Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Microbiological Research 169 (2014) 733–740

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Construction of a food-grade cell surface display system for *Lactobacillus casei*

Jiayang Qin[a,*], Xiuwen Wang[a], Jian Kong[b], Cuiqing Ma[b], Ping Xu[c]

[a] *College of Pharmacy, Binzhou Medical University, Yantai 264003, People's Republic of China*
[b] *State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, People's Republic of China*
[c] *State Key Laboratory of Microbial Metabolism, and School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China*

ARTICLE INFO

Article history:
Received 11 December 2013
Received in revised form 28 January 2014
Accepted 1 February 2014
Available online 10 February 2014

Keywords:
Lactobacillus casei
Food-grade
Surface display
Plasmid elimination

ABSTRACT

In this study, a food-grade cell surface display host/vector system for *Lactobacillus casei* was constructed. The food-grade host *L. casei* Q-5 was a lactose-deficient derivative of *L. casei* ATCC 334 obtained by plasmid elimination. The food-grade cell surface display vector was constructed based on safe DNA elements from lactic acid bacteria containing the following: pSH71 replicon from *Lactococcus lactis*, lactose metabolism genes from *L. casei* ATCC 334 as complementation markers, and surface layer protein gene from *Lactobacillus acidophilus* ATCC 4356 for cell surface display. The feasibility of the new host/vector system was verified by the expression of green fluorescent protein (GFP) on *L. casei*. Laser scanning confocal microscopy and immunofluorescence analysis using anti-GFP antibody confirmed that GFP was anchored on the surface of the recombinant cells. The stability of recombinant *L. casei* cells in artificial gastrointestinal conditions was verified, which is beneficial for oral vaccination applications. These results indicate that the food-grade host/vector system can be an excellent antigen delivery vehicle in oral vaccine construction.

© 2014 Elsevier GmbH. All rights reserved.

Introduction

Approximately 15 million annual deaths worldwide are related directly to infectious diseases, accounting for 25% of the total mortality rate (Morens et al. 2004). Vaccination is effective against many bacteria, viruses, parasites and other infectious diseases in humans. Genetically engineered live vaccine (GELV) is an effective method for vaccine delivery. Non-pathogenic microorganisms are usually used as carriers. The recombinant protective antigen gene fragments of germs are restructured in the vector microbes, and the microbes expressing protective antigens are used as vaccines. Vaccinia virus, adenovirus, and poliovirus are commonly used viral vectors. *Salmonella* and *Bacillus* Calmette-Guérin (BCG) are commonly used bacterial vectors (Detmer and Glenting 2006). The potential for reversion of attenuated strains to virulence is a significant safety concern. Using non-pathogenic bacteria, especially probiotics, as vaccine carriers enhances the safety of vaccines.

Lactobacillus is the largest genus of lactic acid bacteria. Lactobacilli have long been used in food fermentation and preservation, and are generally recognized as safe (GRAS) microorganisms. Lactobacilli strains have attracted attention as antigen carriers for immunization not only for their safety but also for their potential to colonize intestine, tolerate gastric and bile acids, and produce antimicrobial substances (Seegers 2002). Genetically modified strains of lactobacilli carrying important pathogen antigen components can produce specific local or systemic immune responses after oral administration or injection (Detmer and Glenting 2006). Therefore, lactobacilli are a safe and practical choice for GELV.

Lactobacillus strains that have been developed successfully for GELV include *L. casei* (Maassen et al. 1999), *L. plantarum* (Reveneau et al. 2002), *L. johnsonii* (Scheppler et al. 2002), and *L. acidophilus* (Moeini et al. 2011). However, the use of non-food-grade vectors limits their application in humans. Therefore, developing food-grade vaccine delivery systems is essential for expanding the human usefulness of GELVs.

The current study aims to construct a food-grade cell surface display host/vector system for *L. casei* and to provide an alternative antigen delivery vehicle in oral vaccine formulation.

* Corresponding author. Tel.: +86 5356913254.
E-mail addresses: qinjysdu@gmail.com (J. Qin), wangxiuwen0822@163.com (X. Wang), kongjian@sdu.edu.cn (J. Kong), macq@sdu.edu.cn (C. Ma), pingxu@sjtu.edu.cn (P. Xu).

http://dx.doi.org/10.1016/j.micres.2014.02.001
0944-5013/© 2014 Elsevier GmbH. All rights reserved.

Materials and methods

Strains, plasmids, and primers

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *Lactobacillus* strains were grown in MRS medium (De Man et al. 1960) at 37 °C without shaking. *Escherichia coli* strains were aerobically grown in Luria–Bertani medium at 37 °C in a rotary shaker. The antibiotics used for *E. coli* were 100 μg/mL ampicillin and 20 μg/mL chloramphenicol, whereas that used for *L. casei* was 10 μg/mL chloramphenicol.

DNA manipulations

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA). Plasmid DNA was isolated using the TIANprep Mini Kit (TIANGEN, China). The QIAquick Gel Extraction Kit (Qiagen, Germany) was used for DNA purification after digestion. T_4 DNA ligase and Phusion® High-Fidelity DNA polymerase were obtained from New England Biolabs (Beijing, China). Restriction endonucleases were purchased from TaKaRa (Dalian, China). Polymerase chain reaction (PCR) primers were prepared by Sangon (Shanghai, China).

Elimination of plasmid 1 in L. casei ATCC 334

Two methods were employed for plasmid elimination in *L. casei* ATCC 334. The strain was cultured and passaged in MRS broth either for eight subcultures at 42 °C or for eight subcultures at 37 °C in the presence of novobiocin (10 μg/mL) (Kojic et al. 1992). The remaining cultures were plated on MRS solid medium, and single colonies were selected. Two pairs of primers (i.e., yz1 and yz2, yz3 and yz4) were designed to screen plasmid-eliminated strains by PCR. Among the four primers, yz1, yz3 and yz4 binded to the phospho-β-galactosidase gene (*lacG*) on plasmid 1, while yz2 binded to the enzyme IICB gene (*lacE*) on plasmid 1. The binding sites of yz1 and yz2 to plasmid 1 are from nucleotides 6530 to 6544, and 7952 to 7969, respectively. The binding sites of yz3 and yz4 to plasmid 1 are from nucleotides 6353 to 6368, and 6938 to 6954, respectively.

