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BfpB, an Outer Membrane Lipoprotein Required for the Biogenesis of Bundle-Forming Pili in Enteropathogenic *Escherichia coli*

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The bundle-forming pili (BFP) of enteropathogenic *Escherichia coli* are believed to play a role in pathogenesis by causing the formation of bacterial microcolonies that bind epithelial surfaces of the small intestine. This in vivo process is mimicked in vitro by the autoaggregation and localized adherence phenotypes. Expression of BFP, a member of the type IV pilus family, requires the enteroadherence factor (EAF) plasmid, which contains *bfpA*, the gene that encodes the principal structural subunit of BFP. Immediately downstream of *bfpA* are 13 open reading frames transcribed in the same direction as *bfpA*; together with *bfpA*, these compose the *bfp* gene cluster. Disruption of *bfpB*, the second open reading frame downstream of *bfpA*, was performed by allelic exchange. The resulting mutant, B171-8ΔB, did not exhibit the autoaggregation or localized adherence phenotype or produce BFP filaments. Thus, BfpB is required for pilus biogenesis. However, BfpA was produced at wild-type levels and processed normally by B171-8ΔB, indicating that BfpB acts at a step in the BFP biogenic pathway after production and processing of the structural subunit. Biochemical and cell fractionation studies showed that BfpB is a 58-kDa lipoprotein that is located primarily in the outer membrane. Assays of *bfpA* and *bfpB* mRNAs and protein expression showed that both genes are cotranscribed as part of an environmentally responsive operon that is regulated by growth phase and ammonium.

Enteropathogenic *Escherichia coli* (EPEC) is a significant cause of diarrhea among infants living in developing countries (8, 10, 11, 26). EPEC is not believed to cause diarrhea by elaboration of conventional enterotoxins or cytotoxins and does not extensively invade or destroy intestinal epithelial cells. Although the mechanism by which EPEC causes disease is a matter of current investigation, several characteristic features of EPEC infection suggest that EPEC pathogenesis entails the following stages: an early stage characterized by long-range, adhesin-mediated attachment of the bacteria to the host-cell surface and a later stage characterized by "intimate" attachment that leads to changes in the host cell cytoskeleton (20).

Electron microscopic examination of small-bowel biopsies obtained from EPEC-infected children reveals discrete microcolonies of bacteria attached to mucous membranes (32, 36, 37, 44). Beneath the adherent bacteria, close juxtaposition of the bacterial outer membrane and the underlying plasma membrane is evident, and this juxtaposition is associated with localized elevation and invagination of the plasma membrane, effacement of microvilli, and rearrangement of the eukaryotic cytoskeleton. These features compose the attaching and effacing lesion that epitomizes EPEC infection of epithelial surfaces (19, 20, 37). A similar pattern of discrete microcolony adherence can be observed by incubating EPEC with tissue culture cell monolayers in vitro, which results in characteristic circumscribed clusters of bacteria attached to the epithelial cell surface (7, 28). This localized adherence (LA) pattern of attachment is a common property of the classic EPEC serotypes and requires the ~80-kbp enteroadherent factor (EAF) plasmid (3, 21, 24, 26, 27). Strains cured of the EAF plasmid have a dramatically reduced ability to adhere to epithelial cells and, when tested in human volunteers, are found to be less virulent (18, 21). Thus, the EAF plasmid appears to be important for the LA phenotype in vitro and for infectivity in vivo.

The EAF plasmid contains a locus responsible for the elaboration of the bundle-forming pilus (BFP) (41, 42), an environmentally and growth-phase-regulated organelle whose expression is tightly correlated with the ability of EPEC strains to exhibit the localized adherence phenotype (33). This locus, designated the *bfp* operon, is composed of 14 genes; its expression is regulated by a promoter upstream of *bfpA*, the gene encoding pilin, the principal structural subunit of the pilus filament. BFP may mediate the first stage of EPEC pathogenesis, the long-range interaction between the bacteria and eukaryotic cell surfaces (9). In addition, expression of BFP causes formation of dynamic bacterial aggregates, designated the autoaggregation (AA) phenotype (45). These aggregates may constitute the functional infectious units of EPEC within the lumen of the small bowel.

The BfpA pilin protein was shown by sequence homology to belong to the type IV family of pili, whose members include the toxin-coregulated pili of *Vibrio cholerae* and the somatic pili of *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*, proven colonizing factors of these human pathogens (9). In turn, genes involved in type IV pilus biogenesis in these systems have similarity with genes implicated in the assembly and/or transport of certain degradative enzymes, toxins, and DNA-uptake and filamentous-phage transport systems, all of which appear to require the type II, *sec*-dependent general secretory pathway (14, 34, 43). It is not surprising that several of the predicted proteins encoded by downstream open reading frames (ORFs) of the *bfp* operon show sequence homology with type IV pilus biogenic proteins and membrane traffic wardens for the general secretory pathway.

To better understand type IV pilus biogenesis, we have begun to assess the roles of proteins encoded by the *bfp* operon that, in addition to BfpA, might be required for assembly and secretion of this bundle-forming filament. In this report we

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
B171	Wild-type EPEC 0111:NM; carries the 80-kb EAF plasmid and the 90-kb Tet^r-Str^r plasmid	31 and 45
B171-5	B171 lacking the EAF plasmid; retains the 90-kb Tet^r-Str^r plasmid	45
B171-8	B171 lacking the 90-kb Tet^r-Str^r plasmid; retains the EAF plasmid	9
B171-8ΔB	B171-8 EAF plasmid mutant specifically lacking the *bfpB* gene product, BfpB	This work
B171-8dBFP	B171-8 EAF plasmid *bfp* operon mutant	This work
SM10 λpir	*E. coli* laboratory strain carrying functions necessary for autonomous replication of pGP704-based suicide vectors	39
Plasmids		
pBluescript SK$^+$	Cloning vector	Stratagene
pSS9	Subclone of an EAF plasmid *Hin*dIII fragment used to isolate the 5′ end of *bfpB*	40
pERI2493	Subclone of an EAF plasmid *Eco*RI fragment used to isolate the 3′ end of *bfpB*	41
pGP704	Suicide vector for constructing the *bfpB* mutation	25
pWKS130	Low-copy-number cloning vector	46
pLC-AGB	pWKS130 containing the *bfpA* to *bfpB* sequence corresponding to bases 2267 to 5601 (numbering is according to that in reference 41) was used for complementing B171-8ΔB	This work

characterize the protein product of *bfpB*, a gene located downstream of *bfpA* within the *bfp* operon.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains used in this work are described in Table 1. The suicide plasmid used for construction of the *bfpB* mutant was pGP704 (25). Growth conditions were as described previously (33). Briefly, induced cultures were started with a 1:100 dilution of a standard overnight culture (a bacterial suspension in phosphate-buffered saline [PBS] with an optical density at 600 nm of 1.8 of cells from an overnight Luria-Bertani [LB] broth culture) in Dulbecco's modified Eagle's medium (DMEM).

