Molecular analysis of the *cso* operon of enterotoxigenic *Escherichia coli* reveals that CsoA is the adhesin of CS1 fimbriae and that the accessory genes are interchangeable with those of the *cfa* operon

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A deletion mutation in *csoA*, the gene encoding the structural subunit protein of CS1 fimbriae of enterotoxigenic *Escherichia coli* of serotype O6:K15:H16 or H–, was constructed in the subcloned CS1 genetic determinant. The mutation resulted in the abolition of CS1 fimbrial adhesiveness. Complementation, *in trans*, involving the determinant with the *csoA* deletion mutation and the gene encoding the structural subunit protein, CsoA, expressed from compatible plasmids, restored the expression and adhesive ability of CS1 fimbriae. In addition, *trans*-complementation was achieved between the *cso* determinant with the aforementioned deletion mutation and the *cfaB* gene encoding the structural subunit protein (CfaB) of CFA/I fimbriae, resulting in the expression of CFA/I fimbriae. The observation that heterologous assembly was possible between these two fimbrial systems, together with the knowledge that the adhesin of CFA/I fimbriae is the structural subunit, was exploited to investigate whether CsoA had adhering properties. A deletion mutation in *cfaB* was created in the CFA/I fimbrial determinant. Complementation of this mutation with *csoA* *in trans* resulted in expression of the CsoA antigen on the bacterial cell surface and restoration of bacterial adherence. As no minor subunits act as the adhesin in CFA/I fimbriae, adhesion was mediated by CsoA. Nucleotide sequencing of the DNA region downstream from *csoA* confirmed the absence of genes encoding minor subunits which might act as the adhesin. Two open reading frames were revealed which encoded proteins sharing considerable homology with proteins encoded by corresponding ORFs in the CFA/I fimbrial operon. These proteins underlie the functional similarities between the CS1 and CFA/I fimbrial systems, allowing heterologous expression of their respective subunits.

Keywords: CS1 fimbriae, adhesin, CsoA, CFA/I fimbriae, interchangeability in assembly system

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhoea in children in Third World countries, in travellers and in domestic animals. Their virulence is associated with adherence to the intestinal epithelium by means of fimbriae and subsequent production of heat-

labile enterotoxin (LT) or heat-stable enterotoxin (ST) or both (Cravioto *et al.*, 1982). The diversity of serologically distinct fimbrial antigens expressed independently by human ETECs exemplifies bacterial ingenuity in adapting and evading the host's immune responses (de Graaf & Gaastra, 1994).

CFA/I and CFA/II fimbriae are two types of colonization factor antigens expressed independently by different serovars of human ETEC (Evans *et al.*, 1975; Evans & Evans, 1978). CFA/II is a fimbrial complex consisting of

Abbreviations: CFA, colonization factor antigen; ETEC, enterotoxigenic *E. coli*.

three coli surface antigens, designated CS1, CS2 and CS3 (Smyth, 1982). Expression of CS1 or CS2 fimbriae is found almost exclusively on human ETEC strains of serotype O6:K15:H16 or H—. CS1 and CS2 fimbriae are expressed in a biotype-dependent fashion, namely, CS1 fimbriae by strains of biotype A, and CS2 fimbriae by strains of biotypes B, C and F (Smyth, 1986). Therefore, CS1 and CS2 fimbriae cannot be co-expressed, but individually they are co-expressed with CS3 fimbriae. Although these colonization factor antigens exhibit distinct immunological properties, extensive amino acid homology is observed between the subunit proteins of CFA/I and CS1 fimbriae (Perez-Casal *et al.*, 1990; Jordi *et al.*, 1991). The conservation of these regions of homology suggests the possible existence of similarities in the biogenesis systems for these fimbriae. Furthermore, these fimbriae share the property of agglutinating bovine erythrocytes. However, the nature of the receptor or receptors recognized by these fimbriae has not been conclusively determined (de Graaf & Gaastra, 1994).

It has been shown that both fimbrial operons are expressed from large molecular mass plasmids in their respective wild-type strains (Smith *et al.*, 1982; Perez-Casal *et al.*, 1990). Expression from these operons is under environmental regulation mediated by DNA-binding proteins of the AraC family, Rns regulating CS1 fimbrial expression and CfaD regulating the expression of CFA/I fimbriae (Caron *et al.*, 1989; Savelkoul *et al.*, 1990). These proteins are homologous and interchangeable; Rns can regulate expression from the CFA/I operon while CfaD can mediate expression from the CS1 operon.

At the inception of this study the organization of the CS1 operon [designated *csa* by Jordi *et al.* (1991) and agreed with our laboratory, or *coo* by Perez-Casal *et al.* (1990) and Scott *et al.* (1992)] had not been well characterized. CS1 fimbriae are composed of a 16 kDa major subunit protein, CsoA, which is expressed from the CS1 genetic determinant within a 7 kb DNA region (Perez-Casal *et al.*, 1990; Jordi *et al.*, 1991). A 26 kDa protein, CsoB, had also been identified which was proposed to have a role in fimbrial assembly but not in transport of the subunits (Scott *et al.*, 1992). The remaining coding capacity of the CS1 determinant had not been detailed, but was thought to encode additional components necessary for the biogenesis of CS1 fimbriae, by analogy with the organization of other fimbrial operons, such as the *pap* operon (Hultgren *et al.*, 1991).

