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Regulation and structure of an *Escherichia coli* gene coding for an outer membrane protein involved in export of K88ab fimbrial subunits

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ABSTRACT

The nucleotide sequence of the faeD gene of Escherichia coli and the amino acid sequence of its product is presented. The faeD product is an outer membrane protein required for transport of K88ab fimbrial subunits across the outer membrane. The protein is synthesized as a precursor containing a signal peptide, and the tentative mature protein comprises 777 amino acid residues. The distribution of amino acids in the faeD protein is similar to that of other outer membrane proteins; showing a fairly even distribution of charged residues and the absence of extensive hydrophobic stretches. Secondary structure predictions revealed a region of 250 amino acid residues which might be embedded in the outer membrane.

The 5'-end of faeD is located within a region showing dyad symmetry. This region serves to couple translation of faeD to the translation of the gene preceding it (faeC). The 3'-end of faeD shows an overlap of 5 bases with the next gene (faeE).

INTRODUCTION

Fimbriae are extracellular filamentous proteins found on a wide range of Gram-negative bacteria (1). They are mainly composed of a single protein subunit with a molecular weight ranging from 17,000 to 30,000. Fimbriae play an important role in bacterial disease, because they enable bacteria to colonize host-tissues by means of a specific binding to host-receptors. Together with haemolysins and bacteriocins, fimbrial subunits belong to the few proteins of Escherichia coli that pass both the cytoplasmic and the outer membrane. Little is known about the mechanism by which these proteins are transported across the cell-envelope, however it has become clear that for each of these three groups of proteins specific "helper" proteins are required for this process (2,3,4). The genes for these helper proteins appear to be part of the same gene cluster that encodes the exported proteins.

The subject of this paper, the K88ab gene cluster, has been shown to contain at least 5 structural genes (faeC-H, fae: fimbrial adhesin eightyeight) which are located within a single transcriptional unit (Fig.

1)(5,6). The cluster harbors two fimbrial subunit genes. The first (faeC) is expressed at a low level and codes for a small fimbrial subunit. The second (faeG) is expressed at a high level and codes for the large fimbrial subunit which constitutes the major component of the K88ab fimbria. Genetic evidence suggests that the small fimbrial subunit is a minor component of the K88ab fimbria (7). In addition to these two fimbrial subunit genes, the K88ab DNA contains three other genes (faeD, E and F) which are essential for the production of fimbriae. FaeD and E code for an outer membrane and a periplasmic protein, respectively (8). Both proteins are required for transport of fimbrial subunits across the cell-envelope (6,9). The faeF product is located in the periplasmic space, and presumably involved in modification of the fimbrial subunit (9). It is not yet clear what the function of the fifth gene (faeH) is.

For a number of reasons we are particularly interested in the structure of the faeD polypeptide. First, it is involved in transport of proteins (fimbrial subunits) across the cell-envelope, a process about which very little is known. Insight in this phenomenon requires information about the structure of the proteins involved. Second, a large number of fimbrial gene clusters have been analyzed (see 4 and 10 for recent reviews) and, although they may differ in many respects, they invariably code for a similar if not homologous polypeptide. Comparison of the primary structure of this class of proteins may give us insight in molecular evolution and allow us to discern between variable and conserved sequences, and hopefully to correlate these with particular functions.

In this paper we present the sequence of the faeD gene and the primary and secondary structure of its product. Furthermore, we show that efficient translation of faeD requires prior translation of the small fimbrial subunit gene (faeC).

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strains HB101 (11), JM101 (12) and SA11343 (13) were used as bacterial hosts. The plasmid vectors were pBR322 (14), pTG2 (15) and pKG1800 (13).

DNA manipulations

Plasmid isolation, gel electrophoresis of DNA, plasmid transformation and enzymatic manipulations of DNA were carried out by standard methods (16).

DNA sequencing

DNA sequences were determined using the dideoxynucleotide chain termination method (17), combined with the M13 cloning system (12). More than 95% of the sequence was performed on both strands. In addition, all restriction sites used to clone fragments for sequence analysis, were overlapped by sequence determination of different DNA fragments.

Construction of plasmids

Plasmid pFM317 (Fig. 4) was constructed by inserting a 1028 bp HindIII-HincII K88ab DNA fragment (Fig. 1) between the HindIII and SmaI sites of pKG1800. This places the K88ab DNA between the gal promoter and the galK gene of pKG1800. Plasmid pFM317d (Fig. 4) was derived from pFM317, by excission of a 32 bp BstEII fragment. This BstEII fragment contains a SmaI site, and plasmids containing the desired deletion were identified by cleavage with SmaI.

