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Significance of Heat-Stable and Heat-Labile Enterotoxins in Porcine Colibacillosis in an Additive Model for Pathogenicity Studies†

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Although heat-stable (ST) and heat-labile (LT) enterotoxins produced by enterotoxigenic *Escherichia coli* (ETEC) have been documented as important factors associated with diarrheal diseases, investigations assessing the contributions of individual enterotoxins to the pathogenesis of *E. coli* infection have been limited. To address the individual roles of enterotoxins in the diarrheal disease caused by K88-positive ETEC in young pigs, enterotoxin-positive and -negative isogenic *E. coli* strains were constructed by using pBR322 to clone and express LT and STb. Four strains, K88$^+$ *astA*, K88$^+$ *astA*/pBR322, K88$^+$ *astA* STb$^+$, and K88$^+$ *astA* LT$^+$, were constructed and subsequently included in gnotobiotic piglet challenge studies, and their pathogenesis was assessed. The results indicated that all K88$^+$ isogenic strains were able to colonize the small intestines of piglets exhibiting the K88 receptor. However, only LT- and STb-positive strains caused appreciable diarrhea. Piglets inoculated with the K88$^+$ *astA* LT$^+$ strain became dehydrated within 18 h, while those inoculated with the K88$^+$ *astA* STb$^+$ strain did not, although diarrhea developed in several piglets. The changes in the blood packed-cell volume and plasma total protein of gnotobiotic piglets inoculated with the LT-positive strains were significantly greater than those of pigs inoculated with the K88 *astA*/pBR322 strain ($P = 0.012$, $P = 0.002$). Immunochemistry image analysis also suggested that LT enhanced bacterial colonization in a gnotobiotic piglet model. This investigation suggested that LT is a major contributor to the virulence of K88$^+$ ETEC and that isogenic constructs are a useful tool for studying the pathogenesis of ETEC infection.

Escherichia coli strains that colonize the small intestines, invade intestinal epithelial cells, and/or produce one or more toxins are important causes of diarrheal disease in both farm animals and humans. The virulence of enterotoxigenic *E. coli* (ETEC) is believed to be associated with the production of fimbrial adhesins and enterotoxins (1, 19, 35, 36, 51, 54). Fimbrial adhesins mediate the attachment of bacteria to the surface of host epithelium cells and allow bacterial colonization. Fimbriae produced by different ETEC strains are quite diverse (21). In swine, ETEC strains that produce K88 (F4) or F18 are the most common currently associated with diarrheal diseases (19). These fimbriae apparently bind to glycoconjugates in the porcine enterocyte brush borders, and the absence of the respective glycoconjugate renders the animal resistant to bacterial colonization and consequent diarrheal diseases (14, 15, 20, 48, 49).

Enterotoxins, including heat-stable enterotoxins (STa and STb) and heat-labile enterotoxin (LT) (23, 25, 39, 45), have been found to disrupt intestinal fluid homeostasis and to cause hypersecretion of fluid and electrolytes through activation of adenylate cyclase (by LT) or guanylate cyclase (by STa) in small intestinal mucosal cells (26, 34). There are two major serogroups of LT found among *E. coli* strains: LT-I and LT-II. LT-I is associated with diarrheal diseases of both humans and animals, while LT-II is typically associated with diarrheal disease in animals. STs are small and monomeric molecules and may be associated with either human or animal disease (45, 55). STa and STb are the two classes of STs first recognized and differ from each other in both structure and enzyme activity (11, 12). STa is produced by ETEC and other bacteria, while STb is found only associated with ETEC. A third ST, enteroaggregative *E. coli* (EAEC) EAST1, has been more recently identified. It is a plasmid-mediated enterotoxin, of low molecular weight and is frequently but not exclusively associated with EAEC isolated from children with persistent episodes of diarrhea. EAST1 shares about 50% protein identity with STa, and its gene has recently been found in many ETEC strains (9, 44). However, the significance of this enterotoxin in ETEC diarrhea has yet to be determined.

ETEC strains isolated from young animals of a variety of species and from both young and adult humans have shown considerable heterogeneity with regard to the enterotoxins produced. STa is typically the only enterotoxin produced by ETEC strains that infect calves and lambs, and LT is the only toxin found in *E. coli* strains causing diarrhea in chickens. Various combinations of LT, STa, and STb are produced by ETEC strains associated with diarrheal disease in pigs. Indeed, the most common ETEC strain isolated from diarrheic pigs produce LT and STb (with or without STa and/or EAST1) in addition to K88 (F4) fimbriae (36). One survey showed that more than 50% of the ETEC strains isolated from porcine diarrheal disease cases in the United States from 1999

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through 2001 possessed genes for the expression of K88, LT, and STb (19).

The mechanisms whereby enterotoxins elevate either cyclic AMP or cyclic GMP levels in intestinal epithelial cells, stimulate active chloride secretion, inhibit electroneutral sodium chloride absorption in the intestinal epithelium, and subsequently cause unidirectional fluid secretion are well understood (17, 18). However, the significance of the contribution of these enterotoxins to infection remains less clear. Further, the roles these toxins play (if any) in bacterial colonization and enteric infection remain unclear. In addition, whether bacterial colonization mediated by a fimbrial adhesion is sufficient to precipitate diarrhea, as suggested by Smith and Linggood (51), who used a K88 ETEC model, remains unclear and requires testing with currently available tools.

