Escherichia coli K88ac Fimbriae Expressing Heat-Labile and Heat-Stable (STa) Toxin Epitopes Elicit Antibodies That Neutralize Cholera Toxin and STa Toxin and Inhibit Adherence of K88ac Fimbrial E. coli

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Received 18 June 2010/Returned for modification 28 July 2010/Accepted 14 October 2010

Enterotoxigenic Escherichia coli (ETEC) strains are a major cause of diarrheal disease in humans and animals. Bacterial adhesins and heat-labile (LT) and heat-stable (ST) enterotoxins are the virulence determinants in ETEC diarrhea. It is believed that vaccines inducing anti-adhesin immunity to inhibit bacterial adherence and anti-toxin immunity to eliminate toxin activity would provide broad-spectrum protection against ETEC. In this study, an ETEC fimbrial adhesin was used as a platform to express LT and STa for adhesin-toxin fusion antigens to induce anti-toxin and anti-adhesin immunity. An epitope from the B subunit of LT toxin (LTP1, *LCSEYRTNTQYITN*) and an STa toxoid epitope (*CCCELCCNPQCAGCY*) were embedded in the FaeG major subunit of E. coli K88ac fimbriae. Constructed K88ac-toxin chimeric fimbriae were harvested and used for rabbit immunization. Immunized rabbits developed anti-K88ac, anti-LT, and anti-STa antibodies. Moreover, induced antibodies not only inhibited adherence of K88ac fimbrial E. coli to porcine small intestinal enterocytes but also neutralized cholera toxin and STa toxin. Data from this study demonstrated that K88ac fimbriae expressing LT and STa epitope antigens elicited neutralizing anti-toxin antibodies and anti-adhesin antibodies and suggested that E. coli fimbriae could serve as a platform for the development of broad-spectrum vaccines against ETEC.

Enterotoxigenic Escherichia coli (ETEC) strains colonize the host small intestine and produce heat-labile (LT) and/or heat-stable (ST) enterotoxins as a major cause of diarrheal disease in humans and farm animals. The virulence determinants of ETEC in diarrhea are bacterial adhesins and enterotoxins (1, 7, 24, 25, 37, 41, 43). Adhesins mediate the attachment of ETEC bacteria to host epithelium cells in the small intestine and facilitate subsequent bacterial colonization. Enterotoxins, including ST and LT toxins (17, 18, 33), disrupt host fluid homeostasis and cause fluid and electrolyte hypersecretion through activation of adenyl cyclase (by LT) or guanylate cyclase (by STa) in small intestinal epithelial cells (19, 23). Recent experimental studies using a porcine model demonstrated that ETEC strains expressing LT or STa as the only toxin are sufficiently virulent to cause diarrhea (7, 43, 46).

No vaccines are currently available to effectively protect humans and animals against ETEC infections. Experimental oral vaccines carrying adhesin antigens alone showed protection against colonization by ETEC strains expressing the same or homologous adhesins (40). Similarly, experimental anti-toxin vaccines using toxin antigens, mainly LT toxoids or LTp subunits, could not provide effective protection either. Evidence indicated that LT antigen-based experimental vaccines provided protection against only LT-producing ETEC strains but not against ETEC strains that produce STa toxin (13, 14).

As more than two-thirds of human ETEC diarrheal cases and more than one-quarter of porcine ETEC diarrhea cases are caused by STa-producing ETEC strains (15, 16, 28, 31, 35, 42, 48), anti-toxin vaccines must also induce anti-STa immunity in order to provide effective protection against ETEC toxins. It becomes evident that both anti-toxin immunity, including anti-LT and anti-STa immunity, and anti-adhesin immunity are needed for broadly effective protection against ETEC-associated diarrhea (36).

Anti-toxin immunity and anti-adhesin immunity can be simultaneously induced by adhesin-toxin chimeric antigens. When an LT or an LTp subunit was fused to a CFA/I or a CS adhesin, the resultant chimera elicited both anti-LT and anti-adhesin immunity (20, 22, 30). Similarly, chimeric fimbriae with an STa peptide expressed in ETEC adhesin CS31A elicited neutralizing anti-STa antibodies (4, 5). However, no adhesin-toxin chimeric antigens have been constructed for stimulation of both anti-LT and anti-STa anti-toxin immunity. Expressing both LT and STa toxin antigens in one adhesin could produce a single chimeric antigen to induce not only anti-adhesin immunity but also anti-LT and anti-STa immunity. Moreover, as E. coli adhesins bind to host receptors in the small intestine, such adhesin-toxin chimeric antigens, we believe, could have an advantage in directly inducing host mucosal immunity, which is believed to play a critical role in protection against enteric infections.

