The bundle-forming pilus (BFP) of enteropathogenic Escherichia coli (EPEC) is an important virulence factor. We examined the role of divergent alleles of bfpA encoding bundlin, the BFP pilin protein, in pilus biogenesis, pilus interactions, and immune responses. We found that the BFP biogenesis machine from an EPEC strain that expresses one bundlin type is capable of assembling all other bundlin types. Furthermore, we found that EPEC strains expressing divergent bundlin types are capable of forming mixed autoaggregates, suggesting that different pilin types can intertwine. However, we found that there was a marked difference between alleles in immunogenicity in both rabbits and mice of a peptide derived from a region of bundlin undergoing apparent diversifying selection. In addition, despite a high degree of cross-reactivity between divergent bundlin proteins, in both mice and rabbits responses appeared to be stronger against the homologous pilin protein than against the heterologous protein. This result was verified using sera from a volunteer study, which demonstrated that the human antibody responses after an initial challenge with live EPEC were stronger against the homologous bundlin protein than against a divergent bundlin protein. However, a repeat challenge induced equivalent responses. These results are consistent with the hypothesis that human immune responses against bundlin exert selective pressure on bfpA sequence divergence.

Type IV pili (Tfps) are surface appendages that are expressed by diverse gram-negative species. Tfps play numerous roles in pathogenesis, including roles in colonization, adher- ence, autoaggregation, biofilm formation, horizontal gene transfer, motility, and virulence (5, 8, 21, 24, 28, 32, 36, 38, 41–43). The bundle-forming pilus (BFP) of enteropathogenic Escherichia coli (EPEC) is an excellent model for the study of Tfps as expression of the 14-gene bfp cluster in a laboratory strain of E. coli is sufficient for BFP biogenesis and function (30, 40). EPEC is an important cause of serious diarrhea in infants in developing countries (1, 12, 16, 19). Volunteer studies have confirmed the importance of BFP expression for full virulence of EPEC (5). BFP are composed of repeating sub-units of the pilin protein bundlin, the product of the bfpA gene. Antibodies against bundlin are found in volunteers convalescing from experimental EPEC infection and in breast milk of mothers and serum of infants in developing countries (15, 25, 33). Whether these antibodies confer protection against subsequent infection is not known.

The Tfps expressed by different strains in a species may vary in sequence. In Neisseria gonorrhoeae, transformation and recombination from multiple silent pilus loci encoding variant pilin proteins result in abundant antigenic variation in pilin expression (29). This extremely dynamic process can lead to the expression of several pilin variants during infection of a single host and may help the bacteria avoid the immune response and cause persistent infection (39). Pilin sequence variation has also been noted in other species that produce Tfps, including Pseudomonas aeruginosa and Vibrio cholerae (10, 31). We found that EPEC bfpA genes are also variable and defined nine bfpA alleles produced by diverse EPEC strains (6, 7). These alleles could be grouped into two categories based on sequence similarity. The three α alleles are highly similar to one another, resulting in proteins that are 97% identical, while the six β alleles are more divergent, encoding proteins that are 89% identical to one another. In all bundlin proteins 80% of the amino acids are identical (6). The bundlin sequence diversity is concentrated near the carboxyl terminus of the 180- to 182-amino-acid mature proteins, and in particular, the region encoding amino acids 137 to 155 has an excess of nonsynonymo- nous substitutions over synonymous substitutions (Fig. 1) (7). Excess nonsynonymous substitutions imply that evolutionary forces provided a selective advantage to strains that expressed novel amino acids in this region, a phenomenon known as diversifying selection. In contrast, the rest of the protein shows evidence of sequence constraints with an excess of synonymous substitutions. The purpose of this study was to test the hypothesis that variations in bundlin amino acid sequences result in differences in BFP expression, function, and immunogenicity.

**MATERIALS AND METHODS**

**Strains, plasmids, and media.** The strains and plasmids used in this study are listed in Table 1. We employed allelic exchange using suicide vector pCDV442cys1 as described previously (44) to construct strain UMD949, a bfpA null mutant of EPEC strain RN587/1, which, like bfpA mutant strain UMD901, has a mutation that changes cysteine 129 to serine. This mutation renders the endonuclease site at the start codon of bfpA null mutant strain UMD949, a bfpA null mutant of EPEC strain RN587/1, which, like bfpA mutant strain UMD901, has a mutation that changes cysteine 129 to serine. This mutation renders the bundle-forming pilus (BFP) of enteropathogenic Escherichia coli (EPEC) an excellent model for the study of Tfps as expression of the 14-gene bfp cluster in a laboratory strain of E. coli is sufficient for BFP biogenesis and function (30, 40). EPEC is an important cause of serious diarrhea in infants in developing countries (1, 12, 16, 19). Volunteer studies have confirmed the importance of BFP expression for full virulence of EPEC (5). BFP are composed of repeating sub-units of the pilin protein bundlin, the product of the bfpA gene. Antibodies against bundlin are found in volunteers convalescing from experimental EPEC infection and in breast milk of mothers and serum of infants in developing countries (15, 25, 33). Whether these antibodies confer protection against subsequent infection is not known.

