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Permissive Linker Insertion Sites in the Outer Membrane Protein of 987P Fimbriae of *Escherichia coli*

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The FasD protein is essential for the biogenesis of 987P fimbriae of *Escherichia coli*. In this study, subcellular fractionation was used to demonstrate that FasD is an outer membrane protein. In addition, the accessibility of FasD to proteases established the presence of surface-exposed FasD domains on both sides of the outer membrane. The *fasD* gene was sequenced, and the deduced amino acid sequence was shown to share homologous domains with a family of outer membrane proteins from various fimbrial systems. Similar to porins, fimbrial outer membrane proteins are relatively polar, lack typical hydrophobic membrane-spanning domains, and possess secondary structures predicted to be rich in turns and amphipathic β-sheets. On the basis of the experimental data and structural predictions, FasD is postulated to consist essentially of surface-exposed turns and loops and membrane-spanning interacting amphipathic β-strands. In an attempt to test this prediction, the *fasD* gene was submitted to random in-frame linker insertion mutagenesis. Preliminary experiments demonstrated that it was possible to produce *fasD* mutants, whose products remain functional for fimbrial export and assembly. Subsequently, 11 *fasD* alleles, containing linker inserts encoding β-turn-inducing residues, were shown to express functional proteins. The insertion sites were designated permissive sites. The inserts used are expected to be least detrimental to the function of FasD when they are inserted into surface-exposed domains not directly involved in fimbrial export. In contrast, FasD is not expected to accommodate such residues in its amphipathic β-strands without being destabilized in the membrane and losing function. All permissive sites were sequenced and shown to be located in or one residue away from predicted turns. In contrast, 5 of 10 sequenced nonpermissive sites were mapped to predicted amphipathic β-strands. These results are consistent with the structural predictions for FasD.

Many gram-negative bacteria express adhesive fimbriae (39). These organelles consist of helical arrangements of protein subunits (4, 13). Fimbrial biogenesis on the bacterial surface requires subunit export across two membrane barriers. Typically, genes encoding the proteins required for fimbrial biogenesis are clustered together with fimbrial structural genes on a single replicon. Many of the accessory proteins have amino-terminal export signal sequences which are cleaved off by a host- or fimbrial type-specific signal peptidase (25, 46), indicating that they cross the cytoplasmic membrane. Moreover, since the export of the well-studied type 1 fimbriae has been shown to require SecA (10), it is commonly assumed that all fimbria-specific proteins which translocate through the cytoplasmic membrane utilize host components of the general export system (46).

After crossing the cytoplasmic membrane of *Escherichia coli*, the fimbria-specific accessory proteins form a second transport system translocating fimbrial subunits across the outer membrane (9). Genetic studies have indicated that 987P fimbriation and adhesion of porcine enterotoxigenic *E. coli* require the expression of eight genes, six of them, *fasB* to *fasG*, encoding such accessory proteins (9, 48). As with other fimbrial types, some accessory proteins of the 987P system may be incorporated in the fimbrial structure, along with the 987P FasA subunit, as minor components (49). All studied fimbrial systems express at least two proteins which are exclusively involved in export functions. The 987P counterparts of these proteins are FasB and FasD. Functional and structural data on FasB indicate that this protein is a periplasmic chaperone for 987P fimbrial subunits (46a, 48). Fimbrial systems characteristically have one chaperone protein associating with fimbrial subunits in the periplasm, protecting them from proteolytic degradation and preventing them from assembling or aggregating prematurely in the wrong subcellular compartment (9, 18, 30). This is consistent with previous studies which showed that certain fimbrial subunits, 987P subunits included, have the capacity to associate spontaneously into fimbria-like structures in vitro in the absence of other proteins (1, 47).

How fimbrial subunits cross the outer membrane to form the fimbrial thread on the bacterial surface remains to be elucidated. Currently, it is known that each fimbrial gene cluster encodes a single 80- to 100-kDa protein that is required for fimbrial export. This protein is characteristically the largest and the only one of every studied fimbrial system which is stably associated with the outer membrane (9). A recent study with the Pap fimbriae has shown that the various subunits of the fimbriae bind with different affinities to the outer membrane protein (OMP) PapC (11). The respective affinities corresponded to the subunit ordering in the fimbrial structure, the subunit with the highest affinity being the first or tip subunit of the fimbriae. Therefore, PapC was proposed to play an active role in determining the correct order of subunit assembly. As such, PapC was designated a molecular usher (11, 22).

For the 987P fimbrial system, FasD was previously identified as the PapC analog since it was the largest protein of this system (48, 49). In this article, FasD exposure on the bacterial surface is established, confirming that it is an OMP. Its primary structure was determined and was shown to exhibit a significant level of homology with the sequences of the largest

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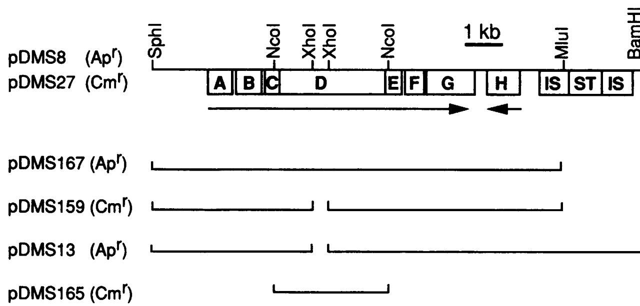

FIG. 1. Physical map of the cloned 12-kb 987P fimbrial gene cluster contained in pDMS8 (pBR322 derivative) or pDMS27 (pACYC184 derivative) and of additional plasmid constructs. The coding region for the eight *fas* genes and the location of the transposon encoding a heat-stable enterotoxin are indicated by boxes. The arrows indicate the directions of transcription of the *fas* genes.

proteins of many other fimbrial systems. This homology suggests the existence of conformational constraints on the structure of FasD and other fimbrial OMPs required for their pivotal role in fimbrial biogenesis. In an attempt to distinguish FasD domains which are required for fimbrial export from less essential sites, the mutagenic approach presented and discussed in this article was carried out.

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MATERIALS AND METHODS

Bacterial strains, media, and reagents. *E. coli* MH6085 (*dcm-6 dam-3 metB galK galT lacY tsx thi ton mtl*?; kind gift from M. Howe), was used to test for *Apa*I linker insertions. Strain CC118 (31) was utilized for detecting and producing FasD-PhoA fusion products. For the immunodetection of FasD, host strain DMS902, a *phoA recA* derivative of nonfimbriated strain MC4100, was used. This strain was constructed by P1 transduction (32) in two steps, preparing phage lysates in SM547 [MC1000 Δ(*phoA-proC*) *phoR tsx*::Tn*5*; kind gift from C. Manoil] and RT628 (JF626 *recA1 srl*::Tn*10*; kind gift from R. Taylor). Nonfimbriated host strain SE5000 (MC4100 *recA56*) (50) was used for all other studies. Cultures for colony isolations or plasmid purifications were grown in LB media (32) with appropriate antibiotics used at the following concentrations: ampicillin, 200 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 45 μg/ml. Media components were purchased from Difco Laboratories (Detroit, Mich.), and, unless specified, reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction and modification enzymes and the *Sma*I linker were from New England BioLabs (Beverly, Mass.), the *Apa*I linker was from Pharmacia LKB Biotechnology (Piscataway, N.J.), and pancreatic DNase was from GIBCO-BRL (Gaithersburg, Md.). Unidirectional removal of DNA was accomplished with the exonuclease III-S1 nuclease kit from Promega (Madison, Wis.).

Plasmid constructs. All plasmid constructs (Fig. 1) originate from pDMS6 and pDMS8 (pBR322 derivatives) or pDMS27 (pACYC184 derivative) containing all of the *fas* genes required for 987P fimbrial expression and adhesion on a 12-kb *Sph*I-*Bam*HI DNA fragment (48, 49). For pDMS8 and pDMS27, the vectors carry T7 promoters upstream of *fasA* to *fasG*, which can be used for specific gene expression as described previously (48). Standard procedures (45) were used to construct the following plasmids. A *Mlu*I-*Bam*HI deletion of pDMS8 (pDMS167) helped to stabilize the plasmid by removing most of the transposon encoding a heat-stable enterotoxin (Fig. 1). This deletion did not affect *fas* gene expression as demonstrated by the fimbriated phenotype of SE5000 carrying pDMS167. Plasmid pDMS13 was derived from pDMS8 as an in-frame *Xho*I deletion in *fasD* (Fig. 1). Plasmid pDMS159 was derived from pDMS27 by deleting both its *Mlu*I-*Bam*HI and *Xho*I fragments as described above. A Klenow enzyme-treated *Nco*I fragment from pDMS8 (Fig. 1) containing *fasD* was inserted, in each orientation, into the *Eco*RV site of the polylinker engineered in the *lacZ* gene of phagemid pKS and pSK (Stratagene, La Jolla, Calif.) to obtain pDMS137, pDMS138, pDMS148, and pDMS149. A vector with a T7 promoter and a chloramphenicol resistance gene was prepared by deleting the *Hin*dIII fragment of pDMS27 containing the *fas* genes (48). This vector was used to prepare pDMS165 by subcloning *fasD* from pDMS148 (*Hin*dIII-*Xba*I fragment) downstream of the T7 promoter. The various *fasD-phoA* fusions originate from a collection of Tn*phoA* mutants of pDMS6 prepared in a previous study (48). The *Hin*dIII-*Xho*I fragment of Tn*phoA* insertion 4.23 encoding the whole fusion product was subcloned into pKS downstream of the T7 promoter to make pDMS168.

