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Cell wall and autolytic system of *Lactobacillus helveticus* ATCC 12046

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The cell wall of *Lactobacillus helveticus* was found to have a three-layered structure when thin sections of whole cells or isolated cell walls were stained by various procedures and examined by electron microscopy. The main feature of the composition of isolated cell walls was a high protein content (about 48% of the cell wall dry weight) with a predominant 52 kDa protein. Peptidoglycan and what was considered as teichoic acids accounted for 36% and 14%, respectively, of the cell wall dry weight. The 52 kDa protein was apparently unglycosylated, located in the outer layer and noncovalently bound to the inner layers. Peptidoglycan was mostly assigned to the innermost layer. The conditions leading to autolysis of whole cells or isolated cell walls were determined, as well as the specificity of the autolysin involved in cell wall degradation.

Introduction

Lactobacillus helveticus is an important lactic acid bacterium that is involved in cheese technology (Accolas *et al.*, 1980; Turner *et al.*, 1983) and that has recently become the object of interest for genetic manipulation (Chassy, 1987; Sandine, 1987; Thomas & Collins, 1988). Its autolysis with the concomitant release of cytoplasmic proteases (Ezzat *et al.*, 1986) is an essential feature of cheese maturation (Bie & Sjoström, 1975*a, b*). A better understanding of the autolytic system of *L. helveticus* could perhaps offer a new way for an accelerated ripening of cheese. Ohmiya & Sato (1970) were the first to follow the autolysis of *L. helveticus* in technological conditions. After adding whole cells to an aseptic rennet curd, the release of DNA was noted and autolysed cells were clearly observed by electron microscopy. More recently, we promoted the autolysis of *L. helveticus* by suspending whole cells in dilute buffers (Lortal *et al.*, 1989). A clear understanding of the autolytic system of *L. helveticus* implies, in particular, a better knowledge of the composition and structure of its cell wall, which have so far been investigated only to a limited extent (Ikawa & Snell, 1960; Baddiley & Davison, 1961; Williams, 1971). The main peptidoglycan amino acids were determined (Glu, Ala, Asp, Lys) as well as their respective percentage in the D-configuration (Ikawa & Snell, 1960). Previous studies also revealed the presence of glycerol-type teichoic acids (Baddiley & Davison, 1961) and

neutral sugars (glucose, galactose but no rhamnose). Wall and membrane teichoic acids of *L. helveticus* were shown to have antigenic properties (Knox & Wicken, 1971). It has also been suggested that the cell wall of *L. helveticus* ATCC 10797 was composed of two layers with the outer layer presumably containing a predominant protein (Masuda & Kawata, 1983).

In the work described here, a more detailed investigation of the morphology and composition of the cell wall of *L. helveticus* ATCC 12046 was undertaken. Moreover, the main characteristics of the autolytic system of this strain were investigated, in particular ways of promoting lysis, the effect of external factors, and the specificity of the autolytic activity involved in degradation of cell wall peptidoglycan.

Methods

Choice of strain. Fifteen strains of *L. helveticus* were considered (14 from the CNRZ collection, Jouy en Josas, France, and ATCC 12046 from the Pasteur Institute, Paris, France). They were harvested by centrifugation at the beginning of the exponential growth phase, washed and suspended in 0.2 M-sodium phosphate buffer (pH 7.0) at 45 °C. The decrease of OD₆₅₀ after 8 and 24 h was noted and a statistical analysis of the results made. *L. helveticus* ATCC 12046 was chosen as a model because of its loss of OD₆₅₀, which was representative of the 15 strains, and its rapid growth (generation time 45 min), and because it belongs to an international collection.

Growth conditions. Cultures of *L. helveticus* ATCC 12046 were grown in MRS medium (De Man *et al.*, 1960). They were incubated at 45 °C

after a 1% (v/v) inoculation with an overnight culture. The strain was kept at -30°C in MRS medium supplemented with glycerol (15%, v/v).

Preparation of cell walls. Cells were harvested in the cold at the beginning of the exponential growth phase (1 mg dry weight ml^{-1}) by centrifugation at 20000 *g* for 15 min, washed once in distilled water, and disrupted in a refrigerated French pressure cell (French & Milner, 1955) [20000 p.s.i. (138 MPa), one run]. The resulting suspension was centrifuged at 1500 *g* for 15 min to discard unbroken cells. The supernatant was centrifuged at 30000 *g* for 15 min, and the pellet was washed four times with distilled water, twice with 0.01 M-sodium phosphate buffer, pH 7, and twice again/with water. The decrease in absorbance of the supernatants was followed at 260 and 280 nm to monitor the efficiency of washing. The final cell wall pellet was suspended in water and lyophilized.

SDS treatment. SDS was obtained from Merck. Whole cells or lyophilized cell walls suspended in 5 ml distilled water (4 mg ml^{-1}) were added to 40 ml of a boiling solution of SDS (4.5%, w/v) and kept at 100°C for 60 min. After centrifugation for 1 h at 50000 *g*, pellets were washed ten times with distilled water to remove all SDS, and lyophilized.

Electron microscopy. Lyophilized cell walls or whole cells were fixed in glutaraldehyde (2.5%, v/v, in 10 mM-sodium cacodylate, pH 7.2) for 15 h at 4°C , and in osmium tetroxide (1%, w/v, in the same buffer) for 15 h at 4°C , dehydrated in acetone and embedded in Araldite (48 h at 64°C). Thin sections were post-stained by three different techniques: (i) lead citrate and uranyl acetate; (ii) phosphotungstic acid (Rambourg, 1969); (iii) silver proteinate (Thiery, 1967). All preparations were observed with a Zeiss EM10 electron microscope.

