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Identification of Major and Minor Chaperone Proteins Involved in the Export of 987P Fimbriae

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The 987P fimbriae of *Escherichia coli* consist mainly of the major subunit, FasA, and two minor subunits, FasF and FasG. In addition to the previously characterized outer membrane or usher protein FasD, the FasB, FasC, and FasE proteins are required for fimbriation. To better understand the roles of these minor proteins, their genes were sequenced and the predicted polypeptides were shown to be most similar to periplasmic chaperone proteins of fimbrial systems. Western blot (immunoblot) analysis and immunoprecipitation of various *fas* mutants with specific antibody probes identified both the subcellular localizations and associations of these minor components. FasB was shown to be a periplasmic chaperone for the major fimbrial subunit, FasA. A novel periplasmic chaperone, FasC, which stabilizes and specifically interacts with the adhesin, FasG, was identified. FasE, a chaperone-like protein, is also located in the periplasm and is required for optimal export of FasG and possibly other subunits. The use of different chaperone proteins for various 987P subunits is a novel observation for fimbrial biogenesis in bacteria. Whether other fimbrial systems use a similar tactic remains to be discovered.

There are many ways in which diarrheogenic enteric bacteria both recognize and attach to specific host receptors and ensure direct delivery of enterotoxins (5) or signal transduction molecules (16) to intestinal epithelial cells. Enterotoxigenic *Escherichia coli* utilize fimbriae which optimize the attachment of this pathogen by bridging the bacteria and the host's enterocytes.

We have studied the 987P fimbriae of enterotoxigenic *E. coli*, which mediate attachment to piglet intestines. The eight genes (*fasA* to *fasH*) which encode all the proteins required for fimbriation have been localized to a plasmid (31), and functions have been identified for five of their products (Fig. 1 and Table 1). These fimbriae consist of a helical repeat of the major subunit, FasA, with FasG and FasF located both at the tip of the fimbriae and at intervals along the fimbriae as minor subunits (3, 12). We have previously shown that FasG is the 987P adhesin and recognizes a glycoprotein on piglet intestinal brush borders. FasF was proposed to be inserted between FasG and FasA during fimbrial biogenesis (3, 17). The usher protein, located in the outer membrane, directs translocation of the subunits from the periplasm during fimbrial biogenesis, and we have previously shown that FasD is the usher for 987P fimbriae (30). It has been demonstrated by others that FasH (FapR) is a transcriptional activator required for the expression of *fasA* and possibly other *fas* genes (19).

A number of additional proteins, apart from the exported subunits, are required for the production of functional fimbriae. Fimbrial subunits are exported by a Sec-dependent mechanism from the cytoplasm to the periplasm and eventually through the outer membrane. After crossing the cytoplasmic membrane, the subunits are thought to be in a quasinative conformation and are able to aggregate in the periplasm. To prevent the aggregation and degradation of subunits, other fimbrial systems have been shown to produce a chaperone protein which shields the various subunits in the periplasm (reviewed in reference 11).

In this study, we identified not only a chaperone for the major subunit, FasA, but an additional chaperone, FasC, which acts to protect the adhesin, FasG, from aggregation and degradation. FasE, which we have termed a chaperone-like protein on the basis of its sequence similarity to other chaperones, could not be detected in 987P fimbriae but was shown to be involved in Fas protein export to the outer membrane. We have sequenced the entire *fas* gene cluster, created mutants, and raised antibody probes to delineate the respective roles and interactions of a major and two minor chaperone proteins of the 987P fimbrial system.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The strains and plasmids used for this study are listed in Table 2. Strain BL21 (λ DE3) was used for the expression of histidine-tagged FasE protein, and strain TB1 was used for the expression of MalE-FasC and MalE-FasE fusion proteins. Strains SE5000 and DMS870 were used for immunoprecipitation and preparation of samples for Western blot (immunoblot) analysis. Plasmid pJC11 was constructed from an approximately 340-bp DNA fragment containing the mature *fasE* gene sequence which was amplified by PCR from pDMS60 (primers, 5′ GCTCGAATTCCATATGAATC CATTACTATATCCATTT3′ and 5′ GCGGGATCCAAGCTTCTATATGAT TTTTAAACGGTAG3′) and cloned into pET16b (Novagen, Madison, Wis.) to express FasE with 10 histidine residues at the N-terminal end. Likewise, plasmid pJC18 was created by cloning the same 340-bp fragment into pMAL-p2 (New England Biolabs, Beverly, Mass.) to create an in-frame *malE-fasE* fusion. Plasmid pJC17 was constructed similarly by amplifying the region of the *fasC* gene corresponding to the mature polypeptide from pDMS57 to give an approximately 350-bp product by PCR (primers, 5′GCTCGAATTCCATATGTCCAGTCTTT CTGACGGAATAGCAATC3′ and 5′GCGGGATCCAAGCTTTCAGTTTAA CGCGCTTAATTTAAC3′) and cloning into pMAL-p2. To create stable mutations in *fasB*, *fasC*, and *fasE*, plasmid pDMS167, which does not contain the transposon adjacent to *fasH* (30), was partially digested with *Nco*I and filled with the Klenow fragment of DNA polymerase, and a 10-bp *Xba*I linker was inserted, resulting in plasmids pDMS187 (*fasE*), pDMS189 (*fasC*), and pDMS191 (*fasB*). None of these mutants were fimbriated; however, fimbriation could be restored by the addition of compatible plasmids encoding the affected genes (i.e., pDMS109 [*fasB*$^+$], pDMS113 [*fasC*$^+$], or pDMS93 [*fasE*$^+$]), indicating that these

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FIG. 1. Arrangement of the *fas* gene cluster. IS, insertion sequence 1; ST, heat-stable enterotoxin StIa.

mutations had no significant effect on the production of the other Fas proteins. Plasmid pDMS203, which contains a *fasD* mutation, was constructed by deleting an *Xho*I fragment which is internal to *fasD* from pDMS167. Bacteria were routinely grown on Luria-Bertani medium (24) supplemented with ampicillin (200 μg/ml), chloramphenicol (30 μg/ml), or kanamycin (45 μg/ml) as required. Medium components were purchased from Difco (Detroit, Mich.), and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes were purchased from New England Biolabs.