Specific labeling of Fas proteins and subcellular fractionation. Fas proteins were specifically labeled for 10 min with a mixture of ^{35}S-methionine and ^{35}S-cysteine (NEN Research Products, Boston, Mass.) by using a T7 expression system as described previously (48, 53). Total protein samples were prepared by centrifuging 0.5 ml of each bacterial culture and solubilizing the pellets in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Outer membrane fractions were prepared from spheroplasts by standard techniques (2). Briefly, 10 ml of labeled bacteria was centrifuged. Pelleted bacteria were resuspended in 90 μl of 30 mM Tris-HCl (pH 8.0)–20% sucrose and transferred to microcentrifuge tubes, and 10 μl of lysozyme (1 mg/ml in 0.1 M EDTA) was added to each sample (40). After 30 min of incubation on ice, the obtained spheroplasts were stabilized with $MgCl_2$ (20 mM final concentration) and centrifuged (15,000 × *g*, 2 min), and periplasmic proteins were removed with the supernatant. The spheroplasts were resuspended in 100 μl of a 10 mM Tris-HCl (pH 8.0)–100 mM NaCl–10 mM $MgCl_2$ solution containing 1 μg of DNase per ml. The spheroplasts were lysed by sonicating twice for 1 min with a Cup Horn accessory at amplitude output 10 (model XL2020; Heat Systems, Farmingdale, N.Y.). To solubilize cytoplasmic membrane proteins, *N*-lauroylsarcosine, sodium salt (ICN Biochemicals, Cleveland, Ohio), was added to a final concentration of 0.5%, and the samples were incubated for 20 min at room temperature. Intact cells were removed by centrifugation at 1,200 × *g* for 10 min at 4°C. Outer membranes were pelleted by high-speed centrifugation in a Beckman JA-18.1 rotor at 17,000 rpm (tube angle, 25°) for 3.5 h in the cold. Pelleted outer membranes were resuspended in SDS-PAGE sample buffer. The quality of the enriched outer membrane fraction was evaluated by comparing the relative signals of labeled FasD with the ones of cytoplasmic chloramphenicol acetyltransferase and periplasmic β-lactamase encoded by the used plasmids (chloramphenicol acetyltransferase by pDMS27 and pDMS159 and β-lactamase by pDMS167).

To localize FasD-PhoA fusion proteins in the periplasm of bacteria grown to the stationary phase, the spheroplasting technique of Witholt et al. was used (57). Briefly, 3 ml of overnight cultures was centrifuged, and pelleted bacteria were resuspended in 50 μl of 200 mM Tris-HCl (pH 8.0). The

following timed additions were made on ice: at 1 min, 1 μl of 0.05 mM EDTA; at 2 min, 50 μl of 200 mM Tris-HCl (pH 8)–1 M sucrose; at 3.5 min, 1 μl of a 6-mg/ml concentration of lysozyme; at 4 min, 100 μl of cold deionized water; at 34 min, 2 μl of 1 M $MgCl_2$. Further steps used to collect periplasmic fractions and isolate outer membranes are described above.

Anti-FasD antibodies. To prepare FasD antigen, the FasD-PhoA fusion protein encoded by pDMS168 was produced in an expression system (53). Since this fusion product was shown to remain soluble in the periplasm, it could be isolated by affinity chromatography as described below. Briefly, strain CC118 (*phoA*) was transformed with pDMS168 and pGP1-2, grown to the late log phase (A_{600} = 1.5), activated for 30 min at 42°C, and grown for an additional 90 min (53). The periplasmic fraction was isolated as described above (40), supplemented with 0.02% azide and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium metabisulfite, 2 μg of aprotinin per ml, 1 mg of leupeptin per ml, 1 mg of pepstatin per ml), and dialyzed overnight at 4°C in phosphate-buffered saline (PBS)–0.1 M EDTA–0.02% azide–0.1 mM phenylmethylsulfonyl fluoride–0.1 mM sodium metabisulfite. The periplasmic fraction was concentrated by ultrafiltration, and the fusion protein was purified by affinity chromatography on an antibacterial alkaline phosphatase antibody column (5 Prime → 3 Prime, Inc., Boulder, Colo.) by conventional procedures (1 to 2 mg protein per liter of culture). Purity was assessed to be >95% by SDS-PAGE and Coomassie blue staining. Rabbits were immunized as described previously. Enzyme-linked immunosorbent assay titers of ≥1/200,000 were reached. Before use, the antibodies were adsorbed with cell lysates of appropriate *E. coli* strains to remove nonspecific antibodies. Moreover, when produced in a *malE* strain, only FasD antigen was detectable by Western blot (immunoblot) analysis.

Protease accessibility to surface-exposed residues of FasD. Intact bacteria and spheroplasts, prepared as described above (40), were submitted to protease treatments. Strain DMS902 with plasmid pDMS165 was grown overnight. Aliquots of 0.5 ml of bacteria were spun, pelleted bacteria were resuspended in 0.1 ml of appropriate buffer, and treated for 30 min at 37°C with either one of three proteases as follows: proteinase K (11.4 U/mg) at 5 mg/ml in PBS; trypsin (9,800 Nα-benzoyl-L-arginine ethyl ester units/mg) at 0.5 mg/ml in 50 mM NH_4HCO_3 (pH 7.8); *Staphylococcus aureus* V8 protease (650 U/mg; Promega) at 0.5 mg/ml in 50 mM NH_4HCO_3 (pH 7.8) for glutamic acid cleavage or at 0.5 mg/ml in 50 mM $NaPO_4$ (pH 7.8) for aspartic and glutamic acid cleavage (17). Reactions were stopped by adding phenylmethylsulfonyl fluoride and 2× sample buffer and by immediately heat treating the samples (100°C, 10 min).

SDS-PAGE and immunoblotting. Protein profiles of whole-cell extracts or of subcellular fractions boiled in sample buffer were determined by SDS-PAGE as described previously (48). Radiolabeled samples were analyzed by fluorography. FasD-PhoA fusion products were analyzed on Western blots by using anti-alkaline phosphatase and peroxidase-conjugated secondary antibodies as described previously (48). Blots were developed by using 3,3′-diaminobenzidine. FasD antigen was detected by Western blotting by using enhanced chemiluminescence (Amersham Corp., Arlington Heights, Ill.). Bacteria replica plated onto nitrocellulose were exposed to chloroform vapor for 15 min and to a lysis buffer (100 mM Tris-HCl [pH 7.8], 150 mM NaCl, 5 mM $MgCl_2$, 1.5% bovine serum albumin (BSA), 1 μg of DNase I per ml, and 40 μg of lysozyme per ml) for 12 to 16 h and rinsed twice with TNT (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) as described elsewhere (45). Rinsed blots were processed for antigen detection by using quaternary structure-specific anti-987P monoclonal antibody E11 (47), peroxidase-conjugated secondary antibodies, and 3,3′-diaminobenzidine as described above.

Seroagglutination. Slide agglutinations were performed with preadsorbed rabbit anti-987P fimbrial antiserum as described previously (47, 49).

DNA sequencing. Collections of subclones of plasmids pDMS137, pDMS138, pDMS148, and pDMS149 were constructed by using the unique restriction sites of the vectors for unidirectional deletions with exonuclease III and S1 nuclease (45). For some constructions, restriction sites in *fasD* were used. Double-stranded plasmid DNA was sequenced by the chain termination method (45) with an Applied Biosystems model 373A sequencer with forward or reverse *lacZ* primers. The *fasD* sequence of both strands was determined. Primers spaced approximately 200 bp over the entire length of *fasD* were prepared with an Applied Biosystems model 380B oligonucleotide synthesizer and used to sequence the linker insertion sites in *fasD*.

