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Enterotoxigenic *Escherichia coli* Adhesin-Toxoid Multiepitope Fusion Antigen CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{G192G/L211A}$-Derived Antibodies Inhibit Adherence of Seven Adhesins, Neutralize Enterotoxicity of LT and STa Toxins, and Protect Piglets against Diarrhea

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ABSTRACT Enterotoxigenic *Escherichia coli* (ETEC) strains are a leading cause of children's diarrhea and travelers' diarrhea. Vaccines inducing antibodies to broadly inhibit bacterial adherence and to neutralize toxin enterotoxicity are expected to be effective against ETEC-associated diarrhea. 6×His-tagged adhesin-toxoid fusion proteins were shown to induce neutralizing antibodies to several adhesins and LT and STa toxins (X. Ruan, D. A. Sack, W. Zhang, PLoS One 10:e0121623, 2015, https://doi.org/10.1371/journal.pone.0121623). However, antibodies derived from His-tagged CFA/I/II/IV-2xSTa$_{A14Q}$-dmLT or CFA/I/II/IV-2xSTa$_{N12S}$-dmLT protein were less effective in neutralizing STa enterotoxicity and were not evaluated *in vivo* for efficacy against ETEC diarrhea. Additionally, His-tagged proteins are considered less desirable for human vaccines. In this study, we produced a tagless adhesin-toxoid MEFA (multiepitope fusion antigen) protein, enhanced anti-STa immunogenicity by including a third copy of STa toxoid STa$_{N12S}$, and examined antigen immunogenicity in a murine model. Moreover, we immunized pregnant pigs with the tagless adhesin-toxoid MEFA protein and evaluated passive antibody protection against STa$^+$ or LT$^+$ ETEC infection in a pig challenge model. Results showed that tagless adhesin-toxoid MEFA CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ induced broad antiadhesin and antitoxin antibody responses in the intraperitoneally immunized mice and the intramuscularly immunized pigs. Mouse and pig serum antibodies significantly inhibited adherence of seven colonization factor antigen (CFA) adhesins (CFA/I and CS1 to CS6) and effectively neutralized both toxins. More importantly, suckling piglets born to the immunized mothers acquired antibodies and were protected against STa$^+$ ETEC and LT$^+$ ETEC diarrhea. These results indicated that tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ induced broadly protective antiadhesin and antitoxin antibodies and demonstrate that this adhesin-toxoid MEFA is a potential antigen for developing broadly protective ETEC vaccines.

KEYWORDS ETEC, enterotoxigenic *Escherichia coli*, diarrhea, fusion antigen, vaccine

Enterotoxigenic *Escherichia coli* (ETEC) bacteria are one of the top five causes of moderate-to-severe diarrhea in children <5 years of age in developing countries and remain a major public health problem (1–3). ETEC bacteria were responsible for an estimated 280 to 400 million diarrheal cases in children <5 years old and 100 million cases in children above 5 years old annually (1), resulting in long-term negative health impacts and the deaths of over 150,000 young children each year (1, 2, 4–7), including nearly 90,000 children >5 years old from South Asia and Africa (8). Additionally, ETEC

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strains are the most common cause of diarrhea in adult travelers (9–13). Currently, there is no licensed vaccine to protect against ETEC-associated children's diarrhea or travelers' diarrhea (14–16).

ETEC bacteria produce immunologically heterogeneous adhesins, including colonization factor antigens (CFA) and coli surface antigens (CS), to attach to host receptors at epithelial cells and to colonize host small intestines. Colonized ETEC bacteria deliver two enterotoxins, heat-labile toxin (LT) and heat-stable toxin type Ib (STa), to small intestinal epithelial cells to disrupt fluid and electrolyte homeostasis, leading to fluid hypersecretion and watery diarrhea (17). It has been realized that an effective ETEC vaccine needs to induce antiadhesin antibodies broadly against the prevalent CFA adhesins and also antitoxin antibodies against both LT and STa toxins (15, 16). Recently, ETEC adhesin antigen CFA/I/II/IV MEFA (multiepitope fusion antigen), carrying epitopes of the seven most prevalent adhesins (CFA/I, CFA/II [CS1, CS2, and CS3], and CFA/IV [CS4, CS5, and CS6]), was demonstrated to induce antibodies against adherence of ETEC and *E. coli* bacteria expressing these seven CFA adhesins *in vitro* (18). Recent studies also showed that genetic fusions of an STa toxoid and a monomeric LT (mnLT; one LT_A domain and one LT_B domain as a single peptide) induced antitoxin antibodies to neutralize both toxins (19–24).

Combining the CFA/I/II/IV MEFA antigen and a toxoid fusion antigen, by either coadministration or genetic fusion of two antigens, should induce antibody responses to these seven CFA adhesins and both toxins. Adhesin-toxoid MEFA CFA/I/II/IV-2xSTa_{N12S}-dmLT and CFA/I/II/IV-2xSTa_{A14Q}-dmLT (where dmLT is double mutant LT), which were constructed by substituting one STa toxoid and a part of the LT_A subunit peptide at the N terminus of 3xSTa_{N12S}-dmLT (renamed 3xSTa_{N12S}-mn$LT_{R192G/L211A}$, since the double mutant LT is a monomer peptide) or 3xSTa_{A14Q}-dmLT with the CFA/I/II/IV MEFA, were demonstrated to induce antibodies against seven CFA adhesins and both toxins in the intraperitoneally immunized mice (25). Unfortunately, both CFA/I/II/IV-2xSTa_{N12S}-dmLT and CFA/I/II/IV-2xSTa_{A14Q}-dmLT carried a 6×His tag (six histidine residues at the N terminus, from protein expression vector pET28α). His-tagged antigens are considered less desirable for human vaccine development, since the 6×His tag potentially can induce antihistidine antibodies (26). Antihistidine immunity induced by vaccine products may risk human health, since histidine is an essential amino acid for human health. Moreover, antibodies derived from His-tagged CFA/I/II/IV-2xSTa_{N12S}-dmLT or CFA/I/II/IV-2xSTa_{A14Q}-dmLT, which carried two copies of STa toxoid STa_{N12S} or STa_{A14Q}, were found to be less effective in neutralizing STa toxin than antibodies derived from toxoid fusion 3xSTa_{N12S}-mn$LT_{R192G/L211A}$ (3xSTa_{N12S}-dmLT), which carried three copies of STa_{N12S} (25). Strongly protective anti-STa antibodies are required for an effective ETEC vaccine to protect against STa-producing ETEC bacteria, which are a top cause of moderate-to-severe diarrhea in children (27). Therefore, those adhesin-toxoid MEFAs should eliminate or minimize antigen safety risk and enhance anti-STa immunogenicity. Moreover, protection of the antibodies induced by CFA/I/II/IV-2xSTa_{N12S}-dmLT or CFA/I/II/IV-2xSTa_{A14Q}-dmLT was evaluated *in vitro* but not *in vivo* against STa-producing (STa^+) ETEC or LT-producing (LT^+) ETEC diarrhea.

