Lipoteichoic acid depletion in *Lactobacillus* impacts cell morphology and stress response but does not abolish mercury surface binding

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Running header: Lipoteichoic acid depletion in *Lactobacillus plantarum*

ABSTRACT

Lipoteichoic acid (LTA) is a key component of the cell wall of most Gram-positive bacteria and plays many structural and functional roles. In probiotic lactobacilli, the function of LTA in mediating bacteria/host cross-talk has been evidenced and it has been postulated that, owing to its anionic nature, LTA may play a role in toxic metal sequestration by these bacteria. However, studies on this last aspect employing strains unable to synthesize LTA are lacking. We have inactivated the LTA polymerase encoding gene *ltaS* in two different *Lactobacillus plantarum* strains. Analysis of LTA contents in wild-type and *ltaS* mutant strains corroborated the role of this gene as a major contributor to LTA synthesis in *L. plantarum*. The mutant strains displayed strain-dependent anomalous cell morphologies that resulted in elongated or irregular cells with aberrant septum formation. They also exhibited higher sensitivity to several stresses (osmotic and heat) and to antimicrobials that target the cell wall. The toxicity of inorganic [(Hg(II))] and organic mercury (methyl-Hg) was also increased upon *ltaS* mutation in a strain-dependent manner. However, the mutant strains showed 0 to 50% decrease in their capacity of Hg binding compared to their corresponding parental strains. This result suggests a partial contribution of LTA to Hg binding onto the cell surface that was dependent on the strain and the Hg form.

Keywords: cell wall, heavy metal, stress response

INTRODUCTION

Lactobacillus is a genus of the so-called lactic acid bacteria, a group encompassing Gram-positive, obligate fermentative bacteria that produce lactic acid as the main end-product of sugar fermentation. *Lactobacillus plantarum* has been isolated from a wide variety of habitats, including fermented foods, silages and the gastrointestinal tract of animals. Comparative genome analyses have revealed a high level of genomic diversity and plasticity within this species (Martino *et al.*, 2016). *L. plantarum* strains play a main role in a number of fermentative processes and some strains have been marketed as probiotics. Due to its applied interest, *L. plantarum* has been the subject of intensive genetic and biochemical studies making it a model organism within the genus *Lactobacillus* (van den Nieuwboer *et al.*, 2016). As a result, the structure and composition of *L. plantarum* cell envelope is well characterized.

Teichoic acids are a major component of the cell wall of most Gram-positive bacteria. They are structurally diverse but share a common linear backbone consisting of phosphate groups linked to different alditol groups by phosphodiester bonds. Therefore, teichoic acids are major contributors to the net negative charge of the cell wall of these organisms. They can be divided into membrane-bound lipoteichoic acids (LTA) and peptidoglycan-bound cell wall teichoic acids (WTA). In addition to the structural diversity of their backbones, teichoic acids can be decorated with additional residues such as D-alanine or sugars (Neuhaus and Baddiley, 2003; Rajagopal and Walker, 2017; van der Es *et al.*, 2017; Weidenmaier and Peschel, 2008). Synthesis of LTA and WTA occurs through different pathways. The typical poly-glycerol phosphate LTA is synthesized on the external side of the cell membrane by the action of the LTA synthase LtaS (Gründling and Schneewind, 2007). The glycolipid anchor is synthesized in the cytoplasm and subsequently translocated to the outer face of the membrane by a flippase (Gründling and Schneewind, 2007; Jorasch *et al.*, 1998). WTAs are fully polymerized in the cytoplasm, exported by an ABC transporter (Swoboda *et al.*, 2010) and linked to the peptidoglycan by LytR-CpsA-Psr (LCP) proteins (Chan *et al.*, 2014; Schaefer *et al.*, 2017).