Homology analysis and structural predictions. The NBRF-PIR Release 35 and the Swiss-Prot Release 24 protein data banks were searched for homologies to FasD, and all homologous proteins were aligned together with the CLUSTAL algorithm (15a) from DNASTAR (Madison, Wis.). The method presented by F. Jähnig to study OMPs was used to predict the structure of FasD (19).

Linker insertion mutagenesis. CsCl pDMS165 DNA was prepared by conventional techniques (45, 51) after plasmid amplification by the addition of 50 μg of spectinomycin per ml at the early to mid-log phase of growth (optical density at 600 nm, 0.4). First, four unique blunt end restriction sites in *fasD* (*Pvu*II, *Stu*I, *Eco*RV, and *Sna*BI) were used to insert a phosphorylated 12-mer *Sma*I linker (TCCCCCGGGGGA) by conventional techniques (45). Second, linkers were inserted randomly into *fasD*. For this, a random population of circularly permuted molecules was obtained by digesting pDMS165 with limiting concentrations of pancreatic DNase I in a mixture of 50 mM Tris (pH 7.6), 10 mM $MnCl_2$, and 50 μg of BSA per ml (14, 45). Because under even optimized conditions, no more than a third of target plasmid linearizes, plasmid cut only once was isolated by agarose gel electrophoresis by using low-melting-agarose and β-agarase I (New England Biolabs). The DNA ends were blunted with Klenow fragment of DNA polymerase I (45). A molar excess of 40 to 400 phosphorylated 6-mer *Apa*I linkers (GGGCCC) was added to linear pDMS165 before ligation with T4 DNA ligase (45). In later experiments, linear DNA was pretreated with shrimp alkaline phosphatase (United States Biochemicals, Cleveland, Ohio) to prevent self ligation. DNA was used to transform competent MH6085 cells by electroporation (12). An additional linearization step was performed to enrich for linker insertions and to remove multiply inserted linkers. For this, approximately 10,000 transformants were pooled and grown to the stationary phase, and plasmid DNA was isolated as described above. The DNA was restricted with *Apa*I, and linear DNA was isolated, ligated, and used to transform MH6085 or SE5000 pDMS13 as described above. Transformants were analyzed by one of two techniques. In the first approach, plasmid minipreps of transformants were analyzed by restriction mapping to determine linker insertion sites in pDMS165. Plasmids with linker inserted in *fasD* were used to transform SE5000 pDMS13 and tested for fimbriation by seroagglutination. With the second approach, distinct colonies of transformed SE5000 pDMS13 were used to inoculate LB broth in microtiter wells (15% glycerol was added to the media for long-term storage at −70°C). Bacteria were replica

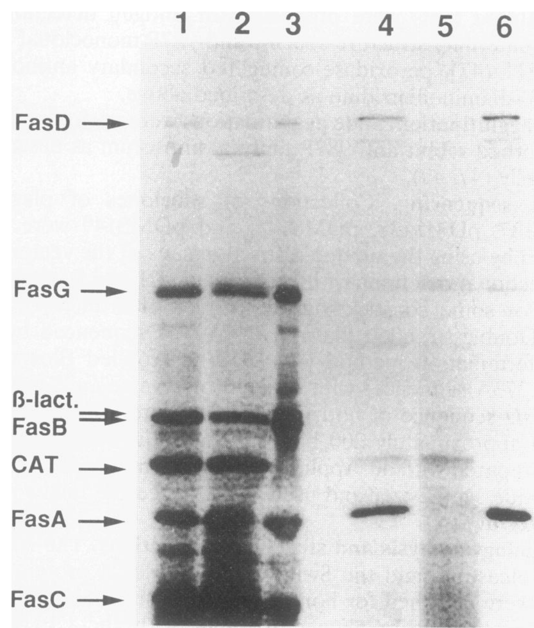

FIG. 2. Proteins labeled in *E. coli* SE5000 after activation of T7 transcription, separated by SDS-PAGE, and analyzed by fluorography. The results with total bacterial extracts (lanes 1 to 3) and outer membrane fractions (lanes 4 to 6) of SE5000 containing pDMS27 (*fasD*$^+$; lanes 1 and 4), pDMS159 (*fasD*; lanes 2 and 5), or pDMS167 (*fasD*$^+$; lanes 3 and 6) are shown. Plasmids pDMS27 and pDMS159 express chloramphenicol acetyltransferase (CAT), and pDMS167 expresses β-lactamase (β-lact.).

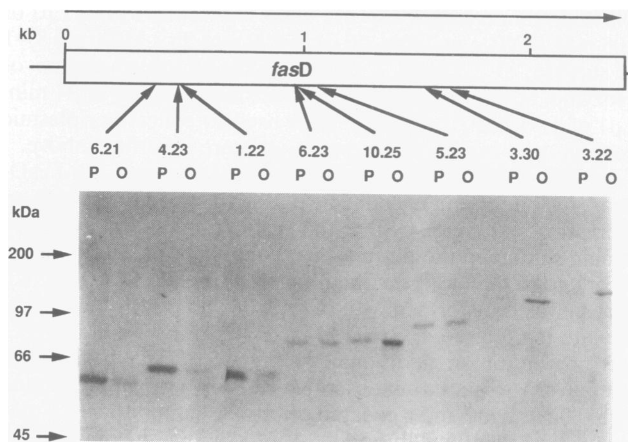

FIG. 3. Physical map and Western blot of Tn*phoA* inserts in the *fasD* gene of pDMS6. Hybrid proteins isolated with periplasmic (P) or outer membrane (O) fractions were visualized with anti-alkaline phosphatase antibodies. The corresponding mutations are indicated at the top of each pair of lanes labeled P and O, and the locations of the in-frame fusion joints between fasD and phoA are indicated on the map by arrows. The direction of transcription of fasD is indicated by an arrow above the map. Molecular masses of standard proteins, given in kilodaltons, are indicated at the left.

plated from the wells to nitrocellulose circles placed on LB agar plates containing ampicillin and chloramphenicol. Bacteria grown on the nitrocellulose circles were analyzed by the immunoblot procedure described above. To identify and map linker insertions, plasmids of positive isolates were studied by restriction analysis after growing bacteria in LB broth with chloramphenicol alone. Plasmid pDMS13 was diluted out by omitting ampicillin from the medium.

Nucleotide sequence accession number. The nucleotide sequence of *fasD* has been deposited in the GenBank data base and given the accession number L22659.

RESULTS

Subcellular localization of FasD. The results of a previous study using protein fusion technology suggested that FasD crosses the cytoplasmic membrane (48). By analogy to other fimbrial systems, but on the sole basis of the size of FasD and its requirement for fimbriation, the protein was proposed to home to the outer membrane (48). In this study, a biochemical approach has been taken to determine directly where FasD localizes. SDS-PAGE and fluorography of *E. coli* SE5000 with plasmids pDMS27 and pDMS167 synthesizing all the Fas proteins indicated that FasD localizes to the outer membrane (Fig. 2, lanes 4 and 6). The data show that the isolated outer membrane fraction was not contaminated by periplasmic β-lactamase and only slightly by the cytoplasmic CAT. In a *fasD* in-frame deletion mutant [SE5000(pDMS159)], the internally truncated FasD protein could not be detected in the outer membrane (Fig. 2, lane 5). The most prominent Fas protein in the outer membrane was the fimbrial subunit FasA. In the *fasD* mutant, FasA, which was absent from the outer membrane (Fig. 2, lane 5), accumulated in the periplasm (data not shown). This suggests that membrane-associated FasD is required for FasA uptake by the outer membrane. Although some of the additional minor bands may result from internal translation start sites in *fasD*, pulse-chase experiments suggested that most of them are due to proteolysis (data not shown). For example, a minor protein migrating between FasD and FasG (M_r = 68,000; data not shown) became the major outer membrane band after pulse-chase labeling. Similar findings have been described for PapC and the CS3 fimbrial OMP (11, 20, 38). FasD has three potential OmpT cleavage sites; the accessibility of any one of two of them (between residues 730 and 731 or 757 and 758) to this outer membrane protease could explain the described band (52). PapC and CS3 have eight and nine, respectively, potential OmpT cleavage sites, which may explain the common findings. The truncation of fimbrial OMPs may have no physiological relevance since all of the studies used multicopy-number plasmids with expression systems or minicells. In any case, our data support previous genetic studies, which suggested that FasD is the 987P OMP.

