

Osmotic stress adaptation in *Lactobacillus casei* BL23 leads to structural changes in the cell wall polymer lipoteichoic acid

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The probiotic Gram-positive bacterium *Lactobacillus casei* BL23 is naturally confronted with salt-stress habitats. It has been previously reported that growth in high-salt medium, containing 0.8 M NaCl, leads to modifications in the cell envelope of this bacterium. In this study, we report that *L. casei* BL23 has an increased ability to form biofilms and to bind cations in high-salt conditions. This behaviour correlated with modifications of surface properties involving teichoic acids, which are important cell wall components. We also showed that, in these high-salt conditions, *L. casei* BL23 produces less of the cell wall polymer lipoteichoic acid (LTA), and that this anionic polymer has a shorter mean chain length and a lower level of D-alanyl-substitution. Analysis of the transcript levels of the *dltABCD* operon, encoding the enzymes required for the incorporation of D-alanine into anionic polymers, showed a 16-fold reduction in mRNA levels, which is consistent with a decrease in D-alanine substitutions on LTA. Furthermore, a 13-fold reduction in the transcript levels was observed for the gene LCABL_09330 coding for a putative LTA synthase. To provide further experimental evidence that LCABL_09330 is a true LTA synthase (LtaS) in *L. casei* BL23, the enzymic domain was cloned and expressed in *E. coli*. The purified protein was able to hydrolyse the membrane lipid phosphatidylglycerol as expected for an LTA synthase enzyme, and hence LCABL_09330 was renamed LtaS. The purified enzyme showed Mn²⁺-ion dependent activity, and its activity was modulated by differences in NaCl concentration. The decrease in both *ltaS* transcript levels and enzyme activity observed in high-salt conditions might influence the length of the LTA backbone chain. A putative function of the modified LTA structure is discussed that is compatible with the growth under salt-stress conditions and with the overall envelope modifications taking place during this stress condition.

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INTRODUCTION

Lactobacilli are normal inhabitants of the human oral cavity and digestive tract where some strains play beneficial roles. They also are used in food fermentation processes and as probiotics in the dairy industry. In most of these habitats, lactobacilli are confronted with salt stress. We have previously reported that *Lactobacillus casei* BL23 shows alterations in the cell wall, when it is subjected to high-salt growth conditions (Piuri *et al.*, 2005). We have observed that growth in the presence of 0.8 M NaCl increases its sensitivity to mutanolysin and lysozyme and

antibiotics that target cell wall synthesis pathways, and also leads to increased sensitivity to positively charged antimicrobial peptides, such as nisin (Piuri *et al.*, 2005). Some of these effects are likely to be a consequence of a decrease in peptidoglycan (PEPG) cross-linkage caused by the altered expression and activity of high and low molecular mass penicillin-binding proteins (PBPs) (Piuri *et al.*, 2005; Palomino *et al.*, 2009). In addition, osmotic stress induced by a high salt concentration has been reported to increase the autolytic activity and survival following lyophilization in *Lactobacillus delbrueckii* subsp. *lactis* (Koch *et al.*, 2007). We have used these properties to develop a new electroporation protocol involving growth in high-salt medium as a cell-wall-weakening agent (Palomino *et al.*, 2010).

Besides PEPG, lactobacilli cell walls contain secondary cell wall polymers named teichoic acids (Debabov *et al.*, 1996;

Abbreviations: CW, total cell wall fraction; Gro-P, glycerolphosphate; LTA, lipoteichoic acid; LtaS, LTA synthase; PBP, penicillin-binding protein; PG, phosphatidylglycerol; PEPG, peptidoglycan; WTA, wall teichoic acid.

Neuhaus & Baddiley, 2003). Together with PEPG, teichoic acids form a polyanionic matrix contributing to the porosity, elasticity and electrostatic steering of the cell envelope. A wide range of biological functions has been attributed to teichoic acids; they have been shown to participate in the modulation of the activity of PEPG hydrolases, the binding of surface proteins, phage adsorption, bacterial adhesion and interaction with the immune system (Neuhaus & Baddiley, 2003; Lebeer *et al.*, 2008; Weidenmaier & Peschel, 2008; Silhavy *et al.*, 2010). Two types of teichoic acid polymers are usually present in Gram-positive bacteria: wall teichoic acid (WTA), covalently linked to the PEPG, and lipoteichoic acid (LTA) tethered to the membrane by a lipid anchor. WTA polymers are commonly made up of glycerolphosphate (Gro-P) or ribitolphosphate subunits, but also sugar combinations have been reported. LTA is usually less diverse and often consists of a glycerolphosphate chain retained by a glycolipid anchor to the bacterial membrane. The backbone chains of both WTA and LTA are partly substituted with D-alanine residues and with additional glycosyl groups in many bacteria. D-Alanine substitutions are directly related to the ionic properties and function of these polymers. The modification of teichoic acids with D-alanine requires the function of four proteins encoded by the *dltABCD* operon, which is present in all sequenced *Lactobacillus* strains (Palumbo *et al.*, 2006; Perea Vélez *et al.*, 2007; Walter *et al.*, 2007). It has been reported that a *dlt* mutant in *Lactobacillus reuteri* displays an increased sensitivity to positively charged antimicrobial peptides, such as nisin (Walter *et al.*, 2007). Furthermore, work by Lebeer *et al.* (2007) showed that inactivation of *dltD* in *Lactobacillus rhamnosus* GG, which increases the negative surface charge, results in increased biofilm formation on polystyrene, suggesting indirectly a function of teichoic acids in biofilm formation.

LTA synthesis in Gram-positive bacteria starts with the biosynthesis of a glycolipid anchor. Once the glycolipid anchor reaches the outside of the membrane, the poly-(glycerolphosphate) backbone chain is produced, which is further decorated with D-alanine residues. The LTA backbone chain is produced by the transfer of Gro-P subunits derived from the membrane lipid phosphatidylglycerol to the tip of the growing chain. This reaction is catalysed by the LTA synthase enzyme (LtaS), which has been characterized in several micro-organisms (Gründling & Schneewind, 2007; Webb *et al.*, 2009; Schirner *et al.*, 2009; Wörmann *et al.*, 2011). An LtaS homologue (LBA0447) has also been identified in *Lactobacillus acidophilus*, and it has been reported that deletion of the gene encoding this protein leads to an absence of LTA in this organism (Mohamadzadeh *et al.*, 2011).

