Biosynthesis of K88 Fimbriae in *Escherichia coli*: Interaction of Tip-Subunit FaeC with the Periplasmic Chaperone FaeE and the Outer Membrane Usher FaeD

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Abstract

K88 fimbriae are ordered polymeric protein structures at the surface of enterotoxigenic *Escherichia coli* cells. Their production and assembly requires a molecular chaperone located in the periplasm (FaeE) and a molecular usher located in the outer membrane (FaeD). FaeC is the tip component of the K88 fimbriae. We studied the expression of the subcloned faeC gene, the subcellular localization of FaeC and its interaction with the chaperone and the outer membrane usher. In the absence of the chaperone or the usher, FaeC could not be detected in *E. coli* cells harbouring the faeC gene and its ribosome binding site under control of the IPTG inducible lpp/lac promoter/operator. The expression of FaeC was detectable in the presence of chaperone FaeE, but a direct interaction between the chaperone and FaeC was not found. The expression of FaeC was also detectable in cells co-expressing the outer membrane usher FaeD. Overexpression of FaeC after changing the faeC ribosome binding site appeared to induce lethality. Expression of subcloned FaeC in the absence of FaeE or FaeD could be detected when faeC was cloned under the tight control of the ara promoter/operator and when lethality induction was avoided. The direct interaction of FaeC with outer membranes containing the usher FaeD was studied by cell fractionation, isopycnic sucrose density gradient centrifugation, SDS-PAGE and immunoblotting. FaeC was found to bind to outer membranes containing FaeD or a FaeD-PhoA hybrid construct containing 215 amino-terminal residues of FaeD. This binding was not observed when control outer membranes without FaeD were used. No other K88 specific proteins were required for this interaction. The direct interaction between FaeC and FaeD in the outer membranes was shown by affinity blotting experiments. FaeE was not required for this interaction.

Together these data indicate that the minor fimbrial subunit FaeC, unlike FaeG, H and F, does not have a strong interaction with the chaperone FaeE in the *E. coli* periplasm, but directly binds to the outer membrane molecular usher FaeD.

Introduction

The production of K88ab fimbriae requires two assembly proteins: FaeE, a periplasmic molecular chaperone (Mooi *et al*., 1983; Bakker *et al*., 1991; Mol *et al*., 1994 and 1995) and FaeD, an outer membrane assembly protein also called molecular usher (Mooi *et al*., 1986; Dodson *et al*., 1993; Valent *et al*., 1995B). These two proteins do not form an integral part of the K88 fimbriae. FaeE in its native state is a homodimer. In the periplasm, the chaperone forms heterotrimeric complexes with FaeG, FaeH and with FaeI. The binding of FaeE to these fimbrial subunits prevents premature polymerisation and provides protection against proteolytic degradation of the subunits. Furthermore, the binding of FaeE might result in a subunit conformation that is required for further steps in the process of fimbriae biosynthesis, as the recognition of and interaction with the outer membrane usher FaeD.

Three possible functions can be assigned to the outer membrane usher FaeD. Firstly, FaeD might form a template at the periplasmic side of the outer membrane on which the fimbrial subunits can assemble into the multimeric fimbrial structure; secondly, the protein probably plays a role in the translocation of the fimbrial subunits across the outer membrane; and finally, the protein may function as an anchor protein. FaeD has been proposed to contain a large central domain of 22 membrane spanning segments and two relatively large periplasmic regions at the amiono-terminal and carboxyl-terminal ends of the protein, respectively (Valent *et al*., 1995B; Harms *et al*., 1999).

Besides FaeE and FaeD, the K88 genetic determinant encodes five fimbrial subunits. FaeG is the so-called major fimbrial subunit, which also carries the adhesive property of the fimbriae (Jacobs *et al*., 1987). The minor subunits FaeC, FaeF, and FaeH form a part of the fimbrial structure, but only in relatively low amounts. FaeC was found at the tip position, whereas FaeH and FaeF were detected along the shaft of the fimbriae. On the basis of the homology between FaeL and FaeH, it has been suggested that Fael is also a minor component of K88 fimbriae (Bakker *et al*., 1992). The minor fimbrial subunits probably play a role in initiation, elongation and/or termination of fimbrial formation (Oudega *et al*., 1989; Bakker *et al*., 1992). The last gene of the K88 operon, faeJ, is probably not expressed (Bakker *et al*., 1992; Mol *et al*., 1995). The topology of the outer membrane usher FaeD