In this study, we expressed an epitope from the LTp subunit designated LTP1 and an epitope from an STa toxoid at the FaeG major subunit of E. coli K88ac fimbriae and examined the ability of these K88ac-toxin fimbrial antigens to induce both anti-adhesin immunity and anti-toxin (anti-LT and anti-STa) immunity. This LTP1 epitope is homologous to an...
epitope of the cholera toxin (CT) B subunit (CTP1; 1LAAEY HNTQ1HTLT21). CT produced by Vibrio cholerae is highly homologous in function and structure to the LT toxin produced by ETEC strains, and CT is commonly used to replace LT in various assays. This CTP1 had been successfully expressed in flagella of Salmonella muenchen. After being immunized with this chimeric flagellum-CTP1 antigen, mice developed anti-CT antibodies (10). The STa epitope used in this study is a shorter peptide of porcine STa toxoid STa13. When its 13th amino acid was substituted, the modified STa protein was no longer toxic but showed anti-STa immunogenicity if carried by an LT toxoid protein (47). Because this LTP1 epitope was from the nontoxic LTB subunit and the STa13 epitope was from the STa13 toxoid, they are considered safe antigens. These two epitopes were expressed individually or combined at a FaeG major subunit of K88ac fimbriniae, and resultant chimeric K88ac fimbriniae were purified and used for rabbit immunization. Serum and fecal samples from the immunized rabbits were examined for anti-LT, anti-STa, and anti-adhesin antibodies, and the abilities of these antibodies to neutralize LT and STa toxins and inhibit K88ac fimbrinial adhesion in vitro were further studied.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli strain TOP10 (Invitrogen, Carlsbad, CA) was used to construct experimental strains and as the negative control in this study. Plasmid p8069 (44), derived from plasmid pBAD88-102, which carries the entire operon expressing K88ac fimbriae (3), was used as a vector in chimeric study. Plasmid p8069 (44), derived from plasmid pBAD88-102, which carries the entire operon expressing K88ac fimbriae (3), was used as a vector in chimeric study. Plasmid p8069 (44), derived from plasmid pBAD88-102, which carries the entire operon expressing K88ac fimbriae (3), was used as a vector in chimeric study. Plasmid p8069 (vector) were digested with EcoRI and SpeI restriction enzymes (Applied Biosystems, Foster City, CA). PCR-amplified products were separated by gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified PCR products of each chimeric faeG gene (insert) and plasmid p8069 (vector) were digested with the EcoR1 and SpeI restriction enzymes (New England BioLabs, Ipswich, MA) and ligated with T4 DNA ligase (Invitrogen) under standard conditions (2). Ligated products were introduced into E. coli TOP10 cells by standard electroporation (2). Positive colonies selected with ampicillin were screened by PCR initially and then sequenced to ensure that the cloned chimeric genes were inserted in the correct reading frame.

Expression of chimeric FaeG major subunit proteins. FaeG-toxin major subunit proteins expressed from the constructed chimeric faeG genes were examined with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Each constructed strain was cultured in LB medium with ampicillin (50 μg/ml) at 37°C overnight. The optical densities (ODs) of overnight-grown bacterial cultures were measured, and equal amounts of cells (based on OD readings) were centrifuged at 3,000 × g for 20 min. Culture pellets were used for total protein preparation using bacterial protein extraction reagent (in phosphate buffer; Pierce, Rockford, IL).

Thirty-microliter volumes of total protein extracts (50 to 100 μg) were used to detect chimeric FaeG major subunit proteins with hybridoma supernatant of anti-K88ac monoclonal antibodies (Mabs) 30/17 and 36/41 (38, 44), rabbit anti-LT antisera, and rabbit anti-STa antisera, respectively. Protein extracts from strain 8069 were used as the positive control for K88ac fimbriniae, and extracts from strain TOP10 were used as the negative control. Total protein extracts were separated in an SDS–10% polyacrylamide gel and transferred to a nitrocellulose membrane. The transferred membrane was blocked with 2% fat-free milk overnight at 4°C and then incubated with anti-K88ac MAb hybridoma supernatant (1:50), anti-CT serum (Sigma, St. Louis, MO) at a dilution of 1:10,000, or 1:3,000-diluted anti-STa serum (a gift from D. C. Robertson, Kansas State University). After three washes, membranes were incubated accordingly with IRDye goat anti-mouse or anti-rabbit IgG (LI-COR, Lincoln, NE) at a dilution of 1:5,000 for 1 h. After final washes, chimeric proteins on the membranes were detected by using LI-COR Odyssey Infrared Gel Imaging System Premium (LI-COR).

Immunolabeling and transmission electron microscopy (TEM) to detect biosynthesized chimeric K88ac fimbriniae. A single K88ac fimbriniae is composed of hundreds of FaeG major subunits. These FaeG major subunits were assembled into a filament structure and displayed at the E. coli cell surface. To verify the biosynthesis of K88ac fimbriniae of chimeric FaeG major subunits, we labeled E. coli cells with anti-K88ac, anti-LT, or anti-STa antibodies and examined them by TEM. Bacteria grown overnight on agar plates were harvested, washed, and

| Primer | Sequence (5’-3’) |
|--------|-----------------|
| Eco811G-F | GTC AGG ATG TGG TGA CCG CTG AGG C |
| SpeG-R | TTA TGG ATC CTG TCT GAT AAC AGA CTA GTA TC |
| K88ac3:LTb-F | TAT CGC AAC ACA CAA ATA TAT AGC ATA AAT GGT TTA GCA TAT TTT GTT |
| K88ac3:LTb-R | TGT TGT GTC GTA ATA TCG CA AAG TAG TAC AGA AGC TTC TCC |
| K88ac4:LTb-F | TAT CGC AAC ACA CAA ATA ATG AGT AAA GAA ATG TCA TCT GTC |
| K88ac4:LTb-R | TGT TGT GTC GTA ATA TCG CA AAG TAG CCG CAG AAC AAA ATA TGC TA |
| K88ac3:STa13-F | GTC TGT AAT CCT CAT TGT GCT GGA TGT TAT GGT TTA GCA TAT TTT GTT |
| K88ac3:STa13-R | ACA CTC AGG ATT ACA AAG TCC ACA AGA GCC TAC TAC AGA AGC TTC TCC |

