X-ray photoelectron spectroscopy and biochemical analysis of the surface of *Lactobacillus helveticus* ATCC 12046

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The cell wall of *Lactobacillus helveticus* ATCC 12046 was investigated: intact cells were compared to cells stripped of their surface layer (S-layer) by treatment with LiCl. The protein profile and the amino acid composition of isolated cell walls were compared with those of LiCl-treated walls. The S-layer-forming-protein was tested for the presence of glycosidic residues. Finally, the overall elemental composition of the surface of intact cells, LiCl-treated cells and isolated S-layer-forming-protein was determined by X-ray photoelectron spectroscopy. The data collected by the ensemble of methods employed indicate that the surface of intact cells consists mainly of proteins, with some polysaccharides and teichoic or lipoteichoic acids. The exposed surface of LiCl-treated bacteria is rich in peptidoglycan, teichoic acids (or lipoteichoic acids) and polysaccharide. The extracted material is practically pure protein with traces of some glycosidic residues.

Keywords: *Lactobacillus helveticus*, S-layer, X-ray photoelectron spectroscopy, cell surface

INTRODUCTION

Lactobacillus helveticus is an important lactic acid bacterium that is involved in cheese technology (Turner *et al.*, 1983). Its autolysis, with the concomitant release of cytoplasmic enzymes (proteases, peptidases, lipases), is an essential feature of cheese ripening (Bie & Sjöström, 1975; Botazzi, 1993). A clear understanding of the autolysis phenomenon requires, among other things, a better knowledge of the composition and structure of the cell wall. Biochemical analysis showed that the overall composition of the crude cell walls of *L. helveticus* ATCC 12046 is 48% protein, 36% peptidoglycan and 14% teichoic acids (Lortal *et al.*, 1991). Electron microscopic examinations of thin sections of whole cells revealed a 40 nm thick cell wall with a three-layered structure. The innermost layer was shown to consist mainly of peptidoglycan (Lortal *et al.*, 1991). The intermediate layer could not be defined with certainty; it was assumed to contain mainly teichoic acids and polysaccharide material as well as some peptidoglycan. The outermost layer was clearly identified as a layer of one predominant protein, which is non-covalently associated

with the underlying layers. Freeze-etched preparations of intact cells have demonstrated that this outermost protein layer is an oblique crystalline surface layer (S-layer) which covers the entire cell surface (Lortal *et al.*, 1992). Crystalline surface layers are a common feature among Eubacteria and Archaeobacteria (Messner & Sleytr, 1992). They are composed of identical proteinaceous subunits that assemble to form tetragonal, hexagonal or oblique crystalline lattices. The presence of S-layers was described in several *Lactobacillus* species by Lortal (1993).

Treatment of *L. helveticus* ATCC 12046 with a 5 M LiCl solution extracted the S-layer-forming-protein from intact cells efficiently. The isolated S-layer subunits aggregated *in vitro* and the self-assembled product exhibited the same oblique symmetry as observed on intact cells *in vivo*. Amino acid analysis of the S-layer protein showed a high content (44%) of hydrophobic amino acids (Lortal *et al.*, 1992). The apparent molecular mass of the purified S-layer protein was first estimated to be 52 kDa by SDS-PAGE (Lortal *et al.*, 1992). Recently, the molecular mass was determined by mass spectrometry, a more precise method, and found to be 43.5 kDa (Lortal, 1993).

In the present work the chemical composition of the cell wall of *L. helveticus* ATCC 12046 was further investigated. The protein profile and the amino acid composition of

Abbreviations: XPS, X-ray photoelectron spectroscopy; PG, peptidoglycan; TA, teichoic acid; HC, hydrocarbon-like compound; Pr, protein; GL, glycosidic residue; S-layer, surface layer.

Fig. 1. SDS-PAGE of 75 µg loads of crude (lane 2) and LiCl-treated (lane 3) cell walls of *L. helveticus* ATCC 12046; the S-layer protein is indicated by an arrow. Molecular mass standards are shown in lane 1.

isolated cell walls were compared with those of LiCl-treated walls. The S-layer-forming-protein was tested for the presence of glycosidic residues. Finally, the overall elemental composition of the surface of intact cells, LiCl-treated cells and isolated S-layer forming protein was investigated by a physical non-destructive method, X-ray photoelectron spectroscopy (XPS) (Rouxhet & Genet, 1991).