The most convincing criterion to determine the contribution of an enterotoxin to colibacillosis perhaps is to demonstrate that the purified toxin causes diarrhea when it is ingested by experimental animals or human volunteers (45). The results of direct ingestion of purified cholera toxin (CT) and the *Clostridium perfringens* enterotoxin by human volunteers clearly indicated the significant contribution of each toxin to diarrhea (31, 47). Alternatively, the significance of a toxin in colibacillosis can be established by constructing bacterial isogenic strains that differ only in the expression of the toxin in question and analyzing challenge results following oral ingestion. In a recent study, Berberov et al. (4) inactivated the *eltAB* gene expressing LT in a wild-type ETEC strain ($K88^+$ STb^+ LT^+ $EAST1^+$) and examined the importance of LT in porcine diarrheal disease. In the present study, we have chosen to take the opposite approach to determine the contribution of enterotoxins to colibacillosis. Instead of inactivating genes for the expression of an enterotoxin, we constructed isogenic strains by cloning genes expressing STb or LT into a plasmid and transforming that plasmid into a nonpathogenic *E. coli* field isolate (1836-2) shown to contain genes for K88ac and EAST1 and to express K88ac fimbriae. Isogenic constructs were tested for expression of the enterotoxins and used for oral inoculation of colostrum-deprived gnotobiotic piglets, and their virulence was assessed. By adding genes expressing individual enterotoxins to a nonpathogenic *E. coli* strain, we developed an additive model to study the pathogenesis of ETEC in gnotobiotic piglets and evaluate the contribution of individual enterotoxins to the porcine colibacillosis caused by ETEC infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. 1836-2, an O8:H4 *E. coli* field isolate, was used as the parental strain to construct isogenic strains. 1836-2 is a nonpathogenic *E. coli* strain that expresses K88 fimbriae and produces no enterotoxins (no fluid accumulated by piglet ligated intestinal loop assay) but has subsequently been found to contain the *astA* gene for EAST1. Plasmid pRAS1 (7, 53) containing the *estB* gene responsible for the expression of STb was kindly provided by T. Casey. Vector pBR322 was used to clone the *eltAB* gene, which was PCR amplified from wild-type ETEC strain 2534-86 and is responsible for expression of LT. Strain 8017 was prepared by transforming vector pBR322 into 1836-2 and served as a negative control. Strains 8015 and 8035 were constructed by transforming plasmids pRAS1 and pWZLT, expressing STb and LT, respectively, into 1836-2 (Table 1).

PCR amplification of *eltAB* gene. To construct strain 8035 ($K88^+$ LT^+), the *eltAB* gene was amplified by PCR and then blunt end ligated into pBR322 at the EcoRV site. PCR was performed at PE 2400 (Applied Biosystems, Foster City, Calif.) in a 50-μl reaction mixture containing 1× *Pfu* DNA polymerase buffer (with Mg^{2+}), 200 nM deoxynucleoside triphosphates, 0.5 μM each forward (5′-CGGATTGTCTTCTTGTATGAT-′3) and reverse primer (5′-GATCGGTA TTGCCTCCTCTAC-′3), and 1 U of *Pfu* DNA polymerase (Stratagene). The PCR program contained 1 cycle of 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and extension for 6 min at 72°C. The PCR product was purified by 1% agarose (FMC Bioproducts, Rockland, Maine) gel electrophoresis with a QIAquick Gel Extraction Kit following the manufacturer's (QIAGEN) instructions.

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

Strain	Relevant properties	Plasmid	Reference or source
1836-2	Field isolate, $K88^+$ $astA^+$		This study
8017	Negative control, $K88^+$ $astA^+$	pBR322	This study
8015	STb^+ strain, $K88^+$ $astA^+$ STb^+	pRAS1	7
8035	LT^+ strain, $K88^+$ $astA^+$ LT^+	pWZLT	This study
3030-2	Wild type, $K88^+$ $astA^+$ STa^+ STb^+ LT^+		Field isolate

[a] Plasmids pRAS1 and pWZLT were derived by cloning the *estB* and *eltAB* genes individually into pBR322. Stains 8017, 8015, and 8035 were constructed by transforming strain 1836-2 with plasmids pBR322, pRAS1, and pWZLT, respectively.

Cloning of *eltAB* gene into plasmid pBR322 and construction of isogenic strains 8015, 8017, and 8035. Plasmid pBR322 was digested with restriction enzyme EcoRV (New England BioLabs, Beverly, MA) in a reaction mixture volume of 25 μl with 1× buffer 3, 1× bovine serum albumin, 1 μg of pBR322 plasmid, and 20 U of EcoRV enzyme, incubated at 37°C for 1 h. Linearized pBR322 DNA was purified by agarose gel electrophoresis with a QIAquick Gel Extraction Kit and subsequently dephosphorylated with calf intestinal alkaline phosphatase (New England BioLabs, Beverly, MA). Blunt-end ligation of the purified, PCR-amplified *eltAB* fragment and the linearized and dephosphorylated pBR322 vector was catalyzed by T4 DNA ligase (Invitrogen) at room temperature overnight. Competent cells of strain 1836-2 were prepared by a standard method (42), and cell transformation was done by electroporation at 2.5 kV with 25 μF of capacitance and 200 Ω of resistance (2). Ampicillin (100 μg/ml) was used to select transformants. Positive colonies were screened by PCR initially and then sequenced to ensure that the *eltAB* gene was inserted correctly. The $K88^+$ LT^+ clone selected for was designated 8035. To construct isogenic strain 8015 ($K88^+$ STb^+), plasmid pRAS1, which was derived from pBR322 by insertion of a HindIII fragment containing the *estB* gene (7, 53), was transformed into 1836-2. For the construction of strain 8017, vector pBR322 (Promega) was directly transferred into 1836-2.

