Cloning of determinants encoding F165<sub>f</sub> fimbriae from porcine septicaemic Escherichia coli confirms their identity as F1C fimbriae

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Cloning of the f165<sub>a</sub> operon that encodes F1C-like fimbriae in Escherichia coli indicates that this operon is a member of the S/Foc family. The genetic determinant coding for the F165<sub>f</sub> fimbriae was cloned from the chromosome of the porcine E. coli wild-type strain 4787 (O115:K<sup>-</sup>:H51:F165). The cloned F165<sub>f</sub> and the wild-type operon expressed a major fimbrial protein subunit of molecular mass 17.2 kDa that was detected by anti-F165 and anti-F1C polyclonal sera. The sequences of the f165<sub>a</sub> and f165<sub>FGH</sub> genes are reported. Major subunit gene f165<sub>a</sub> encodes a mature protein of 156 amino acids. Minor subunit genes F, G and H encode mature proteins of 148, 145 and 276 amino acids, respectively. The amino acid sequences of the four proteins share similarities with those of the known S and F1C fimbrial antigens that are produced by extraintestinal E. coli which is associated with sepsis, urinary tract infections and newborn meningitis. The F165<sub>a</sub> protein was identical to the major subunit protein of F1C, with a difference only at the first position. It was also similar, to a lesser extent, to the major subunit proteins of Sfa1 and Sfa1 fimbriae. F165<sub>f</sub>F was identical to FocF and SfaG/Sfa1G. F165<sub>G</sub> was more closely related to FocG than to Sfa1/Sfa1G, and F165<sub>c</sub>H was more closely related to FocH than to Sfa1H/Sfa1H.

Keywords: extraintestinal Escherichia coli, f165<sub>a</sub> fimbrial operon, fimbrial subunit sequences

INTRODUCTION

Fimbrial antigen F165, which exhibits mannose-resistant haemagglutination (MRHA) of sheep, pig and human type A erythrocytes is produced by Escherichia coli O115 isolates causing septicaemia in young pigs (Fairbrother et al., 1993). In septicaemic E. coli strain 4787 (O115:K<sup>-</sup>:H51), F165 fimbrial antigen complex consists of two fimbrial components, F165<sub>a</sub> and F165<sub>c</sub>, of approximately 18.5 and 17.2 kDa respectively (Fairbrother et al., 1986, 1988; Dubreuil & Fairbrother, 1992). It was suggested that F165 could contribute to the pathogenicity of these isolates (Fairbrother et al., 1989; 1993). The cloned f165<sub>a</sub> operon from strain 4787 expresses a major fimbrial subunit of 18.5 kDa (Harel et al., 1992a). The genetic determinant of fimbrial antigen F165<sub>a</sub> belongs to the P fimbrial class. The f165<sub>c</sub> operon encodes an adhesin whose MRHA and receptor binding properties are similar to those of a Psr-like adhesin recognizing the Forssman antigen of sheep erythrocytes (Harel et al., 1992a; Maiti et al., 1993). TnphoA mutagenesis studies have demonstrated that the presence of the F165<sub>c</sub> fimbrial system is required for complete pathogenicity of at least one F165-positive strain in germ-free pigs (Harel et al., 1992b; Ngeleka et al., 1993). A second fimbrial component of 17.2 kDa, from E. coli 4787 was purified and was named F165<sub>f</sub> (Dubreuil & Fairbrother, 1992). For the first 33 amino acid residues, the F165<sub>f</sub> sequence was identical to that of F1B and very similar to that of F1C fimbriae associated with E. coli isolates from urinary tract infections (UTI) in humans (Klemm et al., 1982; van Die et al., 1984; Dubreuil & Fairbrother, 1992).

Abbreviations: MRHA, mannose-resistant haemagglutination; UTI, urinary tract infection.