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(Valent et al., 1995) and the interaction of the fimbrial subunits FaeG, FaeH and FaeI with the periplasmic chaperone FaeE have been studied (Mol et al., 1994 and 1995). However, relatively little is known about the two minor fimbrial subunits FaeC and FaeF. These two subunits are remarkably smaller than FaeG, FaeH and FaeI (± 15 kDa versus ± 26 kDa, respectively). Another striking property of FaeC and FaeF is that these minor subunits are absolutely required for fimbriae production. FaeC is produced in very low amounts (a few hundred copies per cell, maybe only one subunit per fimbria). By using immunogold-electron microscopy FaeC has been found at the tip of the fimbriae (Oudega et al., 1989). It is not known whether or not the FaeC protein is dependent on FaeE for its folding and/or protection against proteolytic degradation. Inactivation of faeC completely abolishes fimbriae production (Mooi et al., 1984). Based on these data, it has been suggested that FaeC is a tip protein and involved in the initiation of fimbriae production. FaeC might be the first protein to interact with the usher FaeD and to cross the outer membrane.

FaeF is a rather peculiar minor fimbrial protein because it shows a low degree of similarity with other major or minor fimbrial subunit described so far (Bakker et al., 1992). The stability of FaeF in the periplasmic space is apparently not dependent on FaeE (Mooi et al., 1982). The FaeE-FaeG complex, isolated from cells defective in usher FaeD, contains a small amount of FaeF (Mooi et al., 1983). It is not known whether this FaeE-FaeG-FaeF complex is of any biological significance. Indications about the function of FaeF cannot be derived from its primary structure. Deletion of faeF almost completely abolishes fimbriae formation.

Table 1. Plasmids Used in this Study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Ori</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pINIIIA1</td>
<td>B</td>
<td>Expression vector</td>
<td>Masui et al., 1994</td>
</tr>
<tr>
<td>pSV1</td>
<td>A</td>
<td>Expression vector</td>
<td>Bakker, this laboratory</td>
</tr>
<tr>
<td>pMMP-K4</td>
<td>B</td>
<td>Expression vector, ara promoter/operator</td>
<td>Mayer, 1995</td>
</tr>
<tr>
<td>pMMP-K4-FaeC</td>
<td>B</td>
<td>Containing faeC</td>
<td>This study</td>
</tr>
<tr>
<td>pDB88-8</td>
<td>B</td>
<td>Incomplete K88 gene cluster, faeC-faeJ</td>
<td>Bakker et al., 1992</td>
</tr>
<tr>
<td>pDB88-9</td>
<td>B</td>
<td>Complete K88 gene cluster, faeA-faeJ</td>
<td>Huisman et al., 1994</td>
</tr>
<tr>
<td>pDB88-151</td>
<td>B</td>
<td>pDB88-8 derivative, faeC</td>
<td>Bakker, this laboratory</td>
</tr>
<tr>
<td>pDB88-240</td>
<td>B</td>
<td>pDB88-8 derivative, faeD</td>
<td>Bakker, this laboratory</td>
</tr>
<tr>
<td>pDB88-82</td>
<td>B</td>
<td>pDB88-8 derivative, faeF</td>
<td>Bakker, this laboratory</td>
</tr>
<tr>
<td>pDB88-83</td>
<td>B</td>
<td>pDB88-8 derivative, faeG</td>
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<td>pSV88-C</td>
<td>A</td>
<td>Containing faeC</td>
<td>This study</td>
</tr>
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<td>pSV88-CE</td>
<td>A</td>
<td>Containing faeE and faeE</td>
<td>This study</td>
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<td>pSV88-E</td>
<td>A</td>
<td>Containing faeE</td>
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<td>A</td>
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<td>This study</td>
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<td>pOM88-E</td>
<td>B</td>
<td>Containing faeE and faeF</td>
<td>Mol et al., 1994</td>
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<td>pOM88-F</td>
<td>B</td>
<td>Containing faeE</td>
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<tr>
<td>pOM88-FE</td>
<td>B</td>
<td>Containing faeF and faeE</td>
<td>This study</td>
</tr>
<tr>
<td>pDB88-188</td>
<td>B</td>
<td>Containing faeC and faeD</td>
<td>Valent et al., 1995B</td>
</tr>
<tr>
<td>pDB88-188-Tn10</td>
<td>B</td>
<td>Derivative of pDB88-188 with Tn10 transposon in faeC</td>
<td>Valent et al., 1995B</td>
</tr>
<tr>
<td>pQV88D2</td>
<td>A</td>
<td>Containing faeD</td>
<td>Valent, this laboratory</td>
</tr>
<tr>
<td>p88D215PhoA</td>
<td>B</td>
<td>faeD-phoA fusion containing 215 N-terminal residues of FaeD</td>
<td>Valent et al., 1995B</td>
</tr>
</tbody>
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* Origin of replication: A stands for the p15A origin of pACYC184; B indicates the ColE1 origin of pBR322.

