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ORIGINAL PAPER

Strategies to display heterologous proteins on the cell surface of lactic acid bacteria using as anchor the C-terminal domain of *Lactobacillus acidophilus* SlpA

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Abstract

The surface-layer (S-layer) protein of *Lactobacillus acidophilus* is a crystalline array of self-assembling subunits, non-covalently bound to the most outer cell wall envelope, which constitutes up to 20% of the total cell protein content. These attributes make S-layer proteins an excellent anchor for the development of microbial cell-surface display systems. In *L. acidophilus*, the S-layer is formed predominantly by the protein SlpA. We have previously shown that the C-terminal domain of SlpA is responsible for the cell wall anchorage on *L. acidophilus* ATCC 4356. In the present study, we evaluated the C-terminal domain of SlpA of *L. acidophilus* ATCC 4356 as a potential anchor domain to display functional proteins on the surface of non-genetically modified lactic acid bacteria (LAB). To this end, green fluorescent protein (GFP)-CTSlpA was firstly produced in *Escherichia coli* and the recombinant proteins were able to spontaneously bind to the cell wall of LAB in a binding assay. GFP was successfully displayed on the S-layer stripped surface of *L. acidophilus*. Both the binding stability and cell survival of *L. acidophilus* decorated with the recombinant protein were then studied in simulated gastrointestinal conditions. Furthermore, NaCl was tested as a safer alternative to LiCl for S-layer removal. This study presents the development of a protein delivery platform involving *L. acidophilus*, a microorganism generally regarded as safe, which utilizes the contiguous, non-covalently attached S-layer at the cell surface of the bacterium without introducing any genetic modification.

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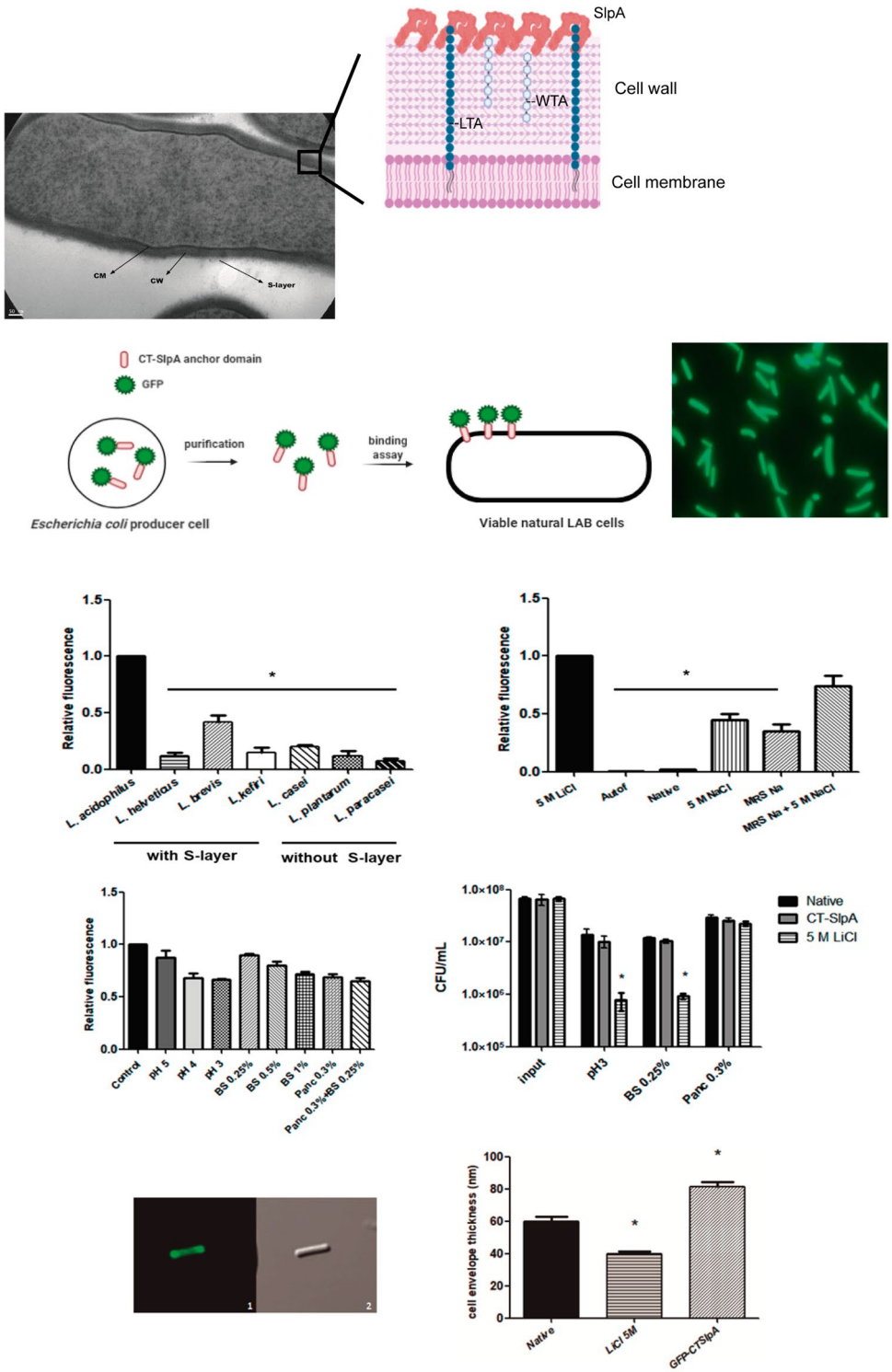
Graphic abstract



Keywords *Lactobacillus* · SlpA · Cell surface display system

Introduction

The S-layer protein (Slp) is a (glyco)-proteinaceous envelope formed of self-assembling subunits, which constitutes the outermost cell surface structure of many Bacteria and Archaea, representing up to 20% of the total cell protein content. Several species of lactic acid bacteria (LAB), including those of the genus *Lactobacillus*, possess an S-layer protein envelope with molecular weights ranging from 25 to 71 kDa. These proteins are typically enriched in basic and hydrophobic amino acids, often exhibiting high isoelectric points (pI) between 9.4 and 10.4 (Malamud et al. 2019).