DNA sequencing and analysis. Double-stranded plasmid DNA was sequenced by the chain termination method (27) with an Applied Biosystems 373A sequencer. The PIR and Swiss Prot protein data banks (releases 91) were searched, and alignment analysis was done by using the Lasergene software package from DNASTAR (Madison, Wis.).

Antigen and antibody preparations. Periplasmic proteins from plasmids containing either the maltose-binding protein (*malE*; pMAL-p2) or *fasC-malE* and *fasE-malE* fusions (pJC17 and pJC18, respectively) were compared by using anti-MalE antibodies. Shifts in the sizes of the MalE fusion proteins indicated that the fusion proteins were produced and exported to the periplasm. These fusion proteins were purified with amylose columns, and the histidine-tagged FasE protein was purified with a nickel column as described previously (3). Rabbits were immunized with the purified proteins (three rabbits per antigen) as described before (3). The anti-987P fimbriae, anti-FasA peptide, anti-MalE-FasG, and anti-FasF antibodies have already been characterized (3, 17).

SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% slab gels and Western blot analysis were performed as described before (32). To ensure the loading of equal samples, protein concentrations were measured before samples were loaded (23), and gels were visualized by Coomassie staining. To adsorb antibodies prior to Western blot analysis, strain DMS870 was resuspended in phosphate-buffered saline and sonicated six times as described before (17), and the supernatants were separated by SDS-PAGE. After the transfer of proteins to nitrocellulose and blocking with 3% bovine serum albumin, antibodies were incubated twice for 1 h at room temperature.

Radiolabeling Fas proteins. Fas proteins were radiolabeled by using [^{35}S]cysteine methionine (NEN-Dupont, Boston, Mass.) and a two-plasmid T7 RNA polymerase-promoter expression system (28, 30, 36). Cells were fractionated as previously described (17). To calculate the ratios of radiolabeled proteins after densitometry, the number of methionines and cysteines in each Fas protein, as deduced from the DNA sequence, and the relative amounts of methionine and cysteine in the labeling mix were taken into account. For comparative purposes, the intensities of the signals of radiolabeled proteins, as determined by densitometric analysis, were normalized to that of an internal standard for each mutant (e.g., β-lactamase or FasB).

Preparation of outer membrane-associated proteins. Like other fimbrial systems, 987P proteins associated with the outer membrane may be removed by treatment at 60°C for 30 min in 75 mM NaCl–0.5 mM Tris-HCl (pH 8.0) (15, 17).

Immunoprecipitation. Antibodies preadsorbed with SE5000 sonicates at room temperature for 30 min were added to radiolabeled periplasmic fractions and incubated at room temperature for a further 2 h with rotation. Meanwhile, 20 μl of 50% protein A-Sepharose beads was preincubated with a sonicate from SE5000, which had not been radiolabeled, for 30 min at room temperature. The beads were collected by centrifugation, resuspended in 0.1% Triton buffer (0.1% Triton X-100, 250 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl [pH 7.5]), and added to the periplasmic fraction containing the antibodies. After a 30-min incubation at room temperature with rotation, the protein A-Sepharose beads were collected by centrifugation, washed three times in 0.1% Triton buffer, and finally resuspended in SDS-sample buffer (27). Samples were incubated either at 100°C for 10 min to dissociate the Fas proteins or at room temperature for 10 min prior to being separated by SDS-PAGE using 15% slab gels. Gels were examined after fluorography (4).

RESULTS

Localization and functional characterization of FasB. FasB was originally identified by using a T7 RNA polymerase-promoter expression system (33); however, FasB was not characterized and its subcellular localization was not studied. We identified the location of FasB by subcellular fractionation of SE5000 with either pDMS167 (Fas$^+$), pDMS203 (*fasD*), or pDMS191 (*fasB*). Comparison of Coomassie-stained SDS-PAGE gels of periplasmic and 60°C extracts identified an approximately 28-kDa protein in the periplasmic fraction which was absent from the *fasB* mutant strain (not shown).

After specific radiolabeling with the T7 RNA polymerase-promoter system, FasB could not be identified in 60°C extracts from a fimbriated (wild-type) strain in which the fimbrial components FasA, FasF, and FasG were visible, suggesting that FasB is compartmentalized to the periplasm (not shown). FasA and FasB were identifiable in radiolabeled periplasmic fractions of the 987P-fimbriated wild-type strain, but periplasmic extracts from a *fasB* mutant strain did not contain FasA, suggesting that soluble periplasmic FasA was stabilized by the presence of FasB (Fig. 2 and Table 3). The amount of cytoplasmic FasA precursor was not altered in a *fasB* mutant (data not shown), indicating that FasB is not involved in FasA synthesis. These results were reproducible, and densitometric analysis did not reveal any significant effect on the localization or quantity of any other Fas protein due to the absence of FasB (Fig. 2 and data not shown).