The GenBank accession numbers for the sequences reported in this paper are U09857 and U09804 for f165<sub>a</sub> and f165<sub>FGH</sub>, respectively.
Fimbrial adhesins produced by extraintestinal *E. coli*, associated with diseases such as UTI sepsis, and newborn meningitis, are important surface antigens and mediate attachment to eukaryotic epithelial cells (Ørskov & Ørskov, 1983; Hacker, 1990; Tennent et al., 1990; Johnson, 1991). Commonly found fimbriae in human extraintestinal *E. coli* are P, S, M and X (Korhonen et al., 1985; Labigne-Roussel & Falkow, 1988). S fimbrial adhesin (Sfa), which is associated to a larger extent with strains causing newborn meningitis and less often with uropathogenic strains, interacts with α-sialyl-(2–3)-β-D-Gal-containing receptors and agglutinates bovine erythrocytes (Hacker et al., 1985). Another type of fimbria, FIC, is associated with UTI (Riegelman et al., 1990). FIC and S fimbriae belong to the same family of fimbrial adhesins, related genetically, immunologically and functionally (Marre et al., 1990; Ott et al., 1988; Riegelman et al., 1990). Although FIC fimbriae lack any haemagglutinating activity, they are able to bind to uropathelial cells. In both *sfa* and *foc* gene clusters, which encode the S and FIC fimbriae respectively, the distal parts encode minor fimbrial subunits (van Die et al., 1991; Hacker, 1985; Schmoll et al., 1989; Riegelman et al., 1990). For the S fimbriae, the 15 kDa protein SfaS is the adhesin that binds to carbohydrate chains terminating with α-sialyl-(2–3)-β-D-Gal-R (Parkkinen et al., 1986; Moch et al., 1987; Morschhäuser et al., 1990), whereas the adhesin of FIC fimbriae is not known. FIC fimbriae as well as S-fimbriated adhesions were shown to mediate binding to renal tubular cell lines (Virkola et al., 1988; Marre et al., 1990). Sfr (for S/FIC related) fimbriae show serological cross-reactivity with S fimbriae and FIC, but fail to exhibit S-specific binding properties (Pawelzik et al., 1988).

The study reports the cloning of a genomic DNA fragment required for the expression of F165 fimbriae, one of the fimbrial components of F165 complex, from a septicaemic porcine F165-positive *E. coli* O115 strain. We also report the nucleotide sequences encoding the major A subunit and minor F, G, and H subunits of F165, and the relationship of this fimbrial antigen, which is expressed by *E. coli* causing septicaemia in pigs, with members of the S/FIC family, which is expressed by *E. coli* causing UTI and newborn meningitis in humans.

**METHODS**

**Bacterial strains and plasmids.** *E. coli* 4787 is a wild-type strain (O115: V165: H51: F165) isolated from the intestinal contents of a diarrhoeic piglet. Strain 4787 was shown to contain P- and S-related nucleotide sequences (Harel et al., 1984), by injecting 10 µg aliquots of purified proteins into rabbits as described by Harel et al. (1992a).

**Cosmid cloning.** Genomic DNA from *E. coli* 4787 was partially cleaved with *Sal*I and ligated into the BamHI site of cosmid vector pMMB33 DNA digested with BamHI, HpaI and SmaI (Frey et al., 1983). Cosmids were packaged into phage λ particles and used to infect *E. coli* HB101.

**Southern hybridization and autoradiography.** Transfer of DNA fragments from agarose gels to nitrocellulose filters, washing and autoradiography were carried out as described by Southern (1975) and Harel et al. (1992a). Stringent conditions were used for the washing procedure. Colony hybridizations were carried out as described by Harel et al. (1991).

**Haemagglutination.** MRHA was carried out essentially as described by Fairbrother et al. (1988) using human A, P, porcine, bovine and ovine erythrocytes.

**Immunoelectron microscopy.** A drop of a washed bacterial suspension was placed on Formvar-coated grids for 1 min. The grids were then placed sequentially on drops of PBS with 1% (w/v) ovalbumin for 5 min and a suitable dilution of rabbit anti-FIC serum for 30 min. Grids were washed and placed on drops of anti-rabbit immunoglobulin with 10 nm gold particles (Sigma) for 30 min. After a final washing step, the grids were stained with 1% (w/v) phosphotungstate (pH 7.0) and examined with an electron microscope (Philip 201) at an accelerating voltage of 60 kV (Harel et al., 1992a).