At least one *ltaS* homolog is present in all genomes of *Lactobacillus* sequenced so far, whereas WTA biosynthetic genes are absent in some species. The structure of LTA has been determined in *Lactobacillus brevis* (Sánchez Carballo *et al.*, 2010), *Lactobacillus casei* (Nakano and Fischer, 1978), *Lactobacillus delbrueckii* (Räisänen *et al.*, 2007), *Lactobacillus plantarum* (Jang *et al.*, 2011) and *Lactobacillus rhamnosus* (Vélez *et al.*, 2007). In all of them, it consists of 1,3-linked poly(glycerol-phosphate) with an anchor of glucose-galactose-glucose-diacylglycerol. In some lactobacilli, such as *L. plantarum* KCTC 10887BP (Jang *et al.*, 2011) or *L. casei* DSM 20021 (Nakano and Fischer, 1978), a third acyl chain is linked to the terminal glucose residue in carbon 6. LTA in lactobacilli are further modified by addition of substituents, particularly D-alanine and glycosidic residues (Neuhaus and Baddiley, 2003; Revilla-Guarinos *et al.*, 2014). Analyses of mutants defective in D-alanylation have shown that the degree of alanylation affects cell morphology, biofilm formation, colonization of the gastrointestinal tract, host immune response and resistance to antimicrobial peptides (Grangette *et al.*, 2005; Revilla-Guarinos *et al.*, 2013; Revilla-Guarinos *et al.*, 2014; Vélez *et al.*, 2007; Walter *et al.*, 2007). Mutants deficient in LTA (knockouts in *ltaS*) have also been obtained in two lactobacilli belonging to the acidophilus

group (*Lactobacillus acidophilus* (Mohamadzadeh *et al.*, 2011) and *Lactobacillus gasseri* (Selle *et al.*, 2014). However, these studies have focused on the role of LTA in bacterial host interactions.

Mercury (Hg) is a toxic trace element that has raised considerable concern as a large part of the world population is exposed to it through foodstuffs, where it is mostly found as divalent inorganic Hg [Hg(II)] or methyl-Hg. This concern has led to exploring strategies aimed to diminish the bioaccessibility of Hg in the gastrointestinal tract. It has been shown that lactobacilli can efficiently bind Hg(II) and methyl-Hg in *in vitro* assays (Alcántara *et al.*, 2017; Jadán-Piedra *et al.*, 2017). Furthermore, a number of studies have also shown that administration of lactobacilli diminish the toxicity associated to Hg exposure (Jadán-Piedra *et al.*, 2019; Jiang *et al.*, 2018; Majlesi *et al.*, 2017). An increased excretion of the toxic through faeces was observed in these studies that has been attributed to Hg binding to the administered bacteria (Jadán-Piedra *et al.*, 2019; Majlesi *et al.*, 2017). However, it has not been clearly determined which cell components are involved in Hg binding. Polyphosphate, an efficient metal chelator, is produced by numerous species of lactobacilli (Alcántara *et al.*, 2014) but has been shown to have no effect in Hg binding (Alcántara *et al.*, 2018). Previous results suggested that Hg mainly bound the cell envelope of *Lactobacillus* cells (Alcántara *et al.*, 2017). In this study, we have explored the possible role of LTA in Hg binding by *L. plantarum*.

MATERIALS AND METHODS

Strains and growth conditions.

Lactobacillus plantarum WCFS1 (Siezen *et al.*, 2012), *L. plantarum* Lpp+ (Alcántara *et al.*, 2018) and derived mutants were grown in MRS medium at 30 °C under static conditions. *Escherichia coli* DH10B was used as a cloning host and it was grown in LB medium at 37 °C under aeration. When required, agar plates were prepared by adding agar at 1.8 % (w/v). Antibiotics used were ampicillin at 100 µg/ml for *E. coli* and erythromycin at 5 µg/ml for *Lactobacillus*.

Mutant construction.

An internal fragment (672-bp) of the *ItaS* gene (LP_1283) was obtained by PCR with oligonucleotides 5'-TTTGAATTCAGACCGAAAAGGAAAGTGC and 5'-TTTAAAGCTTCTTATCGATCGACTGGTTC which incorporated restriction sites (underlined) for EcoRI and HindIII, respectively and chromosomal DNA from WCFS1 strain. The digested PCR product was cloned in EcoRI- and HindIII-digested pRV300 vector (Leloup *et al.*, 1997). As the similarity of the cloned *ItaS* fragment between WCFS1 and Lpp+ was more than 99%, the obtained plasmid was used to transform both strains. To this end, the bacteria were grown in MRS supplemented with 1% glycine to an OD_{595nm} of 0.6 and washed with 1 volume of cold 1 mM MgCl₂ followed by a second wash with half volume of cold 30 % polyethylene glycol 1500. The bacterial pellet was 100-fold concentrated in 30 % polyethylene glycol 1500 and 50 µl aliquots were electroporated with 1.5-2 µg of plasmid in a GenePulser apparatus (BioRad) in 2-mm electrode gap cuvettes at 1500 V, 400 Ohm and 25 µF. Electroporated cells were resuspended in 1 ml of MRS broth and incubated for two hours at 30 °C before being plated on MRS plates with erythromycin. Resistant colonies appeared after three to four days of incubation and they were checked for *ItaS* disruption by PCR with appropriated oligonucleotides. Two mutant strains were selected and named WCFS1 *ItaS* (parental strain WCFS1) and Lpp+ *ItaS* (parental strain Lpp+).

