Chapter 18

Exploring Surface Display Technology for Enhancement of Delivering Viable Lactic Acid Bacteria to Gastrointestinal Tract

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1. Introduction

Anchoring of proteins to the cell surface is a common theme in nature and the processes governed by different surface proteins are bases of many biological phenomena, such as cell-cell recognition, signal transduction, adherence, colonization, and immunoreactions (Westerlund & Korohonen, 1993). The utilization of cellular surface anchoring systems for the display of heterologous proteins on the surface of microbial cells has been developed into an active research area that holds a great promise for a variety of biotechnological applications including the production of whole cell biocatalysts, microbial adsorbents, live vaccines, antibody fragments, and screening of novel proteins (Hansson et al., 2001; Kondo& Ueda, 2004; Lee et al., 2003). Generally construction of these systems is accomplished by the expression of heterologous peptides or proteins as fusions with anchoring domains, which are able to attach to the cell surface (Fig 1.). Anchoring domains are usually cell surface proteins or their fragments. Depending on the characteristics of target and anchor proteins, N-terminal fusion, C-terminal fusion or sandwich fusion strategy can be considered (Lee et al., 2003).

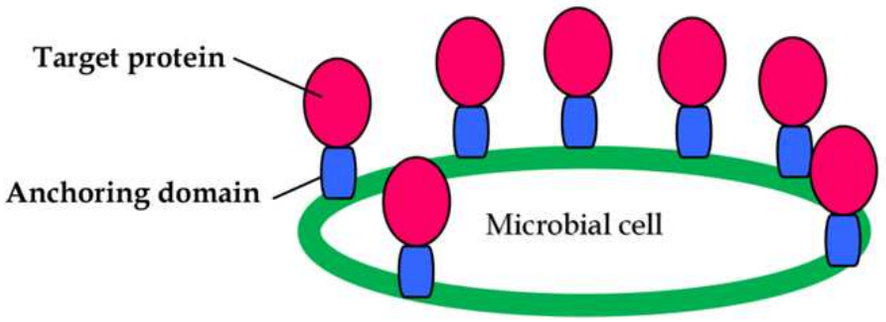

Figure 1. Schematic representation of a microbial surface display system

LAB are gram positive, non-spore forming, fastidious, acid tolerant, and strictly fermentative that secret lactic acid as the major end product of sugar fermentations (Axelsson, 1998). LAB are naturally present in media rich in organic nutrients such as food products and digestive tracts. They are a genetically diverse group of bacteria with GC contents varying from 34 to 53%, including rod shaped bacteria such as lactobacilli and also cocci such as lactococci, enterococci, pediococci, and leuconostoc (Stiles & Holzapfel, 1997). Since time immemorial, LAB have been used for the fermentation and preservation of food products, particularly dairy products, fermented meats, and vegetables. Consequently, several strains of LAB have a long record of safe association with humans and human foodstuffs (Mckay & Baldwin, 1990). The display of proteins on the surface of LAB cells can broaden or improve applications of these bacteria. In this chapter, we intend to describe cell surface anchoring domains used in LAB surface display systems. Then applications of surface engineered LAB are depicted and key factors affecting their performances are highlighted. Moreover, we explained comprehensively a novel application of the protein display in LAB, which is potentially useful for enhancement of the delivery of viable LAB to the gastrointestinal tract (GIT).

2. Anchoring domains in surface display systems of LAB

The cell surface anchoring domains utilized for the development of LAB surface display systems are associated with the cell surface either covalently or noncovalently. Depending on the applied anchoring domains, two modes of the protein display can be considered, including internal and external mode of the protein display. In the case of the internal mode of protein display, fusions of target proteins to anchoring domains are expressed in LAB cells, and therefore target proteins are displayed on the surface of expression hosts, whereas in the case of the external mode of protein display, expression and display hosts are different from each other. If the association of anchoring domains with the cell surface is of a non covalent nature, they can bind to cells when they are added from the outside. Therefore, fusions of target proteins to anchoring domains are produced in suitable expression hosts capable of their correct folding and modifications. The fusion proteins are then purified and incubated with desired display hosts in order to attach to the cell surface. As a result, the external mode of protein display can retain the nongenetically modified status of cells and is valuable for food and vaccine development.

The surface display systems based on the internal mode of protein display are often associated with the limitations in terms of the translocation of target proteins to the cell surface and the control of surface intensity of target proteins. The mislocalization of target proteins can affect their functions negatively(Dieye et al., 2003; Van Der Vaart et al., 1997; Wan et al., 2002). In contrast, the surface display systems based on the external mode of protein display can ensure the full exposure of target proteins outside of the cell wall and the surface intensity of target proteins can readily be adjusted by selecting appropriate display hosts and suitable concentrations for the fusion proteins in the incubation mixture. However, regarding noncovalent interaction of target proteins with the cell surface, the possibility of dissociation of target proteins from the cell surface should be considered.

2.1. Covalent anchors

2.1.1. LPXTG motif containing anchors

The most widely used surface display systems of LAB are based on cell wall anchored proteins that contain an LPXTG motif. These proteins are first synthesized as a preprotein containing an N-terminal signal peptide and a C-terminal cell wall anchor domain. The anchor domain starts at its C-terminus with a short tail of positively charged residues (five to seven amino acids) that remain in the cytoplasm. Upstream of the cytoplasmic domain, a stretch of approximately 30 hydrophobic amino acids is preceded by the highly conserved pentapeptide LPXTG. The charged tail and hydrophobic domain are thought to function as a temporary stop to position the LPXTG motif for proteolytic cleavage. Correct positioning results in cleavage between the threonine and glycine residues followed by amid linkage of the threonine residue to the peptide crossbridge in the peptidoglycan of cell wall, by the action of a sortase (Navarre & Schneewind, 1994). The amino acid composition of the peptide crossbridge varies among the different LAB species and is flexible with respect to the sorting reaction (Strauss et al., 1998; Ton-That et al., 1998). The anchor domain is preceded by a wall associated region of about 50 to 125 residues and is characterized by a high percentage of proline/glycine and/or threonine/serine residues (Fischetti et al., 1990). In surface display genetic constructs, secretion signal peptides are fused to the N-termini of target proteins to transport them to the outside of cell and the LPXTG motif containing anchors are fused to the C-termini of target proteins in order to attach them to the cell surface. The cell surface associated proteinases of *Lactococcus lactis* and *Lactobacilli casei* (PrtPs), M6 protein of *Streptococcus pyogenes*, and Protein A of *Staphylococcus aureus* (SpA) are among LPXTG type anchors, which have mainly been used for the construction of surface display systems in LAB (Maassen et al., 1999; Norton et al., 1996; Piard et al., 1997; Pouwels et al., 1996; Steidler et al., 1998).

Slight deviations from the LPXTG consensus sequence is found in some LAB. Recently Kleerebezem (2003) found that sortase substrates of *Lactobacillus plantarum* contain LPQTXE motifs instead of the LPXTG motifs. In addition, in the carboxy end of cell surface proteinase of *Lactobacillus delbrueckii* (PrtB), a degenerated LPKKT motif is surrounded by two imperfect repeats of 59 residues, which are lysine rich. However, downstream of the LPKKT motif, there is no hydrophobic domain and no charged tail at the extreme C-terminus. It was shown that the C-terminal region of PrtB was able to attach to the cell wall of *L. lactis* and the capacity of attachment was drastically reduced by absence of the duplicated sequences. The high content of total positive charges in the anchoring region of PrtB suggests interactions of the anchor with the negatively charged teichoic acids of the cell wall. The mechanism of PrtB attachment to the cell wall probably implicates electrostatic forces (Germond et al., 2003).