Recombinant DNA techniques. All DNA manipulations were performed by standard molecular and genetic techniques (2, 23). Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's recommendations. PCR amplifications were performed with *Taq* polymerase as recommended by the manufacturer (Perkin-Elmer, Norwalk, Conn.).

Construction of the *bfpB* mutant EAF plasmid. We designed an oligonucleotide primer that corresponds to bases 87 to 134 (relative to the start of translation) of the deduced ORF of *bfpB* with a deletion of bp 108 to 116 and base substitutions of G for C at bp 121, A for T at bp 124, and T for A at bp 129. A second primer corresponded to bp 557 to 585 of the coding sequence. These primers were used to amplify a 498-bp fragment that was then digested with *Ssp*I and *Nde*I. This fragment was used to replace the corresponding wild-type fragment in pSS9 (40), a plasmid that contains the first 939 bp of the *bfpB* coding sequence, to generate plasmid pSS9dbfpB. pSS9dbfpB was digested with *Pst*I and *Hin*dIII, and a 1,281-bp fragment containing the 5′ end of the mutant *bfpB* gene was isolated. pERI2493, a pUC18-based plasmid containing a 7.5-kb *Eco*RI fragment spanning the entire *bfpB* region of the EPEC plasmid (41), was digested with *Hin*dIII and *Cla*I. A 1,813-bp fragment containing the 3′ end of the *bfpB* gene was isolated. The two fragments containing both parts of the *bfpB* gene were ligated into pBluescriptSK$^+$ (Stratagene, La Jolla, Calif.) that had been digested with *Pst*I and *Cla*I. The resulting plasmid was designated pBSdbfpB. pBSdbfpB was digested with *Sph*I, and a 1,641-bp fragment containing the mutant *bfpB* gene was isolated and cloned into the suicide vector pGP704 (25) at the *Sph*I site, generating plasmid pGP704-dbfpB. pGP704-dbfpB and subsequent suicide-vector constructs were propagated in *E. coli* SM10 λpir, which provides functions necessary for autonomous replication of these vectors (39).

A 1,700-bp fragment containing the *sacB* gene from *Bacillus subtilis* was PCR amplified from plasmid pJQ200 (35) with primers that introduced *Sac*I restriction sites at the ends of the fragment. The PCR product was digested with *Sac*I and was ligated into *Sac*I-digested pGP704-dbfpB. A colony that showed differential growth on glucose versus sucrose was selected. Restriction analysis of the plasmid isolated from this colony verified that a 1,700-bp *Sac*I fragment had been inserted into pGP704-dbfpB, generating the gene-disruption suicide plasmid pΔbfpB.

pΔbfpB was conjugated from SM10 λpir into EPEC B171-8, and ampicillin-resistant, sucrose-sensitive colonies, indicating that pΔbfpB had integrated into either the host chromosome or the EAF plasmid, were selected. Plasmid DNA was isolated from several colonies and was screened by Southern analysis for a characteristic hybridization pattern that indicated that pΔbfpB had integrated homologously at the *bfpB* locus: the *bfpB* mutation introduced in pΔbfpB deletes an *Sph*I site within the *bfpB* gene. Colonies that showed this diagnostic pattern were passaged nonselectively in liquid LB medium lacking sucrose for several days to allow loss of the suicide plasmid-borne sequences and were then streaked onto LB sucrose and LB ampicillin plates. Plasmid DNAs isolated from colonies that grew on the sucrose plates and did not grow on the ampicillin plates were subjected to Southern analysis. We identified a colony that was shown by Southern analysis to contain an EAF plasmid in which the wild-type *bfpB* gene had been replaced with the mutant *bfpB* allele.

Construction of complementing plasmid pLC-AGB. A 3,334-bp *Eco*RI-*Msc*I fragment of the EAF plasmid containing *bfpA*, *bfpG*, and *bfpB* as well as the promoter region upstream of *bfpA* was subcloned into the low-copy-number vector pWKS130 (46), which had been digested with *Eco*RI and *Eco*RV.

DNA sequence analysis. The presence of the intended mutation was confirmed by dideoxy sequencing of both strands of the region of the EAF plasmid encompassing the *bfpB* mutation (Sequatech, Mountain View, Calif.). A PCR product of the mutant EAF plasmid spanning 600 bases 5′ and 1,300 bases 3′ of the intended *bfpB* mutation was used as a template for the sequencing reaction.

Preparation and purification of BfpB-specific antiserum. The Chou and Fasman algorithm was used to predict regions of secondary structure in the protein sequence of BfpB that might be antigenic (6). On the basis of this analysis, peptides corresponding to amino acids 132 to 149 (AATVAEKMNSATGGKSTD) and 191 to 253 (ESGRIAFSNEETKRFSISILPGGKYTSKNSISSDSNSSSGSSGSSGSSSSDSGAELKFDSDVD) were synthesized with a Milligen 9050 Pep Synthesizer. The peptides were conjugated to bovine gamma globulin, and the conjugates were used for the production of polyclonal antisera in rabbits (Josman Laboratories, Napa, Calif.).

Absorption of antisera. A total cell extract from the BfpB mutant strain B171-8ΔB was coupled to tresyl-activated agarose (FMC, Rockland, Maine). Rabbit antisera were diluted 1:10 in PBS–1% Tween 20, and the resulting solution was mixed 1:1 with the agarose slurry and incubated at 4°C overnight. The antisera were incubated twice more overnight with fresh aliquots of the agarose slurry before being stored at 4°C with 0.02% sodium azide.

Western blot (immunoblot) analysis. PBS-washed bacterial cell pellets were resuspended in water and sonicated until the opacity cleared. Total protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, Ill.). Total protein (20 μg) from the bacterial lysates was denatured by boiling for 5 min in sample buffer (1.5% sodium dodecyl sulfate [SDS]–2.5% 2-mercaptoethanol–10% glycerol–31.25 mM Tris [pH 6.8]) and then separated by polyacrylamide gel electrophoresis (PAGE) (with a 12.5% polyacrylamide gel). The proteins were then electrophoretically transferred to Immobilon-P membranes. The membranes were blocked and incubated with dilutions of BfpA-specific antiserum as described previously (33) or with the adsorbed BfpB-specific antiserum at a dilution of 1:4,000. Membranes were then incubated with secondary horseradish peroxidase-labeled anti-rabbit antibodies at a dilution of 1:5,000 and developed by enhanced chemiluminescence (Amersham).

