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Mapping the Neutralizing Epitopes of Enterotoxigenic *Escherichia coli* K88 (F4) Fimbrial Adhesin and Major Subunit FaeG

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ABSTRACT Enterotoxigenic *Escherichia coli* (ETEC) strains that produce immunologically heterogeneous fimbriae and enterotoxins are the primary cause of neonatal diarrhea and postweaning diarrhea in young pigs. A multivalent vaccine inducing protective immunity against ideally all ETEC fimbriae and enterotoxins could be effective against diarrhea in young pigs. However, developing a vaccine to broadly protect against various ETEC virulence determinants has proven challenging. Recently developed structure- and epitope-based multiepitope fusion antigen (MEFA) technology that presents neutralizing epitopes of various virulence determinants at a backbone immunogen and that mimics epitope native immunogenicity suggests the feasibility of developing multivalent vaccines. With neutralizing epitopes from ETEC fimbria F18 and enterotoxins being identified, it becomes urgent to identify protective epitopes of K88 (F4) fimbriae, which play a major role in pig neonatal and postweaning diarrhea. In this study, we identified B-cell immunodominant epitopes *in silico* from the K88ac fimbrial major subunit (also adhesin) FaeG and embedded each epitope in a heterogeneous carrier for epitope fusions. We then immunized mice with each epitope fusion protein and examined epitope antigenicity and also neutralizing activities of epitope-induced antibodies. Data showed that while all nine FaeG epitope fusions induced antibodies to K88ac fimbria, anti-K88 IgG antibodies derived from epitopes MTGDFNGSVD (ep1), LNDLTNGGTK (ep2), GRTKEAFATP (ep3), ELRKPDGGTN (ep4), PMKNAGGTKVGAVKVN (ep5), and RENMEYTDGT (ep8) significantly inhibited adherence of K88ac fimbrial bacteria to porcine intestinal cell line IPEC-J2, indicating that these peptides were the neutralizing epitopes of K88ac fimbrial major subunit FaeG and suggesting the future application of FaeG epitopes in ETEC vaccine development.

IMPORTANCE Enterotoxigenic *Escherichia coli* (ETEC) strains producing K88ac fimbriae and enterotoxins are a major cause of porcine neonatal diarrhea and postweaning diarrhea in the United States. Currently, there is no vaccine to induce broadly protective antiadhesin and antitoxin immunity against ETEC-associated diarrhea. To develop a broadly effective ETEC vaccine, we need to target the most important if not all ETEC virulence determinants. While conventional vaccinology approaches encounter difficulties at integrating or including heterogeneous ETEC fimbria and toxin antigens into a vaccine product, multiepitope fusion antigen (MEFA) structural vaccinology provides a new platform to combine neutralizing antigenic elements or epitopes from various heterogeneous virulence factors for broad immunity and protection. Identification of the neutralizing epitopes of K88ac fimbria from this study added the last antigens to an MEFA-based multivalent vaccine against ETEC-associated diarrhea in pigs. An effective vaccine against pig diarrhea

Citation Lu T, Moxley RA, Zhang W. 2019. Mapping the neutralizing epitopes of enterotoxigenic *Escherichia coli* K88 (F4) fimbrial adhesin and major subunit FaeG. Appl Environ Microbiol 85:e00329-19. https://doi.org/10.1128/AEM.00329-19.

Editor Edward G. Dudley, The Pennsylvania State University

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Received 7 February 2019
Accepted 22 March 2019
Accepted manuscript posted online 29 March 2019
Published 16 May 2019

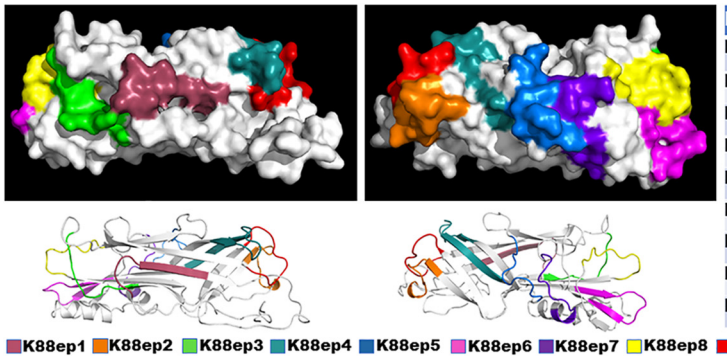
can significantly improve swine health and well-being and reduce economic losses to the swine industry worldwide.

KEYWORDS enterotoxigenic *Escherichia coli*, FaeG, K88 (F4), neutralizing epitope, postweaning diarrhea, vaccine

Enterotoxigenic *Escherichia coli* (ETEC) bacteria that express K88 (F4) or F18 fimbria and enterotoxins, including heat-labile toxin (LT), heat-stable toxin type I (STa), heat-stable toxin type II (STb), and Shiga toxin type 2e (Stx2e), are the primary cause of diarrhea in pigs (1–6). Porcine neonatal diarrhea is largely prevented by passive protection of maternal antibodies through immunization of pregnant sows. Postweaning diarrhea, however, is yet to be effectively controlled (4). Various preventive approaches, including feeding ETEC-specific antibody-containing materials and treatment with prebiotics, probiotics, or dietary supplements, were attempted but found either inconsistent or unviable commercially (4, 7). Developing effective vaccines for postweaning diarrhea has encountered challenges as well. Difficulties include a very narrow window for immunization, the cost of the vaccine and vaccination, and more importantly the need of cross protection against heterogeneous ETEC strains. Ideally, piglets are vaccinated when their maternal antibodies (against neonatal diarrhea) drop to certain levels. These antibody levels can still sufficiently protect against neonatal diarrhea but do not significantly interfere with a vaccine product from stimulation of antigen-specific active immunity. Pig neonatal diarrhea and postweaning diarrhea can be caused by the same ETEC pathogens or strains sharing virulence factors. To be protected against postweaning diarrhea, piglets need to develop active immunity against ETEC infection by the time of weaning and after they are weaned. Vaccines need to be low cost and easy to administer. Furthermore, since ETEC strains produce immunologically different fimbrial adhesins to attach to specific receptors at pig small intestinal epithelia and deliver various toxins to stimulate water and fluid hypersecretion in epithelial cells, an effective postweaning diarrhea vaccine needs to carry antigens from all virulence determinants and to induce broad antiadhesin and antitoxin immunity, thus inhibiting the adherence of immunologically different ETEC fimbriae and neutralizing the enterotoxicity of functionally distinctive ETEC toxins.

