Characterization of a Novel LysM Domain from \textit{Lactobacillus fermentum} Bacteriophage Endolysin and Its Use as an Anchor To Display Heterologous Proteins on the Surfaces of Lactic Acid Bacteria

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The endolysin Lyb5, from \textit{Lactobacillus fermentum} temperate bacteriophage dPYB5, showed a broad lytic spectrum against Gram-positive as well as Gram-negative bacteria. Sequence analysis revealed that the C terminus of the endolysin Lyb5 (Ly5C) contained three putative lysin motif (LysM) repeat regions, implying that Ly5C was involved in bacterial cell wall binding. To investigate the potential of Ly5C for surface display, green fluorescent protein (GFP) was fused to Ly5C at its N or C terminus and the resulting fusion proteins were expressed in \textit{Escherichia coli}. After being mixed with various cells in vitro, GFP was successfully displayed on the surfaces of \textit{Lactococcus lactis}, \textit{Lactobacillus casei}, \textit{Lb. brevis}, \textit{Lb. plantarum}, \textit{Lb. fermentum}, \textit{Lb. delbrueckii}, \textit{Lb. helveticus}, and \textit{Streptococcus thermophilus} cells. Increases in the fluorescence intensities of chemically pretreated \textit{L. lactis} and \textit{Lb. casei} cells compared to those of nonpretreated cells suggested that the peptidoglycan was the binding ligand for Ly5C. Moreover, the pH and concentration of sodium chloride were optimized to enhance the binding capacity of GFP-Ly5C, and high-intensity fluorescence of cells was observed under optimal conditions. All results suggested that Ly5C was a novel anchor for constructing a surface display system for lactic acid bacteria (LAB). To demonstrate the applicability of the Ly5C-mediated surface display system, \(\beta\)-galactosidase (\(\beta\)-Gal) from \textit{Paenibacillus sp.} strain K1, replacing GFP, was functionally displayed on the surfaces of LAB cells via Ly5C. The success in surface display of GFP and \(\beta\)-Gal opened up the feasibility of employing the cell wall anchor of bacteriophage endolysin for surface display in LAB.

Surface display of heterologous proteins or peptides on bacteria is potentially important in several areas of biotechnology, including development of live vaccine delivery systems, diagnostics, whole-cell absorbents, and novel biocatalysts (11). Lactic acid bacteria (LAB) have the status of being generally recognized as safe (GRAS), making them certainly more useful in food and medical applications than other bacterial species. The development of cell surface display systems for LAB has recently become one of the most active research areas. Most of the cell surface display systems for LAB reported thus far have made use of the C terminus of a cell wall-anchoring protein via an LPXTG motif (8, 12, 19, 24). This anchoring mechanism requires processing by a sortase for covalent anchoring of the protein to the cell wall peptidoglycan (15). Various anchoring proteins, such as membrane-spanning protein PgsA (16) and S-layer protein (3), have also been exploited for surface display. However, heterologous proteins have been anchored to the producer cells, and the use of genetically modified organisms is less desirable or at least still being debated. Surface display of heterologous proteins on genetically unmodified Gram-positive bacteria has been successfully carried out using the peptidoglycan binding lysin motif (LysM) domain of the major autolysin AcmA of \textit{Lactococcus lactis} (1, 2, 4, 18, 28).

LysM was first discovered in the lysozyme of \textit{Bacillus} phage d29 as a C-terminal repeat composed of 44 amino acids separated by 7 amino acids (6). LysM is a common module found in more than 4,000 proteins of both prokaryotes and eukaryotes (6). Many bacterial proteins containing LysM are peptidoglycan hydrolases, such as p60 (20), Sep (26), LytF (31), AcmA (5), and Mur (7). The best-characterized LysM-containing protein is the \(N\)-acyetylglucosaminidase AcmA of \textit{L. lactis} subsp. cremoris MG1363. AcmA is the major autolysin and is required for cell separation and cell lysis during the stationary phase of \textit{L. lactis} (5). It contains three domains: the N-terminal signal peptide, an active domain, and a C-terminal peptidoglycan anchor (cA) which consists of three LysM repeats (22). Several functional proteins, including malaria parasite surface antigen, \(\beta\)-lactamase, \(\alpha\)-amylase, and viral capsid proteins, have been covalently bound to cell walls of \textit{Acma}-producing and non-\textit{Acma}-producing \textit{L. lactis} as well as several other Gram-positive bacteria via cA (4, 17, 18, 23, 25).