Subcellular localization of FasD-PhoA fusion products. To confirm the localization of FasD in the outer membrane of *E. coli*, differential compartmentalization of several previously described active FasD fusions to alkaline phosphatase was investigated (48). Periplasmic and outer membrane fractions of host strain CC118 (*phoA*) containing pDMS6 plasmid derivatives with Tn*phoA* inserted in different areas of *fasD* were studied by Western blot analysis using anti-PhoA antibodies. As shown in Fig. 3, the subcellular localization of FasD-PhoA fusions indicated that fusion products which contained at least three-fifths of the FasD sequence were isolated only in outer membrane fractions (mutants 3.30 and 3.22). FasD-PhoA fusion proteins remained in the periplasm when they contained only the NH_2-terminal fifth of FasD (mutants 6.21, 4.23, and 1.22). Fusion products of intermediate sizes were isolated in both fractions (mutants 6.23, 10.25, and 5.23). These results suggest that parts of FasD can direct a periplasmic protein to the outer membrane. Although it is not known

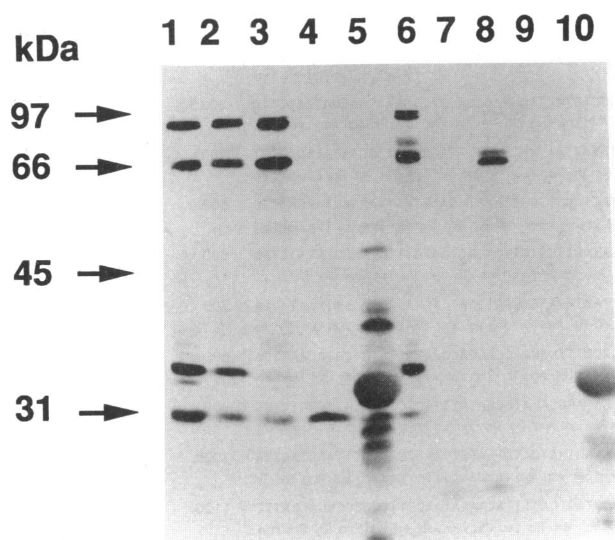

FIG. 4. Western blot of total proteins of *E. coli* SE5000 (pDMS165) probed with anti-FasD antibody and analyzed by enhanced chemiluminescence. Lanes: 1 to 5, intact bacteria; 6 to 10, spheroplasts; 1 and 6, control without proteases; 2 and 7, trypsin-treated bacteria; 3 and 8, bacteria treated with V8 protease used under conditions for cleavage at Glu residues; 4 and 9, bacteria treated with V8 protease used under conditions for cleavage at Asp and Glu residues; 5 and 10, proteinase K-treated bacteria.

whether the folding and insertion of the remaining portion of FasD is the same as those for its native state, our results are at least consistent with the localization studies of native FasD.

Surface exposure of FasD. The potential accessibility of surface-exposed domains of FasD to proteases was determined. The obtained anti-FasD antibody was utilized to identify FasD by Western blot and enhanced chemiluminescence. The antibody recognized FasD and some of its degradation products, which were described above (Fig. 4). Moreover, in addition to submitting intact bacteria to protease treatments, prepared spheroplasts were treated in a similar fashion to identify FasD domains which are exposed on the periplasmic surface of the outer membrane. Trypsin and V8 protease, used under specific conditions for glutamic acid residues, did not affect FasD in intact bacteria (Fig. 4). In contrast, the broad-range protease proteinase K and V8 protease, used under conditions cleaving both aspartic and glutamic acid residues, were able to degrade portions of FasD. Taken together, these data indicate that a portion of FasD is surface exposed and that at least one of its domains includes an aspartic acid residue which is accessible to V8 protease. Interestingly, FasD of spheroplasts was susceptible to all proteases, suggesting that larger domains of FasD are exposed on the periplasmic side of the outer membrane.

DNA sequence of *fasD*. The DNA sequence of the *Nco*I fragment of pDMS8 and the deduced primary structure of FasD are shown in Fig. 5. Initiation of translation of *fasD* is proposed to involve a typical ribosomal binding site (Fig. 5, underlined). The proposed translational start site of *fasD* (Fig. 5) was confirmed by in vivo transcription and translation of two deletion constructs with the T7 expression system (data not shown). The extent of the deletions, which were obtained by exonuclease III treatments, were determined by DNA sequencing. FasD was still expressed after the first 164 bp shown in Fig. 5 were deleted. In contrast, FasD was not detected when the first 222 bp ($\pm$3 bp) were deleted. However, additional bands of lower molecular weights (distinct from the protein band with an M_r of 68,000 mentioned above) were visible, confirming the existence of at least one internal translation start site in *fasD*. A cleavage site for a typical leader sequence of exported proteins in *E. coli* (54) is proposed (Fig. 5, arrow). The calculated molecular mass of the exported FasD (M_r = 92,366) corresponds to that previously calculated for FasD by SDS-PAGE (48). The two most frequently occurring amino acid residues are serines and glycines, accounting for 22% of all residues. FasD has only two cysteines. FasD is hydrophilic and has no apparent hydrophobic transmembrane segment, with the exception of the one contained in the signal sequence. These characteristics are consistent with OMPs (36). The calculated pI is 7.36.

FasD belongs to a new family of OMPs. All of the first proteins aligning to FasD in a homology search of protein data banks were fimbrial proteins of similar size in *E. coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Yersinia pestis*, and *Bordetella pertussis* (3, 20, 24, 27, 34, 38, 42, 43, 56). Several of these proteins have been shown to be OMPs. No other OMPs were included in the next 20 listed proteins of the search. These fimbrial proteins were aligned by penalizing gaps only slightly to better visualize domain alignments. By using the PAM100 residue weight table, the percent similarity varied between 19.2 and 32.7. Homologies were the strongest in the second fifth (Fig. 6) of the multiple alignment. The profiles of residue identities among at least six fimbrial OMPs are shown in Fig. 7A. Some residues are fully conserved among all 10 proteins. Glycine, tryptophan, tyrosine, and proline residues are preferentially conserved over other residues. Residues 302 to 310 in FasD (VPPGPFXIXD) correspond to the best-conserved span of residues among these proteins. All of the fimbrial OMPs have two cysteines aligning at the carboxy-terminal end and, with the exception of FasD, two cysteines aligning around residues 90 and 110. This suggests that the aligning pairs of cysteines make disulfide bridges on either side of the membrane. The similarity in function and structure of these proteins indicates that they should be classified as a new family of OMPs in gram-negative bacteria. Moreover, these proteins can be differentiated from type IV fimbrial OMPs since they share neither homologous sequences nor the consensus sequence of lipoprotein processing sites found with type IV fimbrial OMPs (41).

Structure predictions for FasD. On the basis of two extensively studied groups of OMPs, the porins (including OmpA and LamB) and the active transporters of iron siderophores and vitamin B_{12} (25, 36, 44), OMPs are proposed to be relatively hydrophilic proteins which span the outer membrane as β-strands, in contrast to the hydrophobic α-helical structures of bacterial and eucaryotic cytoplasmic membrane proteins (23, 37, 44). Recently resolved crystal structures of the *Rhodobacter capsulatus* and *E. coli* OmpF and PhoE porins indicated that each subunit of these trimers consists of 16 β-strands forming an antiparallel β-barrel (8, 55). Combinations of certain algorithms, used to identify turns and hydrophobic and amphipathic domains, have been surprisingly correct in predicting most of the membrane-spanning domains of porins (19, 21, 50) and are being used to support structural studies on siderophore receptors (28). Applying Jähnig's predictors, FasD consists mainly of amphipathic β-strands, β-turns, and loops (Fig. 7). Other fimbrial OMPs demonstrate essentially similar predicted structures. Moreover, the size of fimbrial OMPs and the number of predicted β-amphipathic strands are similar to the ones described or proposed for siderophore receptors (28). This suggests a working model in

