Genetic Fusions of Heat-Labile Toxoid (LT) and Heat-Stable Toxin b (STb) of Porcine Enterotoxigenic Escherichia coli Elicit Protective Anti-LT and Anti-STb Antibodies

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Received 11 March 2010/Returned for modification 31 March 2010/Accepted 14 May 2010

Enterotoxigenic Escherichia coli (ETEC) strains that produce heat-labile (LT) and heat-stable (ST) enterotoxins are a major cause of diarrheal disease (27, 32). Bacterial adhesins and enterotoxins are the virulence determinants in ETEC-associated diarrhea (1, 4, 19, 20, 26, 33, 34). Porcine ETEC-associated diarrhea, especially postweaning diarrhea (PWD), causes substantial economic loss to swine producers worldwide (15, 28). Currently, there are no effective vaccines available to protect young pigs against PWD. Antitoxin vaccines are not effective because they are strongly immunogenic. In this study, we used an LT toxoid (LT192 [hereafter, LT192]) isolated from porcine ETEC strains to carry a mature STb peptide for LT192-STb fusions to enhance STb immunogenicity for potential vaccine application. Anti-LT and anti-STb antibodies were detected in immunized rabbits and pigs. In addition, when challenged with an STb-positive ETEC strain, all 10 suckling piglets borne by immunized gilts remained healthy, whereas 7 out of 9 pigs born by immunized gilts developed diarrhea. This study indicates that the LT192-STb fusion enhanced anti-STb immunogenicity and suggests the LT192-STb fusion antigen can be used in future vaccine development against porcine ETEC diarrhea.

MATERIALS AND METHODS

Bacterial strains and plasmids. Two E. coli strains, a nonpathogenic porcine E. coli field isolate 1836-2 (34) and TOP10 (Invitrogen, Carlsbad, CA), were used as host strains in this study. 1836-2, which naturally expresses K88ac fimbriae, was used to construct challenge strains and experimental live attenuated vaccine strains, whereas the TOP10 strain was used for fusion protein expression and purification. Vector pBR322 was used to clone and express LT192-STb fusion proteins, and TOPO TA cloning vector (Invitrogen) was used for cloning of LT192-STb and expression of 6xHis-tagged fusion protein. Strain 8017 (1836-2/pBR322) (34) was used as the negative control. A porcine ETEC field isolate, 3030-2 (11), which expressing K88ac fimbriae and LT and STb enterotoxins, was used to isolate the LT and STb genes and as a positive control. A high-copy vector, pUC19, was used to clone the HindIII fragment of plasmid PRAS1 (5), which carries the xstA gene for LT toxoid expression. All strains were cultured on agar plates or in LB broth at 37°C with 50 μg/ml ampicillin (Table 1).

Mutation of LT genes (eltAB) for toxoid LT192. The porcine-type LT genes were mutated to express LT192 protein that served as a carrier protein to facilitate STb immunogenicity and an antigen to stimulate anti-LT immunity. The eltAB genes of the E. coli strain 8017 were cloned into vector pBR322 at the NheI and Eagl sites and mutated at the nucleotides coding for the 192nd amino acid for toxoid LT192. This mutation was carried out by using two internal, self-complementary PCR primers: LT192-F, 5'-GATTCGAGGACATACACAGGCGGGA-3' (the change from AGA to GGA is underlined), and LT192-R, 5'-CCCTGTTGCATTGCCTGATGAGAC-3' (the change from TCT to GCC is underlined). Briefly, we used primers pBR1900F (5'-CCACCTTACGCGTCTAAACGAAC-3') and LT192-R in one PCR and primers LT192-F and pBR1900R (5'-CGGAAAGCCGGAAAGAATCATA-3') in a second PCR, with development against bacterial and viral pathogens. In this study, we used an analogous detoxified LT protein, designated LT192 as the carrier to enhance STb immunogenicity. This LT192 protein was produced by mutating the porcine-type LT genes (eltAB) isolated from a porcine E. coli strain. We fused the xstB gene coding for the mature STb peptide to the mutated, full-length porcine-type LT192 genes and examined LT192-STb fusion proteins in enhancement of STb immunogenicity and potential vaccine application against porcine diarrhea.
recombinant LT plasmid (pLT) as a DNA template, to amplify the upstream and downstream sequences, at the mutation site, of the eltAB genes. We then used a splice overlapping extension (SOE) PCR to fuse two amplified fragments and mutated the eltAB genes coding for protein LT192.