In this work, we further characterized phenotypic changes and alterations in cell envelope structures in *L. casei* BL23 under salt-stress conditions. Surface envelope properties were evaluated, including the ability to form biofilms in high-salt conditions and the cation-binding capacity of

purified cell walls from this growth condition. The amount of the cell wall polymer LTA, its chain length and levels of D-alanine substitution were determined in the presence of 0.8 M NaCl. We further analysed the correlation with D-alanine levels, chain length and quantities of LTA, with the expression of the *dlt* operon responsible for the D-alanine substitution and a putative *ltaS* gene encoded by LCABL_09330, which showed the expected activity for an LTA synthase enzyme. The extracellular enzymic domain of the LTA synthase (eLtaS) was cloned, expressed and purified and its activity evaluated under high-salt conditions. Based on our findings, we propose a model for how the observed changes in the cell envelope architecture allow bacteria to grow under salt-stress conditions.

METHODS

Growth conditions and constructions of plasmids. *L. casei* BL23 was grown at 37 °C in MRS medium (low-salt condition). MRS broth (BioKar) 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 MgSO₄·7H₂O l⁻¹, 0.05 g MnSO₄·4H₂O l⁻¹, 1 ml Tween 80 l⁻¹ and 20 g glucose l⁻¹. The stress condition was obtained by adding 0.8 M NaCl (high-salt conditions).

Plasmid pProEX-eLtaS_{LC} was constructed for the expression and purification of an N-terminally His-tagged version of the extracellular enzymic domain of the *L. casei* LtaS enzyme (LCABL_09330). The corresponding *ltaS* fragment was amplified from *L. casei* chromosomal DNA using the following primer pair: 5'-*Bam*HI-CGGG-ATCCGATTCGAGTGATGTGAAATCTG-3' and 5'-*Xba*I-GCTCTA-GACTACTTTGACTCGCTACTGCTGC-3'. The resulting PCR product was digested with *Bam*HI and *Xba*I and cloned into plasmid pProEX-HTb (Invitrogen) which was cut with the same enzymes. The resulting plasmid pProEX-eLtaS_{LC} was initially transformed into *E. coli* strain DH5 α . For protein expression and purification the plasmid was introduced into the *E. coli* Rosetta strain. The DNA sequence was verified by fluorescence automated sequencing at the Medical Research Council Clinical Science Centre Sequencing Facility at Imperial College London, UK.

Biofilm formation. The capacity of *L. casei* BL23 to form biofilms on artificial surfaces was evaluated as described in Lebeer *et al.* (2007). Bacteria were grown in MRS (Biokar, 20 g glucose l⁻¹), tryptic soy broth [TSB (Sigma) containing 17 g casein peptone (pancreatic) l⁻¹, 4.5 g glucose l⁻¹, 2.5 g dipotassium hydrogen phosphate l⁻¹, 5 g NaCl l⁻¹ and 3 g soya peptone (papain digest) l⁻¹], TSBm (modified TSB with addition of 20 g Bacto peptone l⁻¹) or FMRS [MRS fermentation broth, 2 g glucose l⁻¹ (ADSA Micro)], all with or without NaCl at the concentrations indicated in the figures. Assays were done in 96-well polystyrene microtitre plates (Greiner Labortechnik). Each well was inoculated with 5 μ l of an overnight culture, which had been adjusted to an OD₆₀₀ of 0.2. After 48 h incubation at 37 °C, growth was determined by OD₆₀₀, and biofilm formation was detected as follows: the supernatant was removed and the adhered cells in each well were stained for 30 min at room temperature with 200 μ l of 0.1% (w/v) crystal violet solution dissolved in 2-propanol/methanol/PBS [1:1:18 (v/v)]. Next, the wells were rinsed with deionized water, air-dried and subsequently 200 μ l 90% (v/v) ethanol was added to each well and the absorbance (A₅₉₅) determined. Data were analysed using the Student's two-tailed *t*-test. Statistically significant differences with *P*-values below 0.005 or between 0.005 and 0.05 are indicated in the figures.

Cell wall purification. Four different treatments were used to prepare and purify cell walls and to preserve their architecture. Briefly, bacterial cells were disrupted by shearing with glass beads in 50 mM Tris/HCl pH 7.5 followed by incubation with DNase (30 µg ml⁻¹) and RNase (5 µg ml⁻¹) for 2 h at 37 °C. The non-lysed cells were removed by a brief centrifugation step (1500 g, 5 min), and cell walls were recovered by centrifugation at 20 000 g for 20 min. This fraction was named the total cell wall fraction (CW); it was treated overnight at 48 °C with 10% (w/v) TCA and washed three times with water. This treatment retains LTA polymers and eliminates WTA (Lambert *et al.*, 1975). In parallel, CW was treated by heating at 100 °C in 2% (w/v) SDS for 15 min, and washed five times in water to remove LTAs and then the SDS-treated CW was incubated with 0.5 mg trypsin ml⁻¹ at 37 °C for 16 h in 0.1 M Tris/HCl (pH 7.5) to remove proteins (Piuri *et al.*, 2005). D-Alanine esters (Lambert *et al.*, 1975) and O-acetyl groups (Billot-Klein *et al.*, 1997) were removed from crude cell walls by alkali treatment with 10 mM NaOH for 1 h at 37 °C and the cell walls were subsequently extensively washed with water.

Cation-binding assays. Analytical-grade copper salt (CuSO₄·5H₂O) (Sigma) was used to prepare a 1000 p.p.m. stock solution in deionized water. For each of the five cell wall fractions, a sample (1 mg) was suspended in MilliQ water and mixed with the Cu²⁺ solution at 100 p.p.m. and the samples were incubated at optimized pH and temperature (pH 5 and 25 °C). After biosorption reached equilibrium, the cell walls were removed by centrifugation at 20 000 g, and the supernatant containing the residual, unabsorbed metal was used to determine the metal concentration by the bicinchoninic acid method (Allievi *et al.*, 2011). The biosorbed metal was calculated as $C_i - C_f$, where C_i and C_f are the initial and equilibrium concentrations of metal in the solution as p.p.m., with $C_i = 100$ p.p.m. Cu²⁺. Data were analysed using the Student's two-tailed *t*-test. Statistically significant differences with *P*-values below 0.005 or between 0.005 and 0.05 are indicated in the figures.