Fas proteins from SE5000(pDMS203) (*fasD*) were radiolabeled prior to periplasmic fractionation, and the previously characterized polyclonal anti-987P antibody, which recognizes fimbriae essentially by binding to the major subunit (FasA) (17), was used to immunoprecipitate proteins from the periplasm as described in Materials and Methods. As shown in Fig. 3, FasA coprecipitated with FasB; under these conditions, no other Fas proteins were precipitated. This finding indicates that FasB interacts directly with FasA in the periplasm. Moreover, since the anti-987P antibody recognizes native FasA (17) and an anti-FasA peptide antibody previously shown to be specific for dissociated and denatured FasA did not immunoprecipitate FasB (not shown), our data suggest that FasA exists in a quasinative state in the periplasm in association with FasB. Immunoprecipitation of the periplasmic fraction after prolonged overexpression [SE5000(pDMS203) (*fasD*) grown for 60 min after induction of the T7 RNA polymerase-promoter system] with the anti-987P antibody identified a second population of FasA subunits associated with minor subunits FasF

TABLE 1. Role of each Fas protein in fimbrial biogenesis

Protein	Molecular mass (kDa)	Location	Function(s)	Reference
FasA	23	Fimbriae	Major subunit	32
FasB	30.5	Periplasm	Chaperone	This work
FasC	18	Periplasm	Chaperone	This work
FasD	92	Outer membrane	Usher	30
FasE	13.5	Periplasm	Chaperone/export	This work
FasF	17.5	Fimbriae	Minor subunit	3
FasG	40	Fimbriae	Adhesin	17
FasH (FapR)	30	Cytoplasm	Transcriptional activator	19

TABLE 2. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic(s)	Reference
E. coli		
SE5000	MC4100 *recA56* (Fim$^-$)	34
BL21(λDE3)	B F$^-$ *ompT hsdSB* (r_B^- m_B^-) *dcm gal* (λ DE3)	35
TB1	K-12 F$^-$ *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [ϕ80 *dlac* Δ(*lacZ*) M15] *hsdR* (r_k^- m_k^-)	1
DMS870	MC4100 Δ*malB zjc*::Tn*5 recA1 srl*::Tn*10*	17
Plasmids		
pDMS6	pBR322 *fas*$^+$ Tn*1681*	31
pDMS6.1.4	pDMS6 *fasG*::Tn*phoA*	32
pDMS8	pDMS6 with a T7 promoter upstream of *fasA*	32
pDMS18	pDMS8 *fasA* frameshift mutation in *fasA*	32
pDMS57	*fasC* in pBluescript KS	This work
pDMS60	*fasE* in pBluescript KS	This work
pDMS93	*fasE* in pACYC184	This work
pDMS109	*fasB* in pACYC184	This work
pDMS113	*fasC* in pACYC184	This work
pDMS167	pDMS8 ΔTn*1681*	30
pDMS187	pDMS167 *fasE* (linker inserted in *fasE*)	This work
pDMS189	pDMS167 *fasC* (linker inserted in *fasC*)	This work
pDMS191	pDMS167 *fasB* (linker inserted in *fasB*)	This work
pDMS200	pKS *fasC*$^+$ *fasG*$^+$	This work
pDMS202	pKS *fasB*$^+$ *fasG*$^+$	This work
pDMS203	pDMS167 *fasD* (linker inserted in *fasD*)	This work
pGP1-2	T7 RNA polymerase	36
pJC11	pET16b *fasE*	This work
pJC17	pMAL-p2 *fasC*	This work
pJC18	pMAL-p2 *fasE*	This work

and FasG (Fig. 3, lane 2). Similar immunoprecipitations of the periplasmic fraction of a *fasA* mutant (pDMS18) did not result in the detection of FasF and FasG (not shown). This suggested that in the absence of subunit export at the outer membrane, subunit accumulation in the periplasm leads to the aggregation of misfolded subunits or premature assembly of subunits, which can be mimicked in vitro with disassociated subunits (29). The alternative possibility that FasB associates as a homopolymeric structure in the periplasm, binding various types of subunits, is unlikely, as shown below. By using the T7 expression system, excess Fas proteins are not degraded efficiently and the difference in the FasA/FasB ratios in the two lanes of Fig. 3 reflects the kinetics of subunit accumulation and degradation in the periplasm of an export-deficient mutant.

Sequence analysis: FasB is the FasA chaperone. The *fas* gene cluster has been sequenced, and the 7,313-kb sequence from *fasA* to *fasG* has been deposited in the GenBank database (accession no. U50547). The predicted *fasB* open reading frame was identified from the *fas* gene cluster sequence by the location of the open reading frame with respect to *fasA* (6) and by the size of its predicted product. Like the exported proteins of other fimbrial systems, the predicted FasB sequence contains a potential signal sequence for the Sec-dependent export system, with a predicted cleavage site 21 amino acids from the N-terminal methionine. The mature polypeptide is predicted to have a molecular mass of 24 kDa and an isoelectric point of 9.11.

Searches of databases using the predicted mature FasB polypeptide sequence revealed homologies to members of the chaperone family of periplasmic proteins, such as PapD (22). There were no other significant homologies, and an alignment of FasB with several other *E. coli* chaperones is shown in Fig. 4. Many of the conserved residues shown to be involved in PapD conformation and subunit recognition, such as the three residues which form the internal salt bridge (E-78, R-120, and D-200 [FasB numbering]), and some of the residues involved in subunit binding, such as R-8 and A-110, are also conserved in FasB (10).