Construction and cloning of LT192-STb fusion genes. The eltAB gene that codes a mature STb toxin was fused at the 3' end of the full-length LT192 genes in this study. To generate such a fusion, we designed two internal PCR primers, STb:LT-F (5'-GCAATACGTTAGGGCGGATCCCTTGCTACAAAATAAAAAGAT-3') and STb:LT-R4 (5'-TTGTGGTAGGGGGCGGCCGCTATGTCGCGCAATGATGG-3'), and conducted a three-step PCR as described previously (37). This STb:LT-F forward primer contains the 3'-end nucleotides (in italic) of the eltAB genes (without the stop codon), a Gly-Pro linker, and 9 nucleotides of the eltAB gene (5'-end nucleotides). The STb:LT-R4 reverse primer contains the 5'-end of the eltAB gene (in italic), the linker (underlined), and the 3'-end of the eltAB genes (without the stop codon). For the first PCR, using pRECorki-F and LT:STb-R4 primers generated a fragment containing the mutated eltAB genes, the Gly-Pro linker, and 9 nucleotides of the eltAB gene (5'-end). A second PCR using the STb:LT-F and STb:EagI-R (5'-GCTGAAATGCTAATCCGCGGGCCATACAGC-3'; with an EagI site underlined) primers generated the fragment that consists of the 3'-end of the eltAB genes, the linker, and the eltAB gene. A third SOE PCR performed on the two amplified products and produced LT192-Gly:Pro-STb fusion genes.

We constructed additional fusion genes by using a longer linker, the L linker (5'-CGAGCTCTGATCCCGGGGATCC-3') (8), in an attempt to increase STb flexibility for greater STb immunogenicity. We substituted for the Gly-Pro linker (4 amino acids) with this L linker by using the following two internal PCR primers and the method described above to construct LT192-L-linker-STb fusion genes: LT192-STb-R3 (5'-CGAAGCTCTGATCCCGGGGATCCGTTTTCCATACGTGATCCGGAGAAT-3'; where the underlined nucleotides represent the L linker, and the italic nucleotides represent the 3'-end of the eltAB genes) and STb:LTF (5'-GATCCGGGAGAACAGCGGAGCTCAGGACATTACAAAGAAATAAAGAAG-3'; where the underlined nucleotides represent the L linker, and the italic nucleotides represent the 3'-end of the eltAB gene).

All PCRs were performed with a PTC-100 thermal cycler (Bio-Rad, Hercules, CA) in a 50-μl reaction mixture containing 1× Pfu DNA polymerase buffer (with Mg2+), 200 nM deoxyguanosine triphosphates (dNTP), 0.5 μM each forward and reverse primers, and 1 U Pfu DNA polymerase (Strategene, La Jolla, CA). The SOE PCR was performed in a reaction mixture of 1× Pfu DNA polymerase buffer (with Mg2+), 200 nM dNTP, 20 μl of each purified PCR product, 1 U Pfu polymerase, and 0.5 U Tag DNA polymerase (Applied Biosystem, Foster City, CA). Final PCR products (inserts) and vector pBR322 were digested with NheI and EagI restriction enzymes (New England Biolabs, Ipswich, MA) and ligated with T4 DNA ligase (Invitrogen) under standard conditions (2). Ligated products were introduced into 1836-2 and TOP10 competent cells with standard electroporation (2). Positive colonies selected by ampicillin antibiotics were screened by PCR initially and then sequenced to ensure that the cloned fusion genes were inserted in the correct reading frame.

**Detection of expressed LT192-STb fusion proteins.** Expressed LT192-Gly:Pro-STb fusion proteins were examined with a GM1 enzyme-linked immunosorbent assay (ELISA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Constructed strains were cultured in Casamino Acids and yeast extract broth with lincomycin (45 μg/ml) and ampicillin (50 μg/ml) at 37°C overnight. TOP10 strains that express 6×His-tagged fusion proteins were cultured with addition of 0.2% L-cysteine (inducer). The overnight-grown culture with equivalent amount of cells (based on optical density [OD] readings) was centrifuged at 3,000 × g for 20 min. Culture supernatant was collected, and culture pellets were further used for total protein preparation using bacterial protein extraction reagent (B-PER; in phosphate buffer [Pierce, Rockford, IL]). Thirty microliters of total protein extracts (50 to 100 μg) was used in a standard 10% SDS-PAGE to detect LT192-Gly:Pro-STb fusion proteins. Transferred nitrocellulose membrane was blocked with 2% fat-free milk overnight at 4°C and then incubated with rabbit anti-cholera toxin (anti-CT) serum (1:3,000 dilution; Sigma, St. Louis, MO) and rabbit anti-STb serum (1:3,000 dilution; a gift from D. Dubreuil, University of Montreal, Montreal, Quebec, Canada), respectively. After three washes, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; Sigma) at a dilution of 1:5,000 for 1 h. After a final round of washes, peroxidase bound to the fusion proteins on the membranes was detected with a SuperSignal West Pico chemiluminescent substrate kit (Pierce).

