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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

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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.

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).

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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.