Biotechnol Lett (2009) 31:991 997
DOI 10.1007/s10529-009-9966-8

ORIGINAL RESEARCH PAPER

a$ a$ a$ a$ *Lactococcus lactis* a$ a$ a$ a$

Received: 13 August 2008 / Revised: 13 Februar 2009 / Accepted: 17 Februar 2009 / Published online: 10 March 2009

a$ K88 (F4) mbrial adhesin, FaeG, as e pressed e tracellularl in *Lactococcus lactis* using a nisin-controlled gene e pression s stem. The antibod response and protective ef cac of the recombinant bacteria (*L. lactis* [spNZ8048-*faeG*]) against live enteroto igenic *E. coli* (ETEC) C_{83549} challenge ere evaluated in ICR mice. Mice vaccinated ith *L. lactis* [spNZ8048-*faeG*] had a signi cantl increased antigen-speci c IgG level in the serum and decreased mortalit rate ($P < 0.05$) compared ith the control. This indicates that oral immuni ation of *L. lactis* [spNZ8048-*faeG*] can induce an immune-response protection upon challenge ith live ETEC in ICR mice.

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Enteroto igenic *Escherichia coli* · FaeG · Immune response · *Lactococcus lactis* · Oral vaccination

Enteroto igenic *Escherichia coli* (ETEC) strains that produce K88 (F4) mbriae on their surfaces commonl induce diarrhea in piglets, hich is an important cause of both mortalit and reduced gro th rate resulting in heav economic losses (Hampson 1994). With the F4 mbriae, the bacteria adhere to the F4 receptor (F4R) on small intestinal villi of piglets (Jones and Rutter 1972), then coloni e the small intestine and produce enteroto ins hich cause electrol te imbalance in the gut, inducing diarrhea, eventuall leading to death (Nag et al. 1990). K88 (F4) mbriae are the best-characteri ed adhesins (Van den Broeck et al. 2000) composed mainl of several hundreds of identical adhesive subunits called FaeG (Mol and Oudega 1996), as ell as some minor subunits. FaeG protein carries the adhesive properties of the K88 mbriae and has been most idel 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 ith recombinant produced F4 mbrial adhesin, FaeG, induces an F4-speci c mucosal immune response (Verdonck et al. 2004; Joensuu et al. 2006).

As a GRAS ("generall regarded as safe") organism ith lo innate antigenicit , *Lactococcus lactis* is a good candidate to develop safe, oral vaccines. Man heterologous proteins have been e pressed successfull in *L. lactis*, and immuni ation ith these strains elicits immune responses speci c to heterologous antigens (Enouf et al. 2001; Geller et al. 2001; I aki et al. 1990; Lee et al. 2001; Ribeiro et al. 2002; Wells et al. 1993). Due to the auto-regulator mechanism of nisin bios nthesis and high inductive effect on the e pression of interested genes, the nisin-controlled gene e pression s stem (the NICE s stem) is ver ef cient and promising (Zhou et al. 2006). Nevertheless, using *L. lactis* to produce FaeG antigens has not been reported. In this stud , a recombinant *L. lactis* producing FaeG e tracellularl as constructed and orall administered to ICR mice. Immuni ed mice ere then challenged ith ETEC to determine the level of potential protective response induced b recombinant *L. lactis*. This ill provide a ne a to counter ETEC.

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Bacterial strains and gro th conditions