SDS-PAGE. SDS-PAGE was performed on slab gels according to the method of Laemmli (1970), using a 15% (w/v) acrylamide separating gel. Cell wall preparations suspended in a 0.05 M-Tris/HCl buffer (pH 6.8) containing SDS (4%, w/v), 2-mercaptoethanol (1%, v/v) and sucrose (12%, w/v), were heated at 100°C for 3 min. After electrophoresis, gels were stained with Coomassie blue G250 for proteins and by the method of Kapitany & Zebrowski (1973) for glycoproteins. The marker protein kit from Pharmacia was used for molecular mass determinations. Relative amounts of the separated proteins were determined by scanning gels with an optical densitometer.

Induction of autolysis of whole cells. (a) Induction by antibiotics. Penicillin G (Serva), D-cycloserine (Sigma), vancomycin (Lilly) and fosfomicin (Sigma) were added to cultures at the beginning of the exponential growth phase (1 mg dry weight ml^{-1}) at final concentrations from 10 to 100 $\mu\text{g ml}^{-1}$. Antibiotic solutions were filtered (Millipore, 0.45 μm) before adding to cultures.

(b) Induction by suspending whole cells in dilute buffers. Exponential-phase cells were harvested in the cold by centrifugation at 20000 *g* for 15 min, and suspended in various buffers at 45°C (0.25 mg ml^{-1}). Phosphate buffers (Merck) were used at various pH values and concentrations, as indicated in the figures.

In all cases, the extent of autolysis was followed by measuring the decrease in OD_{650} with a Beckman spectrophotometer (Acta MIV).

Induction of autolysis of isolated cell walls. Lyophilized cell walls were suspended (0.8 mg ml^{-1}) in Tris/HCl buffers (Merck) (variable pH and concentrations indicated in the text) at 45°C , and autolysis was followed by the decrease in OD_{650} .

Biochemical assays. Protein contents were determined by the Lowry method, using serum albumin (Sigma) as a standard. Amino-terminal residues were detected according to Ghuysen *et al.* (1966), using glycine (Merck) as a standard. Reducing power was quantified following Thompson & Shockman (1968), with muramic acid (Sigma) as a

standard. The release of RNA was estimated by the method of Dubois *et al.* (1956), with RNA from bakers' yeast (Sigma) as a standard. Total neutral sugars in isolated cell walls were estimated by the method of Dubois *et al.* (1956) with glucose (Merck) as a standard. Phosphorus contents were determined as described by De Servio (1969). Quantitative amino acid and hexosamine (muramic acid) analyses were performed with a Beckman 119 CL analyser after hydrolysis of samples in sealed tubes with 5.7 M-HCl at 95°C for 16 h or at 110°C for 24 h. The first conditions were necessary to avoid extensive degradation of muramic acid. Hydrolysates were evaporated to dryness in vacuum over KOH pellets and washed twice with distilled water. After injection into the analyser, detection was carried out with ninhydrin at 570 nm (and 440 nm) (Spackman *et al.*, 1958). An individual calibration was used for each amino acid and hexosamine. Standards of amino acid, glucosamine and muramic acid were from Sigma. In order to determine the possible presence of a muramidase, muramicitol was detected by amino acid analysis (elution before aspartic acid) after reduction of samples with sodium borohydride following Hara & Matsushima (1966). A standard of pure muramicitol was prepared by reduction of muramic acid (0.6 mol) for 2 h with 1.3 mol sodium borohydride (Merck). After adjusting the pH to 5.6, the solution was evaporated and washed three times with methanol. It was finally suspended in methanol and kept at 4°C , where colourless crystals of muramicitol appeared.

Results

Electron microscopy of whole cells

When thin sections of cells of *L. helveticus* ATCC 12046 were stained with lead citrate and uranyl acetate, the cell wall appeared as a three-layered structure of uniform thickness (40 nm) (Fig. 1*a*). The innermost layer (A) was the most dense, and 9 nm thick. The intermediate layer (B) was less dense and 18 to 25 nm thick. The outermost layer (C) was 9 nm thick and only faintly stained. After treatment with 4% SDS at 100°C for 60 min, whole cells appeared distorted and the outermost layer had disappeared (Fig. 1*b*). Treatment of thin sections with phosphotungstic acid at low pH according to the procedure of Rambourg (1969) also led to a three-layered structure for the cell wall, with a large intermediate layer (Fig. 2*a*). However, the extent of staining of the layers differed from what was observed with lead citrate and uranyl acetate. Only the inner and outer layers were stained whereas the intermediate layer was unstained. This three-layered structure with an unstained intermediate layer was also detectable by the Thiery (1967) procedure (Fig. 2*b*), although the innermost and outermost layers were less specifically stained than by the Rambourg technique. Moreover, a thin dark line was observed at the surface of the outermost layer (arrow in Fig. 2*b*). It was noteworthy that the outermost layer remained outside the septum when all three staining procedures were used (Figs 1*a*, 2*a* and 2*b*).