a Nucleotides underlined represent restriction enzyme sites, and nucleotides in italics are of the LTB epitope or the STa13 epitope.
resuspended in phosphate-buffered saline (PBS). A 200-mesh copper grid (EMS, Hatfield, PA) was incubated with a bacterial suspension of each constructed strain by floating a grid on top of a drop of a diluted bacterial suspension (1 x 10^6 to 1 x 10^7 CFU/ml) for 30 to 60 min. Each bacterium-coated grid was separately rinsed in PBST (PBS with 2% BSA and 0.05% Tween 20) three times and then individually incubated with hybridoma supernatants of anti-K888ac MAbs 36/41 and 30/17, rabbit anti-CT serum, or rabbit anti-STa serum for 30 min. After three washes with PBST and another three washes with PBS, each grid was incubated accordingly with gold-conjugated (20-nm particles) goat-anti-mouse or goat-anti-rabbit IgG for 30 min. Bacteria on grids were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (EMS), for 3 min, rinsed in distilled water, negatively stained using 2% phosphotungstic acid, and then air dried. TEM was performed using a JEOL-1210 electron microscope (JEOL Ltd., Tokyo, Japan), and immunolabeling images were examined at a magnification of x30,000.

Porcine brush border bacterial adherence assay to examine chimeric fimbriae in binding. Jejunal small intestine segments were collected from a K888ac receptor-positive piglet to prepare brush border vesicles as described previously (11, 34). Each constructed strain was examined for binding to K888ac receptor-positive brush border vesicles. The numbers of bacteria bound to a single brush border was determined for each bacterial strain.

Chimeric K888ac fimbriae preparation and rabbit immunization. Chimeric K888ac fimbriae of each constructed strain were physically harvested by following the protocol previously reported (11). Briefly, each mutant strain was grown in 5 liters of tryptone soya broth medium overnight at 37°C. Culture growth was centrifuged at 7,000 rpm for 20 min at 4°C, and each pellet was resuspended in 50 ml PBS in a water bath at 65°C for 40 min. While still warm, the suspension was blended in a tissue blender for 2 to 3 min and centrifuged at 10,000 rpm for 20 min. The supernatant was precipitated as the pH was gradually adjusted to 4.0 with 2.5% citric acid and collected by centrifugation at 14,000 rpm for 20 min at 4°C.

One hundred micrograms of purified chimeric K888ac fimbriae from each constructed strain, in an equal volume of Freund's incomplete adjuvant (Sigma), was used to immunize two adult rabbits intramuscularly (i.m.). Two booster injections were administered at biweekly intervals. Blood and fecal samples were collected before and 14 days after immunization. Collected serum samples and fecal samples that were resuspended in fecal reconstitution buffer (10 mM Tris, 100 mM NaCl, 0.05% Tween 20, 5 mM sodium azide, pH 7.4) with the protease inhibitor phenylmethylsulfonyl fluoride (Sigma) at 0.5 mM were stored at −80°C until use.

Animal studies in this project complied with the Animal Welfare Act by following the 1996 National Research Council guidelines (26) and were approved and supervised by a state veterinarian and South Dakota State University's Institutional Animal Care and Use Committee.

Anti-rabbit antibody titration. Rabbit serum and fecal samples were examined for anti-K888ac, anti-CT, and anti-STa antibodies. Purified porcine wild-type ETEC strain 3030-2 K888ac fimbria (12), CT (Sigma), and STa ovalbumin conjugates were used as antigens in enzyme-linked immunosorbent assays (ELISAs) to titrate anti-K888ac, anti-CT, and anti-STa antibodies, respectively. Four hundred nanograms of K888ac fimbriae was used to coat each well of a MaxiSorp ELISA plate (Nunc, Roskilde, Denmark) to titrate anti-K888ac antibodies in a standard ELISA. To titrate anti-LT antibodies, 400 ng GM1 (Sigma) was used to coat each well of a MaxiSorp plate and 40 ng CT was added to each well as described previously (43, 45, 47). For anti-STa antibody titration, we coated each well of a Costar plate (Corning Inc., Corning, NY) with 1.25 ng of STa ovalbumin conjugate (a gift from D. C. Robertson) as described previously (46, 47). Rabbit serum or fecal samples (in triplicate) were used as the primary antibodies (in a binary dilution), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and IgA (1:5,000) were used as the secondary antibodies. The OD at 405 nm was measured after 20 min of development in peroxidase substrates (KPL, Gaithersburg, MD). The titration endpoint was determined as the reciprocal of the interpolated dilution giving an OD of >0.4 after subtraction of the background (22, 45, 47). Antibody titers were calculated on a log2 scale.

