

## Functional Consequences of Sequence Variation in Bundlin, the Enteropathogenic *Escherichia coli* Type IV Pilin Protein<sup>∇</sup>

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**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, adherence, autoaggregation, biofilm formation, horizontal gene transfer, motility, and virulence (5, 8, 21, 22, 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 subunits 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 vari-

ation 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  $\alpha$  alleles are highly similar to one other, resulting in proteins that are 97% identical, while the six  $\beta$  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 nonsynonymous 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 pCVD442cys1 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 protein unstable and subject to rapid degradation (44). For complementation of *bfpA* null mutant strain UMD901, we amplified plasmid pRPA100, which contains *bfpA* from strain E2348/69 under control of its native promoter in a low-copy-number vector, by inverse PCR using primers Donne-362 (5'-AGGTCTG TCTTTGATTGAA-3') and Donne-535 (5'-CGCATCCGGACCATGGAAAC TGTTTTCTTATA-3'). This resulted in insertion of an NcoI restriction endonuclease site at the start codon of *bfpA*. We then amplified each *bfpA* allele

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FIG. 1. Sequence comparison of  $\alpha_1$  and  $\beta_6$  bundlins. An alignment of the sequences of the mature bundlin proteins is shown. Identical residues are connected with lines, and similar residues are connected with two dots. The black background indicates residues that were replaced by a histidine tag to purify the recombinant soluble bundlin proteins. The shaded area indicates the peptides used in this study.

from genomic DNA using conserved primers Donne-536 (5'-GCTCGCCATG GTTTCTAAAATCA-3') and Donne-537 (5'-CGTCGAATTCACAGGGCGT ATTATGTAGATTA-3'), which add a NcoI 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 complementary oligonucleotides Donne-200 (5'-CTGCGACTTGACAGGATCCACGCGT CTAGTTATAAT-3') and Donne-201 (5'-CTAGATTATACTAGACGCGTG GATCCTGTCAAGTCGACGAGCT-3') specifying a consensus sigma-70 promoter, to create pTR102. A *gfp* 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  $-80^{\circ}\text{C}$  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  $\mu\text{g}/\text{ml}$ ; nalidixic acid, 50  $\mu\text{g}/\text{ml}$ ; kanamycin, 50  $\mu\text{g}/\text{ml}$ ; and chloramphenicol, 25  $\mu\text{g}/\text{ml}$ . To induce expression of BFP, overnight cultures of bacteria were diluted 100-fold in Dulbecco modified Eagle medium/F10 and grown at  $37^{\circ}\text{C}$  with shaking (225 rpm) for 3 h.

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

**Bundlin purification and immunization.** Soluble  $\alpha_1$  bundlin with an amino-terminal hexahistidine tag was expressed in the periplasm and purified as previously described (34). Similarly, soluble  $\beta_6$  bundlin was expressed by amplifying the sequences encoding the amino acid glycine 25 of the mature protein through the stop codon from strain RN587/0 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 a NcoI-BamHI fragment into plasmid pPF301, replacing the  $\alpha_1$  allele from this plasmid to form a fusion with the signal sequence of *dsbA* followed by an amino-terminal hexahistidine tag. The resulting plasmid, pPF302, was transferred to strain BL21(AI) *slyD* for purification as described previously (34). Protein concentrations were calculated from extinction coefficients (17).

Bundlin peptides and bundlin peptides conjugated to keyhole limpet hemocyanin (KLH) were purchased from Research Genetics (Huntsville, AL). Female 6- to 8-week-old CBA/J mice (Harlan Sprague Dawley, Indianapolis, IN) were immunized subcutaneously with 100  $\mu\text{g}$  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  $\mu\text{g}$  in incomplete Freund's adjuvant were given on days 21 and 42. Male 1.8- to 2.2-kg New Zealand White rabbits (Covance, Denver, PA) were immunized subcutaneously with 100  $\mu\text{g}$  purified bundlin protein or 200  $\mu\text{g}$  bundlin peptide-KLH conjugate in complete Freund's adjuvant and boosted on days 30 and 60 with 30  $\mu\text{g}$  purified bundlin protein or 200  $\mu\text{g}$  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