METHODS

Bacterial strain and growth conditions. *L. helveticus* ATCC 12046 was obtained from the Pasteur Institute, Paris, France, and stored at -30°C in MRS broth (De Man *et al.*, 1960) containing 15% (v/v) glycerol. The bacteria were grown unstirred in MRS medium at 43°C after inoculation with 1% (v/v) overnight culture. Isolated cell walls were prepared by mechanical disruption of whole cells using a French press as described by Lortal *et al.* (1991).

SDS-PAGE, electroblotting and detection of glycoproteins. SDS-PAGE was performed on slab gels according to Laemmli (1970) using a 15% (w/v) acrylamide separating gel. The samples were suspended in a 0.05 M Tris/HCl buffer (pH 6.8) containing SDS (3%, w/v), 2-mercaptoethanol (1%, v/v), sucrose (15%, w/v) and bromophenol blue (0.025%, w/v), and were heated for 3 min at 100°C . After the electrophoretic separation (180 V, 70 min) the gels were either stained with Coomassie blue R250 for protein detection, or used for glycosidic residue detection. For the latter, immediately after the SDS-PAGE the proteins were electrophoretically (40 V, 2 h) transferred to an Immobilon P sheet (pore size 0.45 µm, polyvinylidene difluoride, Millipore). Glycoproteins were then

revealed using the DIG glycan detection kit (Boehringer Mannheim) with transferrin (3 µg) as a glycoprotein positive standard.

Biochemical assays. Quantitative amino acid and hexosamine analyses were performed as described by Lortal *et al.* (1991). The phosphorus content was determined according to Eckman (1993) using sodium dihydrogen phosphate as standard. The detection limit of the test was 1 µM.

Preparation of samples for XPS analysis. Cells were harvested in the exponential growth phase ($1.5\text{ mg dry weight ml}^{-1}$) and washed three times with distilled water (15000 g , 15 min, 4°C). These samples are designated 'intact cells'. Part of the pellet was suspended in 5 M LiCl solution, incubated for 15 min at 0°C and centrifuged (15000 g , 15 min, 4°C). The cells were then washed three times in distilled water, thus providing the samples referred to as 'treated cells'. The supernatant was dialysed against distilled water overnight at 4°C . A white precipitate appeared and was recovered by centrifugation (30000 g , 15 min, 4°C). This was referred to as the sample 'extract-pct'. Intact cells and treated cells were prepared from two separate cultures. Extract-pct was prepared only from the first culture.

X-ray photoelectron spectroscopy. This technique is based on irradiating a sample by an X-ray beam, analysing the kinetic energy of the ejected photoelectrons and determining their binding energy in the source atom. Each peak of the recorded spectrum is characteristic of a given energy level of an element. The position and the shape of the peaks are influenced by the chemical bonds and the oxidation state of the analysed atom. The method thus provides both an elemental and functional analysis. Due to inelastic scattering, the electrons collected originate from the outermost thin layer (3–5 nm) of the surface. Application of this technique for studying the surface of microbial cells was recently reviewed by Rouxhet *et al.* (1994).

The last pellet of the washed samples (intact and treated cells, extract-pct) was rapidly frozen in a small glass vial immersed in liquid nitrogen. The frozen samples were then stored at -80°C until freeze-drying (0.3 Pa under programmed temperature: 3 h at -50°C , 15 h linear increase from -50°C to -5°C , 6 h at -5°C). Two materials were lyophilized together with the samples and served as contamination controls: sorbitol and silica (Neosil).

The dehydrated cell powder was mounted in a stainless steel trough and pressed to obtain a smooth surface. The analysis was carried out in an SSX-100 model 206 photoelectron spectrometer (Fisons). The X-rays were generated from a monochromatized aluminium anode. The residual pressure in the analysis chamber was about 10^{-6} Pa . The flood gun energy was set at 6 eV with a nickel grid placed 3 mm above the sample surface. Resolution (FWHM_{Au4f_{7/2}}) was 1.0 eV. The size of the analysed area was about 1.4 mm^2 . The order of peak analysis was: C, O, N, P and C again, with a pass energy of 50 eV (high resolution); then C, O, N, P, K, Na, Ca, Mg, S, Cl, Li, C and a general spectrum, with a pass energy of 150 eV (lower resolution). The duplication of C analysis provided an estimate of sample degradation during the period of data accumulation under X-ray irradiation. The analysed peaks corresponded to 1s electrons (main quantic number 1) for C, O, N, Na and Li; to 2p electrons (main quantic number 2 and orbital quantic number 1) for P, S, K, Ca and Cl; and to Auger electrons for Mg. The binding energies were determined by reference to the carbon component due to carbon bound only to carbon or hydrogen, set at 284.8 eV.

