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## Comparison of the Contributions of Heat-Labile Enterotoxin and Heat-Stable Enterotoxin b to the Virulence of Enterotoxigenic *Escherichia coli* in F4ac Receptor-Positive Young Pigs<sup>∇</sup>

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**In swine, the most common and severe enterotoxigenic *Escherichia coli* (ETEC) infections are caused by strains that express K88 (F4)<sup>+</sup> fimbriae, heat-labile enterotoxin (LT), heat-stable enterotoxin b (STb), and enteroaggregative *E. coli* heat-stable toxin 1. Previous studies based on a design that involved enterotoxin genes cloned into a nontoxigenic fimbriated strain have suggested that LT but not STb plays an important role in dehydrating diarrheal disease in piglets <1 week old and also enhances bacterial colonization of the intestine. In the present study, we compared these two toxins in terms of importance for piglets >1 week old with a study design that involved construction of isogenic single- and double-deletion mutants and inoculation of 9-day-old F4ac receptor-positive gnotobiotic piglets. Based on the postinoculation percent weight change per h and serum bicarbonate concentrations, the virulence of the STb<sup>-</sup> mutant ( $\Delta estB$ ) did not significantly differ from that of the parent. However, deletion of the LT genes ( $\Delta eltAB$ ) in the STb<sup>-</sup> mutant resulted in a complete abrogation of weight loss, dehydration, and metabolic acidosis in inoculated pigs, and LT complementation restored the virulence of this strain. These results support the hypothesis that LT is a more significant contributor than STb to the virulence of F4<sup>+</sup> ETEC infections in young F4ac receptor-positive pigs less than 2 weeks old. However, in contrast to previous studies with gnotobiotic piglets, there was no evidence that the expression of LT enhanced the ability of the F4<sup>+</sup> ETEC strain to colonize the small intestine.**

Enterotoxigenic *Escherichia coli* (ETEC) is the most common bacterial cause of diarrhea in the world, annually affecting up to 400,000,000 children under 5 years of age in developing countries and causing 380,000 to 700,000 deaths annually (26, 50). In humans, the clinical appearance of ETEC infection is identical to that of cholera, with severe dehydrating illness not uncommonly seen in adults (39, 47). ETEC infection is also an important cause of disease of animals, especially pigs and calves (34). Economically, enteric colibacillosis with ETEC infection as its principal cause is one of the most important diseases affecting the swine industry (55). The most common and severe ETEC infections in swine are those caused by strains that express K88 (F4) fimbriae (6, 17, 18, 55).

Extensive research has been conducted over the past 4 decades in an attempt to elucidate the virulence factors of ETEC and the associated pathogenesis. To date, these studies have convincingly demonstrated that fimbriae and enterotoxins play important roles in disease (35), but the pathogenesis is much more complicated than previously thought (14). Early studies had shown that the binding of fimbriae to enterocytes is necessary for colonization and is hence a major step in pathogen-

esis (7, 46). In addition, these studies have shown that the susceptibility of swine to F4<sup>+</sup> ETEC is inherited as an autosomal dominant trait and determined by the expression of receptors on enterocyte brush borders (49). Porcine F4ac receptors (F4acR) have been reported to include glycoproteins with relative molecular masses of 45 to 70 kDa (52), intestinal mucin-type sialoglycoproteins (IMTGP) with relative masses of 210 and 240 kDa (IMTGP-1 and IMTGP-2, respectively) (15, 16, 21), and the mucin 4 protein (24, 25, 38). The 45- to 70-kDa glycoproteins are thought to make up a *bcd* receptor that binds to F4ab, F4ac, and F4ad (51). IMTGP-1 and -2 are thought to make up a *bc* receptor that binds to F4ab and F4ac (8, 15, 16). Other receptors exist that bind F4ab and F4ad (22). In the case of F4ab and F4ac, the presence of IMTGP-1 and -2 is more highly correlated with disease susceptibility than brush border adherence (19). However, the 210- and 240-kDa IMTGPs have not been sequenced, and their relationship to mucin 4, if any, has not been determined.

An XbaI polymorphism in intron 7 of the mucin 4 gene (*MUC4*) was shown to be in strong linkage disequilibrium with the ETEC *F4acR* locus, and this finding was used to develop a DNA marker-based (PCR-restriction fragment length polymorphism) test to allow genotyping for F4ab/ac resistance/susceptibility as defined by the brush border adherence assay (24, 25). Three different genotypes were observed and called homozygous resistant (RR), heterozygous susceptible (SR), and homozygous susceptible (SS) (24, 25). In addition, a g.243A→G mutation in intron 17 of *MUC4* was identified as being significantly associated with susceptibility/resistance to

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

Strain	Relevant properties	Plasmid	Source or reference
WAM2317	Nal <sup>r</sup> mutant of wild-type porcine ETEC isolate 2534-86 (O8:K87:NM:F4ac LT <sup>+</sup> STb <sup>+</sup> AstA <sup>+</sup> )		6
DH5 $\alpha$	K-12 laboratory strain		4
JM109	K-12 laboratory strain		44
G58-1	Nonpathogenic, wild type		4
NADC 2290	K-12 strain HB101		9
NADC 2329	HB101; STb <sup>+</sup>	pRAS1	9
NADC 2787	HB101	pBR322	T. Casey
1836-2	Wild-type porcine <i>E. coli</i> isolate; O8:F4ac <sup>+</sup> AstA <sup>+</sup>		54
8035	1836-2; LT <sup>+</sup> (pBR322/ <i>eltAB</i> )	pWZLT	54
MUN283	S17-1 $\lambda$ pir; Km <sup>r</sup> Amp <sup>r</sup> $\Delta$ <i>eltAB::aphA</i>	pMUN283	6
MUN297	Nal <sup>r</sup> Km <sup>r</sup> STb <sup>-</sup> mutant of WAM2317		This study
MUN298	Nal <sup>r</sup> Km <sup>r</sup> Amp <sup>r</sup> STb <sup>-</sup> mutant of WAM2317 complemented with STb	pRAS1	This study
MUN299	Nal <sup>r</sup> Km <sup>r</sup> LT <sup>-</sup> mutant of WAM2317		This study
MUN300	Nal <sup>r</sup> Km <sup>r</sup> Cm <sup>r</sup> LT <sup>-</sup> STb <sup>-</sup> mutant of WAM2317		This study
MUN301	Nal <sup>r</sup> Km <sup>r</sup> Cm <sup>r</sup> Amp <sup>r</sup> LT <sup>-</sup> STb <sup>-</sup> mutant of WAM2317 complemented with LT	pBR322	This study
MUN302	DH5 $\alpha$ ; LT <sup>+</sup>	pBR322	This study
MUN303	DH5 $\alpha$	pBR220	This study
BT340	ts-rep; Amp <sup>r</sup> Cm <sup>r</sup> (FLP recombinase)	pCP20	11
BW25113	ts-rep; Amp <sup>r</sup> (araBp-gam-bet-exo recombinase)	pKD46	13
BW25141	(FRT) Amp <sup>r</sup> Cm <sup>r</sup>	pKD3	13
BW25141	(FRT) Amp <sup>r</sup> Km <sup>r</sup>	pKD4	13

ETEC F4ab/ac infection in pigs based on the brush border adhesion assay (38). Different linkage disequilibrium values between ETEC F4ab and F4ac adhesion phenotypes were detected, suggesting that the inheritance of F4ab and F4ac receptors might be under the control of two closely linked loci (38).