Total cell and membrane fractionation. Total cell fractionation was performed on bacteria grown under optimal *bfpA*-inducing conditions (i.e., 4 h of growth in DMEM at 37°C from a 1:100 dilution of a standard overnight culture) by the procedure of Boeke and Model (4). Each cell fraction was precipitated with 10% trichloroacetic acid, and all fractions were resuspended in the same volume of SDS-PAGE sample buffer (described above).

Membrane fractionation was performed essentially as described previously (43). Briefly, the crude membrane fraction from 600 ml of DMEM-grown bacteria was layered on top of a three-step sucrose gradient (13 ml of 0.77 M sucrose in 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4]–16.8 ml of 1.44 M sucrose in 10 mM HEPES [pH 7.4]–4.8 ml of 2.02 M sucrose in 10 mM HEPES [pH 7.4]) and was then centrifuged at 83,000 × *g* in

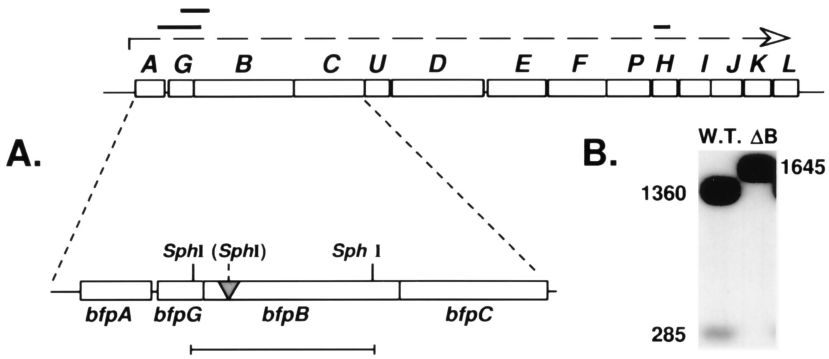

FIG. 1. (A) Schematic of the *bfp* operon. *bfpB* (indicated by *B* in the diagram) is the third gene transcribed in a polycistronic message from a promoter located upstream of *bfpA*. A disruption of *bfpB* that introduced translational stop sites and a 9-bp deletion into the coding sequence of *bfpB* was engineered. The deletion removes a *Sph*I site, the absence of which is diagnostic for the *bfpB* mutation. Bars above the genes indicate regions that were used to generate riboprobes for transcription studies. The line below the expanded *bfpB* region indicates the fragment used as a probe in Southern analysis of the *bfpB* mutant. (B) B171-8ΔB demonstrates an altered Southern hybridization pattern consistent with the predicted loss of a *Sph*I site within the mutated region of *bfpB*. The first lane shows the wild-type (W.T.) pattern of the *Sph*I-digested EAF plasmid isolated from strain B171-8 hybridized with the indicated probe. The second lane shows the pattern obtained when the *Sph*I-digested EAF plasmid from B171-8ΔB (ΔB), the *bfpB* mutant, was hybridized with the same probe. The sizes of the hybridizing fragments are noted at the sides of the gel.

an SW28 rotor for 21 h at 5°C. The cytoplasmic membrane fraction was isolated as a band at the 0.77 M sucrose in 10 mM HEPES–1.44 M sucrose in 10 mM HEPES interface, and the outer membrane fraction was isolated as a band at the 1.44 M sucrose in 10 mM HEPES–2.02 M sucrose in 10 mM HEPES interface. The membrane fractions were recovered in volumes of 1 to 4 ml by puncturing the sides of the centrifuge tubes and extracting the fractions with a needle and syringe. The fractions were then diluted to a final volume of 12 ml in 10 mM HEPES (pH 7.4), pelleted by centrifugation at 230,000 × *g* for 4.5 h, and then resuspended in 12 ml of 10 mM HEPES (pH 7.4) and pelleted again by centrifugation at 230,000 × *g* for 1.5 h. This pellet was resuspended in 500 μl of 10 mM HEPES (pH 7.4) and stored frozen at −20°C.

LA assay. The LA assay was performed as described previously (33).

AA. Cultures were grown for 2 h at 37°C with shaking (200 rpm in an Innova 3000 water bath) and then examined on a hanging-drop slide by phase-contrast microscopy for the presence of bacterial aggregates.

Electron microscopy. Electron microscopic examination of BFP on bacterial surfaces was performed as described previously (9).

Radiolabeling of lipoproteins. A standard overnight culture of B171-8 was diluted 1:100 in DMEM. The cells were grown at 37°C with shaking to an optical density at 600 nm of 0.5. [^{3}H]palmitic acid was added to a final concentration of 50 μCi/ml, and the cells were incubated at 37°C for an additional 2 h. Proteins were precipitated by the addition of ice-cold trichloroacetic acid to a final concentration of 10% (wt/vol). The cells were chilled on ice for 30 min and then microcentrifuged at 4°C for 20 min. The resulting pellet was resuspended in SDS-PAGE sample buffer (described above). Twenty microliters of this sample, representing 0.2 ml of log-phase cell culture, was heated to 95°C for 5 min and then subjected to SDS-PAGE analysis on a 10% gel. The radiolabeled proteins in the dried gel were detected by fluorography after exposure to XAR-5 X-ray film for 2 weeks.

RNase protection assays. RNA was prepared by a modification of the method of Aiba et al. (1) from bacteria grown for different amounts of time in either DMEM, DMEM containing 20 mM ammonium sulfate, or LB broth as previously described (33). DNA fragments corresponding to bases 2352 to 4112 (probe AGB), bases 3658 to 3842 (probe GB), and bases 11546 to 11775 (probe H) were subcloned into T7-T3 expression vectors and used to make riboprobes. All base numbers indicated are relative to the published sequence of the *bfp* locus (41), and probe names indicate the genes detected with each probe. Riboprobes were prepared with a MAXIscript in vitro transcription kit (Ambion, Austin, Tex.) and were gel isolated from 5% denaturing acrylamide gels. RNase protection assays were performed with a HybSpeed RPA kit (Ambion) with 2.5 to 30 μg of RNA per sample.

