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Influence of osmotic stress on the profile and gene expression of surface layer proteins in *Lactobacillus acidophilus* ATCC 4356

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Abstract In this work, we studied the role of surface layer (S-layer) proteins in the adaptation of *Lactobacillus acidophilus* ATCC 4356 to the osmotic stress generated by high salt. The amounts of the predominant and the auxiliary S-layer proteins SlpA and SlpX were strongly influenced by the growth phase and high-salt conditions (0.6 M NaCl). Changes in gene expression were also observed as the mRNAs of the *slpA* and *slpX* genes increased related to the growth phase and presence of high salt. A growth stage-dependent modification on the S-layer protein profile in response to NaCl was observed: while in control conditions, the auxiliary SlpX protein represented less than 10 % of the total S-layer protein, in high-salt conditions, it increased to almost 40 % in the stationary phase. The increase in S-layer protein synthesis in the stress condition could be a consequence of or a way to counteract the fragility of the cell wall, since a decrease in the cell wall thickness and envelope components (peptidoglycan layer and lipoteichoic acid content) was observed in *L. acidophilus* when compared to a non-S-layer-producing species such as *Lactobacillus casei*. Also, the stationary phase and growth in high-salt medium resulted in increased release of S-layer proteins to the supernatant medium. Overall, these findings suggest that pre-growth in high-salt conditions would result in an advantage for the probiotic nature of *L. acidophilus* ATCC 4356 as the increased amount and release of the S-layer might be appropriate for its antimicrobial capacity.

Keywords S-layer proteins · *Lactobacillus* · Osmotic stress

Introduction

Surface-layer (S-layer) proteins are ubiquitous in both Eubacteria and Archaea. S-layers are arrays of a single protein that constitutes the outermost cell envelope and have been considered to function as protective coats, maintenance of cell shape, and adhesion to specific hosts (Avall-Jääskeläinen and Palva 2005; Hynönen and Palva 2013; Gerbino et al. 2015). In addition, we have recently shown that the S-layer of *Lactobacillus acidophilus* has antibacterial endopeptidase activity and antiviral functions (Prado-Acosta et al. 2008, 2010; Martínez et al. 2012). S-layer proteins are present in some *Lactobacillus* species that are normal inhabitants of the oral and genital cavities and the gastrointestinal tract (GIT) of humans and animals and that are generally recognized as safe (GRAS). *L. acidophilus* is one of the species found in the human intestine, and some strains are used as probiotics (Resta-Lenert and Barrett 2003). Probiotics play an important role in the control of the host intestinal microbiota and in the modulation of the host immune response. It is generally assumed that a good adherence capacity is a desirable trait for probiotic lactobacilli, as it can promote the gut residence time, pathogen exclusion, and interaction with host cells for the protection and immunomodulation of the epithelium (Buck et al. 2005; Frece et al. 2005). By proteome analysis, in an adherence assay using Caco-2 cells, Ashida et al. (2011) identified S-layer proteins of *L. acidophilus* L-92, including SlpA. In other studies, Klaenhammer et al. (2005) reported an *slpA* mutant of

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L. acidophilus NCFM as being more resistant to bile and more sensitive to NaCl and ethanol. Furthermore, studies regarding the structure and gene expression of the S-layer proteins of *L. acidophilus* ATCC 4356 (Oling et al. 2001; Smit et al. 2002) and their biological functions have been conducted (Ramiah et al. 2009; Khaleghi et al. 2010; Khaleghi and Kermanshahi 2012), but the effect of salt stress on this strain has not been evaluated. In contrast, in *L. acidophilus* NCFM, the genes responding to salt stress (Weiss and Jespersen 2010) and the effect of GIT conditions on *slp* gene expression have been studied by several authors (O'Flaherty and Klaenhammer 2010; Capozzi et al. 2011).

In our laboratory, we have studied the response to osmotic stress of several gram-positive strains and species and observed several cell envelope modifications when *Bacillus* or *Lactobacillus* strains were exposed to high-salt conditions (Lopez et al. 2000; Piuri et al. 2003, 2005; Palomino et al. 2009, 2010, 2013). In particular, we have shown that growth in high-salt conditions of *Lactobacillus casei* BL23, a species that lacks S-layers, causes several pleiotropic effects, including differences in the susceptibility to enzymatic lysis, increased sensitivity to cationic antimicrobials such as nisin, and increased capacity to form biofilm on artificial surface and transformation ability. These modifications result both from the changes in the peptidoglycan structure and the different zwitterion character of the lipoteichoic acid (LTA) molecule in high-salt conditions (Piuri et al. 2005; Palomino et al. 2010, 2013).

Growth under high-salt conditions is of industrial importance, because NaCl concentration is 0.3 M in the gut when lactobacilli are used as a probiotic and about 0.35 M (2 % *w*/*v*) and up to 0.6 M (about 3.5 % *w*/*v*) in cheeses (Crow et al. 1995; Fox et al. 1996). Pre-growth in high salt prior to lyophilization increases bacterial survival after the process, an important quality in the starter culture industry (Kets et al. 1996; Koch et al. 2007). Although NaCl concentration in food preservation is high, it is mild in the GIT. However, pre-growth of bacteria in hyperosmotic conditions to develop a probiotic formula might result in a better performance for probiotic applications. In fact, evaluation of the LTA content, as that described for *L. casei* (Palomino et al. 2013), is necessary to determine the characteristics of probiotics regarding inflammatory reactions as a consequence of their LTA content (Mohamadzadeh et al. 2011; Lebeer et al. 2012) or anti-inflammatory effects (Kim et al. 2014; Kaji et al. 2010).