Western blotting to confirm the expression of LT. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot assays were performed by standard methods (13, 42, 52). Bacterial strains were grown in Casamino Acids and yeast extract broth overnight at 37°C (40). The overnight-grown culture was centrifuged at 8,000 × *g* for 20 min, and the resultant supernatant was concentrated by vacuum desiccation. The bacterial pellet was lysed in a solution containing 0.25 M sucrose and 0.1 M Na_2HPO_4 with lysozyme and EDTA and then centrifuged at 8,000 × *g* for 15 min. The proteins were separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Rabbit anti-CT (Sigma) was used as the primary antibody, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; Gibco) was used as the secondary antibody. LT protein was visualized with a LumiGLO Reserve Western blotting kit (KPL) and exposure to X-ray film.

GM1 ELISA for detection of LT. LT was also detected by the GM1 enzyme-linked immunosorbent assay (ELISA) method (40). Bacteria were cultured in Casamino Acids and yeast extract broth for 20 h at 37°C and then centrifuged at 8,000 × *g* for 15 min. Bacterial pellets were used to extract periplasmic proteins, which were subsequently assayed for LT as previously described (4, 40). GM1 ganglioside (2.0 μg/ml; Sigma) in 0.1 M phosphate-buffered saline (PBS) was used to coat a polystyrene 96-well microtiter plate (Maxisorb; Nunc, Roskilde, Denmark), which was incubated at room temperature overnight. After three washes with PBS–0.05% Tween 20, the remaining binding sites in the plate wells were blocked by incubation with 5% bovine serum albumin at 37°C for 30 min. Plates were washed three times with PBS-Tween 20 and incubated with the bacterial periplasmic extracts at room temperature for 60 min. Purified CT (20 ng) and periplasmic extract from LT-positive ETEC strain 3030-2 were used as positive controls, and PBS was used as the negative control. Anti-CT rabbit serum (Sigma) diluted 1:2,500 was used as the primary antibody, and horseradish

peroxidase-conjugated goat anti-rabbit IgG (Gibco) at a dilution of 1:1,500 was used as the secondary antibody. Plates were incubated with the primary antibody at room temperature for 30 min, washed three times, and then incubated with the secondary antibody at room temperature for 1 h. After three more washes, plates were incubated with 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) peroxidase substrates (KPL, Gaithersburg, MD) until the color in the positive control reached sufficient density, and absorbance was measured at a wavelength of 405 nm.

STb detection by ELISA. Bacteria were grown overnight in LB broth at 37°C on a shaker at 200 rpm. Cells were pelleted by centrifugation, and culture supernatants were concentrated about 10 times with Amicon filter concentrators. Following concentration, supernatants were used to coat wells in 96-well polystyrene plates at 4°C overnight. Adherent STb was detected with anti-STb rabbit serum kindly provided by T. Casey at a dilution of 1:5,000 and goat anti-rabbit IgG conjugated with HRP (Sigma) at a dilution of 1:10,000. *o*-Phenylenediamine (Sigma) in peroxide buffer (Pierce) was used as the chromogenic substrate, and well optical density was read at 490 nm.

In vivo enterotoxin expression. Ligated ileal loops in neonatal piglets were used to confirm that strains 8015 (K88$^+$ *astA* STb$^+$) and 8035 (K88 *astA* LT$^+$) could produce biologically active enterotoxin. Five-day-old, colostrum-deprived pigs were anesthetized, and 15 to 20 ileal loops were prepared per pig. Each ligated segment intended for inoculation was about 8 cm in length, and loops were separated by an uninoculated interloop segment of about 3 cm. Two milliliters of overnight-grown culture was injected into each loop. After 8 h postinoculation, the pigs were euthanized and subjected to necropsy. Each loop was measured for length (centimeters) and fluid accumulation (grams). The ratio of fluid accumulation to segment length (grams per centimeter) was calculated and used as a measurement of enterotoxin expression.

Oral inoculation of gnotobiotic piglets. Fifty gnotobiotic piglets were derived by closed hysterotomy and reared in sterile isolator units (33). Each litter was randomly divided into three or four groups, depending on the litter size, and each group was reared in a separate isolator unit at a room temperature of 32°C. The piglets were fed Esbilac milk replacer (Pet Ag, Inc.). At 5 days of age, they were orally inoculated with 3×10^9 CFU of either *E. coli* strain 1836-2 (K88$^+$ *astA*), 8017 (K88$^+$ *astA*/pBR322), 8035 (K88$^+$ *astA* LT$^+$), or 8015 (K88$^+$ *astA* STb$^+$) in 50 ml of sterile milk replacer. Piglets were observed to consume the inoculum and monitored for subsequent clinical signs, including vomiting, diarrhea, dehydration, and lethargy. Dehydration levels were graded subjectively as level 0 (undetectable, normal), 1 (skin slightly doughy, backbone slightly prominent), 2 (skin inelastic, eyes slightly sunken in orbits, backbone prominent), 3 (skin turgor, eyes sunken in orbits, prolonged capillary refill time, backbone very prominent), or 4 (shock and imminent death). Piglets were subjected to necropsy at 18 h postinoculation after euthanasia, and samples of the ileum (3 to 5 cm proximal to the ileocecal valve), lower jejunum (one-third to one-half of the distance between the pyloric valve and the ileocecal valve), upper jejunum (one-half to two-thirds of the distance between the pyloric valve and the ileocecal valve), and duodenum (3 to 5 cm distal to the pyloric valve) were collected for bacteriology and histopathology studies.