Figure 1. Expression and detection of subcloned gene products. 14% SDS-PAGE of periplasmic fractions of E. coli C600 harbouring various plasmids. The plasmids used are indicated above the lanes. The equivalent of 0.4 OD660nm units of cells was applied to each lane. Panel A, Coomassie brilliant blue stained gel. Panel B, immunoblot developed with a polyclonal FaeC antiserum. Panel C, immunoblot developed with a polyclonal FaeF antiserum. The positions and size (kDa) of the molecular mass markers and the position of FaeC, FaeE and FaeF are indicated.
production (≤ 1% of the “wild-type” level production). E. coli cells harbouring a fae operon with a mutation in faeF are no longer capable to agglutinate guinea pig erythrocytes and can not adhere to brush border cells of piglets (Mooi et al., 1982; Bakker et al., 1992). These results illustrate the importance of Faef for the biosynthesis of K88 fimbriae and suggest an early role for Faef in the assembly process.

In this study we investigated the initial steps in K88 fimbriae biosynthesis by studying the molecular interactions of the subcloned minor component FaeC with the periplasmic molecular chaperone FaeE and with the outer membrane usher FaeD. Periplasmic complexes of FaeC and FaeE could not be identified but a direct interaction between FaeC and the outer membrane usher FaeD was found. The periplasmic 125 amino acid residues of FaeD (≥ 125 residues) are implicated in this binding interaction.

Results

Subcloning and Expression of faeC

The faeC gene was subcloned in the expression vector pSV1 which resulted in pSV88-C (see Experimental procedures and Table 1). The faeC gene was cloned under control of the IPTG inducible lpp/lac promoter/operator system derived from pINIIA1. Furthermore, the cloned gene contained its homologues faeC ribosome binding site. FaeC is synthesized with an amino-terminal leader peptide required for targeting to and secretion across the cytoplasmic membrane. To investigate whether FaeC is dependent on FaeE for its subcellular localization and stability, the periplasmic contents of E. coli cells cultured to the mid-exponential growth phase of growth were analyzed by SDS-PAGE and immunoblotting (Figure 1). The periplasmic fraction of cells harbouring pOM88-E (a pINIIA1 derivative containing the faeC gene) as well as pSV88-C, contained a protein of about 27 kDa (Figure 1A, lane 3), which was not observed in cells carrying control plasmids pINIIA1 and pSV1 (Figure 1A, lane 1). By immunoblotting this 27 kDa protein was identified as the periplasmic chaperone FaeE. In the periplasmic fraction of cells harbouring pINIIA1 as well as pSV88-C, FaeC could not be detected upon protein staining (Figure 1A, lanes 2). However, immunoblotting with specific FaeC antiserum showed a protein band of about 16 kDa in the periplasmic fraction of cells expressing both FaeC and FaeE (Figure 1B, lane 3), which was not observed after protein staining (Figure 1A, lane 3). No reaction was observed when FaeC was expressed in the absence of FaeE (Figure 1B, lane 2).

Apparently, the expression of native FaeC as a soluble protein in the periplasm, in the absence of FaeE, is too low for detection by immunoblotting. In the presence of the chaperone FaeE, however, FaeC is expressed at a higher level in the periplasmic fraction of the cells. Comparable results with respect to the expression of FaeC were found when whole cells were analysed by immunoblotting, indicating that in the absence of FaeE, FaeC or its precursor did not stably accumulate in the cytoplasm or membranes. This suggested that FaeC in the absence of FaeE is probably degraded. Full IPTG-induction of cells harbouring the indicated plasmids did not result in a significant higher expression level of FaeC. Apparently, the IPTG-inducible promoter is rather leaky in the cells and/or in the constructs we used.

Comparable results were obtained when the faeF gene was subcloned and its expression studied (Figure 1A, lanes 4 and 5; Figure 1C, lanes 4 and 5). The FaeF protein was also poorly expressed in the absence of the chaperone, but the periplasm of cells did contain FaeF when FaeE was co-expressed.

