

# S-layer production by *Lactobacillus acidophilus* IBB 801 under environmental stress conditions

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**Abstract** The ability of microorganisms to synthesize S-layer, the outermost structure of the microbial cell envelope composed of non-covalently bound proteins, has been ascribed to help microorganisms to exert their probiotic properties in the host. In this work, formation of S-layer by the potentially probiotic strain *Lactobacillus acidophilus* IBB 801 under different stress culture conditions (high incubation temperatures, presence of bile salts or NaCl, and acidic pH) was assayed. A marked S-layer synthesis by *L. acidophilus* IBB 801 was detected when the strain was grown at 42 °C and in the presence of 0.05 % bile salts or 2.0 % NaCl. The presence of S-layer proteins was further confirmed by transmission electron microscopy and protein identification by MS/MS. The differential expression of the proteome of this strain at 42 °C, when a marked formation of S-layer was detected, revealed the over-expression of six proteins mainly related to general stress and protein biosynthesis and translation, while four proteins detected in lower amounts were involved in DNA repair and energy metabolism. As *L. acidophilus* IBB 801 produces both a bacteriocin and S-layer proteins, the strain could be of interest to be used in the formulation of functional food products with specific properties.

**Keywords** S-layer proteins · Lactobacilli · Proteomics · Stress

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## Introduction

Lactobacilli are a diverse group of food-grade microorganisms widely applied in the fermented food industry due to their technological and health-promoting properties; these bacteria have been extensively used as starter cultures and as probiotics (Patten and Laws 2015; Leroy and De Vuyst 2004).

Besides gut indigenous lactobacilli, several *Lactobacillus* strains from fermented food products have shown beneficial effects on the gut health (Fuller 1992). In addition, certain strains are able to control undesirable microorganisms not only in the gastrointestinal tract (Hickson 2011) but also in other sites of the human body such as the urogenital tract (Collado et al. 2005; Strus et al. 2005), the oral cavity (Haukioja 2010), and the upper respiratory tract (Kechaou et al. 2013).

Surface properties of lactic acid bacteria (LAB) are relevant in their adhesion to the gastrointestinal epithelium, a fact that is considered a pre-requisite for the exclusion of enteropathogenic bacteria (Mack et al. 1999) and immunomodulation of the host (Blum et al. 2002). Several species of *Lactobacillus*, including mucosal-associated (e.g. *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus*, and *Lactobacillus gallinarum*) and dairy fermentation-associated species (e.g., *Lactobacillus helveticus* and *Lactobacillus kefiranofaciens*) are able to form S-layers (Åvall-Jääskelläinen and Palva 2005; Hynönen and Palva 2013), which constitute the outermost structure of the cell envelope composed of a crystalline array of single non-covalently bound proteins. These proteins are involved in important cell functionalities such as acting as a protective barrier against environmental hazards, controlling the transfer of nutrients and metabolites, maintaining the cell shape and envelope rigidity, and promoting cell adhesion and surface recognition, among others (Vidgren et al. 1992; Buck et al. 2005). In some

*Lactobacillus* species, S-layer proteins mediate bacterial adherence to host cells or to the extracellular matrix (Hynönen et al. 2014). In *L. acidophilus* NCFM, the SlpA protein, one of the three major S-layer proteins of this microorganism, plays a role in adhesion to Caco-2 intestinal epithelial cells in vitro and modulates dendritic cell and T cell functionalities with murine dendritic cells (Johnson et al. 2013). S-layer proteins from *Lactobacillus* are biochemically unique: they do not possess SLH domains (Boot et al. 1996), they are smaller (25–71 kDa) than those of other Gram-positive bacteria, and they are non-glycosylated and highly basic with pI values ranging from 9.35 to 10.4 (Åvall-Jääskelläinen and Palva 2005).

S-layer proteins associated to the peptidoglycan layer of the microbial cell wall can generally be disassembled but not denatured using chaotropic agents such as LiCl or guanidine HCl. Thus, the surface proteins can be separated from cells and media and reassembled in vitro (Sampathkumar and Gilchrist 2004). By means of modern, ultra-structural analysis tools, an accurate image of the assembled layers can be obtained; this study being fundamental for their nano- and biotechnological applications (Sleytr et al. 2001).

There is increasing evidence that S-layer-carrying bacteria may express alternative S-layer protein genes for adaptation to different stress factors such as the immune response of the host for pathogens and to drastic changes in the environmental conditions for non-pathogens (Sára and Sleytr 2000; Scholz et al. 2001; Jakava-Viljanen et al. 2002). In addition, it has been suggested that the surface properties of microorganisms are dependent on the growth conditions and the composition of the fermentation medium (Schär-Zammaretti et al. 2005). However, the relationship between the fermentation and processing (stress) conditions to which microorganisms are exposed during the elaboration of (fermented) food products and the changes in bacterial surface has not been studied in detail. In this study, we aimed to investigate the presence of S-layer proteins in the bacteriocin-producing strain *L. acidophilus* IBB 801 (Zamfir et al. 1999) under stressful culture conditions; proteomic assays were further carried out to gain insight into the bacterial response at 42 °C when S-layer protein production was improved.