The organization of the CFA/I fimbrial operon (*cfa*) had been determined (Jordi *et al.*, 1992). The structural subunit protein, CfaB, together with three other proteins, CfaA, CfaC and CfaE, are expressed from region 1 of the high molecular mass plasmid while CfaD is expressed from region 2 of the same plasmid. A fifth gene is encoded in region 1 which is homologous to the regulatory gene *cfaD*; however, it is non-functional and is called *cfaD'* (Gaastra *et al.*, 1990). The functions of the proteins CfaA, CfaC and CfaE are unknown. It is conceivable that these proteins are the components of a biogenesis system. Indeed, CfaC is a high molecular mass protein with

properties characteristic of an outer-membrane protein and may perform the role of an usher, although it does not show any homology with known ushers which make up the biosynthetic machinery of other fimbriae (Kusters & Gaastra, 1994). In contrast to Pap, S and type 1 fimbriae, minor fimbrial components do not appear to be encoded by the *cfa* fimbrial system (de Graaf & Gaastra, 1994; Smyth *et al.*, 1994). The structural subunit of CFA/I fimbriae was characterized biochemically as the adhesin of this fimbrial system (Bühler *et al.*, 1991). By comparison, the nature of the CS1 fimbrial adhesin is unknown and the possible existence of minor subunits has not been demonstrated. The major subunit gene may in the correct orientation at the fimbrial tip act as the adhesin. Alternatively, the adhesin and the structural subunit may be encoded by separate genes as is the case with P, S and type 1 fimbriae (Smyth *et al.*, 1994). Furthermore, the steps in the biogenesis of CS1 fimbriae remain unelucidated.

This study employed a molecular genetic approach to ascertain the adhesin responsible for CS1 fimbrial adhesiveness. The role of CsoA, the major fimbrial antigen, in the adhering function of CS1 fimbriae was investigated. A deletion mutation in the *csoA* gene was constructed in the subcloned CS1 genetic determinant and the effects of this mutation on the expression and properties of CS1 fimbriae were ascertained. Heterologous subunit expression and assembly between the CS1 and CFA/I fimbrial systems was assessed using genetically constructed strains.

METHODS

Bacterial strains and plasmids. *E. coli* strains E90a and C922a-2 are wild-type enterotoxigenic strains of serotype O6:K15:H16, which are positive and negative, respectively, for CS1 fimbrial expression (Smyth, 1982). *E. coli* strains H10407 and H10407-P are wild-type enterotoxigenic strains of serotype O78:H11, which are positive and negative, respectively, for CFA/I fimbrial expression (Evans *et al.*, 1975). *E. coli* strain 54R118 is an *E. coli* K12 recombinant strain positive for CFA/I fimbriae (McConnell *et al.*, 1981). *E. coli* strain XL1-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F' *proAB lacI^q Z ΔM15, Tn10 (Tet^r)*)] (Stratagene) was used as the host for molecular cloning and expression, and was maintained on LB agar supplemented with the appropriate antibiotics at the following concentrations: ampicillin (100 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹), tetracycline (10 µg ml⁻¹), spectinomycin (50 µg ml⁻¹). Restriction endonucleases were purchased from New England Biolabs and Boehringer Mannheim. The plasmids used in this study are described in Table 1.

Subcloning the CS1 operon and the *csoA* gene separately. *Hind*III digestion of plasmid pSS4180 generates a 13 kb *Hind*III fragment with two internal *Cla*I sites (Fig. 1a). These *Cla*I sites delimit the first gene, *csoB*, of the CS1 operon and, in addition, the second *Cla*I site is positioned 5' to the *csoA* gene, the major subunit gene. Partial digestion with *Cla*I of the 13 kb *Hind*III fragment generates a 7 kb *Cla*I–*Hind*III fragment with the second internal *Cla*I site. This fragment encodes the CS1 fimbrial operon. The 7 kb *Cla*I–*Hind*III fragment was ligated to the plasmid pGEM-9Zf cleaved at its *Acc*I and *Hind*III sites (Fig. 1b). The cohesive ends of the *Acc*I and *Cla*I recognition sites are compatible and are not recleavable with either enzyme. Therefore, the internal *Cla*I site is rendered unique. The