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Plasmids

to create pTR102. A specifying a consensus sigma-70
GATCCTGTCAAGTCGACGAGCT-3 and Donne-201 (5'-H11032
CTAGTTATAAT-3'-CTAGATTATAACTAGACGCGTG
-CTGCGACTTGACAGGATCCACGCGT

tary oligonucleotides Donne-200 (5'-H11032
UMD901 by electroporation. Plasmid pBluescript was modified by insertion
with the alleles to create plasmids pXLW10 through pXLW17. After confirma-
tion by sequencing, these plasmids were introduced into
EcoRI site after the stop codon of 
and Donne-537 (5'-H11032
-GCTCGGCCATG
from genomic DNA using conserved primers Donne-536 (5'-H11032
FIG. 1. Sequence comparison of
proteins. The shaded area indicates the peptides used in this study.
replaced by a histidine tag to purify the recombinant soluble bundlin
residues are connected with lines, and similar residues are connected
identical
of the sequences of the mature bundlin proteins is shown. Identical

from genomic DNA using conserved primers Donne-536 (5'-GCTCGGCCATG
GTTCATATACTCA-3') and Donne-537 (5'-CGTCGATTCAACAGGCGT
ATTATGTAGATTA-3'), which add an Ncol site at the start codon and an
EcoRI site after the stop codon of bfpA, and replaced the bfpA gene of pRPA100
with the alleles to create plasmids pXLW10 through pXLW17. After confirmation
by sequencing, these plasmids were introduced into bfpA mutant strain
UMD901 by electroporation. Plasmid pBluescript was modified by insertion
between the SacI and XbaI sites of an oligonucleotide composed of complemen-
tary oligonucleotides Donne-200 (5'-CTCGCAGTCTACAGGATCCACGGTC
CTAGTATATAA-3') and Donne-201 (5'-CTAGTATATAACTAGACGCGTG
GATCCGTCGACGCGACT-3') specifying a consensus sigma-70
promoter, to create pTR102. A bfpA gene encoding enhanced green fluorescence
protein (eGFP) was cloned from plasmid pKEN into the XbaI site to create
pTR103 for constitutive expression of eGFP.

Bacteria were stored at ~80°C in 50% (vol/vol) Luria broth-50% (vol/vol)
glycerol and grown on Luria plates or in Luria broth with antibiotics at the
following concentrations as needed to maintain plasmids: ampicillin, 200 µg/ml;
nalidixic acid, 50 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 25 µg/ml.
To induce expression of BFP, overnight cultures of bacteria were diluted 100-
fold in Dulbecco modified Eagle medium/F10 and grown at 37°C with shaking
(225 rpm) for 3 h.

**BF phenotypic assays.** Agglutination and disaggregation and localized
adherence assays were performed as previously described (2).

**Bundlin purification and immunization.** Soluble α1 bundlin with an amin-
terminal hexahistidine tag was expressed in the periplasm and purified as previ-
ously described (34). Similarly, soluble β1 bundlin was expressed by amplifying
the sequences encoding the amino acid glycine 25 of the mature protein through
the stop codon from strain RN587/1 with primers Donne-596 and Donne-597.
This strategy deleted the hydrophobic amino terminus from the protein, thereby
making the protein soluble. The PCR product was cloned as an Ncol-BamHI
fragment into plasmid pPF301, replacing the α1 allele from this plasmid to form a
fusion with the signal sequence of dhb4 followed by an amino-terminal hexa-
histidine tag. The resulting plasmid, pPF302, was transferred to strain BL21(AI)
slD for purification as described previously (34). Protein concentrations were
calculated from extinction coefficients (17).

Bundlin peptides and bundlin peptides conjugated to keyhole limpet hemo-
cyanin (KLH) were purchased from Research Genetics (Huntsville, AL). Female
6- to 8-week-old CBAJ mice (Harlan Sprague Dawley, Indianapolis, IN) were
immunized subcutaneously with 100 µg purified bundlin protein or bundlin
peptide-KLH conjugate in complete Freund's adjuvant (1:1 [vol/vol] emulsion;
Difco Laboratories, Detroit, MI). Booster immunizations of 50 µg in incomplete
Freund's adjuvant were given on days 21 and 42. Male 1.6- to 2.2-kg New
Zealand White rabbits (Covance, Denver, PA) were immunized subcutaneously
with 100 µg purified bundlin protein or 200 µg bundlin peptide-KLH conjugate
in complete Freund's adjuvant and boosted on days 30 and 60 with 30 µg purified
bundlin protein or 200 µg bundlin peptide-KLH conjugate in incomplete
Freund’s adjuvant. Serum was harvested on day 72.