## Materials and methods

### Bacterial strains and culture conditions

The strain *L. acidophilus* IBB 801 used in this study, isolated from a Romanian artisan dairy product, has been deposited in the Romanian Collection of Industrial Microorganisms (CMII-ICCF-WFCC 232) under the accession number ICCF 416. The strain was kept at –85 °C in MRS medium in the presence of 25 % (v/v) glycerol as cryo-protectant. To obtain

fresh cultures from the freeze-dried stocks, the strain was transferred (2 % v/v inoculum) twice in MRS broth and incubated at its optimal growth temperature for 24 h or otherwise stated.

### S-layer formation by SDS-PAGE analysis

One milliliter of an overnight culture ( $OD_{600\text{nm}}$  3.0) from the studied strain was centrifuged ( $10,000g \times 10 \text{ min}$ , 4 °C), and the cell pellet washed with distilled water and resuspended in 50  $\mu\text{l}$  Laemmli (1970) buffer. The suspension was heated at 100 °C for 5 min, centrifuged again, and then checked for the presence of S-layer proteins by loading 20  $\mu\text{l}$  in one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were prepared according to Laemmli (1970); briefly, 12 and 4 % (w/v) polyacrylamide were used for the running and stacking gels, respectively. Electrophoresis was conducted in a Compact Dual Plate apparatus V20-CDC (Scie-Plas Ltd., UK) at a constant voltage of 90 V in the stacking gel and of 180 V in the running gel for 2 h. Broad-range protein molecular weight (MW) marker (10–225 kDa; Promega, USA) was used as reference. Gels were stained with Coomassie Brilliant Blue R250 (Carl Roth GmbH, Germany) to visualize the bands. The MW of the bands was calculated by comparison of the mobility of the bands of the MW marker in the gel.

### Growth and S-layer production under different stress conditions

The effect of stress culture conditions on the cell growth and production of S-layer proteins by *L. acidophilus* IBB 801 was studied. Active cultures of the strain were subjected to different growth conditions such as incubation temperature (37 and 42 °C), initial pH (4.5 and 6.2), presence of NaCl (0.6, 2.0, and 3.0 % w/v, final concentration), and bile salts (0.05, 0.1, and 0.2 % w/v, Sigma-Aldrich Chemie GmbH, Germany). Cell growth and survival under the control (37 °C, pH = 6.2, and MRS without NaCl or bile salts) and the mentioned stress culture conditions were followed by measuring the pH, optical density of the cultures at 600 nm ( $OD_{600\text{nm}}$ ), and cell count (CFU/ml) on solid MRS (MRS plus 1.5 % w/v agar) after 24 h of incubation unless otherwise stated. The experiments were done in triplicate and results given are the mean of the three values with SD. The presence of S-layer proteins was checked by SDS-PAGE as described earlier.

### Extraction of S-layer proteins

For the isolation of S-layer proteins, a modified method of Lortal et al. (1992) was used. Briefly, bacterial biomass from 50 ml MRS cultures subjected to different growth conditions were harvested, separately, by centrifugation (10,

000g × 10 min at 4 °C), and washed three times with pH 7.4 phosphate-buffered saline (PBS). The cells were resuspended in 5 ml PBS buffer and S-layer was extracted after adding 5 ml of 5 M LiCl and maintaining at 37 °C for 2 h. Samples were centrifuged and the supernatants containing the S-layer proteins were transferred to a Vivaspın 6 ultrafiltration module with a 10-kDa MM cut-off (Sartorius Stedim Biotech, Goettingen, Germany). Ultrapure Milli-Q water was added to the retentate to 5 ml final volume and then centrifuged again (10,000g × 10 min at 4 °C). Finally, the obtained retentate was transferred to a falcon tube, centrifuged (10,000g × 10 min at 4 °C). The sediment (containing the S-layer proteins) was resuspended in Milli-Q water and analyzed by SDS-PAGE.

### Transmission electron microscopy (TEM)

S-layer formation by *L. acidophilus* IBB 801 was visualized by TEM using an adapted method of that described by Hayat (1972). Bacterial cells were fixed in 3 % (v/v) glutaraldehyde (Merck, Darmstadt, Germany) at 4 °C for 24 h. After fixation, samples were washed in cacodylate buffer, included in agar and washed four times with the same buffer for about 1 h and post-fixed overnight in 1 % (w/v) OsO<sub>4</sub> (SERVA Electrophoresis GmbH, Heidelberg, Germany) at 4 °C. The samples were washed in distilled water and dehydrated in an ethanol series of 10, 30, 50, and 70 % (v/v) and twice in absolute ethanol (1 h in each change). Then, the samples were cleared in propylene oxide, embedded in epoxy resin, and cut with an ultra-microtome (LKB ULTRATOME III®, Sweden). Thin sections were placed on a metal grid (Sigma-Aldrich) and stained using lead citrate and uranyl acetate according to Reynolds (1963). A transmission electron microscope JEOL JEM-1400 (electron microscope, Japan) was used to visualize the cells and S-layer proteins.

### Proteomic analysis

To gain insight in the bacterial response to a selected stress condition, the proteome of *L. acidophilus* IBB 801, which showed a marked S-layer formation under control (37 °C) and stressful (42 °C) culture conditions, was analyzed. The cultures grown at both temperatures were centrifuged and washed three times with 0.1 M Tris-HCl pH 6.8 and the cells resuspended in the same buffer. Protein extracts were obtained by sonication using a LabSonicM apparatus (Sartorius, Germany) at 80 % power, using 4 cycles of 2 min maintaining 1 min in ice between sonications. Cell debris were removed by centrifugation (10,000g, 10 min at 4 °C) and protein concentrations were determined spectrophotometrically using the method of Bradford (1976). The samples were frozen at –75 °C for 24 h and then freeze-dried in a freeze-dryer (CHRIS ALPHA 1-4 LD plus, Germany) at –50 °C, 0.022 mBar for 48 h. The lyophilized samples were

resuspended in adequate volume of 0.1 M Tris-HCl pH 6.8 to reach 600 µg of total protein content for each sample.