2.1.2. Lipoprotein anchors

Lipoproteins are lipid modified proteins produced as secretory precursors with a signal peptide linked to their amino termini. The C-terminal region of their signal peptide contains

a well conserved lipobox motif of four amino acid residues and invariably, the last residue is cysteine. The covalent binding of lipoproteins to the cell membrane is generally achieved via diacylglyceryl modification of the indispensible cysteine residue in the lipobox by a diacylglyceryl transferase. Lipidation of the cysteine residue is a perquisite for cleavage of the signal peptide by a lipoprotein specific signal peptidase (SPase II)(Yamaguchi et al., 1988; Venema et al., 2003). The lipoprotein anchors should be fused at their C-termini to N-termini of target proteins in order to display them on the cell surface. Poquet (1998) identified four lactococcal lipoprotein anchors using nuclease of *S. aureus* as an export specific reporter enzyme. The nuclease activity was shown to require an extracellular location in *L. Lactis* demonstrating its suitability to report the protein export. The enzyme activity was detected in a plate test by the presence of pink halos. Fusions of the lipoprotein anchors to the nuclease expressed in *L. Lactis* were associated with the cell fraction and the recombinant lactococcal cells showed strong nuclease activities indicating the cell surface anchoring function of the lipoproteins. For one of the anchors (NlpI), the surface location of the fusion protein was also confirmed by proteinase K treatment of *L. lactis* cells (Poquet et al., 1998). Basic membrane protein A (BmpA) of *L. Lactis* is a putative lipoprotein that has been used for the protein display on the surface of lactococci (Berlec et al., 2011).

2.1.3. Transmembrane anchors

The strategy to insert target amino acid sequences in the exterior loop between transmembrane spanning domains (TMSs) can limit the insert size in order not to disturb the membrane protein topology. Therefore, a fusion approach is often preferred in which a target protein is simply linked at its N-terminus to one or more TMSs of a cytoplasmic membrane protein. *L. Lactis* bacteriocin transport accessory protein (LcnD) and *Bacillus subtilis* poly-γ-glutamate synthetase A protein (PgsA) are transmembrane proteins, which were fused to the N-termini of target proteins in order to display them on the cell surface of *L. Lactis* and *L. casei*, respectively (Franke et al., 1996; Narita et al., 2006). In addition, in the same random procedure as described above for the lipoprotein anchors Poquet (1998) identified seven lactococcal gene fragments encoding TMSs that function as membrane anchors in *L. Lactis*.

2.2. Noncovalent anchors

2.2.1. S- layer protein anchors

Some LAB strains possess a surface layer (S-layer) of proteins as the outermost structure of the cell envelope. These S-layers are composed of regularly arranged subunits of a single protein (SLP) and may constitute up to 20% of the total cell protein content. S-layers self assemble in entropy driven process during which multiple noncovalent interactions between individual SLPs and the underlying cell surface take place. These two types of interactions in SLPs can be assigned to two separate domains including the self assembly domain and the cell wall binding domain. These domains have been characterized in SLPs of *Lactobacillus acidophilus* ATCC 4356 (S_A), *Lactobacillus crispatus* JCM 5810 (CbsA), and

Lactobacillus brevis ATCC 8287 (SlpA). The C-terminal regions of SA and CbsA showed the cell surface anchoring function and the N-terminal regions were involved in the self assembly process. In contrast, the self assembly domain of SlpA was located in the C-terminal region and its cell wall binding domain was found in the N-terminal region. The (lipo)teichoic acids were identified as the cell wall ligands of SA and CbsA. Moreover, the specific cell wall component that interacts with SlpA was shown to be the neutral polysaccharide moiety of the cell wall (Antikainen et al., 2002; Avall-Jaaskelainen et al., 2008; Smit et al., 2002). Avall-Jaaskelainen (2002) decribed the construction of recombinant *L. brevis* strains expressing poliovirus epitope VP1 of 10 amino acid residues inserted in the *slpA* gene. Insertion sites in the *slpA* gene were selected on the basis of the hydrophilicity profile of the SlpA protein. The four most hydrophilic parts of the SlpA protein were selected for testing because it was expected that parts of them were likely to be sites where the epitope would be accessible to the cell surface. One of the insertion sites was at the N-terminus of SlpA and the others were at its C-terminus. Only one site at the C-terminus showed strong colour response in whole cell enzyme linked immunosorbent assay (ELISA) using anti epitope antibody demonstrating that the epitope was accessible on the surface of the recombinant *L. brevis*. In another study, the C-terminal region of SLP of *L. crispatus* K2-4-3 (LcsB) isolated from the chicken intestine was used for the construction of surface display systems. Green fluorescent protein (GFP) was fused to the N-terminus of LcsB. The fusion protein (GFP-LcsB) was expressed in *Escherichia coli.* It was then purified and mixed with various LAB. The binding of the fusion protein to LAB cells was viewed by the fluorescence microscopy. GFP-LcsB was associated with the cell surface of various LAB including *L. delbrueckii*, *L. brevis*, *Lactobacillus helveticus*, *Lactobacillus johnsonii*, *L. crispatus*, *Streptococcus thermophilus*, *L. lactis* and *Lactobacillus salivarius*. GFP alone did not bind to the cells. These results indicated that binding of GFP to the surface of LAB cells is directed by LcsB. However, the fusion protein could not attach to the cell surface of *L. casei*. The reason for this observation requires further studies to elucidate the target ligand of LcsB on the cell surface of LAB (Hu et al., 2011).

2.2.2. Lysin motif containing anchors

The lysine motif (LysM) was first discovered in the lysozyme of *Bacillus* phage ø29 as a C-terminal repeat composed of 44 amino acids separated by 7 amino acids. The cell wall attachment of several bacterial proteins in both gram positive and gram negative organisms occurs through LysMs, often repeated several times in the protein sequence. Many LysM containing proteins are cell wall hydrolases (Buist et al., 2008). The C-terminal region of peptidoglycan hydrolase (AcmA) of *L. lactis* MG 1363 (CpH) contains three 44 amino acid residue lysMs separated by stretches of 21 to 31 amino acids rich in the serine, threonine, and asparagine residues. CpH is able to bind to the cell surface of lactococci and several strains of lactobacilli. Moreover, CpH is able to bind both to the cell surface of LAB treated with sodium dodecyl sulfate (SDS) to remove cell wall associated proteins and LAB treated with trichloroacetic acid (TCA) to remove carbohydrates and (lipo) teichoic acids. These findings suggest that peptidoglycan is the binding ligand of the CpH domain (Buist et al.,

1995). The C-terminus of endolysin Lyb5 of *Lactobacillus fermentum* bacteriophage øPYB5 (Ly5C) contains three LysMs. Each of LysMs is composed of 41 amino acids and they are separated by intervening sequences varying in length and composition. Ly5C fused to GFP was expressed in *E. coli*. After mixing the fusion protein with various cells *in vitro*, GFP was successfully displayed on the surfaces of *L. lactis, L. casei*, *L. brevis*, *L. plantarum*, *L. fermentum*, *L. delbrueckii*, *L. helveticus*, and *S. thermophilus* cells. Increases in the fluorescence intensities of TCA treated *L. lactis* and *L. casei* cells compared to those of nontreated cells showed that the cell wall peptidoglycan was the cell surface binding target of Ly5C. Concentration of sodium chloride and pH influenced the binding capacity of the fusion protein, and optimal conditions of these factors were determined empirically in order to obtain high fluorescence intensities of *L. lactis* and *L. casei* cells (Hu et al., 2010). N-terminus of putative muropeptidase (MurO) of *L. plantarum* also contains two LysMs composed of about 43 amino acids separated by 22 amino acid residue sequences. The LysM domain fused to GFP was expressed in *E. coli* and it was able to bind to the cell surface of *L. plantarum* after being mixed with the cells (Xu et al., 2011). Examination of supernatant fractions from broth cultures of *L. fermentum*, revealed the presence of a 27-kDa protein termed Sep. The N-terminus of Sep contains a LysM. Sep fused N-terminally to a six histidine epitope was expressed in *L. fermentum*, *Lactobacillus rhamnosus,* and *L. lactis*. The protein was found associated with the surface of the expression hosts. However, it was largely present in the supernatant of the cell cultures (Turner et al., 2004).

