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Chromosomal Insertions in the *Lactobacillus casei upp* Gene That Are Useful for Vaccine Expression

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To develop a stable and marker-free *Lactobacillus* strain useful for the expression of vaccines, we developed a temperature-sensitive suicide plasmid with expression cassettes containing an HCE promoter, a PgsA anchor, the alpha-toxin gene, and an *rrnB* T1T2 terminator (PPαT) that uses a 5-fluorouracil (5-FU) counterselectable marker for *Lactobacillus casei*. Three strains containing the correct PPαT expression cassettes were produced via the selective pressure of 5-FU screening. We confirmed that the *upp* gene was deleted and that the PPαT expression cassettes were inserted into the *upp* site of *L. casei* ATCC 393 by genomic PCR amplification and sequencing. 5-FU resistance in recombinant bacteria could be stably inherited for as long as 40 generations following insertion. However, bacteria containing the integrated DNA grew more slowly than wild-type *L. casei*. An indirect enzyme-linked immunosorbent assay (ELISA) analysis demonstrated that the alpha-toxin gene was expressed. Also, we visualized expression of the protein on the surface of *L. casei* cells using laser confocal microscopy. These results taken together demonstrate that these recombinant bacteria should provide a safe tool for effective vaccine production.

Lactic acid bacteria (LAB) have shown significant potential as vaccine delivery vehicles, primarily through the use of plasmids that express bioactive compounds at mucosal surfaces, where they can stimulate appropriate immune responses (1–3). LAB offer several advantages over current systemic vaccination routes. Several strains may act as natural adjuvants, potentially eliminating the use of toxic adjuvants common in systemic vaccines. Strains have also been identified that protect against degradation during passage of the vaccine through the gastrointestinal tract to the mucosal surface, where they may induce both mucosal and systemic immunity (4).

Lactobacillus casei is a lactic acid bacterium, and its effect on immune cells in the gut has been extensively studied (5–9). It has been reported that this probiotic bacterium interacts with gut-associated lymphoid tissue (GALT) and makes contact with immune cells associated with Peyer's patches and the lamina propria of the intestinal mucosa (10). Cells from the innate immune response have been proposed to be the main target of *L. casei* for the induction of immune stimulation in the gut (9). These observations suggest that probiotic bacteria have the potential for use as vaccine delivery vehicles.

L. casei has been used previously to express plasmid-encoded protective antigens against porcine parvovirus VP2 and *Escherichia coli* K88 and K99, thus conferring on mice protection against both porcine parvovirus and lethal *E. coli* challenge (11, 12). Bioactive compounds must be expressed at a high enough level to elicit the desired immune response (3). While the high copy numbers of some plasmids may seem advantageous for antigen expression, plasmid instability and the selective pressure required for plasmid maintenance complicate their use in human clinical applications (13, 14). Integrating genes into the chromosome for expression is expected to eliminate selection requirements and provide genetic stability (4).

The *upp* gene encodes uracil phosphoribosyltransferase (UPRTase). This enzyme belongs to the pyrimidine salvage pathway and creates UMP from uracil and phosphoribosyl pyrophosphate (15). The toxic antimetabolite 5-fluorouracil (5-FU) is also a substrate of UPRTase and is converted into 5-fluoro-UMP. After conversion, 5-fluoro-UMP acts as a suicide inhibitor of the enzyme thymidylate synthase, which causes cell death. Therefore, microorganisms with active UPRTase are sensitive to 5-FU (16). The *upp* gene has been identified in *L. casei* and is not essential for survival (17). In this study, we describe a gene deletion method where the *upp* gene serves as a counterselection marker. PPαT expression cassettes were created to boost the expression and facilitate antigen expression on the cellular surface. These cassettes contained the HCE constitutive strong promoter of thermostable D-amino acid aminotransferase (D-AAT) from *Geobacillus toebii* and the PgsA anchor protein from *Bacillus subtilis* (18), a poly-γ-glutamate (PGA) synthetase complex. PGA is an unusual anionic polypeptide in which glutamate is polymerized via γ-amide linkages. According to a previous report, PgsA functions to stabilize the complex by anchoring it in the cell membrane (19). PgsA is localized to the membrane and is ideally positioned for the cell surface display of heterologous proteins (20). The alpha-toxin gene from *Clostridium perfringens* serves as an antigen, and the *rrnB* T1T2 terminator from *E. coli* was integrated into the *upp* gene site by site-specific recombination.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Wild-type *L. casei* ATCC 393 was grown in MRS medium (Sigma), and plasmid-free recombinant *L. casei* ATCC 393 PPαT Δ*upp* was grown in SDM medium containing 100