Assessment of piglet dehydration. The level of dehydration following challenge was determined by measuring changes in blood packed-cell volume (PCV) and plasma total protein (TP). Blood samples were drawn from each pig before and 18 h after inoculation and tested for blood PCV and plasma TP as described elsewhere (32). Briefly, blood samples were placed in 75-mm capillary tubes and centrifuged for TP and PCV analysis. PCV was determined with a standard hematocrit total percentage chart. Plasma TP content was determined with a standard medical refractometer. An increase in PCV and plasma TP from preinoculation sample collection to postinoculation sample collection served as an indication of dehydration.

Assessment of bacterial intestinal colonization. Determination of the magnitude and location of bacterial colonization of the small intestine was accomplished by quantitative culture and image analysis of immunohistochemically stained sections of piglet small intestine. The concentration of bacteria in CFU per gram of ileal tissue was determined as previously described (20). Briefly, ileal tissue was weighed, washed, and ground in PBS (at a ratio of N grams of tissue in $9 \times$ N milliliters of PBS), serially diluted, plated on blood agar (brain heart base) or LB agar plates, and incubated overnight at 37°C, after which bacterial colonies were enumerated.

For image analysis, tissue samples from the ileum, lower jejunum, upper jejunum, and duodenum of each challenged piglet were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, mounted on glass slides, and prepared for Giemsa and immunohistochemical staining by standard procedures. Giemsa-stained tissue sections were examined by light microscopy. Four sections each of the ileum, upper jejunum, lower jejunum, and duodenum of each challenged piglet were stained by standard immunohistochemical procedures (41) with rabbit polyclonal antiserum raised against *E. coli* O8 somatic antigen (Denka Seiken, Tokyo, Japan) as the primary antibody, goat anti-rabbit IgG (heavy and light chain specific), avidin-biotin-alkaline phosphatase solution, and Vector Red as the substrate (Vector Laboratories). Microscopic images were taken of the whole area of the entire tissue section on a slide with an Olympus DP70 digital camera on an Olympus AX70 microscope. All images were collected under the same parameters and analyzed by Image Processing Tool kit 5.0 (Reindeer Graphics) for the measurement of bacterial colonization as previously described (4).

Piglet brush border adherence assay for the assessment of K88 receptor expression. Jejunum samples collected from each piglet at necropsy were used to prepare brush border vesicles as described previously (3). Brush border vesicles from each piglet were tested for the adherence of *E. coli* expressing K88ab, K88ac, and K88ad fimbriae as described by Sellwood et al. (46). Suspensions of bacteria mixed with brush borders were examined under a phase-contrast microscope for the adherence of bacteria to brush borders. The numbers of bacteria adhering to individual brush border vesicles were determined, and 10 vesicles from each brush border sample were included for the calculation of bacteria. Individual brush borders were considered adherent if there were more than two bacteria adhering to a brush border vesicle. Pigs without bacterial adherence were excluded from the data analyses.

Statistical analysis. Data were analyzed by the mixed procedure (SAS for Windows, version 8; SAS Institute, Cary, N.C.), and Student's *t* test was used for comparison of the different treatment groups. Calculated P values of <0.05 were regarded as significant.

RESULTS

The constructed strains were initially examined for enterotoxin genes by PCR and DNA sequencing. Expression of cloned toxin genes was confirmed by gut loop assay, ELISA, and Western blotting, and strains were subsequently used in the gnotobiotic piglet challenge studies. Of 50 gnotobiotic piglets inoculated with constructed strains, 37 possessed brush borders that tested positive for K88ac$^+$ *E. coli* adherence. Therefore, data from these piglets were retained in the final analysis. Among those pigs, 11, 11, and 12 piglets were inoculated with constructed strains 8017 (as a negative control), 8015 (STb$^+$), and 8035 (LT$^+$), respectively, and the remaining 4 piglets were challenged with parental strain 1836-2.

Expression of enterotoxins by *E. coli* constructs. When constructs were tested for LT by ELISA with CT as the standard and strain 3030-2 as a positive control, only strain 8035 exhibited positive test results (Fig. 1). LT expression by strain 8035 was also verified by Western blot hybridization. Strain 8015 exhibited positive results when tested for expression of STb by ELISA, but the other constructed strains did not (Fig. 2). Fluid accumulation was observed in ligated gut loops inoculated with constructs 8015 and 8035 and was similar in amount to that of control strain 3030-2, suggesting that each construct produced biologically active enterotoxins (Fig. 3). Strain 8017, constructed as the negative control, did not induce fluid accumulation.

LT and STb expressed by K88ac$^+$ *E. coli* contribute significantly to dehydrating diarrhea in K88 receptor-positive gnotobiotic piglets. All 12 gnotobiotic piglets whose brush borders were adherent for K88ac fimbriae developed diarrhea and exhibited dehydration after oral challenge with LT-positive construct 8035. Dehydration, judged by clinical observation of these pigs, was level 2 to 3. The clinical responses of those 11 piglets to STb-positive construct 8015 were variable, ranging from normal to watery diarrhea and moderate dehydration (level 1 to 2). Six of the 11 K88 receptor-positive piglets de-