Although fimbria-receptor binding plays a key role in ETEC infection, enterotoxins are thought to be the main virulence factors that cause diarrhea and also may play a role in colonization (1, 6, 54). Clinically, ETEC enterotoxin-induced water and electrolyte secretion may result in life-threatening dehydration and metabolic acidosis (6, 23, 35, 39, 45, 47). In the case of ETEC infections in swine, causative strains may express one or more of four different enterotoxins, including heat-labile enterotoxin (LT, encoded by *eltAB*), heat-stable enterotoxin a (STa or STI, encoded by *estA*), heat-stable enterotoxin b (STb or STII, encoded by *estB*), and enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1, encoded by *astA*) (3, 20, 27, 37). Individual F4<sup>+</sup> ETEC isolates from swine commonly produce both LT and STb (30, 33) and are often PCR positive for *astA* (12, 36, 53). One porcine F4ac<sup>+</sup> ETEC strain that expresses LT and STb (WAM2317, serotype O8:K87:NM) was shown to concurrently express EAST1 (6); however, the significance of EAST1 in disease has not been determined.

We have conducted two previous studies with gnotobiotic piglets with isogenic F4ac<sup>+</sup> ETEC strains that varied in their abilities to produce LT and STb in an attempt to clarify the relative importances of these enterotoxins in disease. In the first study, we found that the deletion of *eltAB* in strain WAM2317 resulted in significantly reduced virulence and ability to colonize the small intestine (6). However, this LT<sup>-</sup> strain, which still expressed STb and EAST1, caused diarrhea and a moribund condition within 96 h in 60% of the piglets (6). The second study involved 5-day-old gnotobiotic piglets inoculated with strain 1836-2 (serotype O8:H4:F4ac), which lacks *eltAB* and *estB* but contains *astA*; whether or not this strain

expresses EAST1 has not been determined. Strain 1836-2 was transformed with a plasmid (pBR322) into which *eltAB* or *estB* had been cloned (54). The LT<sup>+</sup> strain caused diarrhea resulting in dehydration within 18 h, whereas the STb<sup>+</sup> strain did not, although diarrhea still developed in some of the piglets; the LT<sup>+</sup> strain colonized the ileum at levels significantly greater than those seen for the F4<sup>+</sup> STb<sup>+</sup> and F4<sup>+</sup> nontoxicogenic strains (54). In both studies, the pigs were confirmed to express F4acR, as evidenced by IMTGP and/or brush border binding, but were not tested for *MUC4*.

Based on these studies, we hypothesized that both LT and STb contribute significantly to virulence in the 9-day-old gnotobiotic piglet model, but LT plays a greater role than STb. In addition, we hypothesized that LT but not STb contributes significantly to colonization of the small intestine. In the present study, we tested these hypotheses by constructing deletion and complemented mutants and inoculating them into 9-day-old F4acR<sup>+</sup> gnotobiotic piglets selected on the basis of the *MUC4* DNA marker test (24, 25, 40). Deletion of *estB* did not significantly reduce and its complementation did not significantly increase virulence. However, deletion of *eltAB* in the STb<sup>-</sup> mutant caused total abrogation of weight loss, dehydration, and metabolic acidosis and LT complementation restored the virulence of the STb<sup>-</sup> LT<sup>-</sup> mutant. Our findings support the hypothesis that LT is a more significant contributor to virulence in young (i.e., <2-week-old) F4acR<sup>+</sup> piglets than is STb; however, in contrast to our previous studies, there was no evidence that LT enhanced colonization of the small intestine.

#### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. Strain WAM2317, a spontaneous nalidixic acid (Nal<sup>r</sup>) mutant of porcine ETEC strain 2534-86 (6) was used as the parent to construct isogenic mutants. Strain 8035 harboring recombinant plasmid pWZLT (54) and strain MUN283 (6) were previously described. STb<sup>+</sup> strain NADC 2329 (9, 51) and STb<sup>-</sup> strains NADC 2290 (4, 51) and NADC 2787 were kindly provided by T.

Casey. Plasmids pCP20, pKD46, pKD3, and pKD4 were purchased from the *E. coli* Genetic Stock Center, Yale University.

**DNA isolation and manipulations.** Plasmid DNA was isolated from bacterial cultures as previously described (4). Standard recombinant DNA procedures and Southern blotting were conducted using standard methods (4, 44). Gene probes for Southern blotting were directly labeled with horseradish peroxidase, and the hybridization products were detected with the ECL direct nucleic acid chemiluminescence detection system (Amersham Pharmacia).

**Mutant construction and verification.** Site-directed mutagenesis was accomplished using the bacteriophage lambda recombinase system ( $\lambda$ -Red) as previously described (13). To construct strain MUN297, the *estB* gene from WAM2317 was disrupted using linear PCR products generated by primers targeting the kanamycin (Km) cassette with FRT sites from the pKD4 template but flanked by 60-nucleotide homologies of either the upstream or downstream region of the *estB* gene. The 81-mer oligonucleotide primers used were StbKU (5'-AAAGGAGAGCTTCGTCACATTTTTTTGACTTGACTCATATAAAG CACCCTGGTATAAGTGTGTAGGCTGGAGCTGCTTCG-3') and StbKL (5'-TGGTGTGAATGCATTGATAAATATATTACCCTCTTTGCTGC AACCATATTGGGCACGGCTGACATGGGAATTAGC-3'). WAM2317 transformants carrying the Red helper plasmid (pKD46) were made electrocompetent as described previously (32) and electroporated with PCR products by use of standard procedures (31). Electroporated cells were spread plated on LB plates containing Km (20  $\mu$ g/ml) to select for Km<sup>r</sup> transformants.