Endolysins from bacteriophages are cell wall hydrolases involved in cell lysis to release the progeny particles from the host cells (9, 30). Most endolysins lack a signal peptide and are translocated across the membrane by the aid of the holin protein. This protein typically contains an N-terminal catalytic domain and a C-terminal cell wall binding domain (33). The endolysins Ply118 and Ply500 of \textit{Listeria monocytogenes} phage share a unique C-terminal cell wall binding domain which establishes specific recognition of and high-affinity binding to bacterial cell wall carbohydrates (13). The temperate bacteriophage dPYB5, isolated from the \textit{Lactobacillus fermentum} YB5 strain, has a hexagonal head, noncontractile tails, and several fibers and belongs to Bradley’s group B as defined by the International Committee on Taxonomy of Viruses (32).
The sequence of the endolysin gene lyb5 from the genome of L. plantarum M1363 was used in this study.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. E. coli BL21(DE3) and E. coli Origami B(DE3) were used as the cloning hosts, and E. coli DH5α was used as the cloning host. E. coli strains were grown aerobically in Luria-Bertani (LB) broth at 37°C. L. lactis and Streptococcus thermophilus were grown anaerobically in M17 broth (Oxoid, Basingstoke, United Kingdom) supplemented with 0.5% (wt/vol) glucose (hereinafter referred to as GM17) at 30 and 42°C, respectively.

<table>
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<th>Strain, plasmid, or primer</th>
<th>Relevant characteristic(s), description, or sequence*</th>
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<tr>
<td><em>E. coli</em> Origami B(DE3)</td>
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*The restriction sites in the primer sequences are underlined. Te’, tetracycline resistance; Kan, kanamycin resistance; Ap’, ampicillin resistance; NICE system, nisin-controlled gene expression system.*

**Plasmids**

- **pBluescript-SK-lysin**
  - pBluescript SK ligated to 1,257-bp PCR product containing lyb5 gene

- **pET-22b (+)**
  - Ap', E. coli expression vector

- **pWaldo-Control**
  - pET-22b ligated to 725-bp GFP gene at Ndel and HindIII sites

- **pUC-bga**
  - pUC ligated to 2,000-bp PCR product containing bga gene

- **pET-ly5C**
  - Ap'; pET-22b (+) carrying HindIII/Xhol-digested product expressing His6-tagged Ly5C

- **pET-gfp**
  - Ap'; pET-22b (+) carrying Ndel/HindIII-digested product expressing His6-tagged GFP

- **pET-gfp-ly5C**
  - Ap'; pET-22b (+) carrying Ndel/HindIII-digested product expressing His6-tagged GFP-Ly5C

- **pET-gal**
  - Ap'; pET-22b (+) carrying Ndel/HindIII-digested product expressing His6-tagged β-Gal

- **pET-gal-ly5C**
  - Ap'; pET-22b (+) carrying Ndel/HindIII-digested product expressing His6-tagged β-Gal-Ly5C

**Primers**

- **LM1**
  - 5'-AATGCTTCTAGAGCAACGCAGCACAC-3'

- **LM2**
  - 5'-GTTCTCTAGAGCTCGAGGATGAGATTTTGCGCCG-3'

- **GFP-HindIII**
  - 5'-GGCGAAGCTTATGGCAAGGAGAAG-3'

- **GFP-Xhol**
  - 5'-ACACCTGAGGTTAGGCTCTCAATCAGT-3'

- **LM-Ndel**
  - 5'-CGACATATGTCTAGACACCAGGACA-3'

- **LM-HindIII**
  - 5'-GTGAGCCTCTGATAGCATGAGAGTGG-3'

- **Gal1**
  - 5'-CAATATAGCTGACGACACACTACC-3'

- **Gal2**
  - 5'-AACAGCTCATTCACAGCACAGC-3'

*Lactobacillus* strains were cultured in MRS broth (Oxoid, Basingstoke, United Kingdom) at 37°C without aeration. Antibiotics were added for *E. coli* as follows: 100 µg/mL ampicillin, 34 µg/mL chloramphenicol, and 12.5 µg/mL tetracycline.