We have recently sequenced the genome of *L. acidophilus* ATCC 4356 (Palomino et al. 2015), a strain whose probiotic nature has been well documented (Resta-Lenert and Barrett 2003). Similarly to the NCFM strain (Goh et al. 2009), we found three S-layer-protein-coding genes: *slpA*, *slpB*, and *slpX*. Ramiah et al. (2009) have shown an increased expression of the *slpA* gene when grown in GIT conditions. However, their study was performed in actively growing cells, a condition that is not found in the GIT where cell division is expected to be low, resembling a stationary phase state. Therefore, when analyzing S-layer and envelope structure biosynthesis that occurs mainly during cell division, they should be evaluated both in the exponential and stationary phases to be able to mimic the GIT environment.

In this work, we studied the influence of the growth phase and osmotic stress on the profile and gene expression of S-layer proteins in the adaptation of *L. acidophilus* ATCC 4356 to high-salt medium.

Materials and methods

L. acidophilus ATCC 4356 and *L. casei* BL23 were grown at 37 °C in de Man, Rogosa and Sharpe (MRS) medium (low salt control conditions). MRS medium (Biokar, Beauvais, France) pH = 6.5 contains 10 g tryptone l^{-1}, 4 g yeast extract l^{-1}, 8 g meat extract l^{-1}, 5 g Na acetate l^{-1}, 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 0.05 g $MnSO_4 \cdot 4H_2O$ l^{-1}, and 1 ml Tween 80 l^{-1} and 20 g glucose l^{-1}. The stress condition was obtained by adding 0.3, 0.4, 0.45, 0.6, or 0.8 M NaCl (high-salt conditions).

Purified S-layer proteins from *Lactobacillus* cultures grown in MRS medium at 37 °C were extracted by using 5 M LiCl, then extensively dialyzed against distilled water overnight at 4 °C and, after centrifugation (10,000×*g*, 20 min), suspended in sterile phosphate-buffered saline (PBS) and stored at −20 °C.

Cells from cultures grown in control or high-salt medium were peeled from their S-layer by treatment with 5 M LiCl. After removal of LiCl by washing with physiological solution, cells were inoculated in MRS medium at 37 °C with or without NaCl and their growth was monitored by optical density at 600 nm (OD600) and viable counts.

Whole cells (20 μl OD600 = 5 overloaded for western blot or 10 μl OD600 = 1 for Coomassie staining or S-layer preparations (20 μg) were heated at 90 °C for 5 min in loading buffer (10 % glycerol, 4 % sodium dodecyl sulfate (SDS), 4 M urea, 2 % β-mercaptoethanol, and 0.05 % bromophenol blue) and subjected to electrophoresis in 12.5 % SDS-PAGE. Gels were stained with Coomassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO, USA). Zymography was performed as described in Prado-Acosta et al. (2008). Polyacrylamide gels were cast with 0.01 % SDS. After the run, hydrolase activity was detected by a clear zone.

For western blot analysis, gels were electrotransferred with a semi-dry blotter (Amersham Biosciences, Chicago, USA) to PVDF membranes (Macherey-Nagel, Düren, Germany) soaked with mouse polyclonal antibodies against the S-layer (diluted 1:5000) (Prado-Acosta et al. 2008) and visualized with biotin-conjugated anti-mouse antibody followed by streptavidin-HRP conjugate (Pierce Biotechnology,

Rockford, IL, USA). Chemiluminescence was detected with luminol substrate ECL (Amersham, GE Healthcare, Chicago, USA). Images were obtained with a G:Box Chemi XT4 digitalizer (Syngene, Cambridge, UK).

Supernatants of exponential and stationary phase cultures were concentrated with Centrisart I SM132 49 devices with 20-kDa cutoff (Sartorius, Göttingen, Germany) by centrifugation at 2500×*g* to achieve a 20-fold concentration. Equal volumes were loaded on SDS-PAGE 12.5 %, and western blot was performed of each condition.