In this study, we produced a tagless adhesin-toxoid MEFA to eliminate antigen safety risks. Additionally, we added a third copy of STa toxoid STa_{N12S} to the tagless MEFA to enhance antigen anti-STa immunogenicity. Adding an extra copy of STa toxoid to an STa-LT toxoid fusion was demonstrated to enhance antigen anti-STa immunogenicity (22). STa_{N12S} was the focus of this study because this STa toxoid was identified to be optimal for inducing neutralizing anti-STa antibodies after being fused to a monomeric LT mutant (21). This new adhesin-toxoid MEFA then was examined for antiadhesin and antitoxin immunogenicity in a murine model. With immunogenicity of the new MEFA protein confirmed, we applied a pig challenge model to evaluate the efficacy of derived antitoxin antibodies against STa^+ ETEC or LT^+ ETEC diarrhea, thus the candidacy of this tagless MEFA antigen for the development of a broadly protective ETEC vaccine.

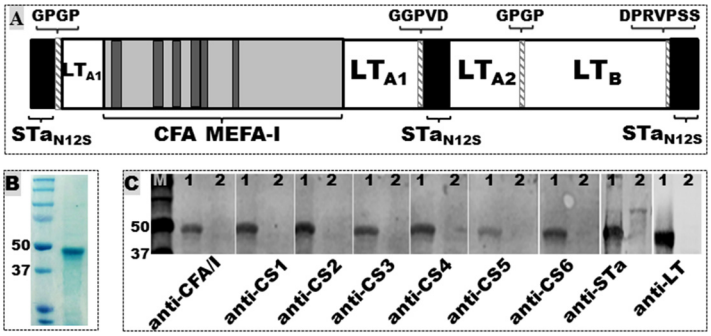

FIG 1 Construction and detection of tagless adhesin-toxoid MEFA CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$. (A) Schematic illustration of the construction of tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA. PCRs were carried out to remove nucleotides coding the 6×His tag, to replace the fragment coding amino acids 31 to 159 of LT$_A$ with nucleotides coding the CFA/I/II/IV MEFA, and to generate an ORF coding a tagless adhesin-toxoid MEFA. (B) Coomassie blue staining of extracted and refolded tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA recombinant protein to assess protein purity. (C) Western blot assays to detect tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA protein with anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5, and -CS6 MAb hybridoma supernatant (1:100; provided by A. M. Svennerholm), and rabbit anti-STa (1:5,000; provided by D. C. Robertson) and anti-CT (1:5,000; Sigma) antisera, in 12% PAGE gel. IRDye-labeled goat anti-mouse and anti-rabbit IgG (1:5,000; LI-COR) were used as the secondary antibodies. Two protein samples were included: 1, the tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA proteins; 2, total proteins of *E. coli* BL21 host strain, with a protein marker (in kilodaltons; Precision Plus Protein prestained standards; Bio-Rad).

RESULTS

The tagless adhesin-toxoid MEFA carried epitopes of the seven CFA adhesins, three copies of STa toxoid STa$_{N12S}$, and a monomeric LT mutant peptide. Overlapping three PCR products generated a single open reading frame (ORF) coding tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA peptide (Fig. 1). The recombinant *E. coli* strain expressing this adhesin-toxoid MEFA protein was designated 9419 (Table 1). DNA sequencing verified that this adhesin-toxoid MEFA gene did not carry 6×His tag nucleotides. This MEFA gene consisted of nucleotides coding three copies of STa$_{N12S}$, 150-amino-acid CFA/I/II/IV MEFA carrying epitopes of CFA/I and CS1 to CS6 major

TABLE 1 *Escherichia coli* strains and plasmids used in the study

Strain or plasmid	Relevant property(ies)	Source or reference
Strains		
BL21	B F$^-$ *ompT hsdS* (r_B^- m_B^-) *gal dcm*	GE Healthcare
H10407	O78:H11; CFA/I, LT, STa	Johns Hopkins University
EL 392-75	O6:H16; CS1/CS3, LT, STa	Johns Hopkins University
UM 75688	CS5/CS6, LT, STa	Johns Hopkins University
E106 (E11881/9)	CS4/CS6, LT, STa	University of Gothenburg
E116 (E19446)	CS3, LT, STa	University of Gothenburg
2423, ETP98066	CS6, LT, STa	Washington University
THK38/pEU405	CS1	Emory University, 35
DH5α/pEU588	CS2	Emory University, 36
9175	6×His-tagged CFA/I/II/IV MEFA + pET28α in BL21	18
9331	6×His-tagged 3×STa$_{N12S}$-mnLT$_{R192G/L211A}$ + pET28α in BL21	21
9401	6×His-tagged pCFA/I/II/IV-2×STa$_{N12S}$-dmLT + pET28α in BL21	25
9419	Tagless pCFA/I/II/IV-3×STa$_{N12S}$-mnLT$_{R192G/L211A}$ + pET28α in BL21	This study
8823	STa$^+$ ETEC challenge strain (987P/pSTa)	24
8819	LT$^+$ ETEC challenge strain (987P/pLT)	This study
Plasmids		
pCFA/I/II/IV 2×STa$_{N12S}$-dmLT	6×His-tagged multiepitope CFA-2×STa$_{N12S}$-dmLT in pET28α at NheI/EagI	25
pCFA/I/II/IV 3×STa$_{N12S}$-mnLT$_{R192G/L211A}$	Tagless multiepitope CFA-3×STa$_{N14S}$-mnLT$_{R192G/L211A}$ in pET28α at NcoI/EagI	This study

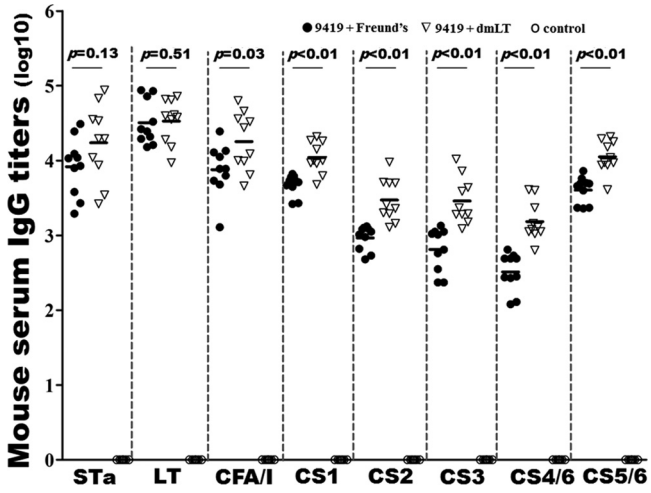

FIG 2 Mouse serum antiadhesin and antitoxin IgG antibody titers (log_{10} scale). Anti-STa, -LT, -CFA/I, -CS1, -CS2, -CS3, -CS4/CS6, and -CS5/CS6 IgG antibodies in the serum samples of each mouse immunized with tagless CFA/I/II/IV-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ MEFA with Freund's incomplete adjuvant (●) or dmLT adjuvant (▽), or serum of each control mouse (○), were titrated in ELISAs. Heat-extracted fimbrial adhesin CFA/I, CS1, CS2, CS3, CS4/CS6, or CS5/CS6 (200 ng per well of a 2HB plate), STa-ovalbumin (10 ng per well of a Costar plate), or LT (100 ng per well of a 2HB plate; List Biological Laboratories, Inc.) and HRP-conjugated goat-anti-mouse IgG (1:3,300; the secondary antibodies) were used to titrate IgG antibodies specific to CFA/I, CS1, CS2, CS3, CS4/6, and CS5/6 and to STa and LT toxins. Each dot represents an IgG titer of a mouse, and bars indicated the mean titer of the group. The *P* values from Student *t* test indicate the significance of differences of antibody titers (log_{10} scale) between two immunization groups.