LTA purification and biochemical analysis. LTA was purified from *L. casei* BL23 grown in MRS medium (low salt) or MRS medium containing 0.8 M NaCl (high salt) by an extraction method using 1-butanol (Morath *et al.*, 2001; Gründling & Schneewind, 2007). LTA-containing fractions were identified in native 25% (w/v) PAGE gel (Tris/borate 0.089 M pH 8.3) followed by staining with alcian blue-silver stain (Wolters *et al.*, 1990), or 25% (w/v) SDS-PAGE (Laemmli buffer) followed by Western blot detection using a polyglycerolphosphate-specific LTA mAb (clone 55 from Hycult Biotechnology) and a HRP-conjugated anti-mouse IgG antibody (Cell Signalling Technologies) at 1:5000 and 1:10 000 dilutions respectively. Fractions containing LTA were pooled and dialysed 7–11 times against double-distilled H₂O and subsequently lyophilized. NMR analysis was used to study the structural modifications of LTA. Purified LTA (2 mg) was suspended in 500 µl D₂O (99.96% purity) and lyophilized. This procedure was repeated once and the lyophilized LTA sample was then suspended in 500 µl D₂O (99.99% purity) and 1d ¹H NMR spectra were recorded at 600 MHz (¹H) and 300 K on a Bruker AvanceIII spectrometer equipped with a TCI cryoprobe. The length of the Gro-P chain as well as the percentage of D-alanine substitution was calculated from the ¹H NMR integrals of the appropriate LTA-specific peaks. The LTA length calculation was based on a C16:0, C18:1 fatty acid composition (62 nonexchangeable protons from CH₂/CH₃ groups), which is the most abundant lipid anchor present in LTA from *L. casei* (Veerkamp, 1971; Machado *et al.*, 2004). The chain length is the ratio of integral values for Gro-P to CH₂/CH₃ groups of the fatty acids, taking into account the number of protons for each signal. The NMR analysis was performed on LTA samples isolated from three independent cultures. In addition, biochemical assays were performed to determine the

phosphate and glucose content of purified LTA samples according to Schnitger *et al.* (1959) and Kunst *et al.* (1984).

Protein purification. The *Staphylococcus aureus* eLtaS protein was used as control protein for enzyme activity assays and the protein expression and purification were performed as previously described (Lu *et al.*, 2009) using strain ANG571. For expression and purification of the eLtaS protein from *L. casei* BL23 strain Rosetta, pProEX-eLtaS_{LC} was used. This strain was grown with aeration at 37 °C in LB medium. Protein expression was induced by adding 0.1 mM isopropyl β-D-thiogalactopyranoside once the culture reached an OD₆₀₀ of 0.5 and the culture was further incubated overnight at 17 °C. The eLtaS_{LC} protein was purified by Ni affinity and size exclusion chromatography as described in Lu *et al.* (2009). Fractions containing the purified protein were pooled and concentrated using an Amicon Centricon concentrator (cut-off, 10 kDa) and the protein concentration determined using the bicinchoninic acid kit from Pierce. The purity of the protein was confirmed by analysis on 10% (w/v) SDS-PAGE gels and Coomassie staining.

In vitro enzyme assay for LtaS. The enzymic activity of purified proteins was measured by following the hydrolysis of phosphatidylglycerol (PG) labelled with NBD [1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycerol-3-[phospho-*rac*-(1-glycerol)] (ammonium salt)] (NBD-PG) using a method described previously (Karatsa-Dodgson *et al.*, 2010). Briefly, 1.8 ml of 10 mM sodium succinate buffer, pH 6.0, ionic strength 50 mM (adjusted with NaCl) was added to 50 µg of TLC-purified NBD-PG lipid (Avanti; order number 810163). Lipids were brought into suspension by sonication for 45 s at an amplitude of 11 using a Soniprep Sanyo sonicator. Next, MnCl₂ or the indicated divalent cation was added from a 1 M stock solution to give a final concentration of 10 mM; the samples were vortexed and 303 µl (~8.333 ng lipid) aliquots transferred into test tubes. Reactions were initiated by the addition of 1.52 µM purified eLtaS enzyme and mixtures were then incubated for 3 h at 37 °C in a water bath. The lipid reaction products were extracted by adding CHCl₃/MeOH to a final ratio of 0.9:1:1. Tubes were vortexed vigorously, centrifuged for 5 min at 17 000 g, and fractions of the bottom chloroform phase were transferred to a new tube and dried under a nitrogen stream. Dried lipids were then suspended in 10 µl chloroform, spotted onto pre-run Å60 silica gel plates (Macherey-Nagel) and separated using a chloroform:methanol:H₂O (65:25:4) solvent system. Plates were dried and subsequently scanned using a Fujifilm FLA-5000 imager equipped with a 473 nm excitation laser and an FITC emission filter. Fluorescent signals of lipid reaction products were quantified using the AIDA software (Raytest Isotopenmessgeräte). The phospholipase PC-PLC (PLC) from *B. cereus* (Sigma) was used as a positive control enzyme and assays were set up as described previously (Karatsa-Dodgson *et al.*, 2010). Reactions were set up in triplicate and mean values and SDs were plotted. Experiments were performed three times and a representative result is shown. To determine the enzyme specificity, the fluorescently labelled lipids 16:0-6:0 NBD-PC (Avanti 810130), 16:0-6:0 NBD-PE (Avanti 810153) and 16:0-6:0 NBD-PS (Avanti 810192) were purified on TLC plates and enzyme reactions set up as described above. At least three independent experiments were performed for all presented data, and a representative graph is shown. Data were analysed using the Student's two-tailed *t*-test. Statistically significant differences with *P*-values below 0.005 or between 0.005 and 0.05 are indicated in the figures.

cDNA synthesis and quantitative real-time PCR analysis. The hot phenol method was used for RNA extraction (Piuri *et al.*, 2003). The concentration of the purified RNA was determined using a Nanodrop2000 spectrophotometer (Thermo Scientific). cDNA synthesis was performed as described previously (O'Rourke *et al.*, 2000) with the following modifications: cDNA was synthesized with 25 pmol reverse primers specific for 16S rRNA, *dlta* or *ltaS*

(LCABL_09330), and 3 µg of RNA. The mixtures were incubated at 95 °C for 10 min, kept 2 min on ice and incubated with 100 U of M-MLV reverse transcriptase (Ambion), according to the manufacturer's recommendations. To confirm the absence of genomic DNA contamination in the RNA samples, reactions were also set up in the absence of reverse transcriptase. cDNA samples were diluted 10-fold and a quantitative real-time PCR run was employed on a MyiQ Real-time thermal cycler (Bio-Rad) using the Real-Mix (Biodynamics) according to the manufacturer's directions. Melting curve analysis and determination of amplicon size verified amplification of the appropriate transcripts. The 16S rRNA gene was used as reference. Results were analysed using the critical threshold (ΔC_T) and the comparative critical threshold ($\Delta\Delta C_T$) method in the IQTM 5 Optical System Software. Each sample was analysed in triplicate, and samples from two independent experiments were analysed for each condition. Reverse primers were as follows: for 16S rRNA 5'-GCGAAGCGGCTGTCTGG-3', for *dltA* 5'-GCCCAACCGAAACGACTGTG-3' for *ltaS* 5'-ATGTGAATCATAA-ATGGTACTCG-3'. Forward primers were: for 16S rRNA 5'-GCAC-TGAAGGGCGGAAACC-3', for *dltA* 5'-GTACCCGCTGACACTTG-ATTGC-3' and for *ltaS* 5'-GCCCGTTATCTTGACGAAGC-3'.