Lethality of faeC Overexpression

The low level expression of the faeC gene in cells containing pSV88-C is not surprising. The fimbrial subunits FaeG, H and I are degraded in the absence of the chaperons FaeE (Mol et al., 1994, 1995). Furthermore, the faeC gene is preceded by a sequence showing little similarity to the consensus Shine-Dalgarno (SD) sequence (Mooi et al., 1984). Furthermore, faeC encodes an unusual hydrophobic signal sequence. The FaeC signal sequence has a so-called von Heijne hydrophobicity of -16.4. Preproteins with such an hydrophobic signal sequence are targeted to the cytoplasmic membrane co-translationally via the SRP (signal recognition particle) route (Giersch, 1989; Davis et al., 1996; Valent et al., 1995). This targeting route can be blocked by overexpression of SRP-dependent preproteins. Upon changing the faeC SD sequence into the consensus sequence and/or exchanging the FaeC signal sequence by that of the less hydrophobic one of the periplasmic protein β-lactamase or that of the outer membrane protein PhoE, the expression of FaeC was found to be raised but also strong lethality was observed upon overexpression of FaeC, also in the presence of FaeE. Cells could hardly be transformed by the newly constructed plasmids containing the (hybrid) faeC gene or the transformants that were obtained grew extremely poor (results not shown).

Expression of faeC in the Absence of FaeE

To avoid lethality induction, stress response and protein degradation, the faeC gene was subcloned under the tight control of the ara promoter/operator of pMPM-K4, a derivative of pBlueScript. This resulted in pMPM-K4.FaeC. E. coli cells were transformed with this plasmid and cultured in the absence of the inducer arabinose. At the mid-exponential phase of growth the cells were shortly (1h) induced for the synthesis of FaeC by the addition of arabinose to the culture medium. Under these conditions the periplasm of these cells did contain FaeC (Figure 2). When FaeE was also expressed in these cells, the level of FaeC in the periplasm was not raised. Apparently, no
degradation of FaeC occurs at these conditions. These results furthermore showed that FaeC can be expressed independently of the chaperone.

**FaeC Does not Form a Complex with the Fimbrial Chaperone FaeE**

FaeE forms complexes with the subunits FaeG, FaeH and Fael, which prevents the proteolytic degradation of these proteins and which might help in the folding process of these fimbrial subunits in the periplasm. These protein complexes could be isolated and analysed by iso-electric focusing, native PAGE and FPLC. To investigate a possible interaction of FaeE with FaeC, periplasmic protein fractions of *E. coli* cells harbouring various plasmids were analysed by native iso-electric focusing (IEF) and immunoblotting. Upon immunoblotting using a monoclonal antibody directed against FaeE, a protein band at pH 9.5 was detected in the periplasmic fraction of cells carrying pOM88-E, pSV88-CE (data not shown). No differences in the running behaviour of FaeE was observed when the protein was expressed alone (plasmid pOM88-E) or in combination with FaeC. In all protein fractions, FaeE migrated at a pH comparable to its calculated pI, indicating that there was no detectable amount of complex formed between FaeE and FaeC (Mol *et al.*, 1995). When the focusing blots were developed with FaeC antiserum, co-migration of FaeC with FaeE was not observed. Attempts to visualise FaeE-FaeC complexes using native gel electrophoresis, native immunoprecipitation, or FPLC-ion exchange chromatography, were not successful. In addition, protein cross-linking techniques were used, but failed to show a direct interaction of FaeC and FaeE in the periplasm.

Similar results were obtained when the interaction of FaeF and FaeE was investigated. No complex formation could be detected.

**FaeC Binds to Outer Membranes Containing FaeD**

By immuno-electron microscopy, FaeC was detected at the tip of the fimbriae (Oudega *et al.*, 1989). As a consequence of this tip position, FaeC might be the first protein which is translocated across the outer membrane during the process of fimbriae formation. Thus, FaeC might be the first protein to interact with the outer membrane usher FaeD in this process.

The possible interaction between FaeC and outer membrane containing FaeD was investigated. The periplasmic fraction and the outer membrane fraction of *E. coli* cells harbouring various plasmids were isolated. The presence of FaeC and FaeD in these fractions was analysed by SDS-PAGE and immunoblotting.

In a first experiment, cells carrying pDB88-83 were used. This plasmid contains the K88 gene cluster with a frameshift mutation in the major subunit gene faeG and cells harbouring this plasmid do not express K88 fimbriae (unpublished results; see also Table 1). The usher FaeD was found in the outer membrane fraction as expected and not in the periplasm (Figure 3A, lanes 7 and 8). Interestingly, FaeC was also detected in the outer membrane fraction, but most of the FaeC protein was found in the periplasm (Figure 3B, lanes 7 and 8). Apparently, a small amount of FaeC co-fractionates with the outer membrane fraction. The periplasmic chaperone FaeE was detected only in the periplasm and not in the outer membranes as expected (data not shown).