Strain or plasmid	Description or genotype	Reference or source
<b>Strains</b>		
E2348/69	Wild-type EPEC strain, serotype O127:H6, produces $\alpha_1$ bundlin	7
UMD901	E2348/69 <i>bfpA</i> C129S	44
RN587/1	Wild-type EPEC strain, serotype O157:H+, produces $\beta_6$ bundlin	6
UMD949	RN587/1 <i>bfpA</i> C129S	This study
RN410/1	Wild-type EPEC strain, serotype O119:H+, produces $\alpha_3$ bundlin	7
RN191/1	Wild-type EPEC strain, serotype O111:H+, produces $\alpha_2$ bundlin	7
B171	Wild-type EPEC strain, serotype O111:H+, produces $\alpha_2$ bundlin	18
E56/54	Wild-type EPEC strain, serotype O128ab:H2, produces $\beta_4$ bundlin	7
012-050982	Wild-type EPEC strain, serotype O142:H+, produces $\beta_1$ bundlin	7
E851/71	Wild-type EPEC strain, serotype O142:H6, produces $\beta_5$ bundlin	7
Z188-93	Wild-type avian EPEC strain, serotype O110:H6, produces $\beta_2$ bundlin	7
CA89-4221	Wild-type canine EPEC strain, serotype NT:H+, produces $\beta_3$ bundlin	7
BL21(AI) <i>slyD</i>	F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> ( $r_B^- m_B^-$ ) <i>gal dcm araB::T7RNAP-tetA <math>\Delta</math>slyD::cat</i>	13
<b>Plasmids</b>		
pRPA100	<i>bfpA</i> gene with native promoter cloned into low-copy-number vector pWKS30	3
pXLW10	pRPA100 with <i>bfpA</i> alpha-3 allele from strain RN410/1	This study
pXLW11	pRPA100 with <i>bfpA</i> alpha-2 allele from strain RN191/1	This study
pXLW12	pRPA100 with <i>bfpA</i> beta-4 allele from strain E56/54	This study
pXLW13	pRPA100 with <i>bfpA</i> beta-1 allele from strain 012-050982	This study
pXLW14	pRPA100 with <i>bfpA</i> beta-5 allele from strain E851/71	This study
pXLW15	pRPA100 with <i>bfpA</i> beta-6 allele from strain RN587/1	This study
pXLW16	pRPA100 with <i>bfpA</i> beta-2 allele from strain Z188-93	This study
pXLW17	pRPA100 with <i>bfpA</i> beta-3 allele from strain CA89-4221	This study
pIL14	AFA-1 afimbrial adhesin genes cloned in a pBR322 vector	23
pPF301	Expression vector for soluble $\alpha_1$ bundlin under control of T7 promoter	34
pPF302	Expression vector for soluble $\beta_6$ bundlin under control of T7 promoter	This study
pKEN	pUC derivative encoding eGFP	11
pTR103	pBluescript derivative with eGFP under control of consensus sigma-70 promoter	This study
pDsRed	Red fluorescent protein under control of <i>lac</i> promoter in pUC19 backbone	Clontech

$\mu\text{l}$ /well of purified soluble  $\alpha_1$  or  $\beta_6$  bundlin, unconjugated bundlin peptides, or KLH ( $2 \mu\text{g ml}^{-1}$  in phosphate-buffered saline [PBS], pH 7.2) and incubated at  $4^\circ\text{C}$  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  $37^\circ\text{C}$  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  $37^\circ\text{C}$  for 1 h. After washing,  $100 \mu\text{l}$  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  $37^\circ\text{C}$  for 1 h. After washing,  $100 \mu\text{l}$  of SureBlue 3,3',5,5'-tetramethylbenzidine peroxidase substrate (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 1:1,000 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 antibundlin 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.** Antisera were affinity purified after conjugation of purified soluble  $\alpha_1$  or  $\beta_6$  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  $\alpha_1$  or  $\beta_6$  bundlin antisera were 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  $37^\circ\text{C}$  with 10% normal goat serum (Gibco) and left overnight at  $4^\circ\text{C}$ . Next, the slides were washed three times with PBS, incubated for 1 h at  $37^\circ\text{C}$  with affinity-purified rabbit anti- $\alpha_1$  or anti- $\beta_6$  bundlin sera at a dilution of 1:1,000 in PBS plus 10% goat serum, washed three times, incubated for 1 h at  $37^\circ\text{C}$  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 Axiocam 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 pTR103 were diluted 1:10, and 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added to the E2348/69(pDsRed) culture to induce expression of DsRed. After 3 h of incubation at  $37^\circ\text{C}$ , 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,  $5 \mu\text{l}$  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 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 pilins (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  $\alpha_1$  bundlin strain E2348/69 and  $\beta_6$  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  $\alpha_1$  bundlin strain E2348/69 expressing DsRed and