Electron microscopy of isolated cell walls

When thin sections of cell walls isolated after mechanical disruption of whole cells were stained by lead citrate and

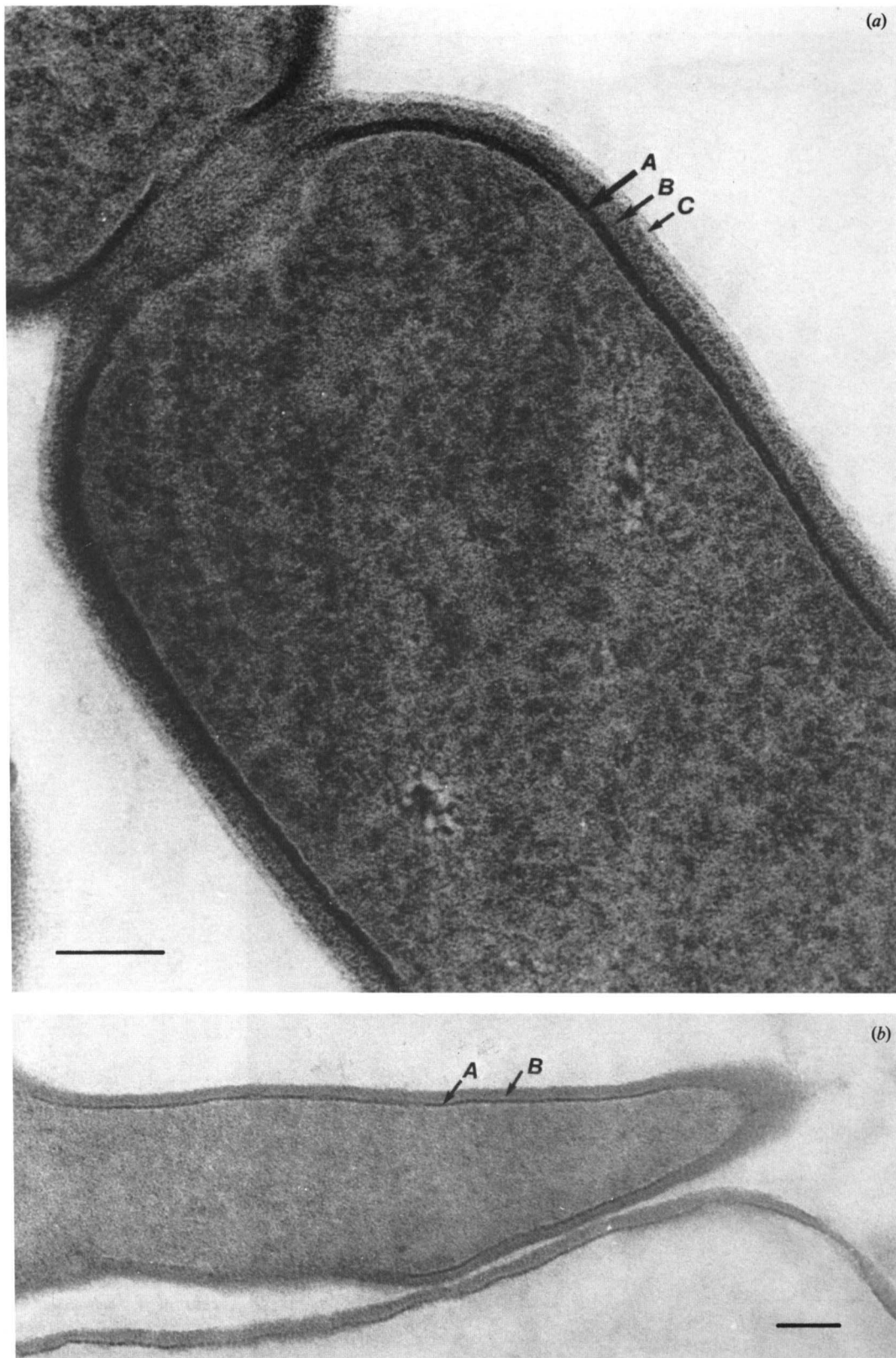


Fig 1. Electron micrographs of whole cells of *L. helveticus* ATCC 12046 stained with lead citrate and uranyl acetate, showing (a) the three-layered structure (A, B, C) of the cell wall and (b) the loss of the external layer C after SDS treatment (4%, 100 °C, 60 min). Bars, 100 nm.