Plasmid pFM315 (Fig. 4) was constructed by inserting a 845 bp EcoRV - HincII K88ab DNA fragment (Fig. 1) between the EcoRI and BstEII sites of pTG2. To this purpose, the single stranded extensions of EcoRI- and BstEII-cleaved pTG2 molecules were removed with S1 nuclease. Cleavage of pTG2 with EcoRI and BstEII removes the part of the bla gene encoding the signal peptide of β-lactamase (15), and the described procedure was expected to result in an in-frame gene fusion between the 5'-end of faeD, and the part of the bla gene coding for the mature β-lactamase. DNA sequence analysis confirmed that the desired fusion was obtained (Fig. 4). Plasmid pFM315d (Fig. 4) was derived from pFM315 by excission of a 32 base pair BstEII fragment as described for pFM317d.

Immunoblotting

Immunoblotting, using antibodies directed against β-lactamase, was performed as described by Krone et al. (18).

Enzyme assays

Galactokinase was essayed as described by McKenney et al. (13). To determine the amount of β-lactamase produced, cells were grown in Trypticase Soy Broth (Oxoid) to an optical density at 660 nm of 0.5. Subsequently, cells were harvested, washed with 0.1 M phosphate buffer pH 7.5, and suspended in the same buffer to an optical density at 660 nm of 2.0. Cells were broken by ultrasonic treatment, and the amount of β-lactamase in the cell-free extracts was determined using the chromogenic substrate nitrocefin (Glaxo) (19).

Computer assisted analysis

The hydrophilicity profile of the faeD protein was determined using

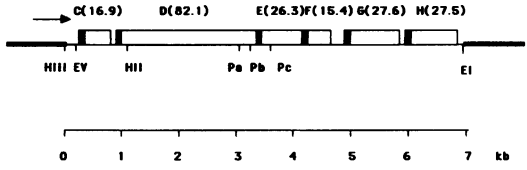


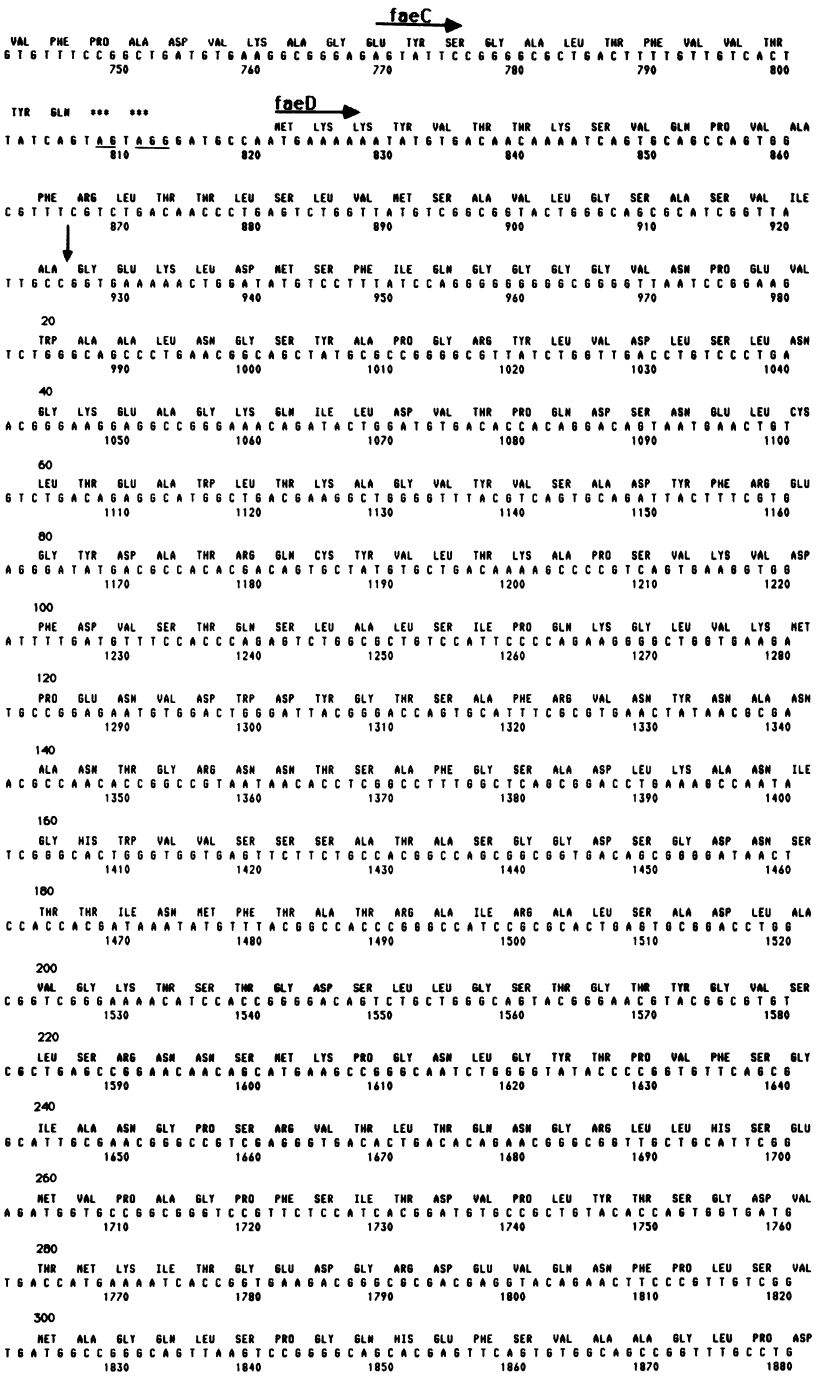
Fig. 1. Genetic and physical map of the K88ab gene cluster. The thick black lines and the thin lines represent pBR322 and cloned K88ab DNA, respectively. The locations of the various structural genes are indicated by boxes. The black ends of the boxes indicate parts of genes coding for the signal peptides. The numbers between parentheses refer to the molecular masses in kilodalton of the gene products. The arrow indicates the direction of transcription. Only relevant restriction sites have been indicated. EI, EcoRI; EV, EcoRV; HII, HincII; HIII, HindIII; Pa, Pb, Pc, PstI; kb, kilobases.