### Two-dimensional electrophoresis (2DE)

Sample preparation and 2DE gels were carried out according to Fadda et al. (2010) with some modifications. Briefly, samples containing 600 µg of proteins from *L. acidophilus* IBB 801 were treated with 1 µl of bezonase (Novagen®) at 37 °C for 30 min and 3 vol of cold acetone were added. After incubation at –20 °C for 16 h, samples were centrifuged (3500g, 10 min), the supernatant was carefully removed, and the protein pellets were air-dried. Proteins were solubilized and rehydrated with specific solutions, centrifuged (3500g, 10 min) and loaded onto immobilized pH gradient strips (pH 4.0 to 7.0, 18 cm, GE Healthcare, Uppsåla, Sweden). Gels were passively rehydrated for 20 h. The isoelectrofocusing assay was performed using IPGphor (GE Healthcare) at 53,500 V h. The second dimension was carried out on an Ettan Dalt six electrophoresis unit (GE Healthcare) using homogenous 12.5 % (w/v) SDS-PAGE. Proteins were resolved overnight at a constant current of 12 mA/gel at 4 °C. Gels were stained with colloidal Coomassie blue, Biosafe™ (BioRad, Hercules, CA, USA) destained with distilled water, and digitized with an Image Scanner III LabScan 6.0 (GE Healthcare).

### Image acquisition and data analysis

Digitalized images (300 dpi) of stained gels were aligned using the SameSpots software version 1.0.3400.25570 (Nonlinear Dynamics, Newcastle, UK), and the data analysis was performed as described by Belfiore et al. (2013); protein expression both at 42 °C (stress temperature) and 37 °C (control) was compared. A protein was considered differentially expressed if the mean normalized spot volume varied at least 1.4-fold between compared spots. The effect was confirmed by analysis of variance at a significance level of  $p < 0.05$ . At least three biological and two technical replicates for each growth condition were performed to obtain six gels for each culture condition.

### Protein identification by mass spectrometry (MS)

The electrophoretic bands obtained in SDS-PAGE corresponding to the presumed S-layer protein as well as the selected spots from two-dimensional electrophoretic (2DE) gels were cut, destained in ACN:20 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8.5 (1:1), dried at room temperature, rehydrated with 25 mM NH<sub>4</sub>HCO<sub>3</sub>, reduced with 10 mM DTT, and alkylated with 55 mM iodoacetamide in 25 mM NH<sub>4</sub>HCO<sub>3</sub>. Tryptic digestion was performed incubating each slice with 120 ng of trypsin (Promega sequencing grade modified) in 20 mM

$\text{NH}_4\text{HCO}_3$  pH 8.5 overnight at 37 °C. Peptides were extracted by incubating each slice with 50 % ACN 0.5 % TFA. Aliquots of the extracted peptides were mixed 1:1 with the matrix (3 mg/ml 4-hydroxy- $\alpha$ -cyano-cinnamic acid in 50 % ACN 0.5 % TFA), loaded onto MTP Anchor Chip Target (Bruker Daltonics) for co-crystallization, and analyzed in an Ultraflex II MALDI-TOF/TOF mass spectrometer for MS and MS/MS analysis in the MS facility CEQUIBIEM (Centro de Estudios Químicos y Biológicos por Espectrometría de Masa; 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 generated peak list was based on signal-to-noise filtering and an exclusion list. The resulting file was then searched by Mascot (Matrix Science, Boston, MA; <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 matched proteins with significant scores ( $p < 0.05$ ) were considered. The obtained hit was validated by MS/MS fragmentation of one or two high S/N peaks per sample.

## Statistics

Data were statistically analyzed using the Infostat Statistical Software (Universidad Nacional de Córdoba, Argentina). One-way analysis of variance (ANOVA) with the post hoc Tukey's test was used to evaluate significant differences among samples.

## Results

### Growth and S-layer formation by *L. acidophilus* IBB 801 under stress culture conditions

*L. acidophilus* IBB 801 grew well in regular MRS at 37 °C (control conditions) reaching cell count values of  $10^9$  CFU/ml and pH values of 4.0 after 24 h (Table 1). This strain showed a marked decrease of more than 2 log CFU/ml in the cell viability ( $1.17 \times 10^7$  CFU/ml) when the strain was incubated at 42 °C after 24 h. The strain was resistant to all the bile salt and NaCl concentrations (up to a 0.2 and 3.0 %, respectively) used in this study, although lower cell count values when grown under the most adverse conditions were obtained.