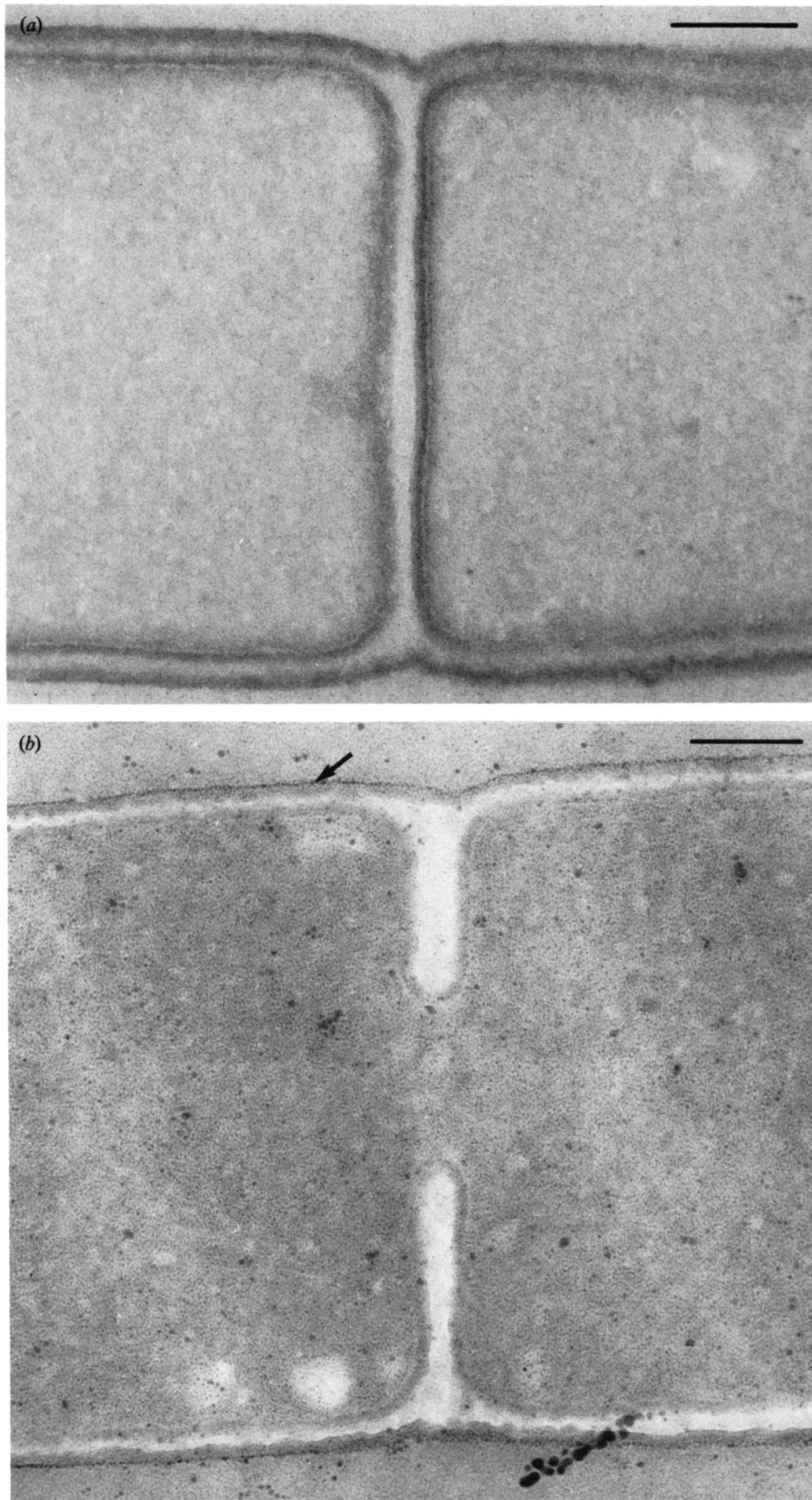


Fig. 2. Electron micrographs of whole cells of *L. helveticus* ATCC 12046 stained with (a) phosphotungstic acid (Rambourg procedure) and (b) silver proteinate (Thiery procedure). Bars, 100 nm.

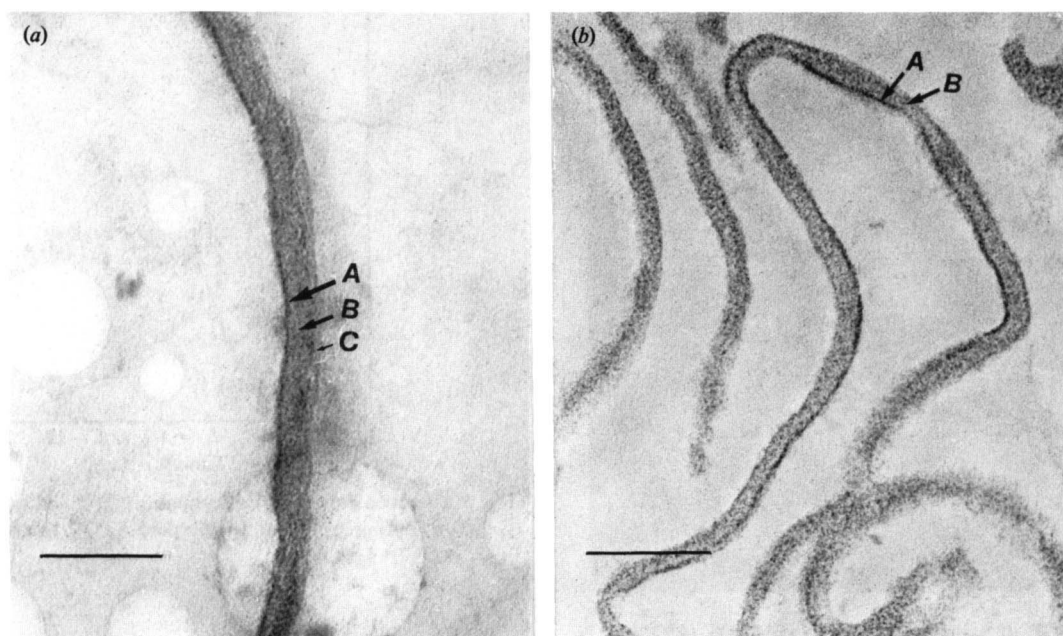


Fig. 3. Electron micrographs of isolated cell walls of *L. helveticus* ATCC 12046 stained with lead citrate and uranyl acetate (a) before and (b) after SDS-treatment (4%, 100 °C, 60 min). Bars, 100 nm.

Table 1. Composition of isolated cell walls of *L. helveticus* ATCC 12046 before and after SDS treatment

Component	Content (mg per 100 mg dry wt) in:	
	Crude cell walls	SDS-treated cell walls
Phosphorus	1.75	2.7
Muramic acid	7.25	12.6
Neutral sugars	3.9	4.8
Protein	48.5	9
Peptidoglycan*	36	63
Teichoic acid†	13.6	21

* Estimated from muramic acid content.

† Estimated from phosphorus content.

uranyl acetate the same three-layered structure was observed (Fig. 3a). However, the innermost layer appeared slightly thinner. As observed with whole cells, the outermost layer was lost after treating isolated cell walls with SDS (Fig. 3b).