The subcellular localization of FaeC was also analysed in cells harbouring pDB88-240. This plasmid encodes all K88 specific gene products except the usher FaeD. Cells harbouring pDB88-240 do not produce any fimbriae, but instead accumulate the fimbrial proteins in the periplasm (Mooi *et al.*, 1983). Of course, no FaeD was detected in cells carrying this plasmid (Fig 3A, lanes 5 and 6). FaeC was only detected in the periplasm and not in the outer membranes (Figure 3B, lanes 5 and 6). Obviously, FaeC does not co-fractionate with the outer membranes, when FaeD is not present. Both pDB88-83 and pDB88-240 are derivatives of pDB88-8 (Table 1). Both plasmids carry nearly identical copies of the K88 gene cluster. They differ only in the frameshift mutations in faeG and faeD, respectively. Because the plasmids are derived from the same parental plasmid, one would expect the expression of the proteins, encoded by the two plasmids, to be identical. Nevertheless, the amount of FaeC present in cells harbouring pDB88-240 appeared to be less than the amount of this protein present in cells harbouring pDB88-83. Apparently, in cells producing the usher FaeD, FaeC interacts with the outer membrane and this positively affects its expression level.

Plasmid pDB88-188 carries the subcloned faeC and faeD genes. Cells harbouring this plasmid overproduce FaeC as well as FaeD, but do not express the chaperon FaeE. Although the overproduction of FaeD might result in localization artefact of the usher (Valent *et al.*, 1995), most
of the FaeD protein was found in the outer membranes, whereas minor amounts were found in the periplasm (Figure 3A, lanes 1 and 2). Also FaeC is produced to a higher level in cells harbouring pDB88-188, than in cells harbouring pDB88-83. The majority of FaeC was detected in the outer membrane fraction, but also the periplasm contained some FaeC (as well as FaeD) (Figure 3B, lanes 1 and 2). Apparently, there is a correlation between the amount of FaeD in the outer membranes and the amount of FaeC that co-fractionates with the outer membranes. Control cells harbouring pDB88-188Tn10 do not produce FaeC because of a transposon insertion in faeC (Fig 3B, lanes 3 and 4), but FaeD was found in the outer membrane fraction of these cells (Figure 3A, lanes 3 and 4). Interestingly, FaeC was expressed in cells harbouring pDB88-188, although the chaperone FaeE was not present. Apparently, in the presence of FaeD, the minor component FaeC is somehow stabilized and not degraded as in K12 C600 E. coli cells expressing FaeC and not FaeD.

Outer membranes of cells harbouring pDB88-188 were also purified by isopycnic sucrose density gradient centrifugation. Analysis of the gradient fractions showed that FaeC fractionated exclusively with the outer membranes present in the gradient fractions. FaeC could not be detected in the fractions containing cytoplasmic membranes (not shown).

From these data it can be concluded that, when FaeD is present in the outer membrane of cells expressing FaeC, the expression level of FaeC is raised and the localization of a significant amount of FaeC is shifted to the outer membranes in a chaperone-independent manner. The amount of FaeC that co-fractionates with the outer membrane seems to be dependent on the amount of FaeD present in the outer membrane. The outer membrane localization of FaeC is completely dependent on FaeD and appeared not to be affected by other fae encoded proteins. These results strongly suggested that FaeC binds directly to the usher FaeD in the outer membranes.

**Direct Binding of FaeC to the Usher FaeD**

To show a direct interaction between FaeC and the usher FaeD in the outer membranes, ligand affinity blotting was used. Outer membranes with and without FaeD were isolated and subjected to SDS-PAGE. The separated proteins were transferred to blotting filter paper. Strips of these blots were then incubated with periplasmic extracts containing FaeC, FaeC and FaeE, or no K88 specific proteins at all. After washing the strips were developed with specific antibodies (Figure 4). FaeC was found to bind to the strips at exactly the same position as FaeD or a FaeD degradation product, irrespective of the presence of the chaperone FaeE. No binding of FaeE to FaeD was found at these conditions. The results indicated a direct binding of FaeC to the outer membrane usher FaeD.

FaeF did not co-fractionate with outer membranes containing FaeD under the same condition at which FaeC did, nor did FaeF bind directly to FaeD in an ligand affinity blotting experiment.

**The Amino-Terminal Part of the Usher FaeD is Sufficient for the Interaction with FaeC**

In the present model for the usher FaeD, the protein has an amino-terminal periplasmic segment, a central membrane embedded domain and a carboxyl-terminal periplasmic part (Valent et al., 1995; Harms et al., 1999). Valent et al. (1995) constructed twelve independent FaeD-

![Image](image_url)
PhoA fusion proteins to study the outer membrane topology of the usher FaeD. One of these FaeD-PhoA fusion protein contains 215 amino-terminal amino acid residues of FaeD. The first 126 amino acid residues of this hybrid form a periplasmic domain, whereas residues 127 - 215 contain the first six outer membrane spanning domains of FaeD, according to the present model. This hybrid protein was found to be exclusively located in the outer membrane of \textit{E. coli} (Valent et al. 1995).