Usually, Slps exhibit two well-defined structural domains: one involved in cell wall anchoring (the C-terminus) and the other required for self-assembly (the N-terminus). The C-terminal domain, which is 60 residues in length, is found in the cell surface proteins of a variety of *Lactobacillus* species, including *L. kefiranofaciens*, *L. crispatus*, *L. helveticus*, *L. amylolyticus*, *L. gallinarum*, *L. acidophilus*, *L. amylovorus* and *L. intestinalis.* In particular, it has been demonstrated that the C-terminus is the most conserved domain in *L. acidophilus* ATCC 4356 (Smit et al. 2001) and *L. crispatus* JCM 5810, ZJ001, K313 and K2-4-3 (Antikainen et al. 2002; Chen et al. 2009; Hu et al. 2011; Sun et al. 2013), and our group has shown that the SlpA C-terminal domain of *L. acidophilus* ATCC 4356 is required for cell wall interaction. This interaction is mediated by the C-terminal domain basic amino acids, mainly lysines and arginines, and the negatively charged secondary cell wall polymers (Fina-Martin et al. 2019). These features of S-layer proteins make them excellent anchors for the development of microbial cell-surface display systems, which have gained increasing attention due to their wide diversity of applications in biotechnology, including vaccine delivery.

For centuries, LAB, many of which have been granted the "generally recognized as safe" (GRAS) status, have been used for the production of fermented food and their preservation. Moreover, many LAB strains harbor probiotic features, can survive the hostile condition of the gastrointestinal tract (low pH, high bile concentration, protease resistance), a feature that allows them to colonize certain intestinal tissues, have intrinsic adjuvant response, and can interact with human immune cells, making them attractive vehicles for vaccine delivery (Matsuguchi et al. 2003; Vizoso Pinto et al. 2009; Rocha-Ramírez et al. 2017; O'Flaherty et al. 2018).

Over the last decades, there has been a growing interest in the application of Slps in the development of oral vaccines. Due to their GRAS status and adjuvant properties, as well as their ability to display antigenic epitopes on bacterial surfaces, Slps have become excellent candidates to be used as antigen carriers, allowing different peptides and proteins to be displayed on the surface of microbial cells. In particular, some researchers have used SlpA as an anchor protein in recombinant LAB, especially *Lactococcus* and *Lactobacillus*. Qin et al. (2014), for example, expressed a food-grade *L. acidophilus* SlpA-based cell surface display vector on *Lactobacillus casei*, whereas O'Flaherty and Klaenhammer (2016) used a vaccine vector containing the SlpA signal peptide from *L. acidophilus* to express *Clostridium botulinum* and *Bacillus anthracis* antigens on the probiotic *L. acidophilus* strain NCFM. However, these genetically designed strains of *Lactobacillus* are DNA-recombinant strains, a fact that affects their GRAS status.

In this line, the C-terminal domain of other Slp proteins has been used as an anchor for heterologous surface display of various proteins on non-genetically modified LAB. Hu et al. (2011), for example, tested the ability of SlpB-mediated surface display by exposing green fluorescent protein (GFP) and β-galactosidase on the surfaces of *Lactococcus lactis* and several *Lactobacillus* species. Zhang et al. (2016) also used this anchor protein to display the carcinoembryonic antigen on the cell surface of *L. lactis*, generating higher levels of antigen-specific secretory IgA in the sera of mice upon oral administration. Other researchers have also used other anchoring domains with the ability to bind to the surface of several Gram-positive bacteria in trans configuration, such as the Lysine motif domain LysM (Hu et al. 2010; Zadravec et al. 2015; Zhang et al. 2016) or the WxL domain (Brinster et al. 2007).

Based on all the above, the aim of the present study was to evaluate the C-terminal domain of SlpA from *L. acidophilus* ATCC 4356 as a potential anchor domain to bind functional proteins to the surface of non-genetically modified LAB. It is important to note that *L. acidophilus* is a LAB species widely used in commercial probiotic products, including cheese, acidophilus milk, and yoghurt as well as in dietary supplements with reported functional effects (Ramachandran et al. 2013; Bull et al. 2014; Guarner et al. 2017). Both the binding stability and cell survival of *L. acidophilus* decorated with the recombinant protein were studied in simulated gastrointestinal conditions. Furthermore, NaCl was tested as a safer alternative to LiCl for S-layer removal.

Materials and methods

Bacterial strains and culture conditions

All *Lactobacillus* strains used in this study (*L. acidophilus* ATCC 4356, *L. kefiri* JCM 5818, *L. brevis* ATCC 14,869, *L. helveticus* ATCC 12,046, *L. casei* BL23, *L. plantarum* ATCC 14,917, and *L. paracasei* 27,092) were propagated in MRS broth (Biokar, Beauvais, France) at 37 °C under static conditions. The MRS broth (pH 6.5) contains 10 g/L tryptone, 4 g/L yeast extract, 8 g/L meat extract, 5 g/L Na

acetate, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $MnSO_4 \cdot 4H_2O$, 1 mL/L Tween 80, and 20 g/L glucose. *Escherichia coli* HMS 174(DE3) (Novagen EMD Biosciences, Madison, WI, USA) was used as the expression host, and *E. coli* Top10 (Thermo Fisher Scientific, Rockford IL, USA) was used as the cloning host. *E. coli* strains were grown aerobically in Luria–Bertani (LB) broth at 37 °C. The antibiotics ampicillin and kanamycin were added at a final concentration of 100 µg/mL and 30 µg/mL respectively.