Assays of sensitivity to high salt concentration, high temperature and antibiotic susceptibility.

The stress conditions assayed were: growth in MRS supplemented with different concentrations of NaCl and in MRS incubated at different temperatures. To this end, cells from frozen stocks were inoculated in MRS agar plates and incubated at 30°C. Cells from single colonies were inoculated in MRS medium and grown at 30°C for 16 h (stationary phase). Subsequently, the cultures were centrifuged, washed twice with one volume of NaCl 0.9 %, and suspended in NaCl 0.9 % to a final OD_{595nm} of 1. For the NaCl tolerance assays, the cell suspensions were used to inoculate to a final OD_{595nm} of 0.05 250 µl aliquots of growth media dispensed in 96 well microtiter plates and incubated for 24 h. Growth was monitored by changes in optical density at 595 nm in a microtiter plate reader POLARStar (BMG Labtech). For growth at different temperatures, cells were inoculated as indicated previously to a final OD_{595nm} of 0.05 in 150 µl of MRS in 200 µl PCR tubes and incubated in a PCR apparatus with block temperature gradient capability (MyCycler™ Bio-Rad) for 24 h and the final OD_{595nm} of the cultures was determined. No antibiotics were used in the growth assays. No revertants to wild-type were detected under

these experimental conditions for the duration of the assay. At least three independent replicates of each growth condition were obtained. For the determination of survival at 55 °C, bacterial cells were grown overnight in MRS, washed two times with 0.9% NaCl and resuspended in the same solution to an OD_{595nm} of 1. Cell suspensions were placed in a water bath at 55 °C and aliquots were withdrawn every 15 min., diluted 1:10 in 0.9 % NaCl and 2 µl samples spotted onto MRS plates that were later incubated at 30 °C. Minimal Inhibitory Concentrations (MIC, expressed in µg/ml) were determined in MRS using serial dilutions of the antimicrobial agents. The assays were performed in 96 well microtiter plates inoculated as indicated in the former section and incubated for 24 h. The MIC was defined as the lowest concentration of antimicrobial agent needed to totally inhibit the growth of the bacterial strain. The following antibiotics were tested: ampicillin, cephalixin, chloramphenicol, penicillin G and tetracycline.

LTA analysis

Crude LTA preparations were obtained and analysed according to Kho and Meredith, 2018. Briefly, bacterial strains were grown to mid-log phase in MRS, washed in one volume of 50 mM sodium citrate pH 4.7 and resuspended in the same buffer before being disrupted with glass beads (0.1 mm) in a FastPrep-24 5G apparatus (3 cycles of 1 min at maximal speed). Cellular debris was removed by centrifugation at low speed (6000 x g 10 min) and bacterial membrane fragments were obtained after centrifugation at 22.000 x g 15 min at 4 °C. The pellets were extracted with water/butanol and the aqueous fractions were treated with resinase HT (Strem Chemicals) at 50 °C and pH 8.5 for 16 h. Samples were loaded onto a PAGE gel (20% T, 6%C in 1 M Tris-HCl pH 8.5) and run in 0.1 M Tris-0.1 M Tricine buffer pH 8.2 at a constant intensity of 30 mA and 4 °C. After electrophoresis, the gel was stained overnight with 0.1% alcian blue in 3% (v/v) acetic acid, followed by destaining in water and an additional staining with silver nitrate.

Cytochrome c binding assay.

The binding assay was performed essentially as previously described (Revilla-Guarinos *et al.*, 2013). Bacterial cultures were grown overnight and washed twice with 20 mM MOPS pH 7. Cells were adjusted to an OD_{595nm} of 4 in 1 ml of the same buffer and incubated with 150 µg/ml of cytochrome c under constant agitation for 10 min. The samples were centrifuged at 12.000 x g for 5 min and the absorbance at 530 nm was determined in the supernatants. The percentages of cytochrome c binding were calculated as $[[1-(A_{530}/A^0_{530})] \times 100$, where A⁰ is the absorbance of the cytochrome c solution without added cells.

Growth inhibition studies.

Bacterial growth in the presence of different concentrations of Hg(II) or methyl-Hg (1.25 to 20 mg/l) was monitored in 96-well microtiter plates containing 200 µl of MRS per well in a POLARstar plate reader (BMG Labtech) at 30 °C.