Composition of isolated cell walls

The composition of isolated cell walls was determined by various classical biochemical assays (Tables 1 and 2). Analyses were carried out before and after SDS treatment. The most striking feature was the high protein content (48%) of untreated cell walls and its

Table 2. Amino acid content of isolated cell walls of *L. helveticus* ATCC 12046 before and after SDS treatment

Amino acid	Content [nmol (mg dry wt) ⁻¹] in:	
	Crude cell walls	SDS-treated cell walls
Asp	780	634
Glu	481	618
Ala	1052	1384
Lys	645	670
Muramic acid	282	502
Glucosamine	285	580
Thr	283	15
Ser	193	21
Gly	179	19
Leu	111	—
Tyr	232	6
Cys	215	—
Pro	82	8
Met	—	—
Ile	149	17
Phe	68	8
Arg	54	7

disappearance after SDS treatment. This clearly indicated that protein material was noncovalently associated with cell walls. Since all muramic acid was recovered in the insoluble fraction after SDS treatment, it was concluded that peptidoglycan material was not released by this treatment. The equal amounts of aspartic acid, glutamic acid, lysine, muramic acid and glucosamine

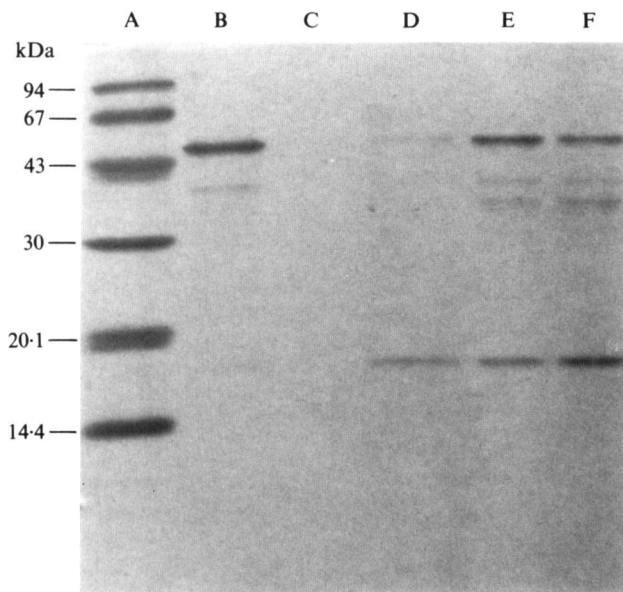


Fig. 4. SDS-PAGE of samples denatured with SDS (4%) and 2-mercaptoethanol (1%) for 3 min at 100 °C (unless otherwise indicated): A, molecular mass standards; B, crude cell walls; C, SDS-treated cell walls; D, supernatant from the SDS-treatment; E, crude cell walls (30 min); F, crude cell walls (60 min).

found in SDS-treated cell walls (Table 2) reflected a peptidoglycan structure typical of lactobacilli (Coyette & Ghuyssen, 1970*a*; Schleifer & Kandler, 1972). Assuming such a structure for *L. helveticus* ATCC 12046, it was possible to estimate the peptidoglycan content at about 36% for the crude cell walls and at 63% for the SDS-treated material.

The phosphorus content of isolated cell walls (1.75%) and its full recovery (2.7%) in the SDS-insoluble material (similar phosphorus/muramic acid ratios) suggested the presence of teichoic acids covalently bound to peptidoglycan. Assuming a composition similar to that reported for another *L. helveticus* strain (Knox & Wicken, 1971), the teichoic acid content was estimated at about 14% for isolated cell walls and 21% for SDS-treated cell walls.

Analysis of the cell wall protein content

SDS-PAGE analysis of isolated cell walls revealed the presence of one predominant protein with an apparent molecular mass of 52 kDa (Fig. 4). This protein appeared to be unglycosylated (Schiff staining procedure with 250 µg protein in the sample; data not shown) and accounted for 70% of the protein material detected after 3 min of denaturation. It was concluded from these results that it accounted for 14% of the total protein cell content. The electrophoretic pattern observed depended greatly on the time of denaturation. With denaturation

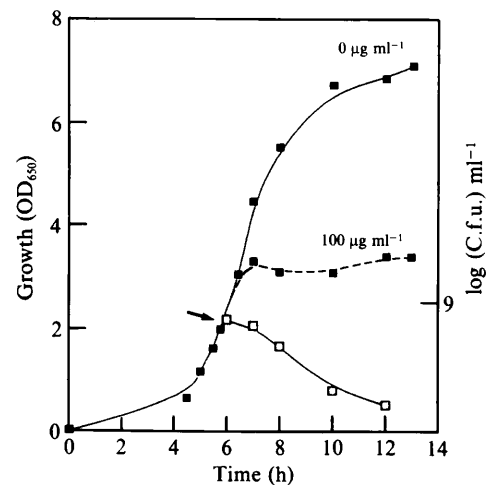


Fig. 5. Effects on growth (■) and viability (□) of addition of penicillin G (arrow) during growth of *L. helveticus* ATCC 12046.

times longer than 3 min the amount of the 52 kDa protein decreased whereas two proteins of lower molecular mass appeared, one of 17 kDa and the other of 40 kDa. After 60 min, the 17 kDa protein became predominant. Minor proteins were also detectable, but it was difficult to assess whether they were true cell wall constituents or merely contaminations with membrane or cytoplasmic material. In agreement with the results of the amino acid analysis (Table 2), SDS-treated cell walls did not contain any detectable protein material (Fig. 4, lane C). The 9% of material still detected by the Lowry procedure in SDS-treated cell walls (Table 1) was perhaps due to peptidoglycan. As expected, protein material was recovered in the supernatant following SDS treatment (Fig. 4, lane D). The 52 kDa protein was also efficiently removed from isolated cell walls by extraction with 4.5 M-lithium chloride at 0 °C for 5 min (10 mg cell walls ml⁻¹) (data not shown).

