</i>	0/4	1/1
	<i>S. virchow</i>	0/31	24/24
C2	<i>S. goldcoast</i>	0/1	1/1
	<i>S. hadar</i>	0/9	1/1
	<i>S. newport</i>	0/10	1/1
C3	<i>S. albany</i>	0/1	1/1
	<i>S. kentucky</i>	0/2	1/1
	<i>S. tado</i>	0/1	1/1
D1	<i>S. berta</i>	1/1 (0/1)	1/1
	<i>S. blegdam</i>	1/1 (1/1)	ND
	<i>S. canastel</i>	0/1	1/1
	<i>S. dublin</i>	216/216 (93/100)	31/31
	<i>S. durban</i>	0/1	1/1
	<i>S. eastbourne</i>	0/1	ND
	<i>S. enteritidis</i>	382/382 (150/150)	52/52
	<i>S. gallinarum</i>	44/44 (0/44)	42/42
	<i>S. kapemba</i>	0/1	ND
	<i>S. miami</i>	0/1	ND
	<i>S. moscow</i>	1/1	1/1
	<i>S. napoli</i>	0/1	ND
	<i>S. panama</i>	0/1	ND
	<i>S. pullorum</i>	12/12 (0/12)	3/3
	<i>S. rostock</i>	1/1 (0/1)	1/1
	<i>S. seremban</i>	1/1 (0/1)	ND
	<i>S. typhi</i>	1/1 (0/1)	ND
	<i>S. wangata</i>	0/1	1/1
D2	<i>S. fresno</i>	0/1	ND
	<i>S. ouakam</i>	0/1	1/1
	<i>S. plymouth</i>	0/1	ND
	<i>S. strasbourg</i>	0/1	ND
E1	<i>S. anatum</i>	0/9	1/1
	<i>S. give</i>	0/1	1/1
	<i>S. lexington</i>	0/1	1/1
	<i>S. london</i>	0/1	1/1
	<i>S. meleagridis</i>	0/9	1/1

Table 1. (cont.)

Serogroup	Serotype	<i>sefA</i> [†]	<i>fimA</i> [†]
E2	<i>S. nchanga</i>	0/1	1/1
	<i>S. orion</i>	0/7	1/1
	<i>S. binza</i>	0/9	1/1
	<i>S. drypool</i>	0/1	1/1
	<i>S. manila</i>	0/1	1/1
E3	<i>S. newington</i>	0/1	1/1
	<i>S. thomasville</i>	0/8	1/1
E4	<i>S. senftenberg</i>	0/9	1/1
	<i>S. taksony</i>	0/9	1/1
F	<i>S. aberdeen</i>	0/1	1/1
G1	<i>S. havana</i>	0/1	1/1
G2	<i>S. ajiobo</i>	0/1	1/1
	<i>S. kedougou</i>	0/10	1/1
	<i>S. worthington</i>	0/1	1/1
K	<i>S. cerro</i>	0/1	1/1
N	<i>S. urbana</i>	0/1	1/1
O	<i>S. adelaide</i>	0/1	1/1
	<i>S. ealing</i>	0/1	1/1
T	<i>S. gera</i>	0/1	1/1

each isolate, PCR using primers Psefa1 and Psefa3 was carried out on a separate occasion to avoid the possibility of cross-contamination. *S. typhimurium* was used as a negative control. PCR-amplified DNA products of 560 bp were obtained from both *S. gallinarum* and *S. pullorum* but not *S. typhimurium*. The 560 bp DNA products derived from *S. gallinarum* and *S. pullorum* were cloned into pUC18 (see Methods), again in separate experiments performed on different occasions, and recombinant clones were identified by colony blot hybridization using the *DraI* *sefA* gene probe. From each of the two cloning experiments, plasmid DNA from three individual recombinants was extracted and the DNA nucleotide sequences of the cloned PCR inserts were determined by double-stranded sequencing. The six DNA sequences, three from each of the serotypes tested, *S. gallinarum* and *S. pullorum*, were identical to the sequence determined for *sefA* in pVW406. Similar experiments using one SEFA-expressing and one SEFA-non-expressing *S. dublin* as templates for PCR also gave identical *sefA* DNA nucleotide sequences.

Discussion

The mAb 69/25 described by Thorns *et al.* (1990) was used to detect expression of the gene encoding the SEF14 antigen from within a *S. enteritidis* pUC gene library. Nucleotide sequence analysis of the minimum DNA fragment allowing expression of the antigen (plasmid

pVW406) identified an open reading frame with two potential f-Met translational start codons, both in the same frame, yielding theoretical translation products of M_r 18000 and 16500. Western blot analysis of minicells harbouring pVW406 gave clear evidence for post-translational processing because two polypeptide products of about M_r 16000 and M_r 14300 were detected, the latter having the same M_r as the mature fimbrial subunit protein isolated from *S. enteritidis*. It thus seems likely that the precursor form of the SEFA subunit arises by translation from the second f-Met codon. The lack of ^{35}S incorporation into the polypeptides in the minicell experiments may reflect the presence of the single N-terminal methionine residue which was likely to be cleaved early during processing.