New strategies need to be explored since conventional vaccine technologies have encountered difficulties in developing a broadly protective multivalent vaccine against postweaning diarrhea (4, 6, 7). Multiepitope fusion antigen (MEFA) vaccinology, an alternative structure- and epitope-based vaccine technology, presents a new platform for multivalent vaccine development (8). By mimicking epitope native antigenicity and presenting multiple heterologous neutralizing epitopes at a backbone immunogen, MEFA vaccinology allows a single immunogen (protein) to carry multiple antigenic elements (epitopes or peptides) from various virulence determinants for broad immunogenicity, thus allowing the development of a broadly immunogenic multivalent vaccine. With this MEFA technology, we have successfully generated ETEC fimbria MEFAs for broadly protective immunity against seven or nine human ETEC virulence determinants (colonization factor antigen [CFA] adhesins) (9–11) and adhesin-toxoid MEFAs for antibodies not only inhibiting adherence of seven human ETEC adhesins and neutralizing LT and STa enterotoxicity but also protecting against ETEC diarrhea in a pig challenge model (12, 13). These MEFAs have become leading antigens for human ETEC subunit vaccines against children's diarrhea and traveler's diarrhea.

Protective epitopes of four toxins (LT, STa, STb, and Stx) produced by ETEC strains associated with pig diarrhea were identified (14–17). MEFA technology has also assisted with the construction of a multivalent toxin antigen and the development of a broadly protective antitoxin vaccine candidate for pig postweaning diarrhea (15). However, a multivalent toxoid MEFA does not induce antiadhesin antibodies to prevent ETEC bacteria from adherence to host receptors. Since ETEC that causes pig postweaning diarrhea produces one, two, three, or four toxins and either K88 or F18 fimbria (5), an effective vaccine ideally needs to induce antitoxin and antiadhesin antibodies against



Ep #	AA sequence
K88ep1	^{23}MTGDFNGSVD^{32}
K88ep2	^{61}LNDLTNGGTK^{70}
K88ep3	^{84}GRTKEAFATP^{93}
K88ep4	^{115}ELRKPDGGTN^{124}
K88ep5	^{134}PMKNAGGTKVGAVKVN^{149}
K88ep6	^{157}LGRGGVTSADGEL^{168}
K88ep7	^{185}PRGSELSAGSA^{195}
K88ep8	^{233}RENMEYTDGT^{242}
K88ep9	^{262}FNQAVTTSTQ^{271}

FIG 1 K88 fimbrial major structure subunit and adhesin FaeG protein model with *in silico* identified epitopes (Ep) and epitope amino acid (AA) sequences. Nine FaeG epitopes were designated as K88ep1, K88ep2, K88ep3, K88ep4, K88ep5, K88ep6, K88ep7, K88ep8, and K88ep9. Phyre3 and PyMOL molecular graphics system (version 2.2) were used to generate the 3-dimensional protein structure of the K88 FaeG subunit and to map each epitope from the FaeG protein model, respectively.

four ETEC toxins and both K88 and F18 fimbriae. As neutralizing epitopes for F18 fimbrial adhesin subunit FedF are also identified (18), K88 (F4) fimbria becomes the only virulence determinant without neutralizing epitopes that are fully determined.

In this study, we identified immune dominant B-cell epitopes *in silico* from FaeG, the major structural subunit as well as the adhesive subunit (adhesin) of K88ac fimbria, genetically embedded each epitope in a heterologous carrier protein, and assessed epitope native topology by examining epitope fusion protein reactivity with anti-K88ac antiserum. We then immunized mice with each epitope fusion protein and measured mouse serum IgG antibodies specific to K88ac fimbria. Furthermore, we examined mouse serum antibodies for inhibition activity against K88ac fimbrial adherence and identified K88ac fimbria-neutralizing epitopes.

(Some of the data described in this manuscript were presented at the Kansas State University Swine Day 2017 [19].)

RESULTS

Immunodominant B-cell epitopes were identified from K88ac fimbrial major subunit FaeG. Nine immunodominant and discontinued epitopes, ranging from 10 to 16 amino acid residues, were identified from the FaeG subunit of K88ac fimbria, designated K88ep1 to K88ep9 (Fig. 1). Protein modeling, based on the K88ac FaeG sequence (GenBank accession number M25302.1) (20), showed that all epitopes were surface exposed at the subunit and were largely located at β sheets or α-helix extensions (Fig. 1).

Nine epitope fusion proteins were expressed and extracted. With the insertion of each K88 fimbrial FaeG epitope (K88ep1 to K88ep9) into protein carrier CfaB, a 17-kDa major structural subunit of human ETEC fimbria CFA/I, nine epitope fusions named ep1 fusion to ep9 fusion were constructed. All 6×His-tagged epitope fusions, at a molecular weight of about 22 kDa, were expressed. Epitope fusion proteins were extracted and refolded at a purity assessed over 95% (Fig. 2). When linearized by SDS-PAGE and examined with anti-K88ac antiserum in a Western blot, however, only ep1, ep2, ep3, ep5, ep6, and ep9 fusions were recognized (Fig. 2).