The production of S-layer proteins by the *L. acidophilus* strain grown under control and different stress conditions is shown in Fig. 1a–c. SDS-PAGE of whole-protein extracts showed changes in the protein patterns when the strain was cultured under different stress conditions. Intense protein

**Table 1** Growth of *L. acidophilus* IBB 801 under different culture conditions after 24 h of incubation

Growth conditions	<i>L. acidophilus</i> IBB 801		
	OD <sub>600nm</sub>	Log CFU/mL	pH
37 °C <sup>a</sup>	2.97 ± 0.15	9.57 ± 0.11	4.04 ± 0.01
42 °C	2.91 ± 0.15	7.07 ± 0.13	4.19 ± 0.00
pH 4.5	0.55 ± 0.03	8.30 ± 0.11	4.31 ± 0.01
0.6 % NaCl	2.60 ± 0.10	9.28 ± 0.11	4.03 ± 0.01
2.0 % NaCl	1.98 ± 0.11	9.42 ± 0.05	4.19 ± 0.02
3.0 % NaCl <sup>b</sup>	0.96 ± 0.01	7.67 ± 0.07	4.46 ± 0.05
0.05 % BS	ND <sup>c</sup>	9.20 ± 0.42	4.17 ± 0.00
0.1 % BS	ND	8.63 ± 0.08	4.42 ± 0.00
0.2 % BS	ND	7.33 ± 0.12	5.63 ± 0.01

Mean values of three measurements with ±SD

<sup>a</sup> Control conditions

<sup>b</sup> Measurements done at 48 h

<sup>c</sup> Not determined

bands of approximately 45 kDa (likely S-layer proteins) of *L. acidophilus* IBB 801 grown at 42 °C, in the presence of 0.05 % bile salts, and of 2.0 % NaCl (Fig. 1a) was detected. When the *Lactobacillus* strain was submitted to a treatment with 5 M LiCl, the presumptive S-layer proteins were successfully removed to indicate the clear 45 kDa bands in the SDS-PAGE profiles (Fig. 1b). The remnant cells of the strains after being treated with 5 M LiCl (without S-layer proteins) are shown in Fig. 1c.

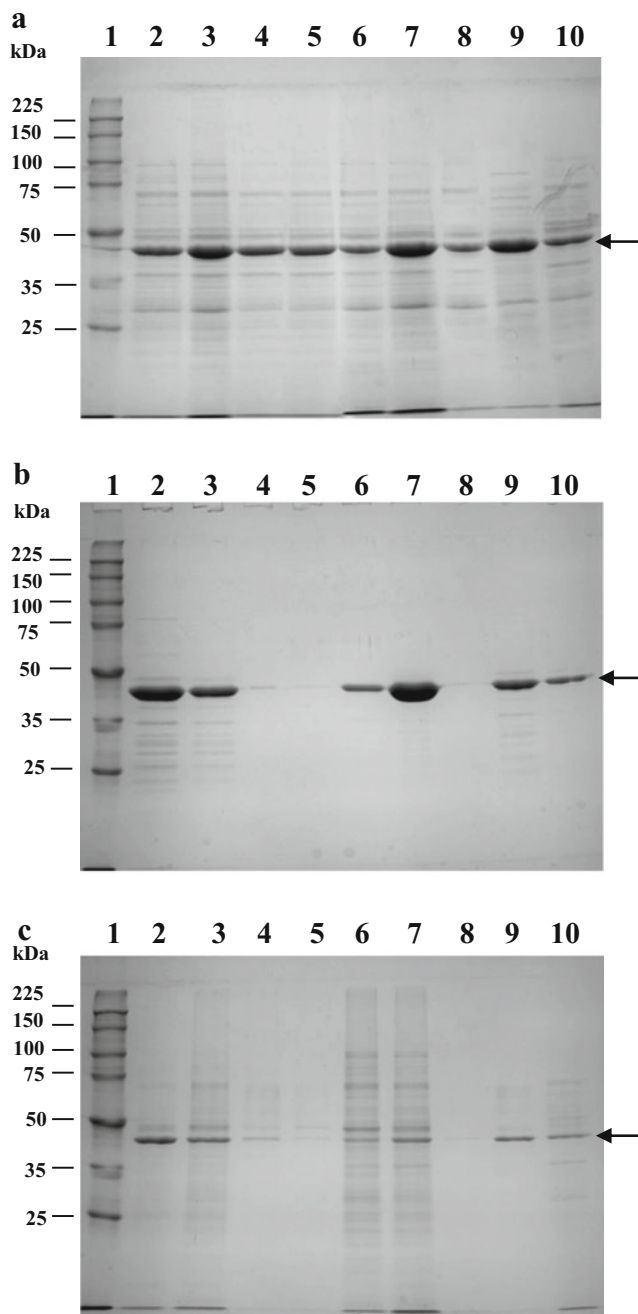
### Transmission electron microscopy (TEM)

The presence of S-layer was additionally evidenced by analyzing the outer surface of the *L. acidophilus* IBB 801 cells by TEM. S-layer proteins covering the cells grown at 37 °C for 24 h (Fig. 2a, b) were detected as a light layer surrounding the cells; cells treated with 5 M LiCl did not show these surface proteins (Fig. 2c).

### S-layer protein identification

To confirm the identity of the S-layer proteins, the intense electrophoretic bands corresponding to a MM of 45 kDa obtained from *L. acidophilus* IBB 801 samples were cut off from the gel and subjected to MALDI-TOF/TOF analysis. The identified protein showed high homology with that of a putative S-layer protein (LBA0221; GI, 489644444) from the strain *L. acidophilus* ATCC 700396/NCK56/N2/NCFM confirming that the 5 M LiCl extraction procedure was successful to remove cell surface proteins of our *Lactobacillus* strain.