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μg/ml of 5-FU (Sigma) at 37°C without shaking. Chloramphenicol (Cm) (Sigma) was used at a concentration of 10 μg/ml for *L. casei* ATCC 393 containing the pGBHCupp-2A2B-PPαT plasmid to create a temperature-sensitive strain. For plasmid propagation, *E. coli* TG1 was grown in LB medium containing 30 μg/ml of Cm.

pGBHCupp plasmid. The temperature-sensitive plasmid pGBHCupp was received from Hongyu Cui (Veterinary Laboratory, Northeast Agricultural University) (17) and contained a pWV01 replicon and the *upp* and chloramphenicol resistance genes. The plasmid pGBWVE1 was received from the Poultry Disease Laboratory, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, and contained the pWV01 temperature-sensitive replicon. Based on the pGBWVE1 plasmid, *upp* and Cm resistance genes were inserted into the pGBWVE1 plasmid while keeping the complete replicon element and the multiple-cloning site (MCS) by digestion with restriction enzymes and ligation with DNA ligase. The primers for the *upp* and Cm resistance genes were as follows (the indicated restriction enzyme sites are underlined): Cm-upper, GGTAAGCTTTATAATGAACTTTAATA AAATTGATTT (HindIII); Cm-lower, TTAACTCGAGTCTCATATTATAA AAGCCAGTCA (XhoI); upp-upper, CGCCATGGGGTTTGCAAACAAAT GATTATC (NcoI); and upp-lower, AGAATTCACCTCCTTACTGAAGTG ACGTCATATCAT (EcoRI).

Construction of a homologous recombination vector. (i) Construction of the pGBHCupp-2A2B temperature-sensitive vector. For homologous recombination in *L. casei* ATCC 393, upstream homologous arm 2A (1,021 bp) and downstream homologous arm 2B (768 bp) were PCR amplified using *L. casei* ATCC 393 genomic DNA as a template to create an internal 651-bp deletion in the *upp* gene site. The sense primer for homologous arm 2A was GATCTAGATCAAAGCGGCGCGCCGGCAG CCCAGTTAGTT (BglII), and the antisense primer was TTGGCGCGCC AAGGCTCCTCCTAAACGCATTC (AscI). The sense primer for homologous arm 2B was ACCTTAATTAAATTCTTTGGCATGTGTAAAA (PacI), and the antisense primer was TCCCCGCGGGGAAGGTTGATC GGAAGCA (SacII). The two PCR fragments were gel purified and inserted into the pMD18-T vector (TaKaRa). Correct inserts were identified by sequencing. The pMD18-T-2A plasmid was digested with BglII (TaKaRa) and AscI (NEB). 2A was inserted into the pGBHCupp vector MCS site using the same restriction enzyme digestion, and this construct was named pGBHCupp-2A. The pMD18-T-2B plasmid was digested with SacII (TaKaRa) and PacI (NEB), and 2B was inserted into the pGBHCupp-2A MCS site via the same restriction enzyme digestion and ligated to create the plasmid pGBHCupp-2A2B.

(ii) Construction of the PPαT expression cassettes. The HCE promoter (218 bp) with the Shine-Dalgarno sequence ----AGGA----, a PgsA anchor (1,140 bp), a multiple-cloning site (MCS), and the *rrnB* T1T2 terminator (434 bp) were synthesized by Shanghai Biological Engineering Co., Ltd., China, with the restriction enzymes AscI and PacI, and the construct was named pUC57–A1821. An alpha-toxin gene containing the restriction endonuclease sites HpaI and BglII was amplified by PCR using *Clostridium perfringens* genomic DNA as a template. The sense primer of the alpha-toxin gene was TACGTAATGTTCTGGGATCCTGATACAGA (HpaI), and the antisense primer was GCTCTAGATTTTATATTATAAG TTGAATTTCCTG (BglII). The PCR fragment was gel purified, inserted into the pMD18-T vector (TaKaRa), and identified by sequencing. The alpha-toxin gene was inserted into the A1821 MCS site to create the final construct, pUC57-PPαT.