subunits (CfaB, CooA, CotA, CstH, CsaB, CsfA, and CssA), and amino acids 1 to 30 and 160 to 192 of LT_{A1} peptide, LT_{A2} peptide, and one LT_B subunit of monomeric LT mutant peptide (Fig. 1A). The 192nd and the 211th residues of this LT monomer were mutated to glycine (LT_{R192G}) and alanine (LT_{L211A}); thus, this monomer LT mutant was designated $mnLT_{R192G/L211A}$. Extracted protein, after solubilization and refolding, showed a purity of over 95% based on Coomassie blue-stained SDS-PAGE (Fig. 1B). The protein recognized by anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5, and -CS6 monoclonal antibody (MAb) hybridoma supernatants, as well as anti-LT and anti-STa rabbit antiserum, was shown to have a molecular mass of about 50 kDa, an expected size of the monomeric tagless CFA/I/II/IV-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ MEFA protein (Fig. 1C). Because $mnLT_{R192G/L211A}$ did not carry the signal peptides and the ribosomal binding sites of the native LT_A subunit domain or the LT_B subunit domain (the LT_A and LT_B subunit genes of native LT have their own signal peptides and ribosomal binding sites; thus, they transcript and translate independently for two proteins to be assembled as a holotoxin), this tagless adhesin-toxoid MEFA gene became a single ORF and encoded a single peptide carrying one B subunit. No proteins of similar size were detected from the host *E. coli* BL21 strain (Fig. 1C).

Tagless adhesin-toxoid MEFA CFA/I/II/IV-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ induced antibody responses to each target adhesin and toxin in mice. Mice intraperitoneally immunized with tagless CFA/I/II/IV-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ MEFA protein, adjuvanted with the AB_5 holotoxin-structured dmLT or Freund's adjuvant, developed IgG antibodies specific to STa, LT, CFA/I, CS1, CS2, CS3, CS4/CS6, and CS5/CS6 (Fig. 2). Mouse serum anti-STa, -LT, -CFA/I, -CS1, -CS2, -CS3, -CS4/CS6, and -CS5/CS6 IgG antibody titers were (in log_{10} scale) 3.9 ± 0.39, 4.5 ± 0.29, 3.9 ± 0.34, 3.7 ± 0.14, 3.0 ± 0.16, 2.8 ± 0.29, 2.5 ± 0.26, and 3.6 ± 0.17, respectively, in the immunized group using Freund's adjuvant, whereas IgG antibody titers to the same toxins and adhesins in the immunization group with dmLT adjuvant were (in log_{10} scale) 4.2 ± 0.51, 4.6 ± 0.23, 4.3 ± 0.39, 4.0 ± 0.21, 3.5 ± 0.28, 3.5 ± 0.31, 3.2 ± 0.26, and 4.1 ± 0.22, respectively. Mice immunized with protein buffer had no antiadhesin or antitoxin antibody detected.

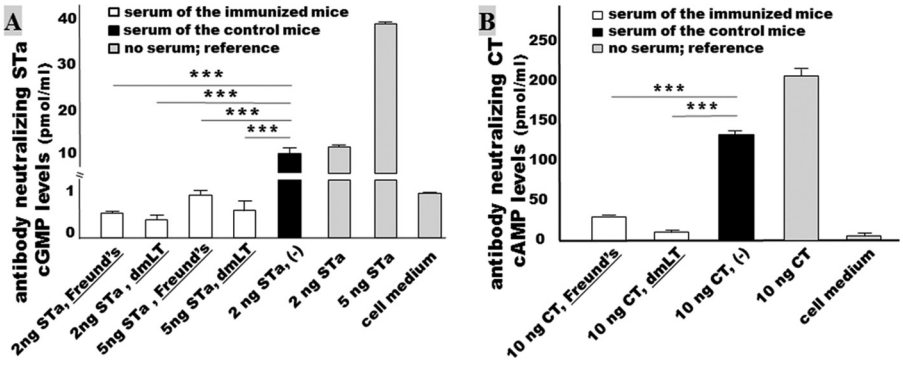

FIG 3 Mouse serum *in vitro* antibody neutralization activity against STa and CT. T-84 cells and EIA cGMP or cAMP ELISA kit (Enzo Life) were used to measure mouse serum antibody neutralizing activities against enterotoxicity of STa and CT. STa toxin elevates intracellular cGMP and CT increases cAMP in T-84 cells, whereas neutralizing anti-STa or anti-LT antibodies neutralize each toxin and prevent them from stimulating cGMP or cAMP. Mouse serum sample (30 μl) from each immunization group or the control group was incubated with STa toxin (2 ng or 5 ng) or CT (10 ng), and then the serum-toxin mixture was added to T-84 cells. Intracellular cGMP or cAMP levels (pmol/ml) in T-84 cells were measured by following the manufacturer's protocol. Columns and bars represent the means and standard deviations of cGMP or cAMP levels. ***, $P < 0.001$.

Adjuvant holotoxin-structured double mutant LT (dmLT; $LT_{R192G/L211A}$) enhanced tagless CFA/I/II/IV-3xSTa_{N12S}-m$nLT_{R192G/L211A}$ MEFA in inducing antiadhesin immune responses. Mice intraperitoneally (i.p.) immunized with tagless CFA/I/II/IV-3xSTa_{N12S}-m$nLT_{R192G/L211A}$ and dmLT adjuvant developed greater serum IgG antibody responses to CFA/I ($P = 0.03$), CS1 ($P < 0.01$), CS2 ($P < 0.01$), CS3 ($P < 0.01$), CS4/CS6 ($P < 0.01$), and CS5/CS6 ($P < 0.01$) than the immunized mice with Freund's adjuvant (Fig. 2).