RESULTS

Lactobacillus casei BL23 grown in high-salt medium shows increased biofilm formation and purified cell walls have increased cation-binding capacity

In previous work from our laboratory several cell envelope modifications were observed when *Bacillus* or *Lactobacillus* strains were exposed to high-salt conditions (Lopez *et al.*, 2000; Piuri *et al.*, 2005; Palomino *et al.*, 2009). A property that is often dependent on surface architecture is the ability of bacteria to form biofilms. To assess if changes caused by salt stress alter the ability of *L. casei* BL23 to form biofilms, biofilm assays were performed in different media and at different NaCl concentrations (0, 0.5 M and 0.8 M). Lebeer *et al.* (2007) established that in lactobacilli the medium components influence biofilm formation and, in particular, high glucose and Tween 80 decrease the yield of biofilm. MRS is not a preferred medium since its high glucose content (20 g l⁻¹) results in high rate of acidification, and the presence of Tween 80, a detergent that decreases the envelope interaction with the polystyrene plaque surface, decreased the biofilm formation, regardless of the salt concentration. Therefore, we lowered the glucose content in MRS to 2 g l⁻¹ (0.2%, w/v) and under these conditions an increase in biofilm formation was observed upon addition of 0.8 M NaCl (Fig. 1a). The ability of *L. casei* BL23 to form biofilms in the presence of varying NaCl concentrations was also assessed in TSB medium (glucose 4.5 g l⁻¹) and a modified version with higher peptone concentration. In all media, the biofilm yield increased proportionally with the NaCl concentration. The highest increase in biofilm formation was obtained in TSBm medium, where a fourfold increase in biofilm formation was observed upon addition of 0.8 M NaCl (Fig. 1a).

Purified cell walls of high-salt-adapted cells also showed an increased ability to bind cations when evaluated for their

capacity to bind Cu²⁺ ions (Fig. 1b). Purification of cell walls was performed using differential treatments to preserve the diverse cell wall components. As shown in Fig. 1(b), increased copper binding was observed for cell walls isolated from bacteria grown under high-salt conditions for all purification methods. These results further support the notion that modification in the cell envelope architecture occur as a consequence of high-salt adaptation.

The LTA content is altered when *L. casei* BL23 is grown in high-salt medium

For a *L. rhamnosus* GG *dlt* mutant lacking D-alanine modifications on teichoic acids, a similar increase in biofilm formation was observed (Lebeer *et al.*, 2007). This led us to analyse the composition and properties of LTA, an important secondary wall component. LTA was purified from *L. casei* BL23 cultures grown in MRS control medium (low-salt conditions) or medium containing 0.8 M NaCl, quantified and further analysed. The mean LTA mass recovery was 9.0 ± 1.8 mg dried weight from 3 l culture (15.9 g total biomass) in the low-salt condition and 4.3 ± 0.8 mg from 6 l culture (11.6 g total biomass) grown in the high-salt conditions. The yield of LTA was, therefore, 0.59 and 0.35 mg g⁻¹ culture, respectively. This result revealed a decrease in the recovery of LTA from strains grown in high-salt conditions. When visualizing LTA-containing fractions during the purification procedure on native and SDS-PAGE gels, we observed an increased mobility of the LTA isolated from *L. casei* BL23 grown in high-salt conditions compared with the low-salt medium. This observation could be explained by a change in the net charge of the polymer, which will be influenced by the degree of D-alanine substitutions and the chain length of the Gro-P backbone.

The LTA is shorter and less D-alanylated when *L. casei* BL23 is grown in high-salt medium

To gain information on the actual structural differences in the LTA polymer under high-salt conditions, LTA was purified and analysed by NMR and the phosphate and glucose content was also determined using biochemical assays. Representative NMR spectra are shown in Fig. 2(a, b). Based on the NMR analysis of three independently isolated LTA samples, mean glycerolphosphate chain lengths of 42 ± 3 in control conditions and 31 ± 4 under high-salt conditions were obtained. LTA in *L. casei* is linked to a trihexosyldiacylglycerol [Glc(β1-6)Gal(α1-2)Glc(α1-3)acyl₂Gro] glycolipid anchor (Fischer *et al.* 1990). Hence, the ratio of the phosphate concentration per two glucose molecules can be used for the chain length calculation. Applying these calculations to purified LTA revealed a mean chain length of 48 ± 4 for the LTA isolated from low-salt conditions and 34 ± 0.8 for the LTA extracted from high-salt conditions, which is consistent with the results of the NMR analysis. Both methods indicate that the LTA