(iii) Construction of the pGBHCupp-2A2B-PPαT homologous recombination plasmid. pGBHCupp-2A2B-PPαT was created by digesting the pUC57-PPαT plasmid with AscI and PacI and inserting PPαT into the pGBHCupp-2A2B vector digested with the same restriction endonucleases. This plasmid was both temperature sensitive and chloramphenicol resistant.

Preparation of competent *Lactobacillus* cells and subsequent transformation. Preparation of competent *Lactobacillus* cells and subsequent transformation were performed as previously described with some modifications (21–23). Briefly, a 100-ml culture of *L. casei* ATCC 393 cells was grown in MRS medium at 37°C for 4.5 h. The cells were placed in an ice bath for 30 min, pelleted at 4°C and 1,717 × *g*, and then resuspended in 40 ml of ice-cold EPWB buffer (0.6 mmol/liter NaH_2PO_4 and 0.1 mmol/liter $MgCl_2$ [pH 7.4] with filter sterilization). The cells were pelleted and washed in EPWB buffer two more times, washed in EPB buffer (EPWB plus 0.3 M sucrose), and then finally resuspended in 1 ml EPB buffer, resulting in competent cells. Approximately 500 ng of plasmid DNA (derived from *E. coli* TG1) was added to a 200-μl aliquot of competent cells on ice and transformed by electroporation in a cold 0.2-cm cuvette at 2.5 kV. Cells recovered in MRS overnight and were then plated on MRS medium containing 5 μg/ml of Cm.

Homologous recombination of *L. casei* ATCC 393. For homologous recombination, cultures were serially transferred three times (1% inoculum) and grown to stationary phase with 24 h of incubation in a 42°C water bath to select for single-crossover integrants of the targeting plasmid, thereby rendering the transformants resistant to 5 μg/ml of Cm in MRS solid medium during 36 h of incubation in a 37°C incubator. To obtain double-crossover recombinant bacteria, single colonies were serially grown to stationary phase in antibiotic-free MRS medium three to five times in a 37°C water bath. The cultures were then serially inoculated three times into fresh antibiotic-free MRS medium and grown for 24 h in a 42°C water bath. Clones that had lost the plasmid as a result of the second homologous crossover event were rendered 5-FU resistant, and dilutions of 1,000- to 10,000-fold were selected by plating on semidefined medium with glucose (GSDM) (4, 24) containing 100 μg/ml of 5-FU (25). Colonies were screened for PPαT integration using primers upstream and downstream of the crossover event that corresponded to the number of the upstream gene in each location. The sense primer for screening was 5′-GGCAGCCCAGTTAGTTTTAGTGGTCAGGAATATCATT, and the antisense primer was 5′-AGGTTGATCGGAAGCAGGATTGCGACCGG TGACGCATA. Integrants were confirmed by sequencing. Sequencing primers included SBF-F-I421 (5′-CAAACCGATTGGAATGTTCC), HCE-F1 (5′-GATCTCTCCTTCACAGATTCC), SBF-F-I437 (5′-TCTCA ACAGCGCCAACAACC), SBF-F-I512 (5′-CGCGAACTGACGAAAGAC TC), SBF-F-I573 (5′-CTAACTCTCAAAAAGGAACAGC), and SBF-F-J021 (5′-GTAGCGCCGATGGTAGTGT).

Growth and hereditary stability of recombinant *L. casei* strains. To determine whether the properties of the recombinant strains changed relative to those of wild-type *L. casei*, growth and hereditary stability were analyzed after the recombinant *L. casei* strains were screened by PCR and sequencing.

Bacterial growth was measured using the optical density at 600 nm (OD_{600}). Recombinant bacteria were transferred into MRS or SDM medium at a dilution of 1:100 and sampled once every 2 h. The OD_{600} was determined after a 10-fold dilution.

Three strains containing PPαT chromosomal integrations were analyzed for stability by serially transferring the cultures after 24 h of incubation into SDM medium containing 5-FU at 37°C (1% inoculum; 40 generations). DNA was extracted from the cells, and genomic PCR was used to confirm the presence of a PPαT integrant band in each strain using screened primers for the integration event.

