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Research Note

Construction of Human Granulocyte-macrophage Colony-stimulating Factor Expression System Using *Lactococcus lactis* MG1363

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Abstract Lactic acid bacteria (LAB) have been used in a wide variety of industrial fermentation processes. Generally, LAB have regarded as safe status, making them potentially useful organisms for the production of commercially important proteins. Here, we developed protein expression systems using *Lactococcus lactis* MG1363 for which the protein of interest is targeted to a defined cell location, e.g., cytoplasm, cell wall, and medium. The expression system of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) was successfully constructed, and the expression of hGM-CSF in the cytoplasm of *L. lactis* was detected by Western blotting. Efficient translocation was obtained using the signal peptide from lactococcal Usp45 protein and covalently anchored to the peptidoglycan by using the cell wall anchor motif of *Streptococcus pyogenes* M6 protein. These results suggest that *L. lactis* containing a new expression system can deliver many other useful genes to the intestine of human and mammalian to stimulate local mucosal immunity.

Keywords: Lactic acid bacteria, *Lactococcus lactis*, human granulocyte-macrophage colony-stimulating factor, GM-CSF, delivery systems

Introduction

Lactic acid bacteria (LAB) including *lactococci*, *streptococci*, and *lactobacilli* because of their unique metabolic characteristics have been used in many fermentation processes of milk, meats, cereals, and vegetables (1). LAB and their products such as bacteriocins are considered as safe additives (Generally Recognized As Safe, GRAS) and a variety of potential beneficial effects have been published. Some of these effects include lactose digestion, cholesterol metabolism, diarrheal disorders, prevention of intestinal infections, immunomodulation, and oral vaccination (2). The intestinal regulation of LAB has been also proven via numerous studies (3). LAB have an ability to recruit bioactive molecules as they pass through gastrointestinal tract. By using these potential probiotic capacity of LAB, foreign gene encoding cytokine or antigen can be introduced into LAB and induce these gene expression in the gut of human or mammalian. Transformed LAB may also be used as oral live vaccine to induce mucosal immunity (4).

Lactococcus lactis is an attractive host for the production of foreign gene because of their food grade status, efficient expression, and metabolic engineering tools. Although *L. lactis* is a non-pathogenic and non-adherent bacteria in the gastrointestinal tract, *L. lactis* when genetically modified with a recombinant plasmid can be used for the oral delivery of certain genes. Mucosal delivery of *L. lactis* expressing interleukin (IL)-2 and IL-6 accelerated immune activity (5) and treatment of murine colitis with *L. lactis* secreting IL-10 alleviated inflammatory bowel disease (6). *L. lactis* producing and secreting IL-12 can be used to enhance an antigen-specific immune response and to stimulate local mucosal immunity (7). To develop a new expression systems that could be delivered to different cell fractions, *L. lactis* MG1363 was modified using hGM-CSF. First, hGM-CSF expressing *L. lactis* was constructed. In this system, expressed proteins were delivered to cytoplasm. To make secreting system, signal peptide from lactococcal Usp45 protein was inserted into 5'-end of hGM-CSF gene. *Streptococcus pyogenes* M6 protein was used to make cell wall anchor motif. The expression system, developed in this study can be used to make commercial LAB strains expressing useful cytokine.

Materials and Methods

Bacterial strains, plasmids, and media Bacterial strains and plasmids used in this study are addressed in Table 1. *E. coli* XL1-blue, DH5α, TOP-10, and SG13009 were used as host strains for the DNA manipulation of hGM-CSF and lactococcal structure gene, and modified plasmids containing hGM-CSF was transformed into *L. lactis* MG1363. *E. coli* cells were cultured in Luria Bertani (LB) medium (1% sodium chloride, 1% tryptone, 0.5% yeast extract) at 37°C, shaking at 250-300 rpm. *L. lactis* cell culture was performed with M17 medium or Todd-Hewitt broth (Becton Dickinson, Franklin Lakes, NJ, USA) medium at 30°C. Erythromycin (150 μg/mL) and ampicillin (100 μg/mL) were used for *E. coli* selection, and erythromycin (5 μg/mL) and chloramphenicol (10 μg/mL) were used for the selection of *L. lactis* containing modified plasmid.