The metabolic ability of the selected strains on lactose was verified by culturing these strains in MRS medium using lactose instead of glucose as the carbon source. The optical density (OD) was measured using a spectrophotometer at 600 nm. Lactose consumption was measured using the Sucrose/Lactose/D-Glucose Kit K-LACSU 01/12 (Megazyme, Ireland). L-Lactic acid production was measured using the L-lactic acid Kit K-LATE 07/11 (Megazyme, Ireland).

To test the stability of the plasmid-eliminated strain, the strain was cultured and passaged in MRS medium at 37 °C every 24 h for 20 cycles. Genomic DNA of the last strain was isolated and used to perform PCR using primers pairs yz1, yz2 and yz3, yz4.

Construction of the food-grade surface display plasmid

The lactose metabolic genes were introduced to pNZ2102. A 3629 bp DNA fragment containing genes of *lacE*, *lacG*, and *lacF* (coding for enzyme IIA) was PCR amplified from the plasmid 1 of *L. casei* ATCC 334 using primers L9 and L8. The purified DNA fragment was digested with *Xho*I and *Pst*I, and then ligated to pNZ2102, which was digested with the same enzymes. The ligation product was transformed into *E. coli* DH5α, and right transformant was selected. This step resulted in the formation of pNZ2102-lacEGF.

The surface layer (S-layer) protein gene *slpA* (Genbank accession no. X71412) was cloned from *L. acidophilus* type strain ATCC 4356 (Boot et al. 1993). The fusion gene of *slpA* and green fluorescent protein gene (*gfp*) was constructed using recombinant PCR. A 1844 bp DNA fragment containing the promoter, signal peptide, and *slpA* gene was PCR amplified from the chromosomal DNA of *L. acidophilus* ATCC 4356 using primers P1 and P2. The 726 bp fragment of the *gfp* gene was PCR amplified from pBAD-GFPuv by using primers P3 and P4. Since P2 and P3 have 21 bp homologous complementary regions, the two purified PCR products were mixed as templates to perform recombinant PCR using primers P1 and P4. The obtained 2585 bp DNA fragment was designated as *slpA-gfp*.

The chloramphenicol acetyltransferase gene (*cm*) of pNZ2102-lacEGF was replaced with *slpA-gfp*. A 5872 bp DNA fragment containing the entire DNA elements of pNZ2102-lacEGF, except

Table 1
Strains, plasmids, and primers used in this study.

Strain, plasmid or primer	Characteristics or sequence	Source or reference
Strains		
E. coli DH5α	Transformation host	Novagen
L. casei ATCC 334	Wild strain isolated from Emmental cheese, Lac^+	ATCC
L. casei Q-5	Plasmid-cured derivative of *L. casei* ATCC 334, Lac^-	This study
L. acidophilus ATCC 4356	Wide type strain, isolated from human, the donor of the signal peptide, promoter, and *slpA* gene	ATCC
Plasmids		
pBAD-GFPuv	Ap^r, the donor of the *gfp* gene	Fisher and Mintz (2000)
pNZ2102	Cm^r, pSH71-derived lactococcal vector harboring the *lacA* promoter	Platteeuw et al. (1996)
pNZ2102-lacEGF	Cm^r, P_{lacA}, pNZ2102 carrying *lacE*, *lacG* and *lacF* from *L. casei* ATCC 334	This study
pQJ-gfp	Cm^s, P_{lacA}, food-grade cell surface display plasmid, the *cm* gene of pNZ2102-lacEGF was replaced by *slpA-gfp*	This study
Primers[a]		
yz1	5′-TTTCCTGCGGTGTCG-3′	This study
yz2	5′-TTCGCCTTTGTTCTACTG-3′	This study
yz3	5′-TGCCATCTGGGAGTTT-3′	This study
yz4	5′-GGCTTATGCGAAGTTTT-3′	This study
L9	5′-GCGCTGCAGACGCTTATGCTTTGGCTTCC-3′	This study
L8	5′-GCGCTCGAGTTACTGCTTGTTCTCAAGTT-3′	This study
P1	5′-TGCAGATCTGGGATGAAATAAAGCCAATA-3′	This study
P2	5′-CCTTTACTCATTCTAAAGTTTGCAACCTTA-3′	This study
P3	5′-CAAACTTTAGAATGAGTAAAGGAGAAGAACTT-3′	This study
P4	5′-CGCGAATTCTTATTTGTATAGTTCATCCAT-3′	This study
C1	5′-GATCTCAGAATTCGAGCT-3′	This study
C2	5′-GCGAGATCTCAATAATCCCTCCTCT-3′	This study

[a] The underlined letters indicate the introduction of restriction sites.

for the *cm* gene, was obtained by PCR using primers C1 and C2, which have the recognition sites of *Eco*RI and *Bgl*II, respectively. The DNA fragment and *slpA-gfp* were digested with *Bgl*II and *Eco*RI, respectively. The two purified DNA fragments were ligated and transformed into *L. casei* Q-5 to obtain the food-grade cell surface display plasmid of pQJ-gfp.

