Epitope Tagging Analysis of the Outer Membrane Folding of the Molecular Usher FaeD Involved in K88 Fimbriae Biosynthesis in *Escherichia coli*

Nellie Harms¹, Wendy C. Oudhuis¹, Eleonora A. Eppens¹, Quido A. Valent¹, Margot Koster², Joen Luirink¹, and Bauke Oudega*¹

¹Department of Molecular Microbiology, Institute of Molecular Biological Sciences, BioCentrum Amsterdam, Faculty of Biology, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands
²Department of Molecular Cell Biology, Institute of Biomembranes, Utrecht University, 3584 CH Utrecht, The Netherlands

Abstract

To analyse the outer membrane folding of the molecular usher FaeD, tagged derivatives were prepared and their expression, tag-localisation and functioning in K88 fimbriae biosynthesis was studied. A semi-random insertion mutagenesis approach with factor X₄ cleavage sites yielded six tagged FaeD derivatives. A site-directed mutagenesis approach in which c-myc epitopes were inserted yielded twenty-one different derivatives. Four tagged FaeD constructs were not expressed in the outer membrane as full-sized proteins to levels that could be detected by using immunoblotting analyses. Two of these had an insertion in the amino-terminal part of FaeD, whereas the other two had a tag inserted in the carboxy-terminal part. The latter ones yielded stable carboxy-terminally shortened truncates of about 70 kDa, as did other mutations in this region. Six tagged derivatives were expressed but the location of the tag with respect to the outer membrane could not be determined, possibly due to shielding. Functional analysis showed that insertion of a tag in two regions of FaeD, a central region of approximately 200 amino acid residues (a.a. 200-400) and the carboxy-terminal region (a.a. 600-end), resulted in a defective K88 fimbriae biosynthesis. In-frame deletions in the amino-terminal region of FaeD abolished fimbriae production. The integrity of these regions is obviously essential for fimbriae biosynthesis. Based on the results and with the aid of a computer analysis programme for the prediction of outer membrane β-strands, a folding model with 22 membrane spanning β-strands and two periplasmic domains has been developed.

Introduction

K88 fimbriae are long and flexible protein structures at the surface of specific enterotoxigenic *Escherichia coli* strains (De Graaf and Mooi, 1986). These K88 fimbriae are composed of different protein subunits. FaeG is the major subunit which also contains the adhesive property of the fimbriae (Jacobs et al., 1987; Bakker et al., 1992). FaeC, FaeF and FaeH are minor fimbrial subunits, that function in initiation, elongation and/or termination of fimbriae formation (Mooi et al., 1982; Oudega et al., 1989; Bakker et al., 1992). The FaeD and FaeJ proteins show significant homology to FaeH and are probably also minor fimbrial components, although it is not clear whether FaeJ is expressed (Bakker et al., 1992).

FaeE and FaeD do not form an integral part of K88 fimbriae. They are cell envelope proteins with specific functions in the biosynthesis of the fimbriae. FaeE is a dedicated periplasmic chaperone, that in its native state is a homodimer (Mol et al., 1994). FaeD is a relatively large protein which is expressed to only a few hundred copies per cell (Mooi et al., 1981). The protein is located in the outer membrane (Valent et al., 1995) and involved in the translocation of the fimbrial subunits across this membrane (molecular usher).

In a previous study, Valent et al. (1995) constructed twelve different FaeD-PhoA fusions and analysed the outer membrane localisation and protease accessibility of these fusion proteins as well as of the wild-type FaeD. The results indicated that FaeD possesses a relatively large central, membrane spanning region inaccessible to proteases and two periplasmic regions, one at the amino-terminal and one at the carboxy-terminal end of the protein. A putative topology model for the membrane-located domain was proposed, but this was only based on computer predictions.

In this study, c-myc-epitope-tagging was employed to study the outer membrane folding of the membrane associated central region of the molecular usher FaeD in more detail. Furthermore, the effects of the different tagging-mutations on the functioning of FaeD in K88 fimbriae biosynthesis was investigated. A folding model with 22 membrane spanning β-strands is presented.