Construction of cytokine expression vector system hGM-CSF was amplified with specific primers (Forward: 5'-GTCGACGCACCCGCCCG CTCG-3'; Reverse: 5'-GATATCCTCCTGGACTGGCTCCCA-3') from pQE/hGM-CSF plasmid (Cytokine Bank). PCR was performed in a total reaction volume of 50 μL containing 5 U of Taq polymerase, 1x PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP mixture, 10 μM of each oligonucleotide primer, and 1 μL of template DNA. Amplification was performed with an initial denaturation at 95°C for 10 min, followed by 25 to 35 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and finalized at 72°C for 10 min. After completion of the cycling process, PCR products were separated by 0.8% agarose gel electrophoresis. Purified PCR product was inserted into pEZ-T vector (RNA Inc., Yongin, Korea), and transformed into *E. coli* XL-1 blue to amplify plasmid DNA using Gene pulser unit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (2.5 kV, 25 μF capacitance, 200 Ω resistance). DNA sequence of inserted hGM-CSF was analyzed after plasmid preparation. pEZ-T/hGM-CSF plasmids prepared from XL-1 blue were digested with *Sal*I and *Eco*RV restriction enzyme, and inserted into the same restriction enzyme sites of pVE5523 (for secretion system), pVE5524 (for cell wall anchoring), and pVE5529 (for expression in cytoplasm). Constructed plasmids were transformed into *L. lactis* using Gene pulser unit (2.5 kV, 25 μF capacitance, 400 Ω resistance).

***L. lactis* cell fractionation** *L. lactis* containing modified plasmid was incubated with 80% ice-cold trichloroacetic acid (TAC: 16% final concentration) in ice for 20 min and centrifuged at 280×*g* for 15 min. The precipitate was washed with 1 mL acetone twice and dried using vacuum centrifuge. Dried pellet was resuspended with 160 μL TES-LMR solution (TES: 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 25% sucrose; LMR: 1 mg/mL lysozyme, 0.1 mg/mL mutanolysin, 0.1 mg/mL RNase) and incubated at 37°C for 30 min with 1% sodium dodecyl sulfate (SDS). The supernatant (containing cell wall digestion) and pellet (containing protoplast) were separated by centrifugation at 4°C, 2,500×*g*. The pellet containing protoplast was washed with TES solution and resuspended with 500 μL distilled water. Resuspended sample was performed 5 times freeze-thawing cycle and centrifuged at 4°C, 21,000×*g* for 45 min to separate cytoplasmic protein and membrane pellet. Membrane pellet was resuspended with 100 μL TE solution containing 1% SDS to get membrane protein fraction. Supernatant containing cytoplasmic protein was precipitated with TCA, and resuspended with 100 μL TE solution. To obtain the cell wall digestion protein fraction, the supernatant was incubated with TCA and precipitated pellet was dissolved in 100 μL 50 mM NaOH. Medium protein fraction was prepared from *L. lactis* culture supernatant. The supernatant was filtered with 0.2 μm pore-size filter, protein precipitation was performed with ice-cold 80% TCA, and the pellet was dissolved in 80 μL 50 mM NaOH.

Western blot analysis Equal amounts of protein were resolved on SDS-polyacrylamide gels (10-15%) and then electrophoretically transferred onto nitrocellulose membrane (100 V, 2 h). Membranes were subsequently blocked with 5% skim milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.05% Tween 20) and incubated with the hGM-CSF antibodies (R and D systems, Minneapolis, MN, USA). Blotting proteins were visualized by enhanced chemiluminescence (ECL) reagents (GE Healthcare, Little Chalfont, UK).

