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ORIGINAL RESEARCH PAPER

Secretory expression of K88 (F4) fimbrial adhesin FaeG by recombinant *Lactococcus lactis* for oral vaccination and its protective immune response in mice

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Abstract K88 (F4) fimbrial adhesin, FaeG, was expressed extracellularly in *Lactococcus lactis* using a nisin-controlled gene expression system. The antibody response and protective efficacy of the recombinant bacteria (*L. lactis* [spNZ8048-*faeG*]) against live enterotoxigenic *E. coli* (ETEC) C_{83549} challenge were evaluated in ICR mice. Mice vaccinated with *L. lactis* [spNZ8048-*faeG*] had a significantly increased antigen-specific IgG level in the serum and decreased mortality rate ($P < 0.05$) compared with the control. This indicates that oral immunization of *L. lactis* [spNZ8048-*faeG*] can induce an immune-response protection upon challenge with live ETEC in ICR mice.

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains that produce K88 (F4) fimbriae on their surfaces commonly induce diarrhea in piglets, which is an important cause of both mortality and reduced growth rate resulting in heavy economic losses (Hampson 1994). With the F4 fimbriae, the bacteria adhere to the F4 receptor (F4R) on small intestinal villi of piglets (Jones and Rutter 1972), then colonize the small intestine and produce enterotoxins which cause electrolyte imbalance in the gut, inducing diarrhea, eventually leading to death (Nagy et al. 1990). K88 (F4) fimbriae are the best-characterized adhesins (Van den Broeck et al. 2000) composed mainly of several hundreds of identical adhesive subunits called FaeG (Mol and Oudega 1996), as well as some minor subunits. FaeG protein carries the adhesive properties of the K88 fimbriae and has been most widely studied as a preventive vaccine against ETEC infection in pigs (Melkebeek et al. 2007; Yahong et al. 2006; Joensuu et al. 2006). Oral vaccination of piglets with recombinant produced F4 fimbrial adhesin, FaeG, induces an F4-specific mucosal immune response (Verdonck et al. 2004; Joensuu et al. 2006).

As a GRAS ("generally regarded as safe") organism with low innate antigenicity, *Lactococcus lactis* is a good candidate to develop safe, oral vaccines. Many heterologous proteins have been expressed successfully in *L. lactis*, and immunization with these strains elicits immune responses specific to heterologous antigens (Enouf et al. 2001; Geller et al. 2001; Iwaki et al. 1990; Lee et al. 2001; Ribeiro et al. 2002; Wells et al. 1993). Due to the auto-regulatory mechanism of nisin biosynthesis and high inductive effect on the expression of interested genes, the nisin-controlled gene expression system (the NICE system) is very efficient and promising (Zhou et al. 2006). Nevertheless, using *L. lactis* to produce FaeG antigens has not been reported. In this study, a recombinant *L. lactis* producing FaeG extracellularly was constructed and orally administered to ICR mice. Immunized mice were then challenged with ETEC to determine the level of potential protective response induced by recombinant *L. lactis*. This will provide a new way to counter ETEC.

Materials and methods

Bacterial strains and growth conditions

According to the designation of the China Institute of Veterinary Drug Control, a standard ETEC strain expressing K88 fimbriae, enterotoxigenic *E. coli* (ETEC) C_{83549} was cultured for 24 h in Luria–Bertani (LB) medium at 37°C. *Escherichia coli* MC1061 was used for plasmid propagation and grown in LB medium at 37°C. *L. lactis* subsp. *cremoris* NZ9800, NZ9700 and plasmid spNZ8048 were kindly provided by NIZO Food Research, Ede, the Netherlands. The *L. lactis* were grown at 30°C without aeration in M17 broth (Difco) (Terzaghi and Sandine 1975) supplemented with 0.5% (w/v) glucose (de Ruyter et al. 1996). Agar (1.5%) was added to GM17 to make solid medium. When required, antibiotics were: chloramphenicol, 5 μg ml^{-1} for *L. lactis*; chloramphenicol, 100 μg ml^{-1} and streptomycin, 10 μg ml^{-1} for *E. coli* MC1061.

Animals and maintenance

Male ICR mice (ICR mouse is short for "Imprinting Control Region" mouse), 4 weeks of age and with an average body weight of 20 g, were procured from Shanghai Laboratory Animal Center, China. Mice were randomly assigned to cages (six per cage) and acclimatized for 1 week in the housing facility maintained at 23 ± 1°C with a 12 h/12 h light/dark cycle. Mice were kept under standard pathogen-free conditions and provided with free access to food and water during the experiments.