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CCATGGGTATAATATATTTAAATGAGGGTGAAGTATTTGACAAAAAAAGAAAATATTATTTGTCGGTTAGTTTCATCCCCAAAAAAAAAGATTCTGATGA   100
TTTAGGTTTCAACATAATATTCACTCAACAAATAGCCGTTAAATTAAGCGCGTTAAACTGATACATATATAAGGAAAATATGAACAAATATCCCCCTTTA   200
                                                                          Met Asn Lys Tyr Pro Pro Leu
TTAACAATGTTGATCATTGGTATTGGTTCCAATGCGGTTGCGGGGGACTATTTCGACCCCTCACTACTTGCAACCGATATTGGAAATAATGATAAGTTAG   300
Leu Thr Met Leu Ile Ile Gly Ile Gly Ser Asn Ala Val Ala↑Gly Asp Tyr Phe Asp Pro Ser Leu Leu Ala Thr Asp Ile Gly Asn Asn Asp Lys Leu
ACTTGTCACTTTTCTCTCATCCAGGGGGGGGAGTCAAAGGAGAGAGAGAGGTTAGTGTATATATTAATGATTTTTTTTACAAAAATGTTACACTGGACTT   400
Asp Leu Ser Leu Phe Ser His Pro Gly Gly Gly Val Lys Gly Glu Arg Glu Val Ser Val Tyr Ile Asn Asp Phe Phe Tyr Lys Asn Val Thr Leu Asp Phe
TGAAAATGGAATTTCCGGAGCCCTAGAGCCAATTTTTCCATCAGGGTTTTTTGATAACATATTAGCTTCGCCATATAGAAGTATTAAAGAGAAAGAACTC   500
Glu Asn Gly Ile Ser Gly Ala Leu Glu Pro Ile Phe Pro Ser Gly Phe Phe Asp Asn Ile Leu Ala Ser Pro Tyr Arg Ser Ile Lys Glu Lys Glu Leu
ATATCAACAGCTGACTTTCTTAGTTTAGTTCCTTATGGTATGGTACGATTTGATCAGGCGATAGCTCGTGTTGACATTAGTATTCCTCAGGCCTATCTAG   600
Ile Ser Thr Ala Asp Phe Leu Ser Leu Val Pro Tyr Gly Met Val Arg Phe Asp Gln Ala Ile Ala Arg Val Asp Ile Ser Ile Pro Gln Ala Tyr Leu
GGCGTGATGCTCAGATGAAATCAGCTCCTGAATCTTGGAATCAGGGTGTTCCTGCATTGTTAATAGATTACCGTTTATCTGGAAGTAAAAATAAATATAA   700
Gly Arg Asp Ala Gln Met Lys Ser Ala Pro Glu Ser Trp Asn Gln Gly Val Pro Ala Leu Leu Ile Asp Tyr Arg Leu Ser Gly Ser Lys Asn Lys Tyr Asn
CTATGGTTCATCACAGAATTTTTATGCTAACGCATTTTTAGGTTTCAACTTAATGGGGTGGCGTTTGAGAACCACCACGAATTACATGTCGTACAATTCA   800
Tyr Gly Ser Ser Gln Asn Phe Tyr Ala Asn Ala Phe Leu Gly Phe Asn Leu Met Gly Trp Arg Leu Arg Thr Thr Thr Asn Tyr Met Ser Tyr Asn Ser
AAAGATTTATACAATAAAGGAGAAAGACAAGGTAGTTTTAACTTTTATAATACATACCTTGAAAAGGATATCGGATATTTACGCTCAACATTGCGTCTGG   900
Lys Asp Leu Tyr Asn Lys Gly Glu Arg Gln Gly Ser Phe Asn Phe Tyr Asn Thr Tyr Leu Glu Lys Asp Ile Gly Tyr Leu Arg Ser Thr Leu Arg Leu
GGGAGCTATCAACTCGAGGGATGATTCTTGAATCATTTAATTTCAAAGGTGGAAAGATTTATAGTAATGATGAAATGTTAAATGACCGTTTACGTAGTTA   1000
Gly Glu Leu Ser Thr Arg Gly Met Ile Leu Glu Ser Phe Asn Phe Lys Gly Gly Lys Ile Tyr Ser Asn Asp Glu Met Leu Asn Asp Arg Leu Arg Ser Tyr
TACTCCAACTGTAAGAGGTATAGCAAGTAGCCAGGCAGTGGTAACTATTAAGCAGGGGGGGGTAGTCATTTTACAAAAAAACGTTCCGCCCGGACCATTT   1100
Thr Pro Thr Val Arg Gly Ile Ala Ser Ser Gln Ala Val Val Thr Ile Lys Gln Gly Gly Val Val Ile Leu Gln Lys Asn Val Pro Pro Gly Pro Phe
GAAATTAATGATTTTTCATTATCAGGATATTCAGGAGACTTATATGTAAACATTAAGGAAGCAGATGGGAGTGAGCATAGTTTTATTCAGCCCTTCTCGA   1200
Glu Ile Asn Asp Phe Ser Leu Ser Gly Tyr Ser Gly Asp Leu Tyr Val Asn Ile Lys Glu Ala Asp Gly Ser Glu His Ser Phe Ile Gln Pro Phe Ser
CGTTACCAGAAATGAAACGCGAGGGTGTTTCTGGATATGAAATTTCATTAGGTCATTATAATAATAGTGGCGCAACTCAATATTACAATGAAAGTCCTTT   1300
Thr Leu Pro Glu Met Lys Arg Glu Gly Val Ser Gly Tyr Glu Ile Ser Leu Gly His Tyr Asn Asn Ser Gly Ala Thr Gln Tyr Tyr Asn Glu Ser Pro Phe
TTTATATGCTTCTTGGTCTCGAGGATACCGTAATGGTATGACATTATATTCTGAAACAATTCAGTCAAGAAAATATCAACTCCTGGGGGTTGGTAGTACA   1400
Leu Tyr Ala Ser Trp Ser Arg Gly Tyr Arg Asn Gly Met Thr Leu Tyr Ser Glu Thr Ile Gln Ser Arg Lys Tyr Gln Leu Leu Gly Val Gly Ser Thr
TTATCTCTTGGGGATTTTGGGGCTGTGTCTGGTGATGCATCATTGTCACGTGCAAATAAATATGACAAAATTCATTCAGGGCAATCTTACGGCTTAAAAT   1500
Leu Ser Leu Gly Asp Phe Gly Ala Val Ser Gly Asp Ala Ser Leu Ser Arg Ala Asn Lys Tyr Asp Lys Ile His Ser Gly Gln Ser Tyr Gly Leu Lys
ATTCGAAAAACAAGGTTGATACTGGAACAACAGTTACATTAGCAACATATAGATATTCTACAAAAGACTTTTACTCATTTAATGACTTTGTATCAAAAAA   1600
Tyr Ser Lys Asn Lys Val Asp Thr Gly Thr Thr Val Thr Leu Ala Thr Tyr Arg Tyr Ser Thr Lys Asp Phe Tyr Ser Phe Asn Asp Phe Val Ser Lys Asn
TGACTCAGTTCAATATGTTTGGGATAACCGATTAAAAAATAGAATTACATTAAGTCTAAATCAATCTCTGGATGATTATGGTTCATTATCTTTAATCGCA   1700
Asp Ser Val Gln Tyr Val Trp Asp Asn Arg Leu Lys Asn Arg Ile Thr Leu Ser Leu Asn Gln Ser Leu Asp Asp Tyr Gly Ser Leu Ser Leu Ile Ala
TCCCAACAAAATTATTGGACGAGTGATTATGTTAGTCGTTCCTTTTCATTATCGCATAGTTTTGGATGGAATGATATATTTTTTTCAACATCCTTTTCTT   1800
Ser Gln Gln Asn Tyr Trp Thr Ser Asp Tyr Val Ser Arg Ser Phe Ser Leu Ser His Ser Phe Gly Trp Asn Asp Ile Phe Phe Ser Thr Ser Phe Ser
TGGACCAAAAGGAAGGTGACAATGCATTAAGAAATAATAACAAAGTATTTGGTTTTTATTCAAGCATACCATTAAGTAAATTAATTGGAAAAAATGAAAG   1900
Leu Asp Gln Lys Glu Gly Asp Asn Ala Leu Arg Asn Asn Asn Lys Val Phe Gly Phe Tyr Ser Ser Ile Pro Leu Ser Lys Leu Ile Gly Lys Asn Glu Ser
CACATATAGTACTTTAAGTTACAATGTGACTAAAATAAATAATCAAGTGCGTAATACCGCCACTCTTGCAGGAAAAGTTCCGGGTTCAATGGCACAGTAT   2000
Thr Tyr Ser Thr Leu Ser Tyr Asn Val Thr Lys Ile Asn Asn Gln Val Arg Asn Thr Ala Thr Leu Ala Gly Lys Val Pro Gly Ser Met Ala Gln Tyr
CGATTCAGTTCAGGATGGGCAAATACGGAGCAAAGTAGTAACAAAGCATTATCTGTAAATTGGGATGGTGACTTATTAGATGGTTCTTTAGGTTATACAA   2100
Arg Phe Ser Ser Gly Trp Ala Asn Thr Glu Gln Ser Ser Asn Lys Ala Leu Ser Val Asn Trp Asp Gly Asp Leu Leu Asp Gly Ser Leu Gly Tyr Thr
GCTCCGGAAAAAATCGAATAACTGACTATAGCCTATCTGGCTCAGCAATTCTGTATCCTTGGCGACTAGCCATTGGGTCTGATAGTGTTATCAACGGTGC   2200
Ser Ser Gly Lys Asn Arg Ile Thr Asp Tyr Ser Leu Ser Gly Ser Ala Ile Leu Tyr Pro Trp Arg Leu Ala Ile Gly Ser Asp Ser Val Ile Asn Gly Ala
CGCGGTAGTTGAGACAGAATTTATTTCTGGTATCAAAGTTCGCCAAGGCGGGGAAACATCTTTACTTGGAACAGCAATTGTAACGTCGATGCAGCCGTAT   2300
Ala Val Val Glu Thr Glu Phe Ile Ser Gly Ile Lys Val Arg Gln Gly Gly Glu Thr Ser Leu Leu Gly Thr Ala Ile Val Thr Ser Met Gln Pro Tyr
ACTGAAAACAGGATAGATCTTGATACACAAAATATACCTGATGATCTTTTTATCAGTAATGCATCCAAAAAAATAGTACCTGAAAAAGGTGCAGTCGTAC   2400
Thr Glu Asn Arg Ile Asp Leu Asp Thr Gln Asn Ile Pro Asp Asp Leu Phe Ile Ser Asn Ala Ser Lys Lys Ile Val Pro Glu Lys Gly Ala Val Val
CGGTTAAATACAATCTCTTTAAAGGTAAGCAAATTGTATTTAGTTTAAAACGTTATGATGGTACTCCATTGCCATTCGGATCTGTTGTTTCTCTTGTGGG   2500
Pro Val Lys Tyr Asn Leu Phe Lys Gly Lys Gln Ile Val Phe Ser Leu Lys Arg Tyr Asp Gly Thr Pro Leu Pro Phe Gly Ser Val Val Ser Leu Val Gly
TAGTGATAGTGAAATTACGGGAATTATTGATGATGCCGGAAGAGTATATTTAGCTGGAATACCAAGTAAAGGAATACTTCATGGTGCTTGGGGATATAAC   2600
Ser Asp Ser Glu Ile Thr Gly Ile Ile Asp Asp Ala Gly Arg Val Tyr Leu Ala Gly Ile Pro Ser Lys Gly Ile Leu His Gly Ala Trp Gly Tyr Asn
AAGTCATGTGAGGTGTCTTTTAATCTTAACGGGAAACCATCAAATAATTCAAATGAAATTATTGAATACGAAGGTGTATGTAAGTAATGAAATATTTATT   2700
Lys Ser Cys Glu Val Ser Phe Asn Leu Asn Gly Lys Pro Ser Asn Asn Ser Asn Glu Ile Ile Glu Tyr Glu Gly Val Cys Lys
CACTATTCTACTAATATTTATATGTAAATATGGATATACGTTTGTTTCAAATCCATTACTATATCCATTTCCTAATGACACAAAGATAATTATTCATTCT   2800
GAAGGAAATGGTAAAATAAAAAAAGGTATTCTTAAAATAACTAATCCGGGGGAAAGCCCATGG   2863
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FIG. 5. Nucleotide sequence of the *Nco*I fragment containing *fasD* and deduced amino acid sequence of FasD. A ribosomal binding site (underlined) and a typical leader sequence cleavage site (arrow) are proposed.