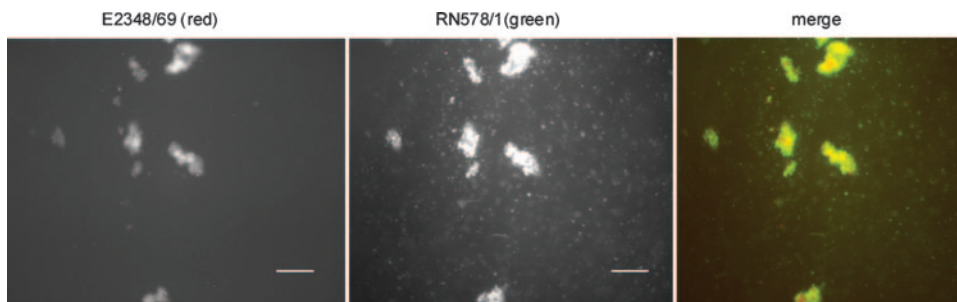


FIG. 2. Mixed autoaggregation of strains expressing  $\alpha_1$  bundlin and  $\beta_6$  bundlin alleles. Strain E2348/69 expressing  $\alpha_1$  bundlin and DsRed and strain RN578/1 expressing  $\beta_6$  bundlin and eGFP were incubated together and examined by immunofluorescence microscopy to detect red (left panel) or green (middle panel) fluorescent bacteria in microcolonies. The right panel shows the merged red and green images. Bar = 20  $\mu\text{m}$ .

prototype  $\beta_6$  bundlin strain RN578/1 expressing eGFP. Once again, we found that autoaggregates were composed of bacteria that expressed both fluorescent proteins and were therefore composed of both strains (Fig. 2). To determine whether these results can be generalized to other bundlin types, we repeated the studies with strains that express  $\alpha_2$  bundlin and  $\beta_1$  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  $\alpha_1$  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  $\beta_5$  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 antiserum raised against the  $\alpha_1$  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  $\alpha_1$  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 di-

vergent 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  $\beta$  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  $\alpha_1$  bundlin and the corresponding region of  $\beta_6$  bundlin. We also raised antisera in 10 mice each to purified soluble (lacking the conserved N-terminal hydrophobic 24 amino acids)  $\alpha_1$  and  $\beta_6$  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  $\alpha_1$  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  $\beta_6$  bundlin the responses against the homologous and heterologous proteins were equivalent. Given the high-titer responses against heterologous proteins, it appears that immunodominant epitopes are conserved among divergent bundlin proteins.

Similar to the responses to the proteins, the  $\alpha_1$  peptide was recognized by the majority of mice immunized with either the homologous peptide or the homologous bundlin protein. In stark contrast, the  $\beta_6$  peptide was not immunogenic, as no mice immunized with either  $\beta_6$  bundlin or  $\beta_6$  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  $\alpha_1$  peptide and 9 of 10 mice immunized with the  $\beta_6$  peptide had significant responses to the KLH carrier protein to which the peptide was conjugated. Curiously, 5 of the 10 mice immunized with the  $\beta_6$  bundlin protein reacted against the  $\alpha_1$  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  $\beta_6$  peptide suggests that there might be differences in the folding of the two peptides used to measure the response. Overall, these