Data treatment was performed with the ESCA 8.3 D software including the elemental sensitivity factors provided by the

spectrometer manufacturer. Atom fractions were calculated from the normalized peak areas after non-linear background subtraction. Major complex peaks (analysed at high resolution) were decomposed by a least-squares best-fitting routine, assuming that the components shape was defined by a Gaussian/Lorentzian ratio of 85/15, using information in the literature concerning component binding energies and a constant value for the full width at half maximum of all the components of a given peak.

RESULTS

Effect of LiCl treatment on the amino acid and hexosamine content of isolated cell walls

Isolated cell walls (3 mg) were submitted to LiCl treatment (5 M, 15 min, 0 °C), centrifuged (20 000 *g*, 15 min) and washed. The last pellet, as well as nontreated cell walls (3 mg), were suspended in 1 ml distilled water. SDS-PAGE and amino acid analysis were performed on equivalent quantities of these two samples.

The results of SDS-PAGE are shown in Fig. 1; the S-layer-forming-protein and the majority of other cell wall proteins were extracted by the treatment.

The results of the amino acid analysis, which also included hexosamines, are presented in Table 1. The data revealed that the relative proportion of muramic acid did not change due to the LiCl treatment. The proportions of the amino acids constituting the peptidoglycan of *L. helveticus*

(Asp, Glu, Ala, Lys) decreased after the LiCl treatment, but the reduction in the proportions of the other amino acids was more pronounced. The difference between the amino acid content of the crude and LiCl-treated cell walls should represent approximately the amino acid composition of the protein that has been released by the LiCl treatment. This difference, expressed as the molar ratio, is indeed quite similar to the amino acid composition obtained after direct analysis of S-layer protein isolated from whole cells (Lortal *et al.*, 1992).

Glycosylation of the S-layer-forming-protein

The extract-pct sample was submitted to SDS-PAGE; a major band and a few smaller bands were revealed (Fig. 2a). After transfer to an Immobilon P sheet and staining, glycosidic residues were found to be associated with the S-layer forming protein (Fig. 2b). Quantities of S-layer protein as low as 150 ng were sufficient to detect the glycosidic residues.

Phosphorylation of the S-layer-forming-protein

The S-layer-forming-protein was extracted directly from the polyacrylamide gel (50 µg protein) and submitted to the colorimetric phosphorus test. The extraction was done by cutting the band corresponding to the S-layer-forming-protein immediately after electrophoresis, suspending it in 500 µl 0.1 M Tris/HCl buffer (pH 7.5),

Table 1. Amino acid (and aminosugar) composition of cell walls of *L. helveticus* before and after LiCl treatment (5 M, 15 min, 0 °C)

Amino acid	Crude cell walls (nmol mg ⁻¹)	LiCl-treated cell walls (nmol mg ⁻¹)	Crude walls – Treated walls	
			(nmol mg ⁻¹)	Molar ratio (%)
Ala*	1372	900	472	17.2
Asp*	615	212	403	14.7
Glu*	604	398	206	7.5
Lys*	475	181	294	10.7
Thr	311	47	264	9.6
Ser	234	56	178	6.5
Val	187	35	152	5.5
Tyr	160	35	125	4.6
Gly	152	40	112	4.1
Pro	152	0	152	5.5
Leu	148	57	91	3.3
Ile	115	27	88	3.2
Cys	68	68	0	0
Arg	66	15	51	1.9
Met	55	35	20	0.7
His	38	11	27	1.0
Phe (+ Glucosamine*)	526	414	112	4.1
Muramic acid*	175	161	14	NI

NI, Not included in the calculation.

* Component of peptidoglycan.