**Extracts from an overnight culture growth of the recombinant LT192 and LT192-Gly:Pro-STb strains were examined for LT proteins in a GM1 ELISA as described previously (34, 35). Briefly, each well of an ELISA plate was coated with 400 ng GM1 (Sigma) at 4°C overnight. The coated plate was blocked with 2.5% casein blocking buffer, washed, and incubated with total protein extracts and supernatant samples. Rabbit anti-CT serum (1:5,000 dilution; Sigma) and HRP-conjugated goat anti-rabbit IgG (1:5,000 dilution; Sigma) were used as the primary and the secondary antibodies. Optical density (OD) was measured at a wavelength of 405 nm after 20 min of incubation in peroxidase substrate (KPL, Gaithersburg, MA).**

**Measuring toxicity of LT192 and LT192-Gly:Pro-STb proteins.** Toxicity of the LT192-Gly:Pro-STb fusion protein was measured in a porcine ligated-loop assay as described previously (34). Briefly, 15 to 20 loops were prepared from the ileum

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**TABLE 1. Escherichia coli strains and plasmids used in this study**

| Strain or plasmid | Relevant properties | Source or reference |
|-------------------|---------------------|---------------------|
| **Strains**       |                     |                     |
| TOP10             | F _mcrA_(mcr-hsdRMS-mcrBC) Δ80lacZ2ΔM15 ΔlacX74 recA1deoR ardA3Δ(ara-ldu)7697 galU galk rpsL (Str') endAI napG | Invitrogen |
| 8527              | TOP10 pLT192-Gly:Pro-STb, 6×His-tagged LT192-Gly:Pro-STb | This study |
| 8542              | TOP10 pLT192-L-linker-STb; 6×His-tagged LT192-L-linker-STb | This study |
| 1836-2            | Porcine _E. coli_ field isolate; K88ac, astA gene | 34 |
| 8523              | 1836-2pBR322; negative control | 34 |
| 8035              | 1836-2pLT; K88ac LT | 34 |
| 8221              | 1836-2pLT192STb; K88ac LT192 | This study |
| 8145              | 1836-2pLT-STb; K88ac LT-Gly:Pro-STb | This study |
| 8488              | 1836-2pLT192STb; K88ac LT192-Gly:Pro-STb | This study |
| 8816              | 1836-2pSTb; K88ac STb | This study |
| 3030-2            | Porcine _ETEC_ isolate; K88ac LT STb | 11 |
| **Plasmids**      |                     |                     |
| pLT               | eltAB gene in pBR322 (NheI/EagI) | This study |
| pLT192            | LT192 in pBR322 (NheI/EagI) | This study |
| pLT192-STb        | LT192-Gly:Pro-STb in pBR322 (NheI/EagI) | This study |
| pLT192-Gly:Pro-STb| LT192-Gly:Pro-STb in pBAD-TOPO, TA clone | This study |
| pLT192-L-linker-STb| LT192-L-linker-STb in pBAD-TOPO, TA clone | This study |
| pLSTb              | LT192-Gly:Pro-STb in pBR322 (NheI/EagI) | This study |
| pRASt             | STb | 5 |
| pSTb              | HindIII fragment of pRASt in pUC19 (HindIII) | This study |

* A nonpathogenic porcine _E. coli_ field isolate, 1836-2, and commercial _E. coli_ TOP10 (Invitrogen) were used as parent strains to express LT, LT192, STb, LT-STb, LT192-STb, and 6×His-tagged LT192-STb proteins.
and jejunum section of a 5- or 7-day-old pig, and each loop was inoculated with 2 × 10^9 CFU of overnight-grown cultures of the LT192 strain, the LT192Gly:Pro-STb strain, the positive control strain 3030-2 (LT7 STb), the STb-positive strain 8186, and the negative control strain 8017 accordingly (in triplicates). At the end of the 8-h postinoculation period, the piglet was euthanized, and each ligated loop was removed and measured for the length (cm) and accumulated fluid (mL). The ratio of fluid accumulated versus the loop length (g/cm) was used as an index of toxic activity.

Toxicity of the LT192 and LT192-Gly:Pro-STb proteins was also examined in animal challenge studies. A group of 5-day-old K88ac receptor-positive gnotobiotic piglets were orally inoculated with 3 × 10^9 CFU of the LT192 strain, LT192-Gly:Pro-STb strain, the positive control, or the negative control strain. During the 24-h postinoculation period, piglets were closely monitored for signs of clinical disease, including vomiting, diarrhea, dehydration, lateral recumbency, and lethargy. All animal studies complied with the Animal Welfare Act, followed the Guide for the Care and Use of Laboratory Animals (20a), and were supervised by South Dakota State University's Institutional Animal Care and Use Committee.

**Purification of 6×His-tagged LT192-STb fusion proteins.** LT192-Gly:Pro-STb and LT192-L-linker-STb fusion proteins were extracted initially according to the method of Grange et al. (14), but a very low yield of the fusion proteins was recovered. We constructed the LT192-STb fusion genes as a single open reading frame (ORF) by removing nucleotides coding the transmembrane signal peptide of the LTA gene, the STb gene, and the STb gene and all nucleotides coding the stop codon of the LTA, LTb, and STb genes. This LT192-STb ORF was cloned into the TOPO TA clone vector pBAD TOPO (Invitrogen) and expressed the LT192-STb as a single 6×His-tagged fusion protein in TOP10 cells. The 6×His-tagged fusion proteins were expressed and purified to an purity of greater than 90% using batch purification of 6×His-tagged proteins from E. coli under native conditions (Qiagen, Valencia, CA). Purified 6×His-tagged fusion proteins (5 μg) were examined with anti-LT and anti-STb antiserum by SDS-PAGE.