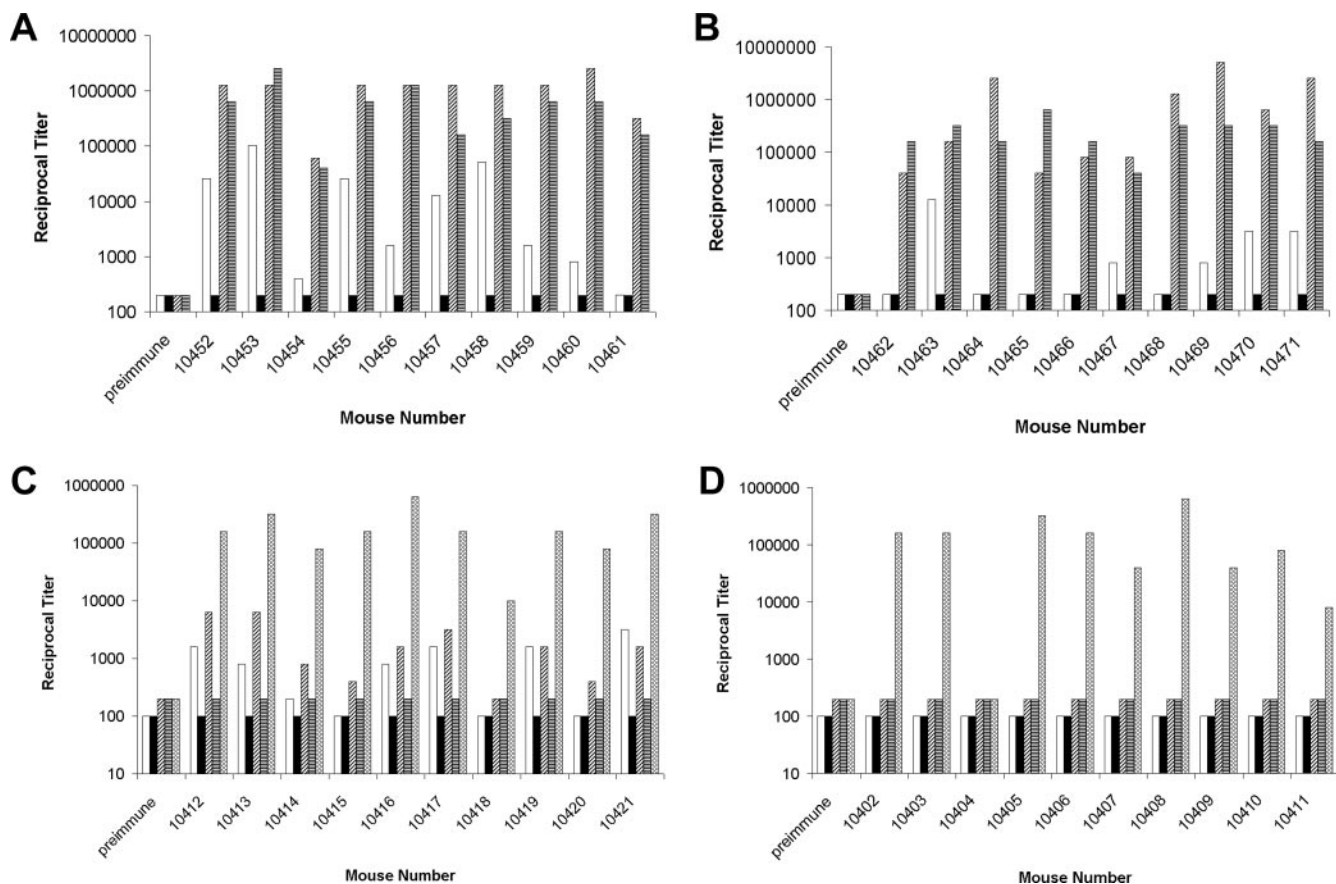


FIG. 3. Immune responses to bundlin proteins and peptides in mice. The antibody titers of individual mice immunized with purified  $\alpha_1$  bundlin protein (A), purified  $\beta_6$  bundlin protein (B),  $\alpha_1$  bundlin peptide conjugated to KLH (C), and  $\beta_6$  bundlin peptide conjugated to KLH (D) are shown. The results for preimmune serum pooled from the mice in each group are also shown. The responses against  $\alpha_1$  bundlin peptide are indicated by open bars, the responses against  $\beta_6$  bundlin peptide are indicated by filled bars, the responses against  $\alpha_1$  bundlin protein are indicated by bars with diagonal strips, the responses against  $\beta_6$  bundlin protein are indicated by bars with horizontal stripes, and the responses against KLH are indicated by cross-hatched bars.