Serum samples of the mice i.p. immunized with tagless CFA/I/II/IV-3xSTa_{N12S}-m$nLT_{R192G/L211A}$ neutralized STa and CT *in vitro*. Mouse serum samples from the immunized group with Freund's adjuvant or dmLT adjuvant showed neutralization activities against enterotoxicity of STa and cholera toxin (CT) (Fig. 3). The intracellular cyclic GMP (cGMP) levels in T-84 cells incubated with 2 ng STa and 30 μl serum of the immunized mice with Freund's adjuvant or dmLT adjuvant were 0.56 $\pm$ 0.05 and 0.41 $\pm$ 0.11 (pmol/ml). These cGMP levels were not significantly different from each other ($P = 0.22$) but were significantly lower than the cGMP levels in T-84 cells incubated with 2 ng STa toxin and the control mouse serum (11.2 $\pm$ 1.10; $P < 0.001$). The cGMP level in T-84 cells incubated with 2 ng STa alone was 12.4 $\pm$ 0.06 pmol/ml (Fig. 3A).

When 5 ng STa toxin was used, the cGMP levels in cells incubated with STa and the serum of the immunized mice with Freund's adjuvant or dmLT adjuvant were 0.96 $\pm$ 0.12 and 0.62 $\pm$ 0.21 (pmol/ml) ($P = 0.19$). Those cGMP levels were significantly lower than the levels in T-84 cells incubated with 5 ng STa alone (39.2 $\pm$ 0.03 pmol/ml; $P < 0.001$). The baseline cGMP level in T-84 cells was 1.1 $\pm$ 0.01 pmol/ml.

The serum samples of the immunized mice neutralized CT as well (Fig. 3B). The cyclic AMP (cAMP) levels in the T-84 cells incubated with 10 ng CT and the mouse serum sample of the immunized group with Freund's adjuvant were 33.5 $\pm$ 0.61, which were different from cAMP levels in T-84 cells incubated with 10 ng CT and the serum of the immunization group with dmLT as adjuvant (12.9 $\pm$ 1.02 pmol/ml; $P < 0.05$). The cAMP level in cells incubated with 10 ng CT and the serum samples of the control mice was 137.1 $\pm$ 3.9 pmol/ml. The T-84 cell cAMP baseline was 5.0 $\pm$ 0.22 pmol/ml.

Serum samples of the immunized mice showed *in vitro* antibody adherence inhibition against ETEC bacteria expressing CFA/I, CS3, CS4/CS6, CS5/CS6, or CS6 and *E. coli* strains expressing CS1 or CS2. Serum samples pooled from mice immunized with tagless CFA/I/II/IV-3xSTa_{N12S}-m$nLT_{R192G/L211A}$ with Freund's adjuvant or dmLT adjuvant exhibited significant inhibition activities against adherence of H10407 (CFA/I, LT^+ STa^+), E116 (CS3, LT^+ STa^+), E106 (CS4/CS6, LT^+ STa^+), UM75688 (CS5/CS6, LT^+ STa^+), and 2423/ETP98066 (CS6, LT^+ STa^+), as well as *E. coli* recombinant strains expressing CS1 adhesin or CS2 adhesin to Caco-2 cells (Table 2).

TABLE 2 Mouse serum *in vitro* antibody adherence inhibition assays[a]

E. coli strain/mouse serum	No. of bacteria ($\times 10^4$) adhered to Caco-2 cells[b] (*P* value)		
	Tagless CFA/I/II/IV 3×STa_{N12S}-$mnLT_{R192G/L211A}$ w/Freund's adjuvant	Tagless CFA/I/II/IV-3×STa_{N12S}-$mnLT_{R192G/L211A}$ w/dmLT adjuvant	Control mice
H10407; CFA/I^+ LT^+ STa^+	4.8 ± 1.75 (0.02)	1.3 ± 0.45 (0.001)	11.1 ± 4.0
THK38/pEU405; $CS1^+$	5.1 ± 0.74 (0.02)	4.4 ± 0.63 (0.003)	6.8 ± 1.1
DH5a/pEU588; $CS2^+$	2.1 ± 0.45 (0.05)	1.9 ± 0.65 (0.035)	3.0 ± 0.7
E116; $CS3^+$ LT^+ STa^+	3.1 ± 0.69 (0.002)	2.76 ± 0.95 (0.002)	5.24 ± 0.79
E106; $CS4^+$ $CS6^+$ LT^+ STa^+	7.32 ± 0.79 (0.047)	7.1 ± 0.95 (0.038)	10.1 ± 2.5
UM 75688; $CS5^+$ $CS6^+$ LT^+ STa^+	4.88 ± 0.47 (<0.001)	6.82 ± 1.33 (0.003)	10.1 ± 1.2
ETP98066; $CS6^+$ LT^+ STa^+	7.88 ± 1.43 (<0.001)	6.9 ± 2.07 (<0.001)	14.7 ± 2.15

[a]The assays used Caco-2 cells and *E. coli* or ETEC bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, or CS6 adhesin and serum samples from the group immunized with tagless CFA/I/II/IV-3×STa_{N12S}-$mnLT_{R192G/L211A}$ with Freund's adjuvant or dmLT adjuvant or from the control mouse group.
[b]The multiplicity of infection was set at 15 bacteria to 1 Caco-2 cell. ETEC or *E. coli* bacteria adhered to Caco-2 cells were counted (CFU) and are presented as means and standard deviations. The *P* values were calculated using nonparametric Wilcoxon matched-pairs test to compare the numbers of bacteria (CFU) adherent to Caco-2 cells treated with the serum samples of the immunized group or the control group.

Serum samples from the immunized group with dmLT adjuvant showed similar levels of antibody adherence inhibition activities against CS1, CS2, CS3, CS4/CS6, and CS6 adhesins but higher levels against adherence of CFA/I ($P < 0.01$) and lower levels against adherence of CS5/CS6 ($P = 0.02$) adhesins than serum samples of the immunized group with Freund's adjuvant.

Tagless CFA/I/II/IV-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ MEFA induced neutralizing antitoxin and antiadhesin antibodies in i.m.-immunized pigs. Anti-LT and anti-STa IgG and IgA antibodies were detected in the colostrum samples of all four intramuscularly (i.m.) immunized pigs (Fig. 4). Colostrum anti-STa IgG and IgA titers were 3.2 ± 0.1 and 1.5 ± 0.2, and anti-LT IgG and IgA titers were 3.8 ± 0.1 and 2.6 ± 0.3, respectively, from the immunized pigs. No anti-LT or anti-STa IgG or IgA antibody was detected from colostrum samples of four control pigs.

IgG antibodies specific to these seven adhesins and two toxins were detected in the serum samples of the immunized pigs (Fig. 5). Pig serum anti-LT, -STa, -CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5, and -CS6 IgG titers were (in $\log_{10}$ scale) 3.8 ± 0.2, 2.6 ± 0.3, 4.4 ± 0.8, 3.7 ± 0.1, 3.5 ± 0.2, 3.7 ± 0.1, 3.3 ± 0.1, 3.8 ± 0.3, and 3.5 ± 0.3 from the immunized pigs. No antiadhesin or antitoxin IgG antibodies were detected in the serum of the control sows.