Transformation of L. casei

Plasmids were transformed into the plasmid-cured strain *L. casei* Q-5 by electroporation. Briefly, *L. casei* cells from an overnight culture were inoculated (2%, v/v) into 50 mL MRS medium in a 125 mL Erlenmeyer flask and then incubated at 37 °C without shaking for 5 h to reach an OD_{600} of 0.5–0.6. Ampicillin was added to obtain a final concentration of 20 μg/mL. Incubation was continued for another hour. The cells were harvested, washed three times using an ice-cold electroporation buffer (0.5 M sucrose, 1 mM ammonium citrate), and then resuspended in 0.5 mL of the same buffer. The ligation solution was mixed with 50 μL ice-cold cell suspension in a 0.1 cm Gene Pulser™ cuvette and then submerged on ice for at least 10 min. Electroporation was carried out at 9 kV/cm in a MicroPulser® supplied by Bio-Rad Laboratories (USA). Following the pulse, 1 mL MRS broth with 10% sucrose at 37 °C was immediately added to the cell suspension and then incubated at 37 °C for 3 h. The diluted suspension was then plated onto MRS agar plates containing 5 μg/mL of chloramphenicol. For food-grade screening, the glucose in MRS agar was replaced with lactose, and no antibiotic was added. After 48 h of incubation, transformants were selected and verified by PCR.

Laser scanning confocal microscopy

L. casei strains were cultured in MRS broth at 37 °C for 24 h. The cells were collected, washed, and suspended in 0.01 mol/L phosphate-buffered saline (PBS). Samples were examined using a Leica TCS SPE confocal microscope equipped with 40 × 1.15 NA oil objective and 488-nm/15 mW Ar laser (Leica, Bensheim, Germany). The green fluorescent signal was acquired at excitation 488 nm laser line and detected at 500–550 nm wavelength range. *L. casei* Q-5 harboring pNZ2102-lacEGF was used as control.

Immunofluorescence tests were performed as follows: *L. casei* cells were blocked for 1 h with 4% goat serum in PBS, stained with anti-GFP mouse monoclonal antibody (TransGen, Beijing, China) for 1 h, and incubated with Cy3-conjugated goat anti-mouse IgG (Sangon, Shanghai, China) for 1 h. The red fluorescent signal was acquired using 550 nm excitation laser line and detected at 560–600 nm.

Survival of recombinant L. casei cells in artificial gastrointestinal juice

Artificial gastric juice (AGJ) and artificial intestinal juice (AIJ) were prepared according to 'Chinese Pharmacopoeia (2010 Edition)' (State Pharmacopoeia Committee 2010). The AGJ contained: 1% (w/v) pepsin, 0.1 M hydrochloric acid, pH 2.0. The AIJ contained: 1% (w/v) pancreatin, 0.05 M potassium dihydrogen phosphate, pH 6.8. *L. casei* Q-5 harboring pQJ-gfp was cultured in MRS broth at 37 °C for 24 h. *E. coli* DH5α was cultured in LB broth at 37 °C for 24 h and used as control. Cells were sequentially exposed to gastric challenge for 2 h and intestinal challenge for 5 h at 37 °C, 60 rpm. The viability of the bacterial cell was measured by the viable plate count technique on MRS agar plates. Survival was calculated using the following equation:

$$\text{Survival} \quad (\%) = \frac{V_i}{V_0} \times 100,$$

where V_0 and V_i are the viability of the bacteria before and after treatment in the artificial gastrointestinal juice, respectively.

Results

Construction of a food-grade host of L. casei by plasmid elimination

L. casei ATCC 334 utilizes lactose via a plasmid-encoded phosphotransferase system, which is consistent with *L. casei* 6H (Alpert and Siebers 1997). The lactose metabolism genes of *lacE*, *lacG*, and *lacF* in strain ATCC 334 are located on plasmid 1 (http://www.ncbi.nlm.nih.gov/nuccore/NC_008502.1). For food-grade cloning, a stable lactose-deficient mutant was created by elimination of plasmid 1.

At 42 °C plasmid elimination, nine colonies were selected and designated as strains 1–9. None of the nine colonies resulted in the formation of a 1440 bp DNA band by colony PCR using primers yz1 and yz2. This result indicates that plasmid 1 in strains 1–9 may be eliminated. Strains 5 and 9 were randomly selected for further investigation. By using novobiocin for plasmid elimination, another nine colonies were selected and designated as strains A to I. A distinct 1440 bp DNA band was obtained using primers yz1 and yz2 in seven of the nine colonies, except for strains A and F. Strains 5, 9, A, and F were cultured in MRS broth for 24 h. Genomic DNAs were isolated and used as templates for PCR with primers yz3 and yz4. The results are shown in Fig. 1. No DNA band was obtained in strain 5, and 600 bp DNA bands were obtained in strains 9, A, F, and *L. casei* ATCC 334 (control). These results indicate that strain 5 was most likely the plasmid-eliminated strain, thus it was used for further investigation.

Strain 5 was cultured in MRS medium with lactose instead of glucose as the carbon source to test its lactose metabolism ability. *L. casei* ATCC 334 was used as control. After 48 h of cultivation, the OD values of *L. casei* ATCC 334 and strain 5 at 600 nm were 3.68 and

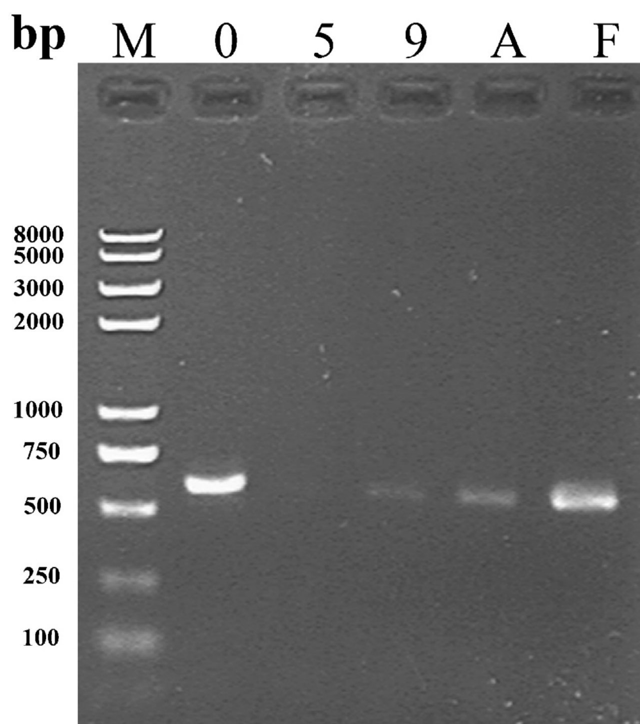

Fig. 1. Screening for plasmid 1-eliminated *L. casei* strains by PCR. Lane M, marker DNAs; Lane 0, *L. casei* ATCC 334; Lane 5, strain 5; Lane 9, strain 5; Lane A, strain A; Lane F, strain F.