RESULTS

Construction of a *bfpB* null mutant. Immediately downstream of *bfpA*, which encodes the major repeating pilus subunit of BFP, is a series of 13 ORFs that are transcribed in the same orientation as *bfpA* (41, 42) (Fig. 1A). *bfpB*, the second ORF downstream of *bfpA*, is predicted to encode a 58-kDa protein. To assess its role in BFP biogenesis, a disruption of *bfpB* was engineered. To avoid polar downstream effects, a gene replacement strategy that was designed to minimally affect the transcription of downstream genes was employed. Primer-directed PCR mutagenesis was used to create a 9-bp deletion corresponding to amino acids 36 to 38 of the predicted protein sequence. This deletion removes a *Sph*I site, the absence of which is diagnostic for the intended mutation. In addition, single base pair substitutions that changed the codons for amino acids 40, 41, and 43 to translational stops were introduced. The mutant *bfpB* gene was introduced on a mobilizable suicide plasmid into an EPEC strain harboring a wild-type EAF plasmid. Allelic replacement by a double-recombination event resulted in the replacement of the wild-type *bfpB* gene with the mutated copy. The presence of the expected deletion and base changes in the mutant plasmid (designated pEAF-ΔbfpB) was verified by Southern analysis (Fig. 1B) and by sequencing. A B171-8 strain harboring this mutant plasmid was designated B171-8ΔB (Table 1).

Preparation of BfpB-specific antibodies. Computer analysis of the predicted protein sequence for BfpB identified several regions of the protein that were potentially antigenic (6). Peptides corresponding to amino acids 132 to 149 and 191 to 253 were synthesized, conjugated to bovine gamma globulin, and used to immunize rabbits. Each peptide conjugate elicited antibodies that recognized a 58-kDa protein present in lysates of the wild-type B171-8 strain and absent in B171-8ΔB and in B171-5 (an EPEC strain that has lost the EAF plasmid [45]) (Fig. 2B), indicating that the synthetic peptides chosen were immunogenic and generated antibodies that cross-react with the BfpB protein. Because reports indicated that some members of the pIV protein superfamily (to which BfpB belongs) form SDS-insoluble complexes (12, 22), the stacking portion of the SDS-polyacrylamide gel was also examined for the presence of BfpB, but no Western blot-reactive species were identified there (data not shown).

Disruption of the *bfpB* coding sequence abolishes LA, AA, and production of BFP. To identify which functional EPEC phenotypes require *bfpB*, the mutant strain B171-8ΔB was compared with its isogenic counterpart B171-8. In an LA assay, B171-8 formed typical adherent microcolonies whereas B171-8ΔB was LA-phenotype negative (Fig. 2A). The AA phenotype was also abolished in B171-8ΔB. Similarly, when negatively stained bacteria were examined by electron microscopy for the presence of BFP, pilus filaments were found associated only with the wild-type B171-8 strain; in contrast, no BFP were

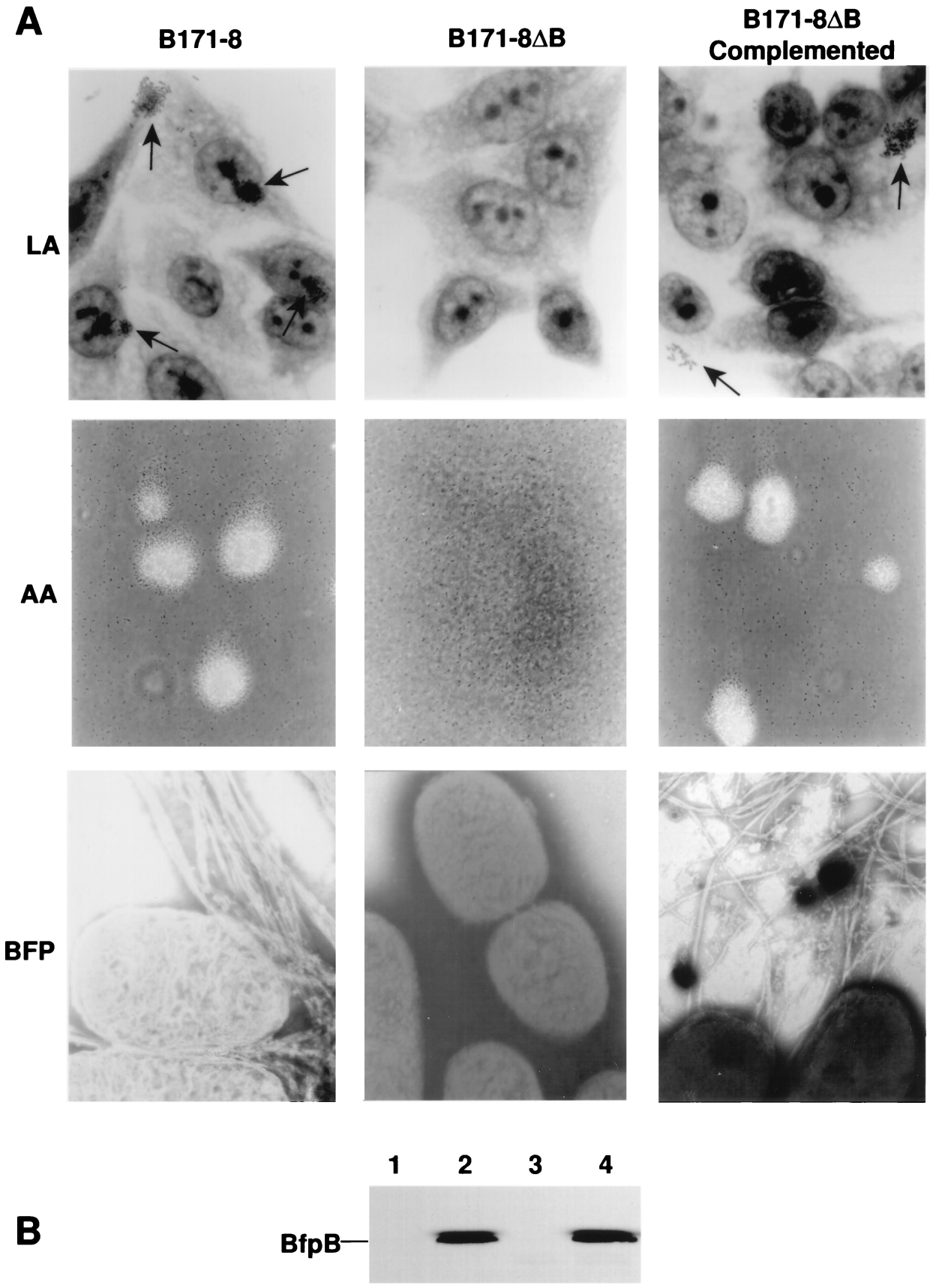
A
B171-8
B171-8ΔB
B171-8ΔB
Complemented
LA
AA
BFP
B
1
2
3
4
BfpB