Fig. 2. SDS-PAGE of extract-pct stained for (a) protein (Coomassie blue) and (b) glycosides (DIG glycan) after electroblotting to an Immobilon P sheet. Lanes: 1 and 6, molecular mass standards; 2, 3 and 4, extract-pct samples (loads of 15, 7.5 and 0.15 µg, respectively); 5, transferrin (3 µg load) as glycoprotein positive standard. The S-layer protein is indicated by an arrow.

crushing and centrifuging (10000 *g*, 15 min). Samples (100 µl) of the supernatant were then submitted to the colorimetric test. No phosphorus could be detected.

Elemental and functional composition of the cell surface

In Tables 2 and 3, showing the results of the XPS analysis, data are presented for each culture, together with the mean values of the duplicates, in order to show the combined reproducibility of culture and spectroscopic analysis. One sample (intact cells, culture a) was analysed twice in the spectrometer and the mean values of these two analyses are given.

The elemental composition of the intact cells, treated cells and extract-pct are summarized in Table 2. A good agreement exists between the duplicates of intact and treated cells. It seems that the surface of treated cells had more oxygen and phosphorus and less nitrogen than the surface of intact cells. The extract-pct sample had higher nitrogen, and lower oxygen and phosphorus than the two other samples. Potassium was detected only on the surface of intact cells; sulfur, on intact cells and in the extract-pct. The concentrations of the other elements that were analysed fell below the detection limit (about 0.05 % atom fraction).

Decomposition of complex peaks supplies information about the chemical function in which a given atom is involved. Four components were defined in the carbon spectra: the component at a binding energy of 284.8 eV was attributed to carbon bound only to carbon or hydrogen, $\text{C}-(\text{C},\text{H})$; the one at a binding energy of 286.3 eV was attributed to carbon singly bound to oxygen or nitrogen, $\text{C}-(\text{O},\text{N})$, as in alcohols or amines; the component at 287.9 eV, to carbon making two bonds with oxygen, $\text{C}=\text{O}$, either two single bonds, as in acetals and hemiacetals, or one double bond, as in carbonyls,

Table 2. Elemental composition of the surface of *L. helveticus* cells (atom fraction, %, excluding hydrogen)

Element	Intact cells			Treated cells			Extract-pct
	Culture a*	Culture b	Mean	Culture a	Culture b	Mean	Culture a
C	60.77	59.31	60.11	57.12	58.46	57.79	62.39
O	29.77	31.07	30.42	35.83	34.63	35.23	21.81
N	8.74	9.15	8.95	5.96	5.90	5.93	15.53
P	0.58	0.46	0.52	1.09	1.00	1.04	0.28
K	D	D	D	BDL	BDL	BDL	BDL
S	D	D	D	BDL	BDL	BDL	D
Na, Ca, Mg, Li, Cl	BDL	BDL	BDL	BDL	BDL	BDL	BDL

D, Detected, but too low to be considered quantitatively; BDL, below detection limit (about 0.05 % for these elements).

* Mean value of two analyses of the same sample, variations were about 5 % of the mean.

Table 3. Functional composition of the surface of *L. helveticus* cells (atom fraction, %, excluding hydrogen)

Peak	Component*	Intact cells			Treated cells			Extract-pct
		Culture a†	Culture b	Mean	Culture a	Culture b	Mean	Culture a
C _{1s}	284.8	23.49	20.43	21.96	15.11	15.89	15.50	27.21
	<u>C</u> -(C,H)							
	286.3	25.58	26.43	26.00	29.15	29.56	29.33	20.48
	<u>C</u> -(O,N)							
	287.9	11.70	12.45	12.14	10.42	11.10	10.76	14.70
	<u>C</u> =O							
O _{1s}	289.0	ND	ND	ND	2.49	1.91	2.20	ND
	<u>C</u> =OOH							
	531.3	10.17	11.40	10.78	8.75	7.64	8.19	15.92
	<u>O</u> =C							
N _{1s}	532.6	19.60	19.67	19.64	27.08	26.99	27.03	5.89
	- <u>O</u> H							
	399.8	8.22	8.57	8.40	5.43	5.55	5.49	14.46
Non-prot N‡	401.4	0.52	0.58	0.55	0.53	0.35	0.44	1.07
	Prot N§							

ND, Not detected.

* Binding energy in eV and attribution.

† Mean value of two analyses of the same sample; variations were up to 10 % of the mean.

‡ Non-protonated nitrogen.