Expression of alpha-toxin protein. (i) Indirect ELISA of alpha-toxin protein. For expression analysis of the PgsA-toxin fusion protein in recombinant bacteria, integrated bacteria were grown overnight in MRS medium at 37°C. Bacterial cells were collected by centrifugation at 3,000 × *g* for 15 min. The pellets were washed twice with sterile phosphate-buffered saline (PBS) (pH 7.4) and lysed in a Bead Beater (Biospec, Bartlesville, OK) by vigorous shaking. The cell debris was centrifuged at 3,000 × *g* for 10 min, and the supernatant was analyzed via indirect enzyme-linked immunosorbent assay (ELISA). ELISAs were performed as follows. Protein extracts (8 μg/ml) were applied to 96-well plates overnight at 4°C. Wild-type *L. casei* protein was used as a negative-control antigen. Plates were washed three times with PBS plus 0.05% Tween 20 (PBST). After the wells were blocked for 2 h at 37°C with PBS containing 5% skim milk, the polyclonal antibody to the alpha-toxin of *Clostridium*

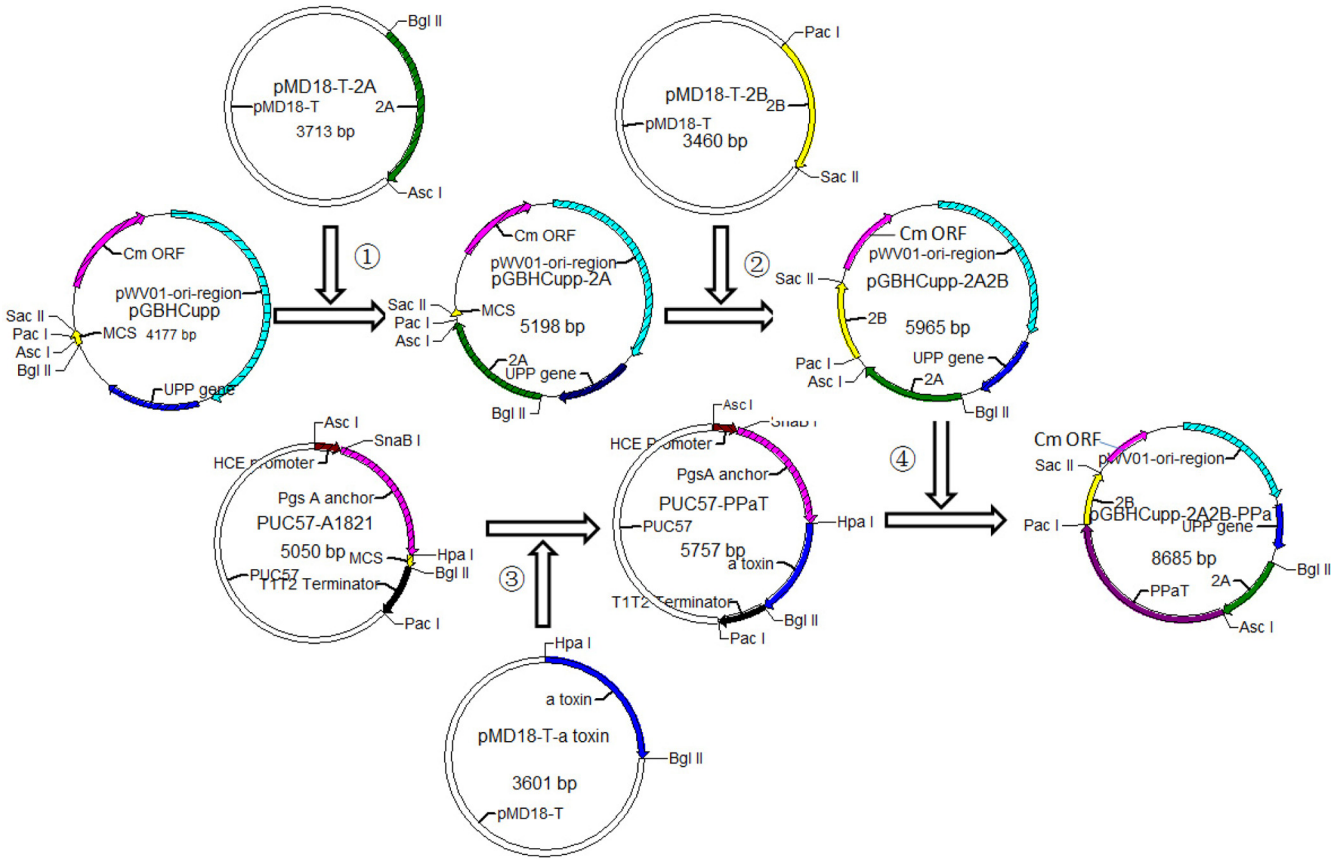

FIG 1 Schematic diagram of vector construction. 1, PCR products of upstream homologous arm 2A with AscI and BglII, cloned into the pMD18-T vector and named pMD18-T-2A. pMD18-T-2A was digested with AscI and BglII endonucleases and inserted into the pGBHCupp vector using the same endonucleases, and this was named pGBHCupp-2A. 2, pMD18-T-2B, containing downstream homologous arm 2B, was digested with PacI and SacII, and 2B was inserted into pGBHCupp-2A; this construct was named pGBHCupp-2A2B. 3, A1821 was synthesized by Shanghai Biological Engineering Co., Ltd., China, and included the HCE promoter, the PgsA anchor, an MCS, and the T1T2 terminator. This construct was inserted into the pUC57 cloning vector and called pUC57–A1821. The alpha-toxin gene cloned into the pMD18-T vector was digested with HpaI and BglII, inserted into the pUC57–A1821 MCS, and named pUC57-PPαT. 4, pUC57-PPαT was digested with AscI and PacI. PPαT was inserted into the pGBHCupp-2A2B vector using the same endonuclease digestion, and this construct was named pGBHCupp-2A2B-PPαT.

perfringens was diluted (1:200) in PBS–1% bovine serum albumin (BSA) in six replicates and incubated for 1 h at 37°C. After the plates were washed three times with PBST, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Invitrogen) was added to each well (1:2,000) and incubated for an additional 1 h at 37°C. After another round of washing, color development was performed using *o*-phenylenediamine dihydrochloride as the substrate, and the absorbance was measured at 490 nm (12).