Anti-LT and anti-STa antibody neutralization. The CT and STa toxins neutralization activity of antibodies in rabbit serum and fecal samples was examined in T84 cells using cyclic AMP (cAMP) and cyclic GMP (cGMP) enzyme immunoassay kits (Assay Design, Ann Arbor, MI) as described previously (47). Briefly, approximately 1 x 10^5 to 2 x 10^5 T84 cells (with greater than 80% confluence) were seeded into each well in Dulbecco's modified Eagle medium and Ham's F12 medium (DMEM/F12; Gibco/Invitrogen, Grand Island, NY). Ten nanograms of CT or 2 ng of STa toxin (diluted in 150 µl of DMEM/F12) was incubated with 150 µl of a rabbit serum or fecal sample (1.5 dilution in DMEM/F12, in triplicate) at room temperature. After 1 h of incubation, the mixture (150 µl of the CT or STa toxins diluted plus 150 µl of the serum or fecal sample dilution) was added to each well, and the plate was further incubated at 37°C in 5% CO2 for 1 h. After a wash, the cells were lysed with 0.1 M HCl (200 µl per well) and then neutralized with 0.1 M NaOH. Cell lysates were collected by centrifugation at 660 x g for 10 min at room temperature. The resultant supernatant was tested for intracellular cAMP or cGMP levels by following the manufacturer's protocols.

FIG. 1. Construction of chimeric faeG genes and detection of chimeric FacG major subunits of E. coli K888ac fimbriae. (A) Construction of the chimeric faeG gene with nucleotides encoding EP3 replaced with a toxin epitope. Internal forward and reverse PCR primers (insert-F and insert-R) were specifically designed to replace EP3 nucleotides from the native faeG gene with nucleotides encoding the LTP1 or the STa13 peptide in a three-step PCR method. The solid black line in each insert primer represents nucleotides complementary to the faeG gene, and the gray line indicates nucleotides of an inserted epitope, including overlapping complementary nucleotides (solid). (B) Constructed single- and double-mutant strains expressing chimeric FacG major subunits with EP3 replaced with LTP1 (strain 8551) or STa13 (strain 8616), EP4 with LTP1 (strain 8549), and EP3 with STa13 and EP4 with LTP1 (strain 8576). (C) Western blot assay for detection of chimeric FacG proteins expressed by the constructed strains 8551, 8549, 8576, and 8616 strains, with strain 8069 as the K888ac FacG-positive control and E. coli TOP10 as the negative control, by hybridoma supernatant of anti-K888ac MAbs 36/41 and 30/17, rabbit anti-CT antisera, and rabbit anti-STa antisera, respectively.
A hundred microliters of a strain 3030-2 bacterial cell suspension was mixed with PBS and incubated at 37°C in 5% CO2 for 1 h. IPEC-J2 cells were washed three times with PBS and dispelled by incubation with 0.25% trypsin (100 μl/106 cells) and resuspended in 1 ml PBS. The resuspension was serially diluted (10^3, 10^4, and 10^5) and spread on LB agar plates, and cultured at 37°C overnight. Colonies were counted, and adherence was calculated as the number of CFU/ml.

**RESULTS**

Four strains were constructed for this study (Table 2). Strain 8551 expressed chimeric K88ac fimbriae with the EP3 epitope at the FaeG major subunit replaced with the LTP1 epitope, and strain 8549 had EP4 replaced with the LTP1 epitope. Strain 8616 produced chimeric K88ac fimbriae that had EP3 replaced with the STa13 toxoid epitope. Strain 8576 was the double mutant that had EP3 replaced with the STa13 toxoid epitope.

**Plasmids**

| Strains or plasmid          | Relevant property(ies) | Source or reference |
|-----------------------------|------------------------|---------------------|
| TOP10                       | F' mcrAΔ(mrr-lhsdRMS_mcrBC) Δ88lacZΔM15 Δ lacX74 recA1 deoR araD139 Δ ara-leu7697 galU galK rpsL (Str') endA1 mupG | Invitrogen          |
| 8851                        | TOP10/pK88ac-LTB@EP3   | This study          |
| 8549                        | TOP10/pK88ac-LTB@EP4   | This study          |
| 8616                        | TOP10/pK88ac-STa@EP3   | This study          |
| 8576                        | TOP10/pK88ac-STa@EP3-LTB@EP4 | This study |
| 8069                        | TOP 10/p8069 (K88ac)   | 44                  |
| 3030-2                      | Porcine ETEC isolate: K88ac LT STb | 12                  |

| Strains or plasmid          | Relevant property(ies) | Source or reference |
|-----------------------------|------------------------|---------------------|
| pBAD88-102                  | K88ac operon on pBAD   | 3                   |
| p8069                       | pBAD88-102 with Spel site downstream of faeG gene | 44                 |
| pK88ac-LTB@EP3              | K88ac with LTB epitope at EP3 of FaeG major subunit | This study |
| pK88ac-LTB@EP4              | K88ac with LTB epitope at EP4 of FaeG major subunit | This study |
| pK88ac-STa@EP3              | K88ac with STa epitope at EP3 of FaeG major subunit | This study |
| pK88ac-STa@EP3-LTB@EP4      | K88ac with STa epitope at EP3 and LTB epitope at EP4 of FaeG major subunit | This study |

| Strains or plasmid          | Relevant property(ies) | Source or reference |
|-----------------------------|------------------------|---------------------|
| Strain or plasmid           | Relevant property(ies) | Source or reference |
| 8069                        | pBAD88-102 with SpeI site downstream of faeG gene | 44                 |
| pK88ac-LTB@EP3              | K88ac with LTB epitope at EP3 of FaeG major subunit | This study |
| pK88ac-LTB@EP4              | K88ac with LTB epitope at EP4 of FaeG major subunit | This study |
| pK88ac-STa@EP3              | K88ac with STa epitope at EP3 of FaeG major subunit | This study |
| pK88ac-STa@EP3-LTB@EP4      | K88ac with STa epitope at EP3 and LTB epitope at EP4 of FaeG major subunit | This study |