**Rabbit immunization with purified 6×His-tagged LT192Gly:Pro-STb fusion proteins.** Two adult rabbits per group were immunized intramuscularly (i.m.) with 100 μg purified 6×His-tagged LT192-Gly:Pro-STb or LT192-L-linker-STb fusion protein in an equal volume of Freund’s incomplete adjuvant (Sigma). Two booster injections of the same dose were followed at biweekly intervals. One rabbit without immunization served as the negative control. Blood and fecal samples were collected before and 14 days after each injection and were stored at ~80°C until use.

**Immunization of pregnant gilts with purified 6×His-tagged LT192-Gly:Pro-STb fusion proteins.** Three pregnant gilts from an isolated hog farm that had no history of ET EC or diarrhea outbreak were used in this study. Two gilts were immunized with 0.5 μg purified 6×His-tagged LT192-Gly:Pro-STb fusion protein in an equal volume of Freund’s complete adjuvant (Sigma). Two booster injections of the same dose were followed at biweekly intervals. One rabbit without immunization served as the negative control. Blood and fecal samples were collected before and 14 days after each injection and were stored at ~80°C until use.

**Anti-LT and anti-STb antibody titration in GM1 and STb ELISA.** GM1 ELISA with CT (Sigma; 200 ng/ml) as the antigen was used to titrate anti-LT antibodies. Anti-STb antibody titers were measured in STb ELISA using recombinant maltose binding protein (MBP)-conjugated STb (a gift from D. Dubreuil; 200 ng/ml) as antigens. Rabbit serum and fecal samples (1:50 dilution), gill colostrum samples (1:10 dilution), and piglet serum samples (1:50 dilution) (in triplicates) were used as the primary antibodies in a binary dilution, and HRP-conjugated goat anti-rabbit or goat anti-porcine (Thermo Fisher Scientific, Rockford, IL) IgG or IgA was used as the secondary antibody. The OD was measured at 405 nm after 20 min of development in peroxidase substrates (KPL). The titration end point was determined as the reciprocal of the interpo- lation dilution giving an OD unit above 0.4 (17, 37). Antibody titers were presented as means and standard deviations at a log_2 scale.

**Anti-LT antibody neutralization.** Anti-LT antibodies in rabbit serum and fecal samples for neutralizing CT were examined using a cyclic AMP (cAMP) enzyme immunoassay (EIA) kit (Correlate EIA; Design, NJ) and T84 cells (ATCC catalog no. CCL-248) as described previously (37). Briefly, each well was seeded with approximately 1 × 10^4 to 2 × 10^5 T84 cells (with more than 80% conflu- ence) in Dulbecco’s modified Eagle’s medium plus Ham’s F-12 medium (DMEM/F12; Gibco/Invitrogen, Grand Island, NY). Ten nanograms of CT (diluted in 150 μl DMEM/F-12 medium) was incubated with 150 μl rabbit serum or fecal sample (1:5 dilution in DMEM/F-12 medium) at room temperature for 1 h, and the mixture was added to each well for incubation at 37°C in 5% CO2 for 2 h. After washes, the cells were lysed with 0.1 M HCl and neutralized with 0.1 M NaOH. Cell lysates were collected with a centrifugation at 660 × g for 10 min at room temperature. Lysate supernatants were used for a cAMP EIA kit by following the manufacturer’s protocol.

We used CT to titrate anti-LT antibodies and test anti-LT antibody neutral- ization in this study. Although CT and LT have antigenic differences, they are highly homologous in structure and function, and the commercially available CT is commonly used in GM1 ELISA to detect LT proteins or to titrate anti-LT antibodies.

**Anti-STb antibodies in protection against infection from an STb-positive ETEC strain.** Ten 3-day-old, K88ac receptor-positive suckling piglets borne by 2 gilts immunized with the 6×His-tagged LT192-Gly:Pro-STb fusion and 9 K88ac receptor-positive piglets borne by the control gilt were taken away from their mothers momentarily, orally inoculated with 2 × 10^9 CFU overnight culture growth of an STb challenge strain, and brought back to their mothers. Piglets were observed every 4 h for the following 72 h. At the end of 72 h, all piglets were necropsied, and their blood and small intestinal samples were collected. Anti- STb antibodies in serum samples from each gilt were examined by STb ELISA described above and were examined for association with clinical outcomes of challenged piglets.

**Statistical analysis.** All piglets were examined with a bacterial adherence assay for brush border binding to K88ac fimbrae as described previously (34). Data only from the challenged piglets whose brush borders bound by K88ac fimbrae E. coli cells were included in final analyses. Data were analyzed by using the mixed procedure involving SAS for Windows, version 8 (SAS Institute, Cary, NC), and Student’s t test. Different treatment groups were compared; the results are expressed as means ± standard deviations. Calculated P values of <0.05 were regarded as significant when treatments were compared at a two-tailed distribution and with two-sample equal or unequal variance.