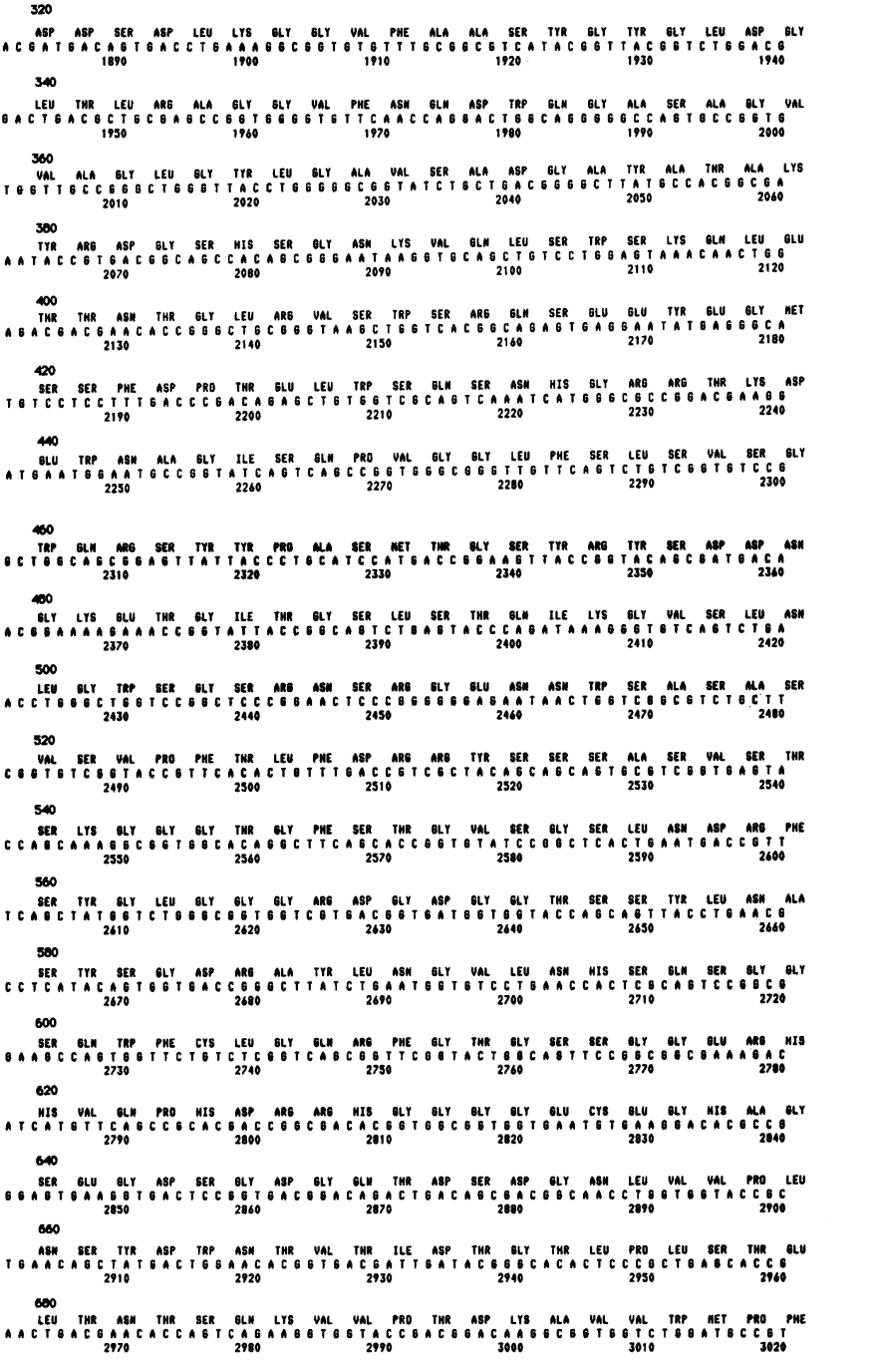
solvent parameter values assigned by Levitt (20). A span setting of seven residues was used for this program. Protein secondary structure analysis was carried out according to the algorithm developed by Chou and Fasman (21).