**Fig. 1** SDS-PAGE profiles of whole cell proteins (**a**), S-layer proteins extracted with 5 M LiCl (**b**), and cell proteins after treatment with LiCl (**c**) from *L. acidophilus* IBB 801 grown under different conditions. Lanes 1: broad range protein molecular weight marker (Promega), 2: cells grown at 37 °C (control), 3: 42 °C, 4: pH 4.5, 5: bile salts 0.2 %, 6: bile salts 0.1 %, 7: bile salts 0.05 %, 8: NaCl 0.06 %, 9: NaCl 2 %, 10: NaCl 3 %. The arrows indicate S-layer protein

### Proteomic response of *L. acidophilus* IBB 801 subjected to 42 °C, an environmental stress condition inducing S-layer protein production

A proteomic approach was conducted to unravel functions involved in the response of *L. acidophilus* IBB 801 to a

non-optimal cell growing temperature (42 °C), a stress condition that promoted a higher S-layer protein formation and that negatively affected the strain cell viability the most among the conditions assayed (Fig. 1b). Differences in protein expression of *L. acidophilus* IBB801 cells growing at 42 °C during 24 h as compared with those obtained in the control (at 37 °C) were analyzed by 2DE (Fig. 3).

The genome data of *L. acidophilus* ATCC 700396 (Altermann et al. 2005) was used to assign genes encoding proteins experimentally obtained by comparison of their peptide mass fingerprinting. The two-dimensional protein patterns revealed a total of 15 spots differentially expressed by a factor greater than 1.4 (Table 2); from the total, ten spots displayed statistically significant differences in their level of expression ( $p < 0.05$ ) including six over-expressed and four under-expressed proteins at 42 °C. The differential expression levels of these proteins ranged from 2.4- to 1.4-fold variations ( $p < 0.05$ ) from which nine proteins were successfully identified (Table 2). The identified 42 °C-responsive proteins were successfully assigned to different functional categories, namely, (i) energy metabolism (mannose 6 P-isomerase, glyceraldehyde-3-phosphate dehydrogenase, L-lactate dehydrogenase, and phosphofructokinase); (ii) protein biosynthesis, processing, and translation (elongation factor G, 50S ribosomal protein L10, and glutamyl-tRNA(Gln) amidotransferase subunit A); (iii) general stress-response (ATP-dependent Clp protease proteolytic subunit); and (iv) DNA repair (exodeoxyribonuclease).

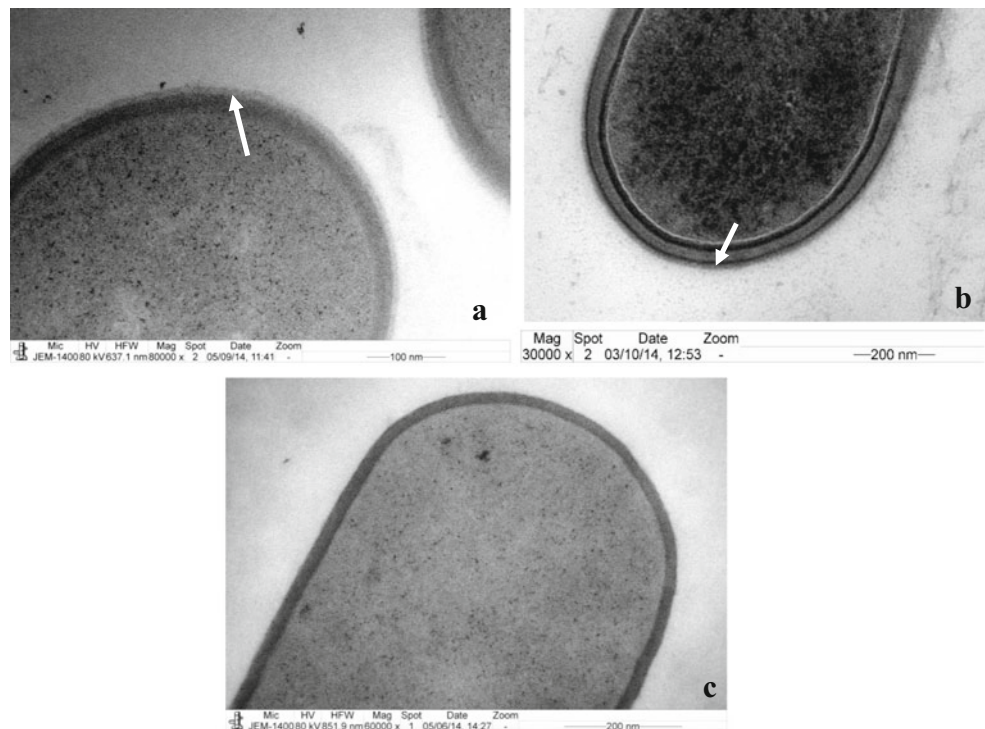
### Discussion

S-layer proteins have been identified in organisms of nearly every taxonomic group of cell-walled bacteria and represent almost a universal feature of archaea (Messner et al. 2010; Sleytr et al. 2005).

It has been claimed that surface properties of microorganisms are dependent on the growth conditions and the composition of the fermentation medium (Schär-Zammaretti et al. 2005; Waar et al. 2002). Schär-Zammaretti et al. (2005) suggested that S-layer proteins are preferentially expressed under different fermentation media. Furthermore, it has been shown that S-layer production varies along the change of medium composition such as the presence of bile salt, penicillin G, etc. (Khaleghi et al. 2010, 2011).

