Chromosomal Insertions in the upp Gene of *Lactobacillus Casei*

Useful for the Expression of Vaccines

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ABSTRACT

To develop a stable and marker-free *Lactobacillus* strain useful for 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-FU counter-selectable 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 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 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.

1. INTRODUCTION

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, 2, 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* (*L. casei*) is a lactic acid bacterium, and its effect on immune cells in the gut has been extensively studied [5, 6, 7, 8, 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 *E. coli* K88 and K99, thus conferring mice with protection against both porcine parvovirus and lethal *E. coli* challenge, respectively [11, 12]. Bioactive compounds must be expressed at a high enough level to elicit the desired immune response [3]. While the high copy number of some plasmids may seem advantageous for antigen expression, plasmid instability and the selective pressure required for plasmid maintenance complicates their use in human
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 counter-selection 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 were integrated into the upp gene site by site-specific recombination.

2. MATERIALS AND METHODS
2.1 Bacterial strains and growth conditions

Wild-type *L. casei* ATCC 393 was grown in MRS medium (Sigma), and plasmid-free recombinant PPαT**upp-L. casei** ATCC 393 was grown in SDM medium containing 100 μg/ml of 5-FU (Sigma) at 37°C without shaking. Chloramphenicol (Cm; Sigma) was used at a concentration of 1μg/ml for *L. casei* ATCC393 containing the pGBHCupp-2A2B-PPαT plasmid to create a temperature sensitive strain. For plasmid propagation, *E. coli* TG1 was grown in LB medium containing 30μg /ml of Cm.

2.2 pGBHCupp plasmid

The temperature-sensitive plasmid pGBHCupp was received from Hongyu Cui at the 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 MCS site by digestion with restriction enzymes and ligation with DNA ligase. The primers for the *upp* and Cm resistance genes were as follows:

- Cm-upper: GGTAAGCTTTATAATGAACCTTTAAATGGATT (Hind III)
- Cm-lower: TTAACTCGAGTCTCATATTATAAGCCAGTCA (Xhol)
- upp-upper: CGCCATGGGGTTTGCAAACAAATGATTATC(NcoI)
- upp-lower: AGAATTCACCTCCTTACTGAAGTGACGTCATATCAT(EcoRI)
2.3 Construction of a homologous recombination vector

2.3.1 Construction of the pGBHCupp-2A2B temperature sensitive vector

For homologous recombination in *L. casei* ATCC 393, upstream homologous arm 2A (1021 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 GATCTAGATCAAGCGGCAGCCGAGCCCAGTTAGTT (Bgl II site underlined) and the anti-sense primer was TTGGCGCGCCAGGGCTCTCTCTAAGCATTG (Asc I site underlined), The sense primer for homologous arm 2B was ACCTTAATTATTCTTT (Pac I site underlined) and the anti-sense primer was TCCCCGCGG GGAAGGTTGATCGGAAGCA (Sac II site underlined). 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 Bgl II (TaKaRa) and Asc I (NEB). 2A was inserted into the pGBHCupp vector MCS site using the same restriction enzyme digestion and named pGBHCupp-2A. The pMD18-T-2B plasmid was digested with Sac II (TaKaRa) and Pac I (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.

2.3.2 Construction of the PPaT expression cassettes

The HCE promoter (218 bp) with the Shine–Dalgano sequence —AGGA—, a PgsA anchor (1140 bp), a multiple cloning site (MCS) and the rrnB T1T2 (434 bp) terminator were synthetized by Shanghai Biological Engineering Co., LTD, China with
the restriction enzymes AscI and PacI and named pUC57-A1821. An alpha toxin gene containing the restriction endonuclease sites HpaI BglII was amplified with PCR using \textit{clostridium perfringens} genomic DNA as a template. The sense primer of the alpha toxin gene was TACGTAATGTTCTGGGATCCTGATACAGA (HpaI site underlined), and the anti-sense primer was GCTCTAGATTATATTTAAGTTGAATTTCC TG (BglII site underlined). The PCR fragment was gel purified and inserted into the pMD18-T vector (TaKaRa) and identified with sequencing. The alpha toxin gene was inserted into the A1821 MCS site to create the final construct, pUC57-PPαT.

2.3.3 Construction of the pGBHCupp-2A2B-PPαT homologous recombination plasmid

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

2.4 Preparation of competent \textit{Lactobacillus} cells and subsequent transformation

Preparation of competent \textit{Lactobacillus} cells and subsequent transformation was performed as previously described with some modification [21, 22, 23]. Briefly, a 100-ml culture of \textit{L.casei} ATCC393 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/L NaH₂PO₄ and 0.1 mmol/L MgCl₂ 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 5μg/ml of Cm.