Anti-K88ac antiserum confirmed FaeG epitope conformation. Antigenic conformation of the identified FaeG epitope carried by epitope fusions was verified in direct and competitive enzyme-linked immunosorbent assays (ELISAs) using anti-K88ac antiserum. Direct ELISAs showed that the ep1, ep2, ep3, ep4, ep5, or ep9 fusion showed significantly greater reactivity with 1:1,600 and 1:3,200 dilutions of anti-K88ac antiserum ($P < 0.05$), the ep7 fusion showed a moderate reactivity, and the ep6 and ep8 fusions showed no reactivity (Table 1).

Competitive ELISAs with heat-extracted K88ac fimbriae (from ETEC strain 3030-2) as the coating antigen showed significantly lower reactivity with anti-K88 antiserum when fusion proteins of epitope 1, 2, 4, or 5 were used as the competing antigen ($P < 0.001$).

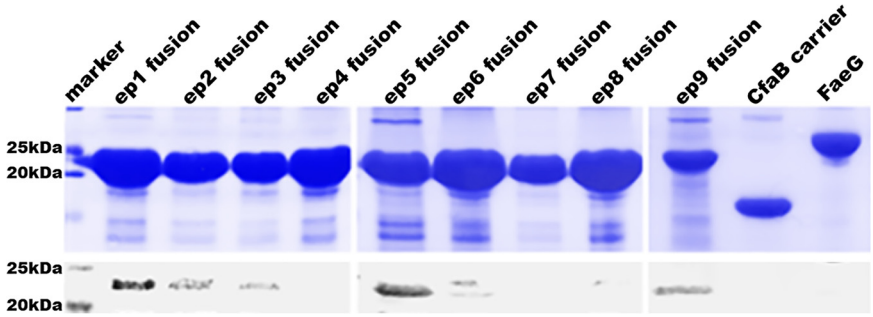


FIG 2 CfaB-epitope fusion (each K88 FaeG epitope carried by backbone protein CfaB) protein extraction and characterization. (Top) Extracted and refolded CfaB-epitope fusion proteins from SDS-PAGE Coomassie blue staining. (Bottom) Reactivity of each epitope fusion protein with anti-K88ac antiserum (antiserum from mice immunized with K88ac fimbriae) from Western blotting.

No reactivity with anti-K88ac antiserum was detected when carrier protein CfaB was used as the coating antigen in direct or competitive ELISAs.

Anti-K88ac IgG antibodies were detected in the mice subcutaneously immunized with each epitope fusion protein. Mice in each group immunized with an epitope fusion protein developed anti-K88ac IgG antibodies (Table 2). Mice immunized with the ep5 fusion protein developed significantly greater anti-K88ac IgG titers than those of the groups immunized with the ep4, ep6, ep7, ep8, or ep9 fusion but not those of the groups immunized with the ep1, ep2, or ep3 fusion. No anti-K88 IgG was detected from the control group.

Mouse antiserum samples from each immunized group were also confirmed for reactivity with K88ac fimbriae or the recombinant FaeG protein in ELISAs and Western blot assays.

Mouse serum samples from the immunized groups showed inhibition of *in vitro* adherence of K88 fimbrial ETEC 3030-2. K88ac fimbrial ETEC 3030-2 bacteria, after being treated with the serum samples from the groups immunized with the ep1, ep2, ep3, ep4, ep5, or ep8 fusion, showed a significant reduction of adherence to porcine cell line IPEC-2 ($P < 0.01$). No significant adherence reduction was observed from 3030-2 bacteria when treated with the serum from the groups immunized with the ep6, ep7, or ep9 fusion protein (Table 3).

DISCUSSION

Data from the current study showed that while all identified *in silico* K88ac FaeG epitopes carried by the CfaB backbone protein induced anti-K88 IgG antibodies in the subcutaneously immunized mice, only antibodies derived from the fusions of epitope 1, 2, 3, 4, 5, or 8 significantly inhibited the adherence from K88ac fimbrial ETEC bacteria to porcine cell line IPEC-J2. This suggested that while all epitopes are immunodomi-

TABLE 1 OD_{650} readings from direct ELISA to measure reactivity between each epitope fusion (CfaB-epitope fusion) protein and anti-K88ac antiserum

	OD_{650} reading for anti-K88 serum dilution:	
Epitope fusion protein	1:1,600	1:3,200
ep1 fusion	1.37 ± 0.00	0.96 ± 0.05
ep2 fusion	1.20 ± 0.11	0.81 ± 0.12
ep3 fusion	1.07 ± 0.03	0.73 ± 0.10
ep4 fusion	0.87 ± 0.04	0.52 ± 0.02
ep5 fusion	1.78 ± 0.23	1.45 ± 0.11
ep6 fusion	0.15 ± 0.03	0.10 ± 0.04
ep7 fusion	0.48 ± 0.04	0.39 ± 0.12
ep8 fusion	0.25 ± 0.03	0.18 ± 0.01
ep9 fusion	1.31 ± 0.04	1.12 ± 0.08
CfaB (–)	0	0

TABLE 2 Mouse serum anti-K88 IgG antibody titers (log_{10}) from groups (5 mice per group) subcutaneously immunized with each epitope fusion protein or the control group[a]

Mouse group	IgG titer	*P* value
ep1 fusion	3.61 ± 0.11	<0.001
ep2 fusion	3.53 ± 0.25	<0.001
ep3 fusion	3.45 ± 0.26	<0.001
ep4 fusion	2.42 ± 0.40	<0.001
ep5 fusion	3.89 ± 0.18	<0.001
ep6 fusion	2.29 ± 0.30	<0.001
ep7 fusion	2.08 ± 0.24	<0.001
ep8 fusion	2.86 ± 0.08	<0.001
ep9 fusion	2.85 ± 0.21	<0.001
Control	0 ± 0	