**General molecular biology.** Molecular cloning techniques were performed essentially as described previously (21). All PCRs were carried out using Taq polymerase (TaKaRa, Tokyo, Japan), and the oligonucleotides used are listed in Table 1. Restriction enzymes and T4 DNA ligase were used according to the instructions of the manufacturer (TaKaRa, Tokyo, Japan).

The Ly5C gene was PCR amplified from the plasmid pBluescript-SK-lysin by using primers LM1 and LM2. The PCR products were digested with HindIII and Xhol and then cloned into similarly digested pET-22b (+) to generate pET-ly5C. The GFP gene was digested from the plasmid pWaldo-Control by using Ndel and HindIII and inserted into the corresponding sites of pET-22b (+) and pET-ly5C, yielding pET-gfp and pET-gfp-ly5C, respectively. The GFP gene was amplified by PCR from the plasmid pWaldo-Control with oligonucleotides GFP-HindIII and GFP-Xhol, and then HindIII and Xhol-digested PCR products were cloned into the corresponding sites of pET-22b (+) to generate pET-gfp2. The Ly5C gene was amplified by PCR with primers LM-Ndel and LM-HindIII and inserted into the Ndel and HindIII sites of the plasmid pET-gfp2, generating the plasmid pET-ly5C-gfp, which could express the fused protein Ly5C-GFP. The β-Gal gene (gal) from Paenibacillus sp. strain K1 (14) was PCR amplified with primers Gal1 and Gal2 using pUC-bga as the template. The PCR products were digested with Ndel and HindIII and ligated into the similarly digested pET-22b...
The resulting plasmids were designated pET-gal and pET-gal-ly5C. *E. coli* BL21(DE3) was transformed with the recombinant plasmids pET-gfp, pET-gfp-ly5C, and pET-ly5C-gfp by the standard CaCl₂ heat shock protocol (21), and pET-gal and pET-gal-ly5C were introduced into *E. coli* Origami B(DE3).

**Overexpression of Ly5C fusion proteins in *E. coli*.** *E. coli* strains harboring the recombinant plasmids constructed as described above were grown overnight at 37°C in LB medium supplemented with 100 μg/ml ampicillin. Subsequently, the overnight cultures were diluted 100-fold in 500 ml of fresh LB broth, and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the cultures reached an optical density at 600 nm (OD₆₀₀) of 0.8. After further incubation for 4 h at 30°C, cells were harvested, washed, and resuspended in 100 ml of phosphate-buffered saline (PBS; 157 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4). Then the cells were disrupted by sonication on ice at 300 W for two cycles (one cycle consists of 50 periods of sonication for 3 s with intermissions of 8 s), and the clear lysates were centrifuged at 12,000 rpm for 30 min and passed through a filter (0.22-μm pore size; Millipore) to remove the cell debris. Next, the clear lysates were applied to HisTrap FF crude columns (GE Healthcare) equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 25 mM imidazole, pH 7.4), the columns were washed with washing buffer (20 mM sodium phosphate, 500 mM NaCl, 25 mM imidazole, pH 7.4), and the His-tagged proteins were eluted in elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Concentrations of purified proteins were determined using the Bradford protein assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze protein expression.