The outer membrane fraction of \textit{E. coli} (pSV88-CE) cells also expressing the FaeD-phoA fusion protein was isolated and analyzed by SDS-PAGE and immunoblotting. The FaeC protein expressed by these cells, co-fractionated with the outer membrane fraction of cells expressing FaeD215PhoA (Figure 5). When pDB88-188, encoding the wild-type FaeD protein, was used, FaeC was also detected in the outer membrane fraction. In control cells, not expressing FaeD (pIIIIA1) or the FaeD-PhoA hybrid protein, FaeC was not detected in the outer membranes (Figure 5). These results implied that the interaction of FaeC with the outer membranes containing FaeD or a FaeD domain is dependent on the presence of the 215 amino-terminal amino acid residues of FaeD of which 125 residues are located in the periplasm (Harms et al., 1999). Apparently, the binding site on FaeD for FaeC is located in the amino-terminal periplasmic arm of the usher.

\section*{Discussion}

For FaeC no direct interaction with the chaperone FaeE could be detected. The amount of FaeC in the periplasm of C600 \textit{E. coli} cells, was enhanced in the presence of the chaperone. The reason for this is not known. A possible explanation might be that FaeE co-expressed with FaeC reduces the periplasmic stress response caused by the expression of a fimbrial subunit and thus the proteolytic degradation of FaeC. Another possible explanation might be that FaeE interacts only transiently with FaeC, which results in a protection against proteolytic degradation. This short-lived interaction might also stimulate the folding of FaeC into a protease-resistant conformation. However, this putative interaction is apparently not essential. In the absence of the chaperone, FaeC can be expressed in \textit{E. coli} cells when the protein is expressed under conditions where stress responses and protein degradation (due to overexpression) are kept as low as possible. FaeC can also be expressed in C600 \textit{E. coli} cells co-expressing the usher FaeD. Apparently, the interaction with the usher results in a reduced degradation or in protection against proteolytic degradation, and thus in an enhanced expression.

If FaeC does transiently form a complex with FaeE, this interaction is not stable under the conditions used for the isolation and analysis of these possible complexes. At the same conditions, the FaeE$_2$-FaeG, FaeE$_2$-FaeH and FaeF$_2$-Fae complexes are detectable (Mol et al., 1994 and 1995). However, the FaeE$_2$-FaeH complex dissociated slowly under these conditions, but could be isolated and purified (Mol et al., 1995). In the Pap system the carboxyterminus of PapG and of some other Pap subunits is important for the interaction with the chaperone PapD. The carboxyterminus of PapG interacts by \textit{β}-zippering with a \textit{β}-strand in the cleft of the chaperone (Soto et al., 1998). A consensus sequence was proposed for the carboxyterminus of fimbrial subunits interacting in a comparable manner with their homologues chaperones. The carboxyterminal part of the K88 fimbrial subunits is shown in Figure 6. The carboxy-terminal structure of FaeF does not at all agree with the consensus sequence for chaperone binding. This could be the reason for the lack of interaction between FaeE and FaeF found in this study. The carboxy-terminal structure of FaeC is in reasonable agreement with the consensus sequence, but some specific differences are apparent. The alanine at position -9 is specific for FaeC and differs from the residues in the other subunits, whereas the negatively charged glutamic acid residue at position -13 and the positively charged lysine residue at -16 are quite unique at these positions. Possibly, this explains the poor or absent interaction with the chaperone.