Results and Discussion

Tagging of FaeD

To study the localisation of the FaeD internal, periplasmic loops and external cell-surface loops, first a semi-random mutagenesis approach was chosen. Plasmid pOV88D was used for these mutagenesis experiments, since *E. coli* HMS174 cells or TOP10F cells harbouring this plasmid were found to express FaeD in the outer membrane to a level that is comparable to the wild-type expression level (Valent et al., 1995). The *faeD* gene, subcloned in pOV88D,
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a The insertion mutations were constructed in pOV88D. The six FaeD constructs with an insertion of a factor Xa-recognition-site are indicated by the code “DXa”. The constructs with the code “Dmyc” contain a c-myc insertion mutation. The number behind the code indicates the amino acid number of the mature FaeD after which the tag was inserted. A * indicates that in this construct two additional c-myc epitopes were inserted at the same position, so in total three epitopes, in order to obtain a stronger tag signal.

b The expression level of FaeD and its tagged derivatives was estimated by denaturing SDS-PAGE and immunoblotting (FaeD antiserum) of various dilutions of isolated outer membrane preparations of cells harbouring a pOV88D-derivative plasmid encoding the tagged FaeD. ++ in the top line, indicates the amount of FaeD found in the isolated outer membranes of E. coli HMS174; ++ for a tagged FaeD derivative indicates a comparable expression level, —tr, no positive immunoblotting signal could be found at the position of FaeD, but in stable truncated proteolytic breakdown products were detected.

c The position of the tag with respect to the outer membrane was investigated as described in experimental procedures. Symbols: nd, not detectable, in, tag located at the periplasmic side of the outer membrane; out, tag found at the cell surface by native immuno-spot-blotting and immunofluorescence, at the outside of the cells, np, non-permissive (proteolytic breakdown of FaeD, no c-myc signal detectable).

d The functioning of the tagged FaeD protein in K88 fimbriae production was studied by complementation analysis (see experimental procedures). Symbols: ++, amount of K88 fimbriae at cell surface (between 10-40% of wild-type); +, strongly reduced level of K88 fimbriae at cell surface (between 1-10% of wild-type); —, no fimbriae found, construct defective in K88 biosynthesis.

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Expression and Topological Analysis of c-myc-Tagged FaeD

Cells harbouring a pOV88D-derivative plasmid encoding one of the tagged FaeD proteins were cultured to the mid-exponential phase of growth. To estimate the expression level of the tagged FaeD mutant proteins, cells and isolated outer membranes were analysed by SDS-PAGE and denaturing immunoblotting. The topology of the c-myc epitopes was investigated as described in experimental procedures. The results of these experiments are summarised in Table 1 and an example of an immunofluorescence assay is shown in Figure 2.

Most of the tagged FaeD proteins were expressed in the outer membrane comparable to the wild-type FaeD encoded by pOV88D. Four c-myc derivatives, could not be detected as full sized FaeD derivatives, however degradation products were detected. Two of these derivatives had an insertion in the amino-terminal part of FaeD, at position 152 and 204, whereas the other two had a c-myc insertion at the carboxyl-terminal end of the protein (number 711 and 727). The latter two formed stable truncates. Obviously, these insertions were not permissive and resulted in a disturbed localisation, insertion and/or folding, followed by proteolytic degradation. Topological studies could not be carried out with these FaeD-derivatives (see Table 1). Two other c-myc derivatives (344 and 595) were poorly expressed, but degradation products were hardly detected. The topological position of these two c-myc epitopes could not be determined.