**Binding of Ly5C fusion proteins to LAB.** LAB strains were grown as mentioned above, and cells from 1-ml aliquots of stationary-phase cultures were collected, washed twice, and resuspended in 100 μl of PBS. The cells were then incubated with 1-ml samples of the clear lysates containing excessive GFP-GFP-Ly5C, Ly5C-GFP, β-Gal, and β-Gal-Ly5C proteins for 30 to 60 min at 37°C. After binding, cells were collected by centrifugation at 10,000 rpm for 5 min and resuspended in 1 ml of PBS with vortex mixing. After being washed five times with PBS, cells were resuspended in 100 μl of PBS. The cells bound with GFP-Ly5C were pelleted and washed with PBS and after being mixed with GFP-Ly5C, cells were pelleted and washed with PBS and the fluorescence level per OD₆₀₀ unit was measured. For 10 min. After the treatments, the cells were washed completely with PBS to remove any residual chemicals. The pretreated cells were subsequently used for Ly5C binding assays as described above.

**Influence of cell growth phase, NaCl, pH, and binding time on Ly5C binding capacity.** *L. lactis* and *Lb. casei* were grown in GM17 and MRS medium, respectively. At regular intervals, the cells were collected and washed twice with PBS and cell samples were diluted to an OD₆₀₀ of 1.0. After being treated with a 0.2 volume of 5% TCA and washed with PBS, the cells from different time points were used for Ly5C binding assays.

The influence of NaCl on the binding of Ly5C to the surfaces of *L. lactis* and *Lb. casei* cells was tested with PBS buffer supplemented with final concentrations of 0, 0.1, 0.2, 0.5, 1.0, and 2.0 M NaCl. Cells of both *L. lactis* and *Lb. casei* pretreated with 5% TCA were mixed with GFP-Ly5C. After incubation for 30 min at 37°C, the cell-associated fluorescence was measured as described above. The effect of pH was evaluated over a range from pH 5 to 12 with 0.5 M NaCl or no NaCl. The GFP-Ly5C binding assay was performed as described above.

The binding time course assay was carried out essentially as the standard GFP-Ly5C binding assay described above, using 5% TCA-pretreated *L. lactis* and *Lb. casei* cells. At different time points (15, 30, 60, 120, 180, and 240 min) after being mixed with GFP-Ly5C, cells were pelleted and washed with PBS and the fluorescence level per OD₆₀₀ unit was measured.

**RESULTS**

**Sequence analysis of endolysin Lyb5.** The endolysin gene lybs5 from *Lb. fermentum* temperate bacteriophage δPYB5 encodes a protein of 418 amino acids with a deduced molecular mass of 45 kDa (30). A signal peptide was predicted by using SOPMA software, and the cleavage site was located between amino acids 29 and 30 at the N terminus of Lyb5 (Fig. 1A). Based on data from reverse position-specific BLAST, two conserved domains in Lyb5 were identified. One, between amino acids 30 and 257, is the active domain and belongs to the GH25 muramidase superfamily, which can degrade cell walls by catalyzing the hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-d-glucosamine residues. The other is the substrate binding domain located in the C terminus (Ly5C) and consists of three putative LysM regions (Lc1, Lc2, and Lc3) between amino acids 258 and 418. Each of the Lc regions is composed of 41 amino acids, and the regions are separated by intervening sequences that vary in length and composition. The sequence between Lc1 and Lc2 consists of 26 amino acids, while the sequence between Lc2 and Lc3 consists of 10 amino acids. The overall level of similarity among the three repeated regions of Ly5C is approximately 70%. Moreover, the LysM repeated regions of Ly5C have about 63% similarity to the pfam01476 LysM domain (Fig. 1B). Comparing Ly5C with the
C termini of AcmA (cA; containing three LysM repeats), Sep (containing one LysM repeat), Mur (containing four LysM repeats), and LAF_0486 from \textit{Lb. fermentum} IFO 3956 revealed the levels of similarity to be 45, 46, 45, and 52%, respectively. The three LysM regions of Ly5C contain YG repeat motifs, which could anchor proteins to the cell walls of LAB.