FIG. 2. (A) Disruption of *bfpB* abolishes BFP filament production and the LA and AA phenotypes. LA, AA, and BFP filament production (BFP) are exhibited by wild-type EPEC B171-8 grown under inducing conditions (B171-8). These phenotypes are abolished in the *bfpB* mutant (B171-8ΔB) but can be reconstituted by supplying the wild-type *bfpB* gene in *trans* (B171-8ΔB Complemented). (B) Expression of BfpB. An antiserum to a synthetic peptide corresponding to amino acids 191 to 253 of BfpB was used to identify the 58-kDa protein encoded by *bfpB* on the EAF plasmid and expressed by the wild-type EPEC strain B171-8 (lane 2) but not by the EAF-plasmid-cured derivative B171-5 (lane 1), or by the *bfpB* mutant B171-8ΔB (lane 3). Complementation of the mutant strain with a wild-type *bfpB* gene restores production of the 58-kDa protein in the mutant strain (lane 4).

found to be produced by B171-8ΔB. Because loss of the EAF plasmid during construction of the mutant would produce these same altered phenotypes, we isolated an EAF plasmid from the bacteria used in these phenotype assays which was then used as a template for PCR amplification with primers spanning the *bfpB* locus. The results verified the presence of the mutated EAF plasmid in B171-8ΔB (data not shown), indicating that the loss of these BFP-mediated phenotypes was most likely due to the *bfpB*-specific mutation.

Expression of the BfpB protein is regulated by environmental signals that affect expression of BfpA, the pilus subunit. Expression of BfpA is regulated at the transcriptional level and is induced during exponential-phase growth; this response is greatly enhanced when the duration of exponential-phase growth is extended by growing the bacteria in a tissue culture medium (DMEM). Conversely, BfpA expression is repressed by addition of ammonium (e.g., 20 mM ammonium sulfate) or the absence of calcium (33). To determine if BfpA and BfpB are coordinately regulated, Western blot analysis of B171-8 lysates was performed with antibodies to BfpA and BfpB. The patterns of expression of the two proteins are quite similar (Fig. 3). Like BfpA (33), BfpB shows a characteristic expression profile in LB broth that is enhanced and extended in DMEM, in which it reaches a maximum at about 4 h. Expression of the proteins is detectable in DMEM for at least 10 h. As is seen for BfpA expression, addition of 20 mM ammonium sulfate to DMEM dramatically reduces expression of BfpB. Thus, BfpB is regulated by the same environmental and growth-phase signals that affect pilus subunit (BfpA) expression.

***bfpB* is transcribed as part of an operon from a promoter located upstream of *bfpA*.** Northern (RNA) and Western blot analyses have shown that *bfpA* mRNA and BfpA protein expression appear to be controlled by the same environmental cues (33), suggesting that regulation of BfpB protein expression might occur at the transcriptional level as well. However, *bfpB* message could not be detected by Northern analysis even when RNA was prepared from cells grown under conditions that favor maximal expression of *bfpA*. Thus, compared with *bfpA*, *bfpB* message is much less abundant. In order to detect this low-abundance message, we employed the RNase protection assay, which offers 10- to 100-fold greater sensitivity than Northern blot analysis as well as the potential to map transcription start sites. When RNase protection was performed with this same RNA and probes AGB and GB as shown in Fig. 4A, both samples showed protection of the full-length probe, indicating that *bfpB* is transcribed as part of a polycistronic message encompassing at least *bfpA*, *bfpG*, and *bfpB*. Probe GB spans the 3′ end of *bfpG* and the 5′ end of *bfpB* and is fully protected in an RNase protection assay. (The shift in size seen between the undigested probe in lane 1 and the protected probes in lanes 4 and 5 reflects digestion of the plasmid-specific 5′ and 3′ ends of the probe, which are introduced during transcription of the riboprobe and which are not protected by *bfp*-specific RNA.) Probe AGB spans the 3′ end of *bfpA* and all of *bfpG* and extends into *bfpB*. The RNase protection pattern for this probe is complicated, showing two major protected bands (Fig. 4B). The predominant band of approximately 115 bp is likely due to transcriptional attenuation at the 3′ end of the *bfpA* gene as predicted by the presence of a potential stem-loop structure at bases 3301 to 3327 (40, 41), which is probably responsible for the dramatic difference in the levels of detectable message between *bfpA* and downstream genes. Such an attenuator is found at a similar position in the *tcp* type IV pilus operon (5). The largest protected band, of approximately 900 bp, is consistent with production of a continuous transcript from *bfpA* through at least *bfpB*.

B171-8dBFP, a deletion mutant of *bfpA* that contains translational stop sites close to the initiation methionine of the gene (Table 1), was found by Western blot analysis to produce neither BfpA nor BfpB (data not shown). Probes AGB and GB were not protected in B171-8dBFP, nor were probes from *bfp* genes extending at least through *bfpH* (Fig. 4A and data not shown), indicating that B171-8dBFP interrupts transcription not only of *bfpA* but of the downstream genes of the cistron as well. These probes were readily protected with RNA from both wild-type and B171-8ΔB samples grown under *bfpA*-inducing conditions (Fig. 4A). Taken together, these data are entirely consistent with the idea that the *bfp* gene cluster is transcribed as an operon from a promoter located upstream of *bfpA* and provide additional evidence that transcription of downstream genes is unaltered in the *bfpB* mutant, as it was designed to be.

As would be predicted from these results, the time course of mRNA expression by *bfpG* and *bfpB* is identical to that of *bfpA* (Fig. 4B) and provides an explanation for the coordinate expression of the BfpB and BfpA proteins shown in Fig. 3. However, the significantly decreased levels of expression of the BfpB and BfpA proteins in the presence of ammonium (below those seen during growth in LB broth despite the presence of detectable, though small, amounts of message) suggest that an additional level of regulation of protein expression may occur posttranscriptionally as well. Decreased levels of transcription of all of these genes are evident in DMEM containing ammonium and in LB broth, although the greater sensitivity of the

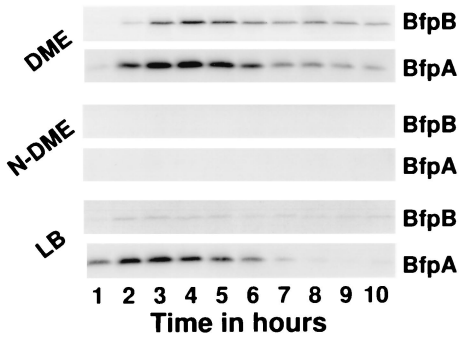

FIG. 3. Regulation of BfpB and BfpA production by environmental and growth-phase signals. Western blot analysis was performed with whole-cell extracts of B171-8 grown in the indicated media for the indicated numbers of hours. The top panel in each set shows the BfpB expression pattern, and the bottom panel shows BfpA expression. Growth media used were as follows: DMEM (DME), LB broth (LB), and DMEM containing 20 mM ammonium sulfate (N-DME). BfpB production is seen to be regulated by the same environmental and growth-phase signals that regulate the production of BfpA.