Autolysis of whole cells

Usually, the inhibition of peptidoglycan synthesis by addition of antibiotics to the growth medium promotes a lytic response in the culture (Rogers, 1980). No detailed studies of the effects of antibiotics on lactobacilli have been made, except for factors affecting lactic acid production (Wilkowske & Krienke, 1951) and bactericidal efficiency (Torre & Davanzo, 1954; Arnold & Hold, 1955). When penicillin G was added to cultures of *L. helveticus* ATCC 12046, non-lytic killing was observed (Fig. 5). With D-cycloserine added at the same final concentrations (10, 40 and 100 µg ml⁻¹) only bacteriostatic effects were noted. Vancomycin and fosfomycin were also inefficient at inducing autolysis (data not shown).

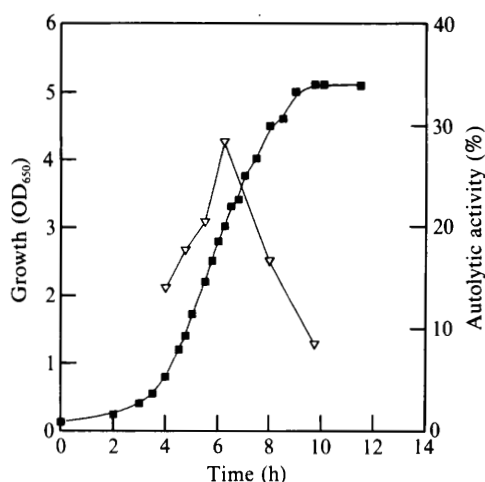


Fig. 6. Changes in autolytic activity (▽) during growth (■). Cells were harvested at different stages of growth, washed and suspended in 0.1 M-sodium phosphate buffer pH 7.0 (or 0.1 M-Tris/HCl buffer pH 7.0; the same results were obtained). The percentage decrease in OD₆₅₀ after 2 h was used to compare autolytic activity.

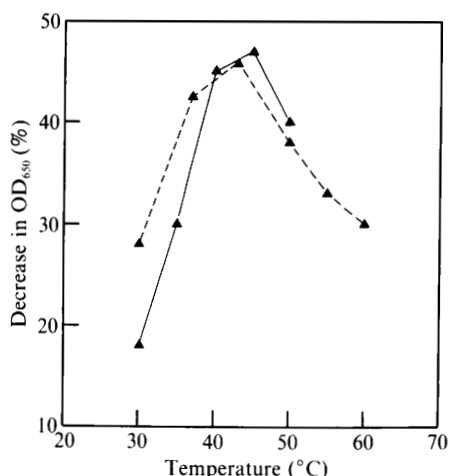


Fig. 7. Effect of temperature on the autolysis of whole cells (▲—▲) (in 0.1 M-sodium phosphate buffer pH 7.0), and of isolated cell walls (▲---▲) (in 0.1 M-Tris/HCl buffer pH 7.0). The percentage decrease (%) in OD₆₅₀ obtained after 24 h is indicated.

The only way to induce the autolytic system of *L. helveticus* ATCC 12046 successfully was to suspend harvested cells in dilute buffers. The autolysis obtained was variable, depending on the conditions used. For example, the harvesting of cells at different states of growth modified the extent of autolysis observed (Fig. 6). The optimum corresponded to the transition between the exponential and stationary growth phases. It was the same whether phosphate or Tris/HCl buffer was used.

A temperature of 40 to 45 °C appeared to be the most efficient for inducing autolysis (Fig. 7). Above 50 °C, autolysis was inhibited. Attempts to determine the effect of buffer pH gave poorly reproducible results and pH 7.0

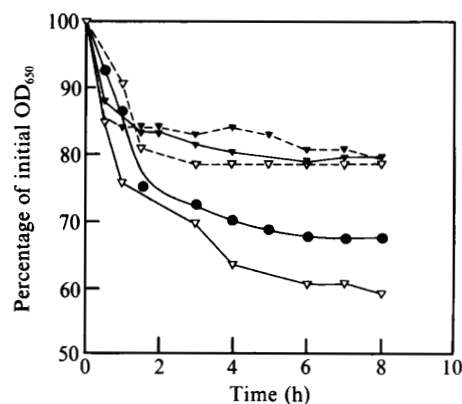


Fig. 8. Effect of calcium and magnesium on autolysis (decrease of OD₆₅₀ in 0.02 M-Tris/HCl buffer pH 7.0). ●, Control; (▽---▽), 10⁻² M-Mg²⁺; (▽-▽), 10⁻³ M-Mg²⁺; (▼---▼), 10⁻² M-Ca²⁺; (▼-▼), 10⁻³ M-Ca²⁺ (final concentrations).

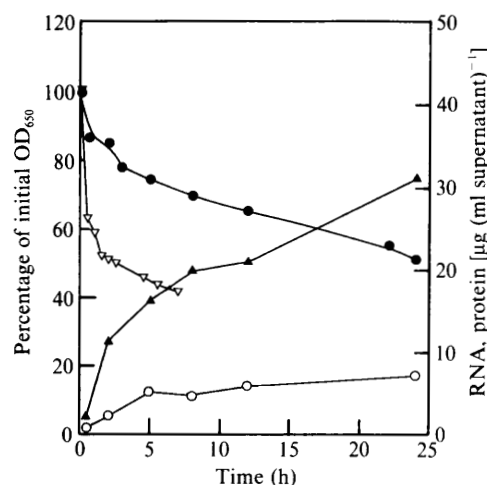


Fig. 9. Concomitant release of RNA (○) and protein (▲) during autolysis (percentage decrease in OD₆₅₀, ●) of whole cells in 0.1 M-sodium phosphate buffer pH 7.0. Improved autolysis was obtained by addition of EDTA (10⁻⁴ M) to the buffer (▽).

was chosen for all subsequent autolyses of whole cells. The molarity of the phosphate buffer had to be between 0.5 and 1 M for optimal autolysis (data not shown). The presence of 10⁻³ or 10⁻² M-CaCl₂ clearly inhibited lysis, whereas MgCl₂ had an effect depending on its concentration (Fig. 8). The presence of 10⁻⁵ M-EDTA in the phosphate buffer increased the rate and extent of autolysis: the decrease of OD₆₅₀ reached 60% after 8 h (Fig. 9).