qPCRs of *slpA*, *slpB*, and *slpX* gene transcription were verified in exponential and stationary phase conditions for control (MRS) and high salt (0.45 and 0.6 M NaCl) from complementary DNA (cDNA) samples, using MyiQ real-time thermal cycler (Bio-Rad, Berkeley, CA, USA). cDNA was synthesized with 25 pmol reverse primers of each gene and 3 mg of RNA. The mixtures were incubated at 95 °C for 10 min, kept for 2 min on ice, and incubated with 100 U of MMLV reverse transcriptase (Promega, Fitchburg, WI, USA), according to the manufacturer's recommendations. For RNA extraction, the hot phenol method was used (Palomino et al. 2013), using the following gene-specific primers designed from unique nucleotide sequences not shared by the *slp* genes, as shown in Supplemental Fig. S1 and Table S1. Forward primers were as follows: 5′-GCGAAGGCGGCTGTCTGG-3′ for *16S* ribosomal RNA (rRNA), 5′-CGGGATCCATGAA GAAAAATTTAAG-3′ for *slpA*, 5′-AGGCCATACTGAAC TT-3′ for *slpB*, and 5′-ATCAAACTGCAACCATTC-3′ for *slpX*. Reverse primers were as follows: 5′-GGCACTGAAG GGCGGAAACC-3′ for *16S rRNA*, 5′-GTTGGTATTGA TTGCTGATG-3′ for *slpA*, 5′-AGTAGCTGAAACCTTG-3′ for *slpB*, and 5′-ACTTGGAATACTTGTCC-3′ for *slpX*. The concentration of the purified RNA was determined using a Nanodrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The 16S rRNA gene was used as reference. Results were analyzed using the critical threshold (ΔC_T) and the comparative critical threshold ($\Delta\Delta C_T$) method in the IQTM 5 Optical System Software (http://www.bio-rad.com/en-us/product/iq5-optical-system-software-version-2-1). Each sample was analyzed in triplicate, and samples from two independent experiments were analyzed for each condition. $\Delta\Delta C_T$ for *slp* genes related to 16S ribosomal housekeeping gene was plotted as relative expression between control and high-salt conditions.

Cell wall purification fractions were prepared using the procedure described in Piuri et al. (2005) from cell cultures grown in MRS medium (low salt control conditions) or MRS medium containing 0.6 M NaCl (high-salt conditions). Briefly, cell walls obtained from bacterial cells were treated by heating at 100 °C in 4 % (*w*/*v*) SDS for 60 min, the non-lysed cells were removed by a short centrifugation step (1500×*g* 5 min), and the cell walls were recovered by centrifugation at 20,000×*g* 20 min. Then, cell walls were washed five times in water and thereafter incubated with DNAse (30 μg/ml) and RNAse (5 μg/ml) for 2 h at 37 °C, followed by 0.5 mg trypsin ml^{-1} at 37 °C for 16 h in 0.1 M Tris–HCl (pH 7.5). Peptidoglycan was obtained by treating cell walls overnight at 4 °C with 10 % (*w*/*v*) trichloroacetic acid to eliminate wall teichoic acid polymers and then washing three times with water. LTA was purified using the 1-butanol extraction method (Palomino et al. 2013).

For the sequential degradation of amino acids from proteins and peptides, we used the Edman degradation method (1949). In this reaction, the N-terminus reacts with phenylisothiocyanate in a basic medium. The derivatized amino acid is identified by reverse-phase liquid chromatography, with detection at 269 nm. This identification is performed by comparison of the retention time with a standard containing all the 20 common derivatized amino acids in a Shimadzu sequencer PPSQ-31 (Kyoto, Japan) at the LANAIS-PROEM facility, CONICET, Buenos Aires, Argentina.

The electrophoretic bands obtained in SDS-PAGE corresponding to S-layer protein were cut, destained in acetonitrile (ACN)/20 mM NH_4HCO_3 pH 8.5 (1:1), dried at room temperature, rehydrated with 25 mM NH_4HCO_3, reduced with 10 mM dithiothreitol, and alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3. Tryptic digestion was performed incubating each slice with 120 ng of trypsin (Promega sequencing grade modified) in 20 mM NH_4HCO_3 pH 8.5 overnight at 37 °C. Peptides were extracted by incubating each slice with 50 % acetonitrile (ACN) 0.5 % trifluoroacetic acid (TFA). Aliquots of the extracted peptides were mixed 1:1 with the matrix (3 mg/ml 4-hydroxy-α-cyanocinnamic acid in 50 % ACN 0.5 % TFA), loaded onto MTP Anchor Chip Target (Bruker Daltonics, Billerica, MA, USA) for co-crystallization, and analyzed in an Ultraflex II MALDI-TOF/TOF mass spectrometer for mass spectrometry (MS) and MS/MS analysis in the MS facility of the Centro de Estudios Químicos y Biológicos por Espectrometría de Masa (CEQUIBIEM), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina. The data were acquired in reflector mode from a mass range of 700 to 4000 Da, and 1250 laser shots were averaged for each mass spectrum. Each sample was internally calibrated with trypsin autolysis and keratin peaks. The peak list generated was based on signal-to-noise filtering and an exclusion list. The resulting file was then searched by Mascot (Matrix Science, Boston, MA, USA; http://www.matrixscience.com/search-form-select.html) with database search parameters, including a mass tolerance of 20–100 ppm, one missed cleavage, oxidation of methionines, and carbamidomethylation of cysteines. Only proteins matched with significant scores ($p < 0.05$) were considered. The hit obtained was validated by MS/MS fragmentation of one or two high S/N peaks per sample.

