Cell wall modifications during osmotic stress in *Lactobacillus casei*

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ABSTRACT

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Aims: To study the modification of the cell wall of *Lactobacillus casei* ATCC 393 grown in high salt conditions.

Methods and Results: Differences in the overall structure of cell wall between growth in high salt (MRS + 1 mol l^{-1} NaCl; N condition) and control (MRS; C condition) conditions were determined by transmission electronic microscopy and analytical procedures. *Lactobacillus casei* cells grown in N condition were significantly larger than cells grown under unstressed C condition. Increased sensitivity to mutanolysin and antibiotics with target in the cell wall was observed in N condition. Purified cell wall also showed the increased sensitivity to lysis by mutanolysin. Analysis of peptidoglycan (PG) from stressed cells showed that modification was at the structural level in accordance with a decreased PG cross-link involving penicillin-binding proteins (PBP). Nine PBP were first described in this species and these proteins were expressed in low percentages or presented a modified pattern of saturation with penicillin G (Pen G) during growth in high salt. Three of the essential PBP were fully saturated in N condition at lower Pen G concentrations than in C condition, suggesting differences in functionality *in vivo*.

Conclusions: The results show that growth in high salt modified the structural properties of the cell wall.

Significance and Impact of Study: Advances in understanding the adaptation to high osmolarity, in particular those involving sensitivity to lysis of lactic acid bacteria.

Keywords: *Lactobacillus casei*, osmotic stress, PBP, peptidoglycan.

INTRODUCTION

Lactobacilli are Gram-positive, nonsporulated, anaerobic bacteria. They are normal inhabitants of the oral cavity and the digestive tract in humans. Some *Lactobacillus* strains are used in food fermentation, and typical examples are found in the dairy industry for the production of cheese, yoghurt and other fermented milk products. As probiotics, these microorganisms may have an effect because of their adhesive properties resulting in a microbial biofilm formation. In most of these habitats, lactobacilli are confronted with salt

stress. Certain bacteria have the ability to respond to environmental conditions by altering the nature of their wall polymers. The cell wall of Gram-positive bacteria is highly resistant to mechanical disruption. In particular, a high resistance to wall hydrolases such as lysozyme or mutanolysin is observed for several lactobacilli species. Osmoadaptation requires the accumulation of osmoprotectors by activation of transport systems. In *L. casei* not only glycine–betaine and carnitine but also peptides can balance the hyperosmotic stress. We show that protease PrtP, the main peptide supplier, was activated during growth in high salt medium. Although genetic expression was not altered by salt, the activity of the enzyme increases (Piuri *et al.* 2003) similar to that of the QacT transporter of *L. plantarum* whose expression was semiconstitutive (Glaasker *et al.*

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1998). These proteins were embedded in the cell envelope (membrane and/or wall) and changes in their environment might influence their activity. In this sense it is worthwhile to remind the variations in membrane lipid and fatty acid composition that were reported in *B. subtilis* and *L. casei* during hypersaline stress (Lopez *et al.* 1998; Machado *et al.* 2004).

In Gram-positive bacteria, the cell wall consists of several distinctive structures. The murein layer is a rigid, shape-determining structure. It contains almost equal amounts of polysaccharides and peptides and can be classified as peptidoglycan (PG) (Delcour *et al.* 1999). PG is the essential structural element that provides shape and stability to most bacterial cells. It is composed of glycan chains of repeating *N*-acetyl-glucosamine and *N*-acetyl-muramic acid residues (linked $\beta 1 \rightarrow 4$) and cross-linked by peptide side chains. The primary structure of the PG of *L. casei* ATCC 393 belongs to subgroup A4 and has a common monomer structure, GlcNAc–MurNAc–L-Ala-g-DGlu–L-Lys–D-Ala, with an asparagine attached to the ω-amino group of lysine (de Ambrosini *et al.* 1996). Polymerization of PG involves the addition of disaccharide pentapeptide subunits onto a growing glycan chain by a glycosyltransferase. The peptide side chains are then cross-linked by a transpeptidase activity. The penicillin-binding proteins (PBP) carry out the glycosyltransf-erase and transpeptidase reactions required for PG synthesis. The PBP can be divided into three classes based on amino acid sequence similarities. Class A high-molecular weight (HMW) PBP are bifunctional PBP that contain an N-terminal glyco-syltransferase domain and a C-terminal transpeptidase domain. Class B high-molecular weight (HMW) PBP are known to have only transpeptidase activity and are, in some cases, required for cell septation and maintenance of cell shape. Finally low-molecular weight (LMW) PBP generally have D,D-carboxy-peptidase activity and, in some cases, are involved in regulating the number of cross-links between the glycan strands. The LMW PBP are subdivided into four enzymatic classes: monofunctional DD-carboxypeptidases, bifunctional DD-carb-oxypeptidase/DD-endopeptidase, DD-endopeptidase, and class C β-lactamases. Although the enzymology of PBP has been studied extensively in many bacteria (Ghuysen 1994; Popham and Young 2003), there is limited information about it in *Lactobacillus* and the *in vivo* functions of these proteins have been poorly reported (Griaznova *et al.* 1990).

Murein represents 50% of the cell wall, although other polymers such as teichoic acids and lipoteichoic acids (LTA) are present in the cell wall of Gram-positive bacteria. Teichoic acids (WTA) and other wall polysaccharides are covalently attached to the PG and they represent 20–50% of the dry weight of the cell wall. Teichoic acids are important antigens and bacteriophage receptors. They are polyanions (large, negatively charged molecules), and may maintain the appropriate ionic environment (especially for divalent cation

sequestration) and pH gradient for membrane synthetic activities, oxidative and fermentation processes. Bacilli, lactobacilli, streptococci, staphylococci, micrococci and *Streptomyces* are especially rich sources of teichoic acids (Neuhaus and Baddiley 2003). The cell wall of Gram-positive bacteria is highly resistant to enzymatic treatment. In particular, high resistance to cell wall hydrolases such as lysozyme or mutanolysin is observed for several lactobacilli species.

Applied aspects involving lysis of lactic bacteria are: (i) intracellular peptidases released into cheese from starter lysis that accelerate the peptidolytic steps and contribute to the higher levels of amino acids decreasing bitterness; (ii) the ability to survive to lytic enzymes as a requirement for probiotic strains in oral administration; (iii) lactic acid bacteria offer an original alternative as antigen expression or delivery vehicles, as they are generally recognized as safe. Lysis for the released of heterologous proteins is an important issue in recombinant DNA technology.

Bacteria have the ability to respond to environmental conditions by altering the nature of their cell wall. In this work we describe their modifications because of growth in high salt of *L. casei* ATCC 393; in particular those related to the structure of PG (degree of cross-linking, presence and behaviour of PBP).

MATERIALS AND METHODS

Micro-organisms, media and growth conditions

Lactobacillus casei ATCC 393 was used in all experiments. Cultures were usually stored at $-70°C$ in 10% w/v glycerol. For cultivation, aliquots were reactivated in MRS broth (BioKar, Beauvais, France) containing 10 g tryptone, 4 g yeast extract, 8 g meat extract, 5 g sodium acetate, 0·2 g $MgSO_4 \cdot 7H_2O$, 0·05 g $MnSO_4 \cdot 4H_2O$ and 1 ml Tween 80, pH 6·5 and 2% w/v glucose l^{-1}, at 37°C for 16 h. N condition was obtained by addition of 1 mol l^{-1} NaCl and pH adjusted with NaOH.