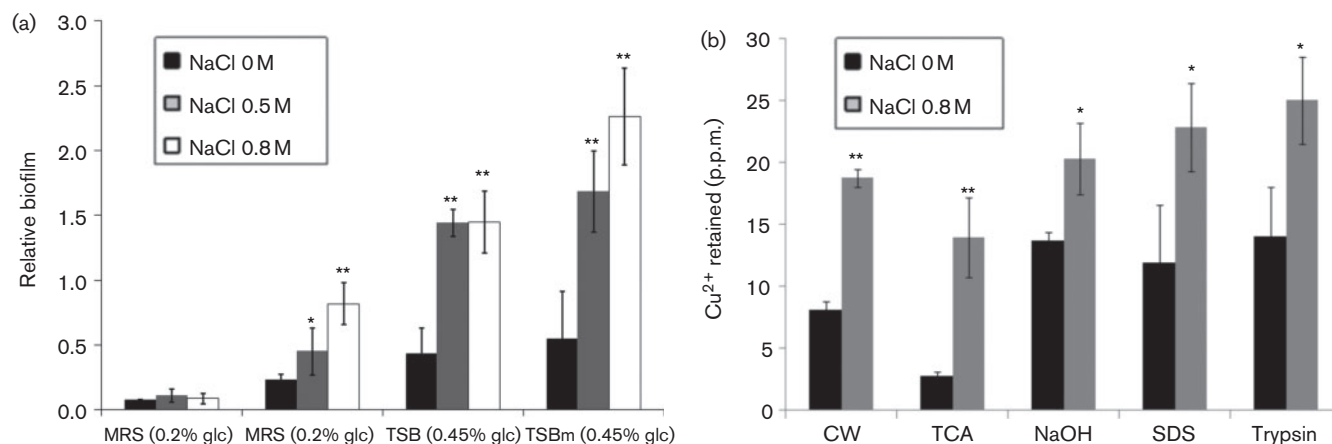


Fig. 1. Surface properties. (a) Relative biofilm formation of strain *L. casei* BL23 when grown in MRS or TSB, 0.2% (w/v) glucose (glc), medium or modified MRS 0.2% (w/v) glc and mTSB (0.45%, w/v, glc, higher peptone concentration) without added NaCl (0 M) or with addition of 0.5 M or 0.8 M NaCl. Results plotted are normalized (OD_{590}/OD_{600}) where the growth was determined as OD_{600} before the crystal violet staining of adherent cells. Four independent experiments with triplicate samples were performed. (b) Cation-binding capacity of purified cell walls. CW (1 mg) prepared from *L. casei* BL23 strains grown in MRS medium (low-salt) or MRS with 0.8 M NaCl (high-salt) was mixed with 100 p.p.m. of a copper solution. Samples were incubated until equilibrium was reached and copper retained within the CW was calculated, as described in Methods. Different treatments, as described in Methods, were used to selectively removed molecules from CW prior to the Cu^{2+} -binding assays: crude stands for no cell walls obtained from disruption and differential centrifugation; TCA to remove WTA; NaOH to remove D-alanyl esters and O-acetylation; SDS to remove LTA and proteins, and trypsin to complete protein removal. Three independent experiments with duplicate samples were performed. Bars show SD. Student's *t*-test was used to determine statistically significant differences between high-salt media and control medium; *, $P 0.005 \leq 0.05$; **, $P < 0.005$.

isolated from *L. casei* BL23, when grown under high-salt conditions, is significantly shorter than the LTA isolated from normal growth conditions. The percentage D-alanine substitution was calculated as the ratio of integral values for D-alanine to Gro-P $\times 100$. Analysis of percentage D-alanine substitution showed means of 64 ± 4 in low-salt and 27 ± 9 in high-salt conditions. Both the differences in chain length and in D-alanine substitution are statistically significant, ($P=0.0039$ and $P=0.0128$ respectively) and are likely responsible for the increased electrophoretic mobility observed in high-salt conditions.

Functionality of the *L. casei* BL23 LTA synthase, LtaS

Biosynthesis of the polyglycerolphosphate LTA backbone in Gram-positive bacteria involves the activity of LTA synthase or LtaS-type enzymes. In *L. acidophilus*, protein LBA0447 has been identified as an LTA synthase enzyme (Mohammadzadeh *et al.*, 2011). The closest homologue in *L. casei* BL23 is LCABL_09330 and to gain insight into the functionality of this enzyme, the enzymic eLtaS_{LC} domain was cloned, expressed and purified as an N-terminal His-tagged version from *E. coli* extracts (Fig. 3a). Functional LtaS-type enzymes are able to cleave the Gro-P head group of the membrane lipid PG, which will result in the production of diacylglycerol (DAG) (Karatsa *et al.*, 2010). This can be readily visualized using fluorescently labelled

lipids and TLC analysis. *In vitro* assays to measure the enzymic activity of the *L. casei* BL32 enzyme were performed as described in Methods. As shown in Fig. 3(b), the *L. casei* BL23 enzyme gave a reaction product, which ran on TLC plates with the same mobility as that observed for *Staph. aureus*, indicating that NBD-DAG is formed upon the hydrolysis of NBD-PG.

The specificity of the enzyme for PG lipid substrate was tested by assessing the ability of the enzyme to cleave different input lipids including NBD-PC, NBD-PE and NBD-PS [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS)]. As can be seen in Fig. 3(c), *L. casei* BL23 enzyme can only hydrolyse the NBD-labelled PG lipid substrate, indicating a narrow substrate specificity.

Next, the metal ion requirement for the enzyme was tested. This analysis revealed that addition of Mn^{2+} resulted in the highest enzyme activity, while Mg^{2+} , Ca^{2+} or Zn^{2+} resulted in low enzyme activity. Furthermore, the metal ion chelator EDTA inhibited the activity of the enzyme (Fig. 4a). Based on these results, which are characteristic for LtaS-type enzymes, the *L. casei* BL23 protein LCABL_09330 was renamed LtaS. Since changes in the chain length of the LTA polymer were observed when cells were grown in high-salt conditions, eLtaS_{LC} activity was assayed in the presence of an increasing concentration of NaCl. As can be seen in Fig. 4(b), high activity was observed at low NaCl

