

Lactococcus lactis

Received: 13 August 2008 / Revised: 13 February 2009 / Accepted: 17 February 2009 / Published online: 10 March 2009
Springer Science+Business Media B.V. 2009

K88 (F4) fimbrial adhesin, FaeG, as expressed extracellularly in *Lactococcus lactis* using a pnisin-controlled gene expression system. The antibody response and protective efficacy of the recombinant bacteria (*L. lactis* [spNZ8048-*faeG*]) against live enterotoxigenic *E. coli* (ETEC) C₈₃₅₄₉ 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.

Enterotoxigenic *Escherichia coli* ·
FaeG · Immune response · *Lactococcus lactis* ·
Oral vaccination

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 (Nag 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 intensively 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).

C. X. Hu · Z. R. Xu · W. F. Li (✉) · N. Dong · L. L. Fu
Key Laboratory of Molecular Animal Nutrition
of Ministry of Education, College of Animal Sciences,
Zhejiang University, Hangzhou, Zhejiang 310029,
People's Republic of China
e-mail: li@ju.edu.cn

C. X. Hu
Department of Life Science, Yuanpei College,
Shaoxing University, Shaoxing, Zhejiang 312000,
People's Republic of China

P. Lu
Institute of Microbiology, College of Life Sciences,
Zhejiang University, Hangzhou, Zhejiang 310029,
People's Republic of China



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.

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) C₈₃₅₄₉ 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) (Teraghi and Sandine 1975) supplemented with 0.5% (v/v) glucose (de Ruiter 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.

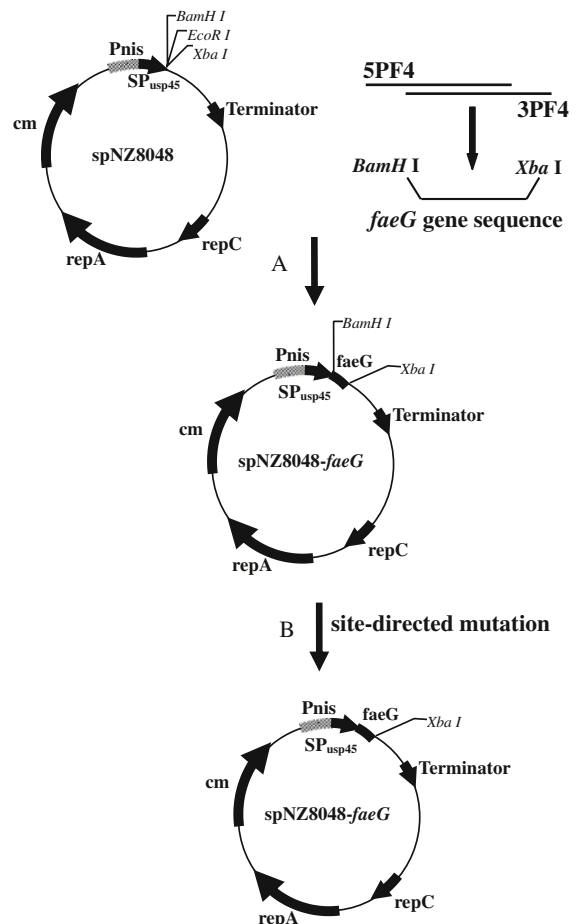
Animals and maintenance

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)



Construction of the recombinant plasmid spNZ8048-faeG. Pnis the nisinA promoter, cm chloramphenicol. faeG gene was inserted into the BamHI/XbaI restriction sites of spNZ8048 and spNZ8048-faeG recombinant plasmid was obtained





centrifuged for 10 min at $10,000\times g$. About 25 μl supernatant was loaded in 12% (v/v) 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/v) 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-indolyl-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 decapitating the animals. The blood samples were centrifuged at $4,000\times g$ and the serum was stored at -20°C .