Molecular cloning, plasmid construction and protein purification

pET28-GFP was used as the N-terminal GFP fusion expression vector (Dieterle et al. 2014). The carboxy terminal domain of SlpA was amplified using the following primers: 5′-AGTGGATCCAACGTTAAAGCAAC-3′ (BamHI restriction site) and 5′-TAGGAGCTCTAATCTAAAGTT TGC-3′ (SacI restriction site). The SlpA CT region was amplified using chromosomal DNA from *L. acidophilus* as template. The DNA sequence was amplified by PCR using GoTaq DNA polymerase (Promega, USA) by following the manufacturer's instructions. pET28-GFP-CTSlpA was transformed into *E. coli* HMS 174 (DE3) cells (Novagen, Merck Millipore, USA) by electroporation for protein expression and further purification. Transformed cells were grown with aeration at 37 °C to an OD_{600} of 0.5 in LB medium. Protein expression was induced by adding 0.1 mM isopropyl-β-d-thiogalactopyranoside and the cultures were further incubated overnight at 19 °C before being harvested. Cell pellets were resuspended in binding buffer (50 mM Tris-HCl pH 8, 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride) and disrupted by sonication (6 cycles of 15 s). The clear lysates were centrifuged at 12,000×*g* for 20 min, and the supernatants were filtered through a 0.22-µm Millipore membrane to remove the cell debris. Subsequently, the clear lysates were applied to HisTrap™ HP columns (GE Healthcare, USA) equilibrated with the binding buffer (20 mM sodium phosphate, 500 mM NaCl, 25 mM imidazole, pH 7.4), then the columns were washed with washing buffer (20 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole, pH 7.4), and the His-tagged protein was eluted in elution buffer (20 mM sodium phosphate, 500 mM NaCl, 200 mM imidazole, pH 7.4). Eluted samples were dialyzed twice against protein buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM dithiothreitol) and once again in the same buffer with 30% glycerol and then stored at − 20 °C. Protein concentration was determined at 280 nm in a Nanodrop 2000 Spectrophotometer (ThermoScientific). SDS-PAGE was used to analyze protein expression.

Binding of the GFP-CTSlpA fusion protein to *Lactobacillus* sp.

Lactobacillus sp. cultures were grown at 37 °C under static conditions in MRS medium. When necessary, stress conditions were obtained by adding 0.6 M NaCl (high-salt conditions). Cells were harvested during the stationary phase. After treating with 5 M LiCl or 5 M NaCl for 45 min at room temperature to remove the S-layer protein from the specific strains (*L. acidophilus*, *L helveticus*, *L. brevis* and *L. kefiri*), cells were washed twice and resuspended in phosphate-buffered saline (PBS) adjusting to an OD_{600} of 1.0.

In a typical binding experiment, 300-µL aliquots of culture were incubated for 60 min at 37 °C with 10 µg of the GFP-CTSlpA chimerical fusion protein. GFP purified protein and PBS were used as negative and autofluorescence controls respectively. After binding, cells were collected by centrifugation at 10,000×*g* for 5 min and washed twice. Cells were resuspended in 100 µL of PBS and whole cells bound to the GFP-CTSlpA fusion protein were analyzed by fluorescence microscopy (Axiostar Plus; Carl Zeiss, Jena, Germany) with a × 100 objective lens with oil immersion, fluorescence confocal microscopy (FV1000 Olympus confocal microscope Olympus Inc., Japan) with an Olympus Plan ApoN 60X oil immersion objective lens (NA = 1.42) and flow cytometry techniques. For the last analysis, fluorescent and non-fluorescent cells within the gated population were distinguished based on fluorescence intensity (GFP-H). Data were acquired by means of the BD FACS Aria software (BD FACSDiva, firmware version 6.1.3; BD Bioscience, San Jose, CA, USA), and subsequently analyzed by the FlowJo 10.0.7 software (https://www.flowjo.com/). Assays were carried out in triplicate and data were normalized to the means of three replicates.

Binding of the GFP-CTSlpA fusion protein and cell viability in gastrointestinal conditions

Lactobacillus acidophilus ATCC 4356 was grown as described above: an aliquot of the stationary phase culture was collected, washed once and removed from its native S-layer by treatment with 5 M LiCl. Binding was carried out as described previously. After binding, cells were pelleted and resuspended in MRS medium for 2 h at 37 °C and adjusted to different conditions that mimic the gastrointestinal environment (pH, concentrations of bile salts and pancreatin). Acidic conditions were achieved by adjusting the pH of MRS broth from pH 5.0 to pH 3.0 with 1 M HCl. The bile and pancreatin conditions were achieved by supplementing the MRS broth with 0.25%, 0.5% and 1% w/v bile salts (Oxgall) or 0.3% pancreatin and without supplement as a control. After incubation, cells were collected by centrifugation and washed twice with PBS. The cell-associated

fluorescence was analyzed by flow cytometry. Survival to acidic conditions, bile salts and pancreatin was tested as follows: *L. acidophilus* was grown overnight in MRS broth at 37 °C, and cells were subjected to three different conditions of cells: native *L. acidophilus*, *L. acidophilus* without the S-layer following removal with 5 M LiCl and *L. acidophilus* bound to GFP-CTSlpA via a binding assay, as described previously. Pellets of OD_{600} 1.0 were resuspended in an equal volume of MRS broth with pH adjusted to 3.0 with 1 M HCl, and MRS supplemented with 0.25% bile salts or 0.3% pancreatin. The survival of the cultures was evaluated by determining the viable counts of the samples after treatments plated on MRS agar and incubated at 37 °C for 48 h and compared with the input.