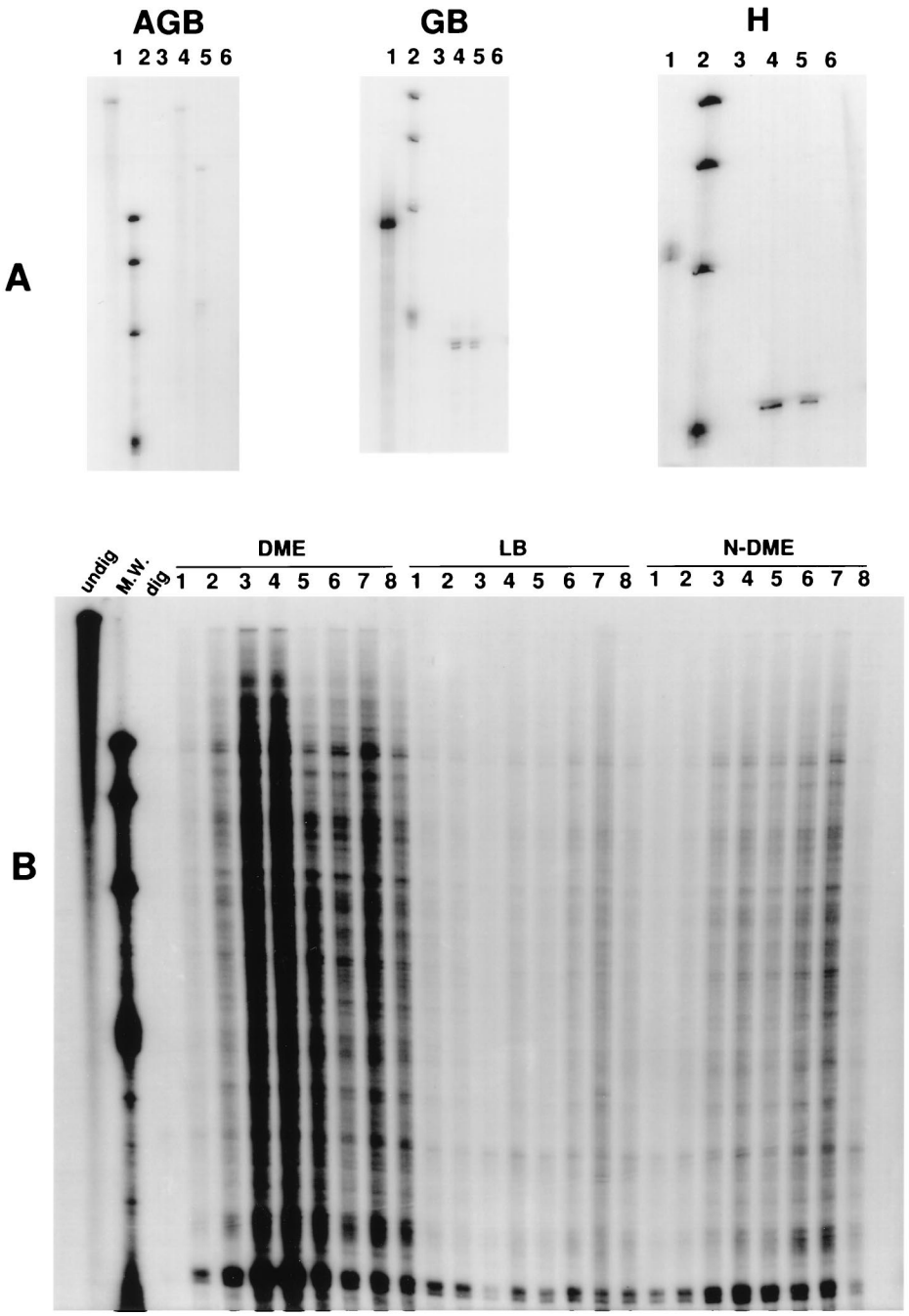

FIG. 4. (A) Transcriptional analysis of *bfpA*, *-G*, *-B*, and *-H* by an RNase protection assay. RNase protection patterns were obtained with the indicated probes and an RNA sample prepared from B171-8, B171-8ΔB, or B171-8dBFP. RNA was prepared from bacteria grown under optimal *bfpA*-inducing conditions. Lanes 1, full-length, undigested probe; lanes 2, molecular weight standards (200 to 500 bases in 100-base increments); lanes 3, digested probe; lanes 4, B171-8 RNA (4-h DMEM time point); lanes 5, B171-8ΔB RNA (4-h DMEM time point); lanes 6, B171-8dBFP RNA (4-h DMEM time point). (Note that for probe AGB, only the upper portion of the gel corresponding to protection of the full-length probe is shown here. For B171-8ΔB, the full-length protected region for probe AGB is expected to be cleaved into two fragments that flank the 9-bp deletion.) (B) Time course and medium profiles of *bfpA*, *bfpG*, and *bfpB* expression. RNA was prepared from wild-type B171-8 bacteria grown for the indicated numbers of hours in the indicated media (see the legend to Fig. 3 for clarification of the media). RNA (2.5 μg) was used in an RNase protection assay with probe AGB. The first three lanes contain undigested, full-length probe (undig), molecular weight standards (M.W.), and digested probe (dig), respectively. These results indicate that *bfpB* is transcribed as part of a polycistronic message from a promoter located upstream of *bfpA*.

RNase protection assay reveals low-level transcription in each of these conditions that was not apparent by Northern blot analysis. Thus, the coordinate expression of the BfpB and BfpA proteins is a consequence, at least in part, of concomitant expression of the *bfpA* and *bfpB* messages from an environmentally responsive operon.