The serum and colostrum samples from the immunized pigs showed neutralization activities against STa and CT enterotoxicity and also inhibition activities against adherence of ETEC or *E. coli* bacteria expressing CFA/I or CS1 to CS6. Intracellular cGMP levels in the T-84 cells incubated with 2 ng STa and the serum or the colostrum samples from the immunized mothers were 5.3 ± 0.4 and 5.7 ± 0.7 pmol/ml, which were significantly lower than those of cGMP in T-84 cells incubated with the toxin and serum (18.9 ± 6.1 pmol/ml) or colostrum (13.3 ± 5.8) of control mothers ($P < 0.01$). cAMP levels in T-84 cells incubated with CT and colostrum or serum of immunized mothers were 19.9 ± 0.1 and 8.2 ± 0.7. These cAMP levels were significantly lower than those in T-84 cells

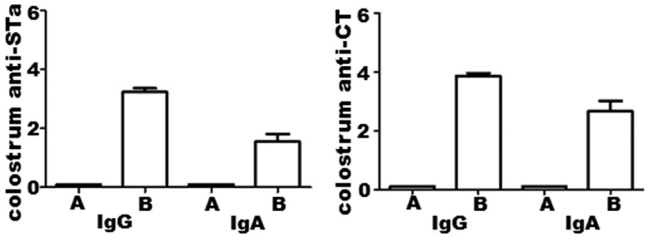

FIG 4 Anti-STa and anti-LT IgG and IgA antibody titers ($\log_{10}$ scale) from the colostrum samples of immunized or control pigs. (A) Control pigs. (B) Pigs i.m. immunized with tagless adhesin-toxoid CFA/I/II/IV-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ MEFA.

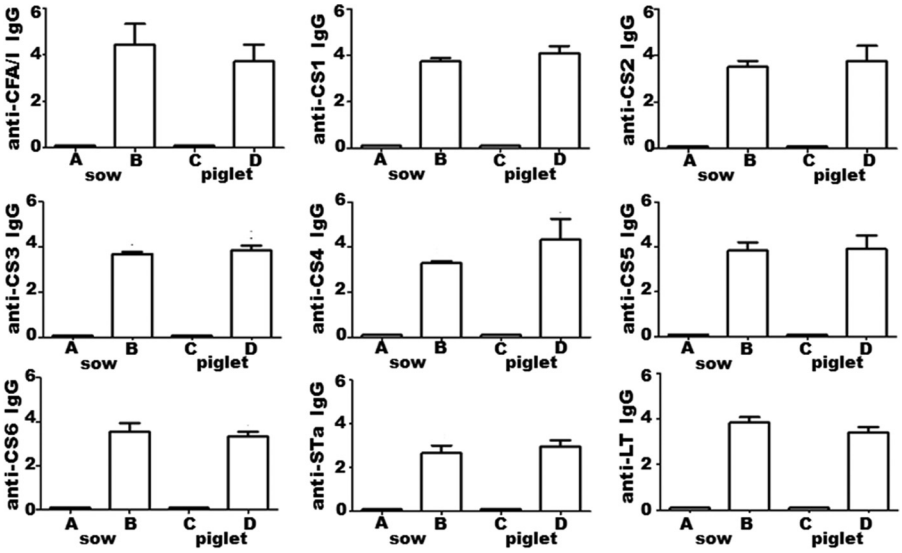

FIG 5 Anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5, -CS6, -STa, and -LT IgG titers (log_{10} scale) from pigs i.m. immunized with tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA (A) or the control sows (B), as well as the piglets born to the immunized mothers (C) and the piglets born to the control mothers (D).

incubated with CT and colostrum (219 ± 7.8) or serum (213 ± 6.2) of control mothers ($P < 0.01$).

The serum and colostrum samples of the immunized pigs significantly inhibited the adherence of ETEC or *E. coli* strains expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, or CS6 to Caco-2 cells compared to the serum or the colostrum samples of the control pigs (Fig. 6).

Piglets born to immunized mothers acquired passive antibodies and were protected against STa$^+$ or LT$^+$ ETEC diarrhea. Piglets born to the immunized sows acquired maternal antibodies through suckling. Anti-LT, -STa, -CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5, and -CS6 IgG levels in serum samples of piglets born to the immunized mothers were (in log_{10} scale) 3.4 ± 0.2, 2.9 ± 0.3, 3.7 ± 0.7, 4.1 ± 0.1, 3.7 ± 0.6, 3.8 ± 0.2, 4.3 ± 0.9, 3.9 ± 0.5, and 3.3 ± 0.2. No antitoxin or antiadhesin IgG antibody was detected from serum samples of piglets born to the control mothers (Fig. 5).

Piglets born to the immunized mothers were protected when they were orally challenged with an STa$^+$ or LT$^+$ ETEC strain. Challenged with STa$^+$ ETEC strain 8823, 17 of the 21 piglets born to three immunized mothers remained healthy (80.9%), 2 piglets had yellow pasty feces (9.5%), and 2 piglets developed watery diarrhea (9.5%). That indicated 89.5% [(90.5 − 9.5)/90.5] protective efficacy against watery diarrhea and

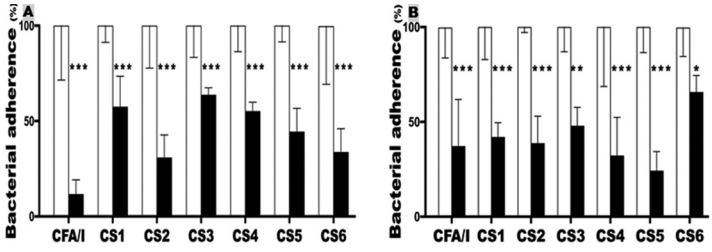

FIG 6 Pig serum and colostrum antibody adherence inhibition against CFA/I and CS1 to CS6 adhesins, with the number of adherent bacteria in the control group set to 100%. (A) Bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, or CS6 adherent to Caco-2 cells (%) after incubation with the serum samples of the pigs i.m. immunized with CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA (black box), compared to the control pigs (white box). (B) Bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, or CS6 adherent to Caco-2 cells (%) after incubation with the colostrum samples of the pigs i.m. immunized with CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA (black box), compared to the control pigs (white box). *, $P < 0.03$; ***, $P < 0.001$.

76.5% [(80.9 − 19)/80.9] against any diarrhea. The challenge outcome of piglets born to the immunized mothers differed significantly from that of piglets born to the control mothers ($P < 0.001$). Of 22 piglets born to three control mothers, 17 developed watery diarrhea (77.3%) after challenge with the STa$^+$ strain. Daily weight gain of piglets born to the immunized mothers was 6.6 ± 3.4 oz, which was significantly different from the daily weight gain of the piglets born to the control mothers (2.6 ± 5.8 oz; $P = 0.01$), 24 h after STa$^+$ ETEC inoculation.

When challenged with LT$^+$ ETEC strain 8819, 9 of 12 piglets born to one immunized mother remained healthy, 1 piglet showed yellow pasty feces, and 2 piglets developed watery diarrhea. In contrast, 7 of 8 piglets born to the control sow developed watery diarrhea 24 h after LT$^+$ ETEC inoculation.