Transmission electron microscopy (TEM)

Three different conditions of *L. acidophilus* cultures were prepared for TEM microscopy analyses: a native *L. acidophilus* culture, a *Lactobacillus* culture removed from its native S-layer by treatment with 5 M LiCl and a *Lactobacillus* culture bound to GFP-CTSlpA in a typical binding assay using a sub-saturating protein concentration. After treatments, samples were prepared according to the following schedule: primary fixation in 2.5% glutaraldehyde in 100 mM phosphate buffer at pH 7.4 for 4 h at 4 °C and secondary fixation in 1% osmium tetroxide in 100 mM phosphate buffer for 1 h at 4 °C. Following two washes with double-distilled water, samples were dehydrated using a series of ascending grades of ethanol (50, 70, 96 and 100%) and then embedded into a Durcupam epoxy resin. After polymerization at 60 °C for 72 h, ultrathin sections of 70–90 nm thickness were obtained using an Ultramicrotome (Reichert Jung Ultracut E). Finally, sections were mounted on copper grids, contrasted with uranyl acetate and lead citrate (Reynolds method) and observed using a TEM Zeiss 109 instrument and photographs were taken with a Gatan W10000 camera. The digital images were used for both cell envelope ultra structural analysis and cell envelope thickness measurements. The thickness measurements were carried out using Image J software (NHI, Bethesda, MD, USA) at a magnification of ×140,000. About 20 randomly selected sections of each type of cell according to the different conditions were used for the measurements at pole regions of each cell.

Statistical analysis

The data were statistically analyzed with Prism 5.0 (GraphPad Inc.). Assays were performed in triplicate and data were normalized to the means of three replicates. Results are presented as means ± standard deviations (indicated by error bars) for replicate experiments. A one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used to estimate statistical significance; a p value of < 0.05 was considered significant. In cell envelope thickness measurements, the mean of each type of cell was determined together with the corresponding errors of the mean. The Student's paired t test was applied and differences were considered to be significant at $p < 0.05$.

Results

GFP display on the cell surface of *Lactobacillus acidophilus* ATCC 4356 mediated by the CT-SlpA

To evaluate the potential of the CT-SlpA portion as a cell wall anchor, the carboxy-terminal region was fused with GFP (Supplementary file 1 Fig. S1), a suitable reporter tool for protein localization in bacteria (Feilmeier et al. 2000). Firstly, the GFP-CTSlpA was heterologously produced in *E. coli* and the purified protein was used in standard binding assays (Supplementary file 1 Fig. S2). Binding was observed by fluorescence microscopy. The results (Fig. 1a) showed equal localization of GFP-CTSlpA on the whole surface of the cells. GFP alone did not display fluorescence to cells, indicating that GFP could be bound only to the *L. acidophilus* cell surface targeted by CT-SlpA. Furthermore, *L. acidophilus* cells conserving the native S-layer showed no fluorescence.

To determine the maximum binding capacity, various amounts of purified GFP-CTSlpA fusion proteins were incubated with an aliquot of S-layer stripped *L. acidophilus* cells with an OD_{600} of 1.0 (approximately 3×10^8 colony-forming units (CFU)/mL). Lactobacilli bound to chimerical proteins were analyzed by flow cytometry and the results showed that the maximum binding capacity of fusion protein was 10 µg (Fig. 1b, c). Regarding the molecular weight of the carrier protein (17.5 kDa) and the number of CFU in the binding assay, it was determined that each cell could bind approximately 1.14×10^6 GFP-CTSlpA fusion proteins. These results showed that the technique used provides the ability to display a large amount of recombinant CTSlpA on the bacterial surface.

SlpA-binding capacity on the *Lactobacillus* surface and analysis of different stripping strategies using NaCl

To study the SlpA binding capacity on the surface of other *Lactobacillus* species, different *Lactobacillus* sp. strains were incubated with purified GFP-CTSlpA and binding was quantified by flow cytometry. Binding to *Lactobacillus* with (*L. helveticus*, *L. brevis*, and *L. kefiri*) and without S-layer (*L. plantarum*, *L. casei* and *L. paracasei*) was compared and