**RESULTS**

Six E. coli strains were constructed to express LT192, LT-STb, and LT192-STb proteins or STb toxin in this study (Table 1). After verification of their expression and reduction in toxicity, purified 6×His-tagged LT192-STb fusion antigens were used to immunize adult rabbits. Rabbit serum and fecal samples were examined for anti-LT and anti-STb antibodies and antibody neutralizing activities. In addition, purified 6×His-tagged LT192Gly:Pro-STb fusion protein was used to immu- nize pregnant gilts for examining anti-STb antibodies in protection against infection from an STb-positive E. coli strain.

**LT proteins were detected in the LT192 and LT192-STb strains.** Expressed LT192 and LT192-STb proteins were examined by GM1 ELISA and SDS-PAGE immunoblot assay. Data from GM1 ELISA detected LT proteins bound to GM1 in the total protein extracts of the wild-type strain 3030-2, the LT recombinant strain 8035, and the LT mutant strain 8221 (LT192). Detection of proteins bound to GM1 from the LT-Gly:Pro-STb strain 8145 and LT192-Gly:Pro-STb strain 8488 was much reduced and was not significantly different (P > 0.05) from that of the negative control strain 8017 (Fig. 1). GM1 ELISA data also showed that LT192 and LT192-Gly:ProSTb bound GM1 equivalently as the LT and LT-Gly:Pro-STb did (P = 0.19 and P = 0.33). SDS-PAGE detected the LT subunit (27 kDa) and LT STb (18 kDa) proteins in both strains 8145 and 8488. Furthermore, expression of the 6×His- tagged LT192-Gly:Pro-STb fusions (28-kDa LT + plus 12-kDa LT STb plus 5-kDa STb plus the linker and the tag) was verified by Western blotting using rabbit anti-CT (Fig. 2A) and rabbit anti-STb antisera (Fig. 2B).

**Toxicity in LT192 and LT192-STb proteins was significantly reduced.** Toxicity reduction in LT192 and LT192-STb proteins...
was suggested in the porcine ligated-loop assay. Although it showed certain variations among replicates, the ligated-loop assay indicated that loops inoculated with strains expressing LT192 or LT192-Gly:Pro-STb had less fluid accumulated and could suggest that toxicity from LT192 or LT192-Gly:Pro-STb was reduced. Fluid accumulation in the loops inoculated with the LT192 and LT192-Gly:Pro-STb strains was measured at 0.079\pm 0.06 and 0.089\pm 0.05 g/cm, respectively, which was not significantly different from that in loops challenged with the negative control strain (0.035\pm 0.03 g/cm; \(P = 0.16\) and \(P = 0.053\)). Fluid accumulation levels in the loops incubated with strain 3030-2 and STb strain 8816 were 0.143\pm 0.08 and 0.115\pm 0.054 g/cm, respectively (Fig. 3).

Toxicity reduction in LT192 and LT192-STb proteins was affirmed in gnotobiotic piglet challenge studies. A total of 24 gnotobiote piglets were inoculated with strains expressing LT192 and LT192-Gly:Pro-STb proteins. Among them, 8 piglets were inoculated with the LT192 strain 8221, 6 piglets with the LT192-Gly:Pro-STb strain 8488, and 5 piglets each with the positive strain 3030-2 and the negative strain 8017. Postmortem phenotyping (brush border adherence assay) showed brush borders of only 14 piglets bound to K88 fimbrial ETEC: 5 in the groups inoculated with the LT192, 2 in the groups with LT192-Gly:Pro-STb, and 3 and 4 in the positive and the negative groups, respectively. During 24 h postinoculation, no piglets developed diarrhea or dehydration in groups challenged with LT192, LT192-Gly:Pro-STb, or the negative control strain. In contrast, all 3 K88-susceptible piglets challenged with positive strain 3030-2 developed diarrhea and showed signs of dehydration.

**STb immunogenicity was enhanced in 6\times His-tagged LT192-STb fusions.** Anti-STb and anti-LT antibodies were detected in serum samples from the rabbits immunized with 6\times His-tagged LT192-Gly:Pro-STb and 6\times His-tagged LT192-L-linker–STb fusions. Serum samples from the rabbits immunized with the 6\times His-tagged LT192-Gly:Pro-STb antigen were titrated at 2.94\pm 0.03 for anti-LT IgG, 3.45\pm 0.06 for anti-STb IgG, and 1.53\pm 0.74 for anti-STb IgA. Serum samples from the rabbits immunized with the 6\times His-tagged LT192–L-linker–STb fusion had log10 titers of 3.25\pm 0.03 in anti-LT IgG, 3.73\pm 0.08 in anti-STb IgG, and 2.02\pm 0.39 in anti-STb IgA antibodies (Fig. 4). Rabbit antibody titration data indicated that the 6\times His-tagged LT192–L-linker–STb fusion elicited greater titers of anti-LT IgG (\(P = 0.01\)) and anti-STb IgG (\(P = 0.04\)) antibodies than the 6\times His-tagged LT192-Gly:Pro-STb antigen did but not of the anti-STb IgA (\(P = 0.50\)). No anti-LT and anti-STb antibodies were detected in the serum sample from the control rabbit.