ELISA for F4-specific antibody

For detecting the specific serum antibody, F4 bacteria (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) C₈₃₅₄₉ 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 ten-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 100LD₅₀ of live enterotoxigenic *E. coli* (ETEC) C₈₃₅₄₉ on the 14th day after last

Mortality of vaccinated mice challenged with virulent enterotoxigenic *E. coli* (ETEC) C₈₃₅₄₉

Group	Immunization	Morbidity	Normal	Mortality	
				No. of deaths/total no.	%
A	<i>L. lactis</i> [spNZ8048- <i>faeG</i>]	1	9	1/10	10
B	<i>L. lactis</i> [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 days, respectively). On the 14th day after last immunization, 10 mice of each group were challenged intragastrically with 100LD₅₀ of live ETEC C₈₃₅₄₉. The mice were then monitored for survival and morbidity for 10 days.



immunization. The gastric acid was neutralized with 1.4% NaHCO₃ 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.

The recombinant plasmid spNZ8048-*faeG* eliminated the *Bam*HI site as constructed (Fig. 1). In spNZ8048 plasmid, promoter P_{nisA} and the signal peptide U_{SP45} 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

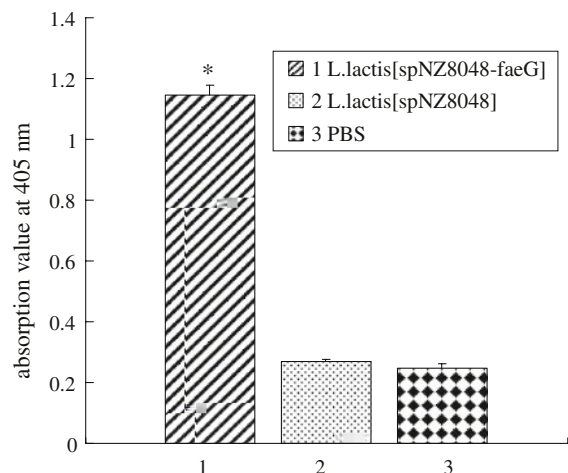


Western blot analysis of FaeG expression with the specific polyclonal antibodies. Lane 1: cell lysate of *L. lactis* NZ9800 [spNZ8048]; lane 2: non-induced recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*]; lane 3: cell lysate of recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*] induced with nisin; lane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

M of FaeG (23.5 kDa) (lanes 3, 4, Fig. 3). FaeG was thus expressed in *L. lactis* NZ9800 by a tight regulator nisin-controlled and secreted into culture medium successfully by the signal peptide U_{SP45}.

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 mbriae 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



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 as 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) C₈₃₅₄₉ 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) C₈₃₅₄₉, a slide agglutination test was conducted. Sera from immunized mice can therefore induce a macroscopic agglutination of C₈₃₅₄₉. In contrast, the sera from unvaccinated mice showed no agglutination (data not shown).

The reason for 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 application in prevention of piglet's diarrhea and has potential for widespread application.

Therefore, 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 method to prevent diarrhea in pre- and post-weaned piglets. Further studies should be conducted to detect the immune

protection of *L. lactis* expressing *FaeG* in pig and other animals.

This study was supported by Zhejiang Provincial Science and Technology Foundation, China (No. 2006C12086).

- Arnaou J, Hjerl-Hansen E, Israelsen H (1997) Heterologous gene expression of bovine plasmin in *Lactococcus lactis*. Appl Microbiol Biotechnol 48:331–338
- Cheun HI, Kamamoto K, Hiramatsu M, Tamaoki H, Shirahata T, Igimi S, Makino SI (2004) Protective immunity of SpaA-antigen producing *Lactococcus lactis* against *Erysipelothrix rhusiopathiae* infection. J Appl Microbiol 96:1347–1353
- Cho HJ, Shin HJ, Han IK, Jung WW, Kim YB, Sul D, Oh YK (2007) Induction of mucosal and systemic immune responses following oral immunization of mice with *Lactococcus lactis* expressing human papillomavirus type 16 L1. Vaccine 25:8049–8057
- de Ruiter PG, Kuipers OP, de Vos WM (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl Environ Microbiol 62:3662–3667
- Enouf V, Langella P, Commissaire J, Cohen J, Corthier G (2001) Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. Appl Environ Microbiol 67:1423–1428
- Geller BL, Wade N, Gilberts TD, Hrub DE, Johanson R, Topisirovic L (2001) Surface expression of the conserved C repeat region of streptococcal M6 protein within the Pip bacteriophage receptor of *Lactococcus lactis*. Appl Environ Microbiol 67:5370–5376
- Gilbert C, Robinson K, Le Page RW, Wells JM (2000) Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in *Lactococcus lactis*. Infect Immun 68:3251–3260
- Hampson DJ (1994) Post-weaning *Escherichia coli* diarrhoea in pigs. In: Giles CL (ed) *Escherichia coli* in domestic animals and humans. CAB International, Oxford, pp 171–191
- Hemsley A, Arnheim N, Tone MD, Cortopassi G, Galas DJ (1989) A simple method for site-directed mutagenesis using the polymerase chain reaction. Nucleic Acids Res 17:6545–6551
- Holo H, Nes IF (1995) Transformation of *Lactococcus* by electroporation. Methods Mol Biol 47:195–199
- Iwaki M, Okahashi N, Takahashi I, Kanamoto T, Sugita-Konishi Y, Aibara K, Koga T (1990) Oral immunization with recombinant *Streptococcus lactis* carrying the *Streptococcus mutans* surface protein antigen gene. Infect Immun 58:2929–2934
- Joensuu JJ, Kotiaho M, Teeri TH, Valmu L, Nuutila AM, Oksanen-Caldente KM, Niklander-Teeri V (2006) Glucosylated F4 (K88) bacterial adhesin *FaeG* expressed in barley endosperm induces ETEC-neutralizing antibodies in mice. Transgenic Res 15:359–373