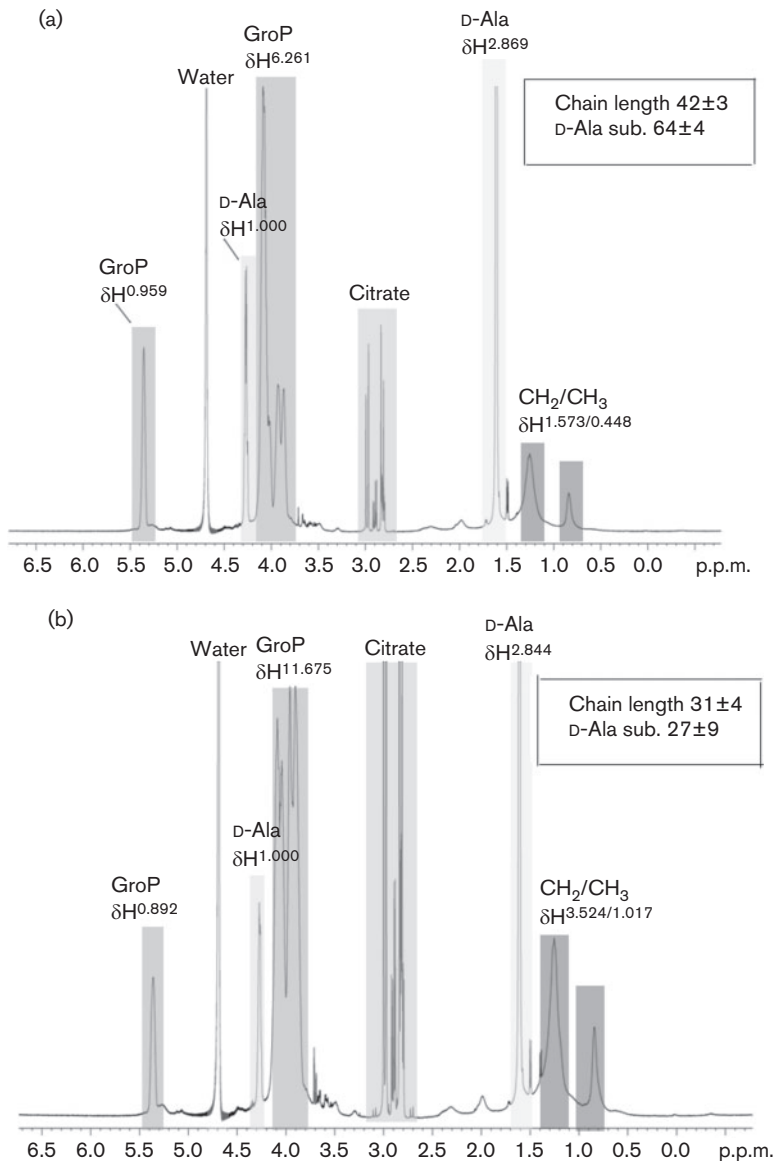


Fig. 2. NMR analysis of purified LTA. LTA (2 mg) purified from *L. casei* BL 23 grown in low-salt condition (a) or high-salt conditions (b) was analysed by NMR. ¹H NMR spectra were recorded at 600 MHz and the peaks corresponding to the D-alanine and GroP components of LTA as well as the CH₂CH₃ groups of the fatty acids are annotated as previously described (Soon Jang *et al.*, 2011; Morath *et al.*, 2001). The signals derived from citrate and water are also marked. The integration values are shown above each signal. Chain length was determined by calculating the ratio of integral values for GroP to CH₂/CH₃ from the fatty acids and percentage D-alanine substitution by calculating the ratio of integral values for D-alanine to Gro-P × 100 and taking into account the number of protons for each signal (5 protons per GroP group; 62 protons for the CH₂/CH₃ per lipid anchor and 4 protons per D-alanine group). The experiment was performed in triplicate and a representative spectrum is shown. The insets show the mean values for chain length and D-alanine substitution of the three assays.

concentrations but at increasing salt concentration the activity decreased abruptly.

High-salt conditions lead to a decrease in *dltA* and *ltaS* gene expression

We next assessed whether the *dlt* operon or *ltaS* gene expression is affected under high-salt conditions. To this end, RNA was extracted from *L. casei* strains grown in low-salt or high-salt conditions and *dltA* and *ltaS* transcript levels were determined by quantitative real-time PCR. No significant differences were observed in the amount of 16S rRNA for the different samples, indicating that all assays were performed with equal amounts of total RNA. The ratio of the transcript level in low-salt versus high-salt conditions for *dltA* was 16 ± 2 , which is consistent with a decrease in D-alanine substitution on LTA under high-salt conditions. Similar results were obtained for *dltC*

expression, a gene further downstream in the *dlt* operon (data not shown). A correlation between environmental changes and LTA D-alanine content has also been reported in *Streptococcus gordonii*, *Staph. aureus* and *Bacillus subtilis* (Ellwood & Tempest, 1972; MacArthur & Archibald, 1984; Lebeer *et al.*, 2007). Moreover, when the *ltaS* gene expression was analysed, the transcription ratio in low-salt versus high-salt conditions was 13 ± 0.7 , consistent with the decrease in activity expected for an LTA chain with a shorter Gro-P backbone.

DISCUSSION

Lactobacillus casei BL23 grown under osmotic stress induced by a high salt concentration shows modifications in LTA polymer resulting in a lower level of D-alanyl-substitution and a shorter mean chain length involving the

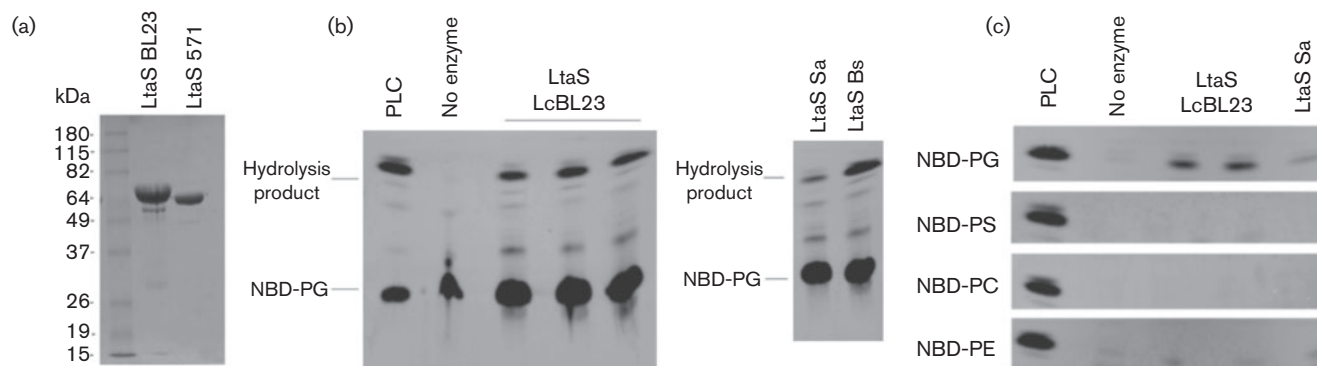


Fig. 3. *In vitro* activity of the *L. casei* eLtaS enzyme. (a) Coomassie-stained gel of purified *L. casei* and *Staph. aureus* eLtaS proteins. Extracellular enzymic domains were purified as N-terminal His-tag fusion proteins and 10 µg purified protein separated on a 10% SDS-PAGE gel and visualized by staining with Coomassie brilliant blue. (b) *In vitro* enzyme activity of the *L. casei* LtaS (LtaS LcBL23), *Staph. aureus* LtaS (LtaS Sa) and *B. subtilis* LtaS (LtaS Bs) enzymes. The NBD-PG lipid substrate was incubated with LtaS LcBL23, LtaS Sa, LtaS Bs enzyme, lipid reaction products extracted and separated by TLC and fluorescent lipid bands visualized by scanning plates with a fluorescence imager. As negative and positive controls, reactions were set up without enzyme or with *Bacillus cereus* PLC enzyme, respectively. Note that only 10% of the PLC reaction was run on the TLC plate. Positions of NBD-PG and hydrolysis product (NBD-DAG) are indicated on the left and proteins added to each reaction are shown at the top of the panel. (c) The substrate specificity of the *L. casei* eLtaS enzyme was tested by setting up enzyme assays with NBD-PG, NBD-PS, NBD-PC or NBD-PE, as indicated. As a control, no enzyme and eLtaS of *Staph. aureus* was added to reaction mixtures, as indicated above the panels. PLC reactions using NBD-PG as a substrate were run alongside to indicate the mobility of the hydrolysis product.