**Isolation of fimbriae, PAGE and immunoblotting.** Crude fimbrial extracts were prepared from bacteria grown on MD-1
plates as described previously (Harel et al., 1992b). After boiling the fimbrial samples for 5 min in buffer [10 mM Tris/HCl (pH 7.8)] containing 4% (w/v) SDS, 0.01 ml β-mercapto-ethanol, 0.2 ml glycerol and 0.002% bromophenol blue, the samples were run on slab gels as described previously (Harel et al., 1992a). Western blotting (immunoblotting) was carried out according to the method of Towbin et al. (1979). Crude fimbrial extracts from wild-type E. coli 4787, E. coli HB101(pIVA4; cosmid positive for F165, expression), and HB101(pPIL110-51) were electrophoresed on SDS-polyacrylamide gels. Following transfer to nitrocellulose filters, the electrophoresed preparations were reacted with anti-F165 fimbrial complex and anti-FIC fimbriae sera to identify the fimbrial bands.

Preparation of minicells and analysis of plasmid-encoded proteins. Plasmid-encoded proteins were expressed in E. coli DS910 minicells, which were prepared by sucrose density centrifugation (Pugsley & Oudega, 1987). Purified minicells were labelled with 50 μCi (1.85 MBq) of [35S]methionine (DuPont). After being labelled for 1 h at 37 °C, proteins from minicells were acetone-precipitated and resuspended in 100 μl sample buffer (Labigne-Roussel et al., 1984). Samples (10⁶ c.p.m.) were loaded on 12% (w/v) gels for SDS-PAGE. The gel was fixed, treated with Enhance (DuPont) and dried. It was then exposed on a XAR-5 film with an intensifying screen at -70 °C.

RESULTS AND DISCUSSION

Cloning of the F165, fimbriae determinant

Genomic DNA was isolated from the E. coli 4787 possessing the F165 fimbrial complex. After cleavage with Sau3A and a packaging procedure, 600 E. coli HB101 colonies were screened for the components of the F165 complex by the immunocolony detection technique. One of the recombinant clones harbouring a cosmid, designated pIVA4, that was found to react with F165 complex antiserum, was also sfa-positive by colony hybridization using an sfa probe derived as a 0.8 kb PstI fragment from pILL1007. The expression by this clone of fimbriae reacting with F165 antiserum was confirmed by the isolation of fimbriae and by subsequent SDS-PAGE and immunoblotting with F165 antiserum. The absence of MRHA properties suggested the presence of foc-like, rather than sfa, coding genes.

The pIVA4 DNA was further cleaved with BamHI, and suitable fragments were ligated into the BamHI site of pACYC184. A recombinant plasmid, pYvan, conferred on strain HB101 expression of fimbriae as demonstrated by electron microscopy, but without specific haemagglutination (Fig. 1). Because of their immunological properties, and their absence of haemagglutination properties, the clones derived from the sfa-positive cosmid were thus described as foc-like clones. The structures could be identified as F165, fimbriae by immunogold labelling experiments using anti-FIC serum (Fig. 1).

Restriction map of pYvan and characterization of the F165, fimbriae

The recombinant plasmid pYvan consisted of the vector pACYC184 and a chromosomal insert of 13 kb. The physical map of pYvan was further determined by digestion with different restriction enzymes. The results indicated that the f165 gene cluster is physically related to both foc and sfa gene clusters (data not shown) (van Die et al., 1984; Hacker et al., 1985, 1993; Ott et al., 1986;
Pawlzik et al., 1988; Schmoll et al., 1990). pYvan encodes at least five different proteins and showed a pattern similar to that of pPIL110-51 which expresses fimbriae F1C which had additional bands of 17, 20, 30 and 100 kDa (Fig. 2, lanes A and B) in comparison with the vector pACYCl84 alone (Fig. 2, lane C). The 17.2 kDa fimbrillin subunit molecule was not labelled since the mature protein does not contain methionine. Some of these proteins are probably involved in transport and biogenesis of F165,, as already demonstrated for several other fimbriae (Tennent et al., 1990).