2.2.3. WxL anchors

The C-terminal cell wall binding domain designated WxL was first identified in proteins of *Lactobacillus* and other LAB based on *in silico* analysis (Kleerebezem et al., 2010). WxL domain contains a WxL motif followed by a proximal well conserved YXXX(L/I/V)TWXLXXXP motif. This domain was found in gene clusters that also encode additional extracellular proteins with C-terminal membrane anchors and LPxTG motif containing anchors, suggesting that they form an extracellular protein complex (Siezen et al., 2006). The C-terminal WxL domains identified in two proteins of *Enterococcus faecalis* were fused at their N-termini to an export reporter enzyme (nuclease of *S. aureus)* and a secretion signal peptide. The fusion proteins expressed in *E. faecalis* were detected in both cell wall and supernatant fractions of the recombinant enterococci. Removal of the WxL domains from the fusion proteins nearly eliminated them in the cell wall. Treatment of the cell wall fractions with SDS disrupted binding of the fusion proteins to these fractions. These results indicated that the fusion proteins had noncovalent interactions with the cell wall of *E. faecalis*. The fusion proteins were able to attach to the cell surface of *E. faecalis* and *L. johnsonii* when they were added exogenously (Brinster et al., 2007).

2.2.4. Other anchors

Basic surface protein A (BspA) is a surface located protein of *L. fermentum* BR11. Sequence comparisons have been shown that BspA is a member of family III of the solute binding

proteins. Most solute binding proteins are lipoproteins. However, BspA is not a lipoprotein and is attached to the cell envelope by electrostatic interactions. It has been used as a fusion partner to direct proteins to the cell surface of *L. fermentum* BR11. In these constructs, BspA was fused at its C-terminus to target proteins and the fusion proteins were expressed in *L. fermentum* BR11 (Turner & Giffard, 1999). The C-terminal region of cell associated dextransucrase of *Leuconostoc mesentroides* IBT-PQ (DsrP) contains five repeats of 65 amino acid residues. The domain expressed in *E. coli* was able to bind to the cell surface of *L. mesentroides* IBT-PQ cells after being mixed with the cells (Olvera et al., 2007). The carboxy end of PrtP of *Lactobacillus acidophilus* was used for the protein display on *L. acidophilus* using the internal mode. The association of this domain with the cell surface was mediated by electrostatic interactions (Kajikawa et al., 2011).

3. Applications of surface engineering of LAB

Research in the field of surface engineering of LAB has mainly been focused on the construction of vaccine delivery vehicles but other interesting applications have also been reported. In this section, we will describe different areas of biotechnology in which surface display of heterologous proteins on LAB have been investigated.

3.1. Development of vaccine delivery vehicles

Vaccination represents one of the most effective public health strategies to combat infectious diseases (Mielcarek et al., 2001). One of the technologies being developed for vaccine production is the use of bacteria as live vectors for the delivery of recombinant vaccine antigens to the immune system. Such vaccines have the potential for the production of protective antigens *in vivo* and are inexpensive to manufacture (Moore et al., 2001).

Most infections affect or initiate infectious processes at mucosal surfaces and mucosal local immune responses can block pathogens at the portal of entry. Live bacterial vaccines can induce mucosal, as well as systemic, immune responses when delivered via mucosal routes, such as oral or intranasal administration (Mielcarek et al., 2001). The mucosal, needle free, administration of vaccines can significantly decrease the need for syringes with their inherent added cost and risk of disease transmission, and it can increase compliance, and consequently the coverage of vaccination programs (Giudice & Campbell, 2006). The first live recombinant bacterial vectors developed were derived from attenuated pathogenic microorganisms. In addition to the difficulties often encountered in the construction of stable attenuated mutants of pathogenic organisms, attenuated pathogens may retain a residual virulence level that renders them unsuitable for the vaccination of partially immunocompetent individuals such as infants, the elderly or immunocompromised patients (Curtiss, 2002). These problems can be addressed by the application of nonpathogenic food grade LAB as antigen delivery vehicles. LAB therefore represent attractive alternatives as antigen carriers and their use has mainly been focused on the construction of mucosal vaccines. The cellular location of antigens can influence the elicited immunological responses. Cell surface anchored antigens are better recognized by the immune system than

those produced intracellularly. Furthermore, intracellular production of antigens may limit their *in vivo* release. The vaccines constructed by the cell surface anchoring of antigens are of particulate nature. In contrast to most soluble antigens, which are ignored by the immune systems, particles are recognized as foreign and as danger eliciting effective immune responses (Storni et al., 2005). Tetanus toxin fragment C (TTFC) is an immunogen protective against tetanus. In a pioneer study by Norton (1996) three recombinant strains of *L. lactis* expressing TTFC in three cellular locations, intracellular, secreted or cell surface anchored via lactococcal PrtP were constructed. The recombinant lactococcal cells were used to immunize mice, which were then challenged by the subcutaneous inoculation of tetanus toxin. When compared in terms of the dose of expressed TTFC required to elicit protection against the lethal challenge, the cell surface displayed form of TTFC was significantly (10-20 fold) more immunogenic than the alternative forms of the protein. The result of this study indicated the advantage of antigen display on the cell surface for the construction of the lactococcal vaccines. In addition to TTFC, several other antigens were displayed on the surface of LAB and the protection studies were carried out to evaluate the efficiency of these vaccines (Bermudez-Humaran et al., 2005; Hou et al., 2007; Kajikawa et al., 2007; Lee & Faubert, 2006; Lee et al., 2006; Li etal., 2010; Lindholm et al., 2004; Liu et al., 2009; Medina et al., 2008; Poo et al., 2006; Tang & Li, 2009; Wei et al., 2010; Xin et al., 2003).

F18 fimbrial *E. coli* strains are associated with porcine postweaning diarrhea and pig edema disease. Adherence of F18 fimbrial *E. coli* to porcine intestinal epithelial cells is mediated by the FedF adhesin of F18 fimbriae. For the development of a mucosal vaccine against porcine postweaning diarrhea and edema disease, different expression cassettes for the display of FedF on the cell surface of *L. lactis* were constructed. Preliminary attempts to express the entire FedF protein as a fusion protein in *L. lactis* resulted in inefficient secretion and degradation of the adhesin. Therefore, only those regions of FedF required for binding specificity to porcine intestinal epithelial cells, were used in the construction of cell surface display systems. Initially, recombinant *L. lactis* clones secreting the partially overlapping receptor binding domains of FedF (42 and 62 amino acid residues) were prepared using two different signal peptides. Substantially higher levels of the fusion proteins (four- to six-fold) were secreted by the clones possessing *L. brevis* SlpA signal peptide than by those possessing *L. lactis* Usp45 signal peptide. In order to enhance the secretion of the fusion proteins, a synthetic sequence encoding the propeptide LEISSTCDA was inserted between the signal sequences and the receptor binding domains of FedF. For the construction of surface display systems, the secreted proteins were anchored to the cell wall of *L. lactis* via the CpH protein or the lactococcal PrtP protein. Three groups of expression vectors with *prtP* spacer sequences of 0.6, 0.8 and 1.5 kb were also designed. The spacers inserted between the receptor binding domains and the anchors. Whole cell ELISA for the detection of cell surface exposure of the FedF receptor binding regions showed that the CpH anchor performed significantly better than the PrtP anchor, particularly in a *L. lactis* mutant devoid of the extracellular housekeeping protease, HtrA. Among the cell surface display systems possessing the CpH anchor, only those with the longest PrtP spacer resulted in efficient binding of the recombinant *L. lactis* cells to porcine intestinal epithelial cells (Lindholm et al., 2004).