Table 1. Plasmids used in this study

Plasmid	Description*	Replicon	Source or reference
pBluescript	Phagemid derived from pUC19, Amp ^r	ColE1	Stratagene
pGEM-7Zf(-)/9Zf(-)	Standard vectors, blue/white selection, Amp ^r	ColE1	Promega
pACYC184	Cloning vector, Cm ^r Tc ^r	p15A	Chang & Cohen (1978)
pCL1921	Low-copy-number cloning vector, Spc ^r	pSC101	Lerner & Inouye (1990)
pLG339	Low-copy-number cloning vector, Tc ^r Kan ^r	pSC101	Stoker <i>et al.</i> (1982)
pSS4180	CS1 operon within a 15 kb <i>SacI</i> DNA region cloned from total DNA of <i>E. coli</i> C921b-1 in pLG339	pSC101	S. G. J. Smith (Dept of Microbiology, University of Dublin), unpublished data
pNTP513	CFA/I region I in pACYC184	p15A	Willshaw <i>et al.</i> (1983)
pMM101	CS1 operon cloned in pGEM-9Zf(-)	ColE1	This study
pMM102	<i>csaA</i> cloned in pBluescript KS	ColE1	
pMM1021	Δ <i>csaA</i> cloned in pBluescript KS	ColE1	
pMM103	pMM101 Δ <i>csaA</i>	ColE1	
pMM104	CS1 operon cloned in pACYC184	p15A	
pMM1041	pMM104 Δ <i>csaA</i>	p15A	
pMM301	<i>cfaB</i> cloned in pGEM-9Zf(-)	ColE1	
pMM7011	<i>cfaC</i> and <i>cfaE</i> cloned in pGEM-7Zf(-)	ColE1	
pMM7013	<i>cfaA</i> Δ <i>cfaB</i> <i>cfaC</i> <i>cfaE</i> cloned in pGEM-7Zf(-)	ColE1	
pMM8102	<i>csaA</i> cloned in pACYC184	p15A	S. G. J. Smith (Dept of Microbiology, University of Dublin), unpublished data
pMM8301	<i>cfaB</i> cloned in pACYC184	p15A	
pSS2192	<i>rns</i> cloned in pCL1921	pSC101	

*Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; Kan^r, kanamycin resistance; Spc^r, spectinomycin resistance.

recombinant plasmid thus generated was designated pMM101. The 7 kb CS1 genetic determinant was then subcloned from pMM101 as an *Ecl136II*-*HindIII* fragment into the *EcoRV*-*HindIII* sites in vector pACYC184 to yield pMM104. This was possible due to the cleavage of the *SacI* recognition site in the multiple cloning site of pGEM-9Zf immediately 5' to the CS1 genetic determinant inserted into pMM101 by the restriction enzyme *Ecl136II* in a blunt-ended manner (Fig. 1b). This achieves compatibility with the blunt-ended *EcoRV* recognition site in pACYC184.

To subclone the *csaA* gene separately, pSS4180 was digested with *ClaI* and *XbaI* and the resulting 700 bp *ClaI*-*XbaI* fragment was ligated with the *ClaI*-*XbaI*-cleaved vector plasmid pBluescript KS (pBKS, Stratagene) to yield the recombinant plasmid pMM102 (Fig. 1c). The orientation of the *csaA* gene in pMM102 ensures that its expression may be directed from the *lacZ* promoter of pBKS when this promoter is derepressed by the inducer IPTG. The *csaA* gene was also subcloned into the vector plasmid pACYC184 to create pMM8102.

Creating a deletion mutation in the CS1 operon. The 700 bp *ClaI*-*XbaI* fragment encoding the *csaA* gene contains four internal *MseI* sites (Fig. 1d). Digestion of this fragment with *MseI* and subsequent ligation of the two end fragments (i.e. the 80 bp *ClaI*-*MseI* fragment and the 51 bp *MseI*-*XbaI* fragment) yielded a 127 bp DNA fragment which was subcloned into pBKS to yield pMM1021 (Fig. 1e). Nucleotide sequencing of

the insert in pMM1021 confirmed that the correct DNA fragments were ligated. The complete *csaA* gene had been deleted. This 127 bp *ClaI*-*XbaI* fragment was used to replace the 700 bp *ClaI*-*XbaI* fragment encoding *csaA* in pMM101. The resultant recombinant plasmid was designated pMM103 (Fig. 1f). Plasmid pMM1041 was produced from pMM103 and pACYC184 by cleavage of the *Ecl136II*-*HindIII* fragment in pMM103 and ligation to the *EcoRV*-*HindIII* sites in the vector pACYC184.

Subcloning the CFA/I fimbrial subunit. Plasmid pNTP513 is a pACYC184 derivative encoding the region 1 genes of the CFA/I fimbrial system within an 8 kb DNA region (Fig. 2a). *cfaB*, the CFA/I fimbrial subunit gene, is encoded on a 1.3 kb *XbaI*-*HindIII* DNA fragment (Fig. 2b) within this 8 kb DNA region. This 1.3 kb *XbaI*-*HindIII* fragment was ligated to vector plasmid pGEM-9Zf to create pMM301, a high-copy-number ColE1 plasmid expressing CfaB. It was also ligated to *XbaI*-*HindIII*-cleaved vector plasmid pACYC184 to create pMM8301.

Creating a deletion mutation in the CFA/I operon. The deletion of the *cfaB* gene in the CFA/I operon was achieved in a three-step process. Firstly, the genetic determinant was removed from pNTP513 by digestion with *EcoRV* endonuclease. This excluded most of the *cfaD'* gene, which is non-functional (Fig. 2a). Secondly, digestion of the unique *Bam*HI site at the 3' end of *cfaB* resulted in a 4033 bp fragment which