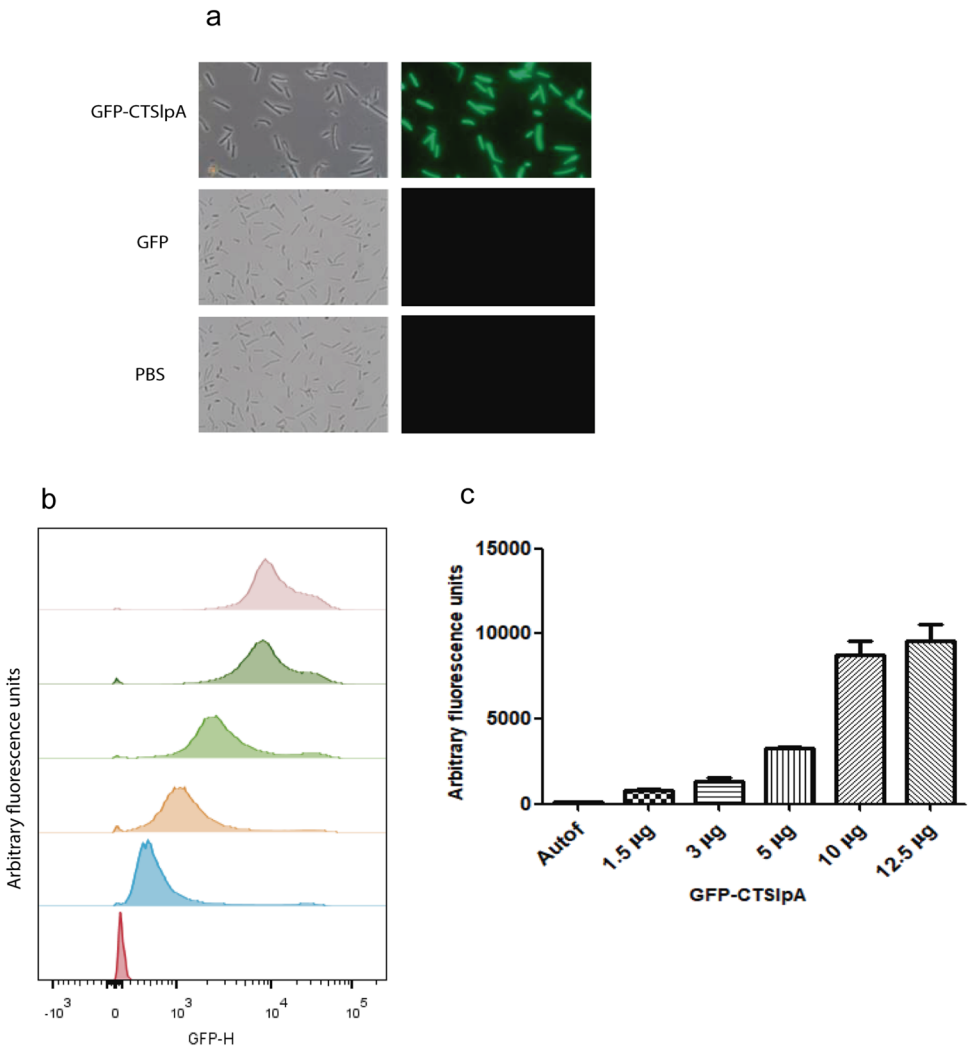


Fig. 1 GFP-CTSlpA binding to the S-layer stripped surface of *Lactobacillus acidophilus*. **a** An aliquot of 5 M LiCl-pretreated *L. acidophilus* was subjected to a binding assay with 10 µg of purified GFP-CTSlpA. Cells were observed by fluorescence microscopy. GFP and PBS were used as negative and autofluorescence controls respectively. Cells were observed by green light (right panels) and white light (left panels). Magnification ×1000. **b** Quantification of the maximum binding capacity by flow cytometry. Different amounts of purified GFP-CTSlpA were added to an equal amount of 5 M LiCl-pretreated *L. acidophilus* cells. **c** Standard curve of the fluorescence and the protein concentration of GFP-CTSlpA. The data points represent the averages and standard deviations from three independent experiments

data were normalized to 100% of the maximum fluorescence provided by *L. acidophilus*. In all cases, binding retention was found to be less than 50% with respect to *L. acidophilus* (Fig. 2a). Although all the species evaluated belong to the same genus, they show substantial differences in their cell wall components, reflected in the molecular mechanisms that support the protein-ligand interaction.

Although the methods to purify S-layer proteins are diverse, they are all based on the fact that Slps are associated with the cell wall in a non-covalent way. Usually, *Lactobacillus* surface-bound proteins are extracted from intact bacteria by the replacement of cations, using Li^+ (Sleytr et al. 2014; do Carmo et al. 2018). Given that remaining traces of the LiCl used for S-layer removal could induce toxicity when administered orally (Sahay et al. 2015), we evaluated NaCl to promote S-layer removal instead of LiCl because it was important to evaluate other stripping strategies to provide a safer protocol. When *L. acidophilus* ATCC 4356 cells were stripped by using 5 M NaCl instead of 5 M LiCl, 45 ± 3.1% of binding retention was observed compared to the control of maximum fluorescence obtained by LiCl S-layer removal, due to a decrease in the depletion of its native S-layer. Besides, in previous studies, we found a variation in the pattern of S-layer expression under osmotic stress due to a high-salt condition in *L. acidophilus* ATCC 4356. Variations in the cell wall and its components might favor detachment of the S-layer when the cells are pre-grown in high-salt conditions (Palomino et al. 2016; Allievi et al. 2019). In this respect, when the microorganisms were pre-grown under high-salt conditions (MRS + NaCl 0.6 M, overnight), 35 ± 3.8% of binding retention, against control, was obtained. Finally, the highest retention (75 ± 7.8% with respect to the control of maximum fluorescence) was obtained when both methods were combined (NaCl pre-growth and stripping) (Fig. 2b).

In summary, these results showed that *L. acidophilus* was the best vehicle to display high amounts of heterologous proteins of all the *Lactobacilli* studied here, when the cells were previously stripped with 5 M LiCl. However,