Results

S-layer content of *L. acidophilus* ATCC 4356 under high-salt conditions

Since we have previously observed the importance of the envelope composition in the adaptation to salt stress of several gram-positive species and strains, here, we studied a *Lactobacillus* strain endowed of S-layers. Klaenhammer et al. (2005) reported that an *slpA* mutant of *L. acidophilus* NCFM is sensitive to NaCl and ethanol and resistant to bile. Here, we studied a different strain (*L. acidophilus* ATCC 4356) and analyzed its response to salt stress during all the growth phases. The S-layer proteins from whole cells were analyzed by SDS-PAGE and western blot. In control conditions, we found only one predominant protein, whereas in high salt, we found two S-layer bands (Fig. 1a). We verified the immunological identity of both protein bands by detection with antibodies against SlpA in western blot (Fig. 1b) and zymogram analysis, which showed peptidoglycan hydrolase activity (Fig. 1c) (Prado-Acosta et al. 2008). As shown in Fig. 1a, for the control condition and the exponential phase, the predominant S-layer protein corresponded to a molecular weight (MW) of 45.9 kDa, representing more than 90 % of the S-layers and a 49.4-kDa S-layer protein representing less than 10 %. In contrast, in high-salt and stationary phase, we observed an increase in the representation of the 49.4 kDa protein. One possible explanation for the 3.5-kDa difference between the two immunologically detected bands could be the processing of the N-terminal 31-amino-acid signal peptide of the 444-amino-acid SlpA protein (predicted ProtParam-ExPASy, http://web.expasy.org/protparam/, MW 46569.8 Da). The S-layer is translocated through the membrane to the extracellular medium by a 31-amino-acid signal peptide, which is cleaved to give a mature S-layer protein, resulting in about 3-kDa difference (Sára and Sleytr 2000). We next sequenced the N-terminus of the 45.9 kDa protein extracted with 5 M LiCl through the Edman degradation method (1949) and found that the processed protein is actually 413 amino acids long, beginning with the sequence ATTINA, having positive

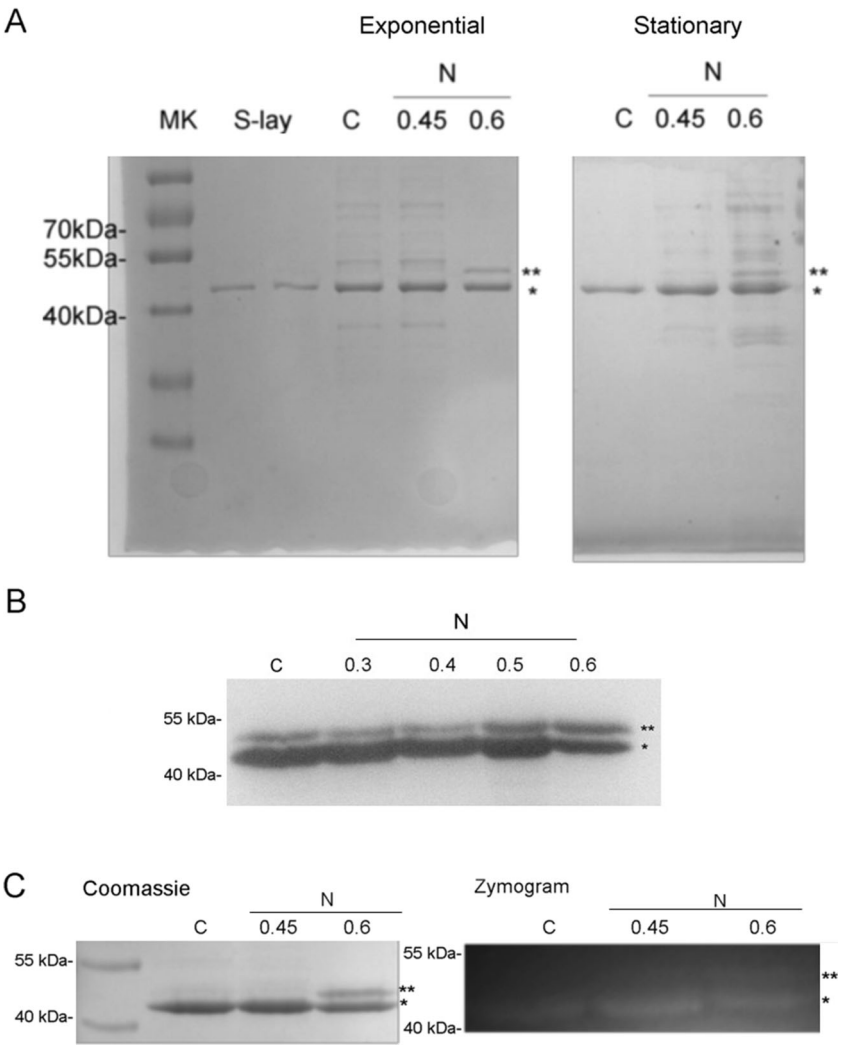

Fig. 1 A 12.5 % SDS-PAGE profile of whole-cell proteins (10 μl OD600 = 1) (**a**), western blot (20 μl OD600 = 5) (**b**), and zymogram analysis of whole-cell proteins (**c**) from *Lactobacillus acidophilus* ATCC 4356 grown under different conditions: control condition in MRS (*C*) or with NaCl (*N*, numbers express molarities) from the exponential and stationary phases. *Double* and *single asterisks* show the 49.4 and 45.9 kDa protein, respectively