which the membrane-spanning domains of FasD adopt a β-barrel structure, with joining surface-exposed β-turns or loops of various lengths.

Identification of permissive sites in FasD. FasD is the only 987P protein which fractionates exclusively with bacterial outer membranes. This suggests that FasD plays a central role in the translocation of fimbrial subunits through the outer membrane of *E. coli*. To better understand how FasD functions, a mutagenesis approach, using in-frame linker insertions, was taken. The first goal was to determine whether specific FasD sites could be altered structurally without affecting bacterial fimbriation. FasD sites which could be mutated without interfering with fimbriation were designated permissive sites. By definition, nonpermissive sites reside in functional domains required either for fimbrial subunit export or for the folding, incorporation, and maintenance of FasD in the outer mem-

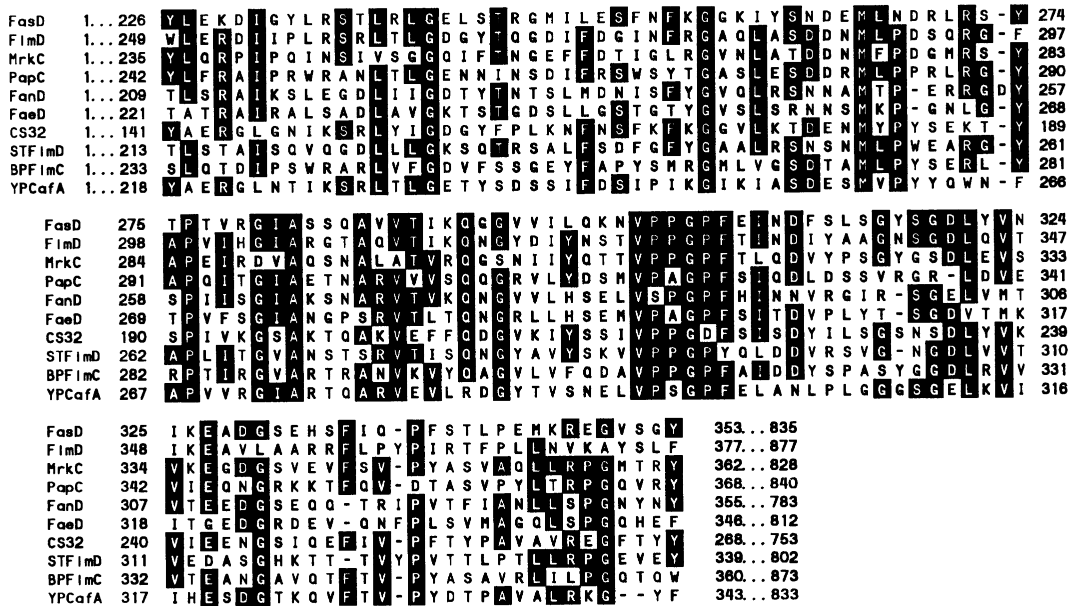

FIG. 6. Regions of FasD and nine additional fimbrial OMPs which gave the highest level of homologies when the CLUSTAL multiple alignment algorithm was used. FimD (27), PapC (38), FanD (43), FaeD (33), and CS32 (20) are *E. coli* proteins; MrkC is from *K. pneumoniae* (3); STFimD is from *S. typhimurium* (42); BPFimC is from *B. pertussis* (56); YPCafA is from *Y. pestis* (24). Amino acid residues are indicated on the left and right sides for each sequence.

brane. As presented above, our working model for FasD assumes that the membrane-spanning domains of this protein consist essentially of amphipathic β-sheets which stabilize the protein in the outer membrane. To optimize inactivation of the function of FasD after linker insertion into membrane-spanning β-sheets, we used linkers encoding residues acting as β-breakers, like proline and glycine. Therefore, the identification of nonpermissive sites should characterize most membrane-spanning domains and the surface-exposed domains which are involved in fimbrial export. In contrast, identified permissive sites should essentially represent connecting surface-exposed turns and loops (i.e., towards the periplasmic or bacterial surface) which are not involved in fimbrial export.

Preliminary experiments were used to determine whether it is possible to engineer in-frame mutations in *fasD* (pDMS165) which can still complement a *fasD* mutant (pDMS13). For this, a *Sma*I linker was inserted in four unique restriction sites of *fasD* with blunt ends (*Pvu*II, *Stu*I, *Eco*RV, and *Sna*BI). To identify a permissive site, complemented *fasD* mutants were screened by seroagglutination with anti-987P fimbrial antibodies. From these constructs, only the insertion into *Eco*RV partially complemented a *fasD* strain (SE5000 with compatible plasmid pDMS13) for 987P fimbriation. Interestingly, this site is located in a predicted turn (Table 1 and Fig. 7), with a highly conserved proline absent only in FasD (Fig. 6, residue 268). In contrast, two of the three other inserts flank or reside in a predicted amphipathic β-strand. This result suggested that the proposed mutagenesis approach could be used on a larger scale to study FasD.

Accordingly, *Apa*I linkers were inserted randomly into pDMS165 previously cut with DNase I. Initially, plasmids from 235 independent colonies were isolated and *Apa*I insertions were mapped by restriction analysis. Plasmids with *Apa*I inserts in *fasD* were tested by complementation analysis for fimbriation by using seroagglutination. Of 74 plasmids with inserts in *fasD*, six were complementing for fimbriation. The corresponding insertion sites were mapped and sequenced (Table 1 and Fig. 7; sites 249, 268, 362 twice, one with a short in-frame duplication of target DNA, 363, and 514). Seroagglutination was evaluated as described previously (49), and comparable strong reactions (+++) were obtained when testing the wild type and four of the five mutated plasmids. Only the construct with an *Apa*I linker in site 268 complemented less efficiently, as shown by a weaker seroagglutination reaction (+). In addition to simple linker insertions, several constructs demonstrated duplications or deletions of one or a few codons at the insertion site of the *Apa*I linker in the *fasD* sequence. These changes probably resulted from using the Klenow fragment of *E. coli* DNA polymerase I to blunt protruding termini frequently created by DNase I (45).