Electron microscopy

To prepare samples for transmission electron microscopy (TEM), cultures were grown until mid-exponential phase and cells were fixed by adding 2·5% (v/v) glutaraldehyde. After 30 min, cells were harvested by centrifugation (5000 *g*, 5 min) and pellet mixed with 1·25% (w/v) water agar, and the agar was allowed to solidify. The agar was then cut into *ca* 1-mm pieces and fixed for additional 30 min in phosphate-buffered 2·5% (v/v) glutaraldehyde. The agar pieces were rinsed three times with phosphate buffer and postfixed in phosphate-buffered 1% (w/v) osmium tetrox-ide for 1 h. The agar pieces were then rinsed with water and

fixed for 1 h in 1% (w/v) aqueous uranyl acetate. All fixations were carried out at room temperature. After dehydration in a graded series of ethyl alcohol and two changes in propylene oxide, the agar pieces containing bacterial cells were embedded in Epon 812 (Spi Supplies, New Chester, PA, USA). Thin sections stained with uranyl acetate and lead citrate were examined in a JEM 1200 EXII Electron Microscope (Jeol, Tokyo, Japan).

Cell diameter measurement

All cell sections for measurements were recorded with Fuji LAS1000 CCD image analyser and software Image Gauge 3.122 (Fuji Film, Tokyo, Japan) from the images obtained with TEM at a magnification of 20 000-fold. Each field to be recorded was selected randomly, making sure that all sections used in area measurements came from one field to prevent us from recording the same cell more than once. The cell sections to be measured were numbered to avoid repeated measurement of the same cell. Oblique cuts or cuts at different depths were considered as those that have diameter value greater or lower, respectively, than the mean ±2 s.d. and discard. Cell areas were determined by tracing the cell outline with a digitizer and the image analysis program Image Gauge 3·122, Fuji, which computed the cell diameter. A total of 20 cells from each group were measured. Data were analysed by analysis of variance.

MIC determination

Broth macrodilution was performed in 1-ml final volume of MRS. Serially diluted antibiotics [vancomycin, bacitracine, penicillin G (Pen G), phosphomycin and nisin] either with or without 1 mol l^{-1} NaCl were inoculated with 1/50 volume of an overnight culture of *L. casei*. After incubation at 37°C for 24–48 h O.D.$_{550}$ was scored for each tube and related to that of MRS broth alone. MIC were calculated by interpolating relative O.D. vs antibiotic concentration in the tendency plots. For ampicillin, *E*-test on MRS agar plates either with or without 1 mol l^{-1} NaCl was used as described by manufacturer (AB Biodisk, Solna, Sweden). Testing was performed in duplicate for each drug–medium combination studied.

Wall isolation, fractionation and modifications

Exponential phase cultures were harvested by centrifugation, and the cells were washed with water at room temperature. The cells were resuspended 1/10 volume of water. Purified PG was prepared from cells boiled in 4% (w/v) SDS for 30 min and then centrifuged to eliminate cell debris and nonlysed cells (3000 *g* 5 min). The crude walls were treated with DNase and RNase in phosphate buffer (pH 6·7) (final concentrations, 5 μg ml^{-1}, 30 μg ml^{-1} and

0·1 mol l^{-1}, respectively). After 2 h incubation at 37°C, cell walls were purified by pronase E treatment (40 units ml^{-1}, muramidase activity inactivated by heating at 60°C for 2 h). Thereafter, they were treated with trypsin (0·5 mg ml^{-1}) at 37°C for 16 h in 0·1 mol l^{-1} Tris-chloride (pH 7·5) containing 0·01 mol l^{-1} MgCl$_2$. Sodium dodecyl sulphate (2% w/v) was also added and samples were heated at 100°C for 15 min. After cooling, the samples were collected by centrifugation (20 000 *g*, 20 min), washed thoroughly with distilled water to remove sodium dodecyl sulphate (SDS) and the purified cell walls obtained were freeze-dried.

To determine whether accessory wall polymers or non-PG wall components are required for PG hydrolases resistance or sensitivity, samples of wall preparations (that had been boiled in SDS and treated with trypsin to remove proteins and lipoteichoic acids, respectively) were treated either with trichloroacetic acid (TCA) to remove teichoic acids, or with NaOH to deacetylate it. TCA-extracted walls were prepared from crude walls (50 mg ml^{-1}) by treatment with 10% (w/v) TCA for 48 h at 48°C followed by extensive washing with cold deionized water (de Ambrosini *et al.* 1996). Deacetylation was carried out by incubating *ca* 1 ml of wet pellets of walls with 10 ml of 0·01 mol l^{-1} NaOH at 37°C for 60 min. The suspensions were then centrifuged and washed with deionized water. Portions of these walls were used directly for incubation with mutanolysin.

Susceptibility of cells and cell wall fractions to hydrolases

The susceptibility of cells or wall fractions to lysis was spectrophotometrically determined. Cells or cell wall fractions were resuspended at an optical density of 0·5 at 550 nm, in 50 mmol l^{-1} NaH$_2$PO$_4$ buffer (pH 6·8) for whole cells and in 0·1 mol l^{-1} KNO$_3$ pH 6·2 for cell walls. Mutanolysin (Sigma Chemical Co., St Louis, MO, USA) was added at 25–100 units ml^{-1} final concentration and samples were incubated at 37°C with constant rotation to keep the contents in suspension. Optical density (550 nm) was measured throughout the experiment. Results were expressed relative to the initial optical density. No correction for changes determined in controls without enzyme was needed as no endogenous lytic enzyme activity was observed in these conditions.

Lysis was also assayed with lysozyme (2 mg ml^{-1}) in 0·1 mol l^{-1} Tris-chloride (pH 7·5) containing 0·01 mol l^{-1} MgCl$_2$. Trypsin (when indicated) was used at 0·5 mg ml^{-1}.

Muropeptide analysis

Peptidoglycan was hydrolysed with mutanolysin. We estimated that more than 95% of the wall from N cultures was hydrolysed by this treatment, while only 30% was obtained with control samples. However, the addition of trypsin

0·5 mg ml^{-1} allowed the increase of lysis because of its reported esterase activity (Logardt and Neujahr 1975). Therefore, samples of both growth conditions were equally treated with mutanolysin and trypsin at 37°C for 16 h. The hexosamine liberated was measured according to Reissig *et al.* (1955). Nonlysed cell wall was removed by centrifugation and the supernatant was freeze-dried.

Muropeptide composition of PG was analysed by fluorescence-assisted carbohydrate electrophoresis (FACE) as described in Young (1996). Derivatization with 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS; Molecular Probes, Eugene, OR, USA) was carried out according to Jackson (1994) in tubes containing dried hydrolysed muropeptide. ANTS was prepared in acetic acid/water (3/17, v/v) at 0·2 mol l^{-1} as final concentration, NaCNBH$_3$ (1 mol l^{-1}, made freshly and used immediately) was solubilized in DMSO for ANTS derivatization. Five microlitres of ANTS solution and 5 μl of NaCNBH$_3$ solution were added to each dry sample. The reagents were mixed, centrifuged and incubated at 37°C overnight. The solution was lyophilized. The derivatized sugars were resuspended in 50 μl of glycerol 5% (w/v) and stored before use at −20°C.