Electron microscopy.

Bacterial cells were cultivated in MRS broth until late exponential phase and they were washed twice with PBS. Cells were fixed with a mix of 2.5% glutaraldehyde plus 2% paraformaldehyde in PBS for 1h at room temperature before being embedded in agarose. Samples were washed with PBS, treated with 2% osmium tetroxide and dehydrated with ethanol. Ultrathin sections (Leica UC6 ultramicrotome) of Spurr resin-included samples were stained with uranyl acetate and lead citrate and they were observed with a FEI Tecnai Spirit G2 transmission electron microscope at the Electron Microscopy Service of the Principe Felipe Research Center (CIPF, Valencia, Spain).

Mercury binding and mercury quantification.

For measuring the mercury binding capacity of the strains the procedure described by Jadán-Piedra *et al.*, 2017 was used with some modifications. Bacterial strains were grown to mid-log phase and washed with PBS before being resuspended in PBS at an OD_{595nm} of 0.15. Hg [Hg(II) or methyl-Hg] was added to a final concentration of 1 mg/l to 1 ml of these cell suspensions which were subsequently incubated at 30 °C for 1 h. After this period, the bacterial suspensions were washed with 1 ml of PBS and Hg contents were determined in the bacterial pellets. For Hg quantification, the pellets were placed in teflon reactors in 4 ml of 14 M HNO₃ (Merck) and 1 ml of 30 % H₂O₂ (Prolabo; VWR International, Barcelona, Spain) and the samples were digested using a microwave accelerated reaction system at 180 °C for 15 min (CEM, Vertex, Barcelona, Spain). The obtained digests were allowed to rest for 12 h to eliminate nitrous vapor and they were made up to volume with 0.6 M HCl. Mercury was quantified by cold vapor atomic fluorescence spectrometry (CV-AFS) (Millennium Merlin PSA 10.025; PS Analytical, Orpington, UK) by using the following analytical conditions: reducing agent, 20 g/l SnCl₂ (Scharlab, Scharlau Chemie, Spain) in 1.8 M HCl (Merck), 4.5 ml/min; reagent blank, 0.6 M HCl, 9 ml/min; carrier gas, argon, 0.3 l/min; dryer gas, air, 2.5 l/min; delay time, 15 s; analysis time, 40 s; memory wash time, 60 s. Quality control for quantification by CV-AFS was performed by analyzing a liquid reference material (QCI-049-1 Trace Metals AA Sample 1; LGC Standards, Barcelona, Spain) with a certified Hg concentration of 40.8 ± 1.19 µg/l.

Statistical analyses

In order to compare the response of the different strains to different growth temperatures, values of final OD were normalized using the min-max method. Average and standard deviations were calculated for the normalized values. Analysis of the difference between means was performed by Student's t-test (Graphpad Prism 4) and statistical significance was set at $p < 0.01$.

RESULTS

L. plantarum strains deficient in LTA synthesis

Insertional mutants defective in *ltaS* (LP_1283; Figure 1A) were obtained in *L. plantarum* WCFS1 and *L. plantarum* Lpp+. LTA was isolated from wild-type strains and their respective *ltaS* mutants and subjected to electrophoretic analysis (Figure 1B). It was evidenced that LTA from Lpp+ and WCFS1 displayed different mobilities, probably reflecting a different degree of polymerization. Inactivation of *ltaS* in Lpp+ strain resulted in the complete absence of LTA. In the WCFS1 *ltaS* strain the LTA signal observed in the parental strain was also absent but, a new slow migrating and less intense signal appeared that could correspond to a different type of LTA that was only produced in the absence of a functional *ltaS*.

We examined the capacity of the *L. plantarum ltaS* strains to bind to the positively-charged molecule cytochrome *c*. The capacity to bind this molecule has been proposed as an indirect measure of the overall negative charge of the cell surface which is partially affected by the presence of LTA. Surprisingly, both *ltaS* mutants showed an increase in the percentage of binding of cytochrome *c* compared to the parental strains (Figure 2). In any case, differential binding of cytochrome *c* by LTA defective mutants possibly indicated a clear alteration in the physicochemical properties of the bacterial surface upon *ltaS* mutation.