charge (pI 9.49) and being still insoluble in aqueous solution by interaction between monomers as described for other S-layers (Avall-Jääskeläinen et al. 2008) with a predicted ProtParam MW (ExPASy) of 43,635.3 Da. Therefore, a reduction of the processing of the signal peptide in the high-salt growth condition might explain the two bands observed. However, no intracellular increase in the precursor accumulated was verified from sonicated whole cells analyzed by western blot (data not shown). When both proteins were analyzed by MS (Supplemental Fig. S2 and Table S2), the predominant SlpA and the auxiliary SlpX were identified. Increasing NaCl in the growth medium changed the S-layer pattern: at 0.6-M NaCl concentration, the proportion of SlpA was up to 60 %, whereas that of SlpX increased to 40 % (Fig. 1b). The 49.4 kDa protein might result from the processing of the signal peptide of SlpX 499-amino-acid protein (ProtParam predicted 54,194 Da), giving a 467-amino-acid protein with a predicted ProtParam MW of 50,946.2 Da, although the discrepancy between the estimated and predicted MW observed would probably be due to an amino acid modification or to its cationic nature. In fact, sequencing of the N-terminus of SlpX was not possible due to a probable modification.

High salt increases *slpA* and *slpX* gene expression

Three *slp* genes, *slpA*, *slpB*, and *slpX*, are annotated in the genome of *L. acidophilus*. Since the three proteins have high amino acid sequence similarity (Supplemental Fig. S1), immunological cross-reactivity is expected and observed. The discrepancy between the estimated and predicted MW is probably due to an amino acid modification and to the cationic nature of these proteins. Therefore, we focused on analyzing the gene expression of *slpA*, *slpB*, and *slpX* to confirm the switch in high salt. The expression of *slpA* and *slpX* increased as the salt concentration of the culture medium increased, whereas that of *slpB* remained silent (Fig. 2), in concordance with previous reports where attempts to demonstrate expression of the *slpB* gene were unsuccessful (Boot et al. 1996; Pouwels et al. 1997).

To verify whether the transcription and translation of the S-layer genes and proteins in the high-salt condition are directly correlated (Fig. 1a), we analyzed the S-layer titer by immunological detection, in each growth condition obtained from the dot blot analysis. The high-salt conditions (0.45 and 0.6 M NaCl) in stationary phase gave the highest titers between 128 and 256, compared to 64 for all the other conditions (Supplemental Fig. S3). However, the transcription level was higher than the protein content. Thus, the correlation seems not to be completely strict in the case of the S-layer protein content, suggesting that the cell wall composition, structure, or additional factors may be involved.

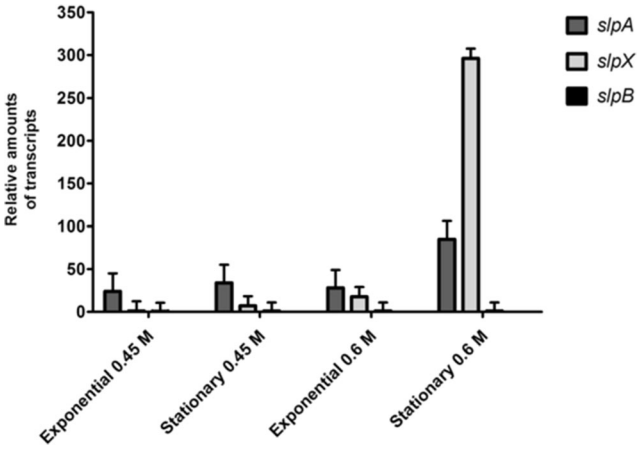

Fig. 2 Expression of *slp* genes by qPCR. Relative transcription between high-salt and control conditions is plotted. *Bars* express the level of expression for each gene normalized to that of rRNA 16S for each condition. *Bars* show standard errors from three independent experiments with duplicate samples

Modification of the cell wall by high salt

We have previously reported modifications of the cell wall structure of *L. casei* in high-salt conditions (Piuri et al. 2005; Palomino et al. 2013). The increase in the S-layer proteins in high-salt medium, involving active transcription of S-layer genes, could be due to a direct stress or to the response of the genes, to the modification of the cell wall (needing more S-layer to counteract its fragility), or to both. Therefore, here, we analyzed the cell wall and peptidoglycan in control and high-salt growth conditions. SDS extraction of total cell wall was significantly lower for *L. acidophilus* than for the non-S-layer-forming *L. casei* species. Cell wall yield obtained from *L. acidophilus* was almost one third and one fifth of that obtained from *L. casei* in control and high-salt growth conditions, respectively (Table 1). In fact, *L. acidophilus* cells grown in high salt showed peptidoglycan to be highly fragile, as verified by rapid autolysis upon successive water washes, with an almost 2-fold decrease in the peptidoglycan layer

Table 1 Cell envelope structure of *Lactobacillus acidophilus* ATCC 4356 in the presence or absence of 0.6 M NaCl and *L. casei* BL23