To facilitate identification of permissive sites, an approach which eliminates unnecessary restriction mapping was used. For this, approximately 90 new colonies were first tested for fimbriation by immunoblotting, using monoclonal antibodies specific for the quaternary structure of 987P fimbriae. Four additional permissive sites were identified in *fasD* by mapping and sequencing *Apa*I inserts in plasmids of colonies that were positive by immunoblot. That the corresponding FasD proteins were functional was confirmed by seroagglutination as described above (Table 1 and Fig. 7; sites 202, 243, 604, and 704).

The occurrence of codon duplications and deletions (substitutions included) among 6 of the 10 *Apa*I insertion mutants encoding a functional FasD protein suggested that, because of the DNase protocol used, many of the mutants encoding a nonfunctional product contained frameshifts. This was confirmed by analyzing the sequence of approximately 20 different *Apa*I insertion sites mapped between nucleotides 600 and 1,200 of *fasD* (by using the coordinates of Fig. 5). In addition to out-of-frame mutants, many mutants with deletions and duplications of large DNA fragments (40 bp and more) were detected. Therefore, to determine the location of nonpermis-

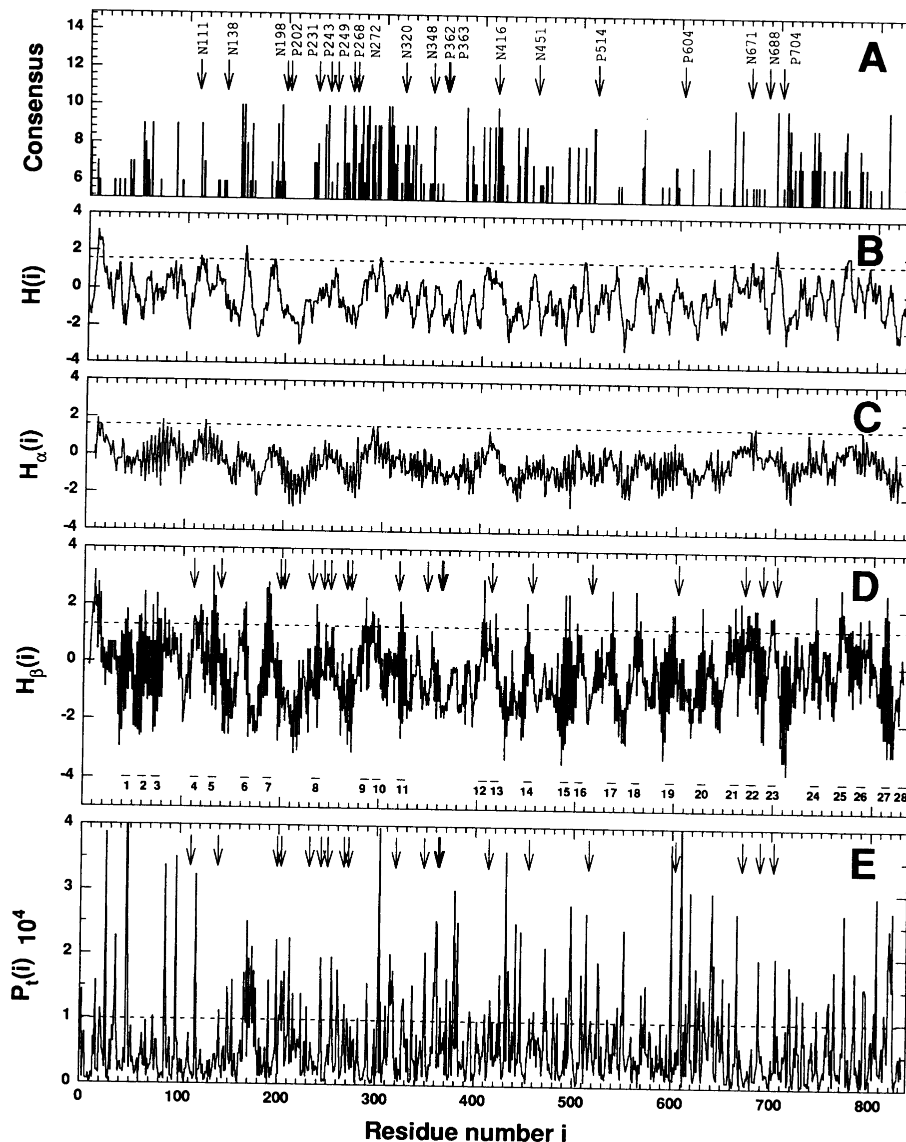

FIG. 7. Conserved residue plot (A) and structure prediction plots (19) (B to E) for the FasD protein: hydrophobicity [H(i); B], α-helix side hydrophobicity [$H_\alpha(i)$; C], β-strand side hydrophobicity [$H_\beta(i)$; D], β-turn potential [$P_t(i)$; E]. Arrows in panels D and E and those with numbers in panel A correspond to the insertion sites, identified as permissive (prefix P) or nonpermissive (prefix N); bars and numbers in panel D indicate 28 proposed β-strands.

sive sites in FasD, in-frame linker insertions, including deletions and duplications of only a few codons, had to be identified by first demonstrating the expression of nontruncated FasD proteins. For this, inactive FasD products of 76 *fasD* mutants with *Apa*I insertions were studied by Western blot analysis and enhanced chemiluminescence by using the anti-FasD antibodies described above. Seven mutants were shown to express a product of the size corresponding to the nonmutated protein. A majority of the remaining mutants expressed no detectable product, and some of the others produced FasD proteins of lower or higher molecular mass, reflecting larger in-frame deletions or duplications. Sequence analysis of the seven nonpermissive insertions in *fasD* (Table 1; sites 198, 320, 348, 416, 451, 671, and 688) showed that six of them included deletions or duplications of several residues.

In summary, 11 permissive sites were identified in FasD, three being only partially permissive since they are associated with a reduced function of FasD (Table 1). The 3 partially permissive sites and 9 of the 10 identified nonpermissive sites interrupt or flank conserved residues. In contrast, six of the eight fully permissive sites are flanked by nonconserved residues on both sides. These data indicate a correlation between permissive sites and nonconserved residues and, conversely, between nonpermissive sites and conserved residues. Moreover, all permissive sites were located in or near predicted turns, whereas 5 of the 10 nonpermissive sites were identified in predicted β-amphipathic strands, suggesting a relationship between the structure and the function of FasD. The proposed nature of this relationship will be elaborated in Discussion.

TABLE 1. Deduced primary structure, predicted secondary structure, and function of mutated FasD proteins

Site and residue[a]	Mutation[b]	Local sequence[c]	Conserved[d]	Prediction[e]	Function[f]
*Pvu*II 111	+4 (I4 + S1)	504 TCA ACA GT*C CCC CGG GGG A*CT GAC 515 109 Ser Thr *Val Pro Arg Gly Thr* Asp 112	+	β	–
*Stu*I 138	+4 (I4 + S1)	585 CCT CAG G*TC CCC CGG GGG A*CC TAT 596 136 Pro Gly *Val Pro Arg Gly Thr* Tyr 139	+	–	–
198	–4 (I2 + De6)	768 TTG A*GG GCC C*TG TCG 794 196 Leu Arg *Ala Leu* Ser 204	+	T	–
202	0 (S3)	783 TAC A*GG GCC C*AC AAT 797 201 Tyr *Arg Ala His* Asn 205	–	T	+++
*Eco*RV 231	+4 (I4)	864 AAG GAT *TCC CCC GGG GGA* ATC GGA 875 229 Lys Asp *Ser Pro Gly Gly* Ile Gly 232	+	T	+
243	0 (S2)	900 GGG GA*G GGC CCA* ACT 914 241 Gly Glu *Gly Pro* Thr 245	+	T	+++
249	+5 (I2 + Du3)	915 CGA GGG ATG *GGG CCC* CGA GGG ATG ATT 926 246 Arg Gly Met *Gly Pro Arg Gly Met* Ile 249	–	T	+++
268	+2 (I2)	975 ATG TT*G GGC CCA* AAT 983 266 Met Leu *Gly Pro* Asn 268	+	T	+
*Sna*BI 272	+4 (I4 + S1)	987 CGT TTA C*TC CCC CGG GGG A*GT AGT 998 270 Arg Leu *Leu Pro Arg Gly Ser* Ser 273	+	T	–
320	–2 (I2 + De4)	1128 TAT TC*G GGC CC*T GTA 1148 317 Tyr Ser *Gly Pro* Val 323	+	β	–
348	+3 (I2 + Du1)	1215 AAA CGC *GGG GCC C*GC GAG 1223 346 Lys Arg *Gly Ala Arg* Glu 348	+	–	–
362	+5 (I2 + Du3)	1257 CAT TAT AAT *GGG CCC* CAT TAT AAT AAT 1265 359 His Tyr Asn *Gly Pro His Tyr Asn* Asn 362	–	T	+++
362	+2 (I2)	1260 TAT AAT *GGG CCC* AAT 1265 360 Tyr Asn *Gly Pro* Asn 362	–	T	+++
363	+2 (I2)	1260 AAT AAT *GGG CCC* AGT 1268 361 Asn Asn *Gly Pro* Ser 363	–	T	+++
416	+2 (I2)	1422 GCT *GGG CCC* GTG 1427 415 Ala *Gly Pro* Val 416	+	β	–
451	–11 (I2 + Del3)	1257 ACG *GGC CCC* TTT 1301 450 Thr *Gly Pro* Phe 464	+	β	–
514	–2 (I2 + De4)	1713 TAT T*GG GCC C*AT GTT 1733 512 Tyr Trp *Ala His* Val 518	+	T	+++
604	+4 (I2 + Du2)	1980 CCG GGT TC*G GGC CC*G GGT TCA ATG 2003 601 Pro Gly Ser *Gly Pro Gly Ser* Met 604	–	T	+++
671	–2 (I2 + De4)	2187 GT*G GGC CCC* GCG 2204 670 Val *Gly Pro* Ala 675	+	β	–
688	–4 (I2 + De6)	2238 GTT *GGG CCC* TCT 2261 687 Val *Gly Pro* Ser 694	–	T	–
704	+2 (I2)	2283 ACG TC*G GGC CC*G ATG 2297 702 Thr Ser *Gly Pro* Met 704	+	T	++