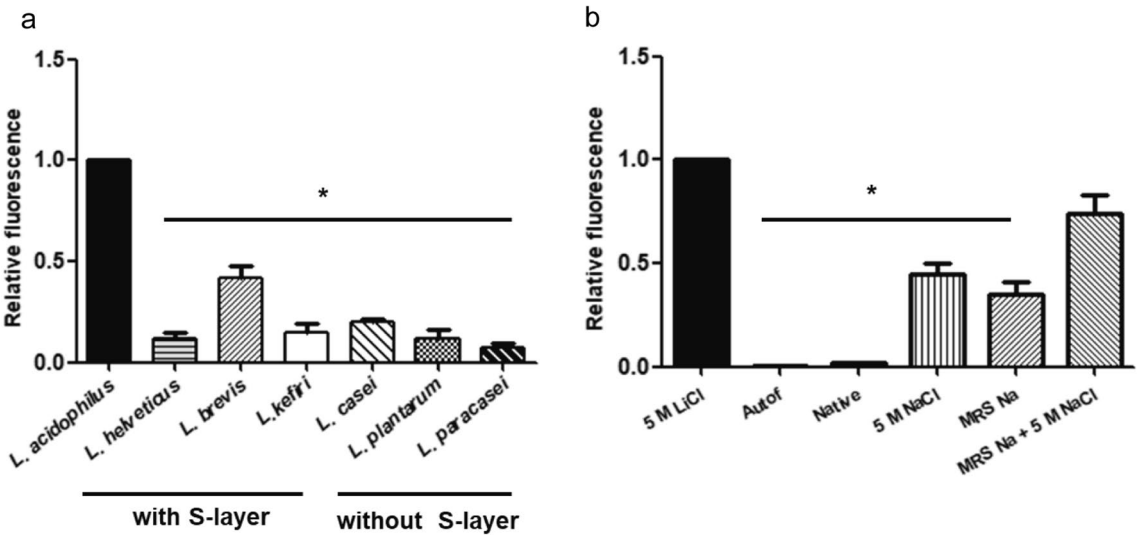


Fig. 2 SlpA-binding capacity on the *Lactobacillus* surface and analysis of different stripping strategies using NaCl. **a** Binding of GFP-CTSlpA to different *Lactobacillus* species by flow cytometry. The error bars represent standard deviations from experiments performed in triplicate. An asterisk (*) denotes a significant difference ($p < 0.05$) in fluorescence intensity between *Lactobacillus sp*. and *L. acidophilus*. **b** Analysis of different stripping strategies using NaCl. Data were normalized to 100% of the maximum fluorescence provided by 5 M LiCl treatment. The error bars represent standard deviations from experiments performed in triplicate. An asterisk (*) denotes a significant difference ($p < 0.05$) in fluorescence intensity between the indicated treatments and 5 M LiCl. *Autof* autofluorescence, *native L. acidophilus* cells with their native S-layer, *5 M NaCl L. acidophilus* cells stripped with NaCl, *MRS Na Lactobacillus* cells growing in the presence of 0.6 M NaCl, and MRS Na + 5 M NaCl denotes both treatments

among the strategies that involved NaCl, the one that yielded the best binding results was the combination of both pre-growth and S-layer removal in the presence of NaCl, which allows us to infer that this could be an interesting tool to explore a safer display system.

Binding retention and viability in simulated gastrointestinal conditions

The release behavior of an oral delivery system strongly depends on the variation of pH along the gastrointestinal tract (Yang et al. 2002). The pH of a stomach can vary between 2 and 6 depending on the fasting state, affecting the efficiency of any pH-sensitive delivery system, whereas that of the small intestine varies between 7 and 8.5, thus being favorable for bacterial survival. However, the presence in the small intestine of pancreatin, which is a mixture of several digestive enzymes (amylase, lipase and protease) produced by exocrine cells of the pancreas, and bile salts (whose concentration ranges between 0.2 and 2% (Whitehead et al. 2008) may have adverse effects on probiotic cells. The survival to bile salts exposure represents an important criterion to define a potential probiotic strain. Different probiotic bacteria show differential resistance to normal concentrations of bile salts and it has been suggested that the survival rate of probiotic strains should vary between 20 and 40%. However, many microorganisms have a survival rate of less than 0.1% (Bezkorovainy 2001; Whitehead et al. 2008).

One potential application of *Lactobacillus* decorated with CT-SlpA as a carrier protein is to deliver antigens to the gastrointestinal mucosa. In this respect, it was important to evaluate the stability of the binding between GFP-CTSlpA and the cell envelope under stress conditions that mimic those encountered during transit through the intestinal tract (pH variations, bile salts or degradation by intestinal proteases). Regarding acid stress, the most adverse condition assayed showed a binding retention of about 66% compared to the input. Interestingly, GFP-CTSlpA association with *Lactobacillus* was slightly affected by increasing the concentration of bile salts, even when the concentration was higher than physiological concentrations. Furthermore, when incubated with pancreatin and pancreatin plus bile salts, the binding retention was $\geq 65\%$ (Fig. 3a).

We further determined how GFP-CTSlpA-decorated *Lactobacillus* could impact cell viability when cells were exposed to hostile gastrointestinal tract conditions. For this purpose, the survival rates of three different conditions (wild-type *Lactobacillus*, *Lactobacillus* without S-layer, and *Lactobacillus* bound to GFP-CTSlpA) were compared with the input after treatments. Acid and bile salts treatments showed significant ($p < 0.05$) log reduction values of 2.13 and 2.06 CFU/mL, respectively, when cells were devoid of their native S-layer. In the other cases, both wild-type cells