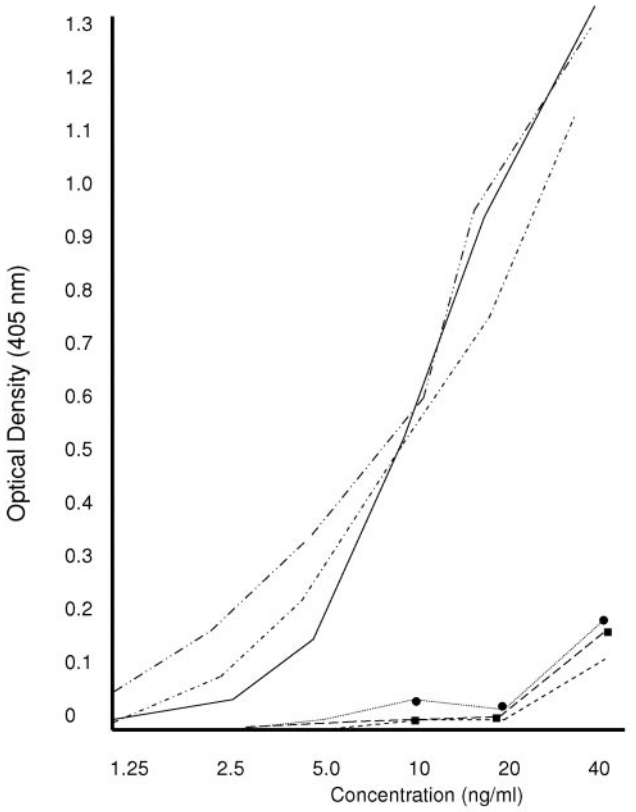

FIG. 1. Detection of LT by GM1 ELISA. Wells of 96-well microtiter plates were coated with the ganglioside GM1 as the ligand for LT. Lysates of pellets from overnight culture were tested for expression of LT with anti-CT as the primary antibodies (1:2,500) and goat anti-rabbit IgG (1:1,500) as the secondary antibodies. Reactions were visualized with peroxidase substrate, and optical densities were measured at 405 nm. Titration curves of commercialized CT (solid line), positive control strain 3030-2 (double-dotted and dashed line), strain 8035 (LT^+) (single-dotted and dashed line), parental strain 1836-2 (circles), strain 8015 (STb^+) (dotted line), and negative control strain 8017 (squares) are shown.

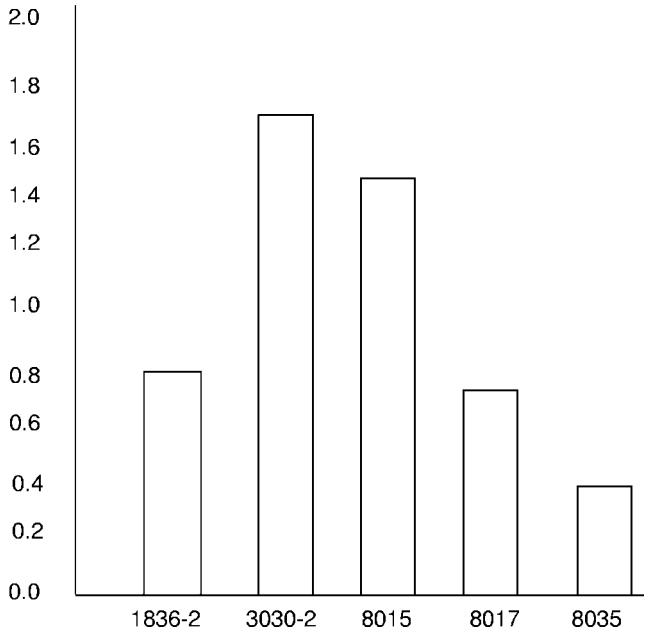

FIG. 2. Detection of STb by ELISA. Supernatants from overnight bacterial culture were concentrated 10-fold with Centricon filters. Anti-STb rabbit serum (1:5,000) was used as the primary antibody, and goat anti-rabbit IgG conjugated with HRP (1:10,000) was used as the secondary antibody. Reactions were visualized with *o*-phenylenediamine and peroxide buffer, and optical densities were read at 490 nm. Student's *t* test indicated that the expression of STb in strains 3030-2 and 8015 was significantly different from that in strain 1836-2 ($P < 0.01$, $P = 0.01$), whereas no significantly differences were found between strain 8035 or 8017 and strain 1836-2 ($P = 0.07$, $P = 0.39$).

veloped diarrhea, while 5 did not. The 11 piglets challenged with LT^- STb^- construct 8017 remained clinically normal, showing neither diarrhea nor dehydration. None of the piglets that lacked K88 receptors developed any clinical signs of diarrhea, regardless of the strain used for inoculation.

Increases in PCV and TP were detected in piglets that became dehydrated as a result of infection with LT- and STb-expressing strains (Table 2). The mean changes in PCV for piglets challenged with strains 8017 ($K88^+$ *astA*/pBR322 vector control), 8015 ($K88^+$ *astA* STb^+), and 8035 ($K88^+$ *astA* LT^+) were −8.81%, −4.47%, and 26.41%, respectively, while the mean changes in TP were −10.48%, 4.21%, and 24.69%, respectively. The increase in PCV of piglets challenged with strain 8035 ($K88^+$ *astA* LT^+) was significantly different ($P < 0.01$) from that of animals challenged with strains 8017 ($K88^+$ *astA*) and 8015 ($K88^+$ *astA* STb^+). However, the change in PCV for animals challenged with strain 8015 ($K88^+$ *astA* STb^+) was not significantly different ($P = 0.33$) from that of animals challenged with control strain 8017. Changes in the plasma TP of piglets challenged with 8035 and 8015 were significantly different ($P < 0.01$ and $P = 0.02$, respectively) from that of piglets challenged with the control construct. The findings indicated that both LT and STb caused dehydration in challenged piglets, but the level of dehydration caused by the LT construct was greater than that caused by the STb construct. The lack of an increase in PCV and plasma TP in animals challenged with strain 8017, which contained the *astA* gene for EAST1, coupled with no clinical observations of disease may suggest that EAST1 does not contribute significantly to the production of piglet diarrhea by ETEC strains. However, since the ability of strain 8017 to express EAST1 has not been determined, no conclusion about the significance of EAST1 can yet be made.