[a] Insertion site in the FasD protein sequence corresponding to the first altered residue in the sequence.
[b] Total number of inserted or deleted amino acid residues. I, number of newly inserted residues; S, number of substituted residues; Du, number of duplicated residues; De, number of deleted residues.
[c] Linker insertion sites with resulting mutations in *fasD* and deduced amino acid changes. Numbers refer to the indicated first and last nucleotides or amino acid residues of the original respective sequences. Linker DNA (*Apa*I or *Sma*I linkers) and new amino acids are in italics.
[d] Conserved nature of the insertion sites. Symbols: +, insertion in a site flanked by conserved residues (defined as identical residues for at least 6 of the 10 compared fimbrial OMPs), or deletion or substitution of conserved residue; –, insertion in a site separated from a conserved residue by at least one residue.
[e] Predicted secondary structure of the insertion sites. Symbols: β, insertions in sites predicted to be involved in forming membrane-spanning β-strands (≥9 residues); T, insertions in sites predicted to form only turns (± 1 residue).
[f] Exported and assembled fimbriae quantified by seroagglutination. Symbols: +++, immediate very strong reaction; ++, strong reaction after 10 s; +, weak reaction after 10 s; –, no reaction for 1 min.

DISCUSSION

In the last few years, studies of fimbrial biogenesis have led to a better understanding of early events occurring in the periplasm. This research has focused on the interactions of fimbrial subunits with a periplasmic chaperone protein (18, 22). In contrast, little concerning the mechanisms of fimbrial subunit export at the outer membrane is known.

Here, we analyzed FasD, a 987P protein previously shown to be required for fimbrial biogenesis. By using two different techniques, we localized FasD in the outer membrane. Moreover, by developing an anti-FasD antibody probe, surface exposure of FasD on either side of the outer membrane could be demonstrated with various proteases. The primary structure of FasD, deduced from DNA sequencing, was shown to share homologous domains with a group of proteins, each of them belonging to another fimbrial gene cluster and some of them having been characterized as OMPs. Finally, we established that linkers can be inserted into certain sites of FasD without affecting the function of its product. Since mutants with FasD$^-$ phenotypes are nonfimbriated (48, 49), the biological function of FasD was tested by detecting fimbriae on the bacterial surface. By screening for fimbriated linker insertion mutants, we identified mutated *fasD* genes encoding functional proteins. Since the only known function of FasD involves fimbrial protein export, the corresponding mutation sites were designated permissive sites, implying permissiveness of fimbriation to linker insertion.

Recent studies with the Pap fimbrial system suggest that fimbrial subunits interact directly with the fimbrial OMP (11). To understand how fimbrial subunits are exported through the outer membrane, many questions remain to be answered, as, for example, the following. (i) What is the overall structure and topology of a fimbrial OMP in the outer membrane? (ii) Which domains of a fimbrial OMP interact with the membrane, and which ones interact with fimbrial subunits. (iii) By which mechanisms are fimbrial subunits translocated? Does the fimbrial OMP participate in the formation of a fimbrial subunit channel, or in a flippase complex (15), translocating subunits through the lipid bilayer? (iv) What energy drives fimbrial export through the outer membrane?

Results of membrane insertion studies, using PhoA fusions to OMPs or secreted proteins, have been used to propose minimal protein domains required for membrane targeting. For example, studies with pullunase and with siderophore and vitamin B_{12} transporters have suggested that PhoA does not cross the outer membrane or that fusions to surface-exposed domains are lethal (7, 23, 29, 35). Therefore, it is tempting to propose that the amino-terminal end of FasD contains specific targeting signals and that the active FasD-PhoA fusions which were isolated with outer membranes correspond to fusions to periplasmic loops. However, this interpretation needs to be tested since it can be argued that the folding and insertion of the FasD portion of the fusion product is not the same as that of native FasD. Because of the limitations concerning the interpretation of results obtained with fusion proteins to study OMPs, we propose another approach as discussed below.

If fimbrial OMPs of different fimbrial systems effectively share similar functions as different chaperones do, this similarity may be translated to the structural level. Fimbrial chaperone proteins share 60% similar sequences considering conservative substitutions (16), whereas fimbrial OMPs share only 20 to 30% similar sequences. Nevertheless, it has been shown that proteins which have low levels of homology (approximately 20% residue identity, by using pairwise comparisons), but share similar functions, can have common core segments which vary only slightly in their geometry (root mean square deviation of the backbone atoms, $\leq$2.4 Å [$\leq$0.24 nm]) (6). Active sites of proteins sharing similar functions can be expected to be located in common core segments. Conserved domains of fimbrial OMPs may be involved in the translocation process of fimbrial subunits through the outer membrane. As such, these domains may include a rigid frame interacting with lipids as well as flexible domains which can be envisioned to undergo a conformational change to accommodate fimbrial subunits during export. In contrast, variable domains may be involved in the specific recognition processes of the different fimbrial subunits.

In this article, we used a random in-frame linker insertion mutagenesis technique to identify permissive sites in FasD. It was proposed that, by incorporating turn-inducing residues in this protein, (i) permissive sites would be preferentially restricted to nonconserved domains or to surface-exposed domains, predicted to form β-turns and loops, and (ii) nonpermissive sites would preferentially include sites in conserved domains or membrane-spanning domains of FasD, predicted to be β-amphipathic. Therefore, nonpermissive sites could represent essential areas in any topological domain involved in exporting fimbrial subunits as well as essential conformational structures of the protein. Such structures should be conserved among the various fimbrial OMPs. Our data support this assumption since they point to 9 conserved sites among the 10 identified nonpermissive sites. This contrasts with the location of the fully permissive sites, six of eight being flanked by nonconserved residues on both sides. Since the proposed β-barrel structure of FasD is expected to be essential for structural stability in the membrane, the function of FasD should be especially sensitive to mutations in its predicted β-amphipathic membrane-spanning strands. In support of the predicted model, we found here that all 11 identified permissive sites are located at predicted turns or at the junctions of predicted β-strands and turns. Moreover, all five linker insertions which targeted predicted β-sheets were identified as nonpermissive. These encouraging findings open new perspectives for experimental testing. For example, it will now be possible to determine whether the obtained permissive linker insertion sites are, in fact, located in surface-exposed domains by introducing reporter epitopes in such sites and probing with specific antibodies (5). Moreover, the extent of the alterations which can be made at these sites without affecting the function of FasD will test the degree of permissiveness more stringently. In a preliminary experiment, we observed that a permissive linker insertion site could tolerate a reporter epitope of 11 residues, the mutated FasD remaining functional (46a).

Finally, it has been proposed that siderophore receptors undergo a conformational change to internalize their ligands (26, 28). However, such changes have not been demonstrated in vivo. Whether FasD undergoes conformational changes during fimbrial export may be more evident since the translocated molecules are much larger. The obtained *fasD* mutants will be useful to study conformational flexibility of OMPs in the presence or absence of the fimbrial subunits and various fimbrial periplasmic proteins.

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