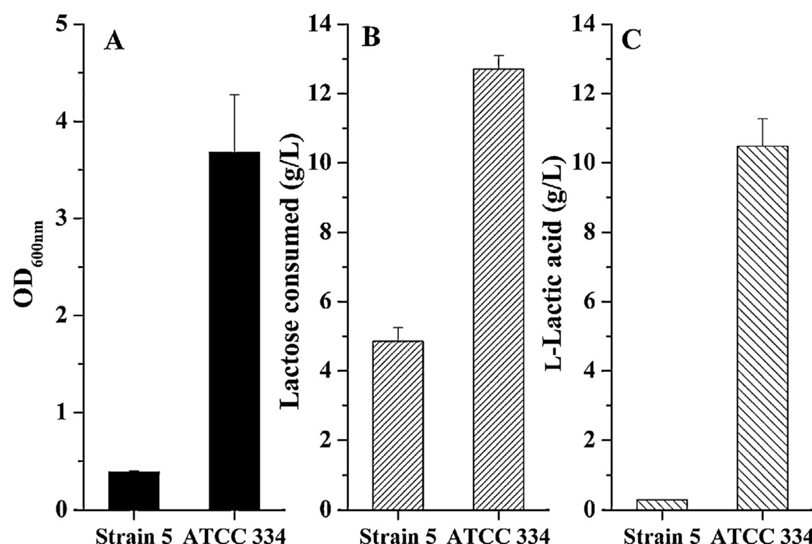

Fig. 2. Cell growth (A), lactose consumption (B) and L-lactic acid production (C) of strain 5 and *L. casei* ATCC 334 in MRS medium with lactose instead of glucose as the carbon source.

0.39, respectively (Fig. 2A). Strains ATCC 334 and 5 consumed 12.7 and 4.9 g/L of lactose, respectively (Fig. 2B). Strains ATCC 334 and 5 produced 10.5 and 0.3 g/L of L-lactic acid, respectively (Fig. 2 C). These data strongly suggest that strain 5 was the Lac- and plasmid 1-eliminated derivative of *L. casei* ATCC 334. To test the influence of plasmid elimination on glucose utilization, strain 5 and ATCC 334 were cultured in MRS medium with glucose as carbon source for 48 h. The OD value, glucose consumption, and L-lactic acid production of strain 5 were 4.0, 14.6 g/L, and 14.1 g/L, respectively. Similar results were obtained with strain ATCC 334, which were 4.2, 16.0 g/L, and 15.5 g/L, respectively. These data suggest that plasmid elimination did not affect the glucose utilization ability of strain 5.

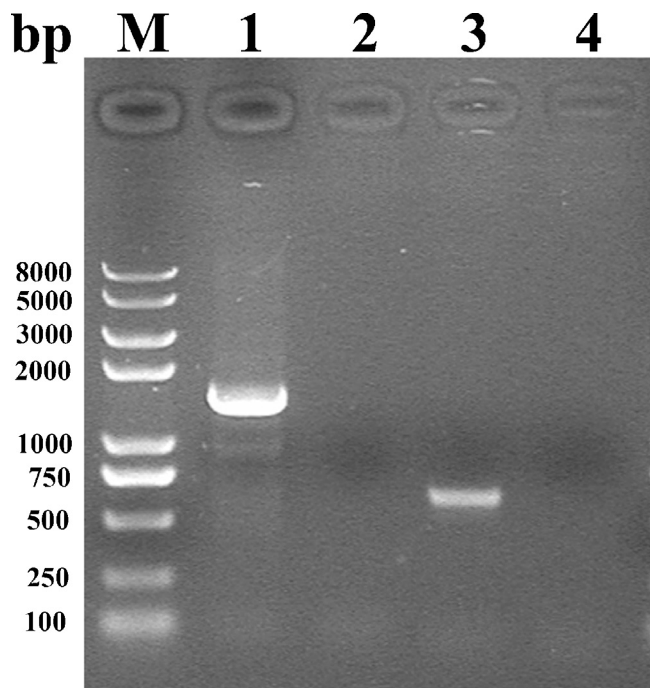

Fig. 3. Detection of plasmid recovery of strain 5 after 20 passages. Lane M, marker DNAs; Lane 1, PCR using primers yz1, yz2 and *L. casei* ATCC 334; Lane 2, PCR using primers yz1, yz2 and strain 5; Lane 3, PCR using primers yz3, yz4 and *L. casei* ATCC 334; Lane 4, PCR using primers yz3, yz4 and strain 5.

The stability of strain 5 was tested by detecting plasmid recovery using PCR. The results are shown in Fig. 3. Using two pairs of primers yz1, yz2 and yz3, yz4, no DNA bands were detected in strain 5 after 20 passage times. The 1440 bp and 600 bp DNA bands could be found using the control strain *L. casei* ATCC 334. These results demonstrate that strain 5 was a stable plasmid-eliminated derivative of *L. casei* ATCC 334 and that no plasmid recovery occurred. Strain 5 was designated as *L. casei* Q-5 and was used for further investigation.

Construction of the food-grade cell surface display vector

The food-grade cell surface display vector was constructed by introducing the lactose metabolic genes of *lacE*, *lacG*, and *lacF* into pNZ2102 under the *PlacA* promoter and replacing the *cm* gene with *slpA-gfp*. The obtained plasmid was designated as pQJ-gfp. The plasmid construction procedures are shown in Fig. 4. The lactose metabolic genes were used as selection markers. The transcription of *gfp* was controlled by the constitutive promoter of S-layer protein. The exporting of GFP to the cell surface of *L. casei* was completed by the signal peptide of S-layer protein.