To construct strain MUN300, the deletion-insertion-inactivated *eltAB* genes from strain MUN283 (6) were PCR amplified using primers LTABU (5'-CGG ATTGCTCTTGTATGAT-3') and LTABL (5'-GATCGGTATTGCCCTCCTC TCAC-3'). The PCR products were used to transform L-arabinose-induced electrocompetent WAM2317 cells carrying pKD46 to generate strain MUN299. Transformants were selected on LB plates containing Km (20  $\mu$ g/ml) at 30°C. Subsequently, the *estB* gene from strain MUN299 harboring pKD46 was disrupted by  $\lambda$ -Red-mediated homologous recombination following transformation of the latter cells with PCR products containing the chloramphenicol (Cm) cassette flanked by 60-nucleotide homologies of either the upstream or downstream region of the *estB* gene, thus generating strain MUN300. The PCR amplicons were generated by primers StbKU and StbKL, employing pKD3 as the template. Transformants were spread plated on LB with chloramphenicol (LB-Cm; 15  $\mu$ g/ml). Constructs were verified by Southern blotting, PCR, and DNA sequencing.

For STb complementation, plasmid pRAS1 (9, 51) was electrotransformed into MUN297, generating isogenic strain MUN298. To complement LT, plasmid pWZLT, containing cloned *eltAB* (54), was BamHI linearized and the 5,635-bp BamHI products were purified, religated using T4 DNA ligase (Invitrogen), and electrotransformed into MUN300, generating strain MUN301. Gene complementation was verified using PCR.

**Y1 mouse adrenal tumor cell assay for LT expression.** The Y1 cell assay for LT activity was conducted as previously described (43). Confluent monolayers of Y1 adrenal cells (ATCCL-79) were exposed to 100  $\mu$ l of cell extract per well from each strain and cells were examined for rounding by phase-contrast microscopy after 6 h of incubation.

**Detection of LT expression by GM<sub>1</sub> ELISA.** Bacterial strains were cultured in brain heart infusion (BHI) broth (Bacto) containing 2% Casamino Acids (Difco) with lincomycin (90  $\mu$ g/ml) in 15-ml tubes for 24 h at 37°C. After the 24-h incubation, polymyxin B (100  $\mu$ g/ml) was added to the different cultures to augment LT release (10) followed by further incubation at 37°C for either 24 or 48 h. Cell-free supernatants were assayed for LT activity by GM<sub>1</sub> enzyme-linked immunosorbent assay (ELISA) as previously described (41).

**Detection of expression of STb by ELISA.** Bacterial strains were cultured overnight in BHI containing 2% Casamino Acids at 37°C, and expression of STb was detected by ELISA using previously published methods (48).

**Genomic characterization of pigs for ETEC F4<sup>+</sup>ab/ac resistance/susceptibility.** Boars mated to prospective gravid specific-pathogen-free purebred Yorkshire donor sows (Gemar Farms, Sutton, NE) for gnotobiotic piglets were screened for the ETEC F4ab/ac resistance or susceptibility allele by use of the DNA marker-based test as described previously (24). This test relies on an XbaI polymorphism in intron 7 of the porcine *MUC4* gene. The PCR-restriction fragment length polymorphism assay was done on DNA extracted from EDTA-anticoagulated blood samples with primers previously described (22). Subsequently, sows that were mated to boars with a homozygous susceptible genotype were screened by the DNA marker test as described above, and those found with the homozygous susceptible genotype after analysis were selected as donors for the gnotobiotic piglets.

**Gnotobiotic piglet experiments.** A total of 25 gnotobiotic purebred Yorkshire piglets from two litters were derived by closed hysterectomy and reared in sterile

isolator units according to standard procedures (28). Each litter was divided at random into five groups, with one group each reared in an individual isolator unit. At 1, 6, and 9 days of age, nasal and rectal swab samples were obtained from each piglet and cultured as described previously (6) to test for preinoculation sterility. When 9 days old, each piglet was examined and weighed, and the rectal temperature was taken. Immediately thereafter, an inoculum of  $5 \times 10^9$  CFU of WAM2317, MUN297, MUN298, MUN300, or MUN301 was poured into 50 ml of sterile milk replacer and fed to each piglet. Only one strain was introduced into each isolator unit.

After inoculation, piglets were examined for decreased rectal temperature, anorexia, vomiting, diarrhea, depression, and a moribund condition. Piglets were monitored for the above parameters every 4 h until 96 h postinoculation (p.i.) or when they became moribund, at which time they were subjected to blood sampling, euthanasia, and necropsy. A moribund condition was defined as previously described (6) with slight modification, viz., severe dehydration (evidenced by loss of skin turgidity and eyes sunken in the orbits), severe (>15%) weight loss, depression, hypothermia, and severe weakness. The rate of weight change for each piglet was calculated by dividing the difference between the preinoculation and preinoculation body weights by the number of hours p.i. at necropsy. Experiments were preapproved by the University of Nebraska—Lincoln Institutional Animal Care and Use Committee.

Preeuthanasia blood samples, collected aseptically, were processed for bacterial culture, hematocrit (HCT) determination, and determination of serum electrolytes and other analytes, as previously described (6). The serum color and transparency were observed grossly for evidence of hemolysis, icterus, and lipemia. Concentrations of the following analytes were measured in serum samples by use of an Olympus AU640e chemistry analyzer: urea nitrogen, creatinine, sodium (Na<sup>+</sup>), potassium (K<sup>+</sup>), chloride (Cl<sup>-</sup>), bicarbonate (HCO<sub>3</sub><sup>-</sup>), total protein, and phosphorus. Anion gap was calculated using the following formula: [Na<sup>+</sup>] - ([HCO<sub>3</sub><sup>-</sup>] + [Cl<sup>-</sup>]). DNA was isolated from preeuthanasia heparinized blood samples from each piglet and the DNA marker test was carried out to confirm that all piglets were of the homozygous susceptible genotype as described above.

Specimens of duodenum (5 cm distal to the pyloric valve), mid-jejunum (half the distance between the pyloric and ileocecal valves), and ileum (5 cm proximal to the ileocecal valve) were collected aseptically at necropsy. These tissues were processed and cultured on LB medium with selective antibiotics to determine the CFU of the respective inocula per g of tissue, as previously described (6). NaI (50  $\mu$ g/ml; LB-NaI) was used for WAM2317, Km (LB-Km; 30  $\mu$ g/ml) was used for MUN297 and MUN300, and ampicillin (Amp) (LB-Amp; 100  $\mu$ g/ml) was used for MUN301. Bacterial isolates from the preeuthanasia blood samples were cultured on antibiotic selection plates as described above. Isolates from the blood and tissues were identified as *E. coli* by standard methods.