Polyacrylamide gel electrophoresis was as follows: ANTS derivatized sugars were separated in 30% (w/v) polyacrylamide gel and 0·8% (w/v) N,N-methylenebisacrylamide with a stacking gel (1·5 cm) of 8% (w/v) polyacrylamide and 0·2% (w/v) N,N-methylenebisacrylamide. The electrophoresis buffer system was 0·1 mol l^{-1} Tris adjusted to pH 8·2 with boric acid (Tris–borate). Bromophenol blue and ANTS-derivatized hydrolysed dextran (2 mol l^{-1} HCl 100°C 2 h) were used as markers. Fluorescence of Bromophenol-blue corresponds to the tetra-maltose as described by Jackson (1994) that migrates as Tetra–Tetra species (Young 1996). The samples were submitted to electrophoresis, initially at 250 V for 30 min and then at 500 V for 60 min. To visualize gels, they were scanned using Fuji CCD luminescent image analyser LAS1000 and software Image Gauge 3.122 (Fuji Film). The exposure time was optimized to increase sensitivity without saturating the intense bands. Fluorescence intensity was calculated for each lane with Image Gauge software 3.122 (Fuji Film) and plotted with the same scale.

Determination of free amino groups

Cell walls from SDS-TCA extraction were acid hydrolysed (6 mol l^{-1} HCl 100°C 16 h) either before or after treatment with 0·5% 2,4-dinitrofluorobenzene (DNFB), and amino acids were determined by thin-layer chromatography with known markers. Fluorescence intensity was recorded using Fuji CCD luminescent image analyzer LAS1000 and software Image Gauge 3.122 (Fuji Film). The exposure time was optimized to increase sensitivity without saturating

the intense spots. Fluorescence intensity was calculated for each spot from samples acid hydrolysed before (post) or after (pre) treatment with DNFB and normalized values for each amino acid were obtained (pre-DNF-aa/post-DNF-aa). A ratio for each labelled amino acid was obtained. Comparison within the condition determines the extent of free amino groups labelled for each condition.

Detection of PBP from bacterial membrane preparations

Membrane was prepared by cell disruption in the presence of glass beads, 50 mmol l^{-1} NaH$_2$PO$_4$ buffer (pH 6·8) and 1 mmol l^{-1} PMSF. They were kept in ice-acetone and submitted to 4 cycles of 1 min sonication (Sonifier model LS 75; Branson Instruments Inc., Stamford, CT, USA) alternated with 1 min cooling. The cell lysates were centrifuged at 12 000 *g* for 10 min. The supernatant fractions were collected and centrifuged at 100 000 *g* for 60 min. The pellets were collected, washed once and resuspended in the same phosphate buffer (1 ml each). The resulting suspensions were designated as membrane preparations and used for the detection of PBP with fluorescent BOCILLIN^{TM} FL (sodium salt; Molecular Probes) which is used as a labelling reagent in binding assays (Zhao *et al.* 1999). The protein concentration of each membrane sample was estimated by using the Bio-Rad protein assay with bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, VA, USA).

Reaction mixtures (50 μl) containing 50 μg of membrane preparation and 30 μmol l^{-1} (final concentration) of BOCILLIN FL were incubated at 35°C for 30 min and denatured with 1% SDS-loading buffer at 100°C for 3 min. Equal amounts of each sample (25 μg of protein per lane) were then loaded onto 8-cm 9·5% SDS-polyacrylamide resolving gels with a 5% stacking gel. Samples were submitted to electrophoresis at 100 V for 90 min or until the tracking dye reached the bottom of the gel. Immediately after electrophoresis, the gels were rinsed with water.

To visualize the labelling of PBP, the gels were directly scanned with a Fuji LAS1000 CCD image analyser and software Image Gauge 3.122 (Fuji Film). Afterwards, gels were stained with Coomassie blue to visualize all membrane proteins, photographed and scanned again.

For the saturation assays of PBP proteins with Pen G, reaction mixtures (50 μl each) containing 25 mmol l^{-1} potassium phosphate (pH 7·5), 50 μg of membrane preparation, and different Pen G concentrations (0–100 μg ml^{-1}), were incubated at 35°C for 20 min. BOCILLIN FL (30 μmol l^{-1}) was then added and the mixtures were incubated additional 20 min, denatured with SDS-loading solution, and subjected to SDS-PAGE as described above. Coomassie blue staining was performed after fluorescence digitalization to ensure equal amounts of protein were

RESULTS

Cell size determination by electron microscopy

Exponential phase cells of *L. casei* grown in MRS (C condition) and MRS medium plus 1 mol l^{-1} NaCl (N condition), were prepared for TEM observation. Growth in 1 mol l^{-1} NaCl resulted in a significant increase in cell size compared with cells grown in medium without NaCl (Fig. 1). The mean, the standard deviation and the distribution of the cell diameters for each growth condition are shown in Fig. 2. A statistically significant difference ($P < 0.05$) was observed in the mean cell diameter between control cells (415 ± 20 nm) and NaCl-grown cells (660 ± 60 nm). A significant enlargement of cells under high ionic stress has been observed in *Staphylococcus aureus* and it has been correlated with slow growing, inhibition of cell division in the absence of effective turgor pressure (Vijaranakul *et al.* 1995). These observations are consistent with our previous findings (Piuri *et al.* 2003) where growth of *L. casei* in N condition was drastically retarded when compared with C growth (division time μ in N was 0.11 h^{-1}, μ in C was 0.48 h^{-1}).
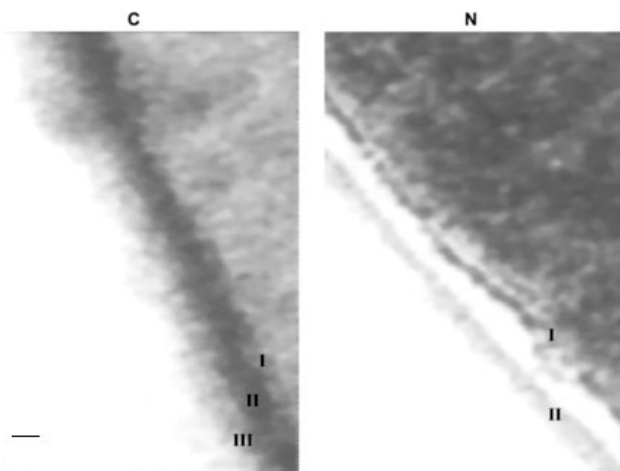
TEM examination of the cell wall with 200 000-fold magnification showed a typical bilaminar structure with a more electron dense inner layer (Fig. 3, II) probably corresponding to the PG. While three layers are observed in C condition, only two appeared in N condition. In NaCl-grown cells, the structure was irregular and resulted in the appearance of a layer detached from the cytoplasmic membrane. The absence of stain adjacent to the internal layer of the wall can be ascribed to the plasmolysis of the
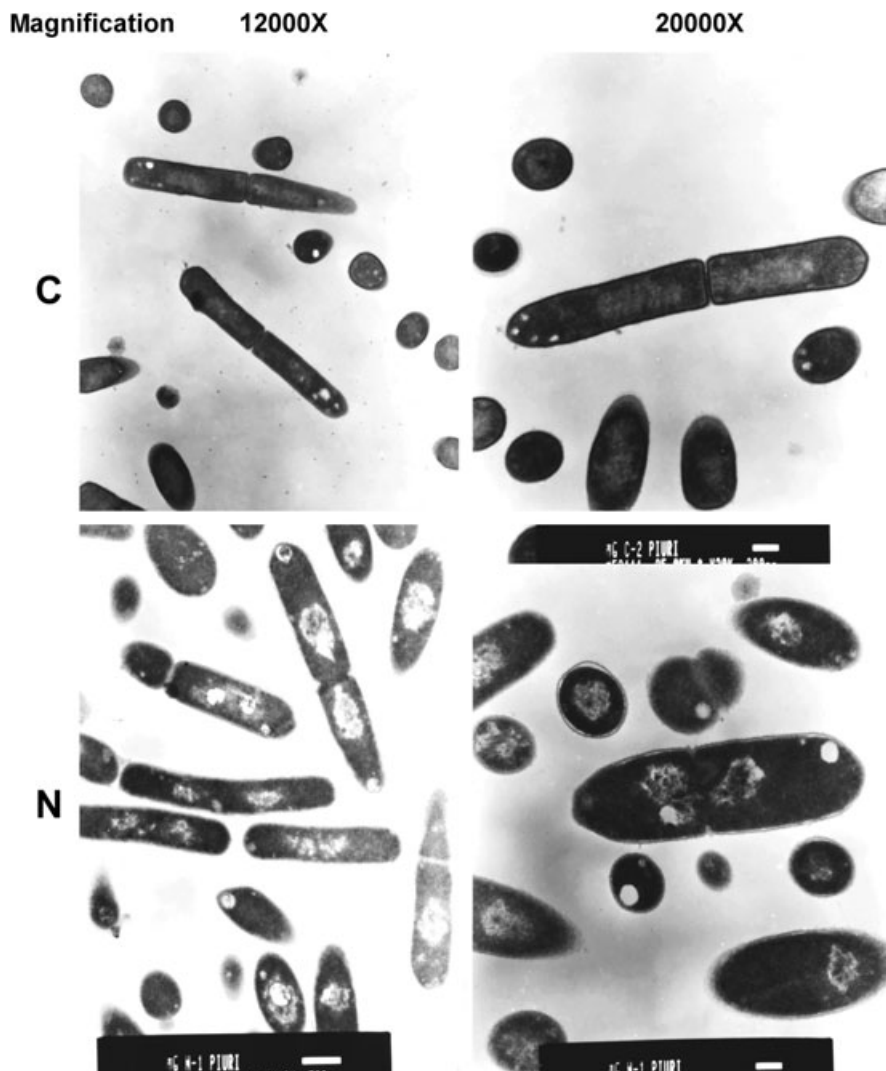
Fig. 1 Transmission electron micrographs (TEM). TEM of thin sections of exponential phase cultures grown in MRS (C), MRS medium containing 1 mol l^{-1} NaCl (N). Magnification of micrographs at the left is 12 000-fold (white bars below 0.5 μm) and at the right is 20 000-fold (white bars below 0.2 μm)
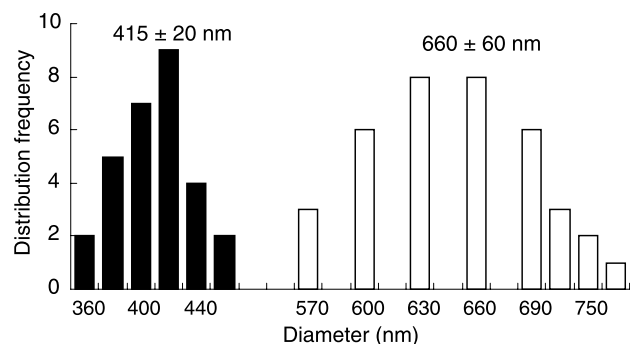
Fig. 2 Frequency distribution. Diameters of *Lactobacillus casei* cells grown in MRS medium (black bars) and MRS containing 1 M NaCl (white bars) are plotted. Diameters from 20 cells from each condition analysed. Mean and standard deviation for each condition are shown by the numbers above the bars. Statistical significance was accepted at the $P < 0.05$ level of probability