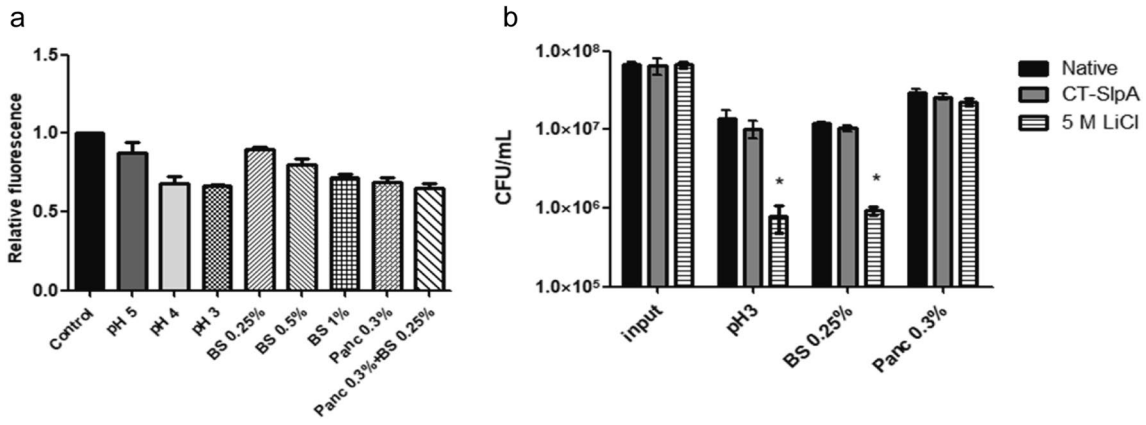


Fig. 3 Binding stability and survival rates in simulated gastrointestinal conditions. **a** Fluorescence data of binding retention in acidic conditions (pH 3–5), bile salts (BS), and pancreatin normalized to control. **b** Survival rates in gastrointestinal conditions. *Native L. acidophilus* cells with their native S-layer. *CT-SlpA* cells bound to chimerical protein. *5 M LiCl Lactobacillus* stripped with 5 M LiCl. The error bars represent standard deviations from experiments performed in triplicate. The asterisk (*) denotes a significant difference with respect to the input ($p < 0.05$)

and those bound to GFP-CTSlpA showed no significant decrease in their survival rates after exposing them to these conditions. Wild-type *Lactobacillus* exhibited log reduction values of 0.73 and 0.93 under pH 3 and bile salt conditions, respectively. A similar trend of log reduction was observed in *Lactobacillus* bound to GFP-CTSlpA (0.95 and 1.02 log reduction). Pancreatin had no statistically significant effect on any type of *Lactobacillus* (Fig. 3b). These results suggest that *Lactobacillus* decorated with GFP-CTSlpA would have a behavior similar to that of the wild type in terms of viability against hostile environmental agents such as acid and bile salts.

The binding of GFP-CTSlpA to *Lactobacillus acidophilus* is localized

A whole-cell-binding assay was performed using a subsaturating concentration of GFP-CTSlpA. Images of confocal microscopy showed that GFP-CTSlpA was able to bind the entire cell although the chimerical protein preferentially bound at the poles of the *Lactobacillus* cell (Fig. 4a).

Wild-type *Lactobacillus*, S-layer stripped *Lactobacillus*, and *Lactobacillus* bound to GFP-CTSlpA were analyzed by TEM. TEM examination of the cell surface with 140,000-fold magnification showed a typical trilaminar structure of *L. acidophilus*. From inside to outside, the bacterial envelope is composed of the cell membrane, a denser inner layer, corresponding to the cell wall composed of peptidoglycan, and the outermost S-layer with an irregular and rough appearance (Fig. 4b1). While control cells presented these three layers, LiCl-treated cells presented only two, with a smoother surface (Fig. 4b2). On the other hand, the *Lactobacillus* bound to the chimerical protein showed the same pattern observed in confocal microscopy, where the poles of the cells presented a higher protein density (Fig. 4b3). Furthermore, the cell wall thickness measurements of the cell poles of *L. acidophilus* bound to GFP-CTSlpA showed a significant increase (83.56 ± 9.86 nm) compared to control cells (60.07 ± 7.4 nm), whereas those of *Lactobacillus* lacking its S-layer showed a significant decrease in cell wall thickness (39.59 ± 5.73 nm) (Fig. 4c).

Discussion

The heterologous display of proteins or peptides on the surface of non-genetically modified organisms has a wide range of biotechnological and industrial applications, including the development of live-vaccine delivery systems, diagnostics, bioconversion, enzyme immobilization, cell adhesion, and oral drug delivery (Bosma et al. 2006; Ribelles et al. 2013; Mao et al. 2016). In this study, we showed that the heterologous protein GFP is able to bind to the surface of *L. acidophilus* depleted of its native S-layer with the aid ofan SlpA anchor, opening up the possibilities for surface display of foreign proteins such as antigens.

The successful development of a cell surface display system requires anchor proteins that show a high binding capacity. Although we analyzed other species, *L. acidophilus* showed the highest affinity to the anchor protein. *L. acidophilus* cells saturated with GFP-CTSlpA bound 1.14×10^6 molecules per cell, displaying high binding capacity comparable to a LysC- or SlpB-mediated surface display system (Hu et al. 2010; Bosma et al. 2006).