**Histopathology, immunohistochemistry (IHC), and image analysis.** At necropsy, specimens were obtained, fixed in 10% neutral buffered formalin for histopathological examination, and processed as previously described (6). Five sections from each of five specific sites in the small intestine (one duodenum, three jejunum, and one ileum) were stained for *E. coli* O8 by an immunohistochemical procedure (6, 42). Microscopic digitalized images of the mucosa of each of three of the five immunohistochemically stained intestinal sections on each area of intestine examined were collected by using a 10 $\times$  lens objective with a color digital camera. All images were recorded under the same microscope and camera settings, i.e., level of transmitted light, image size, contrast, brightness, and exposure time. Four frames of images from each section, each covering actual sample areas of 860 by 650  $\mu$ m (x and y axes, respectively), were recorded with 860- by 650- $\mu$ m intervals between collected images from each of the sections. The bottom edge of the frame in each image (i.e., the x axis) was positioned at the basal lamina of the lamina epithelialis mucosae. All images were saved in the TIFF format and quantitatively analyzed with a SIS AnalySIS Opti imaging analysis program (Soft Imaging System, Inc., Lakewood, CO) for the areas ( $\mu$ m<sup>2</sup>) covered by positive staining of *E. coli* O8<sup>+</sup> bacteria. These areas represented the colonization scores.

**Analysis of brush borders for susceptibility to adhesion of F4<sup>+</sup> *E. coli* and presence of IMTGP.** The F4ac adherence phenotype of each piglet was determined via brush border assays (5). Approximately 10 cm of small intestine, beginning 5 cm distal to the pyloric valve and extending to the ligament of Treitz, was collected for the brush border assay. Brush border specimens were subsequently tested for the presence of IMTGP by the biotinylated adhesin overlay assay as previously described (21).

**Statistical analyses.** Data were analyzed using mixed models which included a fixed effect for strain and random effects for litter and treatment-litter interaction. For the IHC-dependent variable, additional fixed effects were included for position along the intestine (i.e., duodenum, jejunum 1, jejunum 2, jejunum 3,

and ileum) and its interaction with the strain. The IHC analysis also included a random effect for animal within litter. Contrasts were used to test preplanned comparisons. The IHC- and CFU-dependent variables were log transformed prior to analysis. For all parameters, least-squares means for the different treatment groups were compared by using general *t* values. Calculated *P* values of < 0.05 were considered significant.

To test for a linear association between LT and weight change, a second analysis of weight change was carried out which added LT as a covariate. Type I sums of squares were used to test for linear association and lack of fit. To calculate an adjusted *R*<sup>2</sup> value, a null model and a covariate model were fit. The null model included random effects for litter, litter-treatment interaction, and error in addition to the intercept. From the null model, the total residual variance was estimated as the sum of the three variance estimates. A covariate model added the one or more covariates to the null model. From a covariate model, a covariate residual variance was calculated. An adjusted *R*<sup>2</sup> value was then calculated as the reduction in the estimated residual variance in the covariate model as a portion of the estimated total residual variance.

## RESULTS

**Validation of strain constructs.** Mutant strains with deletions in *estB* (MUN297) and both *eltAB* and *estB* (MUN300) and respective complemented mutants (STb<sup>+</sup> MUN298 and LT<sup>+</sup> MUN301) were successfully constructed from the WAM2317 parent; however, attempts to eliminate the antibiotic resistance cassettes with the helper plasmid expressing the FLP recombinase (pCP20) were unsuccessful. Retention of the antibiotic resistance genes did not affect the growth of any of the strains, as growth curves were nearly identical (data not shown). Gene deletions were confirmed by PCR, Southern hybridization, and DNA sequencing of junction sites, as described above. Expression of STb by WAM2317 and MUN298 was demonstrated by ELISA with strain 2329 as a positive control for STb expression (Fig. 1). LT activity was demonstrated in cultures of WAM2317, MUN297, MUN298, and MUN301 but not MUN300 by GM<sub>1</sub> ELISA (Fig. 2) and mouse Y1 adrenal tumor cell assays (data not shown), using cholera toxin as a standard and MUN302 (*E. coli* strain DH5 $\alpha$  containing *eltAB* cloned into pBR322) as a positive control.

**Confirmation of F4acR<sup>+</sup> status and preinoculation sterility cultures of piglets.** Gnotobiotic piglets were derived from parents homozygous for the *MUC4* allele associated with F4acR (24, 25, 40). As a confirmation of F4acR expression, duodenal brush borders of all piglets were retrospectively demonstrated to bind to F4ac ETEC in brush border assays and express IMTGP as detected by the biotinylated adhesin overlay assay (data not shown). In addition, heparinized blood samples from all piglets at the conclusion of the study were confirmed to be homozygous for the *MUC4* allele associated with F4acR, as assessed by the DNA marker assay (data not shown).

Preinoculation cultures of the nares and rectums of all piglets at 1 and 6 days of age were sterile; however, when the piglets were 9 days old, 3 of the 25 had bacterial contaminants. In one isolator unit containing two piglets subsequently inoculated with MUN300, *Pseudomonas aeruginosa* was isolated from both the nares and rectum. In another isolator unit, culture of the nares and rectum yielded a *Micrococcus* sp. in one of three piglets subsequently inoculated with MUN301. Isolates of each contaminant were subcultured on the selective media used for intestinal colonization counts from the piglets (i.e., LB-Km for MUN300 and LB-Amp for MUN301), and neither grew on the respective count plate media. No clinical or histological effects of *P. aeruginosa* were apparent; however,

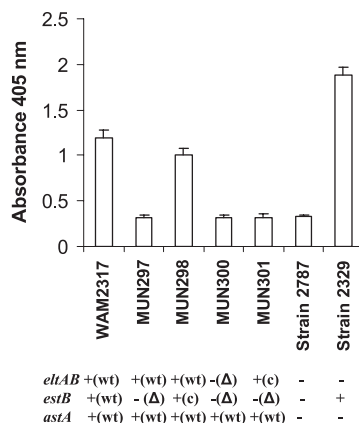


FIG. 1. ELISA to detect STb expression by different isogenic ETEC strains following in vitro culture. The following strains were tested: NaI<sup>+</sup> F4ac<sup>+</sup> ETEC strain WAM2317 (LT<sup>+</sup> STb<sup>+</sup>) and isogenic derivatives STb<sup>-</sup> ( $\Delta$ *estB*) mutant MUN297, STb-complemented  $\Delta$ *estB* mutant MUN298, LT<sup>-</sup> STb<sup>-</sup> ( $\Delta$ *eltAB*  $\Delta$ *estB*) mutant MUN300, and LT-complemented  $\Delta$ *eltAB*  $\Delta$ *estB* mutant MUN301. Other strains used as controls included 2787 [STb<sup>-</sup> laboratory *E. coli* strain HB101(pBR322)] and 2329 [STb<sup>+</sup> clone HB101(pRAS1) (9)]. Strains were grown overnight in BHI containing 2% Casamino Acids and 100  $\mu$ l of cell-free supernatants (1:2 dilutions) were used to coat individual wells in 96-well plates. A 1:2,000 dilution of primary antibodies (rabbit anti-STb serum) and a 1:2,500 dilution of secondary antibodies (alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G) were used. For each experiment, the supernatant from each strain was tested in three different wells (technical replicates). Measurements of optical density at 405 nm (mean  $\pm$  standard error of the mean [SEM]) are shown and represent the outcomes of two independent experiments (biological replicates). wt, wild type; c, complemented.

the MUN301 piglet with *Micrococcus* sp. had a perineal dermatitis.