Complementation of the mutant B171-8ΔB strain. *bfpB* is resident on the low-copy-number EAF plasmid and is transcribed at lower levels than *bfpA* in an operon whose promoter is upstream of *bfpA*. Therefore, to mimic these conditions, a low-copy-number plasmid containing DNA from upstream of *bfpA* through *bfpB* was used to complement B171-8ΔB. The

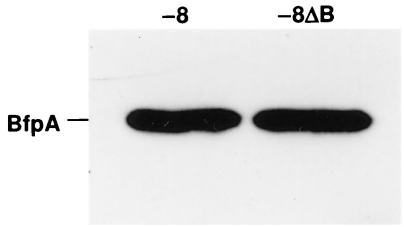

FIG. 5. BfpA, the pilus subunit, is produced at wild-type levels and is processed correctly by prepilin peptidase in a *bfpB* mutant strain. Whole-cell extracts of the wild type (B171-8; lane -8) and the *bfpB* mutant (B171-8ΔB; lane 8ΔB) were prepared from cells grown under BfpA-inducing conditions. Western blot analysis using a BfpA antiserum was performed on these lysates, adjusted to contain equal amounts of total protein.

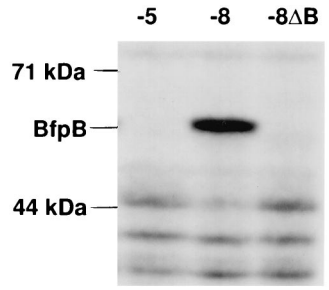

FIG. 6. BfpB is a lipoprotein. EPEC strains were transferred from a standard overnight culture and grown for 2 h in DMEM with shaking. [^{3}H]palmitic acid was added to a final concentration of 50 μCi/ml, and the cells were grown for an additional 2 h. Aliquots representing 0.2 ml of the final culture were subjected to SDS-PAGE analysis on a 12.5% gel. The gel was prepared for fluorography with Amplify (Amersham), dried, and exposed to X-Omat X-ray film at −70°C for 2 weeks. -5, EAF-plasmid-cured derivative of B171 (Table 1); -8, wild-type EPEC B171-8; -8ΔB, the *bfpB* mutant of EPEC B171-8. Molecular mass markers are noted at the left.

resulting complemented strain exhibited the LA and AA phenotypes and produced BfpA and BfpB (detected by Western blot analysis) and BFP filaments (detected by electron microscopy), indicating that the expression of genes downstream of *bfpB* that are required for BFP biogenesis has not been disrupted by the mutation in B171-8ΔB (Fig. 2). This observation and Western blot data indicating that BfpA and BfpG are expressed in wild-type amounts by B171-8ΔB (Fig. 5 and data not shown) demonstrate that the lack of BfpB protein expression by this mutant accounts for its failure to exhibit BFP-mediated phenotypes.

Disruption of *bfpB* does not affect production or processing of the pilin subunit. To examine the effect of the *bfpB* mutation on production of the pilin subunit, a Western blot analysis of pilin expression was performed on a whole-cell lysate of B171-8ΔB with antibodies to purified BFP filaments (9) (Fig. 5). There was no apparent difference in the levels of pilin expression between the wild-type and *bfpB* mutant strains, indicating that the BfpB protein is not required for production of the subunit. In addition, the *bfpB* mutant strain correctly processed pilin from the 22-kDa precursor to its normally processed size of 19 kDa. Thus, BfpB is apparently not required for prepilin peptidase activity and probably acts at a later step in pilus biogenesis.

***bfpB* encodes a lipoprotein.** The predicted amino acid sequence of *bfpB* contains a consensus sequence for a prokaryotic lipid attachment site, suggesting that BfpB might be a lipoprotein. EPEC strains harboring either a wild-type EAF plasmid, no EAF plasmid, or an EAF plasmid containing the mutant *bfpB* gene were radiolabeled in the presence of [^{3}H] palmitic acid. Protein extracts were prepared from the labeled cells and subjected to SDS-PAGE analysis and fluorography. A labeled band of the predicted molecular mass for BfpB (58 kDa) was present in the extract from wild-type cells and absent in the extracts from both the EAF plasmid-negative strain and B171-8ΔB, the strain carrying the *bfpB* mutant EAF plasmid (Fig. 6). Thus, *bfpB* does indeed encode a lipoprotein.

BfpB is located primarily in the outer membrane. Cell-fractionation experiments were performed to localize BfpB. The presence of the lipid moiety suggested that BfpB would localize to either the cytoplasmic or outer membrane. Cell fractionation by the method of Boeke and Model (4) partitions a cell into the cytosol, inner membrane, periplasmic fluid, and outer membrane. Western blot analysis of these fractions with a BfpB-specific antiserum revealed that the majority of the BfpB protein localizes to the outer membrane fraction, with a detectable, but much smaller, amount also being present in the inner membrane fraction (Fig. 7). To determine whether this incomplete separation was an artifact of the fractionation protocol, sedimentation through a sucrose gradient, which utilizes biochemical properties different from those of the differential detergent solubility method employed by Boeke and Model, was used to attempt an alternative separation of inner and outer membranes. By quantitation of 3-deoxyoctulosonic acid (30), a representative outer membrane marker, we estimate that this separation resulted in an inner membrane fraction that was contaminated by approximately 10% with outer membrane components (data not shown). Western blot analysis of these fractions again showed BfpB to be predominantly localized to the outer membrane (with a detectable signal still present in the inner membrane fraction as well).

DISCUSSION

Between 20 and 40 genes have been estimated to be involved in the synthesis, assembly, and export of the *P. aeruginosa* type IV pilus (13). It is likely that production of the BFP of EPEC requires a similar number of proteins, and indeed, sequence analysis of the 14 genes encoded by the *bfp* operon reveals significant homology of some of these gene products with proteins that have been identified as being required for type IV pilus biogenesis in other organisms (40). The functions of most of these protein homologs are completely unknown, however, beyond their strict requirement for biogenesis. With the exception of the *V. cholerae* toxin-coregulated pilus genes (16, 29),

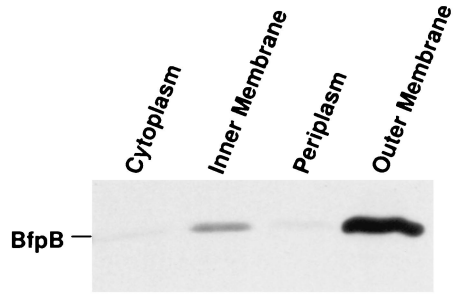

FIG. 7. BfpB is associated primarily with the outer membrane. A culture of B171-8, grown under inducing conditions in DMEM, was fractionated into cytoplasmic, inner membrane, periplasmic, and outer membrane fractions according to the method of Boeke and Model (4). Western blot analysis of aliquots representing equal percentages of the total protein in each cell fraction was performed with a BfpB antiserum.

type IV pilus biosynthetic genes in other organisms are generally not polycistronic and are dispersed throughout the organism's chromosome, making locating and identifying them difficult. Although we cannot rule out that chromosomal genes common to all *E. coli* are also required for type IV pilus production, we have shown previously that the *bfp* locus together with a transcriptional activator locus, *bfpT*, can direct the synthesis of BFP in a non-EPEC strain of *E. coli*, thus delimiting the minimal set of additional genes required for expression of a type IV pilus. The contiguous arrangement of these genes (except *bfpT*) as well as their presence on a naturally occurring, autonomously replicating plasmid in a genetically tractable organism, such as *E. coli*, facilitates molecular genetic approaches in elucidating the specific functions of the genes required for type IV pilus expression.