Table 1 Sensitivity to antibiotics and effectors which target is in the cell wall

| Antibiotic (μg ml^{-1})	MIC	
	C	N
Vancomycin (4)	2274 ± 633	523 ± 6
Bacitracine (2)	>625	270 ± 5
Ampicillin (*E*-test) (2)	1 ± 0·25	0·16 ± 0·05
Penicillin G (3)	1·5 ± 0·5	0·2 ± 0·1
Phosphomycin (2)	22 000 ± 3000	<3000
Nisin (2)	10 ± 0·5	2 ± 0·6

All MIC were determined after 24–48 h of incubation as described in Materials and methods. Number of replicates of independent experiments is in brackets.

Fig. 3 TEM of cell walls. Cell wall putative layers are indicated with numbers I (cytoplasmic membrane), II (peptidoglycan) and III (nonpeptydoglycan). Micrographs magnification 200 000-fold. (black bar below is 25 nm)

cytoplasmic membrane. The increase in electron density from inside to outside gave the appearance of a third layer in the control (Fig. 3, III) and this layer was apparently absent in the N condition (Fig. 3). Other non-PG components such as proteins and teichoic acids might be responsible for that layer observed in the control condition.

Antibiotic susceptibility

In view of the modifications of the cell wall observed here, we investigated the effect of cell wall active agents by measuring their minimum inhibitory concentration (MIC) for growth in both culture conditions (C vs N condition). MIC decreased an average of five times in the presence of

NaCl as shown in Table 1. Cationic peptides such as nisin that generate membrane pores also showed a decrease in the MIC. Modifications of the degree of D-Ala substitution of wall teichoic acids (WTA) have been suggested to account for this observation (Neuhaus and Baddiley 2003).

Increased sensitivity to antibiotics whose target was PG synthesis was observed in N cells. Resistance to glycopeptide antibiotics such as vancomycin has been reported to be intrinsic, chromosomally encoded and not inducible or transferable in *L. casei* (Billot-Klein *et al.* 1994, 1997). Vancomycin inhibits cell growth primarily by interfering with transpeptidation between D-amino acids, and this is supported by the synergic effect between vancomycin and D-analogues (Zarlenga *et al.* 1992). When growth was in medium containing D-alanine, a great decrease in MIC for vancomycin took place in both conditions (Fig. 4), indicating that this activity was equally inhibited and cannot account for the different sensitivity to vancomycin reported here. In contrast, when growth was in medium containing D-lactate, only the C condition showed an increased resistance while in N cells it was almost unchanged (Fig. 4). Thus, in C condition, D-lactate was incorporated efficiently in the pentapeptide termini of the nascent PG impeding the binding of vancomycin while this does not occur in N condition. Resistance depends on mechanisms involving activities of D-lactate dehydrogenase, D-alanine ligase or D,D-carboxypeptidase (Handwerger *et al.* 1994; de Jonge *et al.* 1996) probably linked to PBP. Therefore, our results indicate that at least one of these activities mediating resistance is inhibited during osmotic stress growth.

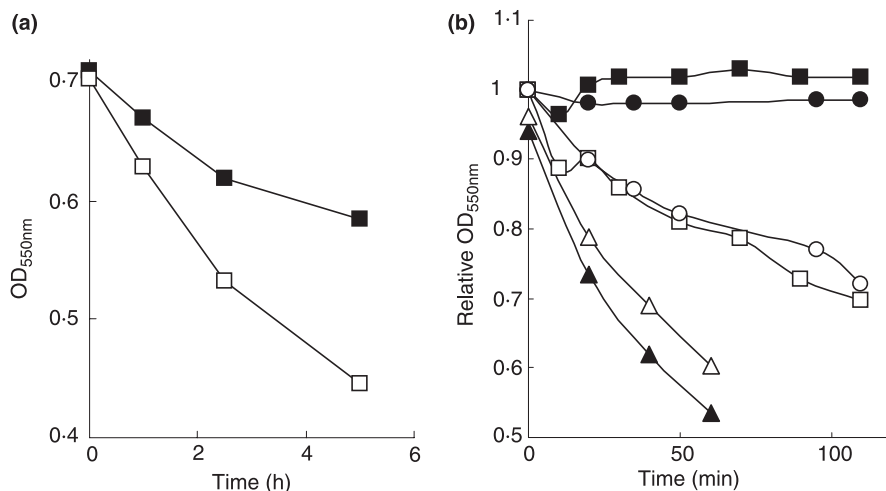## Sensitivity of whole cells and wall preparations to hydrolysis