Construction of recombinant plasmid containing *faeG* gene

The recombinant plasmid construction scheme is summarized in Fig. 1. The *faeG* gene was amplified from the genome of enterotoxigenic *E. coli* (ETEC)

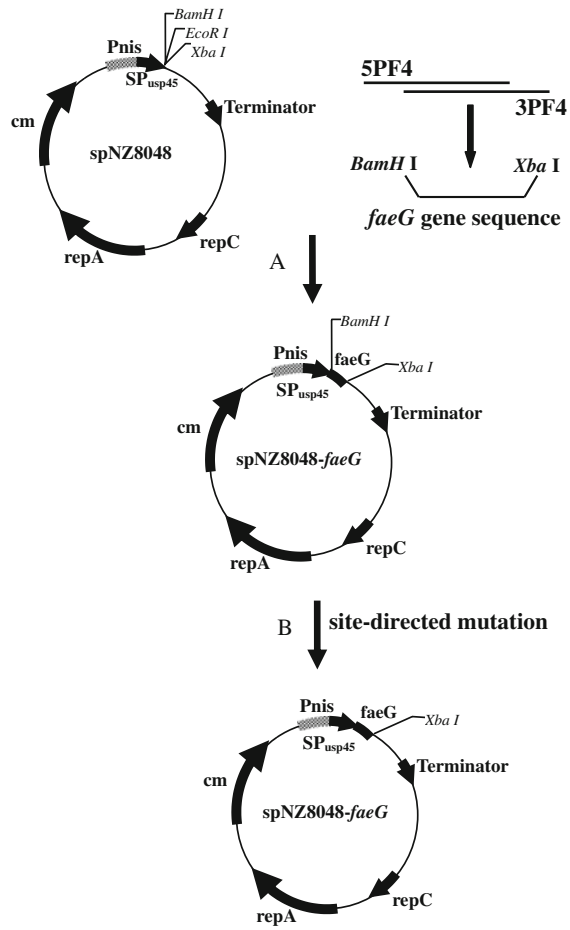

Fig. 1 Construction of the recombinant plasmid spNZ8048-*faeG*. *Pnis* the nisinA promoter, *cm* chloramphenicol. *faeG* gene was inserted into the *Bam*HI/*Xba*I restriction sites of spNZ8048 and spNZ8048-*faeG* recombinant plasmid was obtained

C_{83549} using a forward primer 5PF4 (5′-AGGGGATCCGCCTGGATGACTGGTGATTT-3′) and a reverse primer 3PF4 (5′-GATTCTAGATTAGTAATAAGTTATTGCTAC-3′), with *Bam*HI site (underlined) at the N-terminus and *Xba*I site (underlined) at the C-terminus. PCR (using PTC-200, USA) was performed with 1 cycle at 94°C for 2 min for initial denaturation; 30 cycles at 94°C, 30 s; 55°C, 55 s; 72°C, 30 s for amplification, and a final elongation step at 72°C for 10 min. The PCR product was cloned into pUCm-T vector first, and then the recombinant clone plasmid and spNZ8048 expression plasmid were digested with *Bam*HI and *Xba*I, respectively. The double-digested spNZ8048 and *faeG* fragment were ligated together, and then transformed into *E. coli* MC1061 by the method of Sambrook et al. (1989). The ligation resulted in the expression plasmid spNZ8048-*faeG*. Then, primers 5PB4 (5′-GCCTGGATGACTGGTGATTTCAATGGTT-3′) and 3PB4 (5′-CAGCCTTGGGTGTGGTGGCCTTGGT-3′) were designed to eliminate the *Bam*HI site (GGATCC) located between the signal peptide and *faeG* gene in spNZ8048-*faeG* through PCR-based site-directed mutation method (Fig. 2) (Hemsley et al. 1989). Inverse-PCR was performed with 1 cycle at 94°C for 2 min for initial denaturation; 30 cycles at 94°C, 30 s; 60°C, 60 s; 72°C, 4 min for amplification. Recombinant plasmids spNZ8048-*faeG* eliminated the *Bam*HI site were introduced into *L. lactis* NZ9800 by electroporation using a Bio-Rad Gene Pulsar II Electroporator apparatus at 25 mF, 2 kV and a Gene Controller set at 200 Ω using 0.1 cm gap electroporation cuvettes (Holo and Nes 1995; Geller et al. 2001). Immediately after electroporation, the cells were resuspended in M17 containing 15% (w/v) sucrose, 1% (w/v) glycine and supplemented with 20 mM $MgCl_2$ and 2 mM $CaCl_2$, and incubated at 30°C for 2 h without any agitation to allow the cells to recover from the electroporation. After recovery, the cells were plated on GM17 agar plates containing antibiotics and incubated for 1–2 days at 30°C.