LT enhances ETEC intestinal colonization in gnotobiotic piglets. Quantitative culture of bacteria present in washed ilea indicated a significant difference ($P = 0.01$, $P = 0.003$) in the concentration of adherent bacteria in piglets inoculated with strain 8035 compared with that in those inoculated with strains 8015 and 8017 but no significant difference between the groups inoculated with strains 8015 and 8017 ($P = 0.73$). The mean numbers of CFU per gram of washed ileum from piglets inoculated with strains 8035 ($K88^+$ *astA* LT^+), 8015 ($K88^+$ *astA* STb^+), and 8017 ($K88^+$ *astA*) were 1.2×10^9, 5.7×10^8, and 5.9×10^8, respectively (Table 2).

Histological analysis of four sections of small intestine from pigs challenged with the various constructs also indicated greater bacterial colonization ($P = 0.004$, $P = 0.001$) in piglets

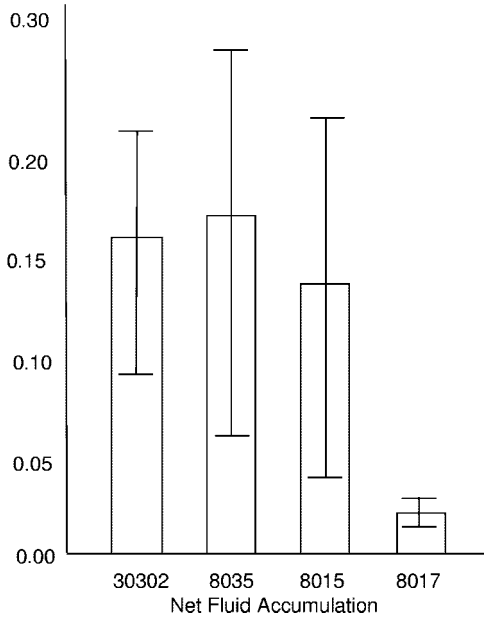

FIG. 3. Ligated ileal loop assay in neonatal pigs. Individual ligated intestinal loops were inoculated with 2 ml of an overnight culture of strain 3030-2 (K88$^+$ LT$^+$ STb$^+$ STa$^+$, positive control), 8035 (K88$^+$ *astA* LT$^+$), 8015 (K88$^+$ *astA* STb$^+$), or 8017 (K88$^+$ *astA*/pBR322, negative control). Fluid accumulation was measured at 8 h postinoculation and expressed as grams per centimeter of ligated loop. Statistical analysis indicated that the fluid accumulation in strains 3030-2 ($P = 0.02$), 8035 ($P = 0.04$), and 8015 ($P = 0.048$) was significantly different from that in strain 8017.

challenged with strain 8035 than in those challenged with strain 8015 or 8017 (Fig. 4). Differences in colonization between piglets challenged with strain 8015 and those challenged with strain 8017 were not significant ($P = 0.774$). Image analysis of immunochemically stained histological sections showed that the means of the areas colonized with bacteria were 429.59, 384.15, and 1,241.31 square micrometers in animals inoculated with strains 8017, 8015, and 8035, respectively. The significant increase in the number of bacteria present on the epithelium of the small intestine in the animals challenged with strain 8035 suggested that LT enhanced bacterial colonization.

DISCUSSION

The results of this study provide direct evidence that both LT and STb contribute to the diarrhea resulting from infection with an ETEC strain. However, the effect of LT on the host was substantially greater than that of STb. These findings are in agreement with those of Berberov et al. (4), who showed that 60% of the piglets inoculated with an STb$^+$ EAST1$^+$ LT$^-$ mutant strain developed severe dehydrating diarrhea, compared to 100% of the piglets inoculated with the STb$^+$ EAST1$^+$ LT$^+$ parent and complemented mutant strains, which suggested that LT is important in pathogenesis. However, the diarrhea seen in the present study was of a less severe form than that of pigs inoculated with the LT-complemented isogenic mutant or the LT$^+$ STb$^+$ parent strain in the study by Berberov et al. (4). Results from both the present study and the study by Berberov et al. (4) also suggest that LT contributes in some way to enhanced colonization of the intestinal epithelium by ETEC strains. Both studies showed enhancement of colonization associated with the presence of LT; the mechanism by which colonization enhancement occurs is unclear but appears to affect bacterial attachment to the epithelium, as well as cause a general increase in the concentration of bacteria in the small intestine.

The ability of the ETEC construct used in this study to cause clinically apparent disease in gnotobiotic piglets suggests that the additive approach of inserting enterotoxin genes into a nonpathogenic, wild-type, fimbria-positive *E. coli* background is a useful model for studying the pathogenesis of ETEC. It may be argued that the addition of enterotoxin genes provides more direct proof of their contribution to virulence than would inactivation or deletion of the same genes from a pathogenic strain. As the strain utilized in constructing the ETEC employed in this study contained genes typical of porcine ETEC, including *astA* (to express EAST1) and those genes essential for the expression of K88 fimbriae, it is possible, indeed likely, that it was a natural derivative of an ETEC strain. Perhaps a more definitive approach to the characterization of the roles of particular genes in pathogenesis would be to construct isogenic strains from an organism containing no known virulence genes and perhaps isolated from an animal species unrelated to the target host of the constructed pathogen. Such a strain would be less likely to contain as-yet-unidentified virulence genes that contribute to the pathogenesis of the disease in question. Nevertheless, by adding LT and STb to a nonpathogenic wild-type *E. coli* strain that expresses K88 fimbriae, we have provided perhaps the clearest evidence that each enterotoxin contributes to the virulence of ETEC strains.