Sample[d]	CW[a]	PG[b]	LTA[c]
L. acidophilus	15.0 ± 1.0 mg	7.5 ± 0.8 mg	0.23 ± 0.02 mg
L. acidophilus NaCl 0.6 M	10.5 ± 1.0 mg	4.5 ± 0.5 mg	0.08 ± 0.01 mg
L. casei	50.0 ± 3.0 mg	35.0 ± 2.0 mg	0.56 ± 0.03 mg

CW cell wall, *PG* peptidoglycan, *LTA* lipoteichoic acid

[a] Insoluble material from cells extracted with 4 % SDS at 100 °C

[b] Insoluble material from cell walls extracted with 10 % *trichloroacetic acid*

[c] Measured as phosphorus from butanol-extracted cells

[d] Results are expressed per gram of dry weight

when compared to the control condition. LTA mass recovery from strains grown in high-salt conditions was 3-fold lower than that in the control condition (Table 1), showing the same adaptation as that described in *L. casei* (Palomino et al. 2013).

A decrease in peptidoglycan might favor the detachment of the S-layer to the growth medium. To verify whether this was true, we determined the amount of S-layers in the supernatant. Western blot analysis of concentrated supernatants showed increased release of S-layer protein to the supernatant in high-salt stationary phase compared to the exponential phase (Fig. 3). This release to the supernatant might partly explain the fate of the protein synthesis by the increased transcription.

Growth and S-layer formation

To verify the influence of the S-layer proteins in the growth capacity in high-salt condition, we compared the growth of the wild-type strain in control medium to that of a strain subjected to the removal of the S-layer with 5 M LiCl. Optimum removal with 5 M LiCl rather than with 1 M LiCl was verified (Fig. 4a). This strategy had been previously used to verify the influence of surface properties in the adhesion capacity and survival to gastric juice conditions for other *L. acidophilus* strains (Kos et al. 2003; Frece et al. 2005). This strategy did not reduce their viability or viable counts correlated with the growth rates (data not shown). However, severe growth defects were observed as salt concentration increased (Fig. 4b). Removal of the S-layer by LiCl treatment caused a delayed growth, even in the absence of NaCl stress, since the treatment may remove, denature, or affect other cell envelope components. Thus, although a differential behavior between control growth conditions and extremely high salt (0.8 M NaCl) is clearly observed, suggesting the influence of the S-layer, the growth defects observed at high NaCl in S-layer-depleted cells cannot be attributed only to the role of the S-layer in the osmoadaptive stress response. As shown in Fig. 4c, delayed recovery of S-layer synthesis at high salinity was correlated with the delayed growth observed under osmotic stress.

Discussion

It has been proposed that the S-layer plays a role as a protective sheath and that it may protect the bacterial cell from various environmental factors such as mechanical and osmotic stresses in *Lactobacillus* (Hynönen and Palva 2013; Gerbino et al. 2015). The need for the cell to increase S-layer gene expression is related to the integrity of the cell envelope structure. Although a correlation between transcription and translation is expected, this was not completely observed in the case of the S-layer proteins. What we found was that a great proportion of the S-layer protein is released to the supernatant. Nevertheless, we cannot discard an increased proteolytic activity in high-salt conditions as that described for *L. casei* (Piuri et al. 2003), which might influence the final concentration of protein located on the surface. This probable event, together with the shedding release to the supernatant, explains that transcription–translation results did not correlate strictly.

The requirement of the S-layer for growth even in normal conditions in the strains carrying S-layer like *L. acidophilus* could be explained by the 3-fold lower amount of peptidoglycan found when compared to a non-S-layer producer as *L. casei* (Table 1). Under high-salt conditions, a 2-fold decrease in peptidoglycan and increased fragility determined the need of the external highly compact S-layer component. The fragility of the cell walls of *Lactobacillus* strains carrying S-layers may be due to the presence of the S-layer or, otherwise, seems to force these strains to be dependent on the presence of the S-layer for growth. In fact, analysis of exoproteome profiles comparing *Lactobacillus* S-layer-containing species with non-forming species showed very few proteins isolated from the non-S-layer-forming species and increased extracellular proteins obtained by LiCl extraction from S-layer-containing species (Johnson et al.

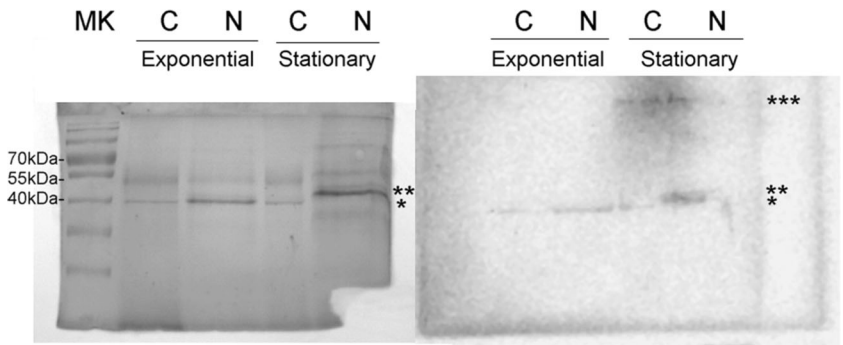

Fig. 3 A 12.5 % SDS-PAGE profile (**a**) and western blot (**b**) of 20-fold concentrated supernatants recovered from *Lactobacillus acidophilus* ATCC 4356 grown under different conditions: control condition (*C*) or with NaCl (*N*, 0.6 M NaCl) from exponential and stationary phases. *Double* and *single asterisks* show the 49.4 and 45.9 kDa protein, respectively. The *triple asterisks* show the position of S-layer multimers