The location of a Shine-Dalgarno site exactly 9 bp upstream of the second f-Met start codon supports the hypothesis that this was the translational start codon. As with other secreted proteins, the deduced N-terminal sequence was hydrophobic, a property common for signal sequences (Silhavy *et al.*, 1983). A number of potential peptidase cleavage sites (Stormo, 1986; Oliver, 1987) were present. An Ala-X-Ala peptidase cleavage motif (Oliver, 1987) was observed in the deduced amino acid sequence immediately before a N-terminal amino acid sequence determined chemically by Feutrier *et al.* (1986). The recombinant SEFA antigen had the same M_r as the native antigen either extracted biochemically or obtained from cell lysis of *S. enteritidis*, suggesting that post-translational processing in *E. coli* and *S. enteritidis* was the same. Our results confirm and add to the information obtained by Feutrier *et al.* (1986) and more recently Müller *et al.* (1991) concerning the nature of this *S. enteritidis* fimbrial antigen; hereafter we refer to the antigen as SEF14 following the nomenclature of Müller *et al.* (1991).

The screening of the *S. enteritidis* pUC gene library with mAb 69/25 was successful because the antigen was expressed in *E. coli*. Sequences upstream of the *sefA* gene in pVW400 contained potential RNA polymerase -10 sites, although no -35 consensus site was identified. Expression appeared to be independent of the inducible *lacZ* system, suggesting that expression of *sefA* was regulated by its own promoter at least in pVW400. Sequences homologous with other known regulatory regions were not identified. SEF14 antigen expression in *S. enteritidis* is dependent upon a number of environmental factors (Thorns *et al.*, 1992), in common with expression of other well-characterized fimbriae (De Graaf *et al.*, 1980; Van Verseveld *et al.*, 1985; De Graaf & Mooi, 1986). A region of significant secondary structure overlapping one potential -10 site was observed. Interestingly, similar 'hairpin' regulatory sequences have been described for other fimbrial antigen

genes (Klemm, 1984; De Graaf & Mooi, 1986). This structure may be involved in *sefA* gene regulation or it may be a rho-dependent stop signal (Yagger & Von Hippel, 1987) of the preceding gene. Without further upstream DNA nucleotide sequence and transcription studies, comments on regulation of *sefA* expression would be speculative. Expression of SEF14 antigen from pVW400 may have been driven by spurious promoter sites in the pUC vector. In pVW406, however, expression of *sefA* should have been dependent on the *lacZ* promoter of pUC18, although no clear evidence for this was gained by IPTG induction experiments.

The limited distribution of *sefA* within group D salmonellae is interesting. Southern hybridization data indicated a chromosomal location for *sefA*, suggesting that the limited distribution pattern was not primarily a consequence of the presence or absence of a plasmid. The apparent inability of some strains to express the antigen as a surface structure, assuming that latex agglutination is an adequate means of testing this, raises questions as to the genetic and physiological competence of these strains to do so. That *E. coli* recombinants did not express the antigen on the cell surface, as tested by IEM, was not unexpected because they probably lacked ancillary genes essential for biogenesis, as described for other fimbrial antigens within the *Enterobacteriaceae* (Orndorff & Falkow, 1984; De Graaf & Klaasen, 1986; Klemm & Christiansen, 1990). Expression of the SEF14 antigen as a surface structure was detected only in *S. enteritidis*, *S. dublin*, *S. blegdam* and *S. moscow*, although the *sefA* gene was present in other group D salmonellae. The nucleotide sequence of *sefA* homologues in individual isolates of *S. dublin*, *S. gallinarum* and *S. pullorum* which did not express the SEF14 antigen was identical to that of *S. enteritidis*, suggesting that lack of antigen detection was not a result of antigenic heterogeneity. In latex agglutination tests with *S. dublin*, 93 out of 100 isolates gave the typical positive reaction seen with *S. enteritidis*. It is possible that the seven non-reacting isolates did not produce the antigen or that its detection was masked by the presence of capsular antigen. In IEM of *S. enteritidis* not all bacteria labelled with mAb 69/25, which suggested that not all cells in a population express the SEF14 antigen. Further genetic analysis is required to understand why certain serotypes of group D salmonellae possessed *sefA* but failed to express SEF14 antigen either as a cytoplasmic antigen or as a fimbrial structure and to understand what modulates antigen expression.

Questions arise as to the significance of the SEF14 antigen in the biology of *S. enteritidis*, particularly whether it may play a role as a virulence factor. Insertion mutants unable to express SEF14 fimbriae may help to answer some of these questions.

The authors wish to acknowledge helpful discussion with C. J. Thorns and Professor I. Smith, technical assistance from Anne Warner and Ian McLaren, the help of Mr A. C. Scott with the electron microscope work, and Dr G. Dougan for the gift of *S. typhi* DNA. C. T. is a visiting fellow from the Ministry of Agriculture, Animal Diseases Research Institute, PO Box 11300, Station 'H', Nepean, Ontario K2H 8P9, Canada.

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