Results and Discussion

L. lactis is a food-grade bacterium that is widely used in the dairy industry. It is a potential candidate for the production of biologically useful proteins and for plasmid DNA delivery to eukaryotic cells. Several delivery systems have been developed to target heterologous proteins to a specific cell location e.g., cytoplasm, cell wall or extracellular medium, and more recently to efficiently transfer DNA to eukaryotic cells (8). Thus, protein secretion and cell wall anchoring system using LAB are key factors to contribute a new functions to probiotics. GM-CSF, a monomeric glycoprotein that function as a cytokine, stimulates immune cells such as neutrophils, eosinophils, basophils, and monocytes, to produce granulocytes (9). GM-CSF also plays a role in embryonic development by functioning as an embryokine produced by reproductive tract (10). To develop a new expression system using hGM-CSF, we modified *L. lactis* MG1363. Amplified hGM-CSF gene was cloned into Gram-positive bacteria expression vectors. LAB expression vectors such as pVE5523, 5524, and 5529 contain P59 promoter and t1t2 and trpA terminators, which were optimized to express heterologous gene expression (11). pVE5523 contains N-terminal signal sequence of lactococcal Usp415 next to P59 promoter, which guides the expressed protein to the extracellular medium (12). pVE5524 contains C-terminal cell wall anchor domain of *Streptococcus pyogenes* M6 protein as well as N-terminal signal sequence, which drives target protein to the peptidoglycan of cell wall (13). The schematic diagram of these three different vector systems was depicted in Fig. 1A.