§ Protonated nitrogen.

carboxylates and amides; and the component at 289.0 eV to carboxylic or ester carbons, C=OOH. The oxygen peaks were tentatively decomposed into two components, one at 531.3 eV, attributed to doubly bound oxygen, O=; and one at a binding energy of 532.6 eV, due to hydroxide, acetal or hemiacetal functions, -OH. The nitrogen peaks consisted of a major component at 399.8 eV, due to non-protonated nitrogen, as in amines or amides, and a very minor component at 401.4 eV, attributed to protonated nitrogen, e.g. ammonium ions or protonated amines.

Table 3 summarizes the results of peak decomposition and thus provides the functional composition of the samples. Again, the reproducibility seems to be satisfactory. The functional analysis reveals more subtle variations between the sample types. When treated cells are compared to intact cells, it becomes obvious that the higher oxygen content mentioned above is mainly due to OH, the lower nitrogen to a non-protonated component, and there is also a decrease of the C-(C,H) component in the carbon peak. The extract-pct sample, which had less oxygen than the two others, is characterized by higher O= and very low OH, as well as by high C-(C,H) components.

The two types of controls employed confirmed that there was no serious contamination problem. The sorbitol control, which is theoretically 100 % C-OH, had only a small (4.9 %) C-(C,H) component. The silica control, in which no carbon is expected, contained only 2.3 %

carbon. The degree of sample degradation was evaluated from comparison of the carbon peaks registered at the beginning and at the end of the data accumulation at high resolution. The shape of these peaks did not alter under irradiation, indicating that there was no damage to the surface during the analysis.

DISCUSSION

The use of XPS in microbiology is not yet widespread. This is partly due to certain doubts concerning the relevance of the (freeze-dried) analysed samples to the cell surface in its natural (hydrated) state. Recent work confirms the reliability of XPS analysis of biosurfaces (P. A. Gerin, P. B. Dengis & P. G. Rouxhet, unpublished) and supply guidelines concerning essential precautions to minimize artefacts (Dengis *et al.*, 1995). The present work is believed to show the potential of XPS in providing significant information about microbial cell surfaces. In this respect, it joins other reports that have accumulated during the last fifteen years and which were reviewed by Rouxhet *et al.* (1994).

Cuperus *et al.* (1992, 1993) reported N/C atomic ratios in the range of 0.042–0.092 for a series of seven lactobacillus strains. The nitrogen concentration of the cell surface found in the present work for *L. helveticus* is much higher (N/C for intact cells 0.149). In other Gram-positive

species values of 0.030–0.140 were revealed (van der Mei *et al.*, 1988a, b, 1989a, 1991, 1992; Büchs *et al.*, 1988; Busscher *et al.*, 1990, 1991; Herben *et al.*, 1990; Cowan *et al.*, 1992a, b). Only in one study, dealing with various staphylococcal strains, were higher values (0.149–0.197) obtained (van der Mei *et al.*, 1989b). To the best of our knowledge, the present study is the first to combine XPS analysis with detection of S-layers in bacteria. Gerin *et al.* (1993) investigated the white-rot basidiomycete *Phanerochaete chrysosporium*; XPS analysis of the dormant conidiospores led them to suspect the presence of a layer of rodlets at the surface. This was later confirmed (Gerin *et al.*, 1994) by freeze-etching and electron microscopy.

Extract-pct

Electrophoretic analysis of the proteins contained in the extract-pct sample revealed that it is composed of a predominant glycoprotein corresponding to the S-layer-forming protein (Fig. 2) as shown by Lortal *et al.* (1992). The presence of glycosidic residues was clearly demonstrated in the present study (Fig. 2b) using the sensitive DIG glycan detection system, though a previous attempt to demonstrate glycosidic residues associated with the S-layer protein failed (Lortal *et al.*, 1991). The method used in that study, based on periodate/Schiff staining reaction after SDS-PAGE, was probably not sensitive enough. S-layer proteins of two strains of lactobacilli (Möschl *et al.*, 1993) and several other bacteria, in particular *Bacillus* and *Clostridium* species, have also been shown to be glycosylated (Messner & Sleytr, 1991).

The results provided by XPS analysis also indicated that the extract-pct sample is principally of a proteinaceous nature. There is good agreement between the three parameters which are involved in a peptide bond:

- (i) non-protonated nitrogen (component of N peak at 399.8 eV): 14.46 %
- (ii) oxygen engaged in a double bond with carbon (component of O peak at 531.3 eV): 15.92 %
- (iii) carbon engaged in a double bond with oxygen (component of C peak at 287.9 eV): 14.70 %

For the extract-pct sample, these parameters could thus be considered as indicators of peptide bonds alone.