A concomitant release of nucleic acids and proteins and a reduction of viability were observed during the decrease of OD₆₅₀ (Fig. 9). Curiously, no muramic-acid-containing material was detected in the supernatant after whole-cell autolysis (data not shown). The degradation of cell structure was followed by electron microscopy (thin sections) at different times of autolysis (Fig. 10). The

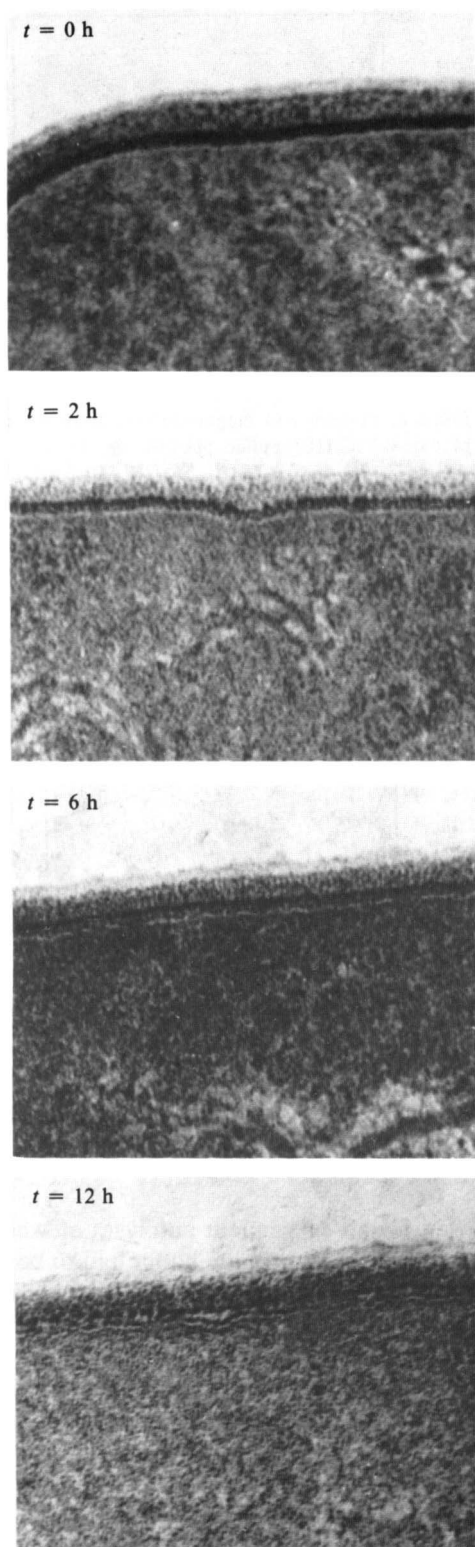


Fig. 10. Electron micrographs showing the degradation of the cell wall during autolysis of whole cells in 0.1 M-sodium phosphate buffer pH 7.0. Magnification $\times 16000$.

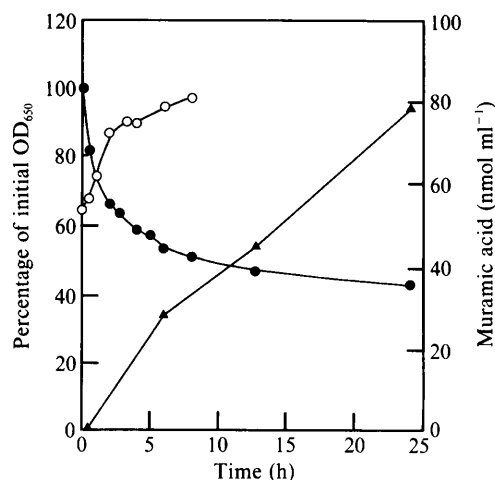


Fig. 11. Autolysis of isolated cell walls (0.75 mg ml^{-1}) in 0.1 M-Tris/HCl buffer pH 7.5 (●). The degradation of peptidoglycan was followed by measurement of muramic acid contents in the supernatant (▲), and by determining the reducing power of crude samples (○).

thickness and density of the inner layer A were clearly reduced after 6 to 12 h of autolysis. Moreover, the degradations appeared randomly distributed around the cell. It should be stressed that the other layers did not seem to be altered and that few empty cells were present.

Autolysis of isolated cell walls

A suspension in dilute buffers of cell walls isolated from *L. helveticus* ATCC 12046 induced the autolytic system. One of the most efficient buffers was 0.1 M-Tris/HCl, pH 7.5 (Fig. 11). The decrease in OD₆₅₀ reached 60% in 24 h and 40% of the total cell-wall peptidoglycan (estimated by muramic acid content) was liberated in the same time. As with whole cells, the optimum temperature was 45 °C (Fig. 7). The optimum buffer concentration was 5×10^{-2} M. It is noteworthy that cell walls prepared by ultrasonication or grinding with alumina showed no autolytic activity under the same conditions.