DISCUSSION

Because ETEC bacteria expressing any one or two (out of at least 23) adhesins and either toxin can cause diarrhea, an effective ETEC vaccine needs to induce broadly protective antibodies against adhesins and both LT and STa toxins. ETEC vaccine candidates under investigation induce antibodies against five or six of the seven most important adhesins and only LT toxin (28, 29) but not against the key STa toxin. Anti-LT (or anti-CT) antibodies were demonstrated previously to protect suckling pigs against diarrhea caused by LT-producing ETEC strains (30–32). That leaves induction of anti-STa antibodies to protect against STa-producing ETEC diarrhea the new focus in ETEC antitoxin vaccine development. Data from this study demonstrated that tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA induced antibodies inhibiting adherence of all seven important adhesins and neutralizing enterotoxicity of LT as well as STa toxin. Additionally, pregnant pigs immunized with CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ MEFA developed antigen-specific IgG and IgA antibodies. Moreover, piglets born to the immunized mothers acquired passive antibodies and were protected against STa$^+$ or LT$^+$ ETEC infection.

A vaccine derived from tagless adhesin-toxoid MEFA CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ should induce broader antiadhesin and antitoxin antibody responses and can be effective against ETEC diarrhea. Recent studies showed that 6×His-tagged toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (previously termed 3xSTa$_{N12S}$-dmLT) induced antitoxin antibodies protecting piglets against STa$^+$ ETEC and LT$^+$ ETEC diarrhea (24), and tagless 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ was found to be as immunogenic as the 6×His-tagged counterpart (data not shown). It was also demonstrated that coadministration of 6×His-tagged toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ and 6×His-tagged CFA/I/II/IV MEFA induced broadly neutralizing antitoxin and antiadhesin antibodies (25). These results suggested that a vaccine carrying 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ and CFA/I/II/IV MEFA, preferably tagless antigens, can induce antibodies to protect against diarrhea caused by ETEC bacteria expressing any of these seven adhesins and either toxin. Although a vaccine combining a tagless toxoid fusion and a tagless CFA/I/II/IV MEFA has flexibility to optimize antigen dose composition, it increases product manufacture cost compared to that of a vaccine consisting of a single protein, CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$. Future studies to directly compare two potential vaccine candidates, one carrying single tagless CFA/I/II/IV-3xSTa$_{N12S}$-dmLT MEFA and the other combining tagless 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ and tagless CFA/I/II/IV MEFA, for induction of neutralizing antitoxin and antiadhesin antibodies and, more importantly, efficacy against ETEC diarrhea, should identify a leading ETEC subunit vaccine candidate.

Double mutant holotoxin-structured LT (dmLT) is demonstrated as an effective adjuvant for 6×His-tagged toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ in parenteral immunization (23). Data from this study confirmed that dmLT was equally as or more effective than Freund's incomplete adjuvant for immunoregulating tagless adhesin-toxoid MEFA CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ for antitoxin and antiadhesin immunity. Mice i.p. immunized with the current adhesin-toxoid MEFA with dmLT adjuvant developed greater amounts of antiadhesin antibodies than the immunized group using Freund's adjuvant. *In vitro* antibody adherence inhibition assays indicated that anti-

bodies in the serum samples from both immunization groups were effective against bacterial adherence. On the other hand, although immunization groups with dmLT or Freund adjuvant developed similar levels of antitoxin antibody responses, serum samples from the immunized mice with dmLT adjuvant showed greater neutralizing activity against cholera toxin. This suggested that anti-LT antibodies derived from holotoxin-structured dmLT or, more likely, the toxic LT A1 peptide of dmLT adjuvant, even at a low dose of 2 μg, enhanced anti-LT antibody neutralization activity.

Data from the current study showed that adhesin-toxoid MEFA CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ without a 6×His tag can be effectively extracted (at a yield of 130 to 150 mg protein per liter of culture medium), and the tagless CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ induced neutralizing antibodies against all seven adhesins and both toxins. Removing the 6×His tag eliminated or at least decreased the risk of recombinant proteins in inducing antihistidine antibodies. The safety of this tagless adhesin-toxoid MEFA, however, would have to be evaluated in future toxicology studies and human volunteer studies.

A pig challenge model was applied to evaluate antitoxin antibodies against ETEC diarrhea in this study. The lack of a suitable animal challenge model to assess the efficacy of ETEC vaccine candidates used to be a major challenge in ETEC vaccine development. Data from a previous study (24) and the current study indicated that pigs immunized with 6×His-tagged 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ or this tagless adhesin-toxoid MEFA developed antitoxin antibodies, and piglets that acquired maternal antibodies were protected against the challenge of an STa$^+$ ETEC or an LT$^+$ ETEC strain. Future studies to immunize piglets and to challenge them directly shall help us to evaluate the efficacy of active acquired antitoxin antibodies against ETEC diarrhea. Although the serum and colostrum antibodies of the immunized pigs inhibited ETEC adherence *in vitro*, this pig model did not allow us to assess the efficacy of the induced antiadhesin antibodies against ETEC bacterial colonization, since ETEC adhesins are host specific. Because the RITARD (removable intestinal tie adult rabbit diarrhea) model has become less practical under current Institutional Animal Care and Use Committee (IACUC) guidelines, the modified rabbit colonization model is under recalibration (15), and the mouse colonization model is currently applicable for CFA/I adhesin only (33, 34). Future studies with a different model will be needed to evaluate additive effects of antiadhesin antibodies (to antitoxin antibodies) against ETEC diarrhea.

In conclusion, results from this study indicate that the tagless adhesin-toxoid MEFA CFA/I/II/IV-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ induced antibodies that neutralized CT and STa enterotoxicity, inhibited adherence of ETEC or *E. coli* bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, or CS6 adhesin to Caco-2 cells, and protected piglets against challenge with an STa$^+$ or LT$^+$ ETEC strain. These results suggest that this tagless adhesin-toxoid MEFA potentially can be a desirable antigen for developing broadly protective ETEC vaccines against children's diarrhea and travelers' diarrhea.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains and plasmids used for this study are included in Table 1. ETEC field isolates producing CFA/I, CS3, CS4/CS6, CS5/CS6, or CS6 (provided by Johns Hopkins University, Washington University, and the University of Gothenburg *E. coli* Reference Strain Center, Sweden) and *E. coli* recombinant strains expressing CS1 or CS2 fimbrial adhesin (35, 36) were used for bacterial fimbria extraction (for coating antigens of antibody titration enzyme-linked immunosorbent assays [ELISAs]) and for *in vitro* antibody adherence inhibition assays. Vector pET28α (Novagen, Madison, WI) and *E. coli* BL21 (GE Healthcare, Piscataway, NJ) were used to express the adhesin-toxoid MEFA recombinant protein.