The expression of the proteins FaeC, D, E, F and G is sufficient for the biosynthesis of K88 fimbriae, although the production of these fimbriae is only about ten percent of the wild-type level. These “stripped” K88 fimbriae are still functional in agglutinating erythrocytes and are able to adhere to porcine epithelial brush borders cells (Mooi et al., 1982; Mooi and De Graaf, 1985). Without FaeC and FaeF, fimbriae production is abolished. This argues for an early role in the biosynthesis of K88 fimbriae for these proteins. Since FaeC is located at the tip of assembled extracellular fimbriae (Oudega et al., 1989) and FaeC co-fractionates with outer membranes containing FaeD independent of the presence of other \textit{fae} encoded proteins, it is likely that FaeC is the first protein that interacts with FaeD in the process of fimbriae biosynthesis. In this study a direct interaction of FaeC and FaeD was observed by using ligand affinity blotting. FaeF did not co-fractionate with outer membranes containing FaeD. But preliminary experiments in our laboratory showed the co-fractionation of FaeF with outer membranes containing FaeD when FaeC was also present. This suggests an interaction between FaeF and FaeD-FaeC complexes at the outer membrane and an ordered binding to FaeD of the two minor components located at the tip of the K88 fimbriae.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Alignment of the carboxy-terminal structure of FaeF, C, G, H and I. Conserved and similar amino acid residues (single letter code) are boxed. Cons., consensus sequence; h, hydrophobic residues A, F, G, I, L, M, P and V; Y, tyrosine; a, aromatic residues tryptophan or tyrosine.}
\end{figure}
As mentioned in the introduction section, small amounts of FaeF were also detected in complex with the FaeE2-FaeG complex (Mooi et al., 1983). This demonstrates the ability of FaeF to interact with the major subunit FaeG. Possibly, in the process of K88 fimbriae biosynthesis, FaeF functions as an adapter molecule, coupling the major subunit to the subunit FaeG interacting with FaeD. Once this initiation complex, consisting of FaeC-F and G, has been formed, more FaeG molecules can bind to the base of the fimbriae. The subsequent binding of FaeG molecules probably results in a step-by-step translocation of the fimbriae across the outer membrane. How this translocation is accomplished is not known. In contrast to the transport of proteins across the cytoplasmic membrane, the periplasmic fimbrial proteins are in a highly folded state, identical or nearly identical to the structure of the polymerised subunits. This is demonstrated by the observations that the periplasmic complex of the major fimbrial subunit FaeG and the chaperone FaeE is able to agglutinate erythrocytes (P.T.J. Willemsen, this laboratory) and is recognised by monoclonal antiserum directed against conformational epitopes of K88 fimbriae (Mol et al., 1994). The FaeD proteins might somehow form a pore that is activated by FaeC and through which the K88 structure is transported.

Experimental Procedures

Bacterial Strains and Culture Conditions

E. coli K12 strain C606 014- (mcrA) supE44 thi-1 leuB6 lacY1 tonA21 was routinely used as a host for plasmids. E. coli TOP10F(+) was obtained from Invitrogen. This strain contains, among other characteristics, the lac repressor and is deficient in the Lom protease. This makes it a rather good (better then C600) cloning strain for vectors containing recombinant genes and the lac promoter/operator. We used this strain in the later stages of this project and that it also appeared to be a good host for the vector containing the ara promoter/operator and for studying the expression of subcloned FaeC at conditions that avoid/reduce induction of expression stress.

YT medium (Miller, 1972) was routinely used for the culturing of E. coli strains. When required, ampicillin (100 µg/ml), tetracycline (10 µg/ml), kanamycin (25 µg/ml) and/or chloramphenicol (30 µg/ml) were added to the culture medium. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM) or arabinose (0.01% w/v) was used for induction of plasmid encoded gene products.

Plasmids

The plasmids pDB88-8 (Bakker et al., 1992), pDB88-9 (Huisman et al., 1994), pDB240 (Mooi et al., 1982), pOM88-E, pOM88-GE (Mol et al., 1994), pDB88-188, pDB88-188-Tn10 and p88D215PhoA (Valent et al., 1995) have been described (see also Table 1). Plasmids pDB88-151, pDB88-240, pDB88-82 and pDB88-63 are derivatives of pDB88-8. Plasmid pDB88-151 contains a stop codon mutation in faeC. pDB88-240 carries a frameshift mutation in faeE. pDB88-82 contains a frameshift mutation in faeF, whereas pDB88-63 contains a frameshift mutation in FaeE. Plasmid pQV88D2 was constructed by cloning the Avil-BamHI DNA fragment of pDB88-151, containing the faeE gene, into the EcoRV-BamHI sites of pACYC184.

For the construction of a compatible plasmid that can coexist with pNIIA1 derivatives (with a pBR322 origin) and that allows a high level of expression of K88 encoded genes, the p15A origin and chloramphenicol resistance marker of pACYC184 and the lpp/lac promoter/operator system laci gene, and multiple cloning site of pNIIA1 (Chang and Cohen, 1978; Masui et al., 1984) were combined in the expression vector pSV1. This vector was constructed by ligating the 4409 bp Xmnl-NruI fragment of pNIIA1 and the 2462 bp XbaI-HindII-Klenow polymerase treated fragment of pACYC184. Subsequently, the XbaI-BamHI DNA fragment of pOM88-E containing the faeE gene was cloned into the corresponding sites of pSV1. This newly constructed plasmid was designated pSV88-E.

Plasmid pSV88-CE, encoding faeC and faeE, was constructed by cloning of a Polymerase Chain Reaction (PCR) amplified fragment of faeC into pSV88-E. Two oligonucleotides, 5′-aag aat tca tct aga taa gct ttt gcc ggc aga tgt gc-3′ and 5′-gca tct aga atg ccc ggt gtc aga cga aac ac-3′, complementary to DNA sequences flanking the faeE gene, were used for the PCR amplification (30 cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min 20 s at 72 °C) of the faeE gene. Plasmid pDB88-9 (Huisman et al., 1994) was used as a template for the PCR. Both primers contain an additional XbaI site which was used to clone the PCR fragment into the XbaI site of pSV88-E. The cloned faeE gene was checked by nucleotide sequencing. Plasmid pSV88-C was constructed by deleting a Psfl fragment, which removed the major part of the faeE gene.