Nisin-controlled expression of FaeG

Overnight cultures of *L. lactis* NZ9800 [spNZ8048-*faeG*] were diluted 1:50 in fresh GM17 medium containing antibiotics and grown until early/mid-growth phase, which corresponds to an OD_{600} of 0.3–0.5. Expression of FaeG in recombinant *L. lactis* NZ9800 were induced for by adding the overnight cultures supernatant of *L. lactis* NZ9700 (containing secreted nisin) at a 1:1,000 dilution. After induction, cells were grown without agitation at 30°C for 3 h.

Western blotting analysis

Expression of FaeG was tested by immunoblotting of the pellet and supernatant fractions of recombinant *L. lactis* culture. The samples were mixed with equal volume of loading buffer and boiled for 10 min, then

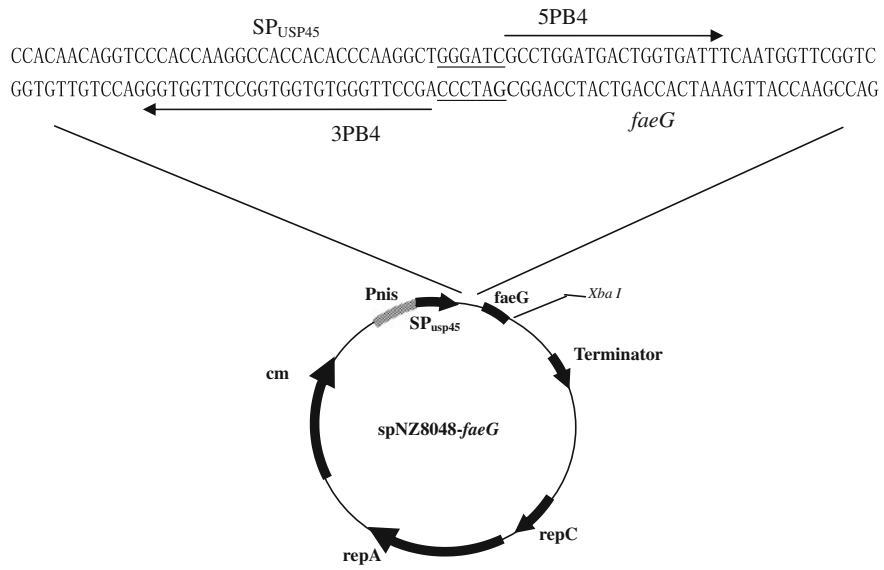

Fig. 2 Scheme of the inverse PCR procedure. The expanded sequence shows the 'back-to-back' positions of the two primers with respect to each other and the site of the introduced mutation (deletion of the *Bam*HI restriction site) in the coding sequence of the *faeG* gene

centrifuged for 10 min at 10,000×*g*. About 25 µl supernatant was loaded in 12% (v/w) polyacrylamide gel according to Sambrook et al. (1989). The proteins checked by SDS-PAGE were electrically transferred onto polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, UK). The membrane was blocked overnight in Tris-buffered saline containing 0.05% Tween20 (TBST) and 5% (v/w) skimmed milk followed by incubation for 2 h in TBST containing polyclonal antibody (1:1,000) raised against FaeG in a rabbit (prepared by our laboratory). After being washed three times in TBST, the membrane was incubated for 1 h with 1:500 diluted goat-anti-rabbit antibodies coupled with alkaline phosphatase (Sino-American Biotech., Luoyang, Henan, China). Then the bands were visualized by the reaction of 5-bromo-4-chloro-3-indol-1-phosphate (BCIP) and Nitroblue Tetrazolium (NBT) solutions on the membrane.

Immunization of ICR mice

Sixty mice were divided into three groups and immunized orally with *L. lactis* [spNZ8048-*faeG*] (Group A), *L. lactis* [spNZ8048] (Group B) and PBS (Group C) up to 30 days (see Table 1). Cell pellets of *L. lactis* were resuspended in sterile phosphate-buffered saline (PBS) at 10^9 c.f.u./ml. Every mouse received 500 µl of either PBS or a cell suspension containing 10^9 c.f.u. (see Table 1). On the 7th day after last immunization, 10 mice of each group were euthanized and the blood was collected by extirpating eyeballs. The blood samples were centrifuged at 4,000×*g* and the serum were stored at −20°C.