(ii) Expression of the PgsA-toxin fusion protein on the cell surface. Laser confocal microscopy was used to confirm the expression of alpha-toxin protein on the bacterial surface. Wild-type *L. casei* ATCC 393 and *L. casei* ATCC 393 PPαT Δ*upp* were grown overnight in MRS medium at 37°C. One milliliter of culture was collected by centrifugation at 1,700 × *g* for 10 min, and the pellets were washed twice with PBS (pH 7.4). The polyclonal antibody to alpha-toxin from *Clostridium perfringens* were diluted (1:200) in PBS, and 200 μl was added and incubated for 1 h at 37°C. After the pellets were washed three times with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Invitrogen, USA) was added to each microtube (1:1,000) and incubated for an additional 1 h at 37°C. Samples were then washed three times with PBS, dyed with 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen, USA) for 30 min at 4°C, washed three times, resuspended in 200 μl PBS, and smeared on a microscope slide. Images were viewed by laser confocal microscopy (model LSM510 META; Zeiss, Germany).

RESULTS

Construction of the pGBHCupp-2A2B-PPαT homologous recombination plasmid. Homologous arms 2A and 2B and the PPαT expression cassettes were amplified by PCR, digested with the relevant restriction endonucleases, and ligated with T4 DNA ligase as indicated in Fig. 1.

Homologous recombination. Genomic PCR was performed after recombinant bacteria containing the pGBHCupp-2A2B-PPαT temperature-sensitive plasmid were screened with temperature elevation and exposure to Cm. This analysis identified a 4,456-bp PCR product from the recombinant bacteria that contained a PPαT expression cassette of 2,721 bp, the deleted *upp* gene of 651 bp, and 2,386 bp from wild-type *L. casei* (Fig. 2). PCR products were sequenced by Genewiz Biological Technology Company, Ltd., Beijing, China. BLAST results demonstrated that the recombinant PCR product included the HCE promoter, a PgsA anchor, alpha-toxin, and the *rrnB* T1T2 terminator. The sequence is precisely the same as that of the expression cassette. These results illustrated that the PPαT expression cassettes had integrated successfully into the genome of *L. casei*.