Plasmid pOM88-8E, encoding faeC and faeE, was constructed by cloning of a PCR-amplified fragment of faeE into pOM88-E. Two oligonucleotides, 5′-aag aat tca tct aga taa gct ttt gcc ggc aga tgt gc-3′ and 5′-gca tct aga atg ccc ggt gtc aga cga aac ac-3′, complementary to DNA sequences flanking the faeE gene, were used for the PCR amplification (30 cycles of 50 s at 95 °C, 30 s at 50 °C and 1 min 20 s at 72 °C) of the gene. Plasmid pDB88-9 (Huisman et al., 1994) was used as a template for the PCR. The first primer contains an EcoRI site which was used to clone the PCR fragment into the EcoRI-SmaI sites of pOM88-E, giving pOM88-8E. The cloned faeE gene was checked by nucleotide sequencing. Plasmid pOM88-8F was constructed by deleting a BamHI fragment, containing the faeE gene. Plasmid pSV88-8F was constructed by cloning the XbaI-BamHI DNA fragment containing the faeE gene into the corresponding sites of pSV1.

DNA Techniques and Sequence Analysis

All basic recombinant DNA procedures were carried out essentially as described (Sambrook et al., 1989). Nucleotide sequence analysis was carried out according to the dideoxy-chain-termination procedure (Sanger et al., 1977) using an semi-automated DNA sequencer (Applied Biosystems/Perkin and Elmer, model 373A).

Gel Electrophoresis, Isoelectric Focusing, Immunoblotting and Antisera

SDS-PAGE was carried out essentially as described by Laemmli (1970). Isoelectric focusing was carried out in vertical mini slab gels using the Bio-Rad mini protein II slab cell. The 5 % acrylamide (5 % T, 3.3 % C) gels contained 2.4 % amphoteries (LKB.). Polyspheric acid (10 mM) was used as the anode buffer and NaOH (20 mM) was used as the cathode buffer. For immunoblotting, proteins were transferred onto nitrocellulose filters essentially as described by Krone et al. (1986). For blotting of native gels, the pH of the blotting buffer was 9.5. The blots were incubated with an appropriate antisera and rabbit anti-mouse or goat anti-rabbit antisem conjugated to horse radish peroxidase. Blots were developed by using an enhanced chemolaminumine (ECL) detection kit (Amersham).

Polyclonal rabbit antisera directed against the major components FaeC (Oudega et al., 1989) and Faef (Bakker et al., 1992) have been described before. A polyclonal mouse antisera directed against the outer membrane protein FaeD has been described by Valent et al. (1995). Polyclonal and monocular mouse antisera directed against the periplasmic chaperone FaeD have been described (Mol et al., 1994).

Subcellular Localization of Proteins

For the subcellular localization experiments, the expression of proteins of interest were not induced or moderately induced to avoid expression phenomena (Valent et al., 1995). The isolation of periplasmic fractions was carried out essentially as described elsewhere (Mol et al., 1995). After centrifugation (5 min, 10,000 x g) the supernatant fractions containing the periplasmic proteins, were collected and stored at -20 °C. The remaining spheroplasts were resuspended in PBS and disrupted by sonification (3 x 30 sec with 1 min intervals at 50 watts). The remaining intact cells were removed by centrifugation (5 min, 8,000 x g). Subsequently, outer membranes were isolated by centrifugation (10 min at 500,000 x g). The pellet fractions were washed and resuspended in PBS. These fractions contain mainly outer membranes contaminated with cytoplasmic membranes; no traces of periplasmic proteins were detected (immunoblotting).

For the separation of cytoplasmic- and outer membranes, the isolated membrane fractions were subjected to isopycnic sucrose density gradient centrifugation, essentially as described elsewhere (Osborn et al., 1972). A 30-65 % (w/v) sucrose gradient was used. After centrifugation, 11 fractions were collected from each gradient. The density and protein contents of the gradient fractions were determined by means of a refractometer and absorption at 280 nm, respectively. Fractions containing outer membranes were pooled. This resulted in purified outer membranes.
**Ligand Affinity Blotting**

The direct interaction between FaeC and the outer membrane usher FaeD was studied by using ligand affinity blotting. This technique was carried out essentially as described by Snyder et al. (1997).

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**References**


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