Construction of the tagless adhesin-toxoid MEFA gene. PCRs with designed primers (Table 3) were used to amplify the CFA/I/II/IV MEFA gene from strain 9175 (18) and the toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ gene from strain 9331 (21). Toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, previously named 3xSTa$_{N12S}$-dmLT, carried three copies of STa toxoid STa$_{N12S}$ and a monomeric LT peptide, which consists of one LT$_A$ subunit domain mutated at the 192th and the 211th residues and one LT$_B$ subunit domain. As described previously (19, 22), splicing overlap extension (SOE) PCR was used to overlap three PCR products into a single chimeric gene (Fig. 1A). The first PCR, using primers STaNcoI-F and LT30CFA-R and toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ DNA template, amplified the first copy of STa$_{N12S}$ and the first 30 amino acids of the LT$_A$ domain. The second PCR, using primers LT30CFA-F and LT160CFA-R and CFA/I/II/IV MEFA DNA template, generated the CFA/I/II/IV fragment. The third PCR, using primers

TABLE 3 PCR primers to construct tagless CFA/I/II/IV-3×STa_{N12S}-$mnLT_{R192G/L211A}$ chimeric gene[a]

Primer	Nucleotide sequence (5′ to 3′)
STaNcoI-F	CATGCCATGGAAATGGCTAGCATGAATAGTAGC
LT30CFA-R	TGCGCTAGCCATGTACTCATTATGCCCTCT
LT30CFA-F	AATGAGTACATGGCTAGCGCAGTAGAGGAT
LT160CFA-R	GTAACCATCGGCTCCCAAAGTCATTACAGG
LT160CFA-F	ACTTTGGGAGCCGATGGTTACAGATTAGCAGGTT
T7-R	TGCTAGTTATTGGTCAGGGGT

[a]PCRs to fuse CFA/I/II/IV MEFA gene (from strain 9175) to 3×STa_{N12S}-$mnLT_{R192G/L211A}$ gene (from strain 9331) by replacing nucleotides coding amino acids 31 to 159 of LT A1 peptide with the CFA/I/II/IV MEFA and also to remove nucleotides encoding the 6×His tag.

LT160CFA-F and T7-R and 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ DNA template, yielded the C-terminal fragment of 3xSTa_{N12S}-$mnLT_{R192G/L211A}$, starting from the 160th amino acid of LT_A. Three PCR products generated with *Pfu* DNA polymerase were purified and then overlapped in a final SOE PCR. The overlapped chimeric gene was further amplified with primers STaNcoI-F and T7-R, digested with NcoI and EagI restriction enzymes (New England BioLabs, Ipswich, MA), and ligated into expression vector pET28α. Since the NcoI restriction site in pET28α expression vector is located upstream of the 6×His tag region and the third copy of the STa_{N12S} toxoid gene (at the 3′ end) has a stop codon, this adhesin-toxoid MEFA chimeric gene did not carry 6×His tag nucleotides. The cloned tagless chimeric gene was verified initially by DNA sequencing.

Expression, extraction, and characterization of the tagless adhesin-toxoid MEFA protein. Plasmids (pET28α) carrying the chimeric adhesin-toxoid MEFA gene were introduced into *E. coli* BL21 bacteria to express tagless adhesin-toxoid MEFA protein. Protein expression, extraction, and refolding followed protocols described previously (37, 38). Briefly, a single colony of the recombinant strain was cultured in 5 ml lysogeny broth (LB) supplemented with kanamycin (30 μg/ml) at 37°C on a shaker (150 rpm). Overnight-grown bacteria were transferred to 500 ml 2× YT (2× yeast extract tryptone) medium broth and incubated continuously until culture optical density at 600 nm (OD_{600}) reached 0.5 and then induced with isopropyl-1-thio-β-D-galactoside (IPTG; 0.5 mM) and cultured for 4 h. Bacteria were collected, lysed in 10 ml bacterial protein extraction reagent (B-PER; Pierce, Rockford, IL) after freeze-thaw, and incubated at room temperature for 30 min. Bacterial lysates were centrifuged, suspended in 10 to 20 ml B-PER with vigorous vortexing and pipetting, mixed with 200 to 400 μl freshly prepared lysozyme (10 mg/ml in B-PER), and incubated at room temperature for 20 min. Suspensions were centrifuged, and pellets were suspended in B-PER and incubated with lysozyme. After centrifugation, inclusion body proteins were suspended in 100 ml 1:10 diluted B-PER, vortexed, and centrifuged. Protein pellets were washed with 100 ml phosphate-buffered saline (PBS) three times, vortexed vigorously, centrifuged at 12,000 rpm for 15 min at 4°C, and finally dissolved in 10 to 20 ml PBS.

Extracted proteins were further solubilized with solubilization buffer and refolded with a protein refolding kit by following the manufacturer's protocols (Novagen). Proteins were refolded in molecular porous membrane tubing (Spectrum Laboratories, Inc., Rancho Dominquez, CA) with refolding buffer (20 mM Tris-HCl supplemented with 0.1 mM dithiothreitol [DTT]) and then dialyzed with refolding buffer without DTT. Refolded and solubilized proteins were centrifuged and collected (in supernatant). Proteins were measured for concentration (milligrams per milliliter) and stored at −80°C.

Refolded protein (10 μg) was examined by 12% SDS-PAGE and immune blot assays with monoclonal anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5, and -CS6 antibody hybridoma supernatant (provided by A. M. Svennerholm at the University of Gothenburg, Sweden), rabbit anti-CT (1:5,000; Sigma, St. Louis, MO), and anti-STa sera (provided by D. C. Robertson at Kansas State University). IRDye-labeled goat anti-mouse or anti-rabbit IgG (1:5,000 dilution; LI-COR, Lincoln, NE) and a LI-COR Odyssey premium infrared gel imaging system (LI-COR) were used for protein detection.

Mouse i.p. immunization with the tagless adhesin-toxoid MEFA protein. Three groups of 8-week-old female BALB/c mice (Charles River Laboratories International, Inc., Wilmington, MA), 10 mice per group, were used in the i.p. immunization study. Each mouse in the first group was i.p. injected with 200 μg tagless adhesin-toxoid MEFA protein CFA/I/II/IV-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (in 200 μl protein buffer, 20 mM Tris-HCl) and 200 μl Freund's incomplete adjuvant (Sigma). The second group was immunized with the same amount of CFA/I/II/IV-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ MEFA protein but with 2 μg dmLT adjuvant (in 200 μl protein buffer; provided by Walter Reed Army Institute of Research, Silver Spring, MD). The third group was injected with 200 μl protein buffer as the negative control. Immunized mice received two booster injections with the same dose of the primary at an interval of 2 weeks. Mice were anesthetized with CO_2 and exsanguinated 2 weeks after the second booster. Blood samples were collected from each mouse before the primary immunization and 14 days after the final booster. Mouse serum samples were stored at −80°C until use. Mouse immunization complied with the Animal Welfare Act by following the 1996 National Research Council guidelines and was approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC).