In another study, pneumococcal surface antigen A (PsaA), a conserved membrane anchored virulence factor, was expressed in different strains of LAB and it was associated with the surface of LAB cells. *L. plantarum* and *L. helveticus* were found to be more effective at inducing mucosal and systemic anti-PsaA immune responses than *L. casei* following intranasal vaccination of mice. Because all three *Lactobacillus* strains expressed almost the same amount of PsaA and were also recovered from mice nasal mucosa in the same period (3 days), the observed differences among their respective antibody responses may reflect their different intrinsic adjuvant properties. PsaA expressed by *L. lactis* at 2×10^{-8} ng/ colony forming unit (CFU), which was about 10% of PsaA amount produced by the *Lactobacillus* strains. The recombinant *L. lactis* remained in the nasal mucosa only 1 day after the inoculation. Therefore, the inability of *L. lactis* expressing PsaA to significantly induce serum IgG or secreted IgA in mice can be explained by the low level of antigen production in this bacterium compared with the *Lactobacillus* strains and also its shorter persistence in the nasal mucosa. Intranasal inoculation of the mice with *L. lactis* expressing PsaA did not exert any effect on *Streptococcus pneumoniae* recovery from the nasal mucosa upon colonization challenge, in comparison with inoculation of saline or the control *L. lactis* carrying the expression vector devoid of the antigen gene. On the other hand, all the recombinant *Lactobacillus* strains showed a significant reduction of *S. pneumoniae* colonization when compared with the saline group ($10^{0.6}$-$10^{1.35}$ CFU). However, only *L. helveticus* expressing PsaA showed a significant reduction of *S. pneumoniae* colonization in relation to control *L. helveticus* (10 CFU) (Oliviera et al., 2006). Among LAB, *L. lactis* and *Lactobacillus* strains have mostly been used for the construction of LAB vaccines. Selection of LAB strains for use as antigen carriers depends on their persistence in the host, capacity to express foreign antigens, and intrinsic adjuvanticity. *L. lactis* does not colonize the internal cavities of man or animals. Therefore, the use of lactobacilli, which are able to colonize the cavities such as the GIT transiently seems more advantages than that of *L. lactis* for developing LAB vaccines because the longer persistence of lactobacilli in the host body may enhance immunological responses. On the other hand, the progress in the genetics of lactobacilli is more recent than that of lactococci. Furthermore, the availability of a commercial powerful gene expression system for *L. lactis*, nisin inducible gene expression system, urged many researchers to establish LAB vaccines based on lactococci. It has been reported that several LAB strains particularly strains from the genera *Lactobacillus* are able to act as adjuvants. This aspect should be considered when selecting a vaccine strain as it is a natural way to potentiate the immune reaction against heterologous antigens produced by recombinant LAB. It might be speculated that a high level of the antigen expression will not be necessary when using immunostimulatory LAB strains. However, studies have not yet been reported for comparison of the adjuvanticity of *L. lactis* and different lactobacilli. *L. casei* and *L. plantarum* are among *Lactobacillus* strains, which can colonize the GIT of human and mice and they show immunostimulatory properties (Pouwels et al., 1998; Wells et al., 1996).

It has been reported that the flagellin of *Salmonella* has significant vaccine potential because it is the only surface antigen of *Salmonella* detected to have a mitogenic stimulatory effect on lymphocytes (Toyota-Hantani et al., 2008). Bacterial flagellins can also induce innate

immune responses through their interaction with Toll-like receptor 5 (TLR5) (Ramos et al., 2004). Recombinant *L. casei* cells expressing the flagellin of *Salmonella* Enteritidis (LCF) on their cell surface via *L. casei* PrtP anchor were constructed. Intragastric immunization of mice with the recombinant lactobacilli resulted in a significant level of protective immunity against an oral challenge with *S.* Enteritidis. There was no significant difference in the level of protection after immunization with the recombinant lactobacilli compared with the free flagellin isolated from *S. Enteritidis*, although the amount of flagellin carried by LCF was less than that of the free flagellin. The immunization of mice with the recombinant lactobacilli did not result in antigen-specific antibody responses in either feces or sera but did induce the release of interferon (IFN)-γ on restimulation of primed lymphocytes *ex vivo*. These results suggested that the protective efficacy provided by flagellin expressing *L. casei* was mainly attributable to cell mediated immune responses. When the levels of IFN-γ produced by primed and flagellin restimulated lymphocytes were compared between the recombinant *L. casei* cells expressing flagellin on their cell surface, and a mixture of the purified flagellin and normal *L. casei*, the results indicated that the *Lactobacillus* strain showed adjuvanticity only when the flagellin was expressed on the cell surface (Kajikawa et al., 2007).

Two recombinant *L. acidophilus* strains displaying flagellin of *Salmonella typhimurium* on the cell surface were constructed using different anchor motifs. In one construct, the flagellin gene was fused at its carboxy end to the C-terminal region of PrtP of *L. acidophilus*. In other construct, the flagellin gene was fused in the same way to the anchor region of mucus binding protein (Mub) of *L. acidophilus* containing an LPXTG motif. The density of the flagellin fused protein at the cell surface of *L. acidophilus* displaying the flagellin by the PrtP protein (FliC-PrtP) was higher than that of *L. acidophilus* displaying the antigen by the Mub anchor (FliC-Mub). Both of the recombinant lactobacilli showed TLR5 stimulating activity, which indicated that the surface associated flagellin was recognized by TLR5. The magnitude of the TLR5 stimulating activity of *L. acidophilus* cells expressing FliC-PrtP was higher than that of *L. acidophilus* cells expressing FliC-Mub and this result showed that the magnitude of the TLR5 stimulating activity was dependent on the quantity of surface located flagellin. Moreover, the two recombinant lactobacilli exhibited dissimilar maturation and cytokine production by human myeloid dendritic cells (Kajikawa et al., 2011).

Human papillomavirus type 16 (HPV-16) has been associated with more than 50% of HPV related cervical cancer (CxCa) (Krinbauer et al., 1992). HPV-16 E7 oncoprotein is constitutively expressed in CxCa cells during malignant progression of HPV-16 induced cervical lesions and is therefore considered as an effective target for the cancer immunotherapy. *L. lactis* was engineered to express HPV-16 E7 on the cell surface (LL-E7) using the M6 anchor and its coadministration with another lactococci secreting IL-12 (LL-IL-12) was investigated for the immunization and immunotherapy of HPV-related CxCa. IL-12 is a heterodimeric cytokine that induces Th1 responses, enhances cytotoxic T-lymphocyte (CTL) maturation, promotes natural killer (NK) cell activity and induces IFN-γ production. Mice were vaccinated intranasally with LL-E7 and were then challenged by injection of tissue culture number 1 (TC-1) tumor cells. Thirty five percent of the vaccinated mice remained tumor free. Coadministration of LL-IL-12 with LL-E7 resulted in higher antitumor

activities as half of the inoculated mice remained tumor free and the tumor median size in the remaining tumor bearing animals was less than that in LL-E7 immunized mice. Antitumoral activity elicited by covaccination with LL-E7 and LL-IL-12 appeared to be long lasting as when the tumor free animals were rechallenged 3 months later with TC-1 cells, they remained tumor free for up to 6 months. To investigate the therapeutic effects of the coadministration of LL-E7 and LL-IL-12, mice were challenged with the TC-1 tumor cell line prior to the initiation of immunotherapy. Once 100% of the mice had palpable tumor, the immunotherapy was started. Only LL-E7/LL-IL-12 treatment resulted in total tumor regression in 35% of the immunized animals. Moreover, the tumor median size in the remaining tumor bearing mice was lower than that measured in mice treated with LL-E7. In contrast, no tumor regression was observed in mice treated with LL-E7 alone. Mice immunized with LL-E7/LL-IL-12 also exhibited both systemic and mucosal humoral responses, which were induced at higher levels than those in mice vaccinated with LL-E7 (Bermudez-Humaran et al., 2005).

Boiling of *L. lactis* cells in TCA followed by washing and neutralization resulted in nonviable spherical peptidoglycan microparticles , which are deprived of surface proteins and their intracellular content is largely degraded. The proteins IgA1 protease (IgA1p), putative proteinase maturation protein A (PpmA) and streptococcal lipoprotein A (SlrA) were bound to the surface of the lactococcal particles after recombinant production of the antigens as hybrids with the CpH domain. TCA removes (lipo) teichoic acids from the lactococcal cell wall that results in enhancement of the binding capacity for CpH fusions. Mice immunized intranasally with the monovalent lactococcal particle based vaccines were not protected against an intranasal pnemococcal challenge. However, intranasal immunization with a trivalent vaccine containing PpmA, SlrA and IgA1p bound to the lactococcal particles by the CpH domain showed protection against fatal pneumococcal pneumonia in mice (Audouy et al., 2007).