The construction, by allelic replacement, of a nonpolar disruption of the *bfpB* gene allowed an analysis of the role of the BfpB protein in BFP biogenesis. This gene disruption demonstrated an absolute requirement for BfpB for the formation of BFP as assessed by the functional assays to determine LA and AA as well as by visual inspection by electron microscopy. No BFP were produced under any of these criteria, despite the fact that the pilin subunit was produced at wild-type levels and processed correctly from its prepilin form in the *bfpB* mutant. Thus, BfpB appears to be required for BFP production at a step subsequent to pilin synthesis and signal-peptide processing.

Expression of the pilin subunit (BfpA) has been shown previously to be regulated by growth-phase and environmental signals (33). To determine whether BfpB expression is similarly regulated, antibodies to synthetic peptides corresponding to segments of BfpB were prepared and used in Western blot experiments. These time course and growth medium studies demonstrated contemporaneous, inducible expression of the two proteins, suggesting a requirement for elevated levels of the BfpB protein only when expression of the pilin subunit is also induced.

These observations and the close, tandem arrangement of the genes in the *bfp* cluster suggested that these genes might be expressed as an operon. We were unable to detect *bfpB* mRNA by Northern blot analysis under any conditions, including in samples in which we could readily detect both BfpB protein and *bfpA* mRNA, indicating that the *bfpB* message is much less abundant than the *bfpA* message under conditions of growth that cause strong expression of BFP. However, we were able to detect *bfpB* mRNA by using RNase protection, a sensitive assay for measuring low-abundance mRNA. With this assay system, a probe containing the 5′ end of *bfpB* was protected in RNA samples from wild-type EPEC grown under conditions known to induce *bfpA* message; a much-reduced level of *bfpB* mRNA was seen in samples grown under noninducing conditions.

The probe used in these RNase protection experiments includes a sequence upstream of *bfpB* that extends into an ORF that does not contain a standard ATG start codon but that may commence with a GTG. By a nomenclature convention agreed upon previously (41, 42), this ORF is designated *bfpG*. The RNase protection pattern observed with this probe suggests that the 3′ end of *bfpG* and the 5′ end of *bfpB* are located on a continuous RNA transcript. Antisera raised to a BfpG peptide were reactive by Western blot analysis with a protein of the size predicted for BfpG present in wild-type, induced EPEC and absent in EAF-plasmid-cured EPEC or wild-type EPEC grown under noninducing conditions (35a). This result indicates that *bfpG* does in fact encode a protein product and, like *bfpB*, is under the control of a promoter upstream of *bfpA*.

When a probe spanning the 3′ end of *bfpA*, all of *bfpG*, and the 5′ end of *bfpB* was used in an RNase protection assay, a very interesting result was obtained. A prominent protected band of 115 bp correlated in size to the distance between the start of the probe (near the 3′ end of the *bfpA* gene) and a potential hairpin-loop sequence between *bfpA* and *bfpG*. These results are very similar to those seen for the *tcp* operon (5), and we hypothesize that this hairpin-loop acts as a transcriptional attenuator and is probably responsible for the dramatic difference in detectable levels of message between *bfpA* and downstream genes. Thus, the *bfp* gene cluster makes up an operon consisting of at least *bfpA*, *bfpG*, and *bfpB*. However, in addition, the close spacing and lack of obvious promoter motifs between downstream *bfp* genes suggest that the entire 14-gene cluster is polycistronic. This conclusion is supported by the observation that a *bfp* operon mutant, B171-8dBFP, produces no detectable message for *bfp* genes as far downstream as *bfpH*, the most distal gene whose transcript we have assayed to date. This mutant requires DNA encompassing the entire *bfpA-bfpL* (inclusive) region in order to restore AA, LA, and BFP production (3a).

In addition to proteins homologous to type IV biosynthetic proteins from other organisms, some of the ORFs in the *bfp* operon are predicted to encode proteins that show homology to membrane traffic wardens involved in the general secretory pathway that are required for the secretion of several extracellular proteins in various gram-negative bacterial species. The first *bfp* operon ORF that we chose for genetic analysis, BfpB, shows sequence homology with PulD (GspD), an integral outer membrane protein component of the general secretory pathway hypothesized to form a channel in the membrane that might allow protein transport across it (12). BfpB also shows homology, particularly in its C-terminal half, with the filamentous phage f1 morphogenetic protein pIV, which is hypothesized to compose a multimeric complex that forms a pore in the outer membrane large enough to permit phage extrusion (17). Neither of these proteins is predicted to be a lipoprotein, although PulD has recently been shown to be dependent on an accessory lipoprotein, PulS, for proper insertion in the outer membrane (12). Incorporation of tritiated palmitate into BfpB indicates that it is indeed a lipoprotein, as was suggested by the presence of a consensus prokaryotic lipid attachment site in its predicted amino acid sequence. It is possible that BfpB has incorporated functions similar to those of PulD and PulS into a single polypeptide. Another member of the pIV-PulD superfamily, XpsD from *Xanthomonas campestris*, has also been demonstrated to be a lipoprotein (15).

Cell-fractionation experiments indicated that BfpB is primarily located in the outer membrane, although a detectable fraction of it is also found associated with the inner membrane. While the level of cross-contamination of membrane fractions makes it difficult to determine whether this inner membrane association occurs in vivo, and a similar difficulty in achieving complete partitioning has been reported for the phage pIV protein as well (38). Russel and Kazmierczak propose that this incomplete partitioning may be due to an association of the outer-membrane-anchored pIV protein with the outer face of the inner membrane; disruption of these interactions during cell fractionation might occur randomly and determine in which membrane fraction the pIV protein segregates (38). If the homology between BfpB and pIV is biologically relevant, a similar mechanism might be responsible for our experimental results as well.

The presence in a *bfpB* mutant strain of wild-type levels of correctly processed pilin subunit suggests a role for BfpB that is carried out temporally distal to events that occur in the cytoplasm or cytoplasmic membrane (protein synthesis and

signal peptidase activity, respectively). BfpB with its outer membrane location is well positioned to be responsible for a later, more cell-surface-proximal step in the biogenesis of BFP.

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