Although there is a large stem-loop structure ($\Delta G = -21.1$) predicted to lie between FasA and FasB, there is no poly(T) region associated with ρ-independent terminator sequences and only very slight homologies with potential σ^{70} promoter sequences. There is also a potential Shine-Dalgarno sequence (GGAG) 7 nucleotides upstream of the initiation codon. Hence, *fasA* and *fasB* are probably transcriptionally coupled

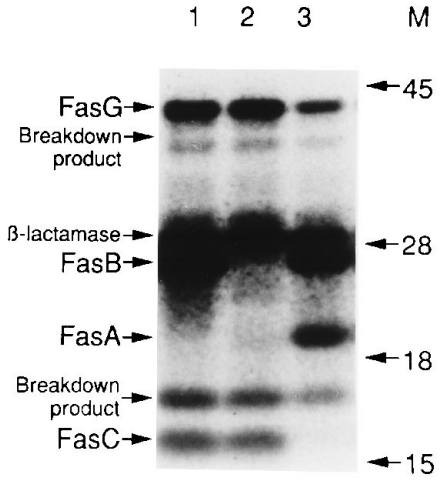

FIG. 2. Fluorograph showing radiolabeled periplasmic Fas proteins after expression with the T7 RNA polymerase-promoter system. Periplasmic fractions of a *fasA* mutant (lane 1), *fasB* mutant (lane 2), and *fasC* mutant (lane 3). The positions of radiolabeled Fas proteins and the molecular masses of standard proteins (M; in kilodaltons) are shown on the left and right, respectively.

TABLE 3. Relative amount of each Fas protein in periplasmic extracts after T7 expression and radiolabeling

Expressed protein	Ratio for mutant[a]						
	fasA	*fasB*	*fasC*	*fasD*	*fasE*	*fasF*	*fasG*
FasA	0	0	0.61	0.69	0.66	0.71	0.67
FasB	0.54	0	0.59	0.66	0.56	0.56	0.55
FasC	0.46	0.40	0	0.31	0.38	0.31	0.55
FasE	0.29	0.30	0.25	0.25	0	0.30	0.37
FasF	0.13	0.12	0.10	0.08	0.09	0	0.12
FasG	0.11	0.12	0.06	0.09	0.09	0.11	0
β-Lactamase	1	1	1	1	1	1	1

[a] Ratios were determined by densitometry of fluorographs from radiolabeled periplasmic extracts. Ratios were calculated from one of two to four reproducible experiments. The intensities of the signals of radiolabeled Fas proteins were normalized to that of β-lactamase, which was standardized to 1 for each mutant.

and less FasB than FasA is produced. This is consistent with the concept that chaperones like FasB are recycled in the periplasm after fimbrial subunit translocation and that, therefore, fewer new chaperone molecules are required.

Taken together, our results indicate that FasB is the 987P periplasmic chaperone for the major subunit, FasA.

FasB is not a chaperone for FasG. In many other fimbrial systems, a single chaperone which protects the different fimbrial subunits has been identified; therefore, we investigated whether FasB was the chaperone for the 987P adhesin, FasG. With anti-FasG antibodies, FasB and FasG could not be coprecipitated (Fig. 5) and a *fasB* mutation had no effect on the amount of periplasmic FasG (Fig. 2 and Table 3), suggesting that FasB is not a periplasmic chaperone for FasG. In contrast, the amount of FasG detected in the periplasmic extracts of the *fasC* mutant was somewhat smaller than that in periplasmic extracts from *fasA* or *fasB* mutants (Fig. 2 and Table 3), suggesting that FasC, not FasB, interacts with FasG to stabilize it in the periplasm; therefore, FasC may be involved in the export of FasG.

Localization and functional characterization of FasC. FasC was previously identified after T7 RNA polymerase-induced expression of the *fas* gene cluster (33). Electron micrograph analysis of a strain lacking FasC showed that fewer than 20% of cells were fimbriated and that those cells which were fimbriated had less than 20 fimbrial structures with rough contours (32). This indicated that FasC was required for the correct formation of fimbriae; however, no role in fimbrial export was hypothesized for this protein.

Previously, two forms of FasC, presumably the precursor and processed forms, were identified (32). We fractionated DMS870 with either pDMS167 (Fas^+), pDMS203 (*fasD*), or pDMS189 (*fasC*), and specific anti-FasC antibodies were used to identify the subcellular localization of FasC by Western blot analysis (not shown). FasC could be detected in the periplasm but not in fimbriae from the wild-type strain, suggesting that FasC is a periplasmic accessory protein which is not part of the fimbrial structure. Confirming results were obtained by immunoprecipitating radiolabeled isolated fimbriae; only FasA, FasF, and FasG proteins were detected (18). Moreover, as mentioned above, subcellular fractions clearly localized FasC in the periplasm (Fig. 2).

Since FasC was not detected on the fimbrial structure, we investigated whether there was any interaction between FasC and other Fas proteins in the periplasm which would explain the role of FasC in fimbriation. The Fas proteins were radiolabeled in a *fasD* mutant background so that fimbriae could not be assembled. Immunoprecipitation of proteins from periplasmic fractions with specific anti-FasC antibodies (Fig. 5) identified only FasC (18 kDa) and FasG (40 kDa). Similarly, anti-FasG antibodies precipitated specifically FasG and FasC (Fig. 5), confirming that there is a direct interaction between FasC and FasG proteins in the periplasm.