3.2. Development of whole cell biocatalysts

Cellulosome is a multi enzyme complex in which various cellulytic enzymes assemble into a macromolecular structure by their attachment to a nonenzymatic central scaffold protein for the efficient degradation of cellulose (Bayer et al., 2004; Demain et al., 2005). Cellulosome of the gram positive thermophile *Clostridium thermocellum* is anchored to the surface of cells, resulting in one of the most efficient bacterial systems for the cellulose hydrolysis. All of the cellulosomal enzymes of *C. thermocellum* contain a twice repeated sequence, usually at their C-termini, called type I dockerin domain. These dockerin domains are considered to bind to the hydrophobic domains termed cohesins, which are repeated nine times in the central scaffold protein (CipA) of the bacterium. CipA also contains a cellulose binding module (CBM3a), allowing the different cellulases to act in synergy on the crystalline substrate, as well as a type 2 dockern domain, which binds the cell surface anchor proteins, ensuring the cellulosome's attachment to the cell surface. Association of the cellulosome with the cell surface yields formation of cellulose-enzyme-microbe ternary complexes and results in enhanced activity and synergy (Begum et al., 1996). The assembly of recombinant

cellulosome inspired complexes on the cell surface of surrogate hosts such as LAB is highly desirable. LAB can produce commodity chemicals such as lactic acid and bioactive compounds (De Vuyst & Leory, 2007; Hofvendahl & Han-Hagerald, 2007; Siragusa et al., 2007). The economics of these processes would be greatly improved if LAB could utilize cellulosic substrates, which are cheap and abundant. While most of LAB can not assimilate cellulose, by the display of cellulosome on the surface of these bacteria, the hydrolysis of cellulosic substrates and the fermentation of hydrolysis products to desirable compounds can be carried out in a single step process, which has economical advantages. As a key step in the development of these recombinant LAB, fragments of CipA were functionally expressed on the cell surface of *L. lactis*. The fragments engineered to contain a single cohesin module, two cohesin modules, one cohesin and CBM3a, or only CBM3a. Cell toxicity from over expression of the proteins was circumvented by use of the nisin A (nisA) inducible promoter. Incorporation of the C-terminal anchor motif of the streptococcal M6 protein in the expression cassette resulted in the successful surface display of the fragments. All of the constructs containing cohesin modules were able to bind to an engineered hybrid reporter enzyme, *E. coli* ß-glucuronidase fused to the type I dockerin domain of a cellusomal enzyme. These results demonstrated that the cohesins were displayed on the cell surface of *L. lactis* cells in the functional form. In addition, the cell surface complex formation was dependent on the presence of both cohesin and dockerin modules (Wieczorek & Martin, 2010).

The process for the conversion of starch to lactic acid by LAB includes the enzymatic hydrolysis of starch followed by the fermentation of resultant oligosaccharides to lactic acid by LAB. These steps can be carried out simultaneously by α-amylase displaying LAB. Therefore, using these whole cell biocatalysts can result in economical benefits for the conversion of starch to lactic acid. The PgsA anchor protein was fused to the N-terminus of α-amylase of *Streptococcus bovis* 148 (AmY). The resulting fusion protein expressed in *L. casei* and it was associated with the membrane and cell wall fractions. However, the status of exposure of AmY outside of the cell wall was not clarified. The constructed whole cell biocatalyst was able to convert starch to lactic acid. Because the lactic acid concentration increased as the total sugar concentration decreased, it was concluded that the lactic acid was produced by simultaneous saccharification and fermentation of starch. The yield of lactic acid was improved by repeated utilizations of the recombinant *L. casei* cells (Narita et al., 2006).

The C-terminal region of peptidoglycan hydrolase (AcmA) of *L. lactis* IL 1403 (CphI) is a homolog of CpH of *L. lactis* MG 1363 and it contains three LysMs. CphI can bind to the surface of various LAB (Tarahomjoo et al., 2008a). We studied the capability of CphI for the development of α-amylase displaying LAB using the external mode of protein display. These whole cell biocatalysts are expected to be effective for the direct fermentation of starch to lactic acid. Starch is a large substrate that is not capable of penetrating the cell wall. For this reason, in order to achieve its efficient hydrolysis, the enzyme must be exposed on the outside of the cell wall such that it is accessible to starch. The display systems based on the internal mode of protein anchoring often have limited ability for the translocation of target

proteins to the cell surface. In contrast, the display systems based on the external mode of protein anchoring can display the enzyme completely outside of the cell wall. Therefore, a whole cell biocatalyst constructed using the externally added cell surface adhesive α-amylase is considered as a suitable selection for our purpose. Moreover, the cell surface adhesive α-amylase can readily be recovered together with LAB cells at the end of starch conversion process for reuse. CphI fused to AmY either at its C-terminus (CphI-AmY) or at its N-terminus (AmY-CphI) was expressed in *E. coli*. Both of the fusion proteins were able to bind to the cell surface of *L. lactis* ATCC 19435. Therefore, CphI is considered as a bidirectional anchor protein. However, the number of bound molecules per cell in the case of CphI-AmY was 3 times greater than that in the case of AmY-CphI. The change in the fusion direction may cause conformational alterations in the fusion protein leading to a better accessibility of CphI for the cell surface binding and an increase in the number of bound molecules. Moreover, the specific activity for starch digestion of CphI-AmY was 11 fold higher than that of AmY-CphI. The starch binding domain of AmY is located at its C-terminus. As a result, the fusion of CphI to the N-terminus of AmY may help improve the adsorption of starch onto the enzyme and enhance starch degradation, resulting in a higher specific activity for starch digestion. In addition to *L. lactis* ATCC 19435, *L. plantarum* NRRL B531, *L. lactis* IL1403, *L. casei* NRRL B441, *L. delbrueckii* ATCC 9649 and *L. casei* NRRL B445 were examined in terms of the binding of CphI-AmY. Of the LAB tested, *L. lactis* ATCC 19435 showed the highest binding capability for CphI-AmY, up to 6×10^4 molecules per cell. The binding of CphI-AmY to *L. delbrueckii* ATCC 9649 cells was very stable and its dissociation rate constant at 37°C was 7×10^{-6} s^{-1} (the half life of binding ($t_{1/2}$) was 28 h). The binding of this protein to cells of *L. lactis* ATCC 19435 was also stable, with a dissociation rate constant of 5×10^{-5} s^{-1} at 30°C ($t_{1/2}$=4 h). Lactate production by lactic acid bacteria is maximal during the exponential growth phase. Therefore, for a successful application of the constructed whole cell biocatalysts in lactate production, suitable fermentation conditions should be specified to adjust the duration of the exponential growth phase with respect to the dissociation rate of the protein. These half lives are long enough for lactic acid fermentation when the inoculm size is adequet and/or suitable growth conditions with high specific growth rates are used (Tarahomjoo et al., 2008a). A CphI mutant devoid of its N-glycosylation sites was expressed extracellularly in *Pichia pastoris*. This domain was able to bind to the cell surface of *L. casei*. However, its dissociation rate constant from the cell surface was 3.5 fold lower than that of CphI. These results indicated that the protein engineering approaches can be useful for increasing the binding stability of noncovalent anchors (Tarahomjoo et al. 2008b).