Immunoblotting of the fimbrial extracts using anti-F165 complex serum revealed two bands, of 18.5 kDa and 17.2 kDa, in parent strain 4787, but only one band of 17.2 kDa in subclone HB101(pYvan) (Fig. 3). Moreover, antiserum directed against F1 C fimbrial extract from HB101(pPIL110-51) recognizes only one band of 17.2 kDa in the fimbrial extracts from wild-type strain 4787, HB101(pYvan) and HB101(pPIL110-51) (Fig. 3). The second band of 18.5 kDa, visible in fimbrial extracts of E. coli 4787 examined with F165 complex antiserum, is the major protein subunit of F165, a Prs-like fimbria (Harel et al., 1991; Maiti et al., 1994). The size of F165 major fimbrial protein is similar to that of the major fimbrial proteins of the S/FIC fimbriae (16–17 kDa). The similarities between the latter subunit proteins have also been demonstrated by serological cross-reactions using anti-F1C serum (Pawlzik et al., 1988).

**Nucleotide sequence of f165,A and f165,F,G,H**

The complete nucleotide sequence of the f165,A gene was determined. This sequence contains a single ORF of 546 bp which encodes a protein of 180 amino acids (Fig. 4). A signal peptide of 24 amino acids is cleaved from the precursor protein to generate the mature F165,A protein of 168 kDa (data not shown). The signal peptide is hydrophobic and has complete sequence similarity with that found in F1C fimbriae (van Die et al., 1984). The f165,A sequence showed an overall 98.9% identity with focA gene (data not shown).

Physical mapping of the f165,A operon showed that the three genes f165,F, G and H were located at the 3' end of the operon. Thus, a fragment carrying the three genes was subcloned to generate the plasmid pF165,F,G,H. An ORF for each of the F (528 nt), G (504 nt), and H (900 nt) genes has been identified. Sequences of f165,F, G and H shared an overall 97.9% identity with those of focF, G, H (van Die et al., 1991). The sequences f165,F/focF and sfaG were found to be identical. Although f165,G and focG were almost identical, they differed greatly from sfaS.

There was only one silent difference between focG and f165,G. Similarly, f165,H and focH differed greatly from sfaH.

Codon usage for f165,A is compatible with genes normally present in E. coli as reported for fimbriae by van

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**Fig. 3.** Western blot of fimbrial subunits from different E. coli strains using (a) anti-F1C serum produced from clone pPIL110-51 and (b) anti-F165 serum produced from strain 4787. Lane A, HB101(pYvan); lane B, HB101(pPIL110-51); lane C, strain 4787.

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**Fig. 4.** Comparison of the deduced amino acid sequence of the major fimbrial subunit protein F165,A with that of FocA, SfaIIA and SfaA fimbriae. The GenBank accession number for amino acids sequences of FocA, SfaIIA and SfaA are M13053, 553211, M35273, respectively (van Die et al., 1984; Hacker et al., 1993; Schmoll et al., 1987). The first amino acid of the mature protein is numbered +1. Blank spaces were introduced to obtain maximum similarity. Amino acid identities with the F165,A sequence are indicated by hyphens.
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**Comparison of F165, major and minor subunit proteins with S/Foc subunit proteins**

The deduced amino acid sequence of the mature F165A major fimbrial subunit gene was compared with the corresponding sequences of other S-like fimbriae (Fig. 4).

F165A protein was almost identical to the corresponding F1C protein and demonstrated less similarity to Sfa, SfAI and F1A (Klemm, 1984; van Die et al., 1984; Schmoll et al., 1987; Hacker et al., 1993). At the amino acid level, only one change was observed in comparison to F1C, in the N-terminal region at position +1 (alanine for valine) (Fig. 4). As shown for many fimbriae, four regions could be distinguished in the F165A protein (Fig. 4): the N-terminal region, the Cys–Cys loop, a variable region, and

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Die et al. (1986). The GC contents of the f165, F, G and H genes were 45, 41.0 and 51 mol % respectively.

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**Fig. 5.** Comparisons of the predicted amino acid sequences among F165,F, FGH and other FocFGH/SfaGSH sequences. The GenBank accession number for amino acids sequences of FocFGH, SfaGSH, are M568237, 553210, and X16664, respectively (van Die et al., 1991; Schmoll et al., 1989; Hacker et al., 1993). (a) F165,F/FocF/SfaG proteins (b) F165,G/FocG/SfaG proteins and (c) F165,H/FocH/SfaH proteins. Blank spaces were introduced to obtain maximum similarity. Amino acid identities with the F165,F sequences are indicated by hyphens.