**LT contributes more significantly to the severity of diarrhea than does STb, and deletion of both *estB* and *eltAB* prevented weight loss in young (<2-week-old) F4acR<sup>+</sup> gnotobiotic piglets.** We hypothesized that both LT and STb contribute significantly to virulence in the 9-day-old F4acR<sup>+</sup> gnotobiotic piglets, with LT playing a greater role than STb. In addition, we hypothesized that LT but not STb contributes significantly to colonization of the small intestine. All piglets inoculated with strains other than the STb<sup>-</sup> LT<sup>-</sup> mutant (MUN300) developed severe dehydrating diarrhea and weight loss and subsequently a moribund condition. Overall, of the 20 piglets that became moribund, this condition was determined to occur over a range of 16 to 96 (mean, 44.2) h p.i. One MUN301 piglet was moribund at 96 h p.i., whereas all others had become moribund by 71 h p.i. Two piglets (one inoculated with WAM2317 and one with MUN301) died before they could be euthanized. The range of weight loss in the piglets that became moribund was 15.1 to 31.1% (mean, 22.8%). All piglets inoculated with MUN300 developed mild diarrhea but gained weight and remained alert and active during the 96-h-p.i. period. Numerically, the mean weight gain per h in the two MUN300 piglets with *Pseudomonas aeruginosa* contamination (0.08% per h) was the same as that of their noncontaminated treatment cohorts (0.08% per h); hence, this contamination was not a significant factor in the study. Interestingly, based on enrichment cultures, 4 of 5 MUN300 piglets were bacteremic with the

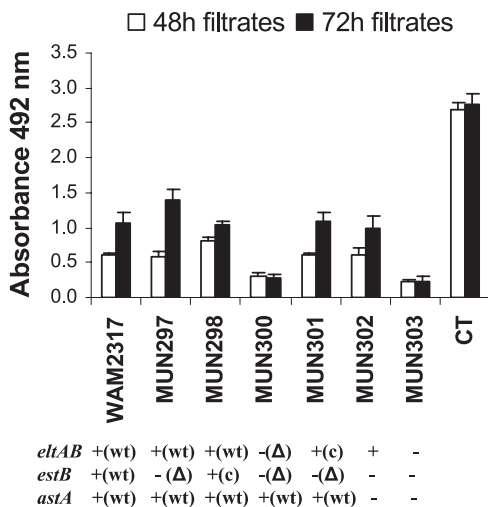
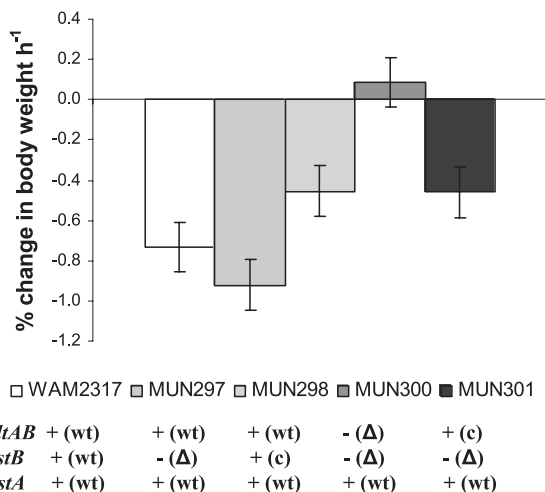


FIG. 2. GM<sub>1</sub> ELISA to detect LT expression by different isogenic ETEC strains following in vitro culture. Individual wells of 96-well plates were coated with 100 µl of GM<sub>1</sub> ganglioside (1.0 µg/ml) in 0.1 M carbonate buffer (pH 9.6). Following overnight incubation, 100 µl of a 1:2 dilution of cell-free supernatant of culture material was dispensed into each well. The following strains were tested: NaI<sup>r</sup> F4ac<sup>+</sup> ETEC strain WAM2317 (LT<sup>+</sup> STb<sup>+</sup>) and isogenic derivatives STb<sup>-</sup> ( $\Delta estB$ ) mutant MUN297, STb-complemented  $\Delta estB$  mutant MUN298, LT<sup>-</sup> STb<sup>-</sup> ( $\Delta eltAB \Delta estB$ ) mutant MUN300, and LT-complemented  $\Delta eltAB \Delta estB$  mutant MUN301. In addition, LT<sup>+</sup> clone MUN302 [*E. coli* DH5 $\alpha$ (pBR322/*eltAB*)] and MUN303 [*E. coli* DH5 $\alpha$ (pBR322)] were used as positive and negative controls, respectively. Strains were cultured in BHI containing 2% Casamino Acids for 48 or 72 h, and cell-free supernatants were collected. The dilution of the primary antibodies (rabbit anti-cholera toxin serum) was 1:2,000, while that of the secondary antibodies (horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G) was 1:2,500. For each experiment, the supernatant from each strain was tested in eight different wells (technical replicates). Mean  $\pm$  SEM measurements of optical density at 492 nm are shown and represent the outcomes of two independent experiments (biological replicates). wt, wild type; c, complemented.

inoculum strain at the time of euthanasia; 13 of 15 piglets in the other treatment groups in which terminal blood samples could be obtained were also bacteremic. The mean weight loss per h in the MUN301 group including the piglet with *Micrococcus* sp. contamination was -0.47%, compared to a mean of -0.46% with this piglet excluded; hence, this contaminant also did not significantly contribute to weight loss in the study. Histologically, all piglets had bacterial colonization of the intestine compatible with enteric colibacillosis. Those that became moribund had mucosal lesions in the small intestine consistent with ischemic bowel necrosis thought to be due to reduced perfusion caused by hypovolemic shock (6). Those in the most severe stages of ischemic bowel necrosis also had fibrin thrombi, hemorrhages, and necrosis in the small intestines, stomach, liver, kidney, and spleen, consistent with disseminated intravascular coagulation (6).