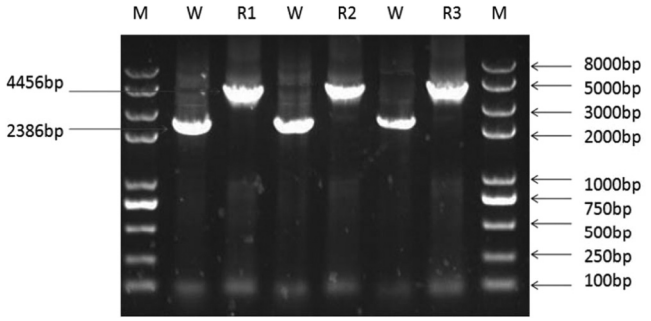

FIG 2 PPαT expression cassettes integrated successfully into the genome of *L. casei*. Lane M, Trans II DNA marker; lane W, wild-type *L. casei* genomic PCR product (the spacer is 2,386 bp in size); lanes R1, R2, and R3, 3, 5, and 12 recombinant *L. casei* (*L. casei* PPαT Δ*upp*) genomic PCR products (the spacer is 4,456 bp in size).

Recombinant *L. casei* strains were obtained by screening. Genomic PCR and sequencing were used to determine the stability of PPαT integrations after 40 generations. These results demonstrated that cassettes were stably inherited over several generations (Fig. 3). However, significant changes occurred in the growth patterns of the recombinants relative to those of the wild type, as all recombinant strains grew more slowly, whether they were cultivated in MRS or SDM culture medium (Fig. 4).

Indirect ELISA. To analyze the expression of the alpha-toxin gene, an indirect ELISA was used. Assay results were repeated six times and showed that the OD_{490} for sample 3 was 0.6962 ± 0.1145, that for sample 5 was 0.4935 ± 0.0239, that for sample 12 was 0.5455 ± 0.0318, and that for the negative control was 0.2468 ± 0.0282. The differences between all groups were very significant ($P \leq 0.01$). These results suggested that the alpha-toxin protein was expressed and that the protein had biological activity.

Expression of the PgsA-toxin fusion protein on the cell surface. Laser confocal microscopy was used to determine whether the fusion protein was expressed on the surface of *L. casei* cells. After cells were treated with FITC-conjugated secondary antibodies and dyed with DAPI, fusion protein was visible on the surface of *L. casei* cells, as shown in Fig. 5.

DISCUSSION

LAB are widely used as live-vaccine vehicles in mucosal immunization because LAB are safe, exhibit adjuvant properties, and are

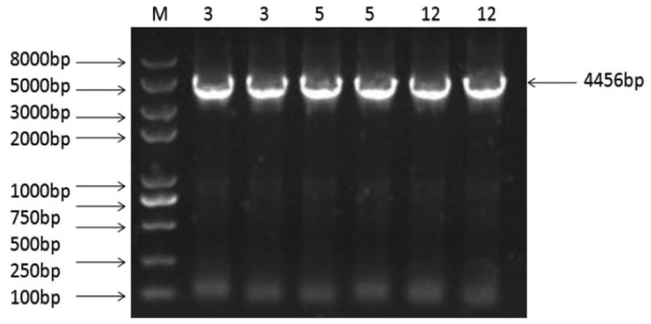

FIG 3 PCR analysis confirms that *L. casei* PPαT Δ*upp* is genetically stable after 40 generations. Lanes 3, PCR product of *L. casei* PPαT Δ*upp* 3 genomic DNA after 40 generations; lanes 5, PCR product of *L. casei* PPαT Δ*upp* 5 genomic DNA after 40 generations; lanes 12, PCR product of *L. casei* PPαT Δ*upp* 12 genomic DNA after 40 generations. Their spacers are 4,456 bp in size.