The STa13 toxoid epitope. Strain 8576 was the double mutant that had EP3 replaced with the STa13 toxoid epitope. Strain 8576 produced chimeric K88ac fimbriae that had EP3 replaced with the STa13 toxoid epitope. Strain 8576 was the double mutant that had EP3 replaced with the STa13 toxoid epitope and EP4 replaced with the LTP1 epitope (Fig. 1B). Each constructed strain was verified for expression of the chimeric FaeG proteins and chimeric K88ac fimbriae. Verified chimeric fimbriae were harvested and used as antigens for rabbit immunization to determine anti-K88, anti-LT, and anti-STa immunogenicity.

**Chimeric FaeG proteins were expressed in each constructed strain.** Chimeric FaeG major subunits were verified with anti-K88ac Mabs and anti-CT and anti-STa antibodies (Fig. 1C). Proteins with a molecular mass of 27 kDa were detected in total protein extracts of strains 8551, 8549, 8576, and 8616 and of positive-control strain 8069 with anti-K88ac MAb hybridoma supernatant but not the negative-control TOP10 strain. When rabbit anti-CT antiserum was used, proteins of the same size (27 kDa) were detected in the 8551, 8549, and 8557 strains but not detected in strain 8616 or TOP10. Accordingly, chimeric proteins expressed by only the 8576 and 8616 strains were recognized by rabbit anti-STa antiserum.

**Chimeric K88ac fimbriae were biosynthesized and assembled at the surface of each constructed strain.** Expression of chimeric K88ac fimbriae was verified by immunolabeling TEM with anti-K88ac Mabs and anti-CT and anti-STa antisera (Fig. 2). Anti-K88ac Mabs detected fimbriae assembled in the 8551, 8549, 8576, and 8616 cell surface, but no gold particle-labeled fimbriae were detected in the TOP10 control strain. Anti-CT antibodies detected assembled fimbriae from the 8551, 8549, 8576, and 8616 cell surface, whereas anti-STa antibodies detected fimbriae displayed at the 8576 and 8616 cell surface.

**RESULTS**

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**Chimeric K88ac fimbriae showed a significant reduction in binding to K88ac receptor-positive brush borders.** Although chimeric fimbriae displayed at the cell surface were clearly visible under immunolabeled TEM, their K88ac receptor-positive brush border binding activity was significantly reduced. The numbers of bacteria of strains 8551, 8549, 8576, and 8616 bound to each brush border were 3.1 ± 1.4, 0.8 ± 1.4, 0.7 ± 1.1, and 4.4 ± 2.1, respectively. The binding activity of these four constructed strains was significantly lower than that of diarrheagenic ETEC strain 3030-2 (13.5 ± 2.8; P < 0.01).

**Chimeric K88ac fimbriae elicited anti-LT, anti-STa, and anti-K88ac antibodies.** Anti-K88ac, anti-LT, and/or anti-STa antibodies were detected in serum and fecal samples from rabbits immunized i.m. with chimeric K88ac fimbriae (Fig. 3A to C). Anti-K88 IgG antibodies were detected at titers (in log2) of 8.46 ± 0.08, 8.95 ± 0.02, 7.66 ± 0.06, and 10.04 ± 0.07, respectively (Fig. 3A). Anti-K88ac secretory IgA (seIgA) antibodies were detected at titers of 6.23 ± 0.08, 6.07 ± 0.13, 6.03 ± 0.04, and 6.23 ± 0.04 in the feces of rabbits immunized with strain 8551, 8549, 8576, and 8616 chimeric fimbriae. In contrast, no anti-K88ac antibodies were detected in the serum or fecal sample from the control rabbit (1.79 ± 0.79 for IgG and 1.31 ± 0.24 for seIgA).

Anti-LT antibodies were developed in rabbits immunized with chimeric K88ac fimbriae carrying the LT1p1 epitope (Fig. 3B). Serum samples from rabbits immunized with strain 8551, 8549, and 8576 chimeric fimbriae had anti-LT IgG antibodies detected at titers of 7.01 ± 0.18, 7.78 ± 0.10, and 6.9 ± 0.02, whereas anti-LT IgA antibodies were detected at 4.1 ± 0.27, 3.9 ± 0.76, and 4.2 ± 0.56, respectively. That was significantly different from anti-LT IgG (1.5 ± 0.4) or IgA (1.3 ± 0.3) antibody detection in serum samples from the control rabbit (P > 0.01). No anti-LT IgG (1.57 ± 0.24) or IgA (1.6 ± 0.34) antibodies were detected in serum samples from rabbits immunized with strain 8616 chimeric fimbriae. Similarly, anti-LT seIgA antibodies were only detected in fecal samples from rabbits immunized with the chimeric fimbriae of strains 8551 (5.1 ± 0.18), 8549 (5.1 ± 0.10), and 8576 (5.1 ± 0.14).