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the C-terminal region. The N- and C-terminal regions of the major fimbrial protein subunits of the S/F1C family and F165α are very homologous and consist of mainly hydrophobic amino acids (Fig. 4). Most differences were observed in two regions, the Cys-Cys loop and the variable regions, which are probably responsible for the divergence of F165αA and FocA from SfaA (Fig. 4) (van Die et al., 1984, 1986; Schmoll et al., 1987; Hacker et al., 1993).

The deduced amino acid sequences of the three genes of the putative minor fimbrial subunits of F165α revealed three precursor proteins, F, G, and H, which have 175, 167 and 299 amino acids, respectively. Each of the three proteins possessed the typical amino terminal signal sequence which is hydrophobic (Fig. 5). The three mature proteins of 148, 145, and 276 amino acids, representing F165αF, G, and H, respectively, had calculated molecular masses of 15.5, 15.1 and 29.5 kDa, respectively. Four cysteine residues were present in the F165αF protein (Fig. 5). Both the G and H proteins contained two cysteine residues in the N-terminal half of the protein which may form an intramolecular disulphide bridge. The penultimate amino acid was tyrosine, as was reported for the other S- and P-related minor fimbrial proteins (van Die et al., 1991; Schmoll et al., 1989; Maiti et al., 1994). As in P fimbriae, the various major and minor proteins of the S/Foc family are highly conserved in their N- and C-termini, which might reflect the involvement of these regions in fimbrial polymerization through subunit–subunit interaction (van Die et al., 1987; Hultgren et al., 1991; Hal Jones et al., 1992).

F165αF was identical to FocF and SfaG/SfaII G. Although F165αG and FocG were identical except for two amino acids difference at positions 86 and 87, these proteins were quite different from SfaS and SfaII S. Similarly F165αH and FocH were identical but differed from SfaH and SfaIII H (Fig. 5). It has been shown that for the S fimbriae, the 15 kDa protein SfaS is the adhesin that binds to carbohydrate chains terminating with α-sialyl-(2-3)-β-D-Gal-R (Parkkinnen et al., 1986; Moch et al., 1987; Morschhäuser et al., 1990), whereas FIC and F165α fimbriae lack that specific adhesive property. S fimbriae and FIC fimbriae adhere to renal tubules. SfaG subunit was shown to contribute to the binding of S fimbriae to renal tubules. It is not clear which of the FIC subunits is responsible for the observed adhesive properties. Moreover, it has been suggested that SfaG acts as a linker molecule for the adhesin (SfaS) which could bind SfaS to fimbriae (Moch et al., 1987). SfaG and SfaH were shown to be involved in modulation of fimbriation by Morschhäuser et al. (1990). We speculate that F165αH or F165αG may be involved in recognition of an unknown receptor.

Septicaemic E. coli strain 4787 expresses Psr-like and F1C-like fimbriae. Studies with uropathogenic E. coli strain KS71 (O4:K12:H1·), whose fimbriae strongly resemble those of another uropathogenic E. coli strain, AD110, have revealed that individual cells express either FIC or P fimbriae (Rhen et al., 1983; Nowicki et al., 1984). These results suggest a mutual regulation of expression as in the uropathogenic strain 536, for which cross-regulation of virulence gene clusters was demonstrated (Morschhäuser et al., 1994). Strains 4787 and KS71 have different O and K antigens and originate from different species, the pig and the human, respectively. These findings suggest that the two isolates are not clonally related. In human UTI, type 1C fimbriae have been associated with groups O2, O6, O18 and O75, with capsular type K5, with mannose-resistant (both P and non-P) adhesins and with haemolysin production (Sitonen et al., 1993).

In conclusion, we have cloned the f165α operon, and sequenced the f165α major fimbrial subunit gene and three minor fimbrial subunit genes. It was previously found that, in strain 4787, f165α was a mosaic operon consisting of sequences related to pap and prs. We have now found that the f165α operon seems to be conserved and highly related to the foc operon and belongs to the S for fimbrial family. The F165α major and minor subunits have the highest identity with the F1C major and minor fimbrial subunits. The contribution of F165α fimbriae to septicaemia in pigs needs to be investigated.

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