Six c-myc derivatives (174, 321, 384, 404, 552 and 613) showed a normal expression level at denaturing conditions, but the topology studies were not successful. The reason for this could be shielding of the epitope at native conditions. To enhance the c-myc signal of these six constructs, two additional c-myc epitopes were constructed next to the first one (see experimental procedures). These FaeD-derivatives, that now contained three c-myc epitopes inserted at the indicated position (indicated by a * in Table 1), were still found to be expressed to a level comparable to wild-type FaeD. In five of these

contains 42 HpaII endonuclease restriction sites. Of these HpaII sites 22 are located in the same reading frame. These 22 HpaII sites were used for the semi-random insertion of a HpaII linker containing the protease factor Xd cleavage site (see experimental procedures). Six FaeD insertion mutants were obtained (Figure 1). Immunoblotting analysis showed that cells harbouring one of these six pOV88D-derivatives expressed full-sized FaeD in their outer membrane (Table 1). It was not clear why the other (sixteen) insertion mutants were not found. One reason could be that these factor Xa cleavage sites were inserted into structural important positions of the FaeD protein, which led to misfolding of the usher and cell-lethality (putative non-permissive sites). A comparable phenomenon has been found by Schifferli and Alnutt (1994) for the 987P molecular usher. Another possibility could be that some of the HpaII restriction sites in faeD were poorly cleaved as compared to the ones that led to a successful linker insertion. Topological studies with the six FaeD-derivative proteins were not successful because treatment of whole cells or isolated outer membranes with Xa protease did not results in cleavage. Possibly, these sites are not accessible due to hindrance of outer membrane components.

Subsequently, a more site-specific approach was chosen. The inserted factor Xa linker in faeD contains a unique SacI cleavage site. This site was used for the insertion of a linker encoding a c-myc epitope. Furthermore, fifteen rather hydrophilic and charged regions of FaeD were selected and a unique SacI site was constructed in either of these regions. These sites were then used for the insertion of a c-myc linker (Figure 1). In total twenty-one myc-tagged FaeD derivatives were obtained.
the orientation of the c-myc epitopes with respect to the outer membrane could now be determined (Table 1).

**Folding Model**

In a previous study, Valent et al. (1995) proposed a preliminary folding model for FaeD based on the analysis of FaeD-PhoA fusions and protease accessibility studies. In this model, FaeD had a relatively large central, membrane spanning, region inaccessible to proteases and two periplasmic regions, one at the amino-terminal and one at the carboxyl-terminal end of the protein, respectively. Twenty four membrane spanning β-strands were predicted in the membrane protected region, only on the basis of computer analysis. Based on the topology studies presented above and by using a computer analysis programme for the prediction of outer membrane β-strands (Gromiha et al., 1997), we developed a modified model for FaeD (Figure 3).

**The Amino-Terminal Region in the Periplasm**

In the model, FaeD has an amino-terminal periplasmic domain of 126 amino acid residues. The computer programme predicted five putative β-strands in this region, at positions 3-11, 18-38 (2 strands), 63-73 and 100-109, respectively (indicated in Figure 3 by the staggered amino acids). However, c-myc epitopes at position 93 and 116 were clearly found at the periplasmic side of the outer membrane. All FaeD-PhoA fusions in this region were also detected as soluble hybrid proteins in the periplasm (Valent et al. 1995). Furthermore, we presume that the two cysteine residues at positions 59 and 87, are also located in the periplasm, possibly forming a cysteine double bond with each other or with one of the cysteine residues located at the carboxyl-terminus. Thus, the positions of these five predicted membrane β-strands are not in agreement with the experimental data. They might fold into periplasmic β-strands required for the structural organisation of this region. Obviously, the computer programme is not capable to discriminate between periplasmic and outer membrane β-strands. We have experienced similar phenomena with other predictive programmes.

Complementation analysis showed that the FaeD derivatives with a c-myc insertion in the periplasmic region were still functioning in K88 fimbriae biosynthesis (Table 1). The mutations in this region had no effect on the structural stability of the protein as did mutations in the carboxyl-terminus (see below). To further test the functioning of this periplasmic segment of FaeD, several derivatives, FaeDΔ26-93, FaeDΔ26-116, FaeDΔ93-116 and FaeDΔ116-131, were constructed with an in-frame deletion in the amino-terminal periplasmic domain (see
Expression and functional analysis experiments showed that these deletions had no effect on the outer membrane localisation of the usher and on its stable expression. However, they did block the functioning of FaeD in fimbriae biosynthesis.