**Enzyme-linked immunosorbent assays (ELISA).** Nunc-Immuno 96-well Maxi-
Sorp plates (Nalge Nunc International, Rochester, NY) were coated with 100

**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Description or genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2348/69</td>
<td>Wild-type EPEC strain, serotype O127:H6, produces α1 bundlin</td>
<td>7</td>
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<tr>
<td>UMD901</td>
<td>E2348/69 bfpA C128S</td>
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<tr>
<td>RN587/1</td>
<td>Wild-type EPEC strain, serotype O157:H+, produces β6 bundlin</td>
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<tr>
<td>UMD949</td>
<td>RN587/1 bfpA C129S</td>
<td>This study</td>
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<td>RN410/1</td>
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</tr>
<tr>
<td>RN191/1</td>
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<td>B171</td>
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<tr>
<td>E56/54</td>
<td>Wild-type EPEC strain, serotype O126ab,H2, produces β6 bundlin</td>
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<td>012-050982</td>
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<td>E851/71</td>
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</tr>
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<td>Z188-93</td>
<td>Wild-type avian EPEC strain, serotype O110:H6, produces β2 bundlin</td>
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</tr>
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<td>CA89-4221</td>
<td>Wild-type canine EPEC strain, serotype NT:H+, produces β1 bundlin</td>
<td>7</td>
</tr>
<tr>
<td>BL21(AI) slyD</td>
<td>F’ompT hsdS6 (rB C126 ) gal dcm araB: T7RNAP-tetA slD::cat</td>
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<table>
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<th>Plasmids</th>
<th>bfpA gene with native promoter cloned into low-copy-number vector pWKS30</th>
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<td>pPIL14</td>
<td>AFA-1 afimbrial adhesin genes cloned in a pBR322 vector</td>
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<tr>
<td>pPF301</td>
<td>Expression vector for soluble α1 bundlin under control of T7 promoter</td>
<td>34</td>
</tr>
<tr>
<td>pPF302</td>
<td>Expression vector for soluble β1 bundlin under control of T7 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pKEN</td>
<td>pUC derivative encoding eGFP</td>
<td>11</td>
</tr>
<tr>
<td>pTR103</td>
<td>pBluescript derivative with eGFP under control of consensus sigma-70 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pDsRed</td>
<td>Red fluorescent protein under control of lac promoter in pUC19 backbone</td>
<td>Clonetech</td>
</tr>
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</table>
µl/well of purified soluble α1 or β6 bundlin, unconjugated bundlin peptides, or KLH (2 µg ml⁻¹ in phosphate-buffered saline [PBS], pH 7.2) and incubated at 4°C overnight. After washing in PBS plus 0.1% Tween 20, plates were blocked in 10% goat serum (Gibco, Paisley, Scotland, United Kingdom) in PBS plus 0.1% Tween 20 (blocking buffer) and incubated at 37°C for 1 h. After washing, six samples of control sera or duplicate samples of twofold serial dilutions of test sera in blocking buffer were added and incubated at 37°C for 1 h. After washing, 100 µl horseradish peroxidase-conjugated secondary antibody (anti-mouse immunoglobulin G [IgG], anti-rabbit IgG, or anti-human IgG, as appropriate) was added to each well at a dilution of 1:5,000 in blocking buffer, and plates were incubated at 37°C for 1 h. After washing, 100 µl of SureBlue 3,3',5,5'-tetramethylbenzidine substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well, and plates were incubated in the dark for 20 min. The reaction was stopped by addition of 1 M sulfuric acid to each plate, and absorbance at 450 nm was recorded. The mean absorbance of four blank wells was subtracted from all other values. Values greater than the mean plus two standard deviations of the mean of six negative control samples were considered positive. Pooled preimmune sera from each group of mice or preimmune rabbit sera at a dilution of 1:10,000 for protein samples or at a dilution 1:2,000 for peptide samples served as negative controls. Pooled serum obtained in a serological study from 20 infants who were 10 to 14 months old from Mali was used at a dilution of 1:5,000 as a positive control for human anti-bundlin antibodies, and serum pooled from 10 infants who were 2 to 4 months old from a Rochester, NY, serological study was used at a dilution of 1:1,200 as a negative control. The use of human sera was approved by the institutional review board of the University of Maryland School of Medicine. Responses were compared using analysis of variance in Microsoft Excel.

Antibodies. Antiserum was affinity purified after conjugation of purified soluble α1 or β6 bundlin using AminoLink Plus immobilization kits as described by the manufacturer (Pierce Biotechnology, Rockford, IL). The concentration of the antibodies was adjusted according to the protein concentration, as estimated by absorbance at 280 nm. Fab antibody fragments were prepared and purified using an ImmunoPure Fab preparation kit according to the manufacturer’s instructions (Pierce). For immunoblotting, whole-cell lysates or purified proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% (wt/vol) nonfat dry milk in PBS (pH 7.4) plus 0.5% (vol/vol) Tween 20. Primary, affinity-purified rabbit α1 or β6 bundlin antiserum was used at a dilution of 1:100,000 in PBS plus 5% nonfat dry milk and 0.1% Tween 20. Bands were detected with horseradish peroxidase-conjugated anti-rabbit serum at a dilution of 1:20,000 and enhanced chemiluminescence (ECL Plus) reagents (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom).