Altered cell morphology in *L. plantarum* lacking LTA

Inspection of the cells of the wild-type and *ltaS L. plantarum* strains by light microscopy evidenced distinct bacterial morphologies. The WCFS1 strain devoid of *ltaS* formed more elongated cells in contrast to the typical short rod-shaped cells of the wild type strain. The cells of this mutant were also characterized for showing bending and curving. In contrast, inactivating the *ltaS* gene in the Lpp+ strain resulted in irregular and engrossed cells (data not shown). Transmission electron microscopy confirmed the evident differences with the parental strains. Mutation of *ltaS* in the Lpp+ strain resulted in an aberrant cell morphology with deformed cells that were bigger in size and did not display the typical rod-like shape (Figure 3). It was also evidenced that most of these bigger cells were the result of lack of daughter cells separation after division, with engrossed septa and thicker cell walls that presented an irregular surface compared to the smooth surface of the wild-type cells. Lack of *ltaS* in the WCFS1 strain resulted in elongated and curved bacilli that were also the result of lack of cell separation after septum formation. Thickening of the cell walls was also detected at some patches of the cell envelope, but no changes in their surfaces were evidenced.

Effect of LTA deficiency on growth under stress conditions

The absence of LTA had a detrimental effect on the growth of *L. plantarum* under different stress conditions. Both *ltaS* mutants displayed a diminished growth in MRS liquid medium under reference conditions (Figure 4), but the impact of elevated NaCl concentrations on growth was higher on the Lpp+ strain. The Lpp+ *ltaS* mutant reached a significantly lower maximal OD at 0.4 M NaCl, that subsequently declined, compared to the culture on MRS. A complete arrest of

growth occurred at 0.8 M NaCl. The WCFS1 *ItaS* mutant also showed a greater sensitivity to NaCl than its parental strain, but it was still able to grow at NaCl 0.8 M.

The mutants also showed a strain-dependent response to temperature. The normalized final OD values of liquid cultures at different temperatures (Figure 5A) showed that increasing the growth temperature resulted in higher reductions of the relative final OD in WCFS1 *ItaS* compared to its parental strain. The Lpp+ strain followed an opposite behavior, and increasing the temperature had a higher impact in reducing the final OD in the wild type compared to its *ItaS* mutant. However, both *ItaS* mutants presented a diminished survival after exposure to high temperatures compared to their parental strains, showing a high mortality rate after 15 min incubation at 55 °C (Figure 5B).

The minimal inhibitory concentration (MIC) of several antibiotics was decreased after *ItaS* deletion, reflecting a higher sensitivity in the absence of LTA (Table 1). The MIC decreased for almost all the tested antibiotics in the *ItaS* mutants, with the exception of chloramphenicol and tetracycline in the Lpp+ background. Of note, the increased sensitivity was maximal for antibiotics targeting the cell wall (up to 8-fold lower MICs for ampicillin, cephalixin and penicillin G).

Inhibition of growth by inorganic and organic mercury

Mercury has a toxic effect on bacterial growth. We showed that the sensitivity to Hg differed between both *L. plantarum* strains and that the capacity to inhibit growth of both inorganic and organic Hg was generally increased in the absence of LTA (Figure 6). The WCFS1 *ItaS* strain was more susceptible to Hg(II) compared to the Lpp+ mutant as complete absence of growth was already detected at 10 mg/l whereas the Lpp+ *ItaS* strain could still grow at this concentration (Figure 6A). Methyl-Hg was more toxic than Hg(II) as the Lpp+ strain growth was arrested at 20 mg/l whereas it had an even stronger inhibitory effect on WCFS1, whose growth was completely inhibited already at 5 mg/l (Figure 6B). However, while the Lpp+ *ItaS* strain did not show a greater sensitivity to methyl-Hg than its parental strain, the WCFS1 *ItaS* strain failed to grow at any methyl-Hg concentration tested.

Mercury binding capacity in *L. plantarum* strains

The capacity to bind inorganic and organic Hg was determined in cell suspensions of the *L. plantarum* strains employed in this study (Figure 7). As observed in the assays reported above, the inactivation of *ItaS* had strain-dependent effects on the binding capacity of each strain. Furthermore, the effect was different for each Hg species. Thus, no clear effect was observed in the capacity to retain Hg(II) of Lpp+, whereas binding of methyl-Hg by the Lpp+ *ItaS* strain was reduced to 50% of that of the parental strain. On the contrary, the WCFS1 *ItaS* mutant showed an around 30% reduction in the binding of Hg(II) but no effect was observed with methyl-Hg.