RESULTS

Nucleotide sequence and identification of the coding sequences.

Fig. 1 shows a genetic and physical map of the cloned K88ab gene cluster. The precise location of the K88ab genes within the cloned fragment was determined previously (5,6), and faeD was shown to be located between faeC and the PstI-c site. The nucleotide sequence of the region located between bases 741 and 3320 (Fig. 1) is presented in Fig. 2. The sequence of the first 817 bases (containing faeC) has been published (7). The DNA sequence shown in Fig. 2 specifies an open translational reading frame between the bases 815 and 3256, whose location is consistent with that of faeD. The 5'-end of faeD contains two potential initiation codons, the second of which is probably positioned more optimally relative to the Shine-Dalgarno sequence (22). The ribosome binding site of faeD is located in an area which has the potential to form a stable secondary structure (Fig. 3), suggesting that this site may not always be accessible to initiating ribosomes (see below). The 5'-end of faeD codes for a signal peptide, which is in agreement with our observation that faeD codes for a precursor which is 2,000 to 3,000 dalton larger than the mature polypeptide (5). The most likely cleavage site for signal peptidase was determined by the method of Von Heijne (23). The tentative mature polypeptide is predicted to comprise 777 amino acid residues and the molecular weight calculated from the amino acid sequence (82,065) agrees well


faeC
faeD


320
ASP ASP SER ASP LEU LYS GLY GLY VAL PHE ALA ALA SER TYR GLY TYR GLY LEU ASP GLY
1890 1900 1910 1920 1930 1940
340
LEU THR LEU ARG ALA GLY GLY VAL PHE ASN GLN ASP TRP GLN GLY ALA SER ALA GLY VAL
1950 1960 1970 1980 1990 2000
360
VAL ALA GLY LEU GLY TYR LEU GLY ALA VAL SER ALA ASP GLY ALA TYR ALA THR ALA LYS
2010 2020 2030 2040 2050 2060
380
TYR ARG ASP GLY SER HIS SER GLY ASN LYS VAL GLN LEU SER TRP SER LYS GLN LEU GLU
2070 2080 2090 2100 2110 2120
400
THR THR ASN THR GLY LEU ARG VAL SER TRP SER ARG GLN SER GLU GLU TYR GLU GLY MET
2130 2140 2150 2160 2170 2180
420
SER SER PHE ASP PRO THR GLU LEU TRP SER GLN SER ASN HIS GLY ARG ARG THR LYS ASP
2190 2200 2210 2220 2230 2240
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GLU TRP ASN ALA GLY ILE SER GLN PRO VAL GLY GLY LEU PHE SER LEU SER VAL SER GLY
2250 2260 2270 2280 2290 2300
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TRP GLN ARG SER TYR TYR PRO ALA SER MET THR GLY SER TYR ARG TYR SER ASP ASP ASN
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GLY LYS GLU THR GLY ILE THR GLY SER LEU SER THR GLN ILE LYS GLY VAL SER LEU ASN
2370 2380 2390 2400 2410 2420
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SER LYS GLY GLY GLY THR GLY PHE SER THR GLY VAL SER GLY SER LEU ASN ASP ARG PHE
2550 2560 2570 2580 2590 2600
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SER TYR GLY LEU GLY GLY GLY ARG ASP GLY ASP GLY GLY THR SER SER TYR LEU ASN ALA
2610 2620 2630 2640 2650 2660
580
SER TYR SER GLY ASP ARG ALA TYR LEU ASN GLY VAL LEU ASN HIS SER GLN SER GLY GLY
2670 2680 2690 2700 2710 2720
600
SER GLN TRP PHE CYS LEU GLY GLN ARG PHE GLY THR GLY SER SER GLY GLY GLU ARG HIS
2730 2740 2750 2760 2770 2780
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HIS VAL GLN PRO HIS ASP ARG ARG HIS GLY GLY GLY GLY GLU CYS GLU GLY HIS ALA GLY
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SER GLU GLY ASP SER GLY ASP GLY GLN THR ASP SER ASP GLY ASN LEU VAL VAL PRO LEU
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660
ASN SER TYR ASP TRP ASN THR VAL THR ILE ASP THR GLY THR LEU PRO LEU SER THR GLU
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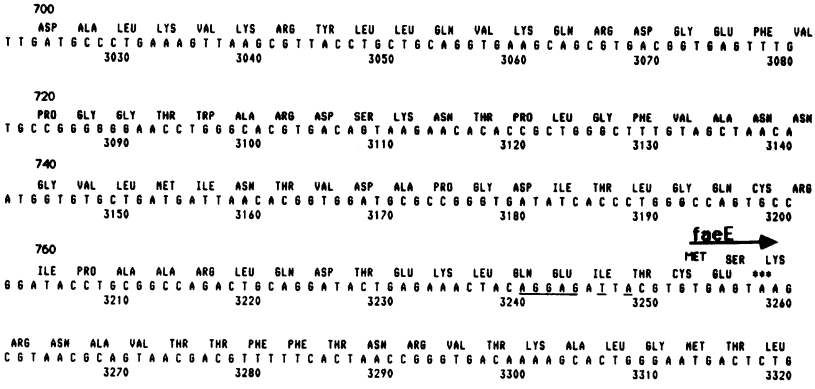