ELISA for F4-specific antibody

For detecting the specific serum antibody, F4 fimbriae (expressed FaeG) was used as the antigen to coat 96-well plates and incubated overnight at 4°C. The plates were blocked with dilution buffer (1% BSA in PBS), then added mouse serum. Alkaline phosphatase-conjugated goat anti-mouse IgG antiserum (Sigma) was used as the secondary antibody (1:10,000 dilution in blocking buffer). The samples were incubated for 1 h at room temperature after each step described above. After incubation, the plates were washed three times with washing buffer (PBS + 0.2% Tween20). An ABTS solution containing H_2O_2 was added and the absorption value was measured at 405 nm by a microplate spectrophotometer.

Slide agglutination test

The slide agglutination assay was conducted essentially as described previously (Lopez-Vidal and Svennerholm 1990). *E. coli* (ETEC) C_{83549} were harvested and adjusted to 10^{10} c.f.u./ml in PBS. About 20 µl of bacterial suspension was then applied to glass slides. One microliter of two-fold dilution series of vaccinated mice antisera were added, and then mixed with a wooden applicator stick. Visible agglutination within 2 min was considered as a positive reaction.

Challenge of vaccinated mice

Ten mice of each group were all challenged intragastrically with $100LD_{50}$ of live enterotoxigenic *E. coli* (ETEC) C_{83549} on the 14th day after last

Table 1 Mortality of vaccinated mice challenged with virulent enterotoxigenic *E. coli* (ETEC) C_{83549}

Group	Immunization	Morbidity	Normal	Mortality	
				No. of deaths/total no.	%
A	*L. lactis* [spNZ8048-*faeG*]	1	9	1/10	10
B	*L. lactis* [spNZ8048]	8	2	5/10	50
C	PBS	9	1	6/10	60

Sixty mice were divided into three groups and immunized by oral gavage. Group A was immunized with recombinant *L. lactis* [spNZ8048-*faeG*]. As negative controls, groups B and C were immunized with *L. lactis* [spNZ8048] and PBS, respectively. Each group received six doses during 30 days (at 1st, 2nd, 3rd, 14th, 15th, 16th, 28th, 29th and 30th day, respectively). On the 14th day after last immunization, 10 mice of each group were challenged intragastrically with $100LD_{50}$ of live ETEC C_{83549}. The mice were then monitored for survival and morbidity for 10 days

immunization. The gastric acid was neutralized with 1.4% $NaHCO_3$ for 15 min prior to the challenge. The mice were then monitored for survival for 10 days. All the surviving mice were sacrificed and the internal lesions were detected.

Statistical analysis

One-way ANOVA (Statistical Analysis System, SAS, version 6.03) was performed to find significant difference among various parameters. A significant level of $P < 0.05$ was used.

Results and discussion

The recombinant plasmid spNZ8048-*faeG* eliminated the *Bam*HI site was constructed (Fig. 1). In spNZ8048 plasmid, promoter PnisA and the signal peptide $U_{SP}45$ were used for nisin-controlled expression and efficient secretion of FaeG by *L. lactis* NZ9800, respectively (Accession numbers of *faeG* and SPusp45 sequences in GenBank were EU570252 and EU382095, respectively) (Zhou et al. 2006). *Bam*HI restriction site encoding two amino acids located between SPusp45 and *faeG* was removed to eliminate its possible effect on the activity of FaeG. The correct orientation and sequence of *faeG* were verified by sequencing.

The *faeG* expression in *L. lactis* [spNZ8048-*faeG*] was induced with nisin. The Western blot results are given in Fig. 3. As expected, no hybridized band was detected from the cell lysates of *L. lactis* [spNZ8048] and recombinant strain without induction by nisin (lanes 1–2, Fig. 3), while *L. lactis* [spNZ8048-*faeG*] with induction showed immunoreactive bands of FaeG in cell lysates and supernatant fractions at the size of about 25 kDa which was consistent with the theoretical

Fig. 3 Western blot analysis of FaeG expression with the specific polyclonal antibodies. *Lane 1*: cell lysate of *L. lactis* NZ9800 [spNZ8048]; *lane 2*: non-induced recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*]; *lane 3*: cell lysate of recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*] induced with nisin; *lane 4*: the supernatant fraction of recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*] induced with nisin

Mw of FaeG (23.5 kDa) (lanes 3–4, Fig. 3). FaeG was thus expressed in *L. lactis* NZ9800 by a tight regulatory nisin-controlled and secreted into culture medium successfully by the signal peptide $U_{SP}45$.