Densitometric analysis of the fluorographs of FasC and FasG immunoprecipitated with either anti-FasC or anti-FasG antibodies indicated that approximately three FasC molecules coprecipitated with each FasG molecule. FasF could also be identified after immunoprecipitation with anti-FasG antibodies. Likewise, FasG could be identified when periplasmic extracts were immunoprecipitated with anti-FasF antibodies (Fig. 5), indicating that under the conditions used to radiolabel the periplasmic fractions there is an interaction between FasF and FasG. However, FasC could not be precipitated with FasF antibodies, indicating that FasG-FasC interaction is specific. The association of FasF and FasG is proposed to be due to the experimental conditions used to optimize Fas protein interactions in the periplasm by overexpressing them briefly and stopping export at the outer membrane with a *fasD* mutant, as described above (Fig. 3).

The FasC sequence: another chaperone. The open reading frame encoding FasC was identified by its location in the *fas* gene cluster sequence and by the size of its predicted product. The predicted FasC polypeptide contains a potential signal sequence for Sec-dependent export (residues 1 to 31). The mature polypeptide is predicted to have a molecular mass of 14 kDa and an isoelectric point of 9.11.

The predicted mature FasC protein sequence was compared with published protein sequences and was seen to be most homologous to other fimbrial chaperones, such as PapD, even though FasC is only half the length of those other chaperones. Most strikingly, FasC aligns best with domain 1 of the two-domain immunoglobulin-like structure of PapD (10) (Fig. 4). There were no other significant similarities, including homologies to other fimbrial subunits in general.

The 5′ 41 bp of *fasC* overlap the 3′ end of *fasB*; hence, these two genes may be translationally coupled. In addition, there is a potential σ^{70} promoter upstream of *fasC* (−35, TTGACA; −10, TTAACA), indicating that *fasC* may be transcribed separately from *fasB*. There is also a potential σ^{70} promoter downstream of *fasC* (−35, TTGACA; −10, TATTA), indicating that *fasD* may be separately transcribed.

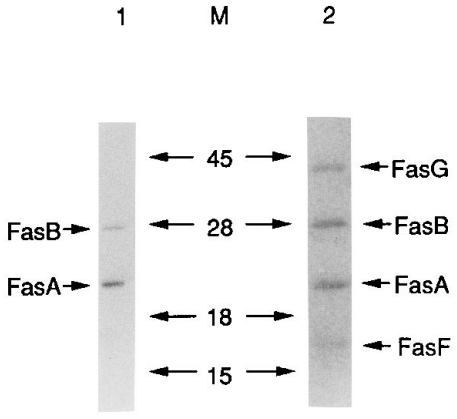

FIG. 3. Immunoprecipitation of radiolabeled FasA and FasB from the periplasm of pDMS203 (*fasD*) using anti-987P polyclonal antibodies. Lane 1, immunoprecipitation after brief radiolabeling (5 min); lane 2, immunoprecipitation after prolonged labeling (60 min). The positions of radiolabeled Fas proteins and the molecular masses of standard proteins (M; in kilodaltons) are shown.

Since FasC, not FasB, stabilizes FasG in the periplasm, FasC and FasG coprecipitate, and FasC is significantly homologous to other fimbrial chaperones, we propose that FasC is the periplasmic chaperone for FasG and that FasC may be involved in optimal delivery of FasG to the outer membrane for effective fimbrial biogenesis.

FasG export in the absence of the chaperones and usher. How fimbrial proteins associate with and are exported through the outer membrane can be evaluated by analyzing heat (60°C) extracts of bacteria. This method was pioneered with the K99 fimbriae (7) and shown to be specific for Pap fimbrial subunits (13), as well as for the 987P proteins (3). By this technique, FasG was previously found to be associated with the outer membrane in the absence of the two other fimbrial subunits, FasA and FasF (3). To determine FasG export efficiency at the outer membrane in the absence of any chaperone or the usher, 60°C extracts from *fasB*, *fasC*, and *fasD* mutants were analyzed. In comparison with that of the *fasB* mutant, there was less export of FasG in the absence of FasC (Fig. 6). This was consistent with our previous data which showed reduced periplasmic FasG in the *fasC* mutant. The smallest amount of FasG protein was found with the *fasD* mutant. FasD has previously been shown to be the usher protein located in the outer membrane (30), and the presence of small amounts of heat-extractable FasG in the absence of the usher may suggest that some FasG can translocate in the absence of FasD. However, whether this export is productive, namely, whether the FasG molecules which do not require the usher to associate with the outer membrane would be assembly competent remains to be shown. In addition to the *fasC* and *fasD* mutants, the *fasE* mutant showed decreased amounts of exported FasG (Fig. 6), suggesting that FasE is involved in fimbrial biogenesis by being required for efficient export of FasG.

FasE is a chaperone-like protein. FasE was originally identified by T7 RNA polymerase-promoter-based overexpression (33), and electron micrograph analysis of strains lacking FasE revealed that 70% of cells contained between one and five very long fimbriae (32); however, no role was postulated for this protein.

The *fasE* gene sequence was identified from the *fas* gene cluster sequence on the basis of its size and position relative to *fasD* and *fasF*. There are only 2 bp between the end of *fasD* and the initial methionine of *fasE*, and there is no ρ-independent transcriptional terminator or putative promoter sequences, indicating that *fasD* and *fasE* are translationally coupled. The *fasE* gene encodes a polypeptide with a proposed signal sequence of 21 amino acids, suggesting that FasE is exported at least to the periplasm. The mature polypeptide has an expected molecular mass of 12.5 kDa, corresponding well to the approximately 13 kDa estimated by SDS-PAGE (this work) (32). The calculated isoelectric point of the predicted mature polypeptide is 9.51.