Displaying GFP-Ly5C on the cell surfaces of LAB. To investigate the potential of Ly5C for displaying heterologous proteins on the cell surfaces of LAB, the GFP which spontaneously emits green light at 508 nm when excited with blue light at 395 nm (GFP<sub>av</sub> was used as a reporter protein (29)). The GFP<sub>av</sub> gene was obtained from pWaldo-Control, and the gene product was fused to Ly5C at its N or C terminus. GFP-Ly5C and Ly5C-GFP (45 kDa) and GFP (28 kDa) were produced in \textit{E. coli} (data not shown). After exogenous addition of cells lysates containing GFP-Ly5C, both \textit{L. lactis} and \textit{Lb. casei} cells were decorated with green fluorescence (Fig. 2A). As a control, no fluorescence was observed on cells incubated with GFP (Fig. 2E), suggesting that Ly5C was able to efficiently direct GFP to the surfaces of \textit{L. lactis} and \textit{Lb. casei} cells. Moreover, the Ly5C-GFP fusion protein was also displayed on the surfaces of \textit{L. lactis} and \textit{Lb. casei} cells (Fig. 2D), indicating that the location of GFP did not influence Ly5C binding. Interestingly, the binding of GFP was located at the specific loci of lactococcus and lactobacillus cells (arrowheads in Fig. 2) where the newly synthesized peptidoglycan was inserted into the cell wall.

To further determine whether Ly5C can serve as an anchor protein with other LAB, TCA-pretreated cells of \textit{Lb. brevis}, \textit{Lb. plantarum}, \textit{Lb. delbrueckii}, \textit{L. buchneri}, \textit{Lb. helveticus}, \textit{Lb. fermentum}, and \textit{S. thermophilus} were tested. As shown in Fig. 3, GFP was presented on the entirety of the surfaces of LAB cells by the direction of Ly5C. This result demonstrated that Ly5C is necessary and sufficient as an anchor for displaying proteins on the cell surfaces of LAB.

GFP-Ly5C binds to peptidoglycan. TCA is known to remove mainly (lipo)teichoic acids from cell walls, which can prevent the C terminus of AcmA from binding to the cell surface (4, 23). To improve the binding capacity of Ly5C, TCA, HCl, HAc, and lactic acid were used to pretreat \textit{L. lactis} and \textit{Lb. casei} cells. Compared with nonpretreated cells of \textit{L. lactis} and \textit{Lb. casei}, TCA-pretreated cells demonstrated a 10-fold increase in fluorescence intensity, indicating that TCA was effective in enhancing Ly5C binding capacity. Moreover, 10% TCA and 5% TCA had the same effect on the binding capacity of GFP-Ly5C, while 0.2% TCA had no impact (Fig. 4A). In addition, fluorescence microscopic observation showed that the 5% TCA treatment left the cellular morphology largely intact and made the binding of GFP-Ly5C occur on the entire cell surface (Fig. 2B).

To identify to which cell wall component Ly5C binds, equal amounts of cells of \textit{L. lactis} and \textit{Lb. casei} were pretreated with SDS or acetone to remove cell wall-associated proteins or lipids, respectively. The results in Fig. 4B showed that GFP-Ly5C could bind to both SDS- and acetone-pretreated cells and that the fluorescence intensity of pretreated cells was improved. These results demonstrated that cell wall-associated proteins or lipids were not the Ly5C binding components. Considering the cell wall components of Gram-positive bacteria, the peptidoglycan was determined to be the binding ligand for Ly5C.