Expression of GFP on L. casei

The food-grade surface display vector pQJ-gfp was transformed into the food-grade host of *L. casei* Q-5 by electroporation. Transformants were selected by food-grade screening method as described in “Materials and methods”. *L. casei* Q-5 cells harboring pQJ-gfp were rod shaped and emitted green fluorescence under laser scanning confocal microscopy (Fig. 5A). No fluorescence was detected in the control strains of *L. casei* Q-5 harboring pNZ2102-lacEGF (Fig. 5B). Immunofluorescence tests using anti-GFP antibody and Cy3-conjugated secondary antibody were performed and detected by laser scanning confocal microscopy. Visible red fluorescence was observed in *L. casei* Q-5 cells harboring pQJ-gfp (Fig. 5C). No fluorescence was detected in the control cells of *L. casei* Q-5 harboring pNZ2102-lacEGF (Fig. 5D). These results indicate that GFP was successfully expressed on the cell surface of *L. casei*.

The survival of recombinant L. casei cells in the artificial gastrointestinal juice

The viabilities of *L. casei* Q-5 harboring pQJ-gfp before incubation, after 2 h of incubation in the AGJ (pH 2.0) and after 5 h of

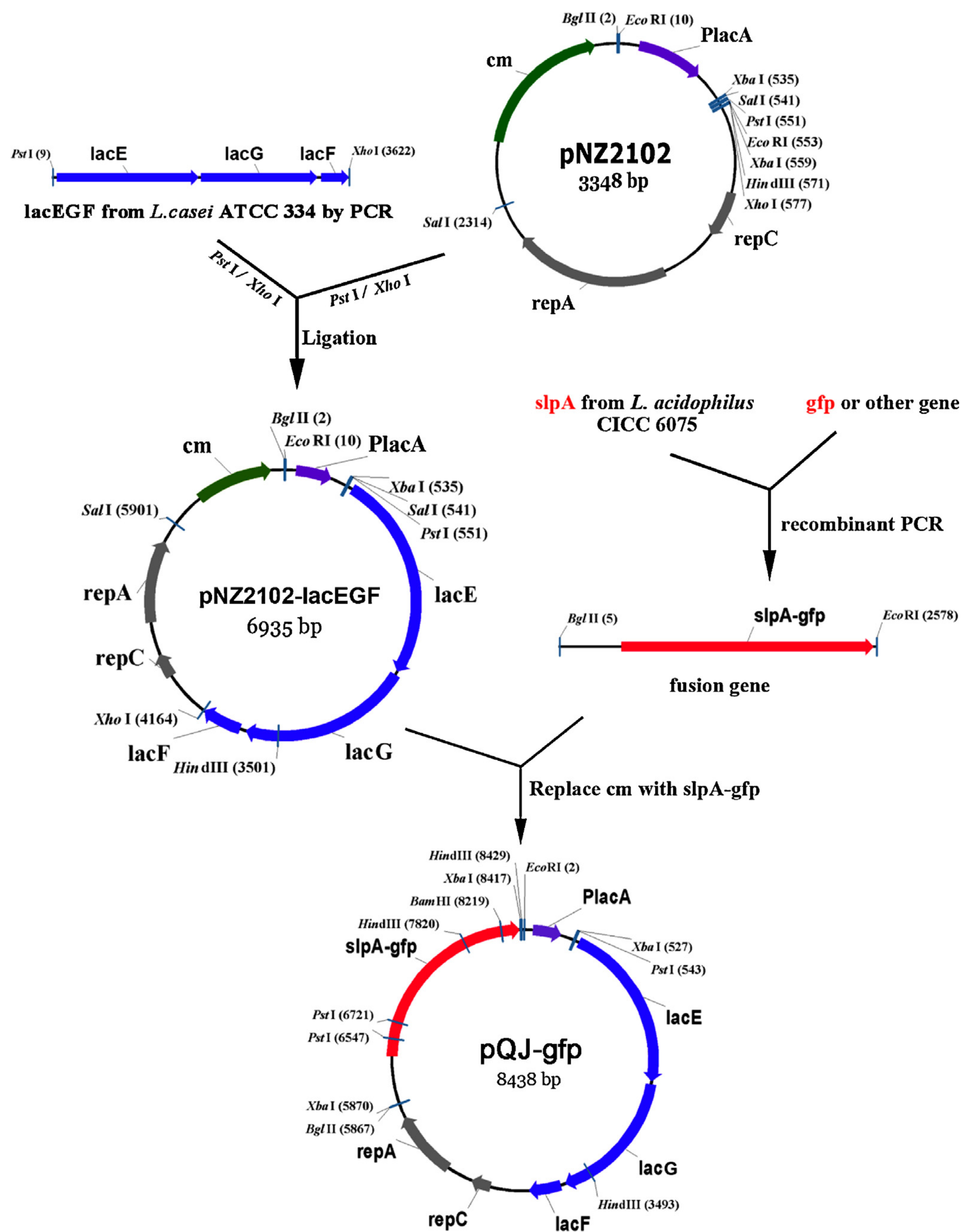

Fig. 4. Construction of the food-grade cell surface display vector.

extended incubation in the AIJ (pH 6.8) were observed. As shown in Table 2, the survival of 79.72% was detected after incubation in the AGJ. The survival increased to 138.48% after incubation in the AIJ. As a control, the survival of *E. coli* DH5α was less than 1%. These results suggest that *L. casei* Q-5 harboring pQJ-gfp was stable in artificial gastrointestinal conditions, which is beneficial for oral vaccination applications.

Discussion

Numerous *L. casei* strains have been developed as oral vaccines to deliver different types of antigen proteins (Table 3). Most previous studies used non-food-grade vectors, in which antibiotic resistance genes such as chloramphenicol and erythromycin were used as selection markers. The products of the resistance

Table 2
Survival of *L. casei* Q-5 harboring pQJ-gfp in artificial gastrointestinal juice.