The Central Membrane Spanning Region

The central region of FaeD, from amino acid residue tyrosine\(^{127}\) to valine\(^{522}\) spans the outer membrane 22 times as predicted by the analysis programme used and in agreement with the experimental data. A relatively high number of aromatic residues, tyrosine, tryptophan and phenylalanine, are found at the lipid-water interface in this central membrane spanning region. The c-myc epitope at position 174 is the first one found outside the cell in external loop 2, whereas the c-myc epitope at position 503, close to the last predicted external loop, is the last epitope detected outside the cell. Furthermore, the c-myc epitopes at position 321, 404 and 478 were also found at the outside. Residues 404 and 503 are not located in predicted external loops as the three other ones (174, 321, 478), but just at...
the membrane-outside interface of β-strands 16 and 21, respectively. The three inserted epitopes at position 404 and the epitope at position 503 are obviously located at the outside of the cells (see experimental data) extending external loops 8 and 11.

The epitope at positions 435 was found in the cells. The epitope(s) at residue positions 344, 384 and 485 could not be located, although the FaeD-derivatives containing these epitopes were expressed. These residues are all located inside membrane spanning β-strands. Obviously the detection of these epitopes is blocked by membrane constituents or segments of FaeD. The proposed membrane spanning strand 13 is apparently important for the structural stability of the usher, since the c-myc insertion in this strand reduced the expression (numbers 344). Two epitope insertions in the membrane spanning region were found to be non permissive (numbers 152 and 204) and resulted in a defective expression and proteolytic breakdown of FaeD. These two positions are obviously of structural importance.

Valent et al. (1995) found a stable protease resistant FaeD fragment of about 45 kDa when trypsin was added from the outside of the cells. This fragment was supposed to be a central membrane protected segment of FaeD. In the present model there is a putative trypsin susceptible site in external loop 1 and one in the last external loop 11. Trypsin cleavage of these sites could give a fragment of approximately the size found by Valent et al. (1995).

Most of the FaeD derivatives with epitopes in the central membrane spanning region were not functional anymore in K88 fimbriae biosynthesis, with the exception of the last four insertions. The latter regions in FaeD are apparently not strictly required for fimbriae expression.

The Carboxyl-Terminal Region
Valent et al. (1995) predicted in the former FaeD folding model a carboxyl-terminal periplasmic fragment of 176 amino acid residues. This prediction was mainly based on a few FaeD-PhoA fusions that localised in the periplasm, the presence of a pair of cysteine residues and the absence of predictable β-strands. In the present model a carboxyl-terminal periplasmic domain of 255 amino acid residues is proposed, mainly based on experimental data. Five c-myc insertions, at positions 529, 552, 570, 613 and 644, were found in the cell, indicating a periplasmic location of this carboxyl-terminal region. Two other c-myc epitopes, at position 711 and 727, could have given more information, but FaeD derivatives with an epitope insertion at these positions were not stable. FaeD with a c-myc epitope inserted at position 644 was stable. Other insertion mutations in the region beyond 644, that we tried to construct, were extremely unstable and could not be recovered. Apparently, this area of the protein is important for the structural organisation of the protein as well as for its functioning in fimbriae biosynthesis. Most of the point mutations, small deletions and insertions that we tried to construct in the area between 644 and 777 resulted in a stable carboxyl-terminally shortened truncate of approximately 70 kDa (± 640 residues, see also Mooi et al., 1982). Apparently, the last 130-140 carboxyl-terminal residues of FaeD are rather susceptible to proteolytic degradation upon structural changes.

The computer programme predicted 10 membrane spanning β-strands in the last 255 amino acid residues of FaeD. These are indicated by the staggered stretches of amino acids in this region in Figure 3. An alternative, membrane spanning model for the carboxyl-terminal region is shown in Figure 4, which is only based on computer predictions. However, since there is no experimental data to support a membrane location of this carboxyl-terminal domain and the computer based models in the periplasmic domain gave also unreliable information, we feel that the model presented in Figure 3 is the most reliable one.