Comparison of the predicted polypeptide sequence with protein sequences in databases showed homologies to the fimbrial chaperones, such as PapD (Fig. 4). Like FasC, FasE is approximately half the length of the chaperones previously identified, and many of the structural motifs identified in PapD are conserved in FasE. There was no significant homology to any other family of proteins.

Localization and functional characterization of FasE. Strain DMS870 with either pDMS167 (Fas^+), pDMS203 (*fasD*), or pDMS187 (*fasE*) was fractionated, and specific anti-FasE antibodies were used to identify the subcellular localization of FasE by Western blot analysis (not shown). FasE could be detected in the periplasm from either the wild type or a *fasD* mutant but not in fimbriae from wild-type strains or in periplasm from a *fasE* mutant (not shown), indicating that FasE, like FasC, is a periplasmic accessory protein which is not part of the fimbrial structure.

Since FasG export was decreased in a *fasE* mutant, as previously shown (Fig. 6), we hypothesized that FasE is involved in the export of FasG and possibly other subunits. Moreover, since the primary structure of FasE is reminiscent of chaperone proteins, FasE may be directly involved in interacting with fimbrial subunits at some point during export. To evaluate how FasE is involved in fimbrial biogenesis, we attempted to identify interactions between FasE and other periplasmic Fas proteins by immunoprecipitation (not shown). However, only FasE was immunoprecipitated with specific anti-FasE antibodies, and FasE was not precipitated with any other Fas protein by using specific antibodies.

Although there is currently no evidence for a direct interaction between FasE and any other Fas protein, this possibility is not unlikely, considering the primary structure of FasE and the reduced export of FasG in a *fasE* mutant. Temporal and spatial constraints may hinder the detection of such an interaction. For example, FasE may interact with FasG during or immediately after the translocation of FasG from the cytoplasm; alternatively, FasE may interact with FasG immediately prior to FasG export but after FasG has interacted in the periplasm with FasC. FasG and FasC were coprecipitated from the periplasm of both wild-type and *fasE* mutant strains, indicating that FasG-FasC interaction is the primary interaction in the periplasm and that FasE does not interact with FasG immediately after FasG translocation across the cytoplasmic membrane or prior to the association of FasG with FasC. In contrast, FasE was not precipitated with FasG from the wild-type or any mutant strain, indicating that the probable association between FasG and FasE is temporary and occurs immediately prior to FasG export from the periplasm through the outer membrane. It is possible that interactions were not detected because anti-FasE antibodies may recognize the site of interaction between FasE and other Fas proteins and therefore precipitate only free FasE; however, FasE was not precipitated with other anti-Fas antibodies.

DISCUSSION

We have previously shown that FasD is the outer membrane-associated usher protein required for export of the Fas proteins from the periplasm and that FasG, FasF, and FasA are the fimbrial components exported in that order (3, 30). In this study, we have initiated characterization of three proteins which are required for 987P fimbrial biogenesis, FasB, FasC, and FasE. FasB and FasC have been shown to be periplasmic chaperones for the major subunit, FasA, and the adhesin, FasG, respectively. Both chaperones interact specifically with a different subunit and protect that subunit from proteolytic degradation in the periplasm. Moreover, we have shown that FasE has sequence similarities with periplasmic fimbrial chaperones and that its presence enhances the association of FasG with the outer membrane.

The archetypal member of the chaperone family of proteins is PapD, which is involved in the export of pyelonephritis-associated pili (Pap). The majority of fimbrial systems studied to date have proteins with homology to PapD, for example, FaeE and FanE in K88 and K99 fimbriae, respectively, and FimC in type 1 fimbriae (Fig. 4) (2, 20). The crystal structure of PapD has been resolved to 2.5 Å (1 Å = 0.1 nm) (9), and a two-domain structure whereby the fimbrial subunit binds in the cleft of the chaperone between the two domains has been predicted (10, 21). The chaperone is essential for fimbriation