Strain	Treatment	Viability (CFU/mL)	Survival (%)
E. coli DH5α	Before treatment	$(2.48 \pm 0.20) \times 10^8$	
	Treated with AGJ[a]	$(7.30 \pm 0.66) \times 10^5$	0.29 ± 0.05
	Treated with AIJ[b]	$(1.13 \pm 0.32) \times 10^6$	0.46 ± 0.09
L. casei Q-5 harboring pQJ-gfp	Before treatment	$(2.42 \pm 0.03) \times 10^8$	
	Treated with AGJ[a]	$(1.93 \pm 0.25) \times 10^8$	79.72 ± 10.88
	Treated with AIJ[b]	$(3.35 \pm 0.76) \times 10^8$	138.48 ± 32.51

Each value is the mean ± standard deviation of three trials.
[a] Incubation in the artificial gastric juice (AGJ), pH 2.0 at 37 °C for 2 h.
[b] Extended incubation in the artificial intestinal juice (AIJ), pH 6.8 at 37 °C for 5 h.

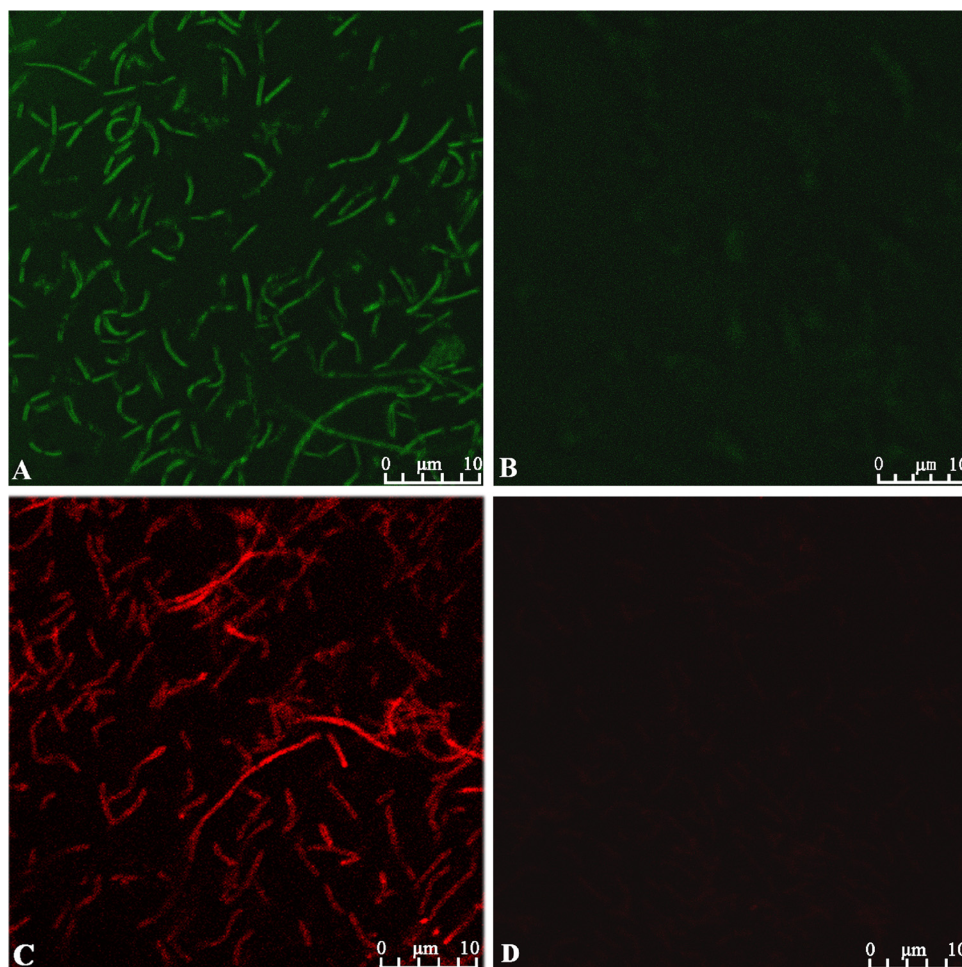

Fig. 5. Detection of fluorescence by laser scanning confocal microscopy (40× magnification objective). (A) Fluorescence detection of *L. casei* Q-5 cells harboring pQJ-gfp; (B) fluorescence detection of *L. casei* Q-5 cells harboring pNZ2102-lacEGF; (C) immunofluorescence detection of *L. casei* Q-5 cells harboring pQJ-gfp treated with anti-GFP antibody and Cy3-conjugated secondary antibody; (D) immunofluorescence detection of *L. casei* Q-5 cells harboring pNZ2102-lacEGF treated with anti-GFP antibody and Cy3-conjugated secondary antibody.

markers may be harmful to human health (Renault 2002), thereby limiting the applications of these vaccines. Therefore, developing food-grade vaccine delivery systems is essential for expanding the human usefulness of genetically engineered live vaccine constructed from lactobacilli strains.

In the present study, *L. casei* ATCC 334 was selected to serve as the host for constructing a new cell surface display system. The strain was isolated from a well-known and highly regarded as safe food, Emmental cheese. *L. casei* ATCC 334 is a well-studied, commercially available genome-sequenced strain (Makarova et al. 2006). Therefore, *L. casei* ATCC 334 has the potential to be a qualified carrier of vaccines. However, plasmid-free strains, such as *L. casei* ATCC 393, are usually used as transformation hosts (Table 3). Meanwhile, *L. casei* ATCC 334 harbors a 29,061 bp plasmid (Makarova et al. 2006), which may affect the replication, stability, and transferability of exogenous plasmids. Hence, the use of strain ATCC 334 in oral vaccine construction is limited. To overcome this problem, the current study applied plasmid elimination to obtain a food-grade derivative of strain ATCC 334. The stability of the plasmid-cured derivative *L. casei* Q-5 was verified by detecting plasmid recovery using PCR methods. We also examined the normal growth ability of *L. casei* Q-5 in MRS medium, and no obvious decrease was

Table 3
Genetically engineered live vaccines developed by *L. casei*.