**FIG. 1.** GM1-ELISA to measure binding of LT192 and LT192-Gly:Pro-STb proteins to GM1. Total proteins extracted from equivalent amounts of cells (determined by OD readings) of each strain (3030-2 [K88ac LT STb], 8017 [1836-2 pBR322], 8035 [1836-2 LT], 8221 [1836-2 LT192], 8145 [1836-2 LT-Gly:Pro-STb], and 8488 [1836-2 LT192-Gly:Pro-STb]) were tested in GM1 binding using anti-CT as the primary antibody (1:5,000) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000) as the secondary antibody. Optical densities, measured at 405 nm, showed significant differences for 3030-2, 8035, and 8221 strains (\(P < 0.01\)). Mean values are shown, and error bars represent standard deviations.

**FIG. 2.** Detection of the 6\times His-tagged LT192-Gly:Pro-STb fusion protein in the Western blot assay. Purified 6\times His tagged proteins of 8527 and total protein extracts of 8017 (–) were separated on 10% SDS-PAGE gel. Rabbit anti-CT (1:3,000) and anti-STb (1:3,000; a gift from D. Dubreuil) sera were used as the primary antibodies, and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG; 1:5,000) was used as the secondary antibody. Proteins bound on the membrane were detected using a SuperSignal West Pico chemiluminescent substrate kit (Pierce). (A) Fusion protein (LT192-Gly:Pro-STb) expressed in the 8488 construct was detected in the 8488 construct was detected using anti-CT antibody. (B) The fusion protein from the same construct was detected by anti-STb antiserum. Molecular mass markers are indicated on the left.
Antibodies in serum and fecal samples of rabbit immunized with the 6×His-tagged LT<sub>192</sub>-STb fusion neutralized CT. Data from the cAMP EIA showed that antibodies in serum and fecal samples of the immunized rabbits neutralized CT and prevented CT from stimulating intracellular cyclic AMP levels in T84 cells. Intracellular cAMP concentrations in the T84 cells incubated with 10 ng CT mixed with the serum samples from the rabbits immunized with 6×His-tagged LT<sub>192</sub>-Gly:Pro-STb and the 6×His-tagged LT<sub>192</sub>-L-linker–STb fusions, CT did not increase cAMP levels in the T84 cells incubated with CT mixed with the serum and fecal sample of the control rabbit were 11.5 ± 0.25 and 12.4 ± 0.49 pmol/ml, respectively (Fig. 5).

Suckling piglets borne by gilts immunized with the 6×His-tagged LT<sub>192</sub>-Gly:Pro-STb fusion remained healthy after being challenged with an STb-positive ETEC strain. Gilts developed anti-STb and anti-LT antibodies after being immunized with the 6×His-tagged LT<sub>192</sub>-Gly:Pro-STb fusion antigen. Anti-STb antibodies in the colostrum samples of two immunized gilts were detected at 1.60 ± 0.01 (IgG) and 0.94 ± 0.03 (IgA), respectively. Anti-LT antibodies were also detected in the same colostrum samples: 1.32 ± 0.05 for anti-LT IgG and 0.88 ± 0.08 for anti-LT IgA. In contrast, no anti-STb antibodies (0.69 ± 0.6 for IgG and 0.176 for IgA) and no or very low anti-LT antibodies (0.79 ± 0.15 for IgG and 0 for IgA) were detected in the colostrum sample of the control gilt (Fig. 6).

Piglets borne by the immunized gilts acquired maternal anti-STb and anti-LT antibodies through suckling and were protected against infection from an STb-positive E. coli strain, 8816. Anti-STb antibodies in serum samples of these piglets
were titrated at $2.21 \pm 0.08$ (IgG) and $1.52 \pm 0.17$ (IgA). When challenged with strain 8816, all 10 K88ac receptor-positive piglets remained healthy. In contrast, 7 out of 9 K88ac receptor-positive piglets born by the unimmunized gilt developed moderate diarrhea after being challenged with the same strain. Serum samples from the piglets born by the unimmunized gilt had no anti-STb antibodies ($0.45 \pm 0.83$ for IgG and $0.15 \pm 0.39$ for IgA) detected (Fig. 6).

**DISCUSSION**

The STb gene (estB) is highly prevalent in *E. coli* strains isolated from pigs with postweaning diarrhea (PWD) (36), and infection with an *E. coli* strain expressing STb as the only toxin caused clinical diarrhea in more than half of gnotobiotic piglets (34). That suggests STb toxin is an important virulence factor in porcine diarrheal disease. Therefore, STb antigens need to be included in developing broad-spectrum vaccines against PWD. However, STb toxin, a small and poorly immunogenic molecule (24), cannot be used as an antigen in vaccine development, unless its immunogenicity is enhanced.