Fluorescence microscopy. HeLa cells were grown in chamber slides and infected with bacteria under blinded conditions as previously described (14). After 3 h, the slides were washed three times with PBS and fixed for 25 min with 4% (vol/vol) formaldehyde. The slides were then blocked for 1 h at 37°C with 10% normal goat serum (Gibco) and left overnight at 4°C. Next, the slides were washed three more times with PBS, incubated for 1 h at 37°C with affinity-purified rabbit anti-α1 or anti-β6 bundlin sera at a dilution of 1:100,000 in PBS plus 10% goat serum, washed three times, incubated for 1 h at 37°C with fluorescein isothiocyanate-conjugated goat anti-rabbit sera (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:320 in PBS plus 10% goat serum, and washed three times.

The slides were covered with antifade reagent (Invitrogen, Carlsbad, CA) and examined with a Zeiss Axioskop microscope. Images were captured with an Axioscam digital 20 camera and analyzed using Axiovision 3.1 software (Zeiss). For mixed autoaggregation assays, overnight cultures of strain E2348/69 carrying plasmid pDsRed and strain RN587/1 carrying prlTr103 were diluted 1:10, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the E2348/69(pDsRed) culture to induce expression of DsRed. After 3 h of incubation at 37°C, the cultures were mixed at a 1:3 ratio and grown under BFP-inducing conditions. This ratio was necessary to compensate for poorer autoaggregation by strain RN587/1. After 2 to 3 h, 5 µl of the culture was placed on a glass slide under a coverslip, examined for red and green fluorescence, and analyzed as described above.

Quantification of bundlin expression. Wild-type strains E2348/69 and RN587/1 were grown under BFP-expressing conditions. The number of CFU present in one half of each culture was determined after serial 10-fold dilution. The other half of each culture was centrifuged, and the pellet was resuspended in 100 µl of 25 mM sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer. Immunoblotting was performed with serial twofold dilutions of the whole-cell lysates along with serial twofold dilutions of purified homologous pilin protein. The densitometry of bands corresponding to bundlin was determined using Kodak ID image analysis software, and a standard curve for known bundlin samples was constructed to determine the concentration of bundlin in each culture using the average density of two dilutions that fell within the range of the known sample densities. The experiment was conducted three times, and the mean and standard deviation of the number of molecules per CFU were calculated and compared using Student’s t test.

RESULTS

Bundlin proteins are functionally interchangeable. Prior studies have demonstrated a considerable degree of sequence variation in the bfpA gene encoding prebundlin, the BFP pilin protein precursor (6, 7). Although some microorganisms, such as P. aeruginosa, are capable of assembling type IV pili upon introduction of highly divergent pilin genes from distantly related species (4, 26, 37), EPEC is unable to assemble heterologous pili when it is transformed with the tcpA pilin gene for the closely related toxin-coregulated pilus of V. cholerae or with gene fusions encoding hybrid bundlin-TcpA pili (27). To determine whether bfpA sequence variation affects pilus biogenesis, we constructed an expression vector containing the native bfpA promoter from EPEC strain E2348/69 followed by restriction sites that would allow cloning of the bfpA gene from any source strain. Amplification of bfpA from strains that express each of the known bundlin variants and subsequent cloning resulted in a series of eight plasmids encoding each alternative bundlin variant. After introduction into strain UMD901, a bfpA null mutant of E2348/69, we monitored pilus biogenesis and function using the autoaggregation/disaggregation assay (2). This assay corresponds with the ability to produce BFP as assessed by electron microscopy (3). We found that each of eight known alternative bfpA alleles was able to complement strain UMD901 to restore both aggregation and disaggregation (data not shown). Thus, the BFP machinery encoded by the bfp operon in strain E2348/69 is able to assemble all known bundlin variants into functional BFP.

EPEC strains expressing divergent bundlin proteins are capable of forming mixed autoaggregates. BFP filaments aggregate longitudinally to form dense bundles (18), an effect that appears to require complementary binding between filaments. As the filaments from many bacteria intertwine, autoaggregation of the bacteria is observed. To determine whether the sequence variation among bfpA alleles affects the ability of bacteria to form heterologous autoaggregates, we used immunofluorescence to follow autoaggregation in mixed cultures of EPEC bacteria after transformation with plasmids that direct the expression of either eGFP or DsRed. We then allowed the bacteria to autoaggregate and examined the aggregates by immunofluorescence to determine whether they were uniform or mixed. We first examined mixtures of homologous strains expressing each fluorescent protein. We found that for both prototypic α1 bundlin strain E2348/69 and β6 bundlin strain RN587/1 autoaggregates displayed both green and red fluorescence with strong colocalization of the fluorescence signals (not shown). This result indicates that autoaggregates are not clonal in origin but are derived primarily from the association of individual or small groups of bacteria. This result is also consistent with the kinetics of autoaggregation, which results in the formation of aggregates containing thousands of bacteria within 3 h, a rate that exceeds the generation time of E. coli.