Fig. 2. Nucleotide sequence of faeD. Bases are numbered starting from the HindIII site (see Fig. 1). The vertical arrow indicates the presumed cleavage site for signal peptidase. The deduced amino acid sequence is numbered from the N-terminus of the mature protein. Nucleotides complementary to the 3'-end of 16 S rRNA are underlined. The DNA and corresponding amino acid sequences of the end of faeC and the start of faeE are also shown.

with the molecular weight estimated from SDS-polyacrylamide gels (81,000)(5). The DNA sequence indicates that there is an overlap of 5 bases between faeD and the next gene, faeE (Fig. 2).

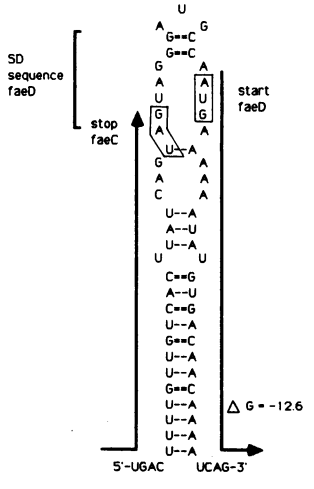

Fig. 3. Possible secondary structure formed by the K88ab mRNA. Terminator (in faeC) and initiator (in faeD) codons have been blocked. ΔG was calculated according to Tinoco et al. (25). SD, Shine-Dalgarno sequence.

Function of the region of dyad symmetry.

As mentioned above, the 5'-end of faeD is located in a DNA region showing dyad symmetry. The transcript of this region has the potential to form a stable stem and loop structure (Fig. 3), and two functions can be envisaged for this structure. First, it may be involved in termination of transcription (24). Second, since the structure sequesters the Shine-Dalgarno sequence as well as the initiator codon of faeD, it may affect initiation of translation of faeD. The 3'-end of faeC extends into the region of dyad symmetry (Fig. 3). Therefore, translation of faeC will disrupt the secondary structure, and might affect transcription termination and/or translation of faeD.

To study the effect of the stem and loop structure on transcription we inserted a K88ab DNA fragment, containing faeC, the region with dyad symmetry and the 5'-end of faeD, between the gal promoter and the galK gene of pKG1800 (Fig. 4). In pKG1800, galK is preceded by stop codons in all three translational frames (13), so that the constructed plasmid (designated pFM317) allowed us to evaluate the effect of signals in the inserted K88ab DNA fragment on transcription only. To study the influence of translation of the 3'-end of faeC on transcription termination, it was necessary to relocate the translation termination codon of faeC so that ribosomes translating faeC could not disrupt the stem and loop structure. The DNA sequence of faeC (7) revealed that this could be accomplished by deleting a 32 bp BstEII fragment from faeC. Excission of the BstEII fragment results in a frame-shift which relocates the stop codon of faeC some 170 bases before the region of dyad symmetry. The plasmid containing this deletion was derived from pFM317 and designated pFM317d (Fig. 4). Plasmid pKG1800 harbors the β-lactamase gene, and to compare the copy numbers of pKG1800 and its derivatives pFM317 and pFM317d, the amount of β-lactamase produced by strains containing these plasmids was also determined (Fig. 4).

Insertion of the K88ab fragment reduced galK expression with 52% (Fig. 4). The reduction in galK expression might be due to termination of transcription or a decrease in plasmid copy number. Since the β-lactamase determinations suggested that insertion of the K88ab DNA fragment in pKG1800 increased its copy number, the latter possibility seems unlikely. Relocation of the translation termination codon of faeC, did not result in a significant change in copy number or galK expression. Thus, it seems unlikely that translation of faeC affects termination of transcription.