The component of the oxygen peak at 532.6 eV (hydroxide) can be attributed both to the glycosidic residues and to serine, threonine and tyrosine of the protein. Indeed, the S-layer-forming-protein is glycosylated and has been reported to have high molar ratios of these amino acids: 9.5 %, 11.1 %, and 3.0 %, respectively (Lortal *et al.*, 1992).

The protonated nitrogen (component of N peak at 401.4 eV) probably represents basic amino acids (lysine, histidine, arginine). The sulfur is attributable to methionine since no cysteine was detected in the amino acid analysis of the S-layer protein (Lortal *et al.*, 1992).

The presence of phosphorus is intriguing. It could be due to phosphorylation of the protein or simply to contamination of the preparation. Direct phosphorus de-

termination in the S-layer forming protein extracted from the gel suggested that the protein is probably not phosphorylated. If it was, the level would be below 0.5 mol phosphate (mol protein)⁻¹. Contamination of the extract-pct sample by phosphorylated molecules must then be considered. The contamination could not be due to inorganic phosphate ions or small molecules containing phosphate since the sample was dialysed before the spectroscopic analysis. It is not likely to be due to teichoic acids as these are covalently bound to the peptidoglycan (Baddiley & Davison, 1961), and therefore would not be extracted by the LiCl treatment. The most probable explanation is that some lipoteichoic acids associated with the extract-pct sample during its preparation for XPS analysis.

Intact cells

The S-layer protein is considered to entirely cover the cell surface. Indeed, XPS data suggest that the major constituent of the intact cell surface is protein even if the similarity of the three 'peptide bond indicators' mentioned above is less evident at that surface (8.40, 10.78 and 12.14 %, respectively) than in the extract-pct sample (14.46, 15.92 and 14.70 %).

The phosphorus present at the intact cell surface can be attributed to teichoic or lipoteichoic acids protruding from the S-layer. Phosphate groups at the cell surface will be ionized at physiological pH and therefore will create a negative electrical charge. Ferritin (a polycation used to probe negatively charged zones) binds homogeneously to the surface of intact cells of *L. helveticus* with a density of about 280 molecules μm^{-2} (Lortal *et al.*, 1992), indicating the presence of areas with high negative electrical charge in the vicinity of the S-layer.

A considerable proportion of $-\text{OH}$ indicating glycosidic residues was also detected. These could be the glycosylated part of the S-layer protein itself, or independent polysaccharides protruding out of the S-layer. Electron microscopic observations after silver protein staining, which is specific for polysaccharides with 1,2-glycol groups (Thiery, 1967), have indicated previously the presence of polysaccharides at the surface of intact cells (Lortal *et al.*, 1991).

Treated cells

LiCl treatment eliminates the outer layer observed by electron microscopy, so the surface exposed after this treatment is considered to be the intermediate layer (Lortal *et al.*, 1991). Given the high surface oxygen and low surface nitrogen of treated cells (Table 2), the high proportion of the carbon peak component at 286.3 eV (Table 3) at the surface of these cells suggests that it is rich in $\text{C}-\text{O}$. The 532.6 eV component of the oxygen peak ($\text{C}-\text{OH}$), which is very high for the surface of the treated cells, indicates abundance of hydroxide functional groups. The complementary information derived from decomposition of the carbon and oxygen independent peaks

Table 4. Chemical composition of the model constituents considered for deduction of the molecular composition of the *L. helveticus* cell surface

Constituent	Atomic ratios relative to carbon			Proportion of carbon and oxygen involved in various chemical functions					Carbon concn (mol g ⁻¹)
	O/C	N/C	P/C	[C-(C,H)]/C	[C-(O,N)]/C	(C=O)/C	(O=C)/C	(OH)/C	
Pr*	0.320	0.270	0	0.410	0.320	0.280	0.275	0.045	0.0424
PG†	0.500	0.200	0	0.275	0.425	0.300	0.250	0.250	0.0410
TAa‡	1.333	0	0.200	0	0.933	0.067	0.200	1.133	0.0249
TAb§	1.067	0.067	0.133	0.067	0.800	0.133	0.200	0.867	0.0278
HC	0	0	0	1.000	0	0	0	0	0.0710
Gl¶	0.833	0	0	0	0.833	0.167	0	0.833	0.0370

* Average protein, calculated by P. Gerin (personal communication) for a collection of bacterial, fungal and mammalian proteins.