results obtained using a larger number of animals, a different species, and a different  $\beta$  bundlin variant are consistent with those obtained in the pilot study with rabbits, indicating that the region of  $\beta$  bundlin representing an area under diversifying selection is poorly immunogenic in comparison to the corresponding region of  $\alpha$  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  $\alpha_1$  and  $\beta_6$  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 re-

activity of the  $\beta_6$  bundlin antisera raised in mice against the corresponding peptide, the antiserum raised in rabbits against the soluble  $\beta_6$  bundlin protein reacted strongly with the homologous peptide. Thus, we concluded that in the context of the entire protein, the  $\beta_6$  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  $\beta_6$  bundlin protein recognized the  $\alpha_1$  peptide at low titer. Similarly, the serum raised against the soluble  $\alpha_1$  protein recognized the  $\beta_6$  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  $\alpha_1$  bundlin recognized BFP expressed by both the prototypic  $\alpha_1$

TABLE 2. Summary of antibody responses in mice to immunization with bundlin proteins and peptides<sup>a</sup>

Immunogen	$\alpha_1$ protein antigen			$\beta_6$ protein antigen			$\alpha_1$ peptide antigen			KLH antigen		
	No. with response/total no.	Geometric mean titer	Geometric mean titer for responding animals	No. with response/total no.	Geometric mean titer	Geometric mean titer for responding animals	No. with response/total no.	Geometric mean titer	Geometric mean titer for responding animals	No. with response/total no.	Geometric mean titer	Geometric mean titer for responding animals
$\alpha_1$ protein	10/10	879,000	879,000	10/10	422,000	422,000	8/10	4,850	9,870	0/10		
$\beta_6$ protein	10/10	368,000	368,000	10/10	211,000	211,000	5/10	696	2,425	0/10		
$\alpha_1$ peptide	7/10	1,299	2,378	0/10			6/10	527	1,425	0/10	10/10	139,000
$\beta_6$ peptide	0/10			0/10			0/10			0/10	9/10	52,900
												98,300

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

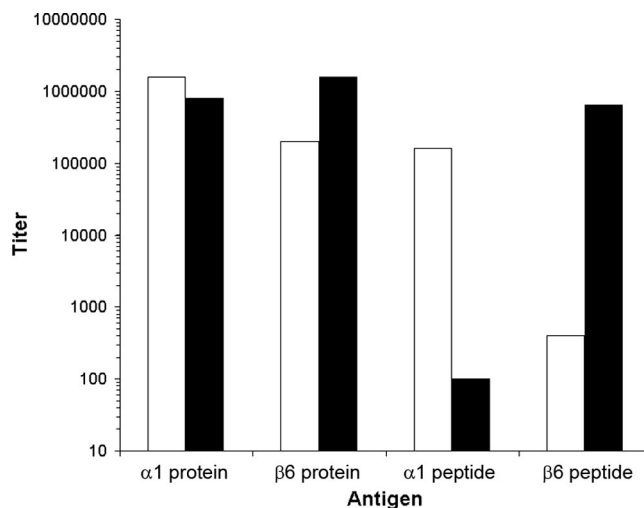


FIG. 4. Reactivity of affinity-purified rabbit antisera against bundlin proteins and peptides. The titers of affinity-purified rabbit sera raised against soluble  $\alpha_1$  bundlin (open columns) and  $\beta_6$  bundlin (filled columns) against  $\alpha_1$  and  $\beta_6$  bundlin proteins and peptides are shown.

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  $\alpha_1$  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.

**Differences in bundlin expression by prototype strains expressing different bundlin alleles.** Despite the fact that affinity-purified antisera against soluble  $\alpha_1$  and  $\beta_6$  bundlins had high ELISA titers against both the homologous and heterologous bundlin proteins, we consistently observed that the rabbit antiserum raised against  $\beta_6$  bundlin produced a band in immunoblots containing whole-cell lysates of the prototype  $\beta_6$  bundlin strain RN587/1 that was less intense than the band produced by the  $\alpha_1$  bundlin antiserum in immunoblots against the prototype  $\alpha_1$  bundlin strain E2348/69 (data not shown). Furthermore, we observed a marked difference in the abilities of these two prototype strains to exhibit autoaggregation (Fig. 6), which correlates with pilin expression. To determine whether these effects were due to differences in pilin expression levels, we determined the number of bundlin molecules per bacterial cell expressed by each wild-type strain. To do this, we grew each strain under BFP-expressing conditions, prepared serial dilutions of the cultures, and subjected these dilutions both to colony counting and to immunoblotting using homologous sera. Serial dilutions of known quantities of the homologous purified bundlin proteins were included in the immunoblots to construct standard curves. 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  $\pm$  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 differ-