Fig. 1. Construction of a deletion mutation in the CS1 operon. The restriction endonuclease map of the 13 kb *HindIII* DNA region encoding the CS1 genetic determinant from pSS4180 is presented (a). The 7 kb *Clal-HindIII* DNA region (b) is the product of the partial *Clal* digestion of the 13 kb *HindIII* DNA region (a). The *SacI* site of the vector multiple cloning site 5' to the CS1 operon is shown when this 7 kb fragment is subcloned in pGEM-9Zf to yield pMM101 (b). The restriction map of the 700 bp *Clal-XbaI* region encoding the *csoA* gene (c) is expanded in (d). This fragment was ligated to vector pBluescript KS to yield pMM102. Deletion of an internal region of this fragment was achieved by digestion with *MseI* (d). The religated fragment (ΔA) was subcloned into the *Clal-XbaI* sites of vector pBK5 to create pMM1021 (e). This 127 bp *Clal-XbaI* fragment was used to replace the intact *csoA* gene in the CS1 operon of pMM101 to yield pMM103 (f). Abbreviations: A, *AccI*; C, *Clal*; EL, *Ecl136II*; ER, *EcoRI*; H, *HindIII*; M, *MseI*; S, *SacI*; X, *XbaI*. *cso* genes are represented by boxes with single-letter designations. The dotted line in (a) represents the 6 kb DNA region upstream from the *cso* operon, which is not drawn to scale.

was subcloned into *BamHI-EcoICRI(SacI)* sites in the vector plasmid pGEM-7Zf. This construct was designated pMM7011 (Fig. 2c). The *KpnI* site 5' to the start of the operon in pNTP513 was digested followed by digestion of a *PvuII* site. The resulting 1.3 kb fragment was ligated to pMM7011 which had been digested with *BamHI*, filled in and then digested at the *KpnI* site in the vector DNA. The resulting plasmid was designated pMM7013 (Fig. 2d). This achieves deletion of the C-terminus of the *cfaB* gene, resulting in translation of 50 amino acids in the N-terminal region, of which 23 constitute the leader sequence. The *KpnI* site is located upstream of the promoter of this operon and, therefore, cleavage at this site should not interfere with expression from the promoter of the *cfa* operon in the presence of the positive regulator CfaD or Rns.

DNA isolation, manipulation and sequencing. All basic DNA techniques were performed as described by Sambrook *et al.* (1989). Nucleotide sequence analysis was carried out by the dideoxy chain-termination method with an automated DNA sequencer (Applied Biosystems 370A) and fluorescent-dye-labelled primers. Deletions were obtained with an Erase-a-Base kit (Promega). The DNA sequencing strategy is outlined in Fig.

Fig. 2. Construction of the deletion in the CFA/I operon. The CFA/I operon is encoded within an 8 kb DNA region of pNTP513 (a). The *cfaB* gene was subcloned as a 1.3 kb *XbaI-HindIII* DNA region (b) into the appropriately cleaved vector plasmids pGEM-9Zf and pACYC184 to create pMM301 and pMM8301, respectively. Construction of the deletion in the *cfaB* gene of the CFA/I operon was achieved as follows. The *BamHI-EcoRV* 4 kb DNA region (c) was subcloned into pGEM-7Zf to create pMM7011, which was cleaved at the unique *BamHI* site and this site then filled in to produce a blunt end. pMM7011 was then cut at the *KpnI* site in the vector DNA and the 1.3 kb *KpnI-PvuII* DNA fragment from the CFA/I region 1 (a) was ligated to these sites in pMM7011 to create pMM7013 (d). This resulted in the deletion of the gene *cfaB* (ΔB). Abbreviations: B, *BamHI*; H, *HindIII*; K, *KpnI*; P, *PvuII*; V, *EcoRV*; X, *XbaI*. *cfa* genes are represented by boxes with single-letter designations.

Fig. 3. Strategy for sequencing the DNA region downstream from the *csoA* gene of the CS1 operon. All DNA regions indicated [the 5.5 kb *XbaI-HindIII* DNA region (1), the 3.7 kb *Clal-EcoRI* DNA region (2) and the 3 kb *EcoRI-XbaI* DNA region (3)] were subcloned separately into the vector pGEM-7Zf and deletions were generated from the appropriate sites, indicated by the direction of the arrows, using the Erase-a-Base kit (Promega). The positions of the open reading frames are indicated above the sequencing strategy diagram. In the DNA approximately 200 bp downstream from the *csoE* gene, two regions (~100 bp apart) of 48 bp and 67 bp share 89% and 84% sequence similarity, respectively, with IS629. Downstream of these sequences (250 bp), approximately 100 bases sequenced showed 100% identity with part of the sequence of IS2. The shaded arrow represents the additional DNA region sequenced by Froehlich *et al.* (1994). This complete region shares 99% identity with the first 877 bases of IS2. Abbreviations: C, *Clal*; ER, *EcoRI*; H, *HindIII*; X₁, *XbaI*; X₂, *XbaI* site 3' to the CS1 operon in pMM104 from which this DNA region was subcloned. Broken lines represent DNA regions which are not drawn to scale.

3. Searches for nucleotide and amino acid sequence similarities were performed with the BLAST family of programs in the EMBL and GenBank databases (Altschul *et al.*, 1990). Com-

parison of protein sequences was performed with the Clustal V program (Higgins & Sharp, 1989).