This is the first report on the secretory expression of FaeG gene in *L. lactis*. Heterologous secretory expression of α-amylase from *Bacillus stearothermophilus* and bovine plasmin in *L. lactis* were also achieved using the same signal peptide (van Asseldonk et al. 1993; Arnau et al. 1997). Additionally, compared to host strain *L. lactis* NZ9800, the recombinant strains harboring empty vector [spNZ8048] or *faeG*-encoding plasmids [spNZ8048-*faeG*] showed similar growth rate (data not shown). This suggested FaeG protein did not influence the growth of *L. lactis*, which was consistent with previous findings (Cho et al. 2007).

The K88 fimbriae antigen was considered as an important component in the development of oral vaccines against ETEC infections. As shown in Fig. 4, the antigen-specific IgG level in the serum of mice orally immunized with *L. lactis* [spNZ8048-*faeG*] was significantly higher than that of the two control groups (immunized *L. lactis* [spNZ8048] or PBS) on the 7th day after last immunization ($P < 0.05$). It indicated that oral immunization with *L. lactis* containing recombinant FaeG could successfully induce the secretion of F4-specific IgG antibodies in mice. The F4-specific IgG response induced by rFaeG

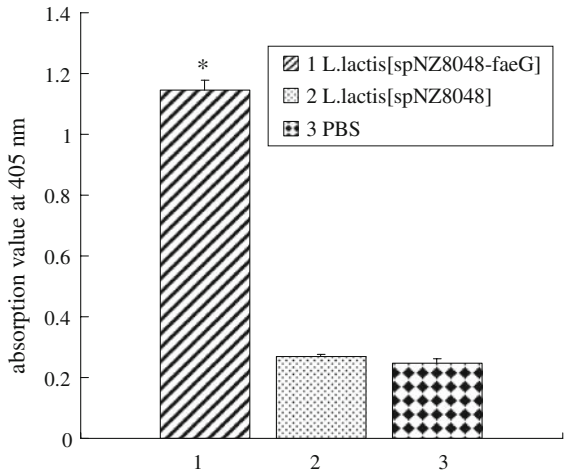

Fig. 4 The absorption value at 405 nm of F4-specific serum antibody in mice. The value was detected on the 7th day after the last immunization with the recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*], *L. lactis* NZ9800 [spNZ8048], or PBS. * Significant difference ($P < 0.05$) compared to group B and C

expressed in *E. coli* or barley was also detected, suggesting the feasibility of developing a safe system which can readily generate K88 adhesin with protective immunogenicity (Verdonck et al. 2004; Joensuu et al. 2006).

The results of immunized mice orally challenged with virulent Enterotoxigenic *E. coli* (ETEC) C_{83549} are shown in Table 1. The mortality rate of group A (immunized with *L. lactis* [spNZ8048-*faeG*]) was lower than that of group B (immunized with *L. lactis* [spNZ8048] and C (immunized with PBS). The vaccinated mice showed only one death among the total 10, while the two control groups showed 50% and 60% mortality rate, respectively. Moreover, most of mice in control groups developed diarrhea on the second day of challenge. Viscera, including liver, spleen, stomach, duodenum and jejunum catarrh, had evident lesions after screening all surviving mice. This indicates that oral immunization of FaeG-expressing *L. lactis* exhibits a protective response against ETEC infection in mice.

Furthermore, in order to determine whether the immunized mice antiserum could interact with the Enterotoxigenic *E. coli* (ETEC) C_{83549}, a slide agglutination test was conducted. Sera from immunized mice can therefore induce a macroscopic agglutination of C_{83549}. In contrast, the sera from unvaccinated mice showed no agglutination (data not shown).

The reason choosing *L. lactis* as expression host is that it is a food-grade bacterium of GRAS status and used as live vaccine which can deliver the recombinant protein to the intestinal mucosa of human/animals. Several proteins from infectious organisms, such as type 3 capsular polysaccharide (CPS) (Gilbert et al. 2000), rotavirus non-structural protein 4 (NSP4) (Enouf et al. 2001) and SpaA of *Erysipelothrix rhusiopathiae* (Cheun et al. 2004), have been expressed in *L. lactis* and induce specific antibodies. Expression of FaeG in *L. lactis* may lead to a promising way in prevention of piglet's diarrhea and has potential for widespread application.

In conclusion, the results showed that recombinant *L. lactis* could express FaeG protein extracellularly and significantly induce protective immune response in mice. Due to the obvious immuno-protective effect of FaeG protein in mice, oral immunization of *L. lactis* expressing FaeG protein is a promising way to prevent diarrhea in pre- and post-weaned piglets. Further study should be conducted to detect the immune protection of *L. lactis* expressing FaeG in pig and other animals.

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