Constructed plasmids were transformed into *E. coli* and erythromycin

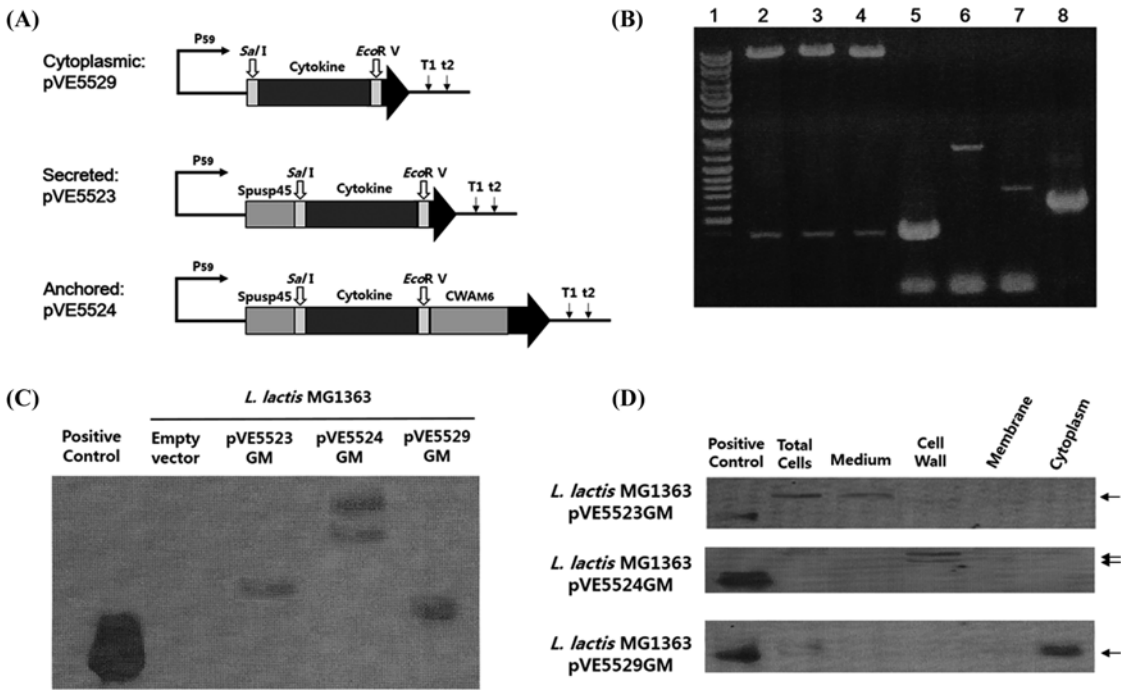

Fig. 1. Construction of expression system using *L. lactis* containing cytokine expression vector. (A) Schematic diagram for vector construction. (B) Confirmation of hGM-CSF cloning into Gram-positive bacteria expression vector by colony PCR and restriction enzyme digestion. 1, 1 kb DNA ladder; 2, pVE5524GM enzyme cut with *Sal*I and *Eco*R V; 3, pVE5523GM enzyme cut with *Sal*I and *Eco*R V; 4, pVE5529GM enzyme cut with *Sal*I and *Eco*R V; 5, PCR product of hGM-CSF; 6, PCR product from pVE5524GM using p59 and Tt1t2 primers; 7, PCR product from pVE5523GM using p59 and Tt1t2 primers; 8, PCR product from pVE5529GM using p59 and Tt1t2 primers. (C) Western blot analysis using anti-hGM-CSF antibody of total cell lysates from *L. lactis* MG1363 containing pVE5523GM, pVE5524GM, or pVE5529GM. (D) Western blot analysis of cell fractions from *L. lactis* MG1363 containing pVE5523GM, pVE5524GM, or pVE5529GM.

resistance gene was used for the selection marker. To confirm the modified plasmid, colony PCR was performed with p59 forward primer and Tt1t2 reverse primer and transformants as a template, and Amplified plasmids in *E. coli* was digested with *Sal*I and *Eco*RV restriction enzyme (Fig. 1B). Colony PCR result shows that pVE5523GM, 5524GM, and 5529GM produced 688, 1220, and 577 bp PCR products, respectively. Digested product from each modified plasmids was 388 bp, whose size is consistent with hGM-CSF. DNA sequencing analysis was also continued. DNA and translated amino acid homologous search have shown that modified plasmids contain hGM-CSF gene (data not shown). The development of reliable systems for the food-grade genetic modification of LAB is a very important in the development of delivery system using microorganisms. Food-grade recombinants can be used as starters in food fermentations and for the safe production of metabolites used as food adjuncts (14). The use of plasmids for food-grade introduction of new or modified genes into *L. lactis* often seems unfavorable because the presence of antibiotic resistance genes in the final constructs is not permitted and the range of alternative selection markers is limited. Several food-grade vectors have been developed by using sugar utilization (15) or the suppression of auxotrophic markers (16) for positive selection of transformants. Unfortunately, selection for these vectors requires special genotypes of the respective host strains. Thus, we need to consider an additional supplementation, e.g., integration of target gene into the bacterial genome, which may be a more reliable approach to stabilizing and maintaining the desired genetic features.

To identify the expression of cloned hGM-CSF, total cell lysates from *L. lactis* containing different modified plasmids were resolved on SDS-PAGE, and then protein expression was detected by Western blotting using anti-hGM-CSF antibody. The positive control band of hGM-CSF was appeared around 24-28 kDa. hGM-CSF protein expressed from pVE5523GM was shifted as compared to positive control band. Multiple protein bands from pVE5524GM were shown around 34 kDa and a single band from pVE5529GM was shown around 28 kDa (Fig. 1C). Next, *L. lactis* cell fractions were subjected to Western blot analysis to identify the protein localization (Fig. 1D). As shown in the figure, major protein bands from *L. lactis* containing pVE5523GM were shown in total cell lysates and medium fraction, multiple bands from *L. lactis* containing pVE5524GM were only shown in total cell lysates and cell wall fraction, and protein bands from *L. lactis* containing pVE5529GM were appeared in total cell lysates and cytoplasm. Constructed pVE5523GM secretes hGM-CSF protein to extracellular medium in *L. lactis* culture. *L. lactis* containing pVE5524GM drives cell wall anchoring, while target protein expressed by pVE5529GM is restricted to cytoplasm. These results suggest that hGM-CSF protein expression was successfully targeted on their right position according to the constructed plasmids, and indicating that the