[a]IgG titers are presented as means and standard deviations. *P* values were calculated from one-way ANOVA by comparing the titers of each immunization group to those of the control group.

nant, epitopes 1, 2, 3, 4, 5, and 8 represent the neutralizing epitopes of the K88ac fimbrial major subunit and adhesin FaeG. It was noted that fusions of epitope 1, 2, 3, or 5 also induced significantly higher anti-K88 IgG antibody titers than the fusions carrying epitope 4, 6, 7, 8, or 9 in the immunized mice. Interestingly, the ep4 fusion induced lower anti-K88 IgG titers, but derived antibodies were the second best in inhibiting 3030-2 adherence to IPEC-J2 cells (28.5%; blocking over 70% of bacterial adherence). Differing from the ep4 fusion, the ep5 fusion stimulated the highest anti-K88ac IgG titer, and antibodies derived from this fusion protein exhibited the best activity in inhibiting the adherence of K88ac fimbrial 3030-2 bacteria (blocking over 75% of bacterial adherence). This suggests that epitope 5 and epitope 4 could be the top candidate antigens to be included in an MEFA for developing a multivalent vaccine against ETEC-associated diarrhea in young pigs. Indeed, peptides surrounding epitopes 5 and 4 were demonstrated to play a crucial role in K88 fimbria binding to host receptors (20–24). A truncation of epitope 5 or epitope 4 resulted in a total loss of K88 fimbria binding to pig small intestinal brush borders (our unpublished data). Therefore, antibodies against these two epitopes can potentially prevent K88 fimbriae from attaching to host receptors and colonizing pig small intestines.

We noted that the ep1, ep2, ep3, and ep5 fusions, which induced greater anti-K88ac IgG titers, strongly reacted with anti-K88ac antiserum, as demonstrated by Western blotting (Fig. 2) and ELISA (Table 3). This likely indicates that these epitopes are well exposed on K88ac fimbria. In contrast, the ep8 fusion did not react with anti-K88 antiserum, although this fusion induced neutralizing anti-K88 antibodies. That may suggest that epitope 8 was poorly exposed on K88 fimbriae. The FaeG protein model

TABLE 3 Mouse serum antibody inhibition against adherence of K88 fimbrial ETEC strain 3030-2 to porcine cell line IPEC-J2[a]

Mouse group	Adherent bacteria (%)	*P* value
Control	100 ± 24.2	
K88 fimbriae	9.8 ± 1.7	<0.001
ep1 fusion	41.5 ± 12	<0.001
ep2 fusion	44.3 ± 8.3	<0.001
ep3 fusion	37.2 ± 12	<0.001
ep4 fusion	28.5 ± 12.3	<0.001
ep5 fusion	22.5 ± 5.0	<0.001
ep6 fusion	87.5 ± 11.7	0.88
ep7 fusion	87.2 ± 15.2	0.86
ep8 fusion	66.7 ± 5.4	0.004
ep9 fusion	88.3 ± 23.5	0.92

[a]Numbers of 3030-2 bacteria (CFU; %) that adhered to IPEC-J2 cells after treatment with mouse serum samples from each group were counted and converted to a percentage, with the number of adherent bacteria treated with the control mouse serum set at 100%. Percentages are presented as means and standard deviations. *P* values were calculated from one-way ANOVA by comparing each immunized group with the control group.

predicted that epitope 8 is located at one end of the FaeG subunit. It is likely that this may be the location where FaeG subunits joined together to assemble K88ac fimbriae. Thus, subunit assembling negatively affects the exposure of epitope 8 on K88ac fimbriae, directly resulting in antibodies derived from K88ac fimbria having poor reactivity with epitope 8 carried by the CfaB backbone. Future protein structural studies can further map the location and posture of epitope 8 and provide more details of the structure of the FaeG subunit and K88ac fimbria.

With the identification of neutralizing epitopes from the K88ac fimbria FaeG major subunit and adhesin during this study, we now have all of the essential antigen elements to construct an MEFA immunogen for the development of a broadly protective vaccine against porcine postweaning diarrhea in the United States. This MEFA can use the A subunit of the LT toxoid as the backbone. By retaining one or two neutralizing epitopes from the A subunit to induce protective antibodies against LT enterotoxicity (14), we can replace other LT A subunit epitopes with the neutralizing epitopes of the K88 FaeG subunit, F18 FedF subunit, and Stx2e A subunit (15), as well as an STb shortened peptide and STa toxoid (15) for a fimbria-toxin MEFA. We have demonstrated that the replacement of an epitope of the LT A subunit with a foreign epitope abolishes LT enterotoxicity but does not alter its formation of LT structure (14). Substitutions of A subunit epitopes of LT toxoid (LT_{R192G} or $LT_{R192G/L211A}$) with the neutralizing fimbria epitopes and toxins Stx2e, STb, and STa peptides should eliminate LT enterotoxicity entirely, resulting in a safe but broadly immunogenic MEFA immunogen for vaccines against porcine postweaning diarrhea.