Host	Vector	Selection marker	Protein expressed	Protein location	References
L. casei ATCC 393	pPG1 and pPG2	Chloramphenicol	Porcine epidemic diarrhea virus S1 region and nucleocapsid protein	Cell surface and extracellular	Liu et al. (2012)
L. casei CICC 6105	pLA	Chloramphenicol	K99, K88 fimbrial protein	Cell surface	Wen et al. (2012)
L. casei ATCC 393	pPG612.1	Chloramphenicol	CSFV-specific CTL epitope and PPV VP2 protein	Extracellular	Xu et al. (2011)
L. casei ATCC 393	pLP401	Erythromycin	Flagellar antigen	Cell surface	Kajikawa et al. (2007)
L. casei ATCC 393	pPG611.1	Chloramphenicol	Porcine parvovirus VP2 protein	Cell surface	Xu and Li (2007)
L. casei ATCC 393	pIAlac	Erythromycin	Human papillomavirus type 16 L1 protein	Intracellular	Aires et al. (2006)
L. casei BLS-S8	pHAT	Erythromycin	Severe acute respiratory syndrome coronavirus spike protein	Cell surface	Lee et al. (2006)
L. casei Shirota	pLP500	Erythromycin	Transmissible gastroenteritis coronavirus spike glycoprotein	Extracellular	Ho et al. (2005)

detected. Therefore, a safe, stable, and plasmid-free strain, *L. casei* Q-5, was constructed to be the host of the food-grade expression system.

By analyzing its genome sequence, we found that the *L. casei* ATCC 334 strain utilizes lactose via a plasmid-encoded phosphotransferase system. This result is consistent with the previous observation in *L. casei* 6H (Alpert and Siebers 1997). The lactose metabolism genes of *L. casei* ATCC 334, which were *lacE*, *lacG*, and *lacF*, are located on its only plasmid. Plasmid elimination resulted in the loss of lactose metabolism ability. By re-introducing *lacE*, *lacG*, and *lacF* into the plasmid-cured derivative of strain ATCC 334, the strain regained lactose-utilization ability. Therefore, the lactose metabolism genes were used as food-grade selection markers.

A well-studied S-layer protein of *L. acidophilus* ATCC 4356 (Boot et al. 1993, 1996; Smit et al. 2001, 2002; Smit and Pouwels 2002) was employed for cell surface display of foreign protein in *L. casei*. S-layers are monomolecular crystalline arrays of proteinaceous subunits and have been identified as the outermost structure of cell envelope in numerous organisms, including many species of *Lactobacillus* (Åvall-Jääskeläinen and Palva 2005; Hynönen and Palva 2013). *L. acidophilus* ATCC 4356 possesses an S-layer composed of a single ~43 kDa hydrophobic, non-glycosylated protein (Boot et al. 1993; Smit et al. 2001). To verify the feasibility of the new host/vector system, GFP was used as a reporter for gene expression. The N-terminus of GFP was fused to the S-layer protein and signal peptide of *L. acidophilus* ATCC 4356. The proved efficient promoter of S-layer protein (Boot et al. 1996) was used to drive the expression of the fusion protein.

The final constructed plasmid pQJ-gfp contained the pSH71 replicon from *Lactococcus lactis* (Platteeuw et al. 1996), lactose metabolism genes from *L. casei* ATCC 334 (as complementary markers), S-layer protein gene from *L. acidophilus* ATCC 4356 (for cell surface display), and *gfp* reporter gene. Except for *gfp*, all the DNA elements of the plasmid originated from safe lactic acid bacteria. For oral vaccine formulation, the *gfp* gene could be replaced by different types of antigen proteins.

The expression of GFP in *L. casei* Q-5 cells harboring pQJ-gfp was verified by laser scanning confocal microscopy. Visible green fluorescence was observed (Fig. 5A). The location of GFP in *L. casei* cells was verified by immunofluorescence tests and laser scanning confocal microscopy. The visible red fluorescence (Fig. 5C) confirmed that GFP was expressed on the cell surface of *L. casei*.

To test the potential of the system constructed here in oral vaccination applications, the tolerance of *L. casei* Q-5 cells harboring pQJ-gfp to artificial gastrointestinal juice was examined. After sequential exposure to AGJ and AIJ, the survival of the strain was even higher than that without treatment. This may be due to the cell growth in AIJ. Therefore, *L. casei* Q-5 cells harboring pQJ-gfp have better ability to tolerate gastrointestinal conditions.

Conclusions

In conclusion, we described the development and application of a food-grade host/vector system. The food-grade host is a plasmid-free derivative of *L. casei* ATCC 334. The food-grade vector was constructed using lactose metabolism genes as selection markers and the regulatory regions of S-layer protein for cell surface expression of foreign proteins. The proposed system is an alternative antigen delivery vehicle in oral vaccine formulation.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31200078) and the Shandong Provincial Natural Science Foundation, China (ZR2001CQ025). We thank the Shandong Province Higher Educational Science and Technology Program (J11LF85), the Science and Technology Development Program of Yantai, China (2010172), and the State Key Laboratory of Microbial Technology, Shandong University (M2012-16).

References

Aires KA, Cianciarullo AM, Carneiro SM, Villa LL, Boccardo E, Perez-Martinez G, et al. Production of human papillomavirus type 16 L1 virus-like particles by recombinant *Lactobacillus casei* cells. Appl Environ Microbiol 2006;72:745–52.

Alpert C-A, Siebers U. The lac operon of *Lactobacillus casei* contains lacT, a gene coding for a protein of the BglG family of transcriptional antiterminators. J Bacteriol 1997;179:1555–62.