To study the effect of the stem and loop structure on the translation of the faeD message, we constructed a gene fusion between the 5'-end of faeD and

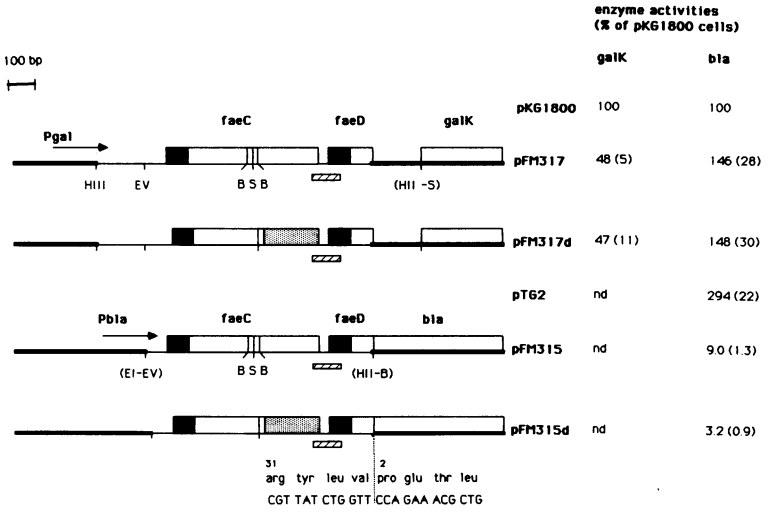

Fig. 4. Plasmids used to study the effect of the K88ab DNA region showing dyad symmetry on transcription and translation. Thick black lines represent pKG1800 DNA (in pFM317 and pFM317d) or pTG2 DNA (in pFM315 and pFM315d). Thin lines represent K88ab DNA. Structural genes are indicated by boxes. The black ends of the boxes indicate parts of genes coding for signal peptides. The location of the region showing dyad symmetry is indicated by the shaded box. The arrows indicate the direction of transcription. Plasmids pFM317d and pFM315d contain a small deletion in faeC, which results in premature termination of translation 170 bases before the region of dyad symmetry. The untranslated region of faeC in these plasmids is indicated by the stippled box. The sequence of the fusion junction between faeD and bla is shown at the bottom of the figure. Numbers above the sequence refer to the amino acid residues of the mature forms of the two proteins. Enzyme activities detected in cells containing the various plasmids are shown on the right. Maps of pKG1800 and pTG2 are not included in the figure. Plasmid pKG1800 contains the bla gene in addition to the galK gene. Galactokinase and β-lactamase activities detected in cells containing pKG1800 have been arbitrarely set at 100%. The values are the mean of at least three independent determinations. The numbers between parentheses indicate standard deviations. Letters between parentheses indicate restriction sites that have been lost due to the cloning procedure. Pgal, galactose promoter; Pbla, β-lactamase promoter; galK, galactokinase; bla, β-lactamase; B, BstEII; EI, EcoRI; EV, EcoRV; HII, HincII; HIII, HindIII; S, SmaI; bp, base pairs; nd, not determined.

the part of the bla gene coding for the mature β-lactamase. To this purpose, a K88ab DNA fragment, containing faeC, the region with dyad symmetry and the 5'-end of faeD, was inserted within the bla gene of pTG2 (see Materials and Methods). The sequence of the fusion junction was determined and it appeared that the expected in-frame fusion was obtained (Fig. 4). The plasmid containing the gene fusion was designated pFM315, and a derivative of pFM315

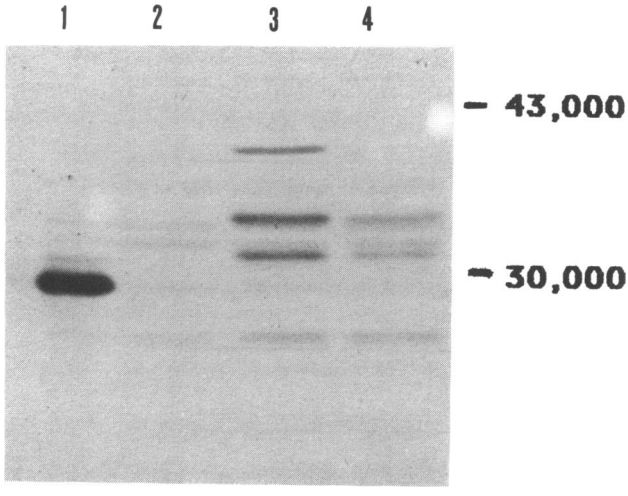

Fig. 5. Identification of faeD-bla fusion polypeptides by immunoblotting. Cells were grown as described for the β-lactamase assay (Materials and Methods), harvested and suspended in sample buffer to an optical density at 660 nm of 20. After heating the cell suspensions for 5 min. at 100°C, polypeptides were separated on a 12.5% SDS-polyacrylamide gel. Equal volumes of the various preparations were applied to the gel, except for the pTG2 preparation, in which case a fourfold smaller volume was used. After electrophoresis, polypeptides were transferred to nitrocellulose and fusion polypeptides were detected with β-lactamase antibodies. Lane 1, strain HB101 with pTG2; lane 2, strain HB101; lane 3, strain HB101 with pFM315; lane 4, strain HB101 with pFM315d. The position of two molecular weight markers is indicated on the right.