† Peptidoglycan (C₄₀H₈₃O₂₀N₈) calculated from data of Coyette & Ghuysen (1970) for *L. acidophilus*, a species phylogenetically close to *L. helveticus*, assuming that all the muramic acid residues are substituted by a pentapeptide and Asp bridges between the peptides.

‡ Teichoic acid a (C_{6.6}H_{13.6}O₈P₁) calculated from data of Knox & Wicken (1971) for *L. helveticus*, assuming 60% of the glycerol C₂ is linked to a glucose residue and 40% to hydrogen.

§ Teichoic acid b (C_{7.8}H_{15.8}O_{8.4}P₁N_{0.4}) calculated from data of Knox & Wicken (1971) for *L. helveticus*, assuming 60% of the glycerol C₂ is linked to a glucose residue and 40% to alanine.

|| Hydrocarbon-like compound (CH₂).

¶ Glycosidic residue (C₆H₁₀O₅).

indicates clearly that the surface of treated cells is rich in C-OH functions, suggesting that it consists mainly of saccharidic material.

The detection of nitrogen and phosphorus suggests the presence of peptidic material (peptidoglycan or protein) and teichoic acids, respectively.

Modelling the surface composition

Interpretation of XPS data concerning the surface composition in molecular terms can be tested in a more formal way by considering model molecules and calculating the atomic ratio of the different elements or functional groups. For a given surface which is expected to consist of a mixture of these model molecules one can construct a set of equations that will include the 'theoretical' and experimental atomic ratios as parameters and the proportions of the types of molecules as the unknown variables. Gerin *et al.* (1993) used this strategy to interpret XPS data concerning the surface of *Phanerochaete chrysosporium* in terms of proteins, carbohydrates (chitin or glucan) and hydrocarbon-like compounds.

The constituents of Gram-positive bacteria cell wall are peptidoglycan (PG), teichoic acid (TA), hydrocarbon-like (HC) moieties linked to TA, proteins (Pr) and glycosidic residues (Gl). Table 4 presents the expected ('theoretical') atom concentration ratios, relative to carbon, for these model constituents. The cell surface can be considered to be composed of a certain combination of all, or some, of these constituents. The proportion of each constituent can be computed by solving a set of equations where the ratios presented in Table 4 and the ratios derived from the XPS data (Tables 2 and 3) are the known parameters, and

the proportions of each constituent are the unknown variables:

$$[N/C]_{\text{xps}} = 0.270 C_{\text{Pr}} + 0.200 C_{\text{PG}} \quad (1)$$

$$[O/C]_{\text{xps}} = 0.320 C_{\text{Pr}} + 0.500 C_{\text{PG}} + 1.333 C_{\text{TAa}} + 0.833 C_{\text{Gl}} \quad (2)$$

$$[P/C]_{\text{xps}} = 0.200 C_{\text{TAa}} \quad (3)$$

$$[C-(C,H)]_{\text{xps}} = 0.410 C_{\text{Pr}} + 0.275 C_{\text{PG}} + 1.000 C_{\text{HC}} \quad (4)$$

$$[C-(O,N)]_{\text{xps}} = 0.320 C_{\text{Pr}} + 0.425 C_{\text{PG}} + 0.933 C_{\text{TAa}} + 0.833 C_{\text{Gl}} \quad (5)$$

$$[(C=O)]_{\text{xps}} = 0.280 C_{\text{Pr}} + 0.300 C_{\text{PG}} + 0.067 C_{\text{TAa}} + 0.167 C_{\text{Gl}} \quad (6)$$

$$[(O=C)]_{\text{xps}} = 0.275 C_{\text{Pr}} + 0.250 C_{\text{PG}} + 0.200 C_{\text{TAa}} \quad (7)$$

$$[(OH)]_{\text{xps}} = 0.045 C_{\text{Pr}} + 0.250 C_{\text{PG}} + 1.133 C_{\text{TAa}} + 0.833 C_{\text{Gl}} \quad (8)$$

$$1 = C_{\text{Pr}} + C_{\text