Åvall-Jääskeläinen S, Palva A. *Lactobacillus* surface layers and their applications. FEMS Microbiol Rev 2005;29:511–29.

Boot HJ, Kolen CP, Andreadaki FJ, Leer RJ, Pouwels PH. The *Lactobacillus acidophilus* S-layer protein gene expression site comprises two consensus promoter sequences, one of which directs transcription of stable mRNA. J Bacteriol 1996;178:5388–94.

Boot HJ, Kolen CP, van Noort JM, Pouwels PH. S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli*, and nucleotide sequence of the corresponding gene. J Bacteriol 1993;175: 6089–96.

De Man JC, Rogosa M, Sharpe ME. A medium for the cultivation of lactobacilli. J Appl Bacteriol 1960;23:130–5.

Detmer A, Glenting J. Live bacterial vaccines – a review and identification of potential hazards. Microb Cell Fact 2006;5:23.

Fisher HE, Mintz CS. Use of the green fluorescent protein as an educational tool. J Ind Microbiol Biotechnol 2000;24:323–6.

Ho PS, Kwang J, Lee YK. Intragastric administration of *Lactobacillus casei* expressing transmissible gastroenteritis coronavirus spike glycoprotein induced specific antibody production. Vaccine 2005;23:1335–42.

Hynönen U, Palva A. *Lactobacillus* surface layer proteins: structure, function and applications. Appl Microbiol Biotechnol 2013;97:5225–43.

Kajikawa A, Satoh E, Leer RJ, Yamamoto S, Igimi S. Intragastric immunization with recombinant *Lactobacillus casei* expressing flagellar antigen confers antibody-independent protective immunity against *Salmonella enterica* serovar enteritidis. Vaccine 2007;25:3599–605.

Kojic M, Vujcic M, Banina A, Cocconcelli P, Cerning J, Topisirovic L. Analysis of exopolysaccharide production by *Lactobacillus casei* CG11, isolated from cheese. Appl Environ Microbiol 1992;58:4086–8.

Lee JS, Poo H, Han DP, Hong SP, Kim K, Cho MW, et al. Mucosal immunization with surface-displayed severe acute respiratory syndrome coronavirus spike protein on *Lactobacillus casei* induces neutralizing antibodies in mice. J Virol 2006;80:4079–87.

Liu DQ, Ge JW, Qiao XW, Jiang YP, Liu SM, Li YJ. High-level mucosal and systemic immune responses induced by oral administration with *Lactobacillus*-expressed porcine epidemic diarrhea virus (PEDV) S1 region combined with *Lactobacillus*-expressed N protein. Appl Microbiol Biotechnol 2012;93:2437–46.

Maassen CBM, Laman JD, Heijne den Bak-Glashouwer MJ, Tielen FJ, van Holten-Neelen JCPA, Hoogteijling L, et al. Instruments for oral disease-intervention strategies: recombinant *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. Vaccine 1999;17:2117–28.

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, et al. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci U S A 2006;103:15611–6.

Moeini H, Rahim RA, Omar AR, Shafee N, Yusoff K. *Lactobacillus acidophilus* as a live vehicle for oral immunization against chicken anemia virus. Appl Microbiol Biotechnol 2011;90:77–88.

Morens DM, Folfers GK, Fauci AS. The challenge of emerging and re-emerging infectious diseases. Nature 2004;430:242–9.

Platteeuw C, van Alen-Boerrigter I, van Schalkwijk S, de Vos WM. Food-grade cloning and expression system for *Lactococcus lactis*. Appl Environ Microbiol 1996;62:1008–13.

Renault P. Genetically modified lactic acid bacteria: applications to food or health and risk assessment. Biochimie 2002;84:1073–87.

Reveneau N, Geoffroy MC, Lotcht C, Chagnaud P, Mercenier A. Comparison of the immune responses induced by local immunizations with recombinant *Lactobacillus plantarum* producing tetanus toxin fragment C in different cellular locations. Vaccine 2002;20:1769–77.

Scheppler L, Vogel M, Zuercher A, Zuercher M, Germond J, Miescher S, et al. Recombinant *Lactobacillus johnsonii* as a mucosal vaccine delivery vehicle. Vaccine 2002;20:2913–20.

Seegers JFML. Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol 2002;20:508–15.

Smit E, Jager D, Martinez B, Tielen FJ, Pouwels PH. Structural and functional analysis of the S-layer protein crystallisation domain of *Lactobacillus acidophilus* ATCC 4356: evidence for protein–protein interaction of two subdomains. J Mol Biol 2002;324:953–64.

Smit E, Oling F, Demel R, Martinez B, Pouwels PH. The S-layer protein of *Lactobacillus acidophilus* ATCC 4356: identification and characterization of domains responsible for S-protein assembly and cell wall binding. J Mol Biol 2001;305: 245–57.

Smit E, Pouwels PH. One repeat of the cell wall binding domain is sufficient for anchoring the *Lactobacillus acidophilus* surface layer protein. J Bacteriol 2002;184:4617–9.

State Pharmacopoeia Committee. Pharmacopoeia of the People's Republic of China; 2010, Appendix XV D.

Wen LJ, Hou XL, Wang GH, Yu LY, Wei XM, Liu JK. Immunization with recombinant *Lactobacillus casei* strains producing K99, K88 fimbrial protein protects mice against enterotoxigenic *Escherichia coli*. Vaccine 2012;30:3339–49.

Xu Y, Cui L, Tian C, Zhang G, Huo G, Tang L, et al. Immunogenicity of recombinant classic swine fever virus $CD8^+$ T lymphocyte epitope and porcine parvovirus VP2 antigen coexpressed by *Lactobacillus casei* in swine via oral vaccination. Clin Vaccine Immunol 2011;18:1979–86.

Xu Y, Li Y. Induction of immune responses in mice after intragastric administration of *Lactobacillus casei* producing porcine parvovirus VP2 protein. Appl Environ Microbiol 2007;73:7041–7.