In order to determine further the cause of differences in antibiotic sensitivities, whole cells and wall preparations were studied. For this purpose cells were grown in MRS
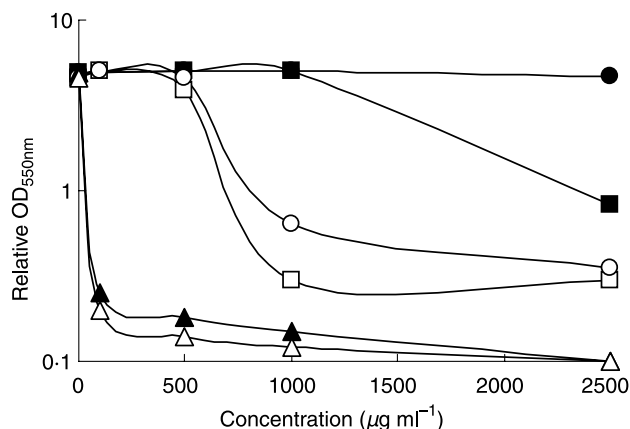
Fig. 4 Effects of D-alanine and D-lactic acid on MIC for vancomycin. Final O.D.$_{550}$ after incubation in the presence of different antibiotic concentrations. Full symbols were used for C conditions, and empty symbols for N conditions. Squares represent data without any addition. Circles were used for data in presence of D-lactate 1·25 mg ml^{-1}. Triangles were used for data in presence of D-alanine 2·5% (w/v)

either with or without NaCl, harvested at the mid-exponential growth phase and resuspended in 50 mmol l^{-1} NaH$_2$PO$_4$ buffer (pH 6·8). Sensitivity to mutanolysin (100 U ml^{-1}), an enzyme that hydrolyses the *N*-acetylmuramyl-1,4–acetylglucosamine bonds in the PG structure, was investigated. Treatments with mutanolysin resulted in cell lysis correlated with a decrease in absorbance at 550 nm. N cells in presence of mutanolysin lysed faster than C cells (Fig. 5a). Lysozyme treatment (2 mg ml^{-1}) showed essentially similar results with increased lysis in N condition. Lysis by treatment with inexpensive egg yolk-lysozyme for 20 min followed by addition of 1% SDS resulted in 70% for N condition compared with only 20% of C condition.

A similar assay was performed with cell wall material (1 mg ml^{-1}). After 1 h treatment with 25 U ml^{-1} of mutanolysin, a 25% decrease took place with N walls, while C walls decreased only 5% even after 4 h incubation (Fig. 5b). These results clearly showed that the differences in sensitivity to lysis of whole cells would be due to differences in their cell wall structure. Modifications of PG can affect sensitivity to PG hydrolases. *Bacillus cereus* PG is resistant to lysozyme because the presence of unacetylated amino groups on the majority of its glucosamine residues, but it can be converted to a lysozyme-sensitive form by acetylation with acetic anhydride (Araki *et al.* 1972). Conversely, lysozyme resistance of the PG of lactobacilli and other organisms is because of O-acetylation of amino sugars, and they can be made lysozyme sensitive by de-O-acetylation with a dilute base (Logardt and Neujahr 1975). Accessory cell wall polymers such as teichoic acids covalently linked to the cell wall (WTA) or lipoteichoic acids linked to the cell membrane (LTA), can also affect the susceptibility to lysis of bacteria. Resistance to lysozyme has been related to the presence of O-acetyl groups, the attachment of teichoic acid (WTA) to the PG or a high degree of peptide cross-linking. To evaluate these possibilities we treated purified cell walls with 0·1 mol l^{-1} NaOH, allowing de-O-acetylation of muramic acid, or 10% TCA allowing the extraction of wall-associated polymers such as WTA. As observed in Fig. 5b, NaOH treatment did not modify the sensitivity of the wall preparation from either condition. However, TCA treatments increased significantly the differences in lysis sensitivity: removing WTA has a more drastic effect in the control condition as calculated from the change in the slope of lysis curves. Such modification increased the lysozyme sensitivity of walls, which were normally quite resistant in C condition. These results indicate that differential teichoic acid content

Fig. 5 Sensitivity of whole cells and wall preparations to lytic enzymes. Relative changes in optical density at 550 nm after incubation of (a) whole cell suspension in the presence of 100 U ml^{-1} mutanolysin. (■) C conditon and (□) N condition. (b) Relative changes in O.D.$_{550}$ after incubation of 1 mg isolated cell wall suspensions in the presence of 25 U ml^{-1} mutanolysin. Symbols: (■) C condition; (□) N condition.; (●) C condition + NaOH; (○) N condition + NaOH; (▲) C condition + TCA; (△) N condition + TCA

might be responsible in part for the increased lysis sensitivity observed in N condition.

Muropeptide analysis

To further investigate the alterations taking place in the cell wall at the structural level, a muropeptide analysis of PG was performed by fluorescence-assisted carbohydrate electrophoresis (FACE; Jackson 1994; Young 1996). This simple method gives information about the extent of mutanolysin hydrolysis, rendering labelled species that migrate in a PAGE gel by charge according to their size. As shown in Fig. 6, the presence of 1 mol l^{-1} NaCl to the growth medium had a profound effect on the muropeptide structure. The amount of oligomer muropeptides decreased in N condition compared with C condition (for mass spectrometry structures, refer to Billot-Klein *et al.* 1997). This result would be in accordance with a reduced degree of cross-linkage as could be estimated according to Driehuis *et al.* (1992). This finding was further corroborated by the analysis of the extent of free amino groups in the cell wall preparations from both conditions using DNFB as described in Materials and methods. The analysis of DNF-derivatives obtained showed that the relative proportion of free amino groups in asparagine related to lysine was 1·5 for C while it was 4·0 for N condition. These results indicate that a larger amount of free amino groups was present in N condition. Therefore, both muropeptide analysis and free amino groups indicated a drop in PG cross-linking in N cells explaining their increased susceptibility to lysis.
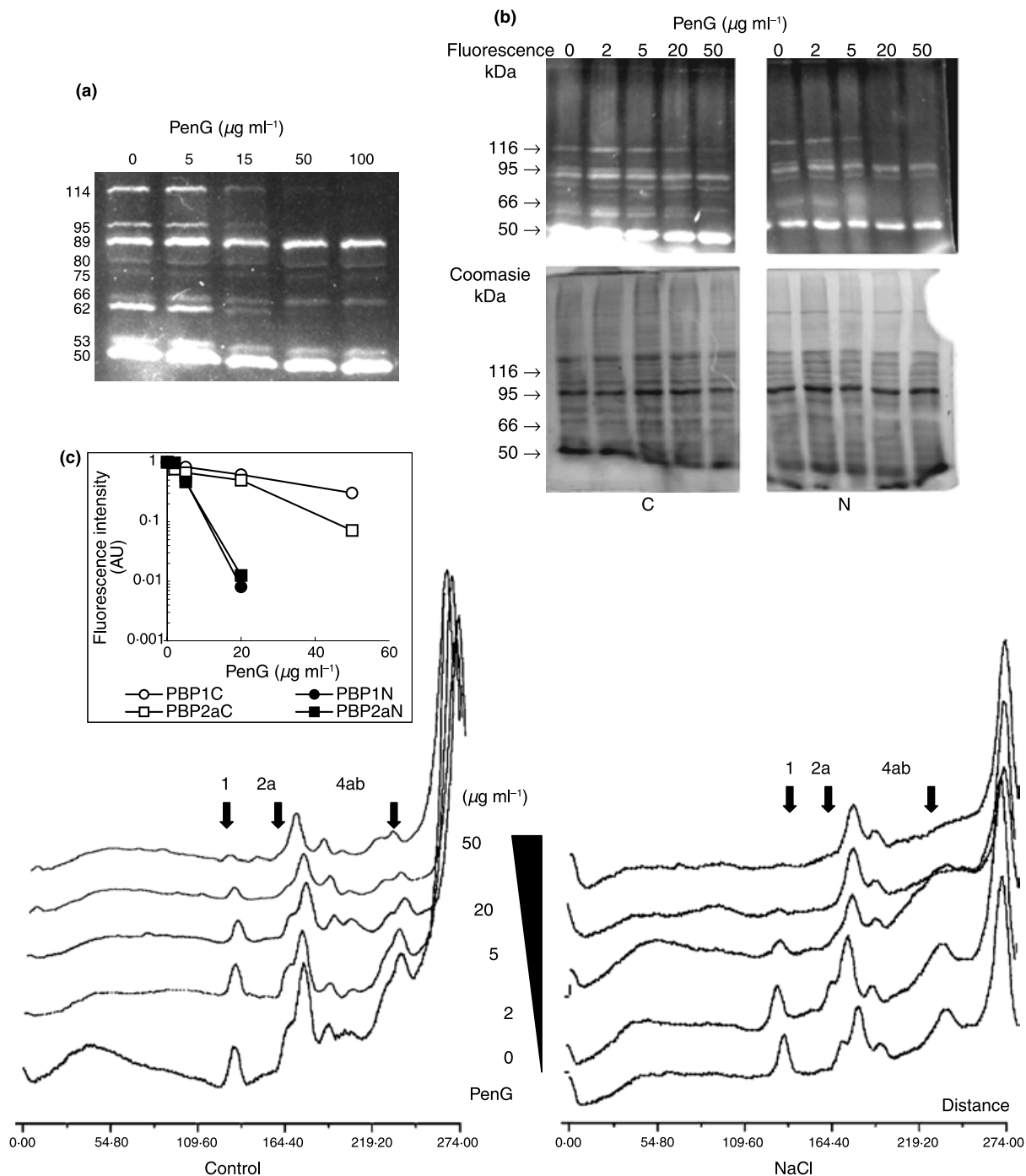
PBP in *L. casei*