Anti-STa antibodies were detected in rabbits immunized with chimeric fimbriae expressing the STa13 toxoid epitope (Fig. 3C). Anti-STa IgG antibodies were detected in serum samples from rabbits immunized with strain 8576 (7.51 ± 0.04) and 8616 (7.49 ± 0.12) chimeric fimbriae. Similarly, only serum samples from rabbits immunized with strain 8576 and 8616 chimeric fimbriae had anti-STa IgA antibodies detected, at titers of 5.88 ± 0.06 and 5.9 ± 0.12, respectively. No anti-STa IgG (1.69 ± 0.52, 1.54 ± 0.40, 1.31 ± 0.18) or anti-STa IgA (1.49 ± 0.13, 1.51 ± 0.38, 1.21 ± 0.16) antibodies were detected in serum samples from rabbits immunized with strain 8551 and 8549 chimeric fimbriae and the control rabbit. Anti-STa seIgA antibodies were detected in fecal samples from rabbits immunized with chimeric fimbriae of strains 8576 (6.08 ± 0.19) and 8616 (6.10 ± 0.14).

**Anti-toxin antibodies in rabbit serum and fecal samples neutralized CT and STa toxins.** CT (Sigma) was no longer able to stimulate an increase in the intracellular cAMP level in T84 cells after incubation with the serum or fecal samples from rabbits immunized with chimeric K88ac fimbriae of the 8551, 8549, and 8576 strains. In contrast, the serum and fecal samples from rabbits immunized with strain 8616, the serum sample of the control rabbit, or culture medium did not prevent the same amount of CT (10 ng) from increasing cAMP levels in T84 cells (Fig. 4A). The cAMP concentrations in the T84 cells treated with CT that had been incubated with the serum of rabbits immunized with strain 8551, 8549, and 8576 fimbriae were 0.52 ± 0.01, 0.44 ± 0.03, and 0.45 ± 0.04 pmol/ml, respectively. The cAMP levels in the T84 cells incubated with CT that was pretreated with the fecal resuspension from rabbits immunized with strain 8551, 8549, and 8576 fimbriae were 1.13 ± 0.06, 1.89 ± 0.13, and 2.12 ± 0.13 pmol/ml. These cAMP levels were significantly lower than those in T84 cells incubated with CT pretreated with serum samples from rabbits immunized with strain 8616 fimbriae (10.85 ± 0.20 pmol/me; P > 0.01) and from the control rabbit (11.75 ± 0.35 pmol/me; P > 0.01) or CT treated with fecal samples from rabbits immunized with strain 8616 fimbriae (10.6 ± 0.75 pmol/me; P > 0.01) and from the control rabbit (13.5 ± 1.22 pmol/me; P > 0.01).
Similarly, antibodies in serum and fecal samples from rabbits immunized with K88ac-STa chimeric fimbriae prevented STa toxin (2 ng) from stimulating cGMP levels in T84 cells (Fig. 4B). The intracellular cGMP concentration in T84 cells treated with STa and serum samples from rabbits immunized with strain 8576 and 8616 fimbriae were 0.74 ± 0.04 and 1.05 ± 0.07 pmol/ml, respectively. These cGMP levels were significantly lower than the cGMP levels in cells incubated with STa incubated with serum samples from rabbits immunized with fim-briae from strain 8551 (54 ± 2.83; P < 0.01). Fecal samples from rabbits immunized with strain 8576 and 8616 fimbriae also prevented STa toxin stimulation of cGMP levels, as T84 cells had cGMP levels of 4.2 ± 0.42 and 5.4 ± 0.57 pmol/ml, respectively. These cGMP levels were significantly lower than the levels in T84 cells when fecal samples from rabbits immunized with strain 8551 (31 ± 2.12 pmol/ml; P < 0.01) or with culture medium alone (38 ± 2.83 pmol/ml; P < 0.01) were used to incubate STa toxin.

To test whether anti-K88ac antibodies interfere with the stimulation of intracellular cAMP or cGMP in T84 cells by LT or STa, we used rabbit anti-K88ac serum samples to preincu-bate CT (10 ng) and STa (2 ng) in a separate study. Data from the cAMP ELISA showed similar cAMP levels (P = 0.14) in T84 cells treated with LT alone (10 ± 0.7 pmol/ml) and those treated with LT plus anti-K88ac serum (8.4 ± 0.7 pmol/ml). Similarly, no significant differences in cGMP stimulation were found when STa (26.8 ± 2.5 pmol/ml) or STa plus rabbit anti-K88ac serum (24.3 ± 2.5 pmol/ml) was used to incubate T84 cells (P = 0.42).

**Antibodies inhibit the adherence of K88ac fimbrial strain 3030-2 bacteria to K88ac receptors.** Antibodies in serum and fecal samples from immunized rabbits reduced the adherence of K88ac fimbrial ETEC strain 3030-2 bacteria to K88ac receptor-positive IPEC-J2 cells (Fig. 5). After incubation with serum samples from rabbits immunized with chimeric fimbriae from the 8551, 8549, 8576, and 8616 strains, the numbers (10^3) of strain 3030-2 bacteria bound to IPEC-J2 cells were 101 ± 21, 142 ± 17, 157 ± 17, and 210 ± 14, respectively. These binding levels were significantly lower than that in strain 3030-2 bacteria incubated with the serum sample from the control rabbit (512 ± 18; P < 0.01) or with cultural medium (550 ± 17; P > 0.01). After incubation with fecal samples from rabbits immunized with chimeric fimbriae from the 8551 and