We need to point out that only K88ac was targeted since K88ac is the predominant variant causing ETEC-associated neonatal and postweaning diarrhea in the United States. Additional studies will be needed to identify neutralizing epitopes from the K88ab variant, which primarily causes pig diarrhea in Europe. However, epitopes identified from this study showed homology between the K88ac and K88ab variants. Indeed, epitopes 1, 2, 3, 5, 8, and 9 are identical, and epitope 6 differs in one amino acid. Whether antibodies induced by these homologous epitopes also inhibit adherence of K88ab ETEC will need to be verified in future studies. Additionally, perhaps pig anti-K88ac and anti-K88ab antiserum (instead of anti-mouse antiserum) should be used to initially characterize antigenicity of these epitopes. We also need to point out that the current study identified K88ac FaeG-neutralizing epitopes based on cell-based *in vitro* antibody adherence inhibition assays. Pig immunization and ETEC challenge studies will be needed to further characterize epitopes for antigenicity and to confirm their candidacy as antigens for vaccine development. Nevertheless, data from this study identified potential neutralizing epitopes for antibodies against K88 fimbria; this can move us one step closer to the development of a broadly protective vaccine against porcine postweaning diarrhea. Additionally, research in identifying neutralizing epitopes and constructing future MEFAs from this study may provide helpful information for multivalent vaccine development against other diseases.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 4 includes the bacterial strains and plasmids used for this study. CfaB recombinant strain 9477 (13) was used as the DNA template in PCRs to amplify the *cfaB* gene and the backbone to carry K88 FaeG epitopes (K88ep1 to K88ep9) for CfaB-epitope fusions. Vector pET28a (Novagen, Madison, WI) and *E. coli* BL21-CodonPlus (DE3) were used to clone CfaB-epitope fusion genes and to express fusion proteins (ep1 fusion to ep9 fusion), respectively. K88ac fimbrial ETEC field isolate 3030-2 (K88ac/LT/STb/STa) was used in antibody adherence inhibition assays to measure mouse serum antibody neutralization activities.

***In silico* identification of K88ac FaeG epitopes and construction of CfaB-epitope fusions.** K88ac fimbrial subunit FaeG immunodominant epitopes were identified using B-cell epitope prediction programs (25, 26). Phyre3 (27, 28) was used to generate a 3-dimensional protein structure of the FaeG subunit (PDB accession number 2J6G), and the PyMOL molecular graphics system (access 106826, version 2.2; Schrödinger, LLC, New York, NY, USA) was used to map each epitope from the FaeG protein model.

Nucleotides coding each FaeG epitope were embedded into carrier gene *cfaB* by replacing the nucleotides coding a CfaB backbone epitope in a splicing overlap extension (SOE) PCR with primers (Table 5) as described previously (14, 29). After digestion with restriction enzymes NheI and EagI (New

TABLE 4 *E. coli* host, field isolate, and recombinant strains as well as plasmids used in this study

Strain or plasmid	Relevant properties	Reference
Strains		
BL21	*huA2* Δ(*argF-lacZ*) *U169 phoA glnV44* φ80 Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	GE Healthcare
3030–2	Pig ETEC field isolate, K88ac/STa/STb/LT	5
9477	6×His CfaB + pET28a in DH5α	9
9503	6×His CfaB + pET28a in BL21	9
9702	6×His FaeG + pET28a in BL21	This study
9675	6×His CfaB-ep1 fusion + pET28a in BL21	This study
9677	6×His CfaB-ep2 fusion + pET28a in BL21	This study
9678	6×His CfaB-ep3 fusion + pET28a in BL21	This study
9679	6×His CfaB-ep4 fusion + pET28a in BL21	This study
9680	6×His CfaB-ep5 fusion + pET28a in BL21	This study
9681	6×His CfaB-ep6 fusion + pET28a in BL21	This study
9682	6×His CfaB-ep7 fusion + pET28a in BL21	This study
9683	6×His CfaB-ep8 fusion + pET28a in BL21	This study
9676	6×His CfaB-ep9 fusion + pET28a in BL21	This study
Plasmid		
pET28a		Novagen

England BioLabs, Ipswich, MA), 6×His-tagged CfaB-epitope fusion genes were individually cloned into the pET28a vector.

CfaB-epitope fusion protein expression and characterization. As described previously (10, 14, 15), a bacterial protein extraction reagent (B-PER; Thermo Fisher Scientific, Rochester, NY) was used to extract epitope fusion proteins (CfaB-FaeG-ep), and protein refolding buffer (Novogen) was used to refold the extracted fusion proteins. Epitope fusion proteins were then assessed for protein purity and integrity in SDS-PAGE with Coomassie blue staining and with anti-K88 mouse antiserum in a Western blot, respectively.

Epitope fusion proteins were further characterized in direct and competitive ELISAs to assess FaeG epitope conformation by measuring reactivity between each fusion protein and anti-K88ac antiserum (antiserum from the mice immunized with K88ac fimbriae). In direct ELISA, each epitope fusion protein (100 ng per well) was coated in the wells of 2HB microtiter plates (Thermo Fisher Scientific). Anti-K88 antiserum and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:3,000; Sigma, St. Louis, MO) were used as the primary antibodies and the secondary antibodies. CfaB protein was used to coat

TABLE 5 PCR primers used to amplify K88 *faeG* gene and protein carrier *cfaB* genes, and for split overlapping extension to insert each K88 FaeG epitope into protein carrier *cfaB* gene