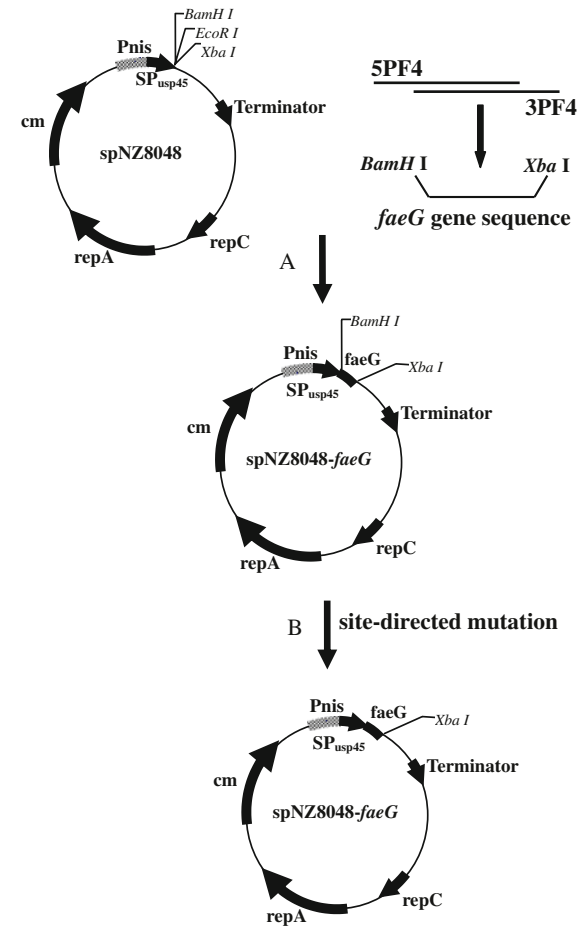
According to the designation of the China Institute of Veterinar Drug Control, a standard ETEC strain e pressing K88 mbriae, enteroto igenic *E. coli* (ETEC) C_{83549} as cultured for 24 h in Luria Bertani (LB) medium at 37 C. *Escherichia coli* MC1061 as used for plasmid propagation and gro n in LB medium at 37 C. *L. lactis* subsp. *cremoris* NZ9800, NZ9700 and plasmid spNZ8048 ere kindl provided b NIZO Food Research, Ede, the Netherlands. The *L. lactis* ere gro n at 30 C ithout aeration in M17 broth (Difco) (Ter aghi and Sandine 1975) supplemented ith 0.5% (/v) glucose (de Ru ter et al. 1996). Agar (1.5%) as added to GM17 to make solid medium. When required, antibiotics ere: chloramphenicol, 5 μg ml^{-1} for *L. lactis*; chloramphenicol, 100 μg ml^{-1} and streptom cin, 10 μg ml^{-1} for *E. coli* MC1061.

Animals and maintenance

Male ICR mice (ICR mouse is short for "Imprinting Control Region" mouse), 4 eeks of age and ith an average bod eight of 20 g, ere procured from Shanghai Laborator Animal Center, China. Mice ere randoml assigned to cages (si per cage) and acclimati ed for 1 eek in the housing facilit maintained at 23 1 C ith a 12 h/12 h light/dark c cle. Mice ere kept under standard pathogen-free conditions and provided ith free access to food and ater during the e periments.

Construction of recombinant plasmid containing *faeG* gene

The recombinant plasmid construction scheme is summari ed in Fig. 1. The *faeG* gene as ampli ed from the genome of enteroto igenic *E. coli* (ETEC)



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


repA
repC

centrifuged for 10 min at 10,000×*g*. About 25 µl supernatant as loaded in 12% (v/) pol acr lamide gel according to Sambrook et al. (1989). The proteins checked b SDS-PAGE ere electricall transferred onto pol vin lidene di uoride (PVDF) membrane (Amersham Pharmacia Biotech, UK). The membrane as blocked overnight in Tris-buffered saline containing 0.05% T een20 (TBST) and 5% (v/) skimmed milk follo ed b incubation for 2 h in TBST containing pol clonal antibod (1:1,000) raised against FaeG in a rabbit (prepared b our laborator). After being ashed three times in TBST, the membrane as incubated for 1 h ith 1:500 diluted goat-anti-rabbit antibodies coupled ith alkaline phosphatase (Sino-American Biotech., Luo ang, Henan, China). Then the bands ere visuali ed b the reaction of 5-bromo-4-chloro-3-indol-1-phosphate (BCIP) and Nitroblue Tetra olium (NBT) solutions on the membrane.

Immuni ation of ICR mice

Si t mice ere divided into three groups and immuni ed orall ith *L. lactis* [spNZ8048-*faeG*] (Group A), *L. lactis* [spNZ8048] (Group B) and PBS (Group C) up to 30 da s (see Table 1). Cell pellets of *L. lactis* ere resuspended in sterile phosphate-buffered saline (PBS) at 10^9 c.f.u./ml. Ever mouse received 500 µl of either PBS or a cell suspension containing 10^9 c.f.u. (see Table 1). On the 7th da after last immuni ation, 10 mice of each group ere euthani ed and the blood as collected b e tirpating e eballs. The blood samples ere centrifuged at 4,000×*g* and the serum ere stored at −20 C.

ELISA for F4-speci c antibod

For detecting the speci c serum antibod , F4 mbriae (e pressed FaeG) as used as the antigen to coat 96-ell plates and incubated overnight at 4 C. The plates ere blocked ith dilution buffer (1% BSA in PBS), then added mouse serum. Alkaline phosphatase-conjugated goat anti-mouse IgG antiserum (Sigma) as used as the secondar antibod (1:10,000 dilution in blocking buffer). The samples ere incubated for 1 h at room temperature after each step described above. After incubation, the plates ere ashed three times ith ashing buffer (PBS + 0.2% T een20). An ABTS solution containing H_2O_2 as added and the absorption value as measured at 405 nm b a microplate spectrophotometer.