Based on the p.i. percent weight change per h, the STb<sup>-</sup> mutant (MUN297) did not have low virulence compared with the parent (Fig. 3). Further, *estB* complementation (MUN298) did not increase and in fact resulted in a significantly decreased rate of weight loss ( $P = 0.047$ ). Piglets inoculated with the LT<sup>+</sup> complemented  $\Delta estB \Delta eltAB$  mutant (MUN301) had a mean rate of weight loss that was 63% of that of the parent. To further assess the relationship between LT and weight loss in the piglets, multiple linear regression between LT or STb expression (based on ELISA measurements in culture supernatants not adjusted for protein concentrations) and p.i. weight change per h was conducted. The analysis indicated that approximately 58% of the variation in the rate of weight loss was explained by the LT expression levels of the respective inoculum strain (adjusted  $R^2 = 0.58$ ,  $P = 0.004$ ) (Fig. 4), whereas no relationship between STb expression and weight change was detected (adjusted  $R^2 = -0.05$ ,  $P = 0.4873$ ). Numerically, the mean rectal temperatures decreased after challenge in all treatment groups except the MUN300 group, but the differences were not significantly different (data not shown). Based



**Pre-planned comparisons:**

Comparison	P value	Effect measured
WAM2317 vs. MUN297	0.320	STb
MUN298 vs. MUN297	0.047*	STb
MUN297 vs. MUN300	0.004*	LT
MUN301 vs. MUN300	0.028*	LT
WAM2317 vs. MUN300	0.007*	LT + STb

FIG. 3. Weight change per h measurements for gnotobiotic piglets inoculated with different isogenic ETEC strains varying in their abilities to express LT and STb. Results are presented as least-squares means  $\pm$  SEM. Groups of piglets were challenged with  $5 \times 10^9$  CFU of NaI<sup>r</sup> F4ac<sup>+</sup> ETEC strain WAM2317 (LT<sup>+</sup> STb<sup>+</sup>), STb<sup>-</sup> ( $\Delta estB$ ) mutant MUN297, STb-complemented  $\Delta estB$  mutant MUN298; LT<sup>-</sup> STb<sup>-</sup> ( $\Delta eltAB \Delta estB$ ) mutant MUN300, or LT-complemented  $\Delta eltAB \Delta estB$  mutant MUN301. Piglets were allowed to progress to a moribund condition or were euthanized at 96 h p.i. if a moribund condition did not develop. wt, wild type; c, complemented; \*, a P value of <0.05 is significant.

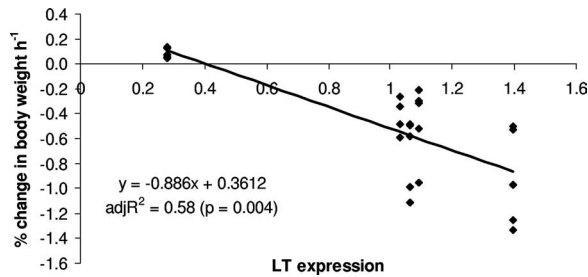


FIG. 4. Linear regression between in vitro LT expression by strains (GM<sub>1</sub> ELISA) and percent weight change per h in piglets after inoculation. To test for a linear association between LT and weight change, an analysis of weight change was carried out which added LT as a covariate. Type I sums of squares were used to test for linear association and lack of fit. To calculate an adjusted  $R^2$  (adj $R^2$ ) value, a null model and a covariate model were fit. The null model included random effects for litter, litter-treatment interaction, and error in addition to the intercept. From the null model, the total residual variance was estimated as the sum of the three variance estimates. A covariate model added the one or more covariates to the null model. From a covariate model, a covariate residual variance was calculated. An adjusted  $R^2$  value was then calculated as the reduction in the estimated residual variance in the covariate model as a portion of the estimated total residual variance.

on p.i. weight change per h, the results indicated that LT is a more significant contributor than STb to the virulence of F4<sup>+</sup> ETEC infection in the young (<2-week-old) F4ac<sup>+</sup> piglet, and deletion of both *estB* and *eltAB* reduced the severity of diarrhea to one in which weight loss did not occur.

**Deletion of *estB* and *eltAB* prevented the development of dehydration and electrolyte imbalances.** We hypothesized that both LT and STb inactivation would reduce the severity of bicarbonate loss in the piglets and therefore attenuate the development of hyperchloremic metabolic acidosis. To address this hypothesis, bicarbonate concentrations were measured for serum samples obtained immediately prior to euthanasia. Serum bicarbonate concentrations in the piglets inoculated with the LT<sup>-</sup> STb<sup>-</sup> mutant (MUN300) were unaffected or minimally affected, whereas all piglets inoculated with the other strains developed severe metabolic acidosis. The mean p.i. HCO<sub>3</sub><sup>-</sup> concentration of the MUN300 group (19.58 ± 2.4 mmol liter<sup>-1</sup>) was significantly higher than that of the WAM2317, MUN297, MUN298, and MUN301 groups (Fig. 5). There were no significant differences in mean p.i. HCO<sub>3</sub><sup>-</sup> concentrations among the other groups, but the lowest mean values occurred with WAM2317 and MUN298 (LT<sup>+</sup> STb<sup>+</sup> groups). The mean Cl<sup>-</sup> concentrations were significantly low in the MUN300 group compared to each of the other groups, consistent with the conservation of Cl<sup>-</sup> to maintain electro-neutrality with the loss of HCO<sub>3</sub><sup>-</sup>. The mean anion gaps for each group were greater than that of the MUN300 group, with the greatest values seen for the WAM2317 and MUN298 groups (data not shown). Serum phosphorus values changed in a similar pattern, but changes were mild and not enough to account for the anion gap changes (data not shown). Increased anion gaps suggested renal retention of anions associated with dehydration, a decreased glomerular filtration rate, and/or an increase in lactate associated with poor tissue perfusion and increased anaerobic glycolysis. Lactate concentrations were not measured.