activity of LTA synthase (LtaS-type enzymes). A correlation between the number of genome-encoded LtaS-like proteins, bacterial shape and complexity of developmental processes has been previously noted (Wörmann *et al.*, 2011; Reichmann & Gründling, 2011). *Staph. aureus*, an organism with a 'simple' round shape, synthesizes LTA with a single enzyme, while rod-shaped *Bacillus* spp., which also undergo the complex developmental process of spore formation, have four LtaS-type enzymes. In *Listeria monocytogenes* a two-enzyme system is used; an LtaS-type

enzyme, LtaP, acts as an LTA primase and produces the Gro-P-glycolipids intermediate while a second enzyme, LtaS, functions as actual LTA synthase and generates the polyglycerolphosphate backbone chain (Webb *et al.*, 2009). A *L. acidophilus* strain with a deletion in LBA0447, coding for an LtaS-type enzyme, has been characterized and shown to lack LTA (Mohamadzadeh *et al.*, 2011). The closest homologue to this protein in *L. casei* BL23 is LCABL_09330 and in this study we show that this protein has the characteristics of an LTA synthase enzyme. However, it is

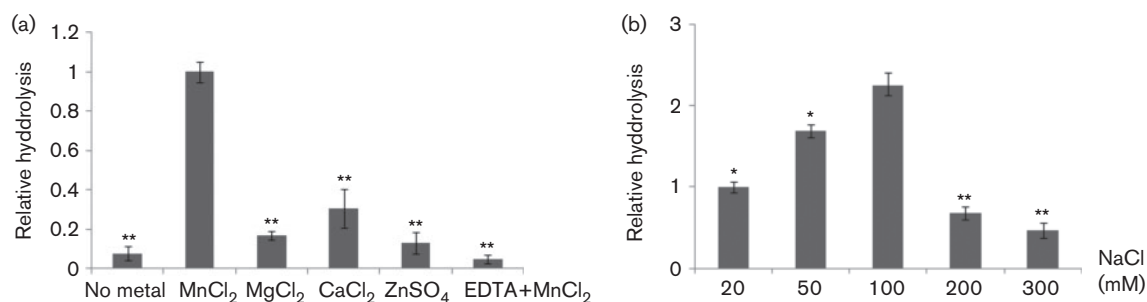


Fig. 4. *L. casei* eLtaS activity is dependent of Mn^{2+} and NaCl concentration. (a) *In vitro* enzyme reactions were set up with purified eLtaS_{Lc} protein in 10 mM sodium succinate (pH 6.0) buffer (ionic strength, 20 mM) in the absence or presence of the indicated metal ion at a final concentration of 10 mM or in the presence of 16 mM EDTA in the presence of 10 mM $MnCl_2$. (b) NBD-PG vesicles were prepared in 10 mM sodium succinate (pH 6) buffer; the ionic strength was adjusted to the indicated value of NaCl (mM). Student's *t*-test was used to determine statistically significant differences between (a) enzyme activity in the presence of $MnCl_2$ and activity with each of the other enzyme reaction conditions and (b) activity in 100 mM NaCl versus the other ionic strengths. Four independent experiments were performed for data presented; *, $P \leq 0.05$; **, $P < 0.005$.

of note that a second LtaS-type enzyme, LCABL_12830, is present in *L. casei* BL23 (Mazé *et al.*, 2010) and perhaps, like *List. monocytogenes*, this strain uses a two-enzyme system for LTA synthesis.

Several pleiotropic effects, including difference in susceptibility to enzymic lysis, increased sensitivity to cationic antimicrobials, such as nisin, and an increased capacity to form biofilm on artificial surface, might not only result from changes in the PEPG structure (Piuri *et al.*, 2005) but, as shown here, also be due to changes to the zwitterionic character of the LTA molecule. The results described here revealed modification of the LTA polymer as a consequence of high-salt stress. A decrease in LTA D-alanylation will result in increased negative charge of the cell wall, and this might help to expel toxic Na^+ , which is present in excess in the high-salt conditions, from the cell, preventing it from reaching the cytoplasm and interfering with metabolic processes. Consistent with this hypothesis, we found an increased cation-binding capacity of purified cell walls obtained from the high-salt conditions (Fig. 1b), involving the different components of the cell envelope. In particular, treatments that mimic cell wall modifications, as seen in high-salt conditions for cell walls isolated from low-salt conditions, such as treatment with NaOH (which removes D-alanyl esters) or boiling in SDS (which removes LTA and proteins) resulted in increased cation-binding ability of cell walls isolated from low-salt conditions (Fig. 1b). Concerning the variations in D-alanine content of LTA, this modification has also been reported in *Strep. gordonii*, *Staph. aureus* and *B. subtilis* (Lebeer *et al.*, 2007; Kiriukhin & Neuhaus, 2001; Ellwood & Tempest, 1972; MacArthur & Archibald, 1984). Cation-induced transcriptional regulation of the *dlt* operon has been reported in *Staph. aureus*. D-Alanylation is diminished and *dlt* expression is repressed at the transcriptional level when this bacterium is grown in medium containing increased NaCl concentrations (Koprivnjak *et al.* 2006).

A model of the proposed overall modifications taking place in the cell envelope as a consequence of the high-salt adaptation is presented in Fig. 5; it involves multiple factors affecting surface properties: (1) as previously reported an increase in cyclic fatty acids is observed under high-salt conditions (Machado *et al.*, 2004); (2) the PEPG is less cross-linked and the number of layers diminished (Piuri *et al.*, 2005; Palomino *et al.*, 2009) and (3) as shown here, LTA chains are shorter and contain a reduced amount of D-alanine substitutions. All these modifications lead to an increase of the negative charges of the outermost cell wall layers that would ensure the extrusion of toxic Na^+ ions and the survival of the bacteria. We also include in the proposed model the WTA polymer attached covalently to PG, which is also subject to D-alanyl substitution. Although further investigation is needed to understand the involvement of WTA in osmotic adaptation, we have already reported that cell walls from low-salt conditions treated with TCA, which removes teichoic acids, behaved similarly to cell walls from high-salt conditions

with respect to sensitivity to lytic enzymes (Piuri *et al.*, 2005). This result suggested that WTA would be reduced or modified in high-salt conditions. In fact, as shown in Fig. 1(b), removal of WTA with TCA treatment modifies the cation-binding capacity of purified cell walls.