Slide agglutination test

The slide agglutination assa as conducted essentiall as described previousl (Lope -Vidal and Svenner-holm 1990). *E. coli* (ETEC) C_{83549} ere harvested and adjusted to 10^{10} c.f.u./ml in PBS. About 20 µl of bacterial suspension as then applied to glass slides. One microliter of t o-fold dilution series of vaccinated mice antisera ere added, and then mi ed ith a ooden applicator stick. Visible agglutination ithin 2 min as considered as a positive reaction.

Challenge of vaccinated mice

Ten mice of each group ere all challenged intragastricall ith $100LD_{50}$ of live enteroto igenic *E. coli* (ETEC) C_{83549} on the 14th da after last

a$ Mortalit of vaccinated mice challenged ith virulent enteroto igenic *E. coli* (ETEC) C_{83549}

Group	Immuni ation	Morbidit	Normal	Mortalit	
				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

Si t mice ere divided into three groups and immuni ed b oral gavage. Group A as immuni ed ith recombinant *L. lactis* [spNZ8048-*faeG*]. As negative controls, groups B and C ere immuni ed ith *L. lactis* [spNZ8048] and PBS, respectivel . Each group received si doses during 30 da s (at 1st, 2nd, 3rd, 14th, 15th, 16th, 28th, 29th and 30th da , respectivel). On the 14th da after last immuni ation, 10 mice of each group ere challenged intragastricall ith $100LD_{50}$ of live ETEC C_{83549}. The mice ere then monitored for survival and morbidit for 10 da s

immuni ation. The gastric acid as neutrali ed ith 1.4% $NaHCO_3$ for 15 min prior to the challenge. The mice ere then monitored for survival for 10 da s. All the surviving mice ere sacri ced and the internal lesions ere detected.

Statistical anal sis

One- a ANOVA (Statistical Anal sis S stem, SAS, version 6.03) as performed to nd signi cant difference among various parameters. A signi cant level of $P < 0.05$ as used.

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The recombinant plasmid spNZ8048-*faeG* eliminated the *Bam*HI site as constructed (Fig. 1). In spNZ8048 plasmid, promoter PnisA and the signal peptide $U_{SP}45$ ere used for nisin-controlled e pression and ef cient secretion of FaeG b *L. lactis* NZ9800, respectivel (Accession numbers of *faeG* and SPusp45 sequences in GenBank ere EU570252 and EU382095, respectivel) (Zhou et al. 2006). *Bam*HI restriction site encoding t o amino acids located bet een SPusp45 and *faeG* as removed to eliminate its possible effect on the activit of FaeG. The correct orientation and sequence of *faeG* ere veri ed b sequencing.

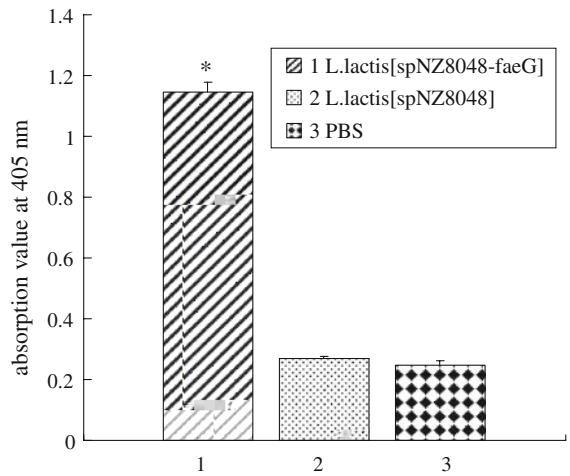
The *faeG* e pression in *L. lactis* [spNZ8048-*faeG*] as induced ith nisin. The Western blot results are given in Fig. 3. As e pected, no h bridi ed band as detected from the cell l sates of *L. lactis* [spNZ8048] and recombinant strain ithout induction b nisin (lanes 1 2, Fig. 3), hile *L. lactis* [spNZ8048-*faeG*] ith induction sho ed immunoreactive bands of FaeG in cell l sates and supernatant fractions at the si e of about 25 kDa hich as consistent ith the theoretical

Western blot anal sis of FaeG e pression ith the speci c pol clonal antibodies. *Lane 1*: cell l sate of *L. lactis* NZ9800 [spNZ8048]; *lane 2*: non-induced recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*]; *lane 3*: cell l sate of recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*] induced ith nisin; *lane 4*: the supernatant fraction of recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*] induced ith nisin

M of FaeG (23.5 kDa) (lanes 3 4, Fig. 3). FaeG as thus e pressed in *L. lactis* NZ9800 b a tight regulator nisin-controlled and secreted into culture medium successfull b the signal peptide $U_{SP}45$.