Antibodies. Anti-CS1 antibodies were raised to CS1 antigen purified from *E. coli* XL1-Blue expressing CS1 fimbriae from the recombinant plasmid pMM101. The antigen was prepared from CFA-broth-grown (Evans *et al.*, 1975) *E. coli* XL1-Blue(pMM101) by homogenization (Ultra-turrax, Janke & Kunkel) followed by ammonium sulphate precipitation and Phenyl-Sepharose (Pharmacia) column chromatography. New Zealand White rabbits were given four injections, intramuscularly, of purified fimbriae (40 µg protein per dose) in Freund's complete adjuvant on day 0, and in Freund's incomplete adjuvant on days 7, 14 and 21. Following absorption with appropriate strains (Smyth, 1982), the specific anti-CS1 antibodies were purified according to Harboe & Ingild (1973). To raise anti-CFA/I antibodies, rabbits were inoculated intravenously, according to the schedule of Evans *et al.* (1975), with formalin-treated whole-cell suspensions of ETEC strain H10407 of serotype O78:H11 expressing CFA/I fimbriae. The serum was absorbed with *E. coli* strain H10407-P, a spontaneous CFA/I-negative variant (Smyth *et al.*, 1979), and the antibodies purified as above. The specificities of these purified immunoglobulins were checked by immunodiffusion and agglutination tests before use.

Immuno-techniques. *E. coli* XL1-Blue recombinants were grown overnight at 37 °C in LB broth supplemented with the appropriate antibiotics. The cultures were standardized and heat-released extracts were obtained as described by Smyth (1982). The heat-released extracts were analysed by SDS-PAGE, which was performed using the method of Laemmli (1970). Molecular mass standards were obtained from Sigma. Electrophoresis was performed according to Towbin *et al.* (1979). For immunodetection, anti-CS1 or anti-CFA/I antiserum was used as the primary antibody and swine anti-rabbit IgG conjugated to peroxidase (DAKO) was used as the secondary antibody. The blots were developed using 4-chloro-1-naphthol (Sigma) as substrate in the peroxidase reaction. Slide agglutination tests were used to rapidly detect expression of fimbriae.

Immuno-electron microscopy. *E. coli* XL1-Blue recombinants were dried onto Formvar-coated 400-mesh gold grids and incubated in anti-CS1 antibodies diluted 1:100 in distilled water. Unbound antibodies were removed with four 1 min washes in distilled water. This was followed by incubation in Protein A-gold conjugates (10 nm particles, Sigma) diluted 1:10 in distilled water. Unbound gold conjugate was removed by four 1 min washes in distilled water. The grids were blotted dry, negatively stained with 1% (w/v) phosphotungstic acid and viewed using a Hitachi H7000 transmission electron microscope.

Haemagglutination. Adherence to bovine erythrocytes was tested in a haemagglutination assay with *E. coli* XL1-Blue recombinants. The assay was done essentially as described by Smyth (1982), in the presence of 1% (w/v) D(+)-mannose.

RESULTS

The deletion mutation in the *csoA* gene is complemented when the *csoA* gene is provided *in trans*

The CsoA antigen was detected by immunoblotting using anti-CS1 antibodies (Fig. 4). The CsoA protein was expressed from the CS1 genetic determinant in pMM104 (lane 3) and from pMM102 expressing the *csoA* gene (lane

Fig. 4. Western blot analysis of expression of the CsoA antigen from recombinant plasmids. Heat-released extracts from wild-type ETEC and *E. coli* XL1-Blue harbouring recombinant plasmids with the DNA inserts described in Methods were probed with anti-CS1 antiserum. Lanes: 1, strain E90a (CS1⁺); 2, strain C922a-2 (CS1⁻); 3, strain XL1-Blue(pMM104); 4, strain XL1-Blue(pMM102); 5, strain XL1-Blue(pMM104); 6, strain XL1-Blue(pMM104 and pMM102). The CsoA antigen is indicated at 16 kDa. Molecular mass standards (Sigma) are indicated on the left. The apparent shadowing effect is caused by protein overloading.

4) in *E. coli* XL1-Blue. Expression was not detected in *E. coli* XL1-Blue harbouring pMM1041 (*csoA* deletion mutant) (lane 5). However, when the *csoA* gene (pMM102) was expressed *in trans* to pMM1041, the CsoA protein was detected (lane 6). The Rns protein, encoded by pSS2192, was not required in these experiments because the DNA region with which this protein interacts to abolish the negative regulation of expression was excluded in the subcloning of the *cso* operon.

To determine how this related to the production of intact fimbriae, immuno-electron microscopy was used. As Fig. 5(a) shows, the CS1 genetic determinant directed the expression of morphologically intact CS1 fimbriae in *E. coli* XL1-Blue. Deletion of the *csoA* gene from the CS1 operon arrested expression of CS1 fimbriae (Fig. 5b). Moreover, CS1 fimbriae were not expressed from the recombinant strain harbouring pMM102, which encodes the *csoA* gene only (data not shown). However, *E. coli* XL1-Blue expresses type 1 fimbriae as detected by immuno-electron microscopy (data not shown). Thus, the biogenesis system of type 1 fimbriae does not direct surface expression of the CsoA antigen on *E. coli* XL1-Blue(pMM102) expressing the *csoA* gene of the CS1 operon. Complementation of the deletion mutation in the *csoA* gene in pMM1041 by expression of CsoA *in trans* from pMM102 restored the biogenesis of morphologically intact CS1 fimbriae (Fig. 5c). This verified that the *csoA* mutation created was non-polar.