DISCUSSION

L. plantarum WCFS1 had previously been reported to bind Hg(II) and methyl-Hg from aqueous solutions (Jadán-Piedra *et al.*, 2017). In order to investigate the likely contribution of LTA from the bacterial cell wall to toxic metal binding by probiotics, we decided to obtain *L. plantarum* strains unable to synthesize LTA. Genome analysis showed that most lactobacilli carry two gene candidates encoding LtaS (lipoteichoic acid synthase enzymes) that share around 50% amino acid identities. Four paralogues of *ItaS* co-exist in *Bacillus subtilis* that encode proteins with 41 to 53% identity to LtaS, but single or combined mutations of them results in no discernible phenotype (Schirner *et al.*, 2009). Only by deleting the four *ItaS* homologues a complete absence of LTA is attained (Wormann *et al.*, 2011). Based on the fact that a previous *L. acidophilus* mutant in the LBA0447 gene was deficient in LTA production (Mohamadzadeh *et al.*, 2011) and in the genome context of LBA0447 homologues (Figure 1A), we decided to mutate the counterparts of this gene in the strains used in this study. We showed that the *L. plantarum* LBA0447 homologue (LP_1283; *ItaS*) played a major role in LTA synthesis in Lpp⁺ and WCFS1 strains, whereas the function of the *L. plantarum* *ItaS* paralogue LP_2580 remains unknown. Whether this gene could be responsible for the LTA signal detected in the *L. plantarum* WCFS1 *ItaS* strain (Figure 1B) needs further investigation.

In lactobacilli, some species synthesize both LTA and WTA, whereas others only produce LTA (Chapot-Chartier and Kulakauskas, 2014). LTA-deficient strains of *S. aureus* (Oku *et al.* 2009) and *B. subtilis* (Schirner *et al.* 2009) have been obtained. Interestingly, both organisms synthesize LTA and WTA and the loss of both polymers was lethal. This may explain our inability to obtain an *ItaS* mutant of *Lactobacillus casei* BL23 (LCABL_09330 gene; data not shown), whose genome inspection revealed the absence of WTA biosynthetic genes (Maze *et al.*, 2010). This, together with the fact that *L. plantarum* (Tomita *et al.*, 2010) and *L. acidophilus* (Altermann *et al.*, 2005) synthesize WTA, suggests that the presence of teichoic acids in the cell wall is necessary for cell viability in lactobacilli and that *ItaS* deficient mutants can only be obtained from WTA-producing strains.

A defect in LTA synthesis (mutation in LP_1283) in *L. plantarum* had drastic consequences in growth and cell morphology. Part of these effects had been previously observed in lactobacilli of *L. acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 lacking the *ItaS* counterparts LBA0447 and LGAS_1586, respectively. Light microscopy inspection of these mutants revealed elongated cells twice as long as the wild type cells, and exhibiting bending and curving (Selle *et al.*, 2017; Selle *et al.*, 2014). However, contrarily to *L. plantarum*, and *ItaS* mutants of other species (Mamou *et al.*, 2017; Oku *et al.*, 2009; Schirner *et al.*, 2009), no relevant growth defect was observed in *L. acidophilus* NCFM upon *ItaS* mutation (Mohamadzadeh *et al.*, 2011). Both *L. plantarum* *ItaS* mutant strains exhibited defects in cell separation and irregularities in their cell walls. Defective septum formation giving rise to long cells with engrossed septa or the appearance of aggregates consisting of multiple non-separated cells have also been observed in *B. subtilis* (Schirner *et al.*, 2009) and *Staphylococcus aureus* *ItaS* mutants (Corrigan *et al.*, 2011; Oku *et al.*, 2009), highlighting the importance of LTA in the structure and functionality of the cell wall. However, differences in the cell morphology were evidenced between both *L. plantarum* mutant strains. The morphology of the *L. plantarum* WCFS1 *ItaS* mutant was similar to that

described for *L. acidophilus* (Selle *et al.*, 2017) and *L. gasseri* (Selle *et al.*, 2014) strains lacking LTA, while the Lpp+ *ItaS* strain was characterized by a non-rod morphology with an irregular surface. A rough surface with protrusions has also been observed in the cells of *tagO* mutants from *S. aureus* unable to synthesize WTA, compared to the smooth surface of wild-type cells (Koprivnjak *et al.*, 2008).