We next examined autoaggregates derived from a mixture of prototypic α1 bundlin strain E2348/69 expressing DsRed and
results can be generalized to other bundlin types, we repeated the studies with strains that express α1 bundlin and β6 bundlin. We found that each strain was capable of forming mixed autoaggregates with the other three strains (data not shown). We concluded, therefore, that pili composed of divergent bundlin proteins are capable of bundling together.

Epitopes of divergent bundlin proteins are conserved and cross-reactive. An analysis of the sequences of bfpA alleles revealed that the region of greatest sequence variation is near the 3′ end of the gene. Furthermore, a region encompassing the codons for amino acids residues 137 to 155 near the C terminus of the mature protein (Fig. 1) has an excess of non-synonymous substitutions, an effect indicating the influence of diversifying selection (7). As this effect may have been exerted by the selective pressure of the immune response from humans infected with EPEC, we developed the hypothesis that this region of bundlin is immunodominant and surface exposed. To test this hypothesis, we raised antisera in rabbits against peptides representing this region. We found that the peptide derived from strain E2348/69, which expresses α1 bundlin and β6 bundlin, was highly immunogenic, inducing antibody responses in two rabbits at a titer of 1:250,000 as determined by ELISA and recognizing bundlin as a single band on an immunoblot of whole-cell lysates at a titer of 1:40,000 (data not shown). To our surprise, however, the corresponding peptide from β6 bundlin was not immunogenic, yielding a titer of 1:50 or less in two different rabbits and failing to recognize specific proteins by Western blotting. Furthermore, despite its high level of reactivity against denatured protein, the antisera raised against the α1 peptide was unable to recognize intact BFP by immunofluorescence, suggesting that the epitopes that it recognizes are either nonconformational or not surface exposed (data not shown). In addition, neither the α1 peptide antiserum nor Fab fragments of this antiserum inhibited autoaggregation (data not shown). These results are consistent with the immunofluorescence data showing that antibodies raised against this highly variable region of bundlin do not recognize intact pili and fail to support (but do not refute) our hypothesis that this region of the pilus is surface exposed.

We conducted further experiments to explore the apparent difference in immunogenicity between cognate regions of divergent bundlin proteins. We decided to raise additional antisera in mice rather than in rabbits, because mice would provide the opportunity to test a greater number of individual animals and to determine whether the observed difference in immunogenicity was limited to a single species. We also elected to test a different β bundlin sequence to determine whether the observed effect was limited to a single bundlin type. Therefore, we raised antisera in 10 mice each to peptides representing amino acids 137 to 155 of α1 bundlin and the corresponding region of β6 bundlin. We also raised antisera in 10 mice each to purified soluble (lacking the conserved N-terminal hydrophobic 24 amino acids) α1 and β6 bundlin proteins. The results of ELISA experiments testing the immunogenicity of these peptides and proteins are shown in Fig. 3 and summarized in Table 2. We found that both bundlin proteins were highly immunogenic, eliciting immune responses in all mice tested with geometric mean titers of >1:200,000 against both the homologous and heterologous pilin proteins. For mice immunized with α1 bundlin, the response against the homologous protein was greater than that against the heterologous protein (geometric mean titers, 879,000 and 422,000, respectively), but not significantly greater (P = 0.09). However, in mice immunized with β6 bundlin the responses against the homologous and heterologous proteins were equivalent. Given the higher titers against heterologous proteins, it appears that immunodominant epitopes are conserved among divergent bundlin proteins.

Similar to the responses to the proteins, the α1 peptide was recognized by the majority of mice immunized with either the homologous peptide or the homologous bundlin protein. In stark contrast, the β6 peptide was not immunogenic, as no mice immunized with either β6 bundlin or β6 peptide developed antibodies against the peptide. The fact that these mice received the immunization was confirmed by the finding that all mice immunized with the α1 peptide and 9 of 10 mice immunized with the β6 peptide had significant responses to the KLH carrier protein to which the peptide was conjugated. Curiously, 5 of the 10 mice immunized with the β6 bundlin protein reacted against the α1 peptide, suggesting a degree of conformational conservation in this region of the pilin proteins that was not expected based on the primary amino acid sequences (Fig. 1). However, the fact that the same sera did not react against the β6 peptide suggests that there might be differences in the folding of the two peptides used to measure the response. Overall, these
results obtained using a larger number of animals, a different species, and a different β bundlin variant are consistent with those obtained in the pilot study with rabbits, indicating that the region of β bundlin representing an area under diversifying selection is poorly immunogenic in comparison to the corresponding region of α bundlin. These results also demonstrate that the bundlin proteins themselves share a considerable degree of immunogenic epitopes.