### 2.5 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 5μg/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. Then cultures were serially inoculated three times into fresh antibiotic-free MRS media 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 1000 to 10,000x were selected by plating on semi-defined medium with glucose (GSDM) [4, 24] containing 100μg/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 anti-sense primer was 5’-AGGTTGATCGGAAGCAGGATTGCGACCGGTGACGCATA. Integrants were confirmed...
by sequencing. Sequencing primers included SBF-F-1421 (5′-CAACCGATTGAATGTTCC), HCE-F1 (5′-GATCTCTCCTCACAGATTCC), SBF-F-I437 (5′-TCTCAACAGGCAACAC), SBF-F-I512 (5′-CGCGAACTGACGAAAGACTC), SBF-F-I573 (5′-CTAACTCTCAAAAAGGACAGC) and SBF-F-J021 (5′-GTAGCGCCGATGGTAGTG).

2.6 The growth property and hereditary stability of recombinant *L. casei* strains

To determine whether the properties of the recombinant strains changed relative to wild-type *L. casei*, growth and hereditary stability were analyzed after the recombinant *L. casei* strains were screened with PCR and sequencing. Bacterial growth was measured using the OD600. Recombinant bacteria were transferred into MRS or SDM medium with a dilution of 1:100 and sampled once every 2 hours. The OD600 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 of the integration event.

2.7 The expression of alpha toxin protein

2.7.1 Indirect enzyme-linked immunosorbent assay of alpha toxin protein

For expression analysis of the Pgs A-toxin fusion protein in recombinant bacteria, integrated bacteria were grown overnight in MRS medium at 37°C. Bacterial cells were collected by centrifugation at 3000×g for 15 min. The pellets were washed twice with sterile phosphate-buffer saline (PBS, pH 7.4) and lysed in a Bead Beater.
biospec, bartlesville, ok) by vigorous shaking. the cell debris was centrifuged at
3000×g for 10 min and the supernatant was analyzed via the indirect enzyme-linked
immunosorbent assay. ELISAs were performed as follows: 8 μg/ml of protein extracts
of were coated onto 96 wells plates overnight at 4°C. Wild-type L. casei protein was
used as a negative control antigen. Plates were washed three times with PBST (PBS +
0.05% tween 20). After the wells were blocked for 2 h at 37°C with PBS containing 5%
skimmed milk, the polyclonal antibody to the alpha toxin of clostridium perfringens
was diluted (1:200) in PBS-1% BSA, added with 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 into
each well (1:2000) and incubated for an additional 1 h at 37°C. After another round
of washing, color development was performed using o-phenylenediamine
dihydrochloride as substrate, and the absorbance was measured at 490 nm [12].

2.7.2 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 PPα T△upp L. casei
ATCC 393 were grown overnight in MRS medium at 37°C. One milliliter of culture was
collected by centrifugation at 1700×g for 10 min, and 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 200μl 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 into
each microtube (1:1000) and incubated for an additional 1 h at 37°C. Samples were then washed three times with PBS, dyed with phenylindole (DAPI) (Invitrogen, USA) for 30min at 4°C, washed three times, resuspended in 200μl PBS, and smeared on a microscope slide. Images were viewed by laser confocal microscopy (Model LSM510 META, Zeiss, Germany).

3. RESULTS

3.1 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 200 μl of the relevant restriction endonucleases and ligated with T4 DNA ligase as indicated in Fig.1.

3.2 The results of 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 4456 bp PCR product from the recombinant bacteria that contained a PPαT expression cassette of 2721 bp in size, the deleted upp gene of 651 bp in size and 2386 bp from wild-type L.casei (Fig.2). PCR products were sequenced by the 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 an expression cassette. These results illustrated that the PPαT expression cassettes had integrated successfully into the genome of L.casei.
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 the wild type, as all recombinant strains grew more slowly, whether they were cultivated in MRS or SDM culture medium (Fig.4).

### 3.3 Indirect ELISA

To analyze the expression of the alpha toxin gene, an indirect ELISA assay was used. Assay results were repeated six times and showed that the OD490 for sample 3 was 0.6962±0.1145, sample 5 was 0.4935±0.0239, sample 12 was 0.5455±0.0318 and the negative control was 0.2468±0.0282. The differences between all groups were very significant (*p*<0.01). These results suggested that the alpha toxin protein was expressed and the protein had biological activity.

### 3.4 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 died with DAPI, fusion protein was visible on the surface of *L. casei* cells, as is shown in figure 5.