CFA/I fimbriae are produced when the deletion mutation in the *csoA* gene is complemented by the *cfaB* gene expressed *in trans*

Because of the extensive amino acid sequence homology between CsoA and CfaB, experiments were performed to determine if the *cfaB* gene encoding the structural subunit

Fig. 5. Immuno-electron microscopy (a–e) of *E. coli* XL1-Blue containing the plasmids (a) pMM104, (b) pMM1041, (c) pMM1041 and pMM102, (d) pMM1041 and pMM301, and (e–f) *E. coli* XL1-Blue(pSS2192) containing the plasmids (e) pMM7013 and pMM8301, (f) pMM7013 and pMM8102. Recombinant strains were incubated in either anti-CS1 antibodies (a–c and f) or anti-CFA/I antibodies (d and e) followed by probing with Protein A–gold conjugates (10 nm). Bars, 200 nm.

protein of CFA/I fimbriae (pMM8301) could complement the *csaA* mutation (pMM103). Heat-released extracts from *E. coli* XL1-Blue harbouring pMM103 complemented *in trans* either with pMM8102 encoding the *csaA* gene or with pMM8301 encoding the *cfaB* gene were analysed by immunoblotting using anti-CS1 antibodies (Fig. 6a) and

anti-CFA/I antibodies (Fig. 6b). The CFA/I subunit protein was expressed from the recombinant strain containing the *csaA* deletion mutation (pMM103) and pMM8301 bearing the *cfaB* gene *in trans* (Fig. 6b, lane 5), while the CS1 antigen was not detected from the same recombinant strain (Fig. 6a, lane 5). A weak reaction of

Fig. 6. Western blot analysis of expression of the CsoA antigen (a) and CfaB antigen (b) from recombinant plasmids expressing the heterologous antigen to the fimbrial genetic determinant. Heat-released extracts from wild-type ETEC and *E. coli* XL1-Blue harbouring recombinant plasmids with the DNA inserts described in Methods were probed with anti-CS1 antibodies (a) or anti-CFA/I antibodies (b). Lanes: 1, strain E90a (CS1⁺); 2, strain 54R118 (CFA/I⁺); 3, strain XL1-Blue(pMM103); 4, strain XL1-Blue(pMM103 and pMM8102); 5, strain XL1-Blue(pMM103 and pMM8301); 6, strain XL1-Blue(pSS2192 and pMM7013); 7, strain XL1-Blue(pSS2192, pMM7013 and pMM8301); 8, strain XL1-Blue(pSS2192, pMM7013 and pMM8102). The position of the CsoA or CfaB antigen is indicated on the right (size in kDa given in parentheses). Molecular mass standards are indicated on the left.

anti-CS1 antibodies with CfaB was observed (Fig. 6a, lane 5). Cross-reactivity between these subunits using polyclonal antibodies raised to either type of whole fimbriae was reported previously (McConnell *et al.*, 1989). The CS1 subunit protein was detected in heat-released extracts from the *csaA* deletion mutant harbouring *in trans* pMM8102 encoding the *csaA* gene (Fig. 6a, lane 4). The CFA/I subunit protein was not detected in heat-released extracts from this recombinant strain (Fig. 6b, lane 4). This confirmed the absence of cross-contaminating subunits in each complemented recombinant strain. Immunoelectron microscopy studies using anti-CFA/I antibodies revealed intact fimbriae labelled with Protein A-gold conjugates assembled on the surface of recombinant strains harbouring the plasmids pMM1041, encoding the deletion mutation in the *csa* operon, and pMM301, encoding the CFA/I subunit protein (Fig. 5d). This recombinant was also probed with anti-CS1 antibodies followed by incubation with Protein A-gold conjugates. The fimbriae observed were not labelled with gold conjugates (data not shown).

The deletion mutation in the *cfaB* gene is complemented when the *cfaB* gene is provided *in trans*

In contrast to the cloned genetic determinant for CS1 fimbriae, the *cfa* genetic determinant possessed its own promoter region and, thus, the regulator CfaD or its homologue Rns, was required for expression. The homologue, Rns, encoded on pSS2192 was used in this study. A deletion was created in the *cfaB* gene encoding the structural subunit protein of the CFA/I operon (pMM7013) (see Methods). The mutation created in the CFA/I operon was characterized using immuno-techniques in a similar manner to the characterization of the deletion mutation in the CS1 operon. *Trans*-complement-

ation experiments were carried out with the CFA/I deletion mutant to assess the polarity of the mutation.

Immunoblot analysis showed that the CfaB antigen was not expressed from the recombinant strain XL1-Blue(pSS2192) containing pMM7013, encoding the deletion in the *cfaB* gene (Fig. 6b, lane 6). The CfaB antigen was detected when this mutation was complemented *in trans* with pMM8301, which encodes the *cfaB* gene (Fig. 6b, lane 7; note also the weak cross-reaction with anti-CS1 antibodies Fig. 6a, lane 7). Agglutination of this recombinant strain by anti-CFA/I antibodies in the slide agglutination test indicated that this antigen was expressed on the cell surface of this strain. Furthermore, CFA/I fimbriae were detected by immuno-electron microscopy (Fig. 5e). These results indicated that this mutation was non-polar with respect to the genes downstream of *cfaB* and that expression of only the structural subunit was abrogated.