(designated pFM315d) (Fig. 4) was constructed to relocate the translational stop codon of faeC, as described for pFM317d. The β-lactamase activity detected in cells containing pFM315 was very low compared to cells with pTG2 (Fig. 4). Furthermore, if the translation of the 3'-end of faeC was prevented a threefold reduction in β-lactamase activity was observed (Fig. 4). This indicates that the region of dyad symmetry is involved in translational control of faeD. Apparently efficient translation of faeD requires prior translation of faeC.

The faeD-bla fusion products were also studied by means of immunoblotting using antibodies directed against β-lactamase. In cells containing pFM315 three bands were observed which reacted specifically with β-lactamase antibodies (Fig. 5). The slowest migrating band presumably contains the unprocessed fusion protein, because it migrated at a position that corresponds with the calculated molecular weight of the unprocessed fusion

product (i.e. 36,002). The two faster migrating bands probably contain the processed fusion protein (calculated molecular weight 32,266), and a degradation product, respectively. In cells harboring pFM315d the putative unprocessed fusion protein was generally not detectable, while the two other fusion proteins were present in much lower amounts compared to cells containing pFM315 (Fig. 5). Thus, the immunoblot experiments also indicated that translation of faeC enhanced translation of faeD.

DISCUSSION

Structure of the faeD polypeptide.

The faeD polypeptide is similar in several respects to other outer membrane proteins (26). It has an appreciable content of charged amino acid residues (19%), and does not exhibit extensive segments of hydrophobic residues. The largest segment devoid of charged or polar groups is 13 residues long and located between residues 357 and 369 (Fig. 2). The hydrophilicity profile of the protein confirms that, like other outer membrane proteins, it is not particularly hydrophobic (Fig. 6).

Secondary structure analyses of outer membrane proteins has shown that they generally have a high β-sheet and a low α-helix content (26). This suggests that regions of proteins which are embedded in the outer membrane have a β-sheet conformation. Paul and Rosenbusch (27) have suggested a folding pattern for outer membrane proteins, in which transmembrane β-sheets of 6 to 24 residues are located between turns. The faeD protein differs from most other analyzed outer membrane proteins, in that Chou and Fasman analysis predicts approximately equal amounts of α-helix and β-sheet (27 and 32%, respectively) (Fig. 6). However, between residues 450 and 700 a region is observed which is devoid of α-helixes, and which shows an alternation of turns and sheets. It is possible that this part of the molecule consists of transmembrane sheets connected by coils or turns which are exposed at the cell surface or in the periplasmic space.

Mutations in the faeD gene abolish fimbria formation, and result in the accumulation of the large fimbrial subunit (pG) in the periplasmic space, where it is found associated with the faeE protein (pE) (9). Apparently, the faeD protein is involved in transport of fimbrial subunits across the outer membrane. We presume that it is a transmembrane protein that binds the pG-pE complexes at the periplasmic side of the outer membrane, transports pG across the membrane, initiates the polymerization of the subunits, and anchors the fimbria to the cell. By examining the topology of the molecule in the outer