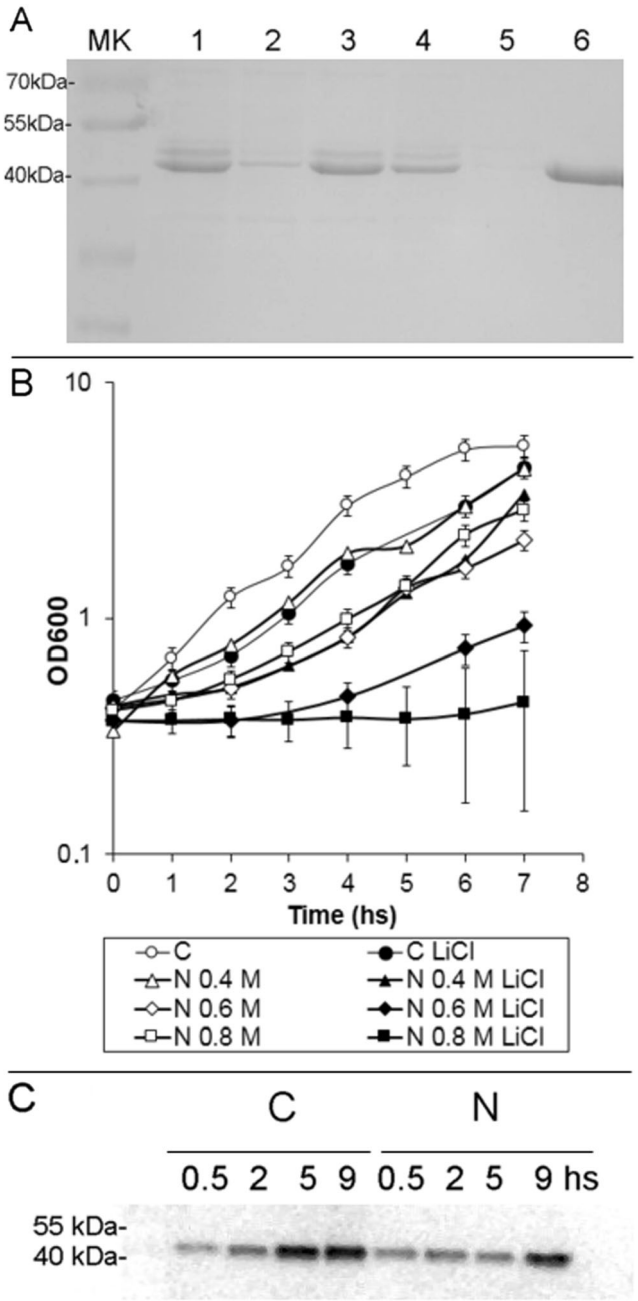

Fig. 4 A **a** 12.5 % SDS-PAGE profile of whole cells extracted with different LiCl concentrations: MW markers (*MK*), whole cells grown in NaCl 0.6 M (*lane 1*), 1 M LiCl-extracted proteins in NaCl 0.6 M condition (*lane 2*), 5 M LiCl-extracted proteins in NaCl 0.6 M condition (*lane 3*), control cells (*lane 4*), 1 M LiCl-extracted control cells (*lane 5*), 5 M LiCl-extracted control cells (*lane 6*). **b** Growth curves of *Lactobacillus acidophilus* ATCC 4356 with (*empty symbols*) and without (*filled symbols*) S-layer. Cells were grown in control medium (*C*) and high-salt medium (*N* = 0.4, 0.6, or 0.8 M NaCl). *Error bars* represent standard errors from three replicate experiments. **c** Effect of NaCl in S-layer synthesis. Cells of *L. acidophilus* ATCC 4356 were treated with LiCl 5 M to remove the S-layer and then recovered in control medium (*C*) or salt medium (0.6 M NaCl). Aliquots (15 μl) were taken at different times and loaded to 12.5 % SDS-PAGE and western blot detection

2015). This observation suggests that an increased association of proteins to the surface layer might participate in maintaining cell wall stability, probably due to the fragility associated with fewer peptidoglycan layers in other S-layer-containing species as that observed for *L. acidophilus*. This hypothesis is the aim of our future work.