The synthesis of PEPG, LTA and WTA occurs within the proton gradient of the membrane-wall matrix of the growing cell. LTA seems to play a crucial part in cell division, which might explain why it is indispensable for viability in some bacteria. The activity of the major autolysin enzymes that cleave PEPG strands to separate daughter cells upon cell division depends on bivalent cations, and it is possible that providing cations could be an important role of teichoic acids in controlling autolysins. LTA has been shown to be involved in controlled binding of autolysins. For Gram-positive bacteria, it has been reported that LTA lacking D-alanine has an improved capacity for autolysin binding that results in an altered PEPG that is more susceptible to lysis (Steen *et al.*, 2005; Weidenmaier & Peschel 2008). Therefore, the increased lysis in high-salt conditions would be the result of modifications of both PEPG (decreased amount and cross-linking) and non-PEPG polymers (fewer D-alanine substitutions).

Growth under high-salt conditions is of industrial importance, with NaCl concentration ranging from 0.3 M in the gut when *L. casei* is 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). These bacteria are subjected to osmotic stress and show increased survival after lyophilization, an important quality in the starter culture industry (Kets *et al.*, 1996; Koch *et al.*, 2007). Also a reduction in the LTA content, as observed in this study, would be welcome in probiotic use since several lactobacilli strains present inflammatory reactions as a consequence of their LTA (Lebeer *et al.*, 2012; Mohamadzadeh *et al.*, 2011).

It has previously been suggested that LTAs are involved in biofilm formation of *Lactobacillus* and adhesion to human enterocyte-like cells or mouse gastric epithelium (Lebeer *et al.*, 2007; Mohamadzadeh *et al.*, 2011). In natural niches, such as the gastrointestinal tract, adherent bacteria can form microcolonies and multicellular structures, recognized as biofilm-like communities. Increased negative surface charges and biofilm formation on polystyrene observed in high-salt situations are features that might favour the adherence and colonization capacity of the gastrointestinal mucosa, characters which are considered to contribute to immune modulation and pathogen exclusion by probiotic bacteria. Lactobacilli genes putatively involved in stress resistance or in adhesion studied by mutant analysis showed the influence of LTA and D-alanylation on probiotic capacity (Lebeer *et al.*, 2008). It was also demonstrated that a *dltD* mutant of the model probiotic *L. rhamnosus* GG having modified LTA molecules has an enhanced probiotic efficacy compared to WT (Perea Vélez *et al.*, 2007). Absence of D-alanyl esters in teichoic acids has

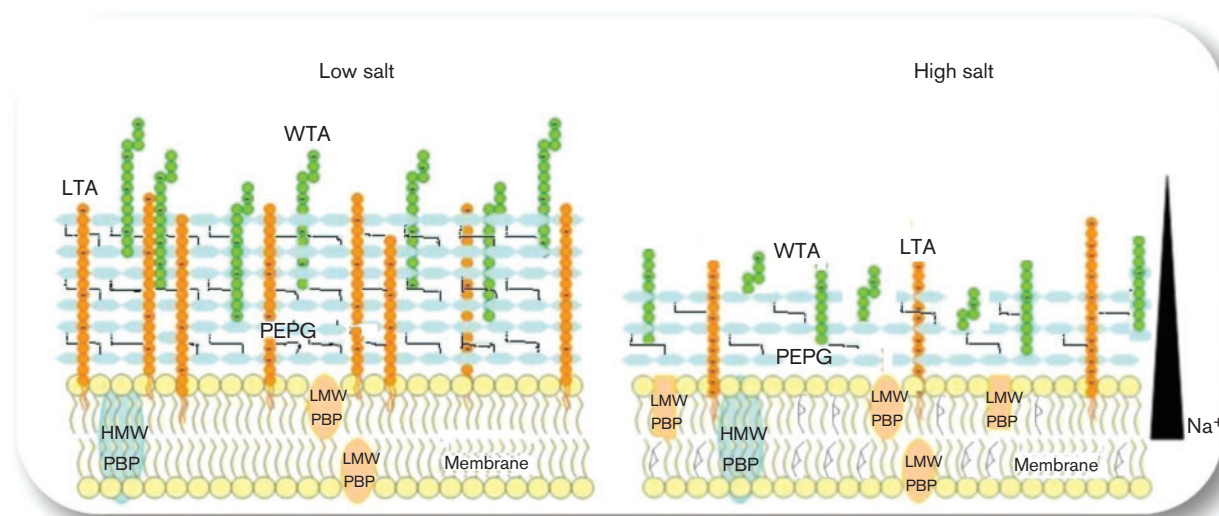


Fig. 5. Proposed model of the cell envelope adaptation under high-salt stress conditions. The Gram-positive cell envelope comprises a thick PEPG layer (light blue hexagons), stabilized by wall teichoic acids (WTA, green circles) and the membrane-linked lipoteichoic acid (LTA, orange circles). D-Alanine substitutions are represented as filled Gro-P monomer circles (orange) and absence of esterification is shown with a minus symbol within the circles. Membrane phospholipid bilayer (yellow and grey) is modified in high-salt conditions by increase in cyclic fatty acids (grey triangles) (Machado *et al.*, 2004). Increased amounts of low molecular mass PBPs (LMW PBP) and decreased amounts of high molecular mass PBPs (HMW PBP) are found in high-salt conditions (Piuri *et al.*, 2005). The PEPG chains are diminished and are less cross-linked (black lines); the secondary wall polymers decrease; LTA chains are shorter and less substituted. All these modifications lead to an increase of the negative charges that would ensure the extrusion of toxic Na^+ ions (black gradient represents Na^+ concentration from the inside to the outside). MRS medium alone, low-salt conditions, versus MRS with 0.8 M NaCl, high-salt conditions.

been shown to alter folding of exoproteins (Hyrylainen *et al.* 2000; Clemans *et al.*, 1999). Altered surface proteins might lead to altered physico-chemical properties of the cell surface. We intend to verify if increased attachment to cells is obtained with high-salt pre-growth which might enhance probiotic efficacy.

Our findings further support a model in which modifications in different cell wall constituents help bacteria to grow under salt stress. Understanding the modification of the surface properties under this growth condition might help to develop technological improvements.

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