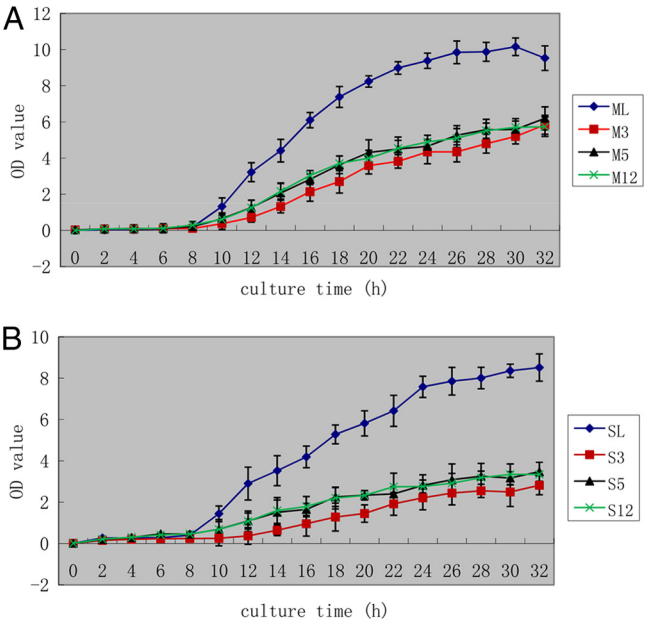

FIG 4 OD_{600} measurements in MRS medium (A) and SDM medium (B). Bacterial growth was measured using the OD_{600}. Recombinant bacteria were transferred into MRS or SDM medium at a dilution of 1:100 and sampled once every 2 h. The OD_{600} was determined after a 10-fold dilution. The horizontal axis indicates the culture time in hours; the vertical axis indicates the OD_{600} value. (A) ML, wild-type *L. casei* grown in MRS medium; M3, *L. casei* PPαT Δ*upp* 3 grown in MRS medium; M5, *L. casei* PPαT Δ*upp* 5 grown in MRS medium; M12, *L. casei* PPαT Δ*upp* 12 grown in MRS medium. (B) SL, wild-type *L. casei* grown in SDM medium; S3, *L. casei* PPαT Δ*upp* 3 grown in SDM medium; S5, *L. casei* PPαT Δ*upp* 5 grown in SDM medium; S12, *L. casei* PPαT Δ*upp* 12 grown in SDM medium.

only weakly immunogenic (23). Integrating genes into the chromosome for expression has the potential to eliminate selection requirements, provide genetic stability (4), and conform to biological safety standards. To integrate sequences into the bacterial chromosome, recombinant plasmids that are suitable for *L. casei* must be constructed. This plasmid can be eliminated when the homologous arm has been integrated into the *L. casei* chromosome using various methods. Therefore, we chose the temperature-sensitive, low-copy-number replicon pWV01. At temperatures of 42°C or higher, plasmid replication failed, and the plasmid underwent homologous recombination with the host chromosome. Systems containing two plasmids have been used for chromosomal integration in some bacteria (4, 25). One of the two plasmids must have a defect that prevents replication in strains lacking the *repA* gene. Therefore, this system requires an *E. coli* strain containing the *repA* gene, such as *E. coli* EC101, for plasmid cloning. A few laboratories possess these *E. coli* strains, where they can be obtained with some difficultly. Two-plasmid systems also require resistance to more than one antibiotic, such as Cm and erythromycin (Em), and thus these systems require more than one transformation. To simplify this process, we employed a single-plasmid system with the temperature-sensitive plasmid pGBHCupp-2A2B-PPαT. Replication failed when the temperature was elevated above 42°C, and the plasmid underwent homologous recombination with the host chromosome. We performed two temperature changes in this experiment. The first temperature rise deleted the plasmid and produced a single recombinant