![FIG. 4. Anti-LT and anti-STa antibody neutralization. Serum and fecal samples (1:5) from rabbits immunized with chimeric K88ac fimbriae were used to neutralize 10 ng CT (A) or 2 ng STa (B). The mixture was added to T84 cells to detect stimulation of intracellular cGMP levels (pmol/ml) with a direct cGMP enzyme immunoassay kit (Assay Designs) to assess anti-STa antibody neutralizing activity and of intracellular cAMP levels with a direct cAMP enzyme immunoassay kit (Assay Designs) to measure anti-LT antibody neutralizing activity. Cell culture medium alone and serum samples from the control rabbit were also included. Error bars indicate standard deviations. ***, P < 0.01.](image1)

![FIG. 5. Bacterial adherence inhibition by serum and fecal samples from rabbits immunized with chimeric K88ac fimbriae. Porcine ETEC strain 3030-2 that expresses K88ac fimbriae and porcine cell line IPEC-J2 expressing K88ac receptors were used in adherence inhibition assays. IPEC-J2 cells (1 x 10^5) were seeded into each well, and the multiplicity-of-infection ratio was preset at 5 bacteria per IPEC-J2 cell. After incubation with rabbit serum or fecal samples (1:5) at room temperature for 1 h, strain 3030-2 bacteria were added to each well containing IPEC-J2 cells and cultured at 37°C in 5% CO2 for 1 h. Cells were dislodged, serially diluted in PBS, plated on LB agar plates, and incubated at 37°C overnight. Colonies were counted and calculated as CFU/ml (in thousands). Error bars indicate standard deviations. ***, P < 0.01.](image2)
8616 strains, strain 3030-2 showed a significantly lower level of binding to IPEC-J2 cells (240 ± 15 and 230 ± 17, respectively; P > 0.01). However, when incubated with fecal samples from rabbits immunized with fimbriae from the 8549 and 8576 strains, strain 3030-2 bacteria showed only a slight reduction in binding to IPEC-J2 cells (470 ± 18 and 460 ± 15, respectively), which was not significantly different from the binding to IPEC-J2 cells by strain 3030-2 bacteria treated with fecal sample of the control rabbit (490 ± 12; P = 0.38, P = 0.21) or with the cultural medium (530 ± 14; P = 0.09, P = 0.06).

DISCUSSION

ETEC adhesins that mediate bacterial attachment to host epithelial cells and LT and ST enterotoxins that induce fluid and electrolyte hypersecretion in the host small intestine play key roles in ETEC-associated diarrhea. Therefore, control or prevention of ETEC infections has largely been based on two strategies: blocking bacterial adherence to host receptors and eliminating enterotoxin activity in host epithelial cells (29, 39). Experimental whole-cell vaccines derived from E. coli strains expressing an adhesin and a toxin antigen, or subunit vaccine candidates combining an adhesin antigen and a toxin antigen or carrying a toxin-toxin fusion protein, could stimulate both anti-adhesin and anti-toxin immunity in hosts. Human volunteers, after administration of a mixture of purified E. coli surface antigen CS6 and LT toxoid LT_{192} proteins, developed anti-CS6 and anti-LT immunity (22). Similarly, after being immunized with a modified Shigella flexneri 2a vaccine strain expressing ETEC CFA/I structural subunits and an LT_{b} subunit, guinea pigs developed anti-CFA/I and anti-LT antibodies that showed the abilities to neutralize toxin and inhibit cell agglutination (30). Even when a native STa peptide was integrated into the ClpG major subunit of E. coli CS31A fimbriae, neutralizing anti-STa antibodies and anti-ClpG antibodies were developed in immunized rabbits and mice (4, 5). Those studies indicated that anti-adhesin and anti-LT or anti-STa immunity can be induced in hosts by the coadministration of purified adhesin antigens and toxin antigens or administration of E. coli cells expressing adhesin and toxin antigens separately. Data from this study demonstrated that administration of chimeric adhesins that had LT and STa epitopes embedded induced not only anti-adhesin but also both anti-LT and anti-STa anti-toxin immunity.

In this study, we genetically integrated the LTP1 epitope and the STa_{13} toxoid epitope into the FaeG major subunit of E. coli K88ac fimbriae for the first time to explore a fimbrial platform vaccine that stimulates both anti-LT and anti-STa anti-toxin immunity and anti-adhesin immunity. As ETEC strains expressing either LT toxin or STa toxin can cause diarrhea (16, 28, 31, 35, 42, 46), inducing both anti-LT and anti-STa immunity is essential for effective protection against ETEC infection. By expressing both LT and STa epitopes at the same major subunit of K88ac fimbriae, we produced a single antigen able to induce anti-K88ac fimbrial immunity, anti-LT immunity, and anti-STa immunity. After being immunized with the chimeric fimbriae from strain 8576 (K88ac-LT_{b} epitope-STA_{13} epitope), rabbits developed antibodies that reduced K88ac fimbrial adherence and neutralized CT and STa toxin stimulation of cAMP and cGMP in T84 cells. Knowing that induced antibodies neutralizing toxins in vitro may not necessarily prevent delivery of toxin by living bacterial, we need to conduct animal challenge studies to assess induced anti-adhesin and anti-toxin immunity for in vivo protection against ETEC diarrhea. Also, further studies are needed to determine the minimum dose of induced antibodies required for toxin neutralization or adherence inhibition. Nevertheless, this study clearly demonstrated that anti-adhesin and anti-toxin (both anti-LT and anti-STa) immunity was induced and suggests the future application of a fimbrial platform in ETEC vaccine development.