The parent strain from which constructs were created was intensively tested for virulence and virulence-associated genes. The strain was tested by PCR for genes encoding LT, STa, STb, StxII, and EAST1; by ligated intestinal loop assay for the ability to stimulate fluid accumulation; and by ELISA for the

TABLE 2. PCV and plasma TP pre- and postinoculation and CFU of inoculum strain cultured per gram of piglet ileum at necropsy[a]

Construct	Preinoculation PCV	Postinoculation PCV	PCV difference (%)	Preinoculation TP	Postinoculation TP	TP difference (%)	No. of CFU/g
1836-2	25.0	25.0	0	3.8	3.3	−9.84	4.44×10^8
8017	25.6	23.1	−8.81	3.7	3.3	−10.48	5.90×10^8
8015	26.7	25.5	−4.47	3.6	3.4	−4.21	5.68×10^8
8035	26.3	33.4	26.41	3.7	4.6	24.69	1.21×10^9

[a] Statistical analysis (Student's *t* test) indicates that changes in the PCV and TP of piglets challenged with strain 8035 were significantly different from those of piglets inoculated with strains 8017, 8015, and 1836-2 and that ileal bacterial colonization in piglets inoculated with strain 8035 was significantly greater than that in piglets inoculated with the other strains.

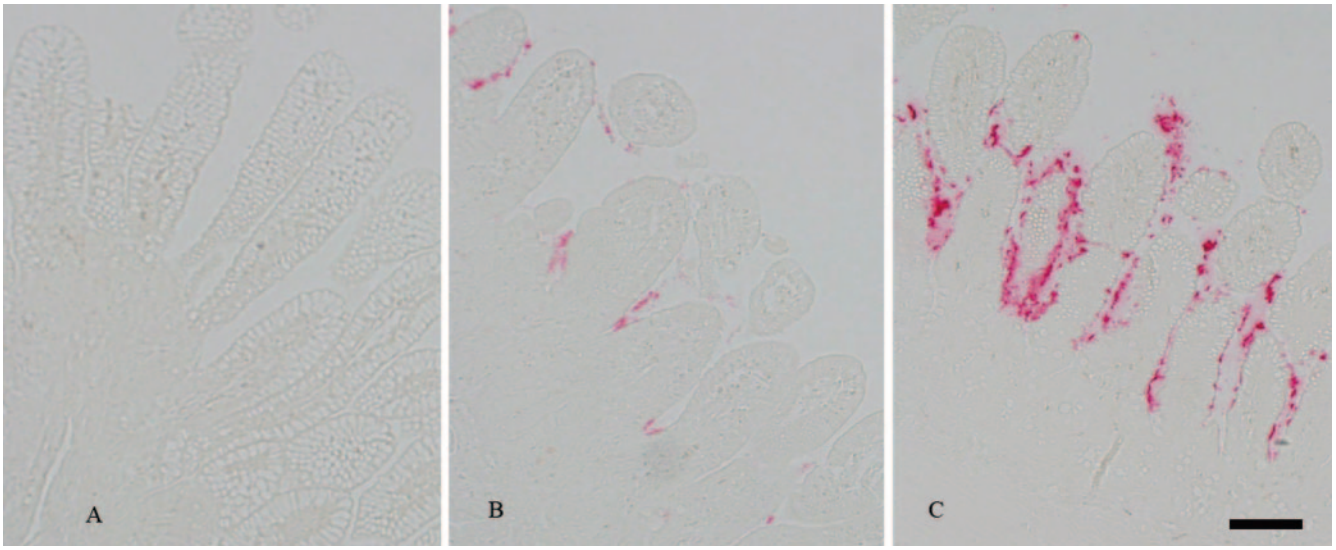

FIG. 4. Histological sections of ileum from challenged gnotobiotic pigs were stained immunohistochemically to demonstrate bacterial colonization on the surface of the mucosal epithelium by strains 8017 (K88$^+$ *astA*) (A), 8015 (K88$^+$ *astA* STb$^+$) (B), and 8035 (K88$^+$ *astA* LT$^+$) (C). Rabbit polyclonal antiserum against *E. coli* O8 somatic antigen was used as the primary antibody, and goat anti-rabbit IgG (heavy and light chain specific) was used as the secondary antibody. Avidin-biotin-alkaline phosphatase and Vector Red as the substrate were used to generate a red reaction product. Microscopic images were digitalized, and red areas were measured by the Image Processing Tool kit. Bar, 100 μm.

LT and STb antigens. The EAST1 gene, *astA*, was the only toxin gene detected by PCR, and all other tests for toxins and/or their activity were negative. The parent strain was tested by PCR for the presence of other virulence-associated genes, including those for K88 (F4), K99 (F5), 987P (F6), F18, and F41 fimbriae, and only the genes for K88 were present. Gnotobiotic piglet challenge with the strain resulted in no clinically apparent disease and limited colonization of the strain to the intestinal epithelium. Changes in virulence in the strain subsequent to introduction of the *eltAB* or *estB* gene, as evidenced by diarrhea, dehydration, and enhanced colonization (*eltAB*), provide strong evidence of the virulence-enhancing characteristics of the genes. Presence of the genes in the appropriate orientation was established by DNA sequencing, and appropriate gene expression was established by ELISA and accumulation of fluid in ligated intestinal loops following the inoculation of loops with constructs containing *estB* or *eltAB*.