Enhanced STb immunogenicity was detected when a mature STb peptide was carried by a maltose-binding protein (MBP) (9). LT protein, like CT of *Vibrio cholerae*, has been regarded as the best adjuvant in eliciting host mucosal immunity which plays a key role in protection against enteric infection. The human-type LT192 protein has been successfully used as an adjuvant to facilitate immunogenicity for small molecules in developing vaccines against bacterial and viral infections (6, 7, 18, 25, 29, 30). In this study, we created a porcine ETEC LT toxoid (LT192) to carry the STb to enhance STb immunogenicity. The porcine-type LT192 should have equivalent adjuvanticity and antigenicity to the human-type LT192 since the human- and porcine-type LTs are highly homologous structurally and functionally (24). Like human type LT192, the porcine type LT192 likely had enterotoxicity reduced, as gnotobiotic piglets were challenged with strain 8816, all 10 K88ac receptor-positive piglets remained healthy. In contrast, 7 out of 9 K88ac receptor-positive piglets born by the unimmunized gilt developed moderate diarrhea after being challenged with the same strain. Serum samples from the piglets born by the unimmunized gilt had no anti-STb antibodies ($0.45 \pm 0.83$ for IgG and $0.15 \pm 0.39$ for IgA) detected (Fig. 6).

**FIG. 4.** Anti-LT and anti-STb antibody titration in serum samples of rabbits immunized i.m. with purified $6 \times$ His-tagged LT$_{192}$-Gly:Pro-STb (open box) and $6 \times$His-tagged LT$_{192}$-L-linker–STb (black box) fusion proteins and of the control rabbit (gray box). A GM1 ELISA with cholera toxin (CT) antigen and an STb ELISA using MBP-STb were used to titrate anti-LT IgG and anti-STb IgG and IgA antibodies. Titers are calculated in log$_{10}$. Mean values are shown, and error bars represent standard deviations.

**FIG. 5.** Cyclic AMP EIA to assess neutralizing anti-LT antibodies in rabbit serum and fecal samples. Serum (open box) or fecal (solid) samples (1:5 dilution in 150 μl DMEM/F-12) from rabbits immunized with purified $6 \times$His-tagged LT$_{192}$-Gly:Pro-STb or LT$_{192}$-L-linker–STb fusion antigens or from the control rabbit were tested to neutralize 10 ng cholera toxin (CT). Cyclic AMP concentrations (pmol/ml) of treated T84 cells were measured using a cAMP EIA kit (Invitrogen). Mean values are shown, and error bars represent standard deviations.
did not show clinical signs of diarrhea after being inoculated with either the LT\textsubscript{192} strain 8221 or the LT\textsubscript{192}-Gly:Pro-ST\textsubscript{b} strain 8488. Therefore, this LT\textsubscript{192} can also serve as a safe antigen to induce anti-LT immunity to protect pigs against infection from LT-producing ETEC strains. Results from this study showed that ST\textsubscript{b} became more immunogenic when fused to the LT\textsubscript{192}, as anti-ST\textsubscript{b} antibodies were detected in rabbits and pregnant gilts immunized with the LT\textsubscript{192}-ST\textsubscript{b} fusion antigens. In addition, anti-LT antibodies were also detected in immunized rabbits and gilts. Together, our data indicate LT\textsubscript{192}-ST\textsubscript{b} fusion antigens not only retain LT immunogenicity but also enhance ST\textsubscript{b} immunogenicity.

Our data suggest that anti-LT and anti-ST\textsubscript{b} antibodies elicited by 6\texttimes{}His-tagged LT\textsubscript{192}-ST\textsubscript{b} fusions neutralize LT and ST\textsubscript{b} toxins. After incubation with fecal or serum samples of the immunized rabbits, CT did not stimulate an increase of intracellular cAMP levels in T84 cells, clearly indicating that the elicited anti-LT antibodies neutralized CT \textit{in vitro}. Moreover, data from the piglet challenge study suggest that elicited anti-ST\textsubscript{b} antibodies are protective against ST\textsubscript{b} toxin. Suckling piglets born by the immunized gilts remained healthy after being challenged with an ST\textsubscript{b}-producing ETEC strain. We noticed that not all piglets in the group challenged with the LT\textsubscript{192}-positive ETEC strain developed diarrhea. That is similar to the results from our previous study in which more than half of the gnotobiotic piglets developed diarrhea after being challenged with a constructed ST\textsubscript{b} ETEC strain, 8015 (1836-2 expressing \textit{estB} in pBR322) (34). In the present study, we found that 7 of 9 K88ac receptor-positive piglets borne by the unimmunized gilt developed moderate diarrhea after being inoculated with the ST\textsubscript{b} strain 8816 (1836 expressing \textit{estB} in pUC19), which had the ST\textsubscript{b} gene expressed by a high-expression vector, pUC19. Since piglets used in this challenge study were naturally born, we suspected fewer piglets would show clinical disease if strain 8015 were used as the challenge strain. Although expression of ST\textsubscript{b} toxin has not been directly compared between strains 8816 and 8015, we found more piglets borne by the control gilt developed moderate diarrhea when strain 8816 was used as the challenge strain. These diarrheal piglets, when further examined for antibodies, had no anti-ST\textsubscript{b} or anti-LT antibodies detected. Apparently, these piglets did not acquire maternal anti-ST\textsubscript{b} antibodies to neutralize ST\textsubscript{b} toxin produced by the challenge strain 8816, and this lack of maternal antibodies thus resulted in diarrheal disease.