**Surface-exposed epitopes of bundlin are cross-reactive.** To determine whether surface-exposed epitopes of bundlin are cross-reactive, given the small quantity of antisera available from mice, it was necessary to raise additional antisera in rabbits against the purified soluble α₁ and β₆ bundlin proteins. We affinity purified these antisera by passage over columns to which the purified bundlin proteins were conjugated. Once again, these antisera were highly reactive as determined by ELISA against both the homologous and heterologous bundlin proteins (Fig. 4). In addition, each affinity-purified antiserum reacted more strongly against the homologous bundlin protein than against the heterologous bundlin protein, again indicating that immunization with bundlin elicits both cross-reactive and type-specific antibodies. However, in contrast to the poor reactivity of the β₆ bundlin antisera raised in mice against the corresponding peptide, the antiserum raised in rabbits against the soluble β₆ bundlin protein reacted strongly with the homologous peptide. Thus, we concluded that in the context of the entire protein, the β₆ peptide is capable of eliciting an antibody response, at least in rabbits. Furthermore, in keeping with the results obtained with mice, the serum raised against the soluble β₆ bundlin protein recognized the α₁ peptide at low titer. Similarly, the serum raised against the soluble α₁ protein recognized the β₆ peptide at low titer. These results confirm that there is a degree of similarity in the conformation of cognate regions of divergent bundlin proteins even though only 8 of 20 residues are identical.

To determine whether the cross-reactive antibundlin antibodies can recognize whole pili and react with surface-exposed epitopes, we performed immunofluorescence assays with EPEC bacteria infecting HeLa cells and exhibiting localized adherence. As controls, we infected cells with bfpA mutant strains transformed with a plasmid encoding an afimbrial adhesin to compensate for the otherwise poor adherence of the mutants. We found that affinity-purified antisera raised against α₁ bundlin recognized BFP expressed by both the prototypic α₁
strain E2348/69 and the prototypic \( \beta_6 \) strain RN587/1 (Fig. 5). The specificity of this result was demonstrated by the absence of staining of the corresponding \( bfpA \) mutant strains. Similarly, affinity-purified antisera raised against \( \beta_6 \) bundlin specifically recognized BFP expressed by both the homologous and heterologous strains. However, the fluorescence intensity observed using both antisera was greater in samples containing the \( \beta_1 \) bundlin strain than in samples containing the \( \beta_6 \) bundlin strain, suggesting either that strain E2348/69 produces more pili than strain RN587/1 or that both antisera reacted more strongly with \( \beta_1 \) pili than with \( \beta_6 \) pili.

**Differences in bundlin expression by prototype strains expressing different bundlin alleles.** Despite the fact that affinity-purified antisera against soluble \( \alpha_1 \) and \( \beta_6 \) bundlin had high ELISA titers against both the homologous and heterologous bundlin proteins, we consistently observed that the rabbit antisera raised against \( \beta_6 \) bundlin specifically recognized BFP expressed by both the homologous and heterologous strains. However, the fluorescence intensity observed using both antisera was greater in samples containing the \( \alpha_1 \) bundlin strain than in samples containing the \( \beta_6 \) bundlin strain, suggesting either that strain E2348/69 produces more pili than strain RN587/1 or that both antisera reacted more strongly with \( \alpha_1 \) pili than with \( \beta_6 \) pili.

![FIG. 4. Reactivity of affinity-purified rabbit antisera against bundlin proteins and peptides. The titers of affinity-purified rabbit sera raised against soluble \( \alpha_1 \) bundlin (open columns) and \( \beta_6 \) bundlin (filled columns) against \( \alpha_1 \) and \( \beta_6 \) bundlin proteins and peptides are shown.](http://iai.asm.org/)

**TABLE 2. Summary of antibody responses in mice to immunization with bundlin proteins and peptides**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>No. with response/total no.</th>
<th>Geometric mean titer for responding animals</th>
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\( \alpha_1 \) protein antigen

<table>
<thead>
<tr>
<th>No. with response/total no.</th>
<th>Geometric mean titer for responding animals</th>
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<tr>
<th>KLH antigen</th>
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No. with response/total no. | Geometric mean titer for responding animals |

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<thead>
<tr>
<th>No. with response/total no.</th>
<th>Geometric mean titer for responding animals</th>
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\( \alpha_1 \) peptide antigen

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<tr>
<th>No. with response/total no.</th>
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<th>No. with response/total no.</th>
<th>Geometric mean titer for responding animals</th>
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\( \beta_6 \) protein antigen

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<tr>
<th>No. with response/total no.</th>
<th>Geometric mean titer for responding animals</th>
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\( \beta_6 \) peptide antigen

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A response was defined as a fourfold increase in the titer compared with pooled preimmune sera.