Factors influencing binding of GFP-Ly5C to lactococcus and lactobacillus cells. To enhance the binding capacity of GFP-Ly5C, some factors that influence GFP-Ly5C binding, such as the cell growth phase, pH, NaCl concentration, and binding time, were tested. As shown in Fig. 5A, \textit{L. lactis} and \textit{Lb. casei} cells incubated for 3 h had the highest fluorescence intensity. The fluorescence intensity then decreased slightly with increasing incubation times. To harvest more cells, both \textit{L. lactis} and \textit{Lb. casei} cells incubated for 12 h were collected and used for the subsequent binding experiments. NaCl could significantly enhance the binding capacity, and the optimal concentration was 0.5 M, indicating that ionic interaction by way of charge was the molecular basis for the binding of Ly5C to the cells (Fig. 5B). The cell-associated fluorescence intensity obtained when binding was carried out at pH 11 was increased 2-fold over that obtained at lower pHs (Fig. 5C). In addition, the fluorescence intensity of cells reached a high level when the cells were mixed with GFP-Ly5C for 30 min, and no obvious increase was observed with prolonged exposure to GFP (Fig. 5D). Fluorescence microscopic observation also showed that both \textit{L. lactis} and \textit{Lb. casei} cells exhibited high-intensity green fluorescent decoration when binding was carried out at pH 11 in the presence of 0.5 M NaCl (Fig. 2C and 3). Furthermore, for the nonpretreated cells of \textit{L. lactis} and \textit{Lb. casei}, an 8-fold increase in fluorescence intensity at pH 11 in the presence of NaCl compared to the intensity under different conditions was also achieved (Fig. 4B). All the results suggested that changing the NaCl concentration or pH was an effective method to improve the capacity of GFP-Ly5C binding to LAB cells.

To determine the maximum binding capacity, different amounts of purified GFP-Ly5C fusion protein were mixed with cells of \textit{L. lactis} and \textit{Lb. casei} at an OD of 1 under various conditions. As shown in Fig. 6, cells of \textit{L. lactis} and \textit{Lb. casei} at an OD of 1 (approximately 10<sup>8</sup> CFU/ml) could bind approximately 35 to 40 μg of GFP-Ly5C fusion protein (45 kDa) under the optimal conditions. Thus, each cell could bind approximately 10<sup>6</sup> GFP-Ly5C molecules.

Targeting galactosidase on the surfaces of LAB cells. To confirm the application of the surface display system constructed using Ly5C, β-Gal was fused to Ly5C at its N terminus and \textit{E. coli} Origami B(DE3) was transformed with the recombinant plasmid expressing the fusion protein. As shown in Fig. 7A, the obvious bands of β-Gal (66 kDa) (lane 1) and β-Gal–Ly5C (85 kDa) (lane 2) were observed at the estimated sizes. The clear lysates containing active β-Gal–Ly5C were added to pretreated cells of \textit{L. lactis}, \textit{Lb. brevis}, \textit{Lb. casei}, and \textit{Lb. plantarum} for 30 to 60 min. β-Gal activity on the surfaces of cells was detected, indicating that β-Gal–Ly5C was associated with the surfaces of LAB cells and retained β-Gal activity (Fig. 7B). As a control, no galactosidase activity from the cells incubated with β-Gal or PBS was detected.

DISCUSSION

Cell surface display in LAB has been studied using various anchor proteins. The LysM domain was a new type of anchoring device and offered an alternative approach for cell surface attachment in LAB (5, 26). AcmA, containing three typical
LysM repeats, can bind noncovalently to the peptidoglycan of Gram-positive bacteria, allowing the display of functional proteins or enzymes on the surfaces of genetically unmodified Gram-positive bacteria (1, 2, 18, 25, 28).

In the present study, the C terminus of Lyb5, containing three LysM motifs, has been proven to have the ability to target different heterologous proteins (GFP and β-Gal) onto the surfaces of LAB cells (Fig. 2 and 7), raising the possibility of cell surface display of other proteins (antigens or functional enzymes) by using Ly5C as the anchoring motif. To our knowledge, this is the first report describing the use of the C terminus of an endolysin from a bacteriophage as an anchor for surface display. This strategy has practical applications for the development of oral vaccines, biocatalysts, whole-cell absorbents, and so on. Moreover, cell-free supernatants containing GFP-Ly5C or β-Gal–Ly5C could be directly mixed with LAB cells,
and heterologous proteins were targeted to the non-genetically modified bacteria. Therefore, the protein-displaying cells could be probiotic wild-type strains which lack an efficient genetic manipulation system, and the concentration of protein displayed on the cells can be controlled by mixing different amounts of purified protein with the cells. As shown in Fig. 2 and 7, the GFP or β-Gal fused to Ly5C at its N or C terminus did not influence the cell wall binding capacity of Ly5C and also maintained its activity, suggesting that Ly5C was compatible with the heterologous protein to be fused. Finally, Ly5C had the ability to direct the GFP or β-Gal to the surfaces of a broad range of LAB cells (Fig. 3 and 7), providing the advan-

FIG. 3. Localization of GFP-Ly5C fusion protein on various TCA-pretreated LAB cells at pH 11 in the presence of 0.5 M NaCl. Scale bars, 10 μm.

FIG. 4. Effects of different chemical pretreatments on the capacities of Ly5C to bind to L. lactis and Lb. casei cells. (A) Effects of pretreatment with different acids on Ly5C binding capacity. Bars: A, no treatment; B, 10% TCA; C, 5% TCA; D, 0.2% TCA; E, 0.01 M HCl; F, 5.6 M HAc; and G, 0.72 M lactic acid. (B) Effects of SDS and acetone on Ly5C binding at pH 11 in the presence of 0.5 M NaCl. The data presented are the averages and standard deviations of results from four independent experiments.
tage of being able to use various probiotic LAB strains isolated from different environments as the host strains to display functional proteins.

For the development of a cell surface display system, it is desirable to obtain anchor proteins that are endowed with a high capacity for cell surface binding. Previous research has demonstrated that TCA treatment is an effective strategy to solve the problem of inefficient binding of the cA domain to the cell wall (4, 23). As shown in Fig. 2, more GFP-Ly5C bound to TCA-pretreated cells than to nonpretreated cells and the

![Image](http://aem.asm.org/)

FIG. 5. Binding of GFP-Ly5C to cells of *L. lactis* and *Lb. casei* under different conditions with the variables of growth phase (A), NaCl concentration (B), pH (C), and binding time (D). The data points represent the averages and standard deviations of results from four independent experiments.

![Image](http://aem.asm.org/)

FIG. 6. (Left) Quantificational detection of surface-displayed GFP-Ly5C fusion protein on cells of *L. lactis* and *Lb. casei* under different conditions; (right) standard curve of the fluorescence intensity and the concentration of GFP-Ly5C fusion protein. Different amounts of purified GFP-Ly5C were added to equal amounts of cells for 1 h, and then the cells were collected, washed, and resuspended in 1 ml PBS. Samples of 200 μl were added to the test plate to measure the fluorescence and cell density at 600 nm. The data points represent the averages and standard deviations of results from four independent experiments.

![Image](http://aem.asm.org/)
binding took place on the entirety of the exposed surfaces of \( L.\) lactis and \( Lb.\) casei cells, while GFP-Ly5C was located mainly at separate sites (e.g., at the midcell and each pole) on non-pretreated cells. However, TCA may be less desirable for use in large-scale production and causes bacterial cell death. As shown in our study, the NaCl concentration and pH had a significant impact on binding (Fig. 5). The highest capacity for GFP-Ly5C binding to the TCA-pretreated LAB cells was obtained under optimum conditions (at pH 11 in the presence of 0.5 M NaCl). Surprisingly, an 8-fold increase in fluorescence intensity of the nonpretreated cells was also achieved under these conditions, and the fluorescence of nonpretreated cells reached the same level as that of TCA-pretreated cells (Fig. 4B). Therefore, we offer an efficient approach to improve the binding capacity of Ly5C for living cells, thus making the surface display system more advantageous for vaccine development purposes.

In conclusion, Ly5C was firstly described as an anchor domain to display heterologous proteins on the cell surfaces of LAB. A versatile, inexpensive, and effective surface display system based on Ly5C was constructed and used to functionally display \( \beta-\)Gal on the cell surfaces of LAB. Additionally, the binding capacity of the heterologous protein on the cell surfaces was significantly enhanced by pretreating cells with TCA or changing the NaCl concentration and pH. Altogether, the novel surface display system based on Ly5C should be useful in enzyme immobilization, the delivery of vaccines, the development of whole-cell absorbents, and so on.

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