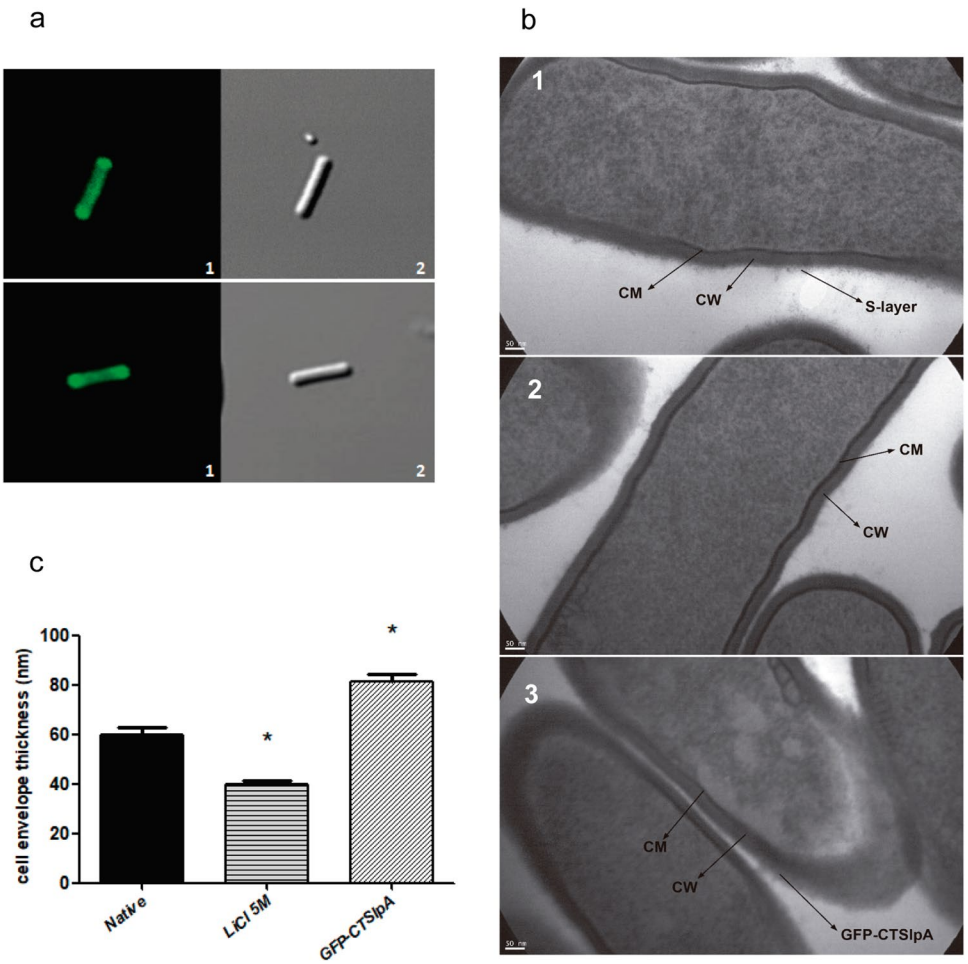


Fig. 4 Localized binding of GFP-CTSlpA to *L. acidophilus*. **a** Confocal microscope observation of L. acidophilus bound to GFP-CTSlpA in a subsaturating binding assay. Cells were observed by green light (1) and white light (2). **b** Representative transmission electron micrographs of native cells (1), stripped cells with 5 M LiCl (2), and cells bound to GFP-CTSlpA (3). *CM* cell membrane, *CW* cell wall. **c** Cell envelope thickness of native cells, stripped cells with 5 M LiCl, and cells bound to GFP-CTSlpA. Values are presented as means ± standard error of the mean. Asterisks (*) denote significant differences vs. native cells

Since LiCl could induce toxicity when orally administered to experimental animals, here we tested NaCl as a more innocuous alternative to remove the native S-layer. It hasalso been reported that growth in high-salt conditions could generate several cell surface modifications, increasing the release of S-layer protein from cells (Palomino et al. 2016; Allievi et al. 2019). Consideringthese two facts, thestrategy based on high-salt pregrowth and NaCl instead of LiCl as an agent to removethe S-layerfor binding high levels of heterologous protein can be considered as safer (Fig. 2b).

Probiotic strains can survive the hostile conditions of the gastrointestinal tract and temporarily colonize certain intestinal tissues. For the development of a successful cell surface display system using the probiotic strain *L. acidophilus* ATCC 4356 (Palomino et al. 2015), desirable features include not only the viability of the bacterium but also the binding stability of the carrier protein. Our results showed that the binding of GFP-CTSlpA to *L. acidophilus* ATCC 4356 at conditions of pH, bile salts, and proteases that emulated the gastrointestinal transit was stable. In this sense, unlike the viability of S-layer-depleted cells, the viability of cells decorated with the carrier protein was not affected, a fact that suggests a protective role against the adverse conditions of the gastrointestinal tract (Fig. 3).This is in line with previous reports that demonstratedhow the *L. acidophilus* ATCC 4356 S-layer protein is stable in simulated gastric fluids (pH > 3) and in all the simulated intestinal fluids (Eslami et al. 2013).

The role of S-layer proteins as carriers for the display of therapeutic biomolecules has opened interesting perspectives due to their GRAS status, intrinsic adjuvant properties and immunomodulatory effects. Moreover, the C-terminal region of *L. acidophilus* ATCC 4356 has murein hydrolase activity against the cell wall ofthe Gram-negative *Salmonella enterica* serovar Newport (Prado-Acosta et al. 2008), *E. coli* (Meng et al. 2015) and Gram-positive pathogens such as *Staphylococcus aureus* and *Bacillus cereus* (Prado-Acosta et al. 2010). Furthermore, *in silico* and experimental analyses have revealed that S-layer proteins present two carbohydrate recognition domains localized in the C-terminus domain. The lectin-like activity described for *L. acidophilus* SlpA would explain the mechanism for the immunomodulatory, anti-viral, and anti-bacterial properties reported for this protein (Martínez et al. 2012; Fina-Martin et al. 2019).

In summary, the goal of this study was to test whether SlpA could be used to generate a surface display system using the C-terminal domain as cell wall anchor for LAB. We showed that, among all LAB studied, *L. acidophilus* displays the highest amount of heterologous protein when treated with LiCl, although the safer approach using NaCl allowed obtaining 75% of bound protein. Moreover, neither the binding stability nor thebacterial survival were affected when *Lactobacillus*was subjected to conditions simulating those of the gastrointestinal transit. The choice of the *L. acidophilus* strain focuses on delivering the decorated strain to the intestine (its niche), where it is expected to transit and be able to adhere for a few days exerting the presentation of the antigen. Thus, the use of the food-grade and probiotic strain *L. acidophilus* as a live vaccine vector to deliver antigen is a promising strategy. The novelty of our work resides in the high performance obtained when stripping with NaCl, compatible with an oral vaccine platform compared to other studies in which non-innocuous stripping strategies such as SDS, acetone or trichloroacetic acid are used.

Finally, we explored the potential use of the GRAS microorganism *L. acidophilus* for the delivery of biomolecules, such as antigens for oral vaccination and therapeutic proteins, tothe gastrointestinal tract. It is important to highlight that the anchor domain used was bound to a non-genetically modifiedbacterium, a fact that did not affect its GRAS status.

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