### 4. DISCUSSION

LAB are widely used as live-vaccine vehicles in mucosal immunization because LAB are safe, exhibit adjuvant properties, and are 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 must be constructed that are suitable for *L. casei*. 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-plasmid 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 difficulty. 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 pGBHCuppp-2A2B-PPαT. Replication failed when the temperature was elevated above 42°C, and the plasmid under gone 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 product that was chloramphenicol resistant. The second temperature rise produced bacteria with two recombination
The 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) [28, 29, 30, 31, 32, 33]. 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 [34]. Christopher J developed a method for marker-less genetic exchange in *Enterococcus faecalis* and used it to construct an *srtA* mutant [34]. Goh YJ used the *upp* gene as a counter-selectable marker-replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM [25]. Joanna A M applied this method to a positive/negative selectable marker system using reverse genetics in *Plasmodium* [35]. In *L. casei*, the *upp* gene is nonessential [17] and can serve as a counter-selectable 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 counter-selectable marker, and PPaT 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 [26]. Various anchor genes have been used for this, including 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 [36, 37, 38, 39]. 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, 27]. 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 body 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 relative to wild-type *L. casei*, both in MRS and SDM medium. This may be related to the insertion of the exogenous gene.

The quantity of protein expressed from genomically integrated genes was less than for gene encoded 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. ELISA results demonstrated that the alpha toxin genes of these three strains were expressed and that the expressed protein had biological activity.

In brief, 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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Fig. 1. schematic diagram of vector construction
Fig.1. schematic diagram of vector construction

①PCR products of upstream homologous arm 2A with Asc I and Bgl II cloned into the pMD18-T vector and named pMD18-T-2A. pMD18-T-2A was digested with Asc I and Bgl II endonuclease and inserted into the pGBHCupp vector using the same endonucleases and named pGBHCupp-2A. ② pMD18-T-2B, containing downstream homologous arm 2B, was digested with Pac I and Sac II, and 2B was inserted into pGBHCupp-2A and named pGBHCupp-2A2B. ③ A1821 was synthesized by the Shanghai Biological Engineering Co., LTD, CHINA and included the HCE promoter, the PgsA anchor, a 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 Hpa I and Bgl II, inserted into the pUC57-A1821 MCS site and named pUC57-PPαT. ④ pUC57-PPαT was digested with Asc I and Pac I. PPαT was inserted into the pGBHCupp-2A2B vector using the same endonuclease digestion and named pGBHCupp-2A2B-PPαT.
Fig. 2. PPαT expression cassettes integrated successfully into the genome of *L. casei*

Band M: DNA Marker trans II; Band W: wild type *L. casei* genomic PCR product, the spacer is 2386 bp in size; Bands R1, R2, and R3: 3, 5, and 12 recombinant *L. casei* (PPαT△upp *L. casei*) genomic PCR products, the spacer is 4456 bp in size.
Fig. 3. PCR analysis confirmed that PPαT△upp L. casei is genetically stable after 40 generations. Three is the PCR product of PPαT△upp L. casei 3 genomic DNA after 40 generations; 5 is the PCR product of PPαT△upp L. casei 5 genomic DNA after 40 generations; 12 is the PCR product of PPαT△upp L. casei 12 genomic DNA after 40 generations. Their spacers are 4456 bp in size.
Fig. 4A. OD600 measurements in MRS medium
Bacterial growth was measured using the OD600. Recombinant bacteria were transferred into MRS or SDM medium with a dilution of 1:100 and sampled once every 2 hours. The OD600 was determined after a 10-fold dilution.

The horizontal ordinate indicates the culture time in hours; the vertical coordinates indicates the OD600 value; (A) ML indicates that wild-type *L. casei* was grown in MRS medium; M3 indicates PPαTΔupp *L. casei* 3 was grown in MRS medium; M5 indicates that PPαTΔupp *L. casei* 5 was grown in MRS medium; and M12 indicates that PPαTΔupp *L. casei* 12 was grown in MRS medium. (B) SL indicates wild-type *L. casei* was grown in SDM medium; S3 indicates that PPαTΔupp *L. casei* 3 was grown in SDM medium; S5 indicates PPαTΔupp *L. casei* 5 was grown in SDM medium; and S12 indicates that PPαTΔupp *L. casei* 12 was grown in SDM medium.
Fig. 5 Detection and localization of PgsA-toxin fusion protein in *L. casei* cells.

Detection of the PgsA-toxin fusion protein localization in *L. casei* cells 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 (indicated by an arrow). The bar for Wild type *L. casei* = 3μm and the bar for PPαT△upp *L. casei* = 2μm.