Primer	Sequence (5′–3′)	Amplified region
CfaB-F	CGGGCTAGCGTAGAGAAAAATATT	Forward primer to amplify *cfaB* gene (NheI site underlined)
CfaB-R	TTACGGCCGGGATCCCAAAGTCAT	Reverse primer to amplify *cfaB* gene (EagI site underlined)
FaeG-F	CGGGCTAGCTGGATGACTGGTGATTTC	Forward primer to amplify *faeG* gene (NheI site underlined)
FaeG-R	TTACGGCCGTTAGTAATAAGTAATTGC	Reverse primer to amplify *faeG* gene (EagI site underlined)
ep1 fusion-R	CGAACCATTGAAATCACCAGTCATTTTTTTAGTTGCATCGTTTGT	Paired with CfaB-F to insert K88ep1 (MTGDFNGSVD) into CfaB
ep1 fusion-F	GGTGATTTCAATGGTTCGGTCGATGATACACCACAGCTTACAGAT	Paired with CfaB-R to insert K88ep1 (MTGDFNGSVD) into CfaB
ep2 fusion-R	TCCACCATTGGTCAGGTCATTCAATTTTTTAGTTGCATCGTTTGT	Paired with CfaB-F to insert K88ep2 (LNDLTNGGTK) into CfaB
ep2 fusion-F	GACCTGACCAATGGTGGAACCAAAGATACACCACAGCTTACAGAT	Paired with CfaB-R to insert K88ep2 (LNDLTNGGTK) into CfaB
ep3 fusion-R	AGCAAATGCTTCTTTGGTTCGGCCTTTTTTAGTTGCATCGTTTGT	Paired with CfaB-F to insert K88ep3 (GRTKEAFATP) into CfaB
ep3 fusion-F	ACCAAAGAAGCATTTGCTACGCCAGATACACCACAGCTTACAGAT	Paired with CfaB-R to insert K88ep3 (GRTKEAFATP) into CfaB
ep4 fusion-R	TCCACCATCAGGTTTTCTGAGTTCTTTTTTAGTTGCATCGTTTGT	Paired with CfaB-F to insert K88ep4 (ELRKPDGGTN) into CfaB
ep4 fusion-F	AGAAAACCTGATGGTGGAACTAATGATACACCACAGCTTACAGAT	Paired with CfaB-R to insert K88ep4 (ELRKPDGGTN) into CfaB
ep5 fusion-R	AACTTTAGTGCCCCCTGCATTTTTCATCGGTTTTTTAGTTGCATC	Paired with CfaB-F to insert K88ep5 (PMKNAGGTKVGAVKVN) into CfaB
ep5 fusion-F	GCAGGGGGCACTAAAGTTGGTTCAGTGAAAGTGAATGATACACCA	Paired with CfaB-R to insert K88ep5 (PMKNAGGTKVGAVKVN) into CfaB
ep6 fusion-R	CGCAGAAGTAACCCCACCTCTCCCTAATTTTTTAGTTGCATCGTT	Paired with CfaB-F to insert K88ep6 (LGRGGVTSADGEL) into CfaB
ep6 fusion-F	GGTGGGGTTACTTCTGCGGACGGGGAGCTGGATACACCACAGCTT	Paired with CfaB-R to insert K88ep6 (LGRGGVTSADGEL) into CfaB
ep7 fusion-R	CCCAGCCGAGAGTTCAGAACCCCTCGGTTTTTTAGTTGCATCGTT	Paired with CfaB-F to insert K88ep7 (PRGSELSAGSA) into CfaB
ep7 fusion-F	TCTGAACTCTCGGCTGGGAGTGCCGATACACCACAGCTTACAGAT	Paired with CfaB-R to insert K88ep7 (PRGSELSAGSA) into CfaB
ep8 fusion-R	ATCAGTGTACTCCATGTTTTCCCTTTTTTTAGTTGCATCGTTTGT	Paired with CfaB-F to insert K88ep8 (RENMEYTDGT) into CfaB
ep8 fusion-F	AACATGGAGTACACTGATGGAACTGATACACCACAGCTTACAGAT	Paired with CfaB-R to insert K88ep8 (RENMEYTDGT) into CfaB
ep9 fusion-R	GCTGGTAGTTACAGCCTGATTAAATTTTTTAGTTGCATCGTTTGT	Paired with CfaB-F to insert K88ep9 (FNQAVTTSTQ) into CfaB
ep9 fusion-F	CAGGCTGTAACTACCAGCACTCAGGATACACCACAGCTTACAGAT	Paired with CfaB-R to insert K88ep9 (FNQAVTTSTQ) into CfaB

the wells of 2HB plates (100 ng per well) as the negative control. In competitive ELISA, K88ac fimbriae (heat-extracted from ETEC strain 3030-2; 100 ng per well) were used to coat the wells of 2HB plates, and each epitope fusion protein (100 ng per well) was used to compete with the coated K88 fimbriae for reaction with anti-K88ac antiserum (diluted from 1:4,000 to 1:32,000), with HRP-conjugated goat anti-mouse IgG as the secondary antibodies. Optical density at 650 nm (OD_{650}) values were measured from a plate reader after exposure to 3,3′,5,5′-tetramethylbenzidine (TMB; KPL, Gaithersburg, MD) for 30 min at room temperature.

Epitope fusion protein mouse immunization. Eight-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA), five mice per group, were subcutaneously immunized with each epitope fusion protein (40 μg) adjuvanted with double mutant heat-labile toxin (dmLT, $LT_{R192G/L211A}$; 1 μg). Two boosters at the same dose as the primary were given at 2-week intervals. The control group received no injection. Mouse immunization protocol was approved by the Kansas State University Institutional Animal Care and Use Committee.

Mouse serum anti-K88ac antibody titration. Mouse serum samples collected before the primary and 2 weeks after the final booster were titrated for anti-K88ac IgG antibodies. As described previously (15, 30, 31), 2HB plates coated with K88ac fimbriae or recombinant FaeG protein (100 ng per well) were incubated with mouse serum binary dilutions (1:400 to 1:128,000) and then secondary antibody HRP-conjugated goat anti-mouse IgG (1:3,000). Anti-K88ac or anti-FaeG IgG antibody titers (log_{10}) were calculated based on OD_{650} values measured after exposure to TMB (KPL). Mouse serum IgA antibody response to K88 was not examined.

Mouse serum antibody adherence inhibition against K88ac fimbrial ETEC strain. Serum samples pooled from each group immunized with an epitope fusion protein and the control group were examined for antibodies inhibiting the adherence of K88ac fimbrial ETEC isolate 3030-2 to IPEC-J2 cells. Briefly, 3030-2 bacteria (3×10^6) premixed with a mouse serum sample (30 μl) from each group were incubated with 90 to 95% confluent IPEC-J2 cells (1.5×10^5 per well) in a CO_2 incubator. After 1 h of incubation, IPEC-J2 cells were washed with phosphate-buffered saline (PBS) to remove nonadherent bacteria and then dislodged with Triton X-100 (0.5%; Sigma). Dislodged cells were collected, diluted, and plated on agar plates. Bacteria were cultured overnight at 37°C and were counted for CFU.