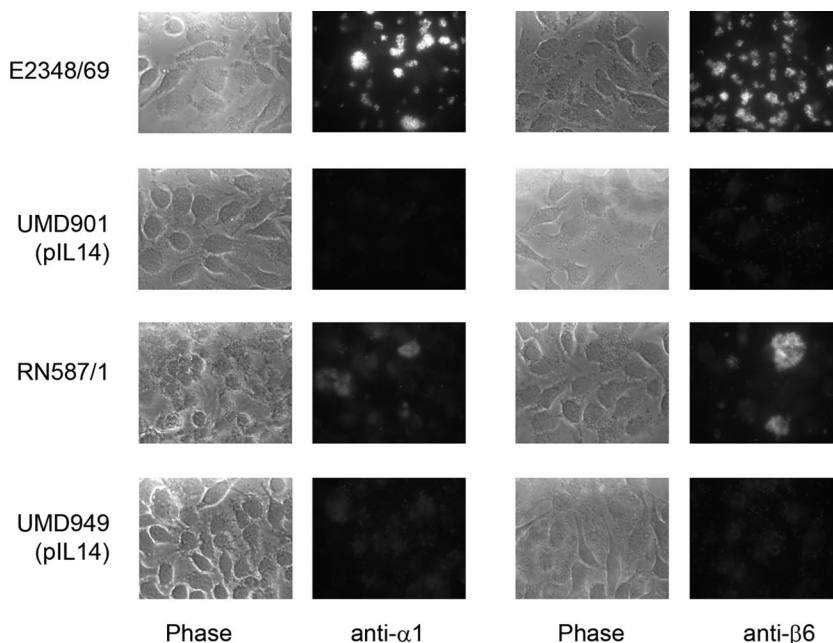


FIG. 5. Immunofluorescence of BFP. HeLa cells were infected with wild-type strain E2348/69, which expresses  $\alpha_1$  bundlin; with wild-type strain RN587/1, which expresses  $\beta_6$  bundlin; or with corresponding *bfpA* mutants UMD901 and UMD949, each transformed with plasmid pIL14 encoding an afimbrial adhesin to compensate for poor adherence. Infected cells were examined by immunofluorescence microscopy using affinity-purified antisera against  $\alpha_1$  and  $\beta_6$  bundlins, as indicated. Corresponding phase-contrast and fluorescence micrographs are shown.

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 prebundlin 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  $\beta_6$

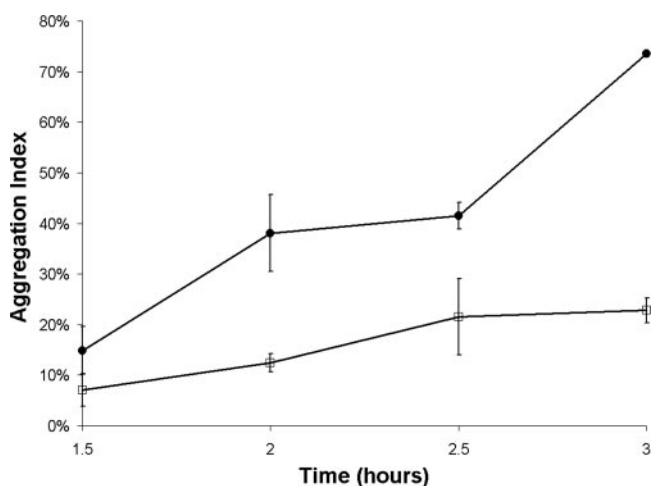


FIG. 6. Aggregation indices of prototype strains. Strain E2348/69 expressing  $\alpha_1$  bundlin (filled circles) and strain RN587/1 expressing  $\beta_6$  bundlin (open squares) were grown under BFP-expressing conditions, and the aggregation indices were determined at the indicated times as described in Materials and Methods. The symbols and error bars indicate the means and standard errors of three experiments. The indices were significantly different ( $P < 0.05$ ) at 2 and 3 h.