Mouse serum antiadhesin and antitoxin antibody titration. IgG antibodies to CFA/I, CS1, CS2, CS3, CS4, and CS5 fimbriae and also to STa and LT toxins from mouse serum samples were titrated in ELISAs as described previously (18, 19, 21, 22, 25). Briefly, wells of 2HB plates (Thermo Scientific, Rochester, NY) were coated with heat-extracted CFA/I, CS1, CS2, CS3, CS4, or CS5 fimbriae (200 ng per

well) and LT (List Biological Laboratories, Inc., Campbell, CA; 100 ng per well) to titrate antibodies to each CFA fimbrial adhesin and to LT, whereas STa-ovalbumin conjugates (10 ng per well) and Costar plates (Corning Inc., Corning, NY) were used to titrate anti-STa IgG antibodies. Serum samples of each mouse were 2-fold diluted (from 1:200 to 1:51,200) and were examined in triplicate. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:3,300; Sigma) and the 3,3′,5,5′-tetramethylbenzidine (TMB) microwell peroxidase substrate system (2-C) (KPL, Gaithersburg, MD) were used to measure optical absorbance (OD_{405}). The highest serum dilution that produced OD readings of >0.3 above the background was calculated for antibody titers. Antibody titers were presented in a $\log_{10}$ scale as previously described (20, 21, 23, 25, 37).

Mouse serum antibody adherence inhibition assay. Mouse serum samples from each group were examined for *in vitro* antibody inhibition activities against adherence of *E. coli* bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, or CS6 to Caco-2 cells (ATCC HTB-37). As described previously (18, 25), overnight-grown ETEC or *E. coli* bacteria scraped off from sheep blood agar plates were gently suspended in sterile PBS (1.05×10^7 bacteria in 100 μl PBS, with a multiplicity-of-infection ratio set at 15 to 1). Bacteria were pretreated with 4% mannose (to prevent type 1 fimbriae from attachment), mixed with 20 μl mouse serum sample from each group, brought to 300 μl with PBS, and then transferred to 90 to 95% confluent monolayer Caco-2 cells (7×10^5 per well in 700 μl cell culture medium). After incubation in a CO_2 incubator (5% CO_2) for 1 h at 37°C, Caco-2 cells were gently washed with PBS to remove nonadherent ETEC or *E. coli* bacteria and then dislodged with 0.25% trypsin (200 μl per well). Bacteria adherent to the Caco-2 cells were collected by centrifugation, suspended in 1 ml PBS, serially diluted, and plated on LB plates. Bacteria were counted for CFU after overnight growth at 37°C. Antibody adherence inhibition activities were reflected by reduction of the numbers of bacteria adherent to Caco-2 cells (in CFU or percentages, with the control set to 100%).

Mouse serum antibody neutralization against CT and STa enterotoxicity. Enzyme immunoassay (EIA) cAMP and cGMP kits (Enzo Life Sciences, Farmingdale, NY) and T-84 cells were used to measure mouse serum antibody neutralization activities against enterotoxicity of STa and CT (cholera toxin; a homologue of LT commonly used in anti-LT antibody neutralization assays). As described previously (19–23, 25), mouse serum samples were examined for prevention of STa toxin from stimulating intracellular cGMP levels or CT from elevating intracellular cAMP levels in T-84 cells. Mouse serum sample (30 μl) from each group was mixed with STa toxin (2 ng or 5 ng) or CT (10 ng). Each serum-toxin mixture was incubated at room temperature for 30 min and then was transferred to 90 to 95% confluent T-84 cells and incubated in a CO_2 incubator (1 h for STa to measure cGMP or 3 h for CT to measure cAMP). Intracellular cGMP or cAMP levels (in picomoles per milliliter) of T-84 cells were measured with an EIA cGMP or cAMP kit by following the manufacturer's protocol (Enzo Life Sciences).

Pig i.m. immunization with the tagless adhesin-toxoid MEFA protein. A total of eight pregnant gilts from the Kansas State University Swine Unit and a local producer, which had no record of diarrhea and were verified not to have preexisting anti-STa and anti-LT antibodies, were raised at a university large-animal research center. As described previously (24), four pregnant gilts each were i.m. immunized with 500 μg tagless adhesin-toxoid MEFA protein and 5 μg dmLT adjuvant 6 to 8 weeks before farrowing, followed by a booster injection at the same dose of the primary 4 weeks later. Four pregnant gilts without immunization were used as the control.

Blood samples were collected from gilts before immunization and after farrowing, and colostrum samples were collected before farrowing.

Pig serum and colostrum antiadhesin and antitoxin antibody titration. Serum samples from each immunized or control gilt were examined for IgG antibodies to STa, LT, CFA/I, and CS1 to CS6, and pig colostrum samples were titrated for IgG and IgA antibodies to each toxin and adhesin. HRP-conjugated goat-anti-porcine IgG and IgA (1:3,000 dilution; Bethyl Laboratories, Montgomery, TX) were used as the secondary antibodies (24). Serum and colostrum samples from each immunized or control pig were examined for antibody neutralization activities against STa and CT, as well as antibody adherence against CFA/I and CS1 to CS6 adhesins as described above.

Piglet ETEC challenge study. Piglets born to immunized and control mothers were used to assess protective efficacy of anti-STa and anti-LT antibodies against ETEC diarrhea. As described previously (24), piglets born to three immunized mothers and three control mothers, after 24 h of suckling, were orally inoculated with 5×10^9 CFU STa^+ ETEC strain 8823 (G58/987P/STa) (24). Suckling piglets born to the fourth immunized sow and the fourth control sow were inoculated with 5×10^9 CFU LT^+ ETEC strain 8819 (G58/987P/LT). Challenged piglets were monitored every 2 to 4 h until 24 h postinoculation and were weighed before challenge and 24 h postchallenge. Clinical symptoms, including diarrhea, dehydration, and lethargy, were recorded from each challenged piglet.

Serum samples were collected from each piglet at necropsy and were titrated for IgG antibodies to each CFA adhesin and toxin as described above. Pig immunization and piglet challenge studies complied with the USDA Animal Welfare Act Regulations and were approved by the Kansas State University IACUC.

Statistical analysis. Mouse and pig antibody titration and antibody neutralization data are presented as means ± standard deviations. Differences in antibody titers (in $\log_{10}$ scale) between the immunized group and the control group were measured using Student's *t* test with SAS for Windows, version 8 (SAS Institute, Cary, NC). Differences of antibody neutralization activities (cAMP and cGMP, in picomoles per milliliter), antibody adherence inhibition activities from serum or colostrum samples between the immunized group and the control group, and piglet challenge outcome data between the piglets born to immunized mothers and piglets born to control mothers were compared with the nonparametric Wilcoxon matched-pairs test. A *P* value of <0.05 indicated significance of differences when two treatments were compared using two-tailed distribution and two-sample unequal variance.

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Senior authors D.S. and W.Z. are developing an ETEC subunit vaccine.

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