Table 1. Bacteria strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Reference
Strain		
E. coli XL1-Blue	Plasmid-free strain	
DH5α	Plasmid-free strain	
TOP-10	Plasmid-free strain	
SG13009	Plasmid-free strain	Qiagen
L. lactis MG1363	Plasmid-free strain	
Plasmid		
pQE/hGM-CSF	Ap^r; ColE1; 3.9 kb :: hGM-CSF	Cytokine Bank
pUC18/hG-CSF	Ap^r; ColE1; 3.5 kb :: hG-CSF	Cytokine Bank
pUC18/hIFN-γ	Ap^r; ColE1; 3.1 kb :: hIFN-γ	Cytokine Bank
pEZ-T	Ap^r; ColE1; 3.0 kb	RNA Inc.
pVE5523	Ap^r; Em^r; ColE1; pAMβ1; pBS::pIL252::t_{trp}A::P_{59}::*SP*Usp45::nucA::t1t2	(11)
pVE5524	Ap^r; Em^r; ColE1; pAMβ1; pBS::pIL252::t_{trp}A::P_{59}::*SP*Usp45::nucA::CWA_{M6}::t1t2	(11)
pVE5529	Ap^r; Em^r; ColE1; pAMβ1; pBS::pIL252::t_{trp}A::P_{59}::nucA::t1t2	(11)
pVE5523GM, G, I	Ap^r; Em^r; ColE1; pAMβ1; pBS::pIL252::t_{trp}A::P_{59}::cytokine::nucA::t1t2	This work
pVE5524GM, G, I	Ap^r; Em^r; ColE1; pAMβ1; pBS::pIL252::t_{trp}A::P_{59}::cytokine::nucA::CWA_{M6}::t1t2	This work
pVE5529GM, G, I	Ap^r; Em^r; ColE1; pAMβ1; pBS::pIL252::t_{trp}A::P_{59}::cytokine::t1t2	This work

ColE1, replicon from *E. coli* plasmid; pAMβ1, replicon from Gram positive bacteria; Ap^r, ampicillin resistance; Em^r, erythromycin resistance

cytokine expression system can be used for the developing of commercially useful LAB strains. Administration of LAB strains carrying the cytokine-cloned plasmid will be effective at diminishing inflammation or stimulating immune cells against infected pathogens.

All of the *L. lactis* strains that have been developed to be used as vaccine DNA delivery vectors are promising for mucosal immunization. Recently, however, several studies have shown that recombinant *L. lactis* can be used as a therapeutic vector such as the treatment of few Crohn's disease patients (17,18). In this study, we also constructed the expression vector system using human Granulocyte-colony stimulating factor (hG-CSF) and human interferon-gamma (hIFN-γ) genes (data not shown). Unfortunately, in this study, we did not confirm the activity of *L. lactis* expressing human cytokines against the immune modulation. Thus, additional experiments should be performed to test activity and safety of invented delivery system. For example, the physiological effect of *L. lactis* expressing hGM-CSF or hG-CSF can be examined with the proliferation of AML-193 cell line and the antiviral activity of IFN-γ expressed *L. lactis* can be tested with A549 cells containing encephalomyocarditis virus (EMCV). Oral administration test using modified *L. lactis* should be carried out to confirm the safety. The delivery of pathogen-derived vaccine through oral or nasal may provoke local immune responses at the entry. However, the use of *L. lactis* to deliver hGM-CSF to a mucosal surface may reduce toxic side effects and have clear advantages of low-cost and simple method of administration. Taken together, our study suggest that *L. lactis* containing a new expression system can deliver many other useful genes to the intestine of human and mammalian.

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Disclosure The authors declare no conflict of interest.

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