Data analyses. Differences among epitope fusion proteins in reactivities with anti-K88ac antiserum in direct ELISAs were compared in two-way analysis of variance (ANOVA). Differences between each immunization group and the control group for IgG antibody titers, antibody adherence inhibition activities, and reactivities with anti-K88ac antiserum in competitive ELISAs were compared in one-way ANOVA. A *P* value of less than 0.05 indicated a significant difference.

ACKNOWLEDGMENTS

We thank B. Schultz (Kansas State University) for providing pig cell line IPEC-J2.

This work was funded by USDA-NIFA Agriculture and Food Research Initiative Competitive Grant no. 2017-67015-26632.

REFERENCES

1. Francis DH. 2002. Enterotoxigenic *Escherichia coli* infection in pigs and its diagnosis. J Swine Health Prod 10:171–175.
2. Frydendahl K, Imberechts H, Lehmann S. 2001. Automated 5′ nuclease assay for detection of virulence factors in porcine *Escherichia coli*. Mol Cell Probes 15:151–160. https://doi.org/10.1006/mcpr.2001.0354.
3. USDA. 2002. Part II: reference of swine health and health management in the United States, 2000. Centers for Epidemiology and Animal Health, Ft. Collins, CO. https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/monitoring-and-surveillance/nahms/nahms_swine_studies#swine2000.
4. Fairbrother JM, Nadeau E, Gyles CL. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim Health Res Rev 6:17–39. https://doi.org/10.1079/AHR2005105.
5. Zhang W, Zhao M, Ruesch L, Omot A, Francis D. 2007. Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. Vet Microbiol 123:145–152. https://doi.org/10.1016/j.vetmic.2007.02.018.
6. Dubreuil JD, Isaacson RE, Schifferli DM. 2016. Animal enterotoxigenic *Escherichia coli*. EcoSal Plus 2016 https://doi.org/10.1128/ecosalplus.ESP-0006-2016.
7. Zhang W. 2014. Progress and challenges in vaccine development against enterotoxigenic *Escherichia coli* (ETEC) -associated porcine post-weaning diarrhea (PWD). J Vet Med Res 1:1001–1013.
8. Duan Q, Lee KH, Nandre RM, Garcia C, Chen J, Zhang W. 2017. MEFA (multiepitope fusion antigen)-novel technology for structural vaccinology, proof from computational and empirical immunogenicity characterization of an enterotoxigenic *Escherichia coli* (ETEC) adhesin MEFA. J Vaccines Vaccin 8:367. https://doi.org/10.4172/2157-7560.1000367.
9. Ruan X, Knudsen DE, Wollenberg KM, Sack DA, Zhang W. 2014. Multiepitope fusion antigen induces broadly protective antibodies that prevent adherence of *Escherichia coli* strains expressing colonization factor antigen I (CFA/I), CFA/II, and CFA/IV. Clin Vaccine Immunol 21:243–249. https://doi.org/10.1128/CVI.00652-13.
10. Nandre RM, Ruan X, Duan Q, Sack DA, Zhang W. 2016. Antibodies derived from an enterotoxigenic *Escherichia coli* (ETEC) adhesin tip MEFA (multiepitope fusion antigen) against adherence of nine ETEC adhesins: CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS21 and EtpA. Vaccine 34:3620–3625. https://doi.org/10.1016/j.vaccine.2016.04.003.
11. Duan Q, Lu T, Garcia C, Yanez C, Nandre RM, Sack DA, Zhang W. 2018. Co-administered tag-less toxoid fusion $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ and CFA/I/II/IV MEFA (multiepitope fusion antigen) induce neutralizing antibodies to 7 adhesins (CFA/I, CS1-CS6) and both enterotoxins (LT, STa) of enterotoxigenic *Escherichia coli* (ETEC). Front Microbiol 9:1198. https://doi.org/10.3389/fmicb.2018.01198.
12. Nandre R, Ruan X, Lu T, Duan Q, Sack D, Zhang W. 2018. Enterotoxigenic *Escherichia coli* adhesin-toxoid multiepitope fusion antigen CFA/I/II/IV-$3\times STa_{N12S}$-$mnLT_{R192G/L211A}$-derived antibodies inhibit adherence of seven adhesins, neutralize enterotoxicity of LT and STa toxins, and protect piglets against diarrhea. Infect Immun 86:e00550-17. https://doi.org/10.1128/IAI.00550-17.
13. Ruan X, Sack DA, Zhang W. 2015. Genetic fusions of a CFA/I/II/IV MEFA (multiepitope fusion antigen) and a toxoid fusion of heat-stable toxin (STa) and heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) retain broad anti-CFA and antitoxin antigenicity. PLoS One 10:e0121623. https://doi.org/10.1371/journal.pone.0121623.
14. Huang JC, Duan QD, Zhang WP. 2018. Significance of enterotoxigenic *Escherichia coli* (ETEC) heat-labile toxin (LT) enzymatic subunit epitopes