In this study, the production of S-layer proteins by the strain *L. acidophilus* IBB 801 was investigated to evaluate whether these proteins were involved in bacterial survival under harsh environmental conditions. This potentially probiotic strain produces acidophilin 801, a bacteriocin with a narrow inhibitory spectrum including other *Lactobacillus* strains and some Gram-negative bacteria (*Escherichia coli* Row, *Klebsiella pneumoniae* K33, and *Salmonella panama* 1467)

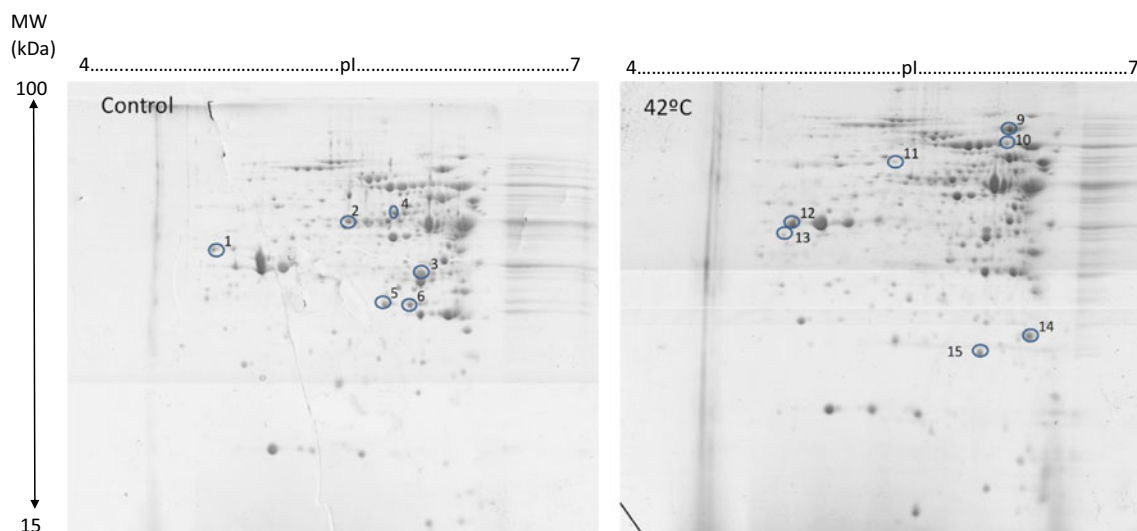
**Fig. 2** Transmission electron micrographs of thin sections of *L. acidophilus* IBB 801 control cells (**a**, **b**) and cells after LiCl treatment (**c**). The *arrows* indicate the presence of S-layer, which is imbedded in the cell wall



(Zamfir et al. 1999). For this purpose, defined adverse conditions of the human gut (presence of bile salts and low pH) and some conditions corresponding to technological food processing (high incubation temperatures and osmolarity) were considered.

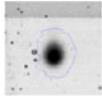
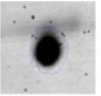
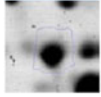
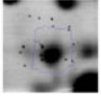
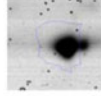
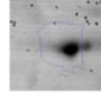
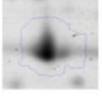
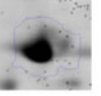
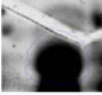
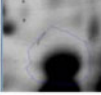
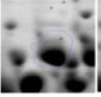
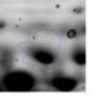
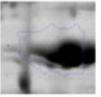
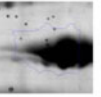
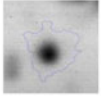
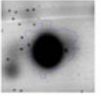
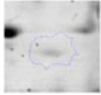
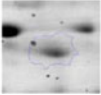
S-layers are not covalently attached to the cell surface and can be extracted either as sheets or as individual subunits in the presence of dissociating agents such as LiCl or EDTA or chaotropic denaturants such as guanidine hydrochloride or urea (Navarre and Schneewind 1999). In our study, S-layer

proteins from *L. acidophilus* IBB 801 were efficiently removed from the cells by using 5 M LiCl, although not a complete extraction was always possible as observed in SDS-PAGE (Fig. 1). This fact has been reported in other lactobacilli strains (Lortal et al. 1992; Frece et al. 2005; Dohn et al. 2011). Interestingly, Frece et al. (2005) directly extracted the S-layer protein from whole cells of *L. acidophilus* M92 using the same concentration (5 M) of LiCl as used in this study. The purified proteins extracted with LiCl showed a single electrophoretic band corresponding to a



**Fig. 3** 2DE gels showing the *L. acidophilus* IBB 801 proteins expressed when grown at 37 °C (control) or 42 °C (stress) in MRS for 24 h. The successfully identified proteins are circled and numbered as shown in Table 1. *MW* molecular weight, *pI* isoelectric point

**Table 2** Identified proteins differentially expressed when *L. acidophilus* IBB 801 was grown at 37 °C (control) or 42 °C (stress) in MRS for 24 h