3.3. Attachment of bacteria to host tissues

Display of adhesins capable of binding to a host tissue on the surface of LAB can provide them with a specific adhesion capability, which can be beneficial when LAB are used as mucosal delivery vehicles of bioactive compounds. Because the displayed adhesins can increase the persistence of bacteria in the host tissue and as a result, the desired effects of the

delivered bioactive compounds can be enhanced. The N-terminal region of SlpA was recently shown to mediate adhesion to human intestinal cell lines *in vitro*. The SlpA adhesion mediating domain fused to the N-terminus of lactococcal CpH protein was expressed in *L. lactis*. To increase the surface accessibility of the hybrid protein, a part of PrtP of *L. lactis* subsp. *cremoris* Wg2 was used as a spacer protein, which could extend the SlpA receptor binding region out of the cell surface. The spacer was inserted between the receptor binding domain and the anchor protein. *In vitro* adhesion assay with the human intestinal epithelial cell line Intestine 407 indicated that the recombinant lactococcal cells had gained an ability to adhere to Intestine 407 cells significantly greater than that of parental nonrecombinant *L. lactis* cells (Avall-Jaaskelainen et al., 2003).

It has been reported that *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) exerts antitumor effects against superficial bladder cancer. These antitumor effects are due to the activation of immune responses, which are mediated by the attachment of BCG to the bladder wall through fibronectin. In addition, *L. casei* strain Shirota showed an antitumor activity when it was administered to the mouse model of superficial bladder cancer. However, the *L. casei* cells exhibits no significant binding to fibronectin. Therefore, higher antitumor activity could be expected for the *L. casei* cells genetically engineered to acquire binding capacity to fibronectin. For this purpose, fibronectin binding domain (FbD) of *S. pyogenes* ATCC 21059 fused to the C-terminal region of PrtP 763 of *L. lactis* NCDO 763 was expressed in *L. casei* strain Shirota. The recombinant *L. casei* cells were able to bind to fibronectin. Furthurmore, FbD expressed on the *L. casei* cell surface promoted the adherence to murine fibroblast STO cells, which secret fibronectin in large amounts (Kushiro et al., 2001).

3.4. Enhancement of delivering viable bacteria to gastrointestinal tract

Probiotics are live microbial food supplements, which benefit the health of consumers by improving their intestinal microbial balance (Fuller, 1989). Since the viability and activity of a probiotic is essential at the site of action, it must survive the harsh environment of the upper GIT, and it must be able to function in the gut environment (Collins et al., 1998). Most commonly used probiotics are lactobacilli and bifidobacteria (Daly & Davis, 1998). However, several studies indicate that most of these bacteria may not be able to withstand the harsh acidity of the upper GIT (Conway et al., 1987; Lankaputhra & Shah, 1995). In a study by Wang (1999), the enhancement of survival of bifidobacteria grown in the presence of starch granules and mixed with them was reported. However, the exact mechanism underlying the protective effect of starch was not clarified. The adhesion of bacteria to starch was considered as a possible explanation for these observations. The results of their study suggested that starch granules can be used to protect living microbes from environmental stress factors.

Microencapsulation is an approach that has been proposed to protect probiotics from environmental stresses. It segregates cells from adverse environments; thus, it minimizes cell injury (Anal & Singh, 2007). Myllaerinen (1999) has recently developed a

microencapsulation technology that involves entrapping bacteria in the hollow cores of partially hydrolyzed starch granules, which are then encapsulated in an outer coating of amylose. The aim of this technology is to protect the probiotic bacteria from adverse environmental conditions during processing, in products during storage, and during passage through the GIT, and it is based on the finding that starch granules can be used to protect living microbes from adverse environments.

We therefore aimed to investigate whether the conferment of starch adhesion ability to cells and using this characteristic to encapsulate the cells between starch granules can enhance their viability in simulated gastric conditions. However, using genetic engineering techniques to confer starch binding ability to probiotics is not favorable because of consumers' concerns about genetically modified food ingredients. CphI of *Lactococcus lactis* IL1403 is an efficient anchoring domain for the display of heterologous proteins on LAB cells, which can bind to the cell surface when added from the outside (Tarahomjoo et al., 2008a). This domain can be used to confer starch binding ability to LAB without making any genetic modifications in them. We therefore studied the capability of the CphI anchor to direct the display of a starch binding domain (SbD) on the surface of *L. casei* cells and the aggregation of cells with starch was examined as an alternative technique of microencapsulation. This is the only available report demonstrating the potential applicability of the cell surface display technology for increasing the delivery of viable microorganisms to the GIT.

3.4.1. Materials and methods

3.4.1.1. Bacterial strains and growth conditions

E. coli XL1-Blue was used for the construction of vectors and the expression of heterologous proteins. It was grown in Luria-Bertani (LB) liquid medium or on LB agar plates at 37°C. *L. casei* subsp. *casei* NRRL B-441 (Agriculture Research Service Culture Collection, Peoria, IL, USA) was used for the binding assay, and it was grown at 37°C in MRS broth (Difco Laboratories, Detroit, MI, USA).

3.4.1.2. DNA manipulation

The gene encoding the linker region and the first nine amino acid residues of SbD of the AmY was prepared by PCR from pQE31amyA (Shigechi et al., 2004) with 5′-aaggatccgggccaagctagccaagcagctc-3′ and 5′-gcgccaattatctgggttttgg-3′ as forward and reverse primers, respectively. The amplified fragment was digested with *Bam*HI and *Bst*XI and inserted at the same restriction sites into pQCA (Tarahomjoo et al., 2008a). The obtained plasmid was designated as pQCLS, in which the gene encoding CphI was fused at its C-terminus to the gene encoding the linker region and SbD of AmY. The correctness of the construct was confirmed by restriction digestion and sequencing.

3.4.1.3. Expression studies

E. coli cells harboring the desired plasmids were grown overnight at 37°C in LB broth supplemented with 100 μg/ml ampicillin and 15 μg/ml tetracycline. The cells were then

harvested by centrifugation and transferred to fresh LB broth containing the antibiotics mentioned above, and incubated at 37°C until the OD_{600} reached 0.5. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce the expression of the target protein. At the same time, ampicillin was added to a final concentration of 400 μg/ml for plasmid maintenance. After further incubation for 4 h, the cells were collected and the expression was studied by resolving the whole-cell extracts by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining the gel using Coomasie Brilliant Blue R250 (CBB).

3.4.1.4. Purification of fusion protein

Proteins were purified under native conditions by metal affinity chromatography, utilizing the interaction between the histidine tag and a nickel chelate column (Ni-NTA superflow column [1.5 ml]; Qiagen GmbH, Hilden, Germany). The induced cells from a 100-ml culture were harvested by centrifugation and were resuspended in binding buffer (50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, 10 mM imidazole). Lysozyme was added to a final concentration of 1 mg/ml and the cell suspension was incubated for 1 h on ice. The cells were disrupted by sonication and the clear supernatant obtained by centrifugation was applied to the Ni-NTA column equilibrated with the binding buffer. The column was washed three times with the same buffer containing 20 mM imidazole and the bound proteins were eluted with the elution buffer, which was the same as the binding buffer except that it contained 250 mM imidazole. The buffer of the eluent was then exchanged to 20 mM Tris-Cl buffer (pH 8.0) by ultrafiltration. The protein preparation was applied to an anion exchange column (SuperQ-5PW, , Tosoh, Tokyo, Japan) equilibrated with 20 mM Tris-Cl buffer (pH 8.0). The absorbed proteins were eluted by a linear NaCl gradient (0–1M). Protein elution was monitored using a UV detector, and the desired fraction was collected and desalted by ultrafiltration. Purified proteins were subjected to 12.5% SDS-PAGE and the bands were visualized by CBB staining. Gels were scanned using a GT-F600 scanner (Epson, Suwa, Japan) and densitometrical analysis was performed with Scion image software (Scion, MD, USA) to quantify the proteins.

3.4.1.5. Cell surface binding assay

L. casei cells were grown as mentioned above until an OD_{660} of 1 was achieved. The cells from a 1.5 ml culture were dispersed in 0.15 ml of de-Man Rogosa Sharp (MRS) medium containing the purified fusion protein at 0.12 mg/ml and incubated at 30ºC for 2 h with gentle shaking. After washing the cells twice with 0.1 M phosphate buffer (PB) (pH 7.0), the cell pellets were resuspended in 2×SDS-PAGE loading buffer containing 20% (w/v) glycerol, 125 mM Tris-HCl (pH 6.8), 4% SDS, 5% (v/v) β-mercaptoethanol, and 0.01% bromophenol blue, and boiled for 5 min. Binding of the protein to the cells was studied by 12.5% SDS-PAGE followed by CBB staining and the amount of the fusion protein bound to the cells was determined by densitometrical analysis of CBB stained gels as mentioned above.