The CsoA antigen is surface expressed when the deletion mutation in the *cfaB* gene is complemented by the *csaA* gene expressed *in trans*

The CsoA protein was expressed from the recombinant strain XL1-Blue(pSS2192) containing plasmids pMM7013 and pMM8102, encoding the *cfaB* deletion mutation and the *csaA* gene, respectively, as shown by immunoblotting (Fig. 6a, lane 8). In addition, the CsoA antigen was expressed on the cell surface as detected by slide agglutination with anti-CS1 antibodies. Immunoelectron microscopy did not reveal this antigen as assembled fimbriae; instead Protein A-gold conjugates which bound to anti-CS1 antibodies interacting with the CsoA antigen appeared to be associated directly with the bacterial cell surface (Fig. 5f). The CFA/I subunit was not expressed from this recombinant strain (Fig. 6b, lane 8).

Table 2. Summary of major antigens expressed from recombinant plasmids in *trans*-complementation experiments using immunotechniques and haemagglutination assays

Plasmid(s) in <i>E. coli</i> XL-Blue	Genes present	Major antigen expressed*	Cell surface expression†	MRHA phenotype‡
pMM102	<i>csoA</i>	CsoA	—	—
pMM104	CS1 operon	CsoA	CS1 fimbriae	+
pMM1041	CS1 operon Δ <i>csoA</i>	—	—	—
pMM1041 + pMM102	CS1 operon Δ <i>csoA</i>	CsoA	CS1 fimbriae	+
pMM1041 + pMM301	CS1 operon Δ <i>csoA</i>	CfaB	CFA/I fimbriae	+
pMM7013	CFA/I operon Δ <i>cfaB</i>	—	—	—
+ pSS2192	<i>rns</i>	—	—	—
pMM7013 + pSS2192	CFA/I operon Δ <i>cfaB</i>	CfaB	CFA/I fimbriae	+
+ pMM8301	<i>cfaB</i>	—	—	—
pMM7013 + pSS2192	CFA/I operon Δ <i>cfaB</i>	CsoA	CsoA antigen	+
+ pMM8102	<i>csoA</i>	—	—	—

* Detected by Western immunoblotting with anti-CS1 or anti-CFA/I antibodies.

† Detected by electron microscopy with anti-CS1 or anti-CFA/I antibodies.

‡ MRHA, mannose-resistant haemagglutination.

The CS1 antigen was not detected in *E. coli* XL1-Blue(pSS2192) containing plasmids pMM7013, encoding the deletion of the *cfaB* gene in the CFA/I operon, and pMM8301, encoding the complementing CfaB antigen *in trans* (Fig. 6a, lane 7).

Trans-complementation of the deletion mutations restores mannose-resistant haemagglutination

Having characterized the deletion mutations in the subunit genes and determined that they were non-polar, we investigated the effect of each mutation on the adhesiveness of the respective fimbriae. The haemagglutination assay (the agglutination of bovine erythrocytes in a mannose-resistant manner) was used to assess the effect of the deletions of the structural subunit genes on fimbrial adhesiveness. Table 2 summarizes the results of the haemagglutination tests. The deletion in the *csoA* gene abolished the adhering ability of CS1 fimbriae, while complementation of this mutation with the *csoA* gene *in trans* restored adhesion. Similarly, deletion of the *cfaB* gene abolished the adhering ability of CFA/I fimbriae, while complementation of this mutation with the *cfaB* gene *in trans* restored adhesion. Thus, complementation of the deletion mutants with their respective structural subunit genes restored adhesion, demonstrating that these mutations do not have a polar effect on the functional expression of their respective fimbriae. Moreover, expression of the heterologous CfaB subunit protein *in trans* to the CS1 operon harbouring the deletion in the *csoA* gene, or expression of the heterologous CsoA subunit protein *in trans* to the CFA/I operon harbouring the deletion in the *cfaB* gene, conferred the adhesion pheno-

type on these recombinant strains. This demonstrated that CsoA, the structural subunit, played a role in the adherence of CS1 fimbriae.

A minor subunit protein is not encoded in the DNA region downstream from the *csoA* gene

Sequencing of the DNA region downstream from the *csoA* gene, which was carried out independently in this study, was reported recently (Froehlich *et al.*, 1994). Our sequence data in these coding regions (*csoC* and *csoE*) are identical to those deposited by Froehlich *et al.* (1994) under accession number X76908 and accordingly are not repeated herein. The salient features of the sequence analysis were highlighted by Froehlich *et al.* (1994). In keeping with the *coo* nomenclature of the CS1 operon, Froehlich *et al.* (1994) have named the open reading frames *cooC* and *cooD*, which correspond to *csoC* and *csoE*, respectively.

Seven differences however, exist between the sequence data published in Fig. 1 of Froehlich *et al.* (1994) and that deposited under accession number X76908. Translation of codons from nucleotides 3791–3805 should read 'Ser Phe Glu Ser Ser' and from nucleotides 3809–3014 should read 'Asn and Glu'. The codon GTG is omitted at nucleotide positions 4042–4044 and an extra codon, AGT, is inserted at position 4051–4053, resulting in the incorrect translation of this region of the published sequence. One hundred and twenty nucleotides have also been omitted from the published sequence at nucleotide positions 4363 through 4482. Finally, two codons, ATC and AAT, were inserted at positions 4843–4848 and the same two codons were omitted at positions 4903–4908. This resulted in the

incorrect translation of this region of the published sequence.