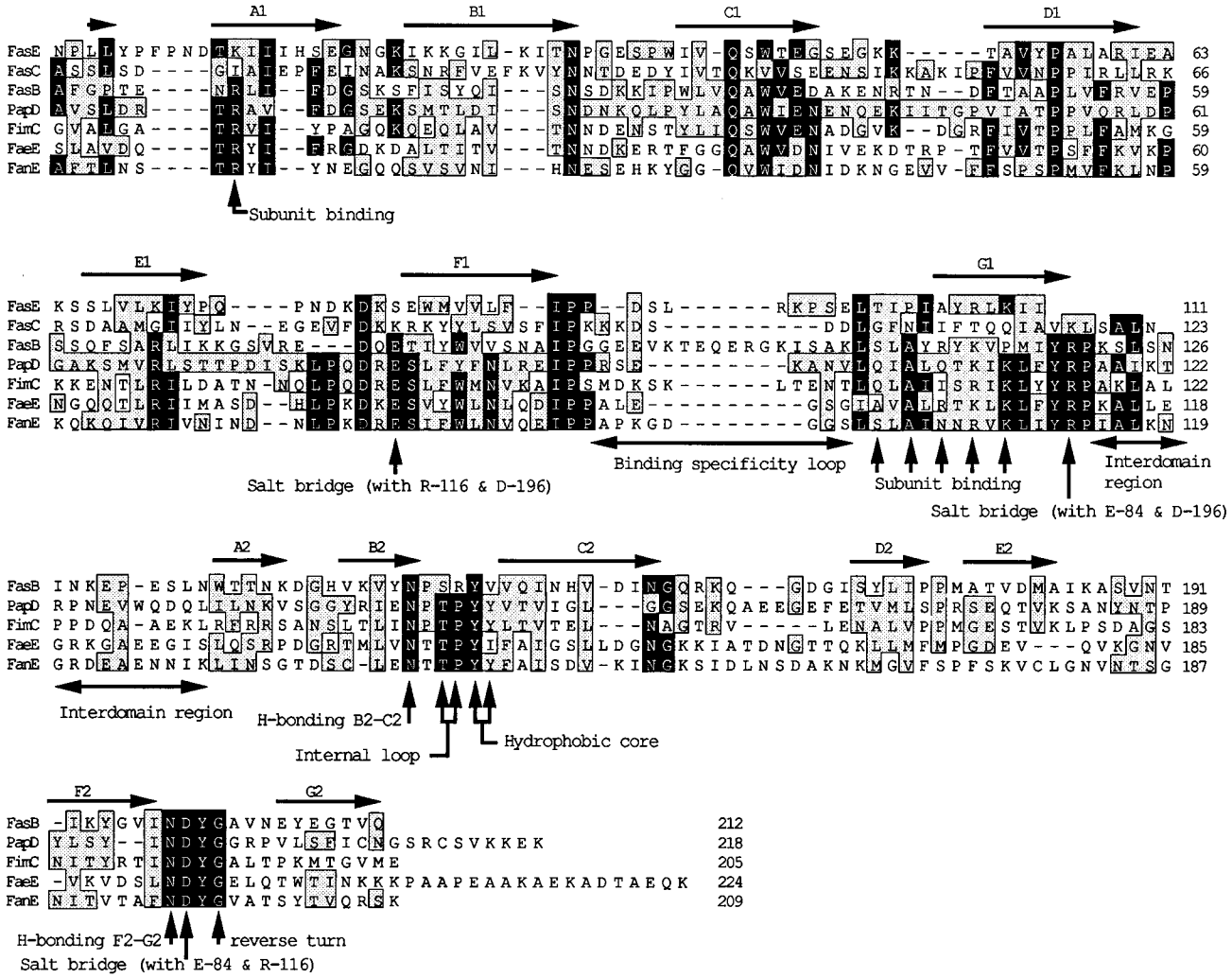

FIG. 4. Alignment of FasB, FasC, and FasE with the *E. coli* fimbrial chaperones PapD, FimC, FaeE, and FanE. The β-sheets, sites of chaperone-subunit interaction, and residues involved in intramolecular salt bridge formation, as determined by crystallography for PapD (9), are indicated. Identical residues are shown as white letters on a black background, while functionally similar residues are shown as black letters on a gray background. Dashes indicate gaps.

being required for efficient delivery of the fimbrial subunit to the usher, and its absence typically results in either the degradation of one or more subunits or premature and unproductive subunit aggregation in the periplasm (8, 20).

The majority of chaperones characterized to date have similar properties. They range in size from approximately 25 to 30 kDa and are all positively charged at physiological pH, having predicted pI values in excess of 9. Likewise, fimbrial subunits are often negatively charged at physiological pH, providing a potential mechanism for stabilizing subunit-chaperone interactions.

FasB appears to be a typical chaperone, associating with FasA and protecting it from degradation in the periplasm. Moreover, it is very similar in sequence to other chaperones, such as PapD. FasB has a molecular mass of approximately 28 kDa and a predicted pI in excess of 9. A number of residues in PapD have been identified as being involved in the interaction of this chaperone with the fimbrial subunits, and some of these are conserved between PapD and FasB. Importantly, a number of subunit binding residues are different; this indicates that alternative interactions are made between FasB and FasA. FasA differs from many other fimbrial subunits at the C-terminal end and does not contain a penultimate tyrosine residue, which has been shown to be important for subunit-chaperone interactions (21).

FasC also acts as a chaperone, associating with FasG and interfering with FasG degradation in the periplasm. Complete degradation of FasG could not be detected in a *fasC* mutant. In such a mutant, FasG may not be degraded as efficiently as FasA in a *fasB* mutant by being a poorer substrate for periplasmic proteases. It may be postulated that efficient elimination of a major subunit is more advantageous for the survival of bacteria than the elimination of an excess of a minor protein. Although FasC has a high pI like other chaperones, it is approximately half the length of previously identified chaperones and does not contain conserved residues at the site of subunit-chaperone interaction. It is noteworthy that this end of FasG is different from those of many other fimbrial subunits and, like the end of FasA, does not contain a penultimate tyrosine residue. On the basis of densitometric analysis and the small

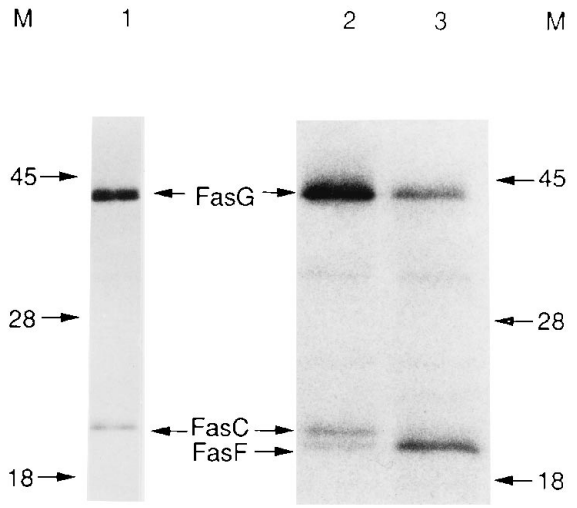

FIG. 5. Immunoprecipitation of radiolabeled periplasmic proteins from pDMS203 (*fasD*) using anti-FasC polyclonal antibodies (lane 1), anti-FasG polyclonal antibodies (lane 2), and anti-FasF polyclonal antibodies (lane 3). The positions of radiolabeled Fas proteins and the molecular masses of standard proteins (M; in kilodaltons) are shown.