In three experiments, we found that strain E2348/69 expressed \( 9.99 \times 10^5 \pm 0.43 \times 10^5 \) bundlin molecules per CFU (mean ± standard deviation). In contrast, strain RN587/1 expressed \( 1.73 \times 10^5 \pm 0.56 \times 10^5 \) molecules per CFU (P = 0.01). Thus, the difference...
ences that we observed between these prototype strains in BFP immunofluorescence (Fig. 5), autoaggregation (Fig. 6), and immunoblotting were due to significant differences in BFP expression levels rather than to differences in recognition of surface epitopes by antisera raised against the divergent bundlin proteins.

Human responses to homologous and heterologous bundlin proteins after experimental EPEC infection. In mice and rabbits immunized with \( \alpha_1 \) bundlin we consistently found stronger responses against the homologous bundlin protein than against the heterologous (\( \beta_6 \)) bundlin protein. To determine whether such differences are also found in humans infected with EPEC, we examined archived sera from a volunteer challenge study (15). This study involved a comparison of volunteers who had received wild-type EPEC strain E2348/69 (\( n = 6 \)) or isogenic eae mutant strain CVD206 (\( n = 5 \)) 70 days earlier or who were naïve prior to EPEC challenge (\( n = 6 \)). All volunteers were then challenged with an oral dose of live E2348/69 bacteria. We tested prechallenge sera and sera from 27 days postchallenge for antibodies against purified \( \alpha_1 \) and \( \beta_6 \) bundlin proteins using ELISA. As in the earlier study using a less-highly-purified prepurified molecule with an uncertain degree of proper folding (15), we found that the responses to bundlin were modest (Fig. 7). As there were no detectable differences in titers between veteran volunteers previously challenged with wild-type bacteria and veteran volunteers previously challenged with eae mutant bacteria, we pooled these groups for further analysis. We found that the day 27 geometric mean titer against \( \alpha_1 \) bundlin was significantly higher than the prechallenge titer in naïve volunteers (\( P = 0.025 \)). In contrast, the day 27 titer against \( \beta_6 \) bundlin was not significantly higher than the prechallenge titer in this group. These results are consistent with the results obtained with mice and rabbits, indicating that the antibody responses after exposure to bundlin are greater against the homologous protein than against a divergent bundlin protein. Furthermore, these results extend the findings obtained with animals immunized with purified protein to humans infected with wild-type EPEC. Consistent with these results is the finding that the veterans had significantly higher prechallenge titers against \( \alpha_1 \) bundlin than against \( \beta_6 \), bits immunized with \( \alpha_1 \) bundlin.
bundlin (P = 0.006). These higher titers reflect the exposure of the subjects to α₁ bundlin 70 days earlier. In contrast, there was no difference among the naïve veterans in the prechallenge titers against α₁ and β₁ bundlins. However, there was no evidence of an amnestic response in the veteran volunteers, as there was no significant rise in titers against either α₁ or β₁ bundlin in these volunteers. Nevertheless, the day 27 titers against β₁ bundlin in the veterans were significantly higher than those in the naïve group (P = 0.027), possibly reflecting a broadening of the immune response with repeated exposure to favor conserved epitopes.

**DISCUSSION**

The EPEC BFP is an important virulence factor required for full pathogenicity in an experimental human model of disease (5). Previous studies have demonstrated a considerable degree of sequence variation in bfpA alleles specifying bundlin, the major subunit of the BFP (6, 7). In particular, a region encompassing the codons for amino acid residues 137 to 155 near the C terminus of the mature protein (Fig. 1) has an excess of nonsynonymous substitutions, indicating the influence of diversifying selection (7). As this effect may have been exerted by the selective pressure of the immune response from humans infected with EPEC, we developed the hypothesis that this region of bundlin is recognized by host antibodies and is surface exposed. As an initial step in testing this hypothesis, we examined antibody responses in mice, rabbits, and humans against divergent bundlin peptides, proteins, and pili. Our results supported some aspects of the hypothesis and failed to support others. In particular, we could not verify that the divergent region is surface exposed. In addition, we found that antibodies raised against one bundlin type reacted strongly with a divergent type. However, we found dramatic differences in immunogenicity between cognate peptides from divergent types, and we determined that responses were stronger against the homologous proteins than against the heterologous proteins. Given the high degree of overall sequence conservation between divergent pilin proteins, the ability of sera from volunteers to distinguish between two types suggests an important degree of type-specific responses that may be relevant to subsequent susceptibility and may provide the basis for bfpA sequence divergence. It is possible that alternative forces contribute to bundlin sequence variation as well. For example, pressure due to pilus-specific bacteriophages could lead to sequence diversity, although BFP phages have not yet been described.