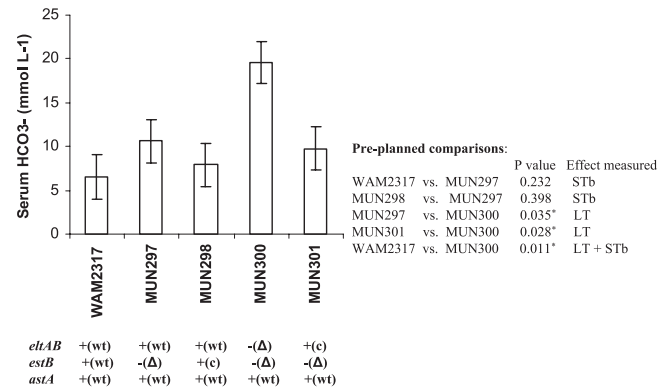


FIG. 5. Serum bicarbonate measurements for gnotobiotic piglets inoculated with different isogenic ETEC strains varying in their abilities to express LT and STb. Results are presented as least-squares means ± SEM. Groups of piglets were challenged with  $5 \times 10^9$  CFU of NaI<sup>r</sup> F4ac<sup>+</sup> ETEC strain WAM2317 (LT<sup>+</sup> STb<sup>+</sup>), STb<sup>-</sup> ( $\Delta$ *estB*) mutant MUN297, STb-complemented  $\Delta$ *estB* mutant MUN298, LT<sup>-</sup> STb<sup>-</sup> ( $\Delta$ *eltAB*  $\Delta$ *estB*) mutant MUN300, or LT-complemented  $\Delta$ *eltAB*  $\Delta$ *estB* mutant MUN301. Piglets were allowed to progress to a moribund condition or were euthanized at 96 h p.i. if a moribund condition did not develop. Serum analyte concentrations were determined from blood samples obtained immediately prior to euthanasia. wt, wild type; c, complemented; \*, a  $P$  value of <0.05 is significant.

The effects of LT and STb expression on the development of dehydration were further assessed by measurement of the HCT, serum total protein, blood urea nitrogen concentration, and serum creatinine concentration in the preeuthanasia blood samples. Increases in each of these parameters were expected to reflect the level of dehydration due to water loss, greater increases being expected with more dehydration (6). Significant differences in the HCT, total protein, and creatinine concentrations between the WAM2317 and MUN300 groups were detected ( $P$  values were 0.0095, 0.0284, and 0.0070, respectively), whereas the differences in urea nitrogen concentrations approached significance ( $P = 0.0536$ ). No significant differences in any of these parameters were detected among the other groups. However, mean values for each analyte were greatest in the WAM2317 and MUN298 groups, lowest in the MUN300 group, and intermediate in the MUN297 and MUN301 groups. This pattern mirrored that of bicarbonate concentrations. Since piglets inoculated with MUN300 had normal HCT, total protein, urea nitrogen, and creatinine concentrations, it was demonstrated that deletion of both *estB* and *eltAB* completely prevented dehydration as well as the development of metabolic acidosis.

**Effect of LT and STb expression on bacterial colonization of the small intestine.** We hypothesized that expression of LT, but not STb, by the respective bacterial strain would enhance its ability to colonize the small intestine (6, 54). No significant differences in mean plate counts were detected, with the exception of those inoculated with MUN298, which were significantly less than MUN297 (Fig. 6A). However, based on the results of IHC, there were no significant differences in colonization scores among any of the strains (Fig. 6B). In addition to the appropriate antibiotic resistance patterns, all bacterial isolates from count plates had the expected phenotype for the respective *E. coli* inoculum strain (beta-hemolytic, lactose fer-



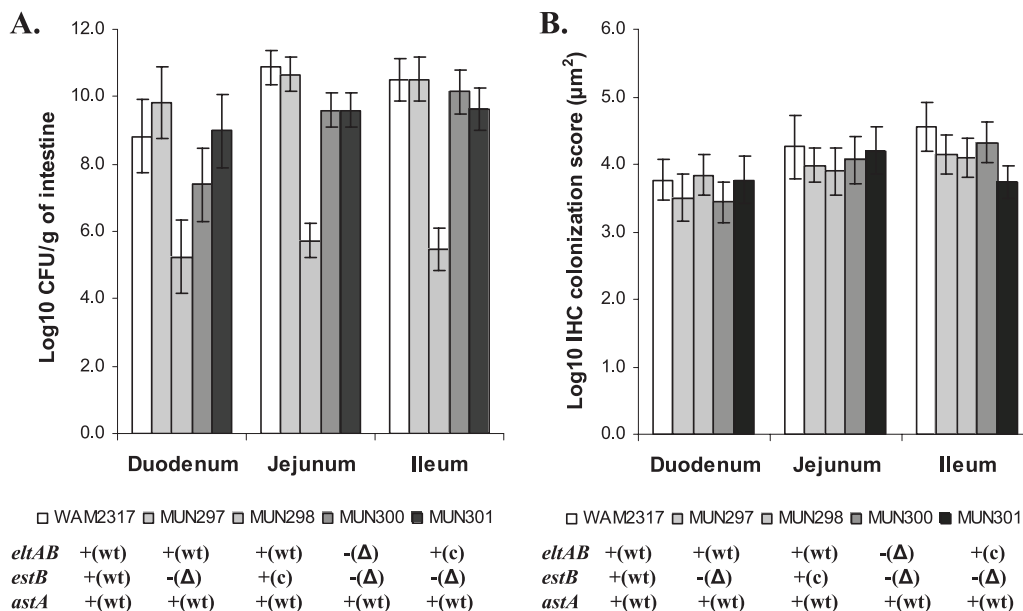


FIG. 6. Small intestinal mucosal bacterial colonization measurements for gnotobiotic piglets inoculated with different isogenic ETEC strains varying in their abilities to express LT and STb. Results are presented as log<sub>10</sub> least-squares means ± SEM. Groups of piglets were inoculated with 5 × 10<sup>9</sup> CFU of NaI<sup>r</sup> F4ac<sup>+</sup> ETEC strain WAM2317 (LT<sup>+</sup> STb<sup>+</sup>), STb<sup>-</sup> ( $\Delta$ *estB*) mutant MUN297, STb-complemented  $\Delta$ *estB* mutant MUN298, LT<sup>-</sup> STb<sup>-</sup> ( $\Delta$ *eltAB*  $\Delta$ *estB*) mutant MUN300, or LT-complemented  $\Delta$ *eltAB*  $\Delta$ *estB* mutant MUN301. Piglets were allowed to progress to a moribund condition or were euthanized at 96 h p.i. if a moribund condition did not develop. Mucosal colonization measurements were determined on proximal duodenal, mid-jejunal, and distal ileal specimens obtained at necropsy. (A) CFU per g of intestine. (B) Area ( $\mu$ m<sup>2</sup>) of mucosa colonized based on image analysis of tissue sections immunohistochemically stained with anti-O8 *E. coli* antiserum. wt, wild type; c, complemented.

menting, indole positive, and acid/acid in triple sugar iron medium tubes).