size of FasC, this chaperone may multimerize around the C-terminal end of FasG. We suggest that on average three FasC molecules bind to each FasG molecule in the periplasm, although it is not clear whether the FasC-FasG complex is a heterotetramer or some other permutation that results in a 3:1 average ratio. It has previously been shown that FaeE, the periplasmic chaperone for K88 fimbriae, binds Fae subunits in a 2:1 or 1:1 ratio (25, 26), and it is possible that the FasC-FasG complex may exist as heterotrimers (2 FasC:1 FasG) and heteropentamers (4 FasC:1 FasG).

In various experiments, FasA, FasF, and FasG were shown to aggregate in the periplasm. Overexpression of these proteins under conditions in which they cannot be exported are expected to result in free subunits in the periplasm, as it appears that the chaperone proteins are transcribed and translated less efficiently than are the subunits. Under these conditions, the observed formation of subunit aggregates may be driven by the difference in the free energy of fimbrial subunits before and after aggregation. This difference in free energy is thought to be responsible for the driving force normally required to translocate subunits to the growing fimbriae (14).

On the basis of sequence similarity, FasE also appears to be a chaperone-like protein. However, FasE was not shown to interact with any of the fimbrial subunits in the periplasm. It is postulated that FasE optimizes the export of at least FasG from the periplasm to the outer membrane since the amount of FasG associated with the outer membrane is reduced in a *fasE* mutant. It has previously been shown that in the absence of FasE, approximately 70% of cells make a few, very long fimbriae, and it was also speculated that FasG creates weak points along the fimbriae that provide adhesive breakage points (3, 33). If FasE regulates the export of FasG into the outer membrane to initiate fimbrial biogenesis or into growing fimbriae to provide breakage points, then a few long fimbriae would be expected in a *fasE* mutant. The identification of FasG export in the absence of all other Fas proteins has led us to speculate that FasG may be able to enter the membrane in the absence of FasD, probably with the aid of FasE in vivo. It remains to be determined, however, whether the exported FasG in the absence of other Fas proteins is in an assembly-competent form. For example, it is possible that this export is a means of reducing the burden of excess FasG in the periplasm. We propose that once in the membrane, FasG, FasE, and FasD associate to form a structure which allows the ordered export of fimbrial subunits (Fig. 7). No other radiolabeled periplasmic Fas protein could be precipitated with FasE by using anti-FasE antibodies, which indicates that FasE interactions are either transitory or occur in the outer membrane.

Although both FasC and FasE are only half the size of previously characterized chaperones, both of them are homologous to PapD and align to the first domain of PapD. These alignments suggest that FasC, FasE, and domain 1 of PapD all have similar tertiary structures essential for their function as chaperones.

This is the first identification of multiple chaperones for a fimbrial system. Similarly, each of the excreted *Yersinia pestis* Yop proteins have a separate chaperone (37). It is likely that other systems that have more than one chaperone will be identified.

Our data show that FasB is the periplasmic chaperone for the 987P major subunit, FasA, and that FasC is a chaperone for the adhesin, FasG. Our preliminary results suggest that the chaperone-like protein FasE is required for the association of FasG with the outer membrane. Future investigations will examine whether FasE is directly involved in subunit transloca-

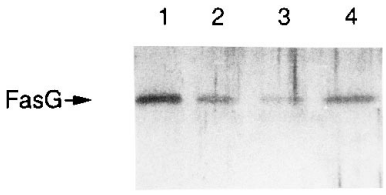

FIG. 6. Western blot of outer membrane-associated proteins from overnight cultures using anti-FasG antibodies and visualized by enhanced chemiluminescence. Equal amounts of protein were loaded in all lanes. Lane 1, *fasB* mutant; lane 2, *fasC* mutant; lane 3, *fasD* mutant; lane 4, *fasE* mutant. Normalized ratios of FasG export, as measured by densitometry, correspond to 1 (*fasB*), 0.5 (*fasC*), 0.26 (*fasD*), and 0.65 (*fasE*). The position of FasG is shown on the left.

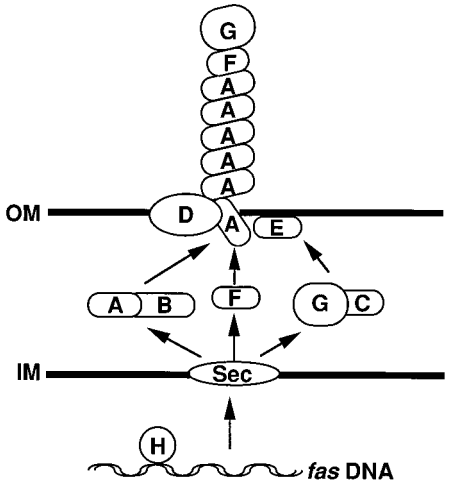

FIG. 7. Model for 987P fimbrial subunit translocation. After export into the periplasm via a Sec-dependent mechanism, subunits FasA and FasG interact with their respective chaperones, FasB and FasC. FasE is required for efficient translocation of FasG and may form a complex with the membrane-bound usher, FasD. OM, outer membrane; IM, inner membrane.

tion and how the proposed interactions among FasE, FasD, and FasG result in the initiation of fimbrial biogenesis.

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