Specificity of the autolysin(s)

Since no amino groups appeared during autolysis of cell walls, it was concluded that neither an amidase nor a peptidase was involved in this autolytic system (data not shown). The clear increase of reducing power of the suspension and the increase of muramic-acid-containing material in the supernatant strongly suggested the occurrence of a glycosidase (Fig. 11). Moreover, the doubling of muramicitol found after 24 h of autolysis indicated the presence of at least an *N*-acetylmuramidase in the autolytic system (data not shown).

Discussion

The three-layered structure of the cell wall of *L. helveticus* ATCC 12046 was clearly established by different staining procedures applied to thin sections of whole cells or isolated cell walls. Among the other lactobacilli examined to date (Kakefuda *et al.*, 1967; Hungerer *et al.*, 1969; Wallinder & Neujahr, 1971; Higgins *et al.*, 1973), a similar structure was observed only in *L. brevis* (Masuda & Kawata, 1979). The disappearance of the outer layer from the isolated cell walls after SDS treatment and the concomitant loss of all protein material showed that the predominant 52 kDa protein was located in this layer and that it was in some way noncovalently associated with the underlying layer. Such a 50 kDa protein had previously been encountered in another *L. helveticus* strain and negative staining experiments had suggested the possibility of a regular surface arrangement (Masuda & Kawata, 1983). In our case no regular arrangement was detected by negative staining (data not shown). Perhaps, as with *L. fermenti* (Kawata *et al.*, 1985), the presence of a regular arrangement could be demonstrated by freeze-etching experiments. The PAGE experiments involving prolonged denaturation times suggested that the 52 kDa protein might be a trimer involving disulphide bridges between 17 kDa monomers. Such associations of cell wall proteins have been observed in other Gram-positive bacteria (Sleytr & Messner, 1988).

The assignment of peptidoglycan mostly to the inner cell wall layer was substantiated by several observations. This layer was strongly stained by the procedure of Rambourg (1969), which stains peptidoglycan material efficiently in various bacteria (Rousseau & Hermier, 1975; Frehel *et al.*, 1982; Frehel & Ryter, 1982; Leduc *et al.*, 1989). Moreover, peptidoglycan totally remained in the insoluble fraction after removal of the outer layer by SDS. Finally, it was the only layer clearly damaged after 12 h of autolysis of cells suspended in phosphate buffers.

The intermediate layer was the most puzzling. Proteins and peptidoglycan were the main constituents of isolated cell walls (Table 1). If they were assigned mostly to the outer and inner layers, respectively, a limited amount of material remained available for the intermediate layer, which would thus contain mainly teichoic acid and polysaccharide material. Considering that the intermediate layer was much thicker than the inner layer in SDS-treated cell walls, but that there was a predominance of peptidoglycan (63%), it could not be excluded that the intermediate layer contained some peptidoglycan material. To clarify this would require a more thorough biochemical analysis of the SDS-treated cell walls and an electron microscopy study of the effects of specific enzymic degradation of peptidoglycan. Another interesting feature was the thin dark line observed at the outside of the outer layer revealed by the

Thiery staining procedure, which is specific for polysaccharides with 1,2-glycol groups. It was apparently not due to sugar material bound to the 52 kDa protein, which appeared unglycosylated. Further work will be necessary to establish complete correlation between morphological features and the biochemical structure of the cell wall of *L. helveticus* ATCC 12046.

By varying external factors, conditions leading to 60% autolysis of whole cells of *L. helveticus* in 8 h were defined. Few autolytic systems have been described among *Lactobacillus* species. Whole cells or isolated cell walls of *L. acidophilus* (Coyette & Ghuysen, 1970*b*; Coyette & Shockman, 1973; Higgins *et al.*, 1973) and *L. fermentum* (Neujahr & Logardt, 1973) lyse readily when suspended in dilute buffers. The citrate buffer (0.01 M, pH 5) used for exponential-phase cells of *L. acidophilus* led to a 90% decrease of OD₆₅₀ in 3 h. On the other hand a 90% decrease in OD₆₅₀ was observed 90 min after suspending exponential-phase cells of *L. fermentum* in Tris/HCl buffer (0.1 M, pH 7.2). The autolytic system of *L. helveticus* ATCC 12046 thus appeared much less efficient than those of *L. acidophilus* and *L. fermentum*. This was substantiated by the slow appearance of visible structural alterations of the peptidoglycan layer and by the absence of lysis after treatment with antibiotics specifically inhibiting peptidoglycan biosynthesis. The possibility of promoting a better autolysis of *L. helveticus* cells is being investigated further. In a preliminary experiment, the addition of glycine (0.5 to 1%) to the growth medium led to cells becoming more prone to lysis. Glycine is presumed partly to replace alanine in peptidoglycan and thus to interfere with the formation of peptide cross-bridges (Hammes *et al.*, 1973).

Several features of the autolytic system of *L. helveticus* have already been encountered with *L. acidophilus* or *L. fermentum*: maximal autolytic activity at the end of the exponential phase, the inhibition by calcium ions and random degradation of the cell wall. As far as the specificity of the autolytic activity is concerned, both *L. acidophilus* and *L. fermentum* possess an *N*-acetylmuramidase (Coyette & Ghuysen, 1970*b*; Neujahr & Logardt, 1973). In *L. acidophilus* this activity was extracted from cell walls. *L. fermentum* also contained an amidase activity, both autolysins being released during degradation of the cell wall. In *L. helveticus* the glycosidase bound to the cell walls is probably an *N*-acetylmuramidase. Its limited activity might be an intrinsic property or due to low amounts, tight controls or particular structural features of peptidoglycan. Further characterization will require its extraction and the study of its interaction with isolated cell walls or peptidoglycan material.

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