Functional category	Score	Spot N°/Protein name/ gene <sup>a</sup> /access number	Access number	MW/pI <sup>b</sup>	MS-MM/MS <sup>c</sup>	Regulation at 42 °C relative change	Average normalized volumes 37 °C	42 °C
General stress	181	15. ATP-dependent Clp protease proteolytic subunit Gene: <i>clpP</i>	gi 489642157	21400/5.26	8/3	1.9-fold		
		Ordered locus name: LBA0694					14775986,03	27971294,62
DNA repair	231	6. Exodeoxyribonuclease Gene: <i>exoA</i>	gi 546530435	29660/5.02	28/4	-1.8-fold		
		Ordered locus name: LBA1699					35848572,78	20236728,58
	151	1. Mannose 6P isomerase Gene and ordered locus names: LBA0745	gi 489644697	36677/6.02	22/4	-2.1-fold		
Energy metabolism: Metabolism of carbohydrates	384	12. Glyceraldehyde-3-phosphate dehydrogenase Gene and ordered locus names: LBA0698	gi 489642161	36643/5.92	30/6	1.5-fold		
		Ordered locus name: LBA0698					40247500,96	62447524,08
	219	3. l-lactate dehydrogenase Gene: <i>ldhI</i> Ordered locus name: LBA0271	gi 489644560	35074/5.05	32/3	-2.0-fold		
	78	4. Phosphofructokinase Gene: <i>fruK</i> Ordered locus name: LBA1778	gi 489645356	32968/5.30	1/1	-1.7-fold		
Protein biosynthesis processing and translation	392	9. Elongation factor G Gene: <i>fusA</i> Ordered locus name: LBA0289	gi 489644582	76806/4.93	21/5	1.3-fold		
		Ordered locus name: LBA0289					56561670,16	75266114,15
	307	14. 50S ribosomal protein L10 Gene: <i>rplJ</i> Ordered locus name: LBA0369	gi 489644665	18602/4.72	14/5	2.4-fold		
	60	11. Glutamyl- tRNA(Gln) amidotransferase subunit A Gene: <i>gata</i> Ordered locus name: LBA0532	gi 546522134	51870/5.74	3/1	2.1-fold		

<sup>a</sup>Accession number in the UniProtKB (proteome:up000006381) for *Lactobacillus acidophilus* (strain ATCC 7000396/NCK56/N2/NCFM)

<sup>b</sup>Calculated molecular mass (Da) and isoelectric point

<sup>c</sup>N° of peptides obtained by MS and MS/MS, respectively.

MM of ~45 kDa for both strains, which is within the typical range of S-layer proteins for most lactobacilli (Åvall-

Jääskelläinen and Palva 2005; Messner et al. 2010; Dohn et al. 2011). Also, Yasui et al. (1995) found that 36 out of 41

strains of *Lactobacillus brevis* possessed S-layer proteins with MM values in a range of 38 and 55 kDa. Mass spectrometer analysis of the protein band extracted with LiCl confirmed the S-layer protein formation by *L. acidophilus* IBB 801, which showed high homology with surface proteins of other *L. acidophilus* strain (Johnson et al. 2013). When studying S-layer protein formation by strains of *L. acidophilus* and *Propionibacterium freudenreichii*, several other proteins were identified by proteomic approaches; however, many of these proteins showed unspecific/unknown functions; the identification of these proteins could provide information to better understand mechanisms of cell envelope biology and immunomodulation of probiotic microorganisms (Johnson et al. 2013; Le Maréchal et al. 2015).

In this study, it was found that S-layer proteins in *L. acidophilus* IBB 801 are present in both cells grown under optimal culture conditions (control) and in cells subjected to stress conditions although at variable extent. High concentrations of S-layer proteins were extracted when the strain grew at 37 and 42 °C but also when 0.05 % bile salts or 2 % NaCl were added to the growth medium. Khaleghi et al. (2010, 2011) showed that S-layer production by the *L. acidophilus* ATCC 4356 was increased under stress culture conditions. In addition, other authors reported that S-layer proteins were present during all growth phases of *L. acidophilus* M92 under heat stress conditions (Frece et al. 2005); the authors suggested that S-layer protein was preferentially expressed under conditions not optimal for bacterial growth.

The presence of S-layer on the surface of *L. acidophilus* IBB 801 grown at optimal culture conditions (37 °C) was clearly visualized by TEM. S-proteins appeared as a thin layer completely covering the thick peptidoglycan-containing layer. In addition, the extracted S-layer proteins removed from the SDS-PAGE were successfully identified by MALDI-TOF/MS/MS as S-layer proteins from the *L. acidophilus* species. The presence of S-layer in bacteria must provide a selective advantage since maintenance of surface proteins requires a large energy input and may constitute up to 14 % of the total cell protein content in the exponential growth phase (Boot et al. 1993; Powels et al. 1998; Sára and Sleytr 2000). Since no morphological cell changes could be detected after removal of the S-layer with 5 M LiCl, a cell-shape-determining function of the crystalline array can be excluded in the *L. acidophilus* IBB 801 strain (Beveridge et al. 1997; Sleytr and Messner 1998). However, some studies suggested that S-layer proteins of lactobacilli were important for hydrophobicity, auto-aggregation, and bacterial adherence to different host surfaces (Frece et al. 2005; Greene and Klaenhammer 1994; Hynönen et al. 2002; Kos et al. 2003; Vadillo-Rodríguez et al. 2004, 2005).