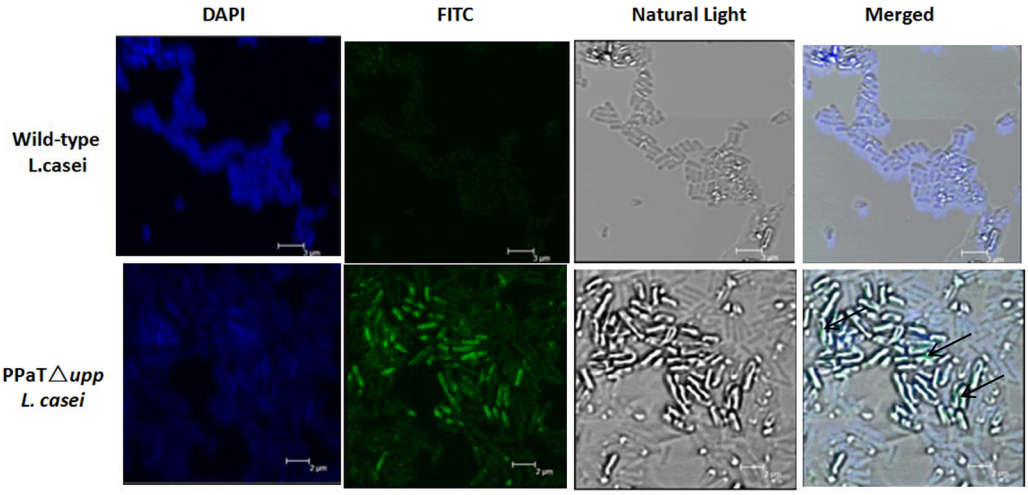

FIG 5 Detection and localization of PgsA-toxin fusion protein in *L. casei* cells. The PgsA-toxin fusion protein localization in *L. casei* cells was detected by laser confocal microscopy. All the cells were probed with FITC-conjugated goat anti-rabbit IgG antibody and stained with DAPI. Merged images showed green light (FITC) from the PgsA-toxin fusion protein on the surface of *L. casei* cells (arrow). Bars, 3 μm for wild-type *L. casei* and 2 μm for *L. casei* PPαT Δ*upp*.

product that was chloramphenicol resistant. The second temperature rise produced bacteria with two recombination events that were chloramphenicol sensitive and 5-FU resistant. The double-recombinant product was selected on 5-FU-containing SDM plates.

The *upp* gene encodes a phosphoribosyl transferase (PRTase) (26–31). PRTases recycle free purine or pyrimidine bases by converting them into the corresponding nucleotide monophosphates, thereby sparing the cell the burden of synthesizing these molecules *de novo*. However, PRTases may also act on base analogs, creating unnatural nucleotides that can be toxic to the cell. Accordingly, PRTase-defective mutants are resistant to the toxic effects of analogs such as 5-FU (32). Kristich et al. developed a method for markerless genetic exchange in *Enterococcus faecalis* and used it to construct an *srtA* mutant (32). Goh et al. used the *upp* gene as a counterselectable marker replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM (25). Braks et al. applied this method to a positive/negative selectable marker system using reverse genetics in *Plasmodium* (33). In *L. casei*, the *upp* gene is nonessential (17) and can serve as a counterselectable marker because the cell becomes 5-FU resistant if the *upp* gene is deleted. In this study, we took advantage of the deletable nature of the *upp* gene to create a counterselectable marker, and PPαT expression cassettes were used to replace the *upp* gene.

In addition, antigens expressed on the surface of bacteria are better recognized by the immune system than those that are intracellular (34). Various anchor genes have been used for this, including those for OprF (a major outer membrane protein of *Pseudomonas aeruginosa*), FadL (an outer membrane protein involved in long-chain fatty acid transport in *Escherichia coli*), and PgsA (35–38). PgsA is considered to be a better anchor for LAB. Therefore, we used the PgsA gene product in this study as an anchor for surface display of antigens on LAB. PgsA is a transmembrane protein derived from the poly-γ-glutamic acid synthetase complex (the Pgs-BCA system) of *Bacillus subtilis* (19, 39). Indeed, the surface display of antigens on the surface of *L. casei* cells has been confirmed by laser confocal microscopy. Green fluorescence was detected on the surface of *L. casei* cells when probed with FITC-conjugated goat anti-rabbit IgG antibody, and blue fluorescence was observed in the bodies of cells stained with DAPI.

In an analysis of the properties of the three new recombinant strains, all strains were genetically stable. However, the recombinant strains grew more slowly than wild-type *L. casei*, in both MRS and SDM media. This may be related to the insertion of the exogenous gene.

The quantity of protein expressed from a genomically integrated gene is less than that from a gene carried by a plasmid, as the genome is present as a single copy and multiple copies of a plasmid are present in the cell. Therefore, we used an ELISA to detect protein expression. The ELISA results demonstrated that the alpha-toxin genes of these three strains were expressed and that the expressed protein had biological activity.

In summary, a single-plasmid system was suitable for the construction of recombinant strains of *L. casei*. We successfully integrated expression cassettes into the *L. casei* chromosome and detected expressed protein for the preparation of food-grade vaccines.

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