Unlike LT or heat-stable toxin type a (ST\textsubscript{a}), ST\textsubscript{b} toxin does not increase intracellular cAMP or cGMP levels in T84 or other cells, and thus anti-ST\textsubscript{b} antibody neutralization cannot be tested in cell lines. However, early studies showed that ST\textsubscript{b} toxin stimulates fluid secretion in the ligated loops of weaned or neonatal pigs (10, 21, 31, 34). Therefore, we first tested anti-ST\textsubscript{b} antibody neutralization with a loop assay. We found the loops of a 5- or 7-day-old pig after 8 h of incubation with mixture of culture filtrate of strain 8816 and serum and fecal samples from the immunized rabbits had significantly less fluid accumulated compared to the loops incubated with 8816 culture filtrate alone, respectively ($P < 0.02$ and $P < 0.04$). That indicated that rabbit anti-ST\textsubscript{b} antibodies neutralized some ST\textsubscript{b} toxin in culture filtrates. Then we tested anti-ST\textsubscript{b} antibodies in protection against infection from ST\textsubscript{b}-producing strain 8816 in challenge studies. Data from the challenge studies showed that
all piglets born by the immunized gilts remained healthy, whereas a majority of the piglets born by the unimmunized gilt developed clinical disease after infection with 8816. Together, these data suggest that anti-STb antibodies induced by 6×His-tagged LT192-Gly-Pro-STb fusion antigens are protective against STb toxin.

It has been reported that STb is more potent to older piglets and tends to stimulate more fluid in ligated loops (10, 21, 31). Considering the piglets that we used in challenge studies were naturally born and raised in nonsterile environment, we realized having kept them for a few weeks in such an environment certainly increases their chance of exposure to ETEC and other pathogens. Therefore, we used piglets 5 or 7 days old in the gut loop assay and challenge studies. Using young piglets in our challenge studies could contribute to the lack of a large amount of fluid accumulated in ligated loops or the more severe diarrhea that developed in the control pigs during the challenge study. Future studies using older piglets, and perhaps including an additional control group immunized with LT192 antigen alone, will be needed to further assess anti-STb antibodies in protection against STb-producing ETEC. In addition, studies assessing host anti-STb mucosal immunity in protection against ETEC will be more informative.

A 4-amino-acid Gly-Pro linker and a 7-amino-acid L linker were used to link the STb and LT192 in this study. We hypothesized that fusing STb at the C terminus of LT192 with a longer hinge could display STb antigen better and thus had greater enhancement of STb immunogenicity. Data from antibody titration suggest that rabbits immunized with the LT192-L-rich LT-subunit STb fusion had significantly greater titers of anti-LT IgG and anti-STb IgG antibodies, but not anti-STb IgA antibodies. On the other hand, fusion of STb at the C terminus of LT192 resulted in low OD readings when crude extracts from strains expressing the LT192-Gly-Pro-STb fusion were used in GM1 ELISA (Fig. 1). It has been reported that the LTb subunit and LT192, when an STa protein was fused at the C terminus, showed a reduction in GM1 binding (8, 37). However, it is unclear whether the lower binding to GM1 is caused by a lower level of protein expression, alteration of the LTb structure, stability, secretion, or pentamer formation after fusion of the STb antigen. It is also unknown whether such alteration would affect efficacy when purified LT192-Gly-Pro-STb or LT192-L-rich STb fusion antigen is used in an intranasal immunization or a live attenuated strain expressing this fusion is given orally. Clearly, further studies are needed to better quantify and characterize the LT192-STb fusion antigens.

ACKNOWLEDGMENTS

We thank Daniel Dubreuil (Universite de Montreal, Canada) for providing anti-STb serum and purified MBP-STb protein and Thomas Casey (National Animal Disease Center, Ames, IA) for providing the STb plasmid pRAS1; Eric Nelson and C. Welbon for assistance with rabbit immunization; and M. Zhao, C. Zhang, X. Ruan, M. Liu, K. Mateo, D. Baker, and M. Mucciante for assistance with animal studies. Financial support for this study was provided by USDA NRI SD9902298 (D. Francis), NIH AI068766 (W. Zhang), NPB 07-006 (W. Zhang), and the South Dakota Agricultural Experiment Station.

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