Similar to *S. aureus* cells unable to produce LTA, the *L. plantarum ItaS* strains were more sensitive to heat and osmotic stress than their corresponding parental strains. In fact, *S. aureus ItaS*-deficient strains could only be obtained at 30 °C and under osmotically stabilizing conditions (Corrigan *et al.*, 2011; Oku *et al.*, 2009). LTA has been postulated to play a role in cell-wall cation homeostasis, especially of Mg^{2+} . *L. plantarum* strains lacking *ItaS* showed increased sensitivity to Hg cations. In *B. subtilis* (Schirner *et al.*, 2009) and *L. acidophilus* (Selle *et al.*, 2017), *ItaS* mutants are more susceptible to the toxic effects of Mn^{2+} and a *B. subtilis ItaS* deletion strain had reduced requirements of Mg^{2+} in minimal medium (Schirner *et al.*, 2009). It is assumed that the loss of LTA-cations interactions at the cell wall allows better cation access to the membrane, which results in increased toxicity (Mn^{2+}) or lower requirements (Mg^{2+}) for cations (Schirner *et al.*, 2009). In the case of Hg, increased surface accessibility may also explain its higher toxicity in *L. plantarum ItaS* strains.

Similar to *L. plantarum*, hypersensitivity to antibiotics has also been described in a *B. subtilis ItaS* mutant, suggesting again an increased access of the antimicrobial agents to their action sites in the cell envelope (Schirner *et al.*, 2009). LTA can be decorated by alanine residues via Dlt activity, which reduces the negative charge density of the polymer. Enhanced sensitivity to cationic antimicrobial peptides has also been observed in *L. casei* mutants defective in D-alanylation of LTA (mutants in the *dltA* gene) (Revilla-Guarinos *et al.*, 2013). Mutation of *dltA* in *L. casei* resulted in increased sensitivity to Hg (Alcántara *et al.*, 2017) and higher binding of cytochrome *c* (Revilla-Guarinos *et al.*, 2013). If the increased cytochrome *c* binding would be explained only by electrostatic interactions at the cell envelope, inactivation of *ItaS* or *dltA* would be expected to have opposite effects, as the absence of LTA would result in cell wall decreased negative charge whereas lack of D-alanyl residues would result in increased negative charge. Previous studies have shown that a correlation between cytochrome *c* binding and negative charge density is not straightforward as other interactions and changes in cell wall structure could facilitate cytochrome *c* access to its binding sites (Revilla-Guarinos *et al.*, 2014; Saar-Dover *et al.*, 2012). In fact, the microscopic observation of *L. plantarum ItaS* mutants indicates a substantial alteration of the cell wall structure.

An *L. casei dltA* strain also had increased capacity to bind Hg(II) (28% higher than the wild type (Alcántara *et al.*, 2017)), which initially pointed to LTA as a contributor to surface binding of this cation. However, despite the drastic effect of LTA depletion in the characteristics of *L. plantarum*, it had variable consequences on the capacities of the strains to bind Hg(II) or methyl-Hg which, again, were strain-dependent. Furthermore, no clear relation appeared to exist between toxicity and the capacity to bind mercury. The strain-dependence of the *ItaS* phenotype in *L. plantarum* could indicate heterogeneity in the cell wall composition, where differences in LTA polymer length and substitutions (e.g. degree of glycosylation and D-alanylation) may exist (Neuhaus and Baddiley, 2003). They also suggest that the effect of the

lack of LTA on Hg binding would be mostly indirect, through alterations of the cell wall structure as both strains differed in morphology and phenotypic traits. Recently, a derivative of *Lactobacillus pentosus* MP-10 (a phylogenetically closely related species to *L. plantarum*) cured from its five endogenous plasmids was shown to reduce by 22% its capacity for Hg [Hg(II)] sequestration and exhibited a decreased MIC for this compound (Abriouel *et al.*, 2019). DNA sequences in the *L. pentosus* MP-10 plasmids related to metal physiology comprise a region containing a Cd(II) detoxification pump with homology to the previously characterized pWCFS103 plasmid of *L. plantarum* WCFS1 (van Kranenburg *et al.*, 2005). However, it is very unlikely that lack of detoxification pumps results in decreased Hg binding ability, for which it is expected that other plasmid-encoded characteristics that contribute to Hg binding are implicated. As an example, other bacterial surface polymers such as exopolysaccharides (EPS) may contribute to Hg binding. In fact, *L. plantarum* WCFS1 contains four gene clusters involved in EPS synthesis (Remus *et al.*, 2012) and EPS-mediated metal removal has been described in lactobacilli (Polak-Berecka *et al.*, 2014). Previous studies showed that lactobacilli bind Hg(II) and methyl-Hg at their surface via mechanisms that do not require cell viability and possess high affinity for the metal, as the bound Hg cannot be removed by strong treatments with chelants (Alcántara *et al.*, 2017). Inorganic and organic Hg shows high reactivity with sulfhydryl residues (e.g. Cys from proteins), for which it is expected that complex covalent and non-covalent interactions with proteins and other cell-wall polymers/components, including LTA, contribute to the overall capacity of Hg binding in lactobacilli. The absence of LTA may possibly affect the abundance and distribution of proteins in the cell envelope in a strain-specific manner, thus accounting for the differences observed in Hg binding capacity.