The alterations in PG here described are qualitative and these could be attributed to the biosynthetic process dependant on the PBP. As far as we know, in contrast to lactococci and bacilli, little is known about PBP and PG synthesis in lactobacilli (Griaznova *et al.* 1990; Delcour *et al.* 1999). For the detection of PBP, we carried out fluorescent BOCILLIN FL binding assays with membrane preparations. We analysed the PBP profile and found 9 PBP. They are here first described and classified according to their calculated MW in kDa: 114 (PBP1), 95, 89 (PBP2 a,b), 80, 75 (PBP3 a,b), 66, 62 (PBP4 a,b) and 53, 50 (PBP5 a, b) (Fig. 7a). As shown in Fig. 7a, PBP saturation assays with increasing Pen G showed that three of them were fully saturated (molecular mass 114, 95 and 62 kDa), suggesting that at least in C condition these are the essential PBP. Membranes preparations containing the PBP from both C and N growth conditions were submitted to analysis to determine saturation patterns (Fig. 7b). In the absence of PenG PBP2b, PBP3a,b, PBP4a,b and PBP5 a,b of N membranes were less represented compared with the C condition (see fluorescence patterns in Fig. 7c, data of PenG 0). It is possible then to speculate that if certain PBP are less expressed in the N condition, there would be a decrease in cross-linking. However, no difference was observed for the HMW PBP, PBP1 and PBP2a. In Fig. 7c the saturation profile is shown for PBP1 and PBP2a at lower Pen G concentrations in N than in C condition; in particular 50% saturation takes place at 5 μg ml^{-1} in N while
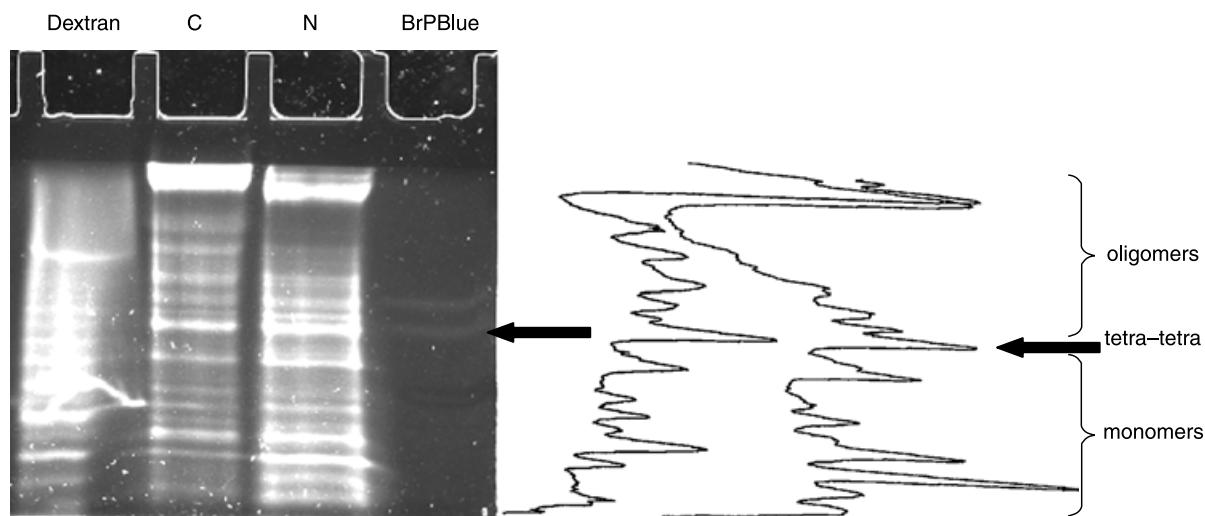
Fig. 6 Muropeptide profiles analysed by FACE. Muropeptides were prepared and labelled with the fluorescent dye ANTS, and separated by electrophoresis through a 30% polyacrylamide gel as described in the text. Fluorescent-labelled muropeptides were visualized and photographed in CCD camera. According to their electrophoretic mobility, muropeptides monomers and oligomers are indicated on the right with Fluorescence intensity pattern for C and N condition. Arrow indicates the tetra-tetra species

(a)

PenG (μg ml^{-1})

0   5   15   50   100

114
95
89
80
75
66
62
53
50

(b)

PenG (μg ml^{-1})

Fluorescence   0   2   5   20   50   0   2   5   20   50
kDa

116 →
95 →
66 →
50 →

Coomasie
kDa

116 →
95 →
66 →
50 →

C   N

(c)

Fluorescence intensity (AU)

PenG (μg ml^{-1})

—○— PBP1C   —●— PBP1N
—□— PBP2aC   —■— PBP2aN

1   2a   4ab   1   2a   4ab

(μg ml^{-1})

50

20

5

2

0

PenG

Distance

0·00   54·80   109·60   164·40   219·20   274·00   0·00   54·80   109·60   164·40   219·20   274·00

Control   NaCl

Fig. 7 PBP profiles and saturation assay. Membrane were purified from cultures grown in C and N conditions. Membranes were incubated with BOCILLIN and proteins separated on 9·5% SDS–PAGE; (a) C condition membrane (20 μg of proteins) incubated for the detection of PBP by fluorescence using a CCD imager. Molecular mass of the nine resolved PBP is indicated in numbers (kDa). PenG was added in increasing concentrations indicated on top of each lane. (b) Saturation assay with increasing PenG with 25 μg of proteins of C and N membranes. Fluorescence and Coomassie-stained gels are shown. No resolution of the two PBP5 proteins was observed in this saturation assay. Molecular mass markers are indicated in numbers. (c) Fluorescence intensity of saturation assays. Upper Plot of the fluorescence intensity for PBP1 and PBP2a in both conditions is shown. Bottom shows fluorescence pattern for each PenG concentration that increases from the bottom to the top. Arrows indicate the position of bands corresponding to essential PBP1, PBP2a and PBP4ab in the control condition

20–25 μg ml^{-1} are needed for the C condition. This observation showed that the affinity for penicillin of the HMW PBP from N cells was increased, suggesting that differences in functionality *in vivo* must occurs. This result also agrees with the differential antibiotic sensitivity described above (Table 1; Fig. 4).