The LTP1 epitope was selected in chimeric K88ac fimbria construction because a homologous nontoxic CTP1 epitope elicited anti-CT antibodies when it was integrated into bacterial flagella (10). Likewise, the selection of the STA_{13} epitope was based on the fact that the detoxified STa_{13} protein showed anti-STa immunogenicity when it was fused to a carrier protein, LT_{192} (47). Insertion of LTP1 either at EP3 (strain 8551) or at EP4 (strain 8549) of FaeG of K88ac apparently did not affect chimeric fimbrial antigen elicitation of anti-LT antigenicity, as rabbits immunized with chimeric fimbrial antigens from strains 8551 and 8549 developed nearly identical titers of anti-LT IgG and IgA antibodies. Furthermore, chimeric fimbriae of the LTP1 single mutants (strain 8551 and 8549) and the LTP1/STA_{13} double mutant (strain 8576) elicited equivalent titers of anti-LT antibodies. Similarly, the same titers of anti-STa antibodies were elicited by chimeric fimbriae expressing the STA_{13} epitope alone at EP3 (strain 8576) or with LTP1 at EP4 (strain 8616). These data indicate that the additional expression of the STA_{13} epitope at EP3 did not negatively affect the immunogenicity of the inserted LTP1 epitope, suggesting that this K88ac fimbria could serve as a platform to express multiple foreign epitopes.

E. coli strains expressing chimeric K88ac fimbriae that had EP3 and EP4 of the FaeG subunit replaced with the LTP1 and STA13 epitopes can be used in future live attenuated vaccine development if expressed chimeric K88ac fimbriae maintain host receptor binding activity. However, expression of multiple foreign epitopes could alter the K88ac fimbrial structure or/and biological activity, thus limiting its application in live vaccine strain development. Our previous study indicated that removal of EP3 or EP4 did not affect K88ac fimbria in binding to K88ac receptor-positive porcine brush borders (data not shown). It has been reported that the chimeric flagella of Salmonella cells maintained mobility when CTP1 was expressed by the flagella (10), and K88ab fimbriae kept their binding function after their native epitopes were replaced with epitopes from the VP1 coat protein of the foot-and-mouth disease virus or a peptide from an envelope glycoprotein of human Immunodeficiency virus type 1 (3, 27). After replacing EP3 and/or EP4 with LTP1 and/or STA13 peptides, we found, however, that the resultant chimeric K88ac fimbriae showed a significant reduction in binding activity compared to the native K88ac fimbriae in strain 3030-2. A reduction in binding to K88ac receptors located on small intestinal epithelial cells likely could significantly decrease the stimulation of host local mucosal immunity by these chimeric fimbriae. Although their application in subunit vaccines, at least through the i.m. route, will not be affected, these K88ac-toxin chimeric fimbriae may not be suitable for use as oral vaccine antigens. In addition,
epitope replacement could also influence fimbrial structure or stability, as it was shown in Fig. 1C that four chimeric fimbriae had differences in interaction with anti-K88ac MAbs, although these differences could also be caused by the specificity of the MAbs used in this study.

It has been noticed that anti-K88ac antibodies in serum samples from the immunized rabbits showed a similar inhibition of the adherence of strain 3030-2 bacterium to IPEC-J2 cells but antibodies in fecal samples from the immunized rabbits exhibited a significant difference in adherence inhibition. Fecal samples from rabbits immunized with chimeric K88ac fimbriae having EP3 replaced with the LTP1 or STA13 epitope showed significant adherence inhibition, but antibodies in fecal samples from rabbits immunized with chimeric K88ac with EP4 substituted showed little adherence-inhibiting activity (Fig. 5). It is unclear what caused this variation in adherence inhibition, but we speculate that the replacement of EP4 likely altered the chimeric K88ac fimbrial structure. Future studies to construct alternative chimeric K88ac fimbriae by replacing EP4 with the STA13 toxoid peptide or a different LT B epitope and to evaluate the elicited antibodies in fecal samples in adherence inhibition will help us to construct a double mutant that elicits antibodies for better adherence inhibition.

Expression of two or more toxin epitopes in K88ac fimbriae could lead to the application of K88ac fimbriae as a platform for developing effective multivalent vaccines against porcine ETEC diarrhea. All of the ETEC strains associated with porcine diarrhea express LT and STa or STb toxins, and nearly 70% of these strains express K88ac fimbriae (48). In addition, K88ac fimbriae, like other bacterial flagella or fimbriae, are excellent mucosal adjuvants and protein carriers (3, 4, 6, 8–10, 32). Thus, K88ac fimbriae can be used as protein carriers to enhance the immunogenicity of otherwise non- or poorly immunogenic epitope antigens and to induce host mucosal immunity. K88ac fimbriae, like other bacterial adhesins, may also serve as platforms for developing epitope vaccines against other enteric diseases.

ACKNOWLEDGMENTS

We thank Eric Nelson and Craig Welbon for their assistance in rabbit immunization, David Francis for providing anti-K88ac MAb hybridoma supernatant, Don Robertson for STa conjugates and anti-STa antisera, and Robert Jans for assistance with TEM.

Financial support for this study was provided by NIH AI068766 (W. Zhang), the Center for Infectious Disease Research and Vaccinology, and the South Dakota Agricultural Experiment Station.

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