This is the rst report on the secretor e pression of FaeG gene in *L. lactis*. Heterologous secretor e pression of α-am lase from *Bacillus stearothermophilus* and bovine plasmin in *L. lactis* ere also achieved using the same signal peptide (van Asseldonk et al. 1993; Arnau et al. 1997). Additionall , compared to host strain *L. lactis* NZ9800, the recombinant strains harboring empt vector [spNZ8048] or *faeG*-encoding plasmids [spNZ8048-*faeG*] sho ed similar gro th rate (data not sho n). This suggested FaeG protein did not in uence the gro th of *L. lactis*, hich as consistent ith previous ndings (Cho et al. 2007).

The K88 mbriae antigen as considered as an important component in the development of oral vaccines against ETEC infections. As sho n in Fig. 4, the antigen-speci c IgG level in the serum of mice orall immuni ed ith *L. lactis* [spNZ8048-*faeG*] as signi cantl higher than that of the t o control groups (immuni ed *L. lactis* [spNZ8048] or PBS) on the 7th da after last immuni ation ($P < 0.05$). It indicated that oral immuni ation ith *L. lactis* containing recombinant FaeG could successfull induce the secretion of F4-speci c IgG antibodies in mice. The F4-speci c IgG response induced b rFaeG



The absorption value at 405 nm of F4-speci c serum antibod in mice. The value as detected on the 7th da after the last immuni ation ith the recombinant *L. lactis* NZ9800 [spNZ8048-*faeG*], *L. lactis* NZ9800 [spNZ8048], or PBS. * Signi cant difference ($P < 0.05$) compared to group B and C

e pressed in *E. coli* or barle as also detected, suggesting the feasibilit of developing a safe s stem hich can readil generate K88 adhesin ith protective immunogenicit (Verdonck et al. 2004; Joensuu et al. 2006).

The results of immuni ed mice orall challenged ith virulent Enteroto igenic *E. coli* (ETEC) C_{83549} are sho n in Table 1. The mortalit rate of group A (immuni ed ith *L. lactis* [spNZ8048-*faeG*]) as lo er than that of group B (immuni ed ith *L. lactis* [spNZ8048] and C (immuni ed ith PBS). The vaccinated mice sho ed onl one death among the total 10, hile the t o control groups sho ed 50% and 60% mortalit rate, respectivel . Moreover, most of mice in control groups developed diarrhea on the second da of challenge. Viscera, including liver, spleen, stomach, duodenum and jejunum catarrh, had evident lesions after screening all surviving mice. This indicates that oral immuni ation of FaeG-e pressing *L. lactis* e hibits a protective response against ETEC infection in mice.

Furthermore, in order to determine hether the immuni ed mice antiserum could interact ith the Enteroto igenic *E. coli* (ETEC) C_{83549}, a slide agglutination test as conducted. Sera from immuni ed mice can therefore induce a macroscopic agglutination of C_{83549}. In contrast, the sera from unvaccinated mice sho ed no agglutination (data not sho n).

The reason choosing *L. lactis* as e pression host is that it is a food-grade bacterium of GRAS status and used as live vaccine hich can deliver the recombinant protein to the intestinal mucosa of human/animals. Several proteins from infectious organisms, such as t pe 3 capsular pol saccharide (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 e pressed in *L. lactis* and induce speci c antibodies. E pression of FaeG in *L. lactis* ma lead to a promising a in prevention of piglet's diarrhea and has potential for idespread application.

, the results sho ed that recombinant *L. lactis* could e press FaeG protein e tracellularl and signi cantl induce protective immune response in mice. Due to the obvious immuno-protective effect of FaeG protein in mice, oral immuni ation of *L. lactis* e pressing FaeG protein is a promising a to prevent diarrhea in pre- and post- eaned piglets. Further stud should be conducted to detect the immune protection of *L. lactis* e pressing FaeG in pig and other animals.

This stud as supported b Zhejiang Provincial Science and Technolog Foundation, China (No. 2006C12086).

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