DISCUSSION

Bacteria undergo morphological transformations as part of their normal life cycle. In addition, stress effectors lead to similar variations. In this paper we describe the cell wall modifications taking place in *L. casei* during growth in high salt medium.

Retardation of growth in high salt can be associated with an increase in the cellular size (cells in N condition are 60% larger than in C condition) and modifications in the cell envelope structure as visualized by TEM (Figs 1–3). These results are in agreement with the observations in *S. aureus* where salt, but not glycerol stress, increased cell size, suggesting that high ionic strength and not osmotic strength *per se*, was responsible for the size effects (Vijaranakul *et al.* 1995). In *Escherichia coli*, ionic strength also triggers the cells to lengthen, perhaps via an inhibition of cellular division, suggesting that turgor may control DNA replication and cell division (Meury 1988). In *B. subtilis*, hypersaline medium resulted in filamentation growth (Lopez *et al.* 1998) and division abnormalities such as a polar septation (Ruzal *et al.* 1998).

The modified cell wall structure observed by TEM could be correlated with a decrease in PG crosslinking. In N cells, the wall was detached from the cytoplasmic membrane, indicating plasmolysis. Higher sensitivity to lysis by mutanolysin (Fig. 5) and to antibiotics whose target is the cell wall biosynthetic pathway (Fig. 4; Table 1) was observed.

As the MIC for more than one antibiotic decreased significantly, all with target in the cell wall, it could be due to the greater access of antibiotics to their targets when cells are grown in high salt. This is suggested by the fact that the cells were more sensitive to nisin, which has to pass through the cell wall to reach its membrane target. However, this might not be the only factor. The different behaviour observed for vancomycin resistance by the addition of D-lactate suggested modifications in PBP functionality *in vivo* (D,D-carboxypeptidase or other ligase dehydrogenases).

Resistance to lysis by hydrolases has also been related to the presence of O-acetyl groups in PG. It has been suggested that it protects the PG strands from the hydrolytic activity of muramidase-type lysins. High degree of O-acetylation was described for *L. casei* (Billot-Klein *et al.* 1997) but our results of mild basic hydrolysis of cell wall preparations showed no differential behaviour of wall between C and N conditions (Fig. 5b). Moreover, the naturally vancomycin-

resistant *L. casei* ATCC 393 strain grown in the presence of Pen G showed a decreased O-acetylation (39% compared with 69% in the absence of antibiotic) as well as a decrease in PG cross-linking (an increase in the amounts of monomer, from 45 to 64% of all muropeptides) (Billot-Klein *et al.* 1997) similar to that described here for the N condition. Mass spectrometry analysis of the muropeptides species is needed to corroborate this.

C walls treated with TCA, which removes teichoic acids, behaved as N walls (Fig. 5b), suggesting that WTA would be low or modified in this growth condition. Teichoic acids with their different substitutions have been involved in the lysis sensitivity in other micro-organisms (Peschel *et al.* 2000). Therefore, the increased lysis in N condition reported here would be more complex than only modifications of PG; other non-PG polymers would be involved and they will be investigated further.

We have described nine PBP for *L. casei* ATCC 393 (Fig. 7), three of which would be the essential PBP (PBP1, PBP2a and PBP4b; MW 114, 95 and 62 kDa, respectively). The changes observed by TEM with *L. casei* resemble the morphological changes described in mutated PBP cells of *B. subtilis* (Popham and Young 2003). Differences in concentration and affinity of PBP in N cultures were observed (Fig. 7b,c). The increased monomer fraction observed by FACE in N condition (Fig. 6) involved decreased PG-cross-linking. Two possible phenomena might explain these results: a lower expression of PBP genes and/or differences in their correct assembling in the envelope. If fewer HMW PBP molecules are expressed, a decrease in cross-linking would occur. Moreover, if transpeptidase activity, normally attributed to PBP of HMW, was inhibited during growth in high salt this can also lead to a decrease in PG cross-linking. The two possibilities are in fact taking place. While the PBP1 and PBP2a content was almost the same in both conditions, their saturation patterns were different and this behaviour can be related to a modification in the enzyme affinity during the growth in high salt and hence its activity. However, for the rest of the PBP a decrease in their content was observed in N cells (compare C and N conditions at PenG 0 profile in Fig. 7c). Although the number of PBP is not modified by salts as described in *Enterococcus faecalis* (Mainardi *et al.* 1998), the essential PBP were either less represented or their affinity for PenG was modified.

To date, nothing is known about the control of PG synthesis in lactobacilli, so we can only speculate that PBP (transpeptidases, D,D- or LD-carboxypeptidase), which are normally active during the synthesis of PG, are inhibited by growth in high salt. We hypothesize that variations in the surrounding environment reported here would hide (or expose) not only sites for substrates but also the configuration of envelope proteins as suggested for PrtP (Piuri *et al.* 2003) and QacT (Glaasker *et al.* 1998). *Lactobacillus casei*

ATCC 393 changes its cell wall structure as a function of external osmolarity. This, together with the modifications in membrane biochemical composition (Machado *et al.* 2004), affects the lipid wall–protein interactions involved in stress adaptation and affects protein assembly and folding as well as signal transduction and transport activities. Recent reports on the ABC transport system of *Lactococcus lactis* show that this system senses osmotic stress through alterations in the physicochemical properties of membranes, this being the electrostatic interactions between ionic lipids and the transporters essential to the osmosensing mechanism (Van der Heide *et al.* 2001). Modification as a result of the adaptation to high external osmolarity affects all membrane proteins including PBP and those that are translocated through the membrane such as PrtP proteinase, which modifies its activity (Piuri *et al.* 2003). These changes are likely to be essential to deal with the stress conditions produced by the osmotic imbalance between the cells and their environment.

Our results have great impact not only as part of the general understanding of the osmotic adaptation but also in the application field. In the field of recombinant DNA technology and the new vaccine delivery vectors that involve preparation of large amounts of nucleic acids such as plasmids and antigen expression, pregrowth in high salt enables to obtain high yields of DNA and proteins. We have observed that lysis by treatment with inexpensive egg yolk lysozyme (1 mg ml^{-1} 20 min) followed by 1% SDS resulted in threefold higher yields of DNA for the N condition compared with the C condition.

Lysis of lactic acid bacteria is also essential for the development of the texture and flavour of fermented products. Intracellular enzyme release is essential for complete conversion of protein into growth-promoting amino acids and precursors for flavour. A balance of both autolysed and intact cells of lactic acid bacteria is important for the desired ripening events (Fox *et al.* 1996).

For lactic acid bacteria used in cheese manufacturing, cooking and salting treatments are used to increase autolysis of the dairy starter bacteria in order to obtain a balance between lactic acid production (by viable bacteria) and peptidolytic activity by intracellular peptidases released from lysed cells (Crow *et al.* 1995). Lysis of lactic acid bacteria is essential for the development of the texture and flavour of cheeses. For example, in Cheddar cheese-ripening conditions 4% NaCl is needed. Intracellular enzymes of the starter LAB are essential for complete conversion of the milk protein casein into the growth-promoting amino acids and the precursors for flavour. A balance of autolysed and intact cells of lactic acid bacteria is important for the desired cheese-ripening events (Fox *et al.* 1996). We hypothesize that a preadaptation to high salt of starter strains would result in an increased sensitivity to lysis of a proportion of the inocula with provision of peptidases, release of higher levels of amino acids and acceleration of the overall industrial process.