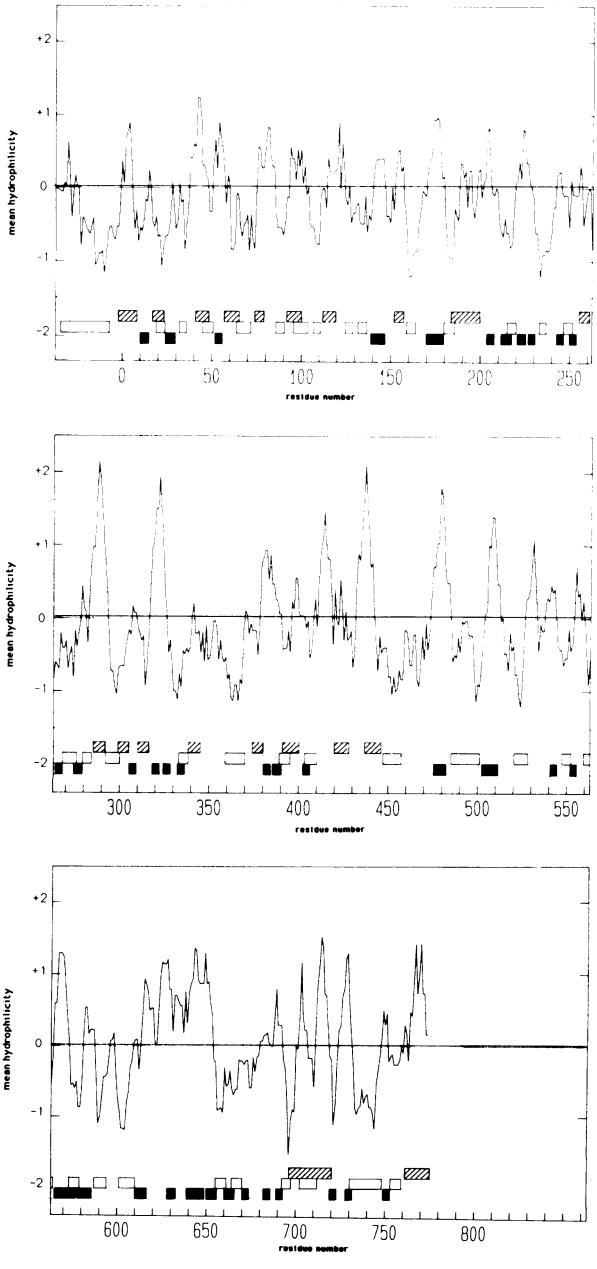

Fig. 6. Hydrophilicity profile and secondary structure prediction of the faeD polypeptide. In the hydrophilicity profile, relatively hydrophilic regions appear above the zero line. Amino acid residues are numbered from the N-terminus of the mature protein. Black boxes, β-turns,; open boxes, β-sheet; shaded boxes, α-helix.

membrane and by means of site-specific mutagenesis, we hope to be able to assign these functions to various domains of the molecule.

Regulation of the faeD gene.

The 3'-end of faeC and the 5'-end of faeD are located in a region showing dyad symmetry (Fig. 3). Thus, depending on whether or not the faeC gene is translated, the transcript of this region may form a secondary structure. The secondary structure might be involved in termination of transcription or, since it sequesters the ribosome binding site of faeD, in translational control of faeD.

To study the effect of this DNA region on transcription, it was inserted between the gal promoter and the galK gene of pKG1800. Insertion of the K88ab DNA fragment reduced galK expression with 52% (Fig. 4), indicating that transcription is terminated within the K88ab DNA fragment. When translation of the 3'-end of faeC was prevented, no further reduction in galK expression was observed. This might indicate that translation of faeC occurs too infrequently to significantly affect the formation of the secondary structure, and thus transcription termination. It is also possible that the reduced galK expression is not due to this secondary structure, but to a decreased mRNA stability, or to the presence of as yet unidentified transcriptional terminators within the K88ab DNA fragment.

A gene fusion between the 5'-end of faeD and the β-lactamase gene was used to determine whether the region showing dyad symmetry was involved in translational control. To this purpose, the amount of fusion protein produced was determined by means of a β-lactamase assay and immunoblotting. These experiments revealed that, if translation of the 3'-end of faeC was prevented, synthesis of the fusion protein decreased. The most likely explanation for this phenomenon is, that the ribosome binding site of faeD is masked by mRNA secondary structure if the 3'-end of faeC is not translated. A similar mechanism controls the translation of the lysis gene of bacteriophage MS2 (28). It should be noted that translation of faeC is terminated within the Shine-Dalgarno sequence and very close to a start codon of faeD (Fig. 3). Therefore, it is conceivable that (components of) a ribosome terminating translation of faeC could reinitiate translation at faeD without being released from the mRNA molecule. Such a mechanism has been suggested for genes which have overlapping stop and start codons (21), or stop and start codons that are separated by only a few bases (30).

It is not clear to what extent translation of faeD is enhanced by prior translation of faeC. The β-lactamase assay suggested that translation of faeC

increased translation of faeD only threefold. However, the immunoblot experiments suggested a much larger increase (Fig. 3). This discrepancy might be caused by the fact that increased synthesis of the fusion protein does not result in a proportional increase in the amount of its enzymatically active form. This assumption is confirmed by immunoblotting experiments, which show that increased synthesis of the fusion protein leads to a disproportional large increase in the amount of its unprocessed form, which is probably enzymatically inactive (31). It has been shown that sequestration of a ribosome binding site in a secondary structure with approximately the same thermodynamic stability as the K88ab structure, may reduce translation of a gene twentyfold (32). This suggests that unmasking of the ribosome binding site of faeD, by ribosomes translating faeC, may enhance translation of faeD approximately twentyfold.

Fusion of the translational initiation signals of faeD to the bla gene dramatically reduces its expression. This indicates that faeD is translated infrequently, which is consistant with the observation that the faeD protein is present in low amounts in the cell (5).

Thus, faeC and faeD form a translationally coupled gene pair. Translational coupling may be a means to ensure a proper molar production of polypeptides that are part of the same structure (29). We have not yet been able to locate the product of faeC, but in view of the afore mentioned, it seems likely that it is part of a structure that also contains the product of faeD. It is possible that pC is part of the basal structure of the fimbria where it might form a link between pD and the filamentous structure composed of pG. This assumption is supported by the observation that, in the absence of pC, pG is not assembled into fimbriae but accumulates in the periplasmic space (unpublished experiments).

There is an overlap of 5 bases between faeD and faeE (Fig. 2). It is not clear what the function of this overlap is. It seems unlikely that the overlap is involved in translational coupling, since the faeD protein is produced in much lower amounts than the faeE protein (5). Furthermore, a mutation which relocates the stop codon of faeD and abolishes the overlap between the two genes, does not alter the amount of the faeE protein synthesized in minicells (not shown).

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