Conclusion

We have obtained *L. plantarum* strains defective in LTA synthesis. These mutants displayed growth and morphological characteristics common to other related bacteria lacking LTA, but they showed strong strain-dependent behaviour, which probably indicates differences in the structure/chemical composition of the cell wall, including LTA, between *L. plantarum* strains. The overall Hg binding capacity of lactobacilli is probably multifactorial and it may be affected by the presence of other binding molecules. Additionally, LTA absence could give rise to changes in the distribution of these molecules or contribute to alterations in the cell wall that modify the accessibility of Hg to them.

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Figure legends.

Figure 1. The *ItaS* genes in lactobacilli (A) Genome context of *ItaS* genes in *L. acidophilus* and *L. plantarum*. The two *ItaS* (phosphoglycerol transferase or lipoteichoic acid synthase) genes from *L. acidophilus* NCFM (LBA0447 and LBA0750) and *L. plantarum* WCFS1 (LP_1283 and LP_2580) are depicted together with their surrounding genes. In the clusters containing LBA0447 and LP_1283, genes encoding diacylglycerol glucosyl transferases and a putative transporter, possibly involved in the synthesis of LTA, are present. (B) Detection of LTA in *L. plantarum* strains. LTA from wild-type and *ItaS* mutant strains was isolated, subjected to PAGE and stained with alcian blue/silver nitrate.

Figure 2. Binding of cytochrome *c* by *L. plantarum* strains. The graph represents the percentages of cytochrome *c* bound to bacterial cells (OD of 4 in 20 mM MOPS buffer pH 7). Mean plus standard deviations from four determinations are presented. Asterisks represent significant differences between wild-type and mutant strains at $p < 0.001$.

Figure 3. Transmission electron microscopy images showing the cellular structure of wild-type and *ItaS* *L. plantarum* strains. Abnormal thickening of the cell walls in the *ItaS* mutants is indicated by black arrowheads. The black electrodense granules detected in the bacterial cytoplasm correspond to polyphosphate inclusions, whereas the white holes are positions that were occupied by these polyphosphate granules and that were chipped or torn out during the preparation of ultrathin sections. The horizontal scale bars represent 500 nm.

Figure 4. Osmotic stress sensitivity in *L. plantarum* *ItaS* mutants. Bacterial cells were cultured in MRS medium without added NaCl or with NaCl at 0.4 and 0.8 M and growth was monitored at OD_{595nm}. wt corresponds to the wild-type strains.

Figure 5. Sensitivity to thermal stress in *L. plantarum* *ItaS* strains. (A) Normalized (min-max method) final OD_{595nm} values reached by cultures of *L. plantarum* strains and their derived *ItaS*-defective mutants grown for 24 h at different temperatures. (B) Survival at 55 °C. Wild-type strains and their corresponding *ItaS* mutants were subjected to incubation at 55°C and at several time intervals aliquots of the cell suspensions were spotted onto MRS plates that were incubated at 30 °C.

Figure 6. Sensitivity to mercury of *L. plantarum* strains. Growth curves of *L. plantarum* strains in MRS broth with Hg(II) (A) or methyl-Hg (B) concentrations ranging from 1.25 to 20 mg/l are represented.

Figure 7. Capacity of mercury binding in *L. plantarum* strains. The capacity to bind inorganic [Hg(II)] and organic (methyl-Hg) Hg was measured in wild-type and *ItaS* strains from *L. plantarum* Lpp+ (A) and *L. plantarum* WCFS1 (B). Results are expressed as the percentage of binding relative to the wild-type strains. The experiment was repeated five times with cells coming from independent cultures. Asterisks represent significant differences at $p < 0.001$.

441 **Table 1.** MICs ($\mu\text{g/ml}$) for different antimicrobials in *L. plantarum* strains.

Strain	WCFS1	WCFS1 <i>ltaS</i>	Lpp+	Lpp+ <i>ltaS</i>
Ampicillin	4	0.5	2	1
Cephalexin	>16	2	8	2
Chloramphenicol	16	8	8	8
Penicillin G	>3.2	0.4	>3.2	0.8
Tetracycline	8	2	4	4

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Figure 1

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Figure 6A

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Figure 6B

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