In addition, sequencing of approximately 800 bp of one strand of the DNA region downstream from the *csoE* gene (Fig. 3, the region between *EcoRI* and *HindIII*) revealed that approximately 200 bp downstream from the 3' end of *csoE*, insertion sequences are found. These sequences, similar to IS629 and IS2, are detailed by Froehlich *et al.* (1994), who sequenced both strands in this region. CsoC and CsoE do not share similarity with the structural subunit protein, CsoA, a property associated with minor subunit proteins of other fimbrial systems. Thus, this DNA region does not contain an open reading frame encoding a minor subunit protein which could act as an adhesin.

DISCUSSION

The interchangeability of genes of the CS1 and CFA/I fimbrial systems in mediating surface expression of their heterologous structural subunits in this study suggests a relatedness between these two fimbrial systems and certainly that similar modes of fimbrial biogenesis may exist. Three putative accessory proteins in each fimbrial system, CsoB/CsoC/CsoE and CfaA/CfaC/CfaE, respectively, have been identified to be involved in assembly of fimbrial subunits. Of these, CsoE shows the lowest degree of amino acid sequence identity and similarity with its homologue protein, CfaE (see below). The putative accessory proteins of the CS1 fimbrial system could interact productively with the CfaB protein and direct its assembly into functional CFA/I fimbriae. In contrast, the surface expression, but not assembly, of the CsoA protein mediated by the accessory proteins of the CFA/I fimbrial system implies some functional deficiency which may relate to the interchangeability of the E proteins. Alternatively, this result may reflect a property of the deletion mutation created in the CFA/I operon. A recent study examining the interchangeability of the type 1 and F1C fimbrial export proteins demonstrated that while replacement of the type 1 biogenesis apparatus with that of F1C fimbriae was successful in restoring the wild-type phenotype, the reverse was not as successful (Klemm *et al.*, 1995).

The observation of heterologous assembly of fimbrial subunits was utilized to investigate if there was a similarity in structural organization between CS1 and CFA/I fimbriae. It was previously demonstrated, through biochemical studies, that the structural subunit protein, CfaB, is the adhesin of the CFA/I fimbria (Bühler *et al.*, 1991). Therefore, no adhesin is expressed from the CFA/I fimbrial system when the structural subunit protein is deleted. The absence of a minor subunit protein acting as an adhesin in this system allowed the examination of the role of the CsoA antigen in adherence, when it is expressed *in trans* to the plasmid expressing only the accessory components required for the biogenesis of CFA/I fimbriae.

In our study, the adhering property of CS1 fimbriae was shown to be conferred by its structural subunit protein,

CsoA, as is the case with CFA/I fimbriae. Deletion of the gene encoding the structural subunit of CS1 fimbriae and subsequent *trans*-complementation with the *csoA* gene demonstrates that the structural CsoA subunit makes a contribution to the adhesive property of CS1 fimbriae. However, the surface expression of the CsoA protein in a functionally adhesive manner, mediated by a heterologous fimbrial system (CFA/I) lacking its own adhesin, confirms that the CsoA protein specifies adherence.

Sequencing of the entire CFA/I genetic determinant did not reveal any gene encoding a minor protein subunit which could represent a putative adhesin (Jordi *et al.*, 1992), thus confirming the biochemical study of Bühler *et al.* (1991). By analogy, in this study, and independently in that of Froehlich *et al.* (1994), the downstream DNA region of the CS1 genetic determinant was sequenced and revealed two open reading frames and sequences similar to insertion sequences. The proteins encoded by these open reading frames do not resemble a putative subunit protein which could act as an adhesin. Instead, the translated product of the *csoC* gene shares 63% amino acid identity and 84% amino acid similarity with the CfaC protein of the CFA/I fimbrial system, while the translated product of the *csoE* gene shows 53% amino acid identity and 75% amino acid similarity with the CfaE protein of the CFA/I fimbrial system (this study and Froehlich *et al.*, 1994). Together with the observation that the CsoB, CsoC and CsoE proteins of the *cso* operon have been shown to be able to substitute for their CFA/I homologues, this strongly suggests that these proteins represent the biogenesis system of these fimbriae. However, these proteins do not show any similarity with the components of known biogenesis systems (Hultgren *et al.*, 1991), even though CsoC has a molecular mass in the range of identified molecular ushers, and properties consistent with it being an outer-membrane protein. Therefore, the ancillary proteins of these two fimbrial systems may represent prototypes of an as yet undocumented assembly apparatus.

Recent studies on the immunogenicity of CFAs expressed by human ETEC demonstrate the ability of monoclonal antibodies against CFA/I to cross-react with and inhibit the haemagglutination property of such heterologous CFAs as CS1 and CS4 (Rudin & Svennerholm, 1994; Rudin *et al.*, 1994). It was concluded that a vaccine composed of bacteria expressing selected CFAs could induce protection against ETEC expressing a broader range of CFAs. The ability of the ancillary proteins of one such fimbrial system to mediate the surface expression of a subunit of another CFA fimbrial system, demonstrated in this study, opens up the possibility of the creation of a recombinant vaccine strain which expresses a number of heterologous CFAs by utilizing just one biogenesis apparatus.

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