As we have described earlier, an *slpA* mutant of *L. acidophilus* NCFM has been reported to be osmosensitive (Klaenhammer et al. 2005). In addition, it has been suggested that the auxiliary S-layer component SlpX of *L. acidophilus* NCFM affects the permeability of the S-layer as the *slpX*-negative mutant is more susceptible to SDS and more resistant to bile than the wild type (Goh et al. 2009). Challenging of the *slpX*-negative mutant with 10 % NaCl gave no significant difference in the survival rate, probably because the absence of one *slp* gene was compensated by the overexpression of another (Goh et al. 2009). The presence of multiple S-layer protein genes in the same strain is a common feature in lactobacilli. The strict requirement of the S-layer for growth in normal conditions has been stated by Goh et al. (2009); even if an *slp* mutant was obtained in the *L. acidophilus* NCFM background, the absence of one *slp* gene was compensated by the overexpression of another *slp* not usually expressed. A knockout mutant of *slp* genes of *L. acidophilus* NCFM expressed *slpA* and *slpX* or *slpB* and *slpX* simultaneously (Goh et al. 2009). This argues in favor of the statement by Hynönen and Palva (2013) based on their unpublished results about the difficulty or impossibility to create completely S-layer-negative *Lactobacillus* mutants, indicating that at least one functional S-layer protein gene is essential for the growth of S-layer-carrying lactobacilli. Therefore, since double mutants are probably non-viable (Hynönen and Palva 2013), it is difficult to firmly conclude that S-layers are essential for the osmoadaptation of *L. acidophilus* ATCC 4356.

As shown in the present study, S-layer proteins are present during all growth phases and the genes are preferentially expressed in the stationary phase and in the presence of 0.6 M NaCl (Figs. 1 and 2). Schär-Zammaretti et al. (2005) showed that the expression of the S-layer protein gene of *L. acidophilus* NCC 2628 is induced when the strain is grown under conditions of limited protein supply. Other authors have shown that S-layer production in *L. acidophilus* is increased under other stress conditions (Khaleghi et al. 2010; 2011). However, these authors used mild stress conditions (1 to 3 % NaCl) and focused only on *slpA* gene expression, without analyzing *slpX*. In the present study, we found that increasing NaCl concentration over 0.6 M (3.5 % *w*/*v*) is required to verify the role of the auxiliary S-layer component SlpX. Similarly, in *Lactobacillus brevis* ATCC 14869, Jakava-Viljanen et al. (2002) found that the differential expression of the *slpB* and *slpD* genes is related to the oxygen content of the growth medium and the growth stage.

This upregulated expression of *slpA* and *slpX* in high-salt conditions agrees with an increase in S-layer protein synthesis, modifying the proportion between SlpX and SlpA in the S-layer profile in high-salt growth conditions. In fact, our results show a switch to increase the quantity of S-layer proteins due to the high-salt conditions, rendering a modified S-layer profile between SlpX and SlpA when both conditions are compared. This modified S-layer profile resembles the developmental switch or antigenic variation of S-layer protein synthesis in *Bacillus anthracis* (Fouet 2009; Mignot et al. 2002). Since it has been verified that the inversion of the *slp* segment between *slpA* and *slpB* takes place only in very few cells (Boot et al. 1996), it is not surprising that *slpB* was not detected in the present study.

We also found that the processing of the signal peptide of the SlpA protein renders a 413-amino-acid mature protein beginning with the sequence ATTINA, as described for other *Lactobacillus* species (Lortal et al. 1992; Vidgrén et al. 1992; Sillanpää et al. 2000; Ventura et al. 2000; Sára and Sleytr 2000; Hynönen et al. 2014).

Recently, the adaptation of probiotics to stress conditions has been analyzed (Khaleghi 2010; 2011) and reviewed (Mills et al. 2011), and results have shown that general stress response, DNA repair, and energy metabolism favor increased survival during exposure to stressful conditions. We propose, for biotechnological applications, that pre-growth of lactobacilli in high-salt conditions would result in an advantage for the probiotic nature of cells: the increased production and release of the S-layer might be appropriate for their antimicrobial capacity (Prado-Acosta et al. 2008, 2010; Martínez et al. 2012; Meng et al. 2015). Also, the reduction of the LTA content needs to be evaluated for possible anti-inflammatory (Kim et al. 2014; Kaji et al. 2010) or inflammatory effects (Lebeer et al. 2012; Mohamadzadeh et al. 2011; Zadeh et al. 2012). This is necessary to determine the benefit of pre-growth in high-salt conditions for the design of a probiotic formulation. We are currently evaluating the influence of high-salt stress on the expression of LTA genes in *L. acidophilus* and the survival to gut-simulated conditions of cells pre-grown in high-salt conditions.

In summary, in this work, we found that S-layer proteins are involved in the adaptation of *L. acidophilus* to osmotic stress. We showed, for the first time, an increased expression of the SlpX protein due to high-salt conditions, accompanying the predominant S-layer protein SlpA. Our results suggest that a variation in the pattern of S-layer expression in stress conditions (salt growth phase) would be linked to the overall stress responses of this species. Moreover, the importance of the fragility of the cell wall observed for *L. acidophilus*, when compared to other non-S-layer-forming *L. casei*, would strongly support the need of this additional envelope.

The transcriptional and translational regulation mechanisms of *Lactobacillus* S-layer protein genes on a molecular level are unexplored. Overexpression of *slp* genes might be attributed to the release of a genetic regulation, and this will be the aim of our future investigations.

Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of interest The authors declare that they have no competing interests.

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