3.4.1.6. Starch binding assay

The fusion protein (0.06 mg/ml in PB) was mixed with an equal volume of a suspension of starch granules (Corn starch, Sigma-Aldrich Tokyo, Japan) (10 mg/ml) in the same buffer

and incubated at 37°C for 2 h with gentle shaking. After centrifugation, the supernatant was examined for the presence of unbound proteins by 12.5% SDS-PAGE and CBB staining.

3.4.1.7. Aggregation of bacteria with starch and microencapsulation

After performing the cell surface binding assay as described above, the cells were washed and resuspended in PB to a final density of 10^9 cells/ml. The suspension of the starch granules in PB was mixed with an equal volume of the cell suspension for 30 min. The mixture was then allowed to stand at room temperature for 1 h. The formation of aggregates was studied both visually and with phase contrast microscopy. To determine the percentage of cells adhering to starch, a 0.5 ml sample was taken from below the liquid surface after the sedimentation. Optical density at 540 nm was measured and compared with those of controls including bacteria without starch and starch without bacteria to calculate the starch adhesion percentage as described by Crittenden (2001). For coating of the aggregates with amylose, a 1% solution of amylose in water (amylose from potato, Sigma –Aldrich, Tokyo, Japan) was prepared by heating it to a temperature of 170°C in a pressure heater (Taiatsu Techno, Tokyo, Japan), which was then cooled down to about 37°C. The aggregates were mixed gently with 0.5 ml of the amylose solution and the coating was allowed to form overnight at 4°C.

3.4.1.8. Survival of cells in simulated gastric juice

Simulated gastric juice (SGJ) was prepared as described by Lian (2003), which was a pepsin solution (3 g/*l*) in saline (0.5% NaCl). The SGJ was prepared freshly and its pH was adjusted to 2.0 or 3.0 with 5 M HCl. The cells (5×10^7 cfu) were mixed with 1 ml of the filter sterilized SGJ and incubated at 37°C. At specified time intervals, the gastric juice was removed following centrifugation and the cells were washed once with PB following with two washes with saline. Amylose coated cells were then resuspended in PB containing 30 U/ml α-amylase (Megazyme, Bray, Ireland) and incubated at 40°C for 20 min to aid the release of cells from the encapsulating materials. Viable bacteria were enumerated on MRS-agar after incubation for 24 h at 37°C and survival percentage was determined by dividing the final viable population (cfu/ml) with the initial viable population (cfu/ml) of *L. casei* cells exposed to the SGJ.

3.4.2. Results

3.4.2.1. Expression and purification of fusion protein

To investigate the capability of CphI for the construction of a cell surface adhesive SbD, this domain was fused at its C-terminus to the linker region and SbD of AmY (Fig. 2). The fusion protein (CphI-SbD) was expressed intracellularly in *E. coli* using the T5 promoter at 0.35 g/*l*. The molecular size was 56 kDa as expected and 75% of the protein was present in the soluble form. When the protein was purified under native conditions by the histidine tag affinity chromatography, two additional protein bands corresponding to 73 and 71 kDa were present in the protein preparation (Fig. 3, lane 1). After incubation of the protein preparation with corn starch, no band for CphI-SbD was detected in the supernatant

indicating its adsorption to the starch granules (Fig. 3, lane 2). CphI-SbD was further purified using an anion exchange chromatography (Fig. 3, lane 4). The result of starch binding assay showed that the purified fusion protein was in the active form and it was able to adhere to the starch granules (data not shown).

Figure 2. Structure of expression cassette

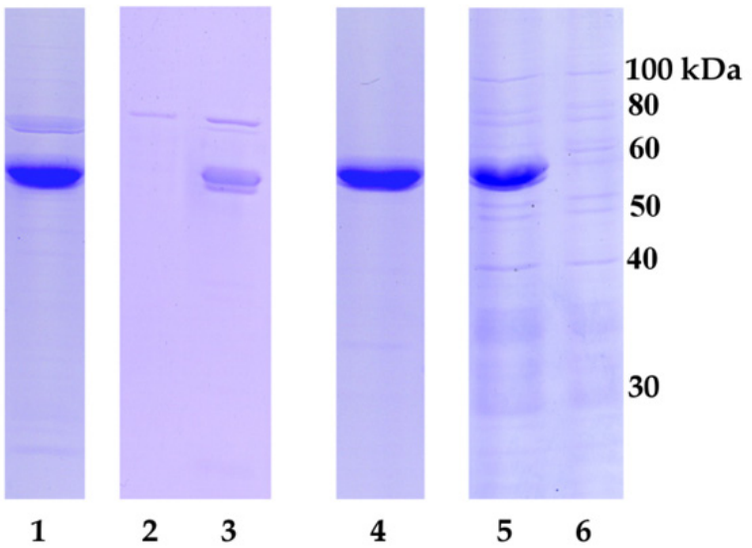

Figure 3. Purification of CphI- SbD and its binding to corn starch and *L. casei* cells. Lane 1, nickle chelate column purified protein preparation; lane 2, supernatant after starch binding assay; lane 3, control without starch; lane 4, ion exchange chromatography purified CphI-SbD; lane 5, cells bound to CphI-SbD; lane 6, cells only.

3.4.2.2. Binding of CphI-SbD to L. casei cells

L. casei cells were incubated with the purified fusion protein and studied in terms of the binding of the fusion protein by SDS-PAGE. As shown in Fig. 3 (lane 5), the fusion protein was associated with the cells. The result of the densitometrical analysis showed that 6×10^4 molecules of CphI-SbD were bound to each cell of *L. casei*.

3.4.2.3. Aggregation of bacteria with starch

For aggregation of bacteria with starch, an optimal ratio between bacteria and starch must be determined. Therefore, we examined the dependence of aggregate formation on the final starch concentration in the mixture at a constant cellular density. Free cells mixed with starch, and starch without cells were used as controls. For each concentration of starch, we compared the volume of sediment formed in the sample containing the mixture of bacteria bound to CphI-SbD and starch with those of the controls visually. The result is shown in Table 1. The volumes of the sediments formed in the controls after 1 h standing at room

temperature, were almost the same at all the starch concentrations tested. When the starch concentration was 5 mg/ml, the volume of formed sediment in the sample containing the mixture of bacteria bound to CphI-SbD and starch was markedly larger than those of the controls. The adhesion percentage of *L. casei* cells to the starch granules under these conditions was determined by cosedimentation assay, which measures the reduction in optical density in bacterial suspensions after the addition of starch (Crittenden et al., 2001), and it was 32% for the bacteria bound to CphI-SbD and 4% for the free cells.

Starch concentration (mg/ml)	BPS[1]	BS[2]	S[3]
1	+	+	+
2	++	+	+
5	+++	+	+
10	+	+	+

[1]: Mixture of *L. casei* cells displaying CphI-SbD and starch
[2]: Mixture of *L. casei* cells and starch
[3]: Starch only

Table 1. Comparison of sediment formation at different starch concentrations.

Mixing of the bacteria bound to CphI-SbD with starch at a final concentration of 5 mg/ml resulted in the crosslinking of starch granules and the formed aggregates were observed by phase contrast microscopy (Fig. 4). In contrast, when starch was mixed with free cells or when no bacteria were present in the mixture, the starch granules were found to be separated from each other and no aggregates were observed by phase contrast microscopy (data not shown).