Individual piglets showed variation in illness when they were orally inoculated with constructed strain 8015 (STb$^+$), ranging from no to moderate diarrhea. Previous studies also showed inconsistency in diarrhea associated with STb in various animal models. STs have been reported to stimulate fluid accumulation in suckling mice (26) and found to contribute to the severity of disease in rabbits (10). Kennedy et al. (28) concluded that STb did not cause fluid secretion in suckling mice up to 12 days old or ligated intestinal loops in rats or rabbits. However, STb-producing strains induced significant fluid secretion in ligated intestinal loops of piglets compared to STb-negative strains (28). Other studies clearly indicated that STb caused fluid secretion, but whether it also caused diarrhea remains controversial (7, 16, 27, 53). Fairbrother et al. (16) observed watery diarrhea in 2 of 12 piglets inoculated with STb-producing strains, and an additional piglet developed moderate diarrhea. Six of these 12 piglets showed no sign of diarrhea, and none exhibited evidence of intestinal epithelial colonization (16). Casey at al. (7) constructed ETEC strains expressing either STa or STb. While both constructs stimulated fluid accumulation in intestinal loops of neonatal piglets <24 h old, diarrhea was only demonstrated following the inoculation of piglets of the same age with the STa construct. The fimbriae expressed by the constructs in that study were F41, which is known to mediate colonization limited to the ileum (i.e., the terminal small intestine), whereas K88 mediates colonization of the entire small intestine (5, 24). Taken together with our own study, these observations suggest that STb is weakly diarrheagenic in young piglets <5 days old. Piglets in the study by Berberov et al. (4) were 9 days old at challenge and developed severe dehydrating diarrhea following inoculation with an LT$^-$ mutant that expressed STb and EAST1. STb is universally present in ETEC strains that cause diarrhea in weaned pigs (19); hence, it likely has an important role in pathogenesis of disease, but this remains to be elucidated. Its relative significance could vary, depending on the age of the pigs, the fimbrial background of the strain, and other factors.

EAST1 was originally recognized in EAEC strains that had infected humans (43, 44). The toxin is substantially different genetically and antigenically from STa of ETEC, despite their 50% homology at the amino acid level. Surveys have detected the EAST1 gene by PCR in >40% of ETEC strains (44), and >22% of the *E. coli* strains isolated from piglets with diarrhea carried the *astA* gene as the only PCR-detected virulence gene (8). However, the significance of EAST1 in ETEC infection and clinical disease is unclear. Whether porcine *E. coli* strains possessing *astA* but lacking other enterotoxin-encoding genes are diarrheagenic in piglets has not yet been determined. Ngeleka et al. (38) found that 13.5% of isolates from piglets with diarrhea contained only the *astA* gene, but at the same

time, 10.8% of the isolates from piglets without diarrhea contained *astA* as the only toxin-encoding gene. Although our observations that strain 1836-2 neither causes fluid accumulation in ligated porcine intestinal loops nor causes diarrhea in challenged piglets, whether EAST1 was expressed from the *astA* gene in strain 1836-2 has not been verified; therefore, the notion that EAST1 alone is insufficient to cause diarrhea in pigs is unable to be confirmed or denied by this study.

It has been reported recently that essentially all K88-positive ETEC strains isolated from diarrheic pigs express both STb and LT (19). The contribution of LT to diarrheal disease in pigs has been the subject of limited investigation. Early studies with crude whole-cell lysates and broth culture supernatant fluids from LT-positive or LT- and STb-positive strains in ligated intestinal assays suggested a correlation between the presence of LT or LT-STb and diarrheal disease (22, 29, 30, 37, 50), but none of these studies compared the effects of LT-positive and LT-negative isogenic strains. Broes et al. (6) found that piglets challenged with *E. coli* strains of serotype O8:KX105 that expressed both STb and LT developed diarrhea, but no diarrhea was shown following inoculation with STb-positive but LT-negative strains. The authors (6) noted the high positive association between the production of LT and the virulence of the *E. coli* O8:KX105 strain and suggested that LT may be an important virulence factor. However, the lack of extensive colonization (only about 10^7 to 10^8 CFU/10 cm of ileum and jejunum, respectively) may have compromised any conclusion from the study regarding the virulence potential of STb-positive but LT-negative strains. Berberov et al. (4) constructed isogenic strains by inactivating the *eltAB* gene in wild-type ETEC strain 2534-86 ($F4^+$ LT^+ STb^+ *astA*) and evaluated the significance of LT in diarrheal disease in gnotobiotic pigs. The investigators reported that all piglets developed severe dehydrating diarrhea and septicemia after they were inoculated with LT-positive strains and suggested the importance of LT in the pathogenesis of the disease.

The results of this study are in agreement with those reported by Berberov et al. (4) in that LT significantly enhanced the colonizing ability of the challenge strain. Quantitative culture assessment and histological image analysis performed in both studies support the notion that LT enhances bacterial colonization. The mechanism by which LT has such an effect remains unclear. However, it is speculated that LT released by colonized bacteria may alter the enterocyte brush border glycocalyx or microvillus cytoskeleton and subsequently increase the availability of fimbrial receptors.

In this study, an additive model for investigating the contributions of genes to the pathogenesis of ETEC disease was described and tested. This model enabled us to assess the contributions of individual enterotoxin genes to disease with minimal potential for polar effects or other regulatory interference. This additive model provided a good tool to evaluate the contribution of individual enterotoxins to ETEC colibacillosis in gnotobiotic piglets. This approach enabled us to rule out influence of the plasmid vector of animal disease. Future opportunities made available with this model include the possibility of investigating possible synergy between various enterotoxins in the development of diarrheal disease.

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