The structure of soluble α₁ bundlin has been determined by nuclear magnetic resonance, and a model that fits the bundlin monomers into the BFP has been proposed (35). According to this model, amino acids corresponding to the amino acids under the influence of diversifying selection are predicted to be surface exposed. However, we could not verify this prediction, as antiserum raised in rabbits against a peptide from this region of α₁ bundlin did not recognize intact pili and did not block autoaggregation, despite the high titer of this serum when it was used in ELISA or immunoblotting assays. Failure to interact with intact pili should not be interpreted as an indication that this region of the protein is not surface exposed. The relevant epitopes may not be recognized in the context of the intact pilus due to differences in three-dimensional conformation. For example, the solution structure of the isolated receptor binding peptide from the type IV pilin of P. aeruginosa is not identical to that of the intact pilin (9, 20).

In addition to an effect on immunogenicity, we examined whether bfpA sequence diversity could influence pilus biogenesis or function. To determine whether the biogenesis machine from a strain that produces α₁ bundlin can assemble divergent BFP, we complemented a bfpA null mutant of this strain with plasmids encoding each alternative bfpA allele. We found that each plasmid restored the ability to produce functional BFP, demonstrating that BFP biogenesis machines are capable of assembling pili composed of all bundlin types. Thus, the sequence divergence among these alleles is not critical to BFP biogenesis.

BFP are so named because of their tendency to interact longitudinally to form rope-like structures (18). This property, combined with the ability of the bacteria to retract the pili, endows EPEC with the autoaggregation and disaggregation phenotypes. To determine whether BFP composed of divergent bundlin proteins are capable of forming mixed bundles, we examined autoaggregation using pairs of strains expressing four different bundlin types and two different fluorescent proteins. We found that aggregates were composed of mixtures of both strains of bacteria with colocalization of the fluorescence signals. Thus, the sequence divergence does not preclude interpilus bundle formation. These experiments also provide further evidence that autoaggregates result from recruitment of planktonic bacteria rather than from proliferation of a progenitor (5).

One of the striking findings of this study was the marked difference in immunogenicity of cognate peptides from α₁ and β₁ bundlin molecules representing the region under apparent diversifying selection. Whereas the α₁ bundlin peptide was immunogenic in mice and rabbits, the corresponding β₁ bundlin peptide was not. However, rabbits immunized with soluble β₁ bundlin produced antibodies against the β₁ bundlin peptide,

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**FIG. 7.** Antibody responses to bundlin in volunteers challenged with wild-type EPEC strain E2348/69. The geometric mean prechallenge (Pre) and day 27 postchallenge titers of veteran volunteers (open columns) or naïve volunteers (filled columns) against α₁ and β₁ bundlin proteins are shown. The error bars indicate the standard errors of the geometric means. Asterisks indicate significant differences (P < 0.05) between pairs of data.
indicating that in the context of the full protein, epitopes from this region can be recognized. We were also surprised to find that 5 of 10 mice immunized with the soluble β_n bundlin protein were able to recognize the α bundlin peptide, despite conservation of only 8 of 20 amino acids. This result was verified in rabbits. Thus, it appears that cross-reactive epitopes exist even in this highly divergent region, indicating perhaps that similar three-dimensional conformations are adopted by the divergent proteins. This result may help explain how the α_t strain is able to assemble all other pilin types.

To determine whether there are cross-reactive surface-exposed epitopes in BFP composed of divergent bundlin molecules, we tested affinity-purified rabbit anti-α bundlin and affinity-purified rabbit anti-β bundlin sera by fluorescence microscopy against strains expressing homologous and heterologous BFP. We found that both sera were able to recognize BFP from both strains. Any apparent differences in signal intensity were accounted for by the fact that the strain expressing α bundlin produced almost sixfold more bundlin protein than the strain expressing β bundlin. Thus, animals immunized with one bundlin protein can recognize pili as well as pilin from a strain expressing a divergent bundlin type.

We took advantage of archived sera from a human EPEC rechallenge study to determine whether people infected with one EPEC strain develop antibodies against homologous and heterologous bundlin types. The results were enlightening. Although the responses of individual volunteers against bundlin were modest, the responses of veteran volunteers against bundlin produced almost sixfold more bundlin protein than the strain expressing β bundlin. Thus, veterans infected with one bundlin type against the homologous bundlin strain is able to assemble all other pilin types.

In the current study we determined that initial exposure to a subsequent strain if the naïve volunteers developed titers against the homologous bundlin, to which they were previously exposed, than against a divergent bundlin type. These results are consistent with our hypothesis that the human immune response may exert selective pressure that favors bfpA sequence variation. According to this hypothesis, individuals exposed to one EPEC strain are more likely to be infected and transmit the bacteria to other individuals upon exposure to a subsequent strain if the latter strain has a bfpA allele specifying a different sequence in this region. Whether the difference in initial response correlates with differences in subsequent susceptibility to infection with strains expressing homologous and heterologous bundlin types cannot be determined from the current study. A longitudinal birth cohort study that examines infection with EPEC, development of antibodies against EPEC antigens, and signs and symptoms of EPEC infection may help unravel the relative roles of exposure, age, and immunity in susceptibility to EPEC disease.

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REFERENCES