- Jones GW, Rutter JM (1972) Role of the K88 antigen in the pathogenesis of neonatal diarrhea caused by *Escherichia coli* in piglets. *Infect Immun* 6:918–927
- Lee MH, Roussel Y, Wilks M, Tabaqchali S (2001) Expression of *Helicobacter pylori* urease subunit B gene in *Lactococcus lactis* MG1363 and its use as a vaccine delivery system against *H. pylori* infection in mice. *Vaccine* 19:3927–3935
- Lopez-Vidal Y, Svennerholm AM (1990) Monoclonal antibodies against the different subcomponents of colonization factor antigen II of enterotoxigenic *Escherichia coli*. *J Clin Microbiol* 28:1906–1912
- Melkebeek V, Verdonck F, Goddeeris BM, Coenraets E (2007) Comparison of immune responses in parenteral FaeG DNA primed pigs boosted orally with F4 protein or reimmunized with the DNA vaccine. *Vet Immunol Immunopathol* 116:199–214
- Mol O, Oudega B (1996) Molecular and structural aspects of bacterial biosynthesis and assembly in *Escherichia coli*. *FEMS Microbiol Rev* 19:25–52
- Nag B, Case TA, Moon HW (1990) Phenotype and genotype of *Escherichia coli* isolated from pigs with post-weaning diarrhea in Hungary. *J Clin Microbiol* 28:651–653
- Ribeiro LA, Azevedo V, Le Loir Y, Oliveira SC, Diez Y, Piard JC, Gruss A, Langella P (2002) Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microbiol* 68:910–916
- Sambrook J, Fritsch EF, Maniatis T (eds) (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Teraghi BE, Sandine WE (1975) Improved medium for lactic streptococci and their bacteriophages. *Appl Microbiol* 29:807–813
- van Asseldonk M, de Vos WM, Simons G (1993) Functional analysis of the *Lactococcus lactis* usp45 secretion signal in the secretion of a homologous proteinase and a heterologous α -amylase. *Mol Gen Genet* 240:428–434
- Van den Broeck W, Coenraets E, Oudega B, Goddeeris BM (2000) The F4 bacterial antigen of *Escherichia coli* and its receptors. *Vet Microbiol* 71:223–244
- Verdonck F, Coenraets E, Van der Stede Y, Goddeeris BM (2004) Oral immunization of piglets with recombinant F4 bacterial adhesin FaeG monomers induces a mucosal and systemic F4-specific immune response. *Vaccine* 22:4291–4299
- Wells JM, Wilson PW, Norton PM, Gasson MJ, Le Page RW (1993) *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Mol Microbiol* 8:1155–1162
- Yahong H, Liang W, Pan A, Zhou Z, Wang Q, Huang C, Chen J, Zhang D (2006) Protective immune response of bacterially derived recombinant FaeG in piglets. *J Microbiol* 44:548–555
- Zhou XX, Li WF, Ma GX, Pan YJ (2006) The nisin-controlled gene expression system: construction, application and improvements. *Biotechnol Adv* 24:285–295