in LT enterotoxicity and immunogenicity. Appl Environ Microbiol 84: e00849-18. https://doi.org/10.1128/AEM.00849-18.
15. Rausch D, Ruan X, Nandre R, Duan Q, Hashish E, Casey TA, Zhang W. 2017. Antibodies derived from a toxoid MEFA (multiepitope fusion antigen) show neutralizing activities against heat-labile toxin (LT), heat-stable toxins (STa, STb), and Shiga toxin 2e (Stx2e) of porcine enterotoxigenic *Escherichia coli* (ETEC). Vet Microbiol 202:79–89. https://doi.org/10.1016/j.vetmic.2016.02.002.
16. Ruan X, Robertson DC, Nataro JP, Clements JD, Zhang W, The STa Toxoid Vaccine Consortium Group. 2014. Characterization of heat-stable (STa) toxoids of enterotoxigenic *Escherichia coli* fused to a double mutant heat-labile toxin (dmLT) peptide in inducing neutralizing anti-STa antibodies. Infect Immun 82:1823–1832. https://doi.org/10.1128/IAI.01394-13.
17. Zhang W, Francis DH. 2010. Genetic fusions of heat-labile toxoid (LT) and heat-stable toxin b (STb) of porcine enterotoxigenic *Escherichia coli* elicit protective anti-LT and anti-STb antibodies. Clin Vaccine Immunol 17: 1223–1231. https://doi.org/10.1128/CVI.00095-10.
18. Lu T, Seo H, Moxley RA, Zhang W. 2019. Mapping the neutralizing epitopes of F18 fimbrial adhesin subunit FedF of enterotoxigenic *Escherichia coli* (ETEC). Vet Microbiol 230:171–177. https://doi.org/10.1016/j.vetmic.2019.02.015.
19. Lu T, Zhang W. 2017. Identifying immuno-dominant and neutralizing epitopes from K88 fimbriae of enterotoxigenic Escherichia coli (ETEC). Kansas Agric Exp Station Res Rep 3:54. https://doi.org/10.4148/2378-5977.7507.
20. Thiry G, Clippe A, Scarcez T, Petre J. 1989. Cloning of DNA sequences encoding foreign peptides and their expression in the K88 pili. Appl Environ Microbiol 55:984–993.
21. Jacobs AA, Roosendaal B, van Breemen JF, de Graaf FK. 1987. Role of phenylalanine 150 in the receptor-binding domain of the K88 fibrillar subunit. J Bacteriol 169:4907–4911. https://doi.org/10.1128/jb.169.11.4907-4911.1987.
22. Bakker D, van Zijderveld FG, van der Veen S, Oudega B, de Graaf FK. 1990. K88 fimbriae as carriers of heterologous antigenic determinants. Microb Pathog 8:343–352. https://doi.org/10.1016/0882-4010(90)90093-6.
23. Bakker D, Vader CE, Roosendaal B, Mooi FR, Oudega B, de Graaf FK. 1991. Structure and function of periplasmic chaperone-like proteins involved in the biosynthesis of K88 and K99 fimbriae in enterotoxigenic *Escherichia coli*. Mol Microbiol 5:875–886. https://doi.org/10.1111/j.1365-2958.1991.tb00761.x.
24. Zhang W, Fang Y, Francis DH. 2009. Characterization of the binding specificity of K88ac and K88ad fimbriae of enterotoxigenic *Escherichia coli* by constructing K88ac/K88ad chimeric FaeG major subunits. Infect Immun 77:699–706. https://doi.org/10.1128/IAI.01165-08.
25. Larsen JE, Lund O, Nielsen M. 2006. Improved method for predicting linear B-cell epitopes. Immunome Res 2:2. https://doi.org/10.1186/1745-7580-2-2.
26. Saha S, Raghava GPS. 2007. Prediction methods for B-cell epitopes. Methods Mol Biol 409:387–394. https://doi.org/10.1007/978-1-60327-118-9_29.
27. Bennett-Lovsey RM, Herbert AD, Sternberg MJE, Kelley LA. 2007. Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins 70:611–625. https://doi.org/10.1002/prot.21688.
28. Kelley LA, Sternberg MJE. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4:363–371. https://doi.org/10.1038/nprot.2009.2.
29. Duan QD, Zhang WP. 2017. Genetic fusion protein 3×STa-ovalbumin is an effective coating antigen in ELISA to titrate anti-STa antibodies. Microbiol Immunol 61:251–257. https://doi.org/10.1111/1348-0421.12494.
30. Ruan X, Liu M, Casey TA, Zhang W. 2011. A tripartite fusion, FaeG-FedF-LT(192)A2:B, of enterotoxigenic *Escherichia coli* (ETEC) elicits antibodies that neutralize cholera toxin, inhibit adherence of K88 (F4) and F18 fimbriae, and protect pigs against K88ac/heat-labile toxin infection. Clin Vaccine Immunol 18:1593–1599. https://doi.org/10.1128/CVI.05120-11.
31. Ruan X, Zhang W. 2013. Oral immunization of a live attenuated *Escherichia coli* strain expressing a holotoxin-structured adhesin-toxoid fusion (1FaeG-FedF-LTA2:5LTB) protected young pigs against enterotoxigenic *E. coli* (ETEC) infection. Vaccine 31:1458–1463. https://doi.org/10.1016/j.vaccine.2013.01.030.

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ERRATUM

Erratum for Lu et al., "Mapping the Neutralizing Epitopes of Enterotoxigenic *Escherichia coli* K88 (F4) Fimbrial Adhesin and Major Subunit FaeG"

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Volume 85, no. 11, e00329-19, https://doi.org/10.1128/AEM.00329-19. Page 6, Materials and Methods, paragraph 1, line 6: "3030-2 (K88ac/LT/STb/STa)" should be "3030-2 (K88ac/LT/STb/EAST1)."

Page 7, Table 4: In the "Relevant properties" column for strain 3030-2, "K88ac/STa/STb/LT" should be "K88ac/LT/STb/EAST1."

Citation Lu T, Moxley RA, Zhang W. 2019. Erratum for Lu et al., "Mapping the neutralizing epitopes of enterotoxigenic *Escherichia coli* K88 (F4) fimbrial adhesin and major subunit FaeG." Appl Environ Microbiol 85:e01946-19. https://doi.org/10.1128/AEM.01946-19.

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Published 1 October 2019