DISCUSSION

In this study, the contributions of LT and STb to the virulence of an F4ac<sup>+</sup> ETEC strain were studied using F4acR<sup>+</sup> gnotobiotic piglets by use of isogenic deletion mutants constructed via the bacteriophage lambda recombinase system ( $\lambda$ -Red). This study is the first to compare the contributions of these two enterotoxins to virulence using isogenic single- and double-deletion mutants of a naturally occurring porcine pathogen. The data confirm and extend the results of previous studies in that they highlight the importance of LT as a virulence factor and provide evidence that it is a greater contributor to virulence in F4acR<sup>+</sup> piglets <2 weeks old than is STb. Deletion of *eltAB* resulted in significantly reduced ability of the strain to cause weight loss, dehydration, metabolic acidosis, and electrolyte imbalance; complementation restored these effects. By contrast, *estB* deletion did not significantly reduce any of these parameters of virulence, and piglets inoculated with the STb-complemented  $\Delta$ *estB* mutant (MUN298) had a significantly low rate of weight loss compared to the  $\Delta$ *estB* mutant (MUN297). Although we did not detect a significant contribution of STb to virulence based on weight change per h, we cannot conclude that there were no effects, since we did not construct or inoculate piglets with an STb<sup>+</sup>-complemented LT<sup>-</sup> STb<sup>-</sup> strain. The reason for this was a limitation in the number of available isolator units; we had only five available at one time and were forced to use only five treatment groups and the contrasts shown in Fig. 3 and 5. Three piglets in the study

had either *Pseudomonas aeruginosa* (two inoculated with MUN300) or *Micrococcus* sp. (one MUN301 piglet) contamination; however, there was no evidence that these organisms had any significant effect on weight change or any other measured parameter.

Significant differences in clinical pathology parameters of dehydration and electrolyte imbalance were limited primarily to comparisons between the LT<sup>+</sup> STb<sup>+</sup> parent (WAM2317) and the LT<sup>-</sup> STb<sup>-</sup> (MUN300) group; however, there was a pattern in that the most severe alterations were seen in those inoculated with LT<sup>+</sup> STb<sup>+</sup> strains (WAM2317 and MUN298), intermediate changes were seen in the LT<sup>+</sup> STb<sup>-</sup> (MUN297 and MUN301) groups, and complete lack of dehydration or electrolyte imbalance occurred in the LT<sup>-</sup> STb<sup>-</sup> (MUN300) group. The MUN298 group, having a significantly low rate of weight loss compared to the MUN297 group, became moribund on the average at 55.2 h p.i., compared to 30.3 h p.i. for MUN297. Hence, the collection of serum samples at a later time p.i. may have had an impact on the results but was actually less on the average than that of the MUN301 group (57.6 h p.i.). These findings suggest that STb plays an additive role to the effects of LT in young pigs, with the relative contributions to virulence being LT > STb. Two previous studies using different designs found a lack of a significant contribution of STb to the causation of severe diarrhea (9, 54). In those studies, the piglets were inoculated at <1 day and 5 days of age, respectively, and involved different fimbrial types (9) or strains (54). In addition, those studies utilized an additive (gene addition) instead of a subtractive (gene deletion) approach.

In a previous study, we demonstrated that WAM2317 and isogenic LT<sup>-</sup> (MUN285) and an LT-complemented mutant (MUN287) derivatives expressed EAST1 (6). We did not test for EAST1 expression in the present study but have no reason to suspect that WAM2317 or its derivatives had lost their ability to express this toxin. If that is the case, the fact that the LT<sup>-</sup> STb<sup>-</sup> (MUN300) group did not lose weight or become dehydrated or acidotic after inoculation suggests that EAST1 may not contribute significantly to the virulence of the organism. Confirmation of this will require further experimentation with  $\Delta astA$  and complemented mutants or studies using *astA* clones in an F4ac<sup>+</sup> AstA<sup>-</sup> background.

In contrast to previous studies by Zhang et al. (54) and Berberov et al. (6), data from the present study do not support the hypothesis that LT enhances ETEC intestinal mucosal colonization in young swine. Like these previous studies, we used both quantitative culture (CFU per g of intestine) and image analysis of the mucosae in immunohistochemically stained tissue sections (colonization score in  $\mu\text{m}^2$ ) to measure the effects of LT and STb expression on colonization. The reason for the differences in the results of these studies is unknown. It is also unclear why MUN298 yielded significantly lower quantitative culture results, although no differences were found by IHC. A study in mice infected with human ETEC isolates (1) and recent in vitro studies using a porcine jejunal cell line (29) have provided evidence that LT expression enhances bacterial adherence and colonization. In the latter study, it was found that ETEC strains lacking LT were inhibited in the ability to attach to IPEC-J2 cells, LT<sup>+</sup> strains bound to IPEC-J2 cells with higher affinity than LT<sup>-</sup> strains, F4ac<sup>+</sup> ETEC strains could prevent adherence of F4ac<sup>+</sup> LT<sup>+</sup> STb<sup>+</sup> strain 3030-2 only when they expressed LT, and the enhanced adherence phenomenon was not dependent upon toxigenic activity (i.e., a biologically active A-subunit moiety) (29).

This is the first study to both characterize the *MUC4* genotype and the IMTGP phenotype of piglets. All piglets used in this study were both of a homozygous susceptible *MUC4* genotype and phenotypically positive for IMTGP. Both mucin 4 (24) and IMTGP (19) have been implicated as F4acRs; however, the relationship, if any, between these proteins has not been determined. Characterization of the F4acR phenotype is of paramount importance in the conduct of studies addressing the roles of enterotoxins in virulence, since they play such an important role in determining the susceptibility of the animal to disease. We believe that the fact that we characterized each animal as being both homozygous susceptible *MUC4* genotype and IMTGP positive helped reduce the potential for variability in the expression of F4acR as a confounding variable in the study.

In summary, based on p.i. percent weight change per h and serum bicarbonate concentrations, deletion of the STb gene ( $\Delta estB$ ) in an F4ac<sup>+</sup> LT<sup>+</sup> ETEC strain did not significantly reduce the virulence of the organism in young piglets (<2 weeks old), although clinical pathology parameters of dehydration and acidosis suggested that STb may provide an additive effect to that of LT. Deletion of the LT genes ( $\Delta eltAB$ ) in the STb<sup>-</sup> mutant resulted in a complete abrogation of the ability of the strain to cause weight loss, dehydration, and metabolic acidosis in inoculated pigs, and LT complementation restored the virulence of this strain. These findings confirm that LT is a

more significant contributor than STb to the virulence of F4ac<sup>+</sup> ETEC in F4acR<sup>+</sup> piglets <2 weeks old. In contrast to two previous studies with gnotobiotic piglets, there was no evidence that the expression of LT enhanced the ability of the F4ac<sup>+</sup> ETEC strain to colonize the small intestine. Our finding that the LT<sup>-</sup> STb<sup>-</sup> (MUN300) group did not lose weight or become dehydrated or acidotic after inoculation suggests that EAST1 may not contribute significantly to the virulence of the organism. More studies are needed to address the potential role of LT expression on colonization and also to address the role of EAST1 in disease.

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