Channel Function of FaeD
Thanassi et al. (1998) showed that the molecular usher PapC, involved in the Pap pilus biogenesis across the E. coli outer membrane forms an oligomeric channel. This channel, possibly a hexamer, has a diameter of approximately 2 nm, large enough to accommodate a pilus subunit or the linear form of the-tip fibrillum. PilQ is involved in type IV pilus biogenesis and constitutes a homologue of XcpQ and PulD which are required for protein transport across the outer membrane (Bitter et al., 1998). PilQ and XcpQ were also found to form large oligomeric structures of 10-12 monomers with large central diameters of approximately 5.3 nm (Bitter et al., 1998). These data suggest a common structure and organisation for the members of these protein transporter families. We carried out various isolation procedures for the K88 usher FaeD, which were comparable to the procedures used for the isolation of the oligomeric structures of PapC, PilQ and XcpQ. However, we were not able to detect oligomeric FaeD complexes. Furthermore, various protein cross-linking experiments were carried out, as were done for PapC (Thanassi et al., 1998). Also, these experiments did not result in the identification of large oligomeric FaeD structures. Possibly, the putative quaternary structure of FaeD in the outer membrane is less stable than that of PapC, PilQ and XcpQ, or FaeD is different from the other molecular ushers studied so far in that it does not function as an oligomer. The latter statement might be true, since the estimated copy number of FaeD per cell (± 200-400) and the number of fimbriae per cell (a few hundred) does not support a model in which the functional FaeD unit in the outer membrane contains 6 or 10-12 FaeD subunits.

A model for the functioning of FaeD in fimbriae biosynthesis might be that FaeD forms a pore by itself. Recently, the structures of two outer membrane proteins with a pore function, FhuA (Locher et al. 1998; Ferguson et al. 1998) and FepA (Buchanan et al. 1999), have been solved. These proteins have a size similar to FaeD and each of these proteins have 22 membrane spanning antiparallel β-strands organized in a barrel structure and a compact, amino-terminal barrel-filling domain. In our present model, FaeD has 22 membrane spanning β-strands, like FhuA and FepA and two periplasmic domains. One of these domains (possibly the amino-terminal domain) could form a barrel-filling domain, like in FhuA and FepA, whereas the other domain might function in subunit sorting, binding and fimbriae formation.

Experimental Procedures

Bacterial Strains, Plasmids and Culture Conditions
Escherichia coli strains HMS174 F-, recA hsdR recF1774 F- (Campbell et al., 1978) and TOP10F' (Invitrogen, Ca USA) were used as hosts for the subcloning of the faeD gene or a mutated faeD gene into the pET9a expression vector without hindrance of possible lethal background
expression. E. coli strain BL21(DE3) FompTmrecO (DE3) harbouring pLYSE (Studier et al., 1990) was used for the expression of faeD cloned in pQV88D.

Plasmid pT9a (Studier et al., 1990) was used as an expression vector. Plasmid pQV88D contains the complete gene (faeD) encoding the K88 fimbral operon. The construction of this plasmid has been described (Valent et al., 1995). Plasmid pFM205, a pBR322-derivative containing the K88 fimbral operon has been described (Mooi et al., 1979). Plasmid pFM240, containing the same operon as pFM205 but with a mutation in the faeD gene that results in a large carboxy-terminal deletion in Faed has been described by Mooi et al. (1982). Cells containing pFM205 produce K88 fimbriae, whereas carrying pFM240 do not. A pACYC184 derivative containing the K88 operon of pFM240 was constructed during the course of this study.

A S. typhimurium strain (Miller, 1972) was routinely utilized for the culturing of E. coli strains. When necessary, ampicillin (100 µg/ml), kanamycin (40 µg/ml), tetracycline (12 µg/ml) and/or chloramphenicol (25 µg/ml) were added to the culture medium. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.4 mM) was used for induction of the faeD gene encoded by the plasmid pQV88D.

DNA Manipulations, Linkers and Sequence Analysis
All basic recombinant DNA procedures were carried out essentially as described (Sambrook et al., 1989).