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REFERENCES

de Ambrosini, V.M., Gonzalez, S., Perdigon, G., de Ruiz Holgado, A.P. and Oliver, G. (1996) Chemical composition of the cell wall of lactic acid bacteria and related species. *Chemical and Pharmaceutical Bulletin (Tokyo)* **44**, 2263–2267.

Araki, Y., Nakatani, T., Nakayama, K. and Ito, E. (1972) Occurrence of N-nonsubstituted glucosamine residues in peptidoglycan of lysozyme-resistant cell walls from *Bacillus cereus*. *Journal of Biological Chemistry* **247**, 6312–6322.

Billot-Klein, D., Gutmann, L., Sable, S., Guittet, E. and van Heijenoort, J. (1994) Modification of peptidoglycan precursors is a common feature of the low-level vancomycin-resistant VANB-type *Enterococcus* D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. *Journal of Bacteriology* **176**, 2398–2405.

Billot-Klein, D., Legrand, R., Schoot, B., van Heijenoort, J. and Gutmann, L. (1997). Peptidoglycan structure of *Lactobacillus casei*, a species highly resistant to glycopeptide antibiotics. *Journal of Bacteriology* **179**, 6208–6212.

Crow, V.L., Coolbear, T., Gopal, P.K., Martley, F.G., McKay, L.L. and Riepe, H. (1995) The role of autolysis of lactic acid bacteria in the ripening of cheese. *International Dairy Journal* **5**, 855–875.

Delcour, J., Ferain, T., Deghorain, M., Palumbo, E. and Hols, P. (1999) The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek* **76**, 159–184.

Driehuis, F., de Jonge, B. and Nanninga, N. (1992) Cross-linkage and cross-linking of peptidoglycan in *Escherichia coli*: definition, determination, and implications. *Journal of Bacteriology* **174**, 2028–2031.

Fox, P.F., Wallace, J.M., Morgan, S., Lynch, C.M., Niland, E.J. and Tobin, J. (1996) Acceleration of cheese ripening. *Antonie Van Leeuwenhoek* **70**, 271–297.

Ghuysen, J.M. (1994) Molecular structures of penicillin-binding proteins and beta-lactamases. *Trends in Microbiology* **2**, 372–380.

Glaasker, E., Heuberger, E.H., Konings, W.N. and Poolman, B. (1998) Mechanism of osmotic activation of the quaternary ammonium compound transporter (QacT) of *Lactobacillus plantarum*. *Journal of Bacteriology* **180**, 5540–5546.

Griaznova, N.S., Subbotina, N.A., Beliavskaia, I.V., Taisova, A.S., Afonin, V.I., Tiurin, M.V., Shenderov, B.A., Sazykina, Iu.O. *et al.* (1990) Penicillin-binding proteins of various strains of *Lactobacillus*. *Antibiot Khimioter* **35**, 15–19.

Handwerger, S., Pucci, M.J., Volk, K.J., Liu, J. and Lee, M.S. (1994) Vancomycin-resistant *Leuconostoc mesenteroides* and *Lactobacillus casei* synthesize cytoplasmic peptidoglycan precursors that terminate in lactate. *Journal of Bacteriology* **176**, 260–264.

Jackson, P. (1994). High-resolution polyacrylamide gel electrophoresis of fluorophore-labeled reducing saccharides. In *Guide to Techniques in Glycobiology. Methods in Enzymology*, vol. 230, ed. Lennarz, W.J. and Hart, G.W. pp. 250–265. San Diego, CA: Academic Press.

de Jonge, B.L., Handwerger, S. and Gage, D. (1996) Altered peptidoglycan composition in vancomycin-resistant *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy* **40**, 863–869.

Logardt, I.M. and Neujahr, H.Y. (1975) Lysis of modified walls from *Lactobacillus fermentum*. *Journal of Bacteriology* **124**, 73–77.

Lopez, C.S., Heras, H., Ruzal, S.M., Sanchez-Rivas, C. and Rivas, E.A. (1998) Variations of the envelope composition of *Bacillus subtilis* during growth in hyperosmotic medium. *Current Microbiology* **36**, 55–61.

Machado, M.C., Lopez, C.S., Heras, H. and Rivas, E.A. (2004) Osmotic response in *Lactobacillus casei* ATCC 393: biochemical and biophysical characteristics of membrane. *Archives of Biochemistry and Biophysics* **422**, 61–70.

Mainardi, J.L., Billot-Klein, D., Coutrot, A., Legrand, R., Schoot, B. and Gutmann, L. (1998) Resistance to cefotaxime and peptidoglycan composition in *Enterococcus faecalis* are influenced by exogenous sodium chloride. *Microbiology* **144**, 2679–2685.

Meury, J. (1988) Glycine-betaine reverses the effects of osmotic stress on DNA replication and cellular division in *Escherichia coli*. *Archives in Microbiology* **149**, 232–239.

Neuhaus, F.C. and Baddiley, J. (2003) A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiology and Molecular Biology Reviews* **67**, 686–723.

Peschel, A., Vuong, C., Otto, M. and Gotz, F. (2000) The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. *Antimicrobial Agents and Chemotherapy* **44**, 2845–2847.

Piuri, M., Sanchez-Rivas, C. and Ruzal, S.M. (2003) Adaptation to high salt in *Lactobacillus*: role of peptides and proteolytic enzymes. *Journal of Applied Microbiology* **95**, 372–379.

Popham, D.L. and Young, K.D. (2003) Role of penicillin-binding proteins in bacterial cell morphogenesis. *Current Opinion in Microbiology* **6**, 594–599.

Reissig, J.L., Strominger, J.L. and Leloir, L.F. (1955) A modified colorimetric method for the estimation of N-acetylamino sugars. *Journal of Biological Chemistry* **217**, 959–966.

Ruzal, S.M., Lopez, C., Rivas, E. and Sanchez-Rivas, C. (1998) Osmotic strength blocks sporulation at stage II by impeding activation of early sigma factors in *Bacillus subtilis*. *Current Microbiology* **36**, 75–79.

Van der Heide, T., Stuart, M.C. and Poolman, B. (2001) On the osmotic signal and osmosensing mechanism of an ABC transport system for glycine betaine. *EMBO Journal* **20**, 7022–7032.

Vijaranakul, U., Nadakavukaren, M.J., de Jonge, B.L., Wilkinson, B.J. and Jayaswal, R.K. (1995) Increased cell size and shortened peptidoglycan interpeptide bridge of NaCl-stressed *Staphylococcus aureus* and their reversal by glycine betaine. *Journal of Bacteriology* **177**, 5116–5121.

Young, K.D. (1996) A simple gel electrophoretic method for analyzing the muropeptide composition of bacterial peptidoglycan. *Journal of Bacteriology* **178**, 3962–3966.

Zarlenga, L.J., Gilmore, M.S. and Sahm, D.F. (1992) Effects of amino acids on expression of enterococcal vancomycin resistance. *Antimicrobial Agents and Chemotherapy* **36**, 902–905.

Zhao, G., Meier, T.I., Kahl, S.D., Gee, K.R. and Blaszczak, L.C. (1999) BOCILLIN FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins. *Antimicrobial Agents and Chemotherapy* **43**, 1124–1128.