To promote their survival, bacteria react to stress conditions by multiple adaptive mechanisms. Environmental signals induce quick and complex responses under growth-restrictive conditions such as starvation, salinity, pH changes, and high

temperatures. These stress responses are known by the transient induction of general and specific proteins and by physiological changes that enhance the ability of an organism to cope with more adverse environmental conditions. Heat causes protein denaturation and subsequent aggregation, destabilization of macromolecules, and membrane fluidity modification (Van de Guchte et al. 2002; Champomier Vergés et al. 2010). Temperature up-shifts induce expression of genes coding for heat shock proteins (HSPs), some of which are molecular chaperones (GroEL, DnaK, small heat shock proteins, and ClpATPases) that assist refolding of damaged cellular proteins (Van de Guchte et al. 2002); others (ClpP and FtsH) act as proteases and degrade irreversibly damaged proteins. Since S-layer protein formation by *L. acidophilus* IBB 801 was increased under stress incubation temperature (42 °C) when compared to the optimal (37 °C) for growth, the proteomic response of this strain subjected to both temperatures was investigated. In this work, an ATP-dependent Clp protease proteolytic subunit (spot n° 15) was 1.9-fold overexpressed during growth of *L. acidophilus* IBB 801 at 42 °C; this general stress protein has chymotrypsin-like activity that cleaves protein in various peptides in an ATP-dependent hydrolysis reaction playing a key role in degradation of misfolded proteins. In addition, Russo et al. (2012) reported the upregulation of three proteins ClpP, ClpE, and ClpB belonging to Clp family in the strain *Lactobacillus plantarum* WCFS1 after the exposure at 42 °C for 30 min. On the other hand, the exodeoxyribonuclease (spot n° 6) protein was downregulated 1.8-fold in cells growing at 42 °C; this enzyme has DNA binding, endonuclease and exodeoxyribonuclease activities and it is involved in DNA repair being essential for cell survival. The downregulation observed when cells were grown at 42 °C suggests a less efficient DNA repair during cell adaption evidenced by the lower cell viability found at 42 than at 37 °C (Table 2). A varied expression of proteins related to the carbohydrate metabolism in the IBB 801 proteome patterns was observed at 42 °C, from which one protein (spot n° 12) involved in glycolysis (glyceraldehyde-3-phosphate dehydrogenase) showed increased relative volumes (1.5-fold) at 42 °C. In this sense, a high synthesis rate of glycolytic enzymes was reported for a *Lactococcus lactis* strain subjected to heat treatments and to osmotic stress (Zhang et al. 2010). On the contrary, other three proteins also related with energy metabolism, mannose 6 P-isomerase (spot n° 1) involved in mannose/fructose, amino sugar, and nucleotide sugar metabolisms; phosphofructokinase (spot n° 4) involved in mannose/fructose metabolism; and L-lactate dehydrogenase (spot n° 3) implicated in glycolysis and in the metabolism of cysteine/methionine and pyruvate/propanoate, were significantly downregulated at 42 °C. These results suggest a decreased metabolic activity of *L. acidophilus* IBB 801 during its growth at 42 °C in correlation with the lower cell viability observed at this temperature



(Table 1). Similarly, a differential under-regulation of several enzymes involved in the glycolytic pathway was reported in *Lactobacillus rhamnosus* HN001 (DR20) in response to heat and osmotic stress (Prasad et al. 2003) as well as in *Lactobacillus sakei* 23K during its adaption to cold temperatures and the presence of NaCl (Marceau et al. 2004). In fact, important pathways in *Lactobacillus* carbohydrate metabolism are generally impaired when they are exposed to stressful or not optimal growing conditions (Belfiore et al. 2013).

More interestingly, proteomic analyses of *L. acidophilus* IBB 801 growing at 42 °C allowed detecting significant up-regulation of three proteins involved in translation and protein biosynthesis; these proteins being deeply related to each other. The elongation factor G protein (spot n° 9) catalyzes the GTP-dependent ribosomal translocation step during translation, elongation, and the coordinated movement of the two tRNA molecules, the mRNA and conformational changes in the ribosome. The 50S ribosomal protein (spot n° 14) forms part of the ribosomal stalk, playing a central role in the interaction of the ribosome with GTP-bound translation factors. Finally, the glutamyl-tRNA(Gln) amidotransferase subunit A (spot n° 11) allows formation of correctly charged Gln-tRNA(Gln) through the transamidation of misacylated Glu-tRNA(Gln) in organisms lacking glutamyl-tRNA synthetase. This reaction takes place in the presence of glutamine and ATP through an activated gamma-phospho-Glu-tRNA(Gln). Increased protein synthesis could be a positive strategy ensuring bacterial survival under harmful conditions as it was previously proposed for other biological reactions such as the enhanced synthesis of nucleotide and nucleic acids in *L. sakei* subjected to osmotic stress (Belfiore et al. 2013). In addition, improved protein synthesis by the studied strain observed at 42 °C could signify, consequently, an increased expression of S-layer proteins as observed by SDS-PAGE analyses (Fig. 1). Although in the present study the expression of S-layer protein genes was not evaluated, an induction of the S-layer protein gene of *L. acidophilus* NCC 2628 was observed when the strain was cultured under limited protein supply conditions (Schär-Zammaretti et al. 2005) or by the presence of 0.01–0.05 % of bile salts in *L. acidophilus* ATCC 4356, while the expression of the *slpA* gene in this strain was decreased in presence of 0.1 % bile with concomitant changes in colony morphology and cell surface hydrophobicity (Khaleghi et al. 2010).

In conclusion, we found in this study that certain environmental stress conditions could induce S-layer production by *L. acidophilus* IBB 801, which most probably helped the strain to maintain cell viability under detrimental culture conditions. The proteomic studies provided information on the proteome changes when *L. acidophilus* IBB 801 was subjected to a stress incubation temperature. Finally, as this strain produces both a bacteriocin with quite narrow inhibitory spectrum and S-layer proteins, this strain could be of interest to be

used in the formulation of functional food products with specific properties.

#### 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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