The factor Xε linker was constructed by annealing two custom-made oligonucleotides, 5′-CTG GAT ATT TTA ATA GAA GGA GAA GCT ATT TA-3′ and 5′-C GTA ATT AAG AGC TTC TCT TTC TCC TAT TAT AA-3′. An alternative linker was described by Osterlund et al., 1982. Insertion of this linker in the wrong orientation results in stop codons in all reading frames and in the synthesis of truncated FaeD polypeptides. The linker also contains a unique SacI restriction site. The SacI linker encoding the c-myc epitope was constructed by annealing two custom-made oligonucleotides, 5′-TGA CAG CAT GTA AGC TTC TCT TTC TCC TAT TA-3′ and 5′-CGG AGG ATG CCT ACC CTT TCT TTC TCC TAT AA-3′. An alternative linker was described by Osterlund et al., 1982. Insertion of this linker in the correct orientation into faeD results in the synthesis of a full-sized FaeD protein with 11 additional amino acids (Val-Ile-Leu-Ile-Glu-Gly-Ala-Leu-Ser-Tyr). Four of these amino acid residues form the factor Xε cleavage site Ile-Glu-Gly-Arg (cleavage after Arg). Insertion of this linker in the correct orientation results in stop codons in all reading frames and in the synthesis of truncated FaeD polypeptides. The linker contains a unique BglII restriction site. A comparable linker but containing two c-myc epitopes and BglII restriction sites at both ends was also constructed. This linker was used to enhance the reaction of c-myc antisera with several Faed derivatives that constructed one copy of the c-myc epitope but failed to give a reasonable signal in the assays used.

Nucleotide sequence analysis was carried out according to the dideoxy chain-termination procedure (Sanger et al., 1977), using the semi-automated DNA sequencer of Applied Biosystems (model 373A) and custom-made oligonucleotides.

Tagging of FaeD
For random linker insertion mutagenesis of faeD using the factor Xε linker, plasmid pQV88D was linearised by partial digestion with the restriction endonuclease HpaII II, in the presence of ethidium bromide (Österlund et al., 1982). Plasmid pQV88D contains 64 SacI sites; 42 of these sites are randomly located in faeD; 22 of these 42 are in the same reading frame. The full-sized, linear form of the plasmid was isolated and ligated with the unphosphorylated factor Xε linker (Lathe et al. 1984). The Xε linker was constructed to give only in-frame insertions in one of the 22 HpaII sites of faeD mentioned above. The ligation mixture was then used to transform competent HMS174 cells with selection for kanamycin resistance. Subsequently, transformants were screened for the presence of the Xε linker in pQV88D by colony hybridisation using a digoxigenin-labelled factor Xε primer. Selected clones were further analysed for the presence of the linker in faeD by restriction analysis of isolated plasmid DNA. Finally, clones which contained the linker in the correct orientation were selected by immunoblotting using specific FaeD antisera. The nucleotide sequence of the mutated faeD gene present in the selected plasmids was then checked by nucleotide sequence analysis.

For site-specific tagging of Faed two approaches were used. The faeD constructs containing the Xε linker were inserted into the c-myc linker in the unique SacI site of the Xε linker. In another approach several unique SacI sites were created in the faeD gene of pQV88D by site-directed mutagenesis using custom made mutagenesis primers and the Quickchange™ site-directed mutagenesis kit of Stratagene Cloning Systems, CA, USA. These SacI sites were thus used to insert the c-myc epitope linker. Selection of faeD insertion derivatives was carried out essentially as described for the selection of the Xε tagged derivatives. Correct clones were then further identified by immunoblotting with c-myc antiserum and FaeD antiserum, and by nucleotide sequence analysis.

The expression level of the tagged FaeD derivatives was estimated by SDS-PAGE and immunoblotting of whole cells and isolated outer membranes, using FaeD and c-myc antisera.

Construction of Amino-Terminal Deletion Derivatives
For the construction of FaeD derivatives with a deletion in the amino-terminal periplasmic region, unique Sad sites at amino acid position 26, 83, 116 and 131 were pFM240 in the faeD gene of pQV88D by site-directed mutagenesis using custom made mutagenesis primers and the Quickchange™ site-directed mutagenesis kit. By using other unique restriction sites on both ends of the faeD gene in pQV88D and the created Sad sites, the in-frame deletion Δ26-93, Δ26-116, Δ83-116 and Δ116-131 were prepared. The constructed deletion derivatives were checked by nucleotide sequencing.

Topological Analysis of faeD Insertion Mutations
To study the location of the factor Xε cleavage site in mutated Faed, cells were cultured to the mid-exponential phase of growth and then collected by centrifugation. Next, the cells were washed and resuspended in factor Xε incubation buffer (30 mM Tris-HCl pH 8.0, 0.12 M NaCl, 1 mM CaCl2). Factor Xε (Boehringer Mannheim) was added together with 10 mM MgCl2 to stabilise the outer membranes or in combination with 5 mM EDTA to destabilise the membranes. Following incubation (2 h, 25 °C) proteolytic degradation was stopped by the addition of the protease inhibitor phenylmethylsulfonylfluoride (PMSF). Cells were then collected, solubilised in solubilisation mixture for denaturing SDS-PAGE, and analysed by immunoblotting. Essentially the same procedure was used for the analysis of isolated outer membranes containing mutated Faed. Outer membranes were isolated as described before (Valent et al., 1995).

For the topological analysis of c-myc tagged Faed, dilutions of whole cells were analysed by native blotting using the c-myc antisiser (Boehringer, Mannheim). As a control serum specific antibodies directed against the outer membrane protein OmpA and recognising only the periplasmic domain of OmpA was used. This serum was kindly provided by Tommassen, Utrecht, The Netherlands. Whole cells that reacted with c-myc antisiser and not with the OmpA antisiser were supposed to expose the c-myc epitope at the cell surface. Whole cells that did not react with the c-myc epitope were permeabilised using an EDTA treatment and analysed by native spot blotting. In addition, isolated outer membranes were analysed by native blotting. Cells that reacted positively in the latter two assays and negatively in the assays using intact, whole cells were supposed to have the c-myc epitope at the periplasmic side of the outer membrane. Cells that did not react in any of these tests were supposed to have a c-myc epitope buried in the membrane or heavily shielded by outer membrane components.

In another approach cells were analysed by immunofluorescence microscopy in collaboration with Tommassen and Koster (Utrecht University) essentially as described by Merck et al. (1997).

Gel Electrophoresis, Immunoblotting and Antiseras
Denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially as described (Laemmli, 1970). Proteins were transferred onto 0.45 µm nitrocellulose filters as described (Towbin et al., 1979). The blots were then incubated with an appropriate antiserum and rabbit anti-mouse antibodies conjugated to horse radish peroxidase (DAKO-immunoglobulins, Denmark) and subsequently developed by enhanced chemiluminescence (ECL, Amersham).

Polyclonal mouse antibodies against Faed have been described before (Valent et al., 1995). Monoclonal antibodies against K88 fimbiare have been characterised by Van Zijlderveld et al. (1990). Antibodies against the c-myc epitope were obtained from Boehringer, antibodies against the periplasmic domain of OmpA were a gift from Tommassen (UU, The Netherlands).

K88 Fimbriae Production and Complementation Analysis
The K88 fimbriae production of cells was either tested by native spot blotting of dilutions of cells by or ELISA using whole cells. For K88 fimbiare detection, the monoclonal K88 antisiser conjugated to horseradish peroxidase was used (Pol et al., 1994; Valent et al., 1995).

For the functional analysis of the constructed faeD insertion mutants a complementation assay was carried out. Cells containing a pACYC184 derivative (pACYC184-240) with the K88 operon of mutant plasmid pFM240 (defective in Faed usher production) were also transformed with one of the constructed pQV88D mutant plasmids and the K88 fimbiare production was tested by ELISA and/or native spot blotting.
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References