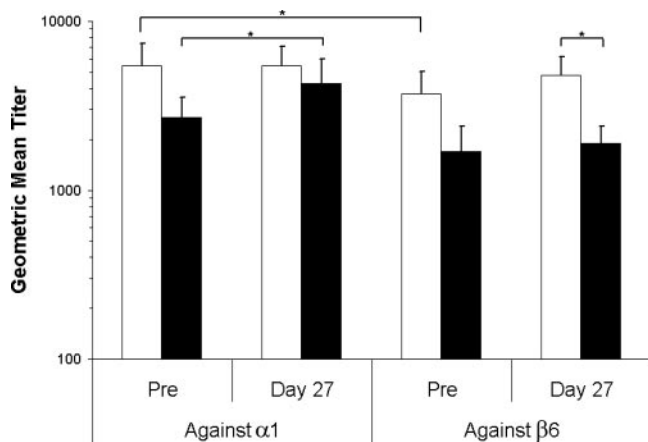


FIG. 7. Antibody responses to bundlin in volunteers challenged with wild-type EPEC strain E2348/69. The geometric mean prechallenge (Pre) and day 27 postchallenge titers of veteran volunteers (open columns) or naïve volunteers (filled columns) against  $\alpha_1$  and  $\beta_6$  bundlin proteins are shown. The error bars indicate the standard errors of the geometric means. Asterisks indicate significant differences ( $P < 0.05$ ) between pairs of data.

bundlin ( $P = 0.006$ ). These higher titers reflect the exposure of the subjects to  $\alpha_1$  bundlin 70 days earlier. In contrast, there was no difference among the naïve veterans in the prechallenge titers against  $\alpha_1$  and  $\beta_6$  bundlins. However, there was no evidence of an anamnestic response in the veteran volunteers, as there was no significant rise in titers against either  $\alpha_1$  or  $\beta_6$  bundlin in these volunteers. Nevertheless, the day 27 titers against  $\beta_6$  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 pro-

teins. 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  $\alpha_1$  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  $\alpha_1$  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  $\alpha_1$  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 inter-pilus 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  $\alpha_1$  and  $\beta_6$  bundlin molecules representing the region under apparent diversifying selection. Whereas the  $\alpha_1$  bundlin peptide was immunogenic in mice and rabbits, the corresponding  $\beta_6$  bundlin peptide was not. However, rabbits immunized with soluble  $\beta_6$  bundlin produced antibodies against the  $\beta_6$  bundlin peptide,



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  $\beta_6$  bundlin protein were able to recognize the  $\alpha_1$  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  $\alpha_1$  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- $\alpha_1$  bundlin and affinity-purified rabbit anti- $\beta_6$  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  $\alpha_1$  bundlin produced almost sixfold more bundlin protein than the strain expressing  $\beta_6$  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 undergoing rechallenge were significantly different than those of naïve volunteers infected with EPEC for the first time. First, the veterans had higher initial titers of serum IgG against  $\alpha_1$  bundlin, to which they were previously exposed, than against  $\beta_6$  bundlin, to which they were not previously exposed. Similarly, the naïve volunteers developed titers against the homologous  $\alpha_1$  bundlin that were significantly higher than their prechallenge titers. Despite these differences, the pre- and postchallenge titers against both bundlin types were similar for the veteran and naïve groups with one exception: the postchallenge titers against  $\beta_6$  bundlin were significantly higher in the veterans than in the naïve group. This result may indicate that repeated exposure to EPEC can lead to an increase in the production of cross-reactive antibodies.

It has long been known that EPEC disease almost exclusively afflicts children under 2 years of age, particularly the very youngest infants (1, 12, 16, 19). Whether this unique predilection is the result of differences in exposure to the pathogen, inherent susceptibility to its effects, or acquired protective immunity from repeated infection has never been ascertained. In the current study we determined that initial exposure to a strain of EPEC expressing one type of bundlin results in stronger responses against the homologous bundlin protein 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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