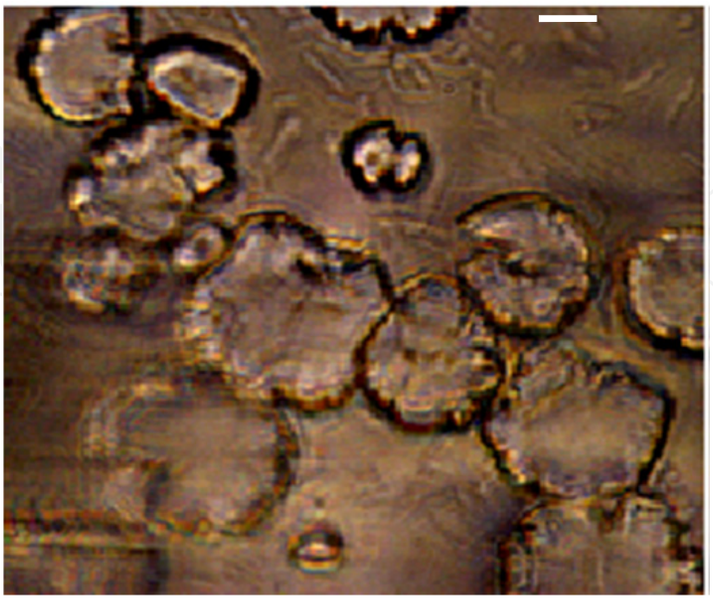

Figure 4. Aggregation of *L. casei* cells displaying CphI-SbD with starch granules (scale bar=5 μm)

3.4.2.4. Survival of encapsulated cells under simulated gastric conditions

When *L. casei* free cells were exposed to the SGJ at pH 3.0 and 2.0 for 1 h, the survival percentages of the cells were 0.074% and 0.002%, respectively. However, the survival percentages of amylose coated bacterial aggregates after 1 h incubation in the SGJ were 63.9% and 6.03% at pH 3.0 and 2.0, respectively (Fig. 5).

We studied the effects of starch, amylose coating and CphI-SbD on cell survival in the SGJ (pH 3.0, 1 h) (Table 2). It was observed that when free cells were mixed with the starch granules, the survival percentage was 3.1%, and the survival percentage of amylose coated free cells in the SGJ was 7.2%. The survival of the CphI-SbD displaying bacteria (0.093%) was not significantly different from that of free cells (0.074%) and the survival of amylose coated fusion protein displaying bacteria was comparable to that of amylose coated free cells. However, when fusion protein displaying bacteria were aggregated with the starch granules, the survival percentage was 7.7% higher than that of free cells mixed with the starch granules, and when the aggregates were coated with amylose, the survival percentage was 27.6% higher than that of the amylose coated mixture of the starch granules and free cells.

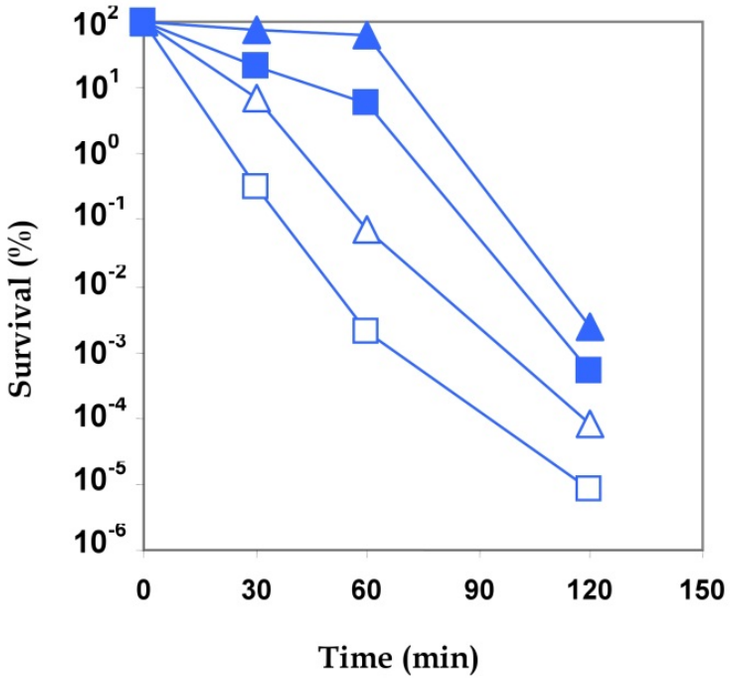

Figure 5. Time course of survival of *L. casei* cells under simulated gastric conditions. Open and filled triangles are free cells and amylose coated bacterial aggregates, respectively at pH 3.0; Open and filled rectangles, those at pH 2.0. The Data represent the means of two independent experiments.

Bacteria	CphI-SbD	Starch	Amylose	Survival (%)
+	–	–	–	0.074 ± 0.012
+	+	–	–	0.093 ± 0.018
+	–	+	–	3.1 ± 0.4
+	+	+	–	10.8 ± 2.0
+	–	–	+	7.2 ± 1.7
+	+	–	+	6.6 ± 2.5
+	–	+	+	36.3 ± 5.4
+	+	+	+	63.9 ± 2.7

Table 2. Effects of protective systems components on cell survival. Data represent means± standard deviations of three independent experiments.

3.4.3. Discussion

The objective of this study was to evaluate the possibility for enhancement of the delivery of viable microorganisms to the GIT through the conferment of starch binding ability to them. In this way, the bacteria are entrapped between starch granules to use the protective effect of starch for maintaining the cell viability under adverse conditions. The surface display technology based on the external mode was used to provide starch adhesion ability for the cells while retaining their nongenetically modified status because consumption of genetically modified microorganisms is not favorable for consumers. It was observed that CphI-SbD was able to bind both to the cell surface of *L. casei* and to the starch granules. Therefore, the fusion protein was able to mediate the adhesion of cells to the starch granules. We examined the aggregation of cells with starch as an alternative protective strategy, which entraps bacteria between starch granules. Compared with the previous method of entrapping bacteria within the porous starch granules prepared by an enzymatic digestion (Myllaerinen et al., 2001), our technique, is much simpler and faster. Moreover, starch granules can be used in their intact forms without any modifications.

When the aggregates of CphI-SbD displaying bacteria with starch were coated with amylose and exposed to the SGJ, there were significant increases in the survival percentage (63.9% at pH 3.0, and 6.03% at pH 2.0) with respect to those of free cells (0.074% at pH 3.0, and 0.002% at pH 2.0). The pHs of the SGJ measured for all the combinations of bacteria, fusion protein, starch and amylose coating (Table 2), in addition to that of the SGJ without any of these components both before and after the incubation at 37°C, were in the range of 3.00- 3.04. Therefore, the observed increases in the survival were not due to the modifications of the pH of SGJ.

Analysis of the effects of fusion protein, starch and amylose coating on the cell survival showed that the binding of fusion protein to *L. casei* cells did not have a significant effect on the cell survival (Table 2). When free cells were mixed with the starch granules, their survival percentage was 3.03% higher than that of free cells, which indicated the protective effect of starch on the cell survival. It was observed that, the entrapment of bacteria between

the starch granules with the aid of CphI-SbD (the aggregation of bacteria with the starch granules) enhanced the protective effect of starch, and the survival percentage was increased to 10.8%. The effect of the amylose coating on the survival of CphI-SbD displaying bacteria was comparable to that of the free cells (6.6 and 7.2% respectively), and the observed difference was not statistically significant. Incorporation of the fusion protein in the protective system composed of the starch and amylose resulted in a 27.6% increase in the cell survival percentage, which showed that the simultaneous application of two protective strategies (the aggregation of bacteria with starch and the amylose coating) resulted in the highest cell survival percentage (63.9%). In conclusion, in this study we showed the potential usefulness of the cell surface display technology for protection of cells under adverse gastric conditions.

4. Conclusion

Surface display is an attractive technology that can be used to confer new functions to LAB. The effectiveness of these systems depends on the appropriate selection of several factors including the anchoring domains, secretion signals, and host strains. Moreover, a proper strategy for the fusion of anchoring domains to target proteins should be determined to protect the functionality of target proteins. So far, a limited number of surface display systems have been developed. The characterization of anchoring, secretion, and regulatory signals from genome sequences can expand the surface display systems. The low transformation efficiency of LAB is a major obstacle for the construction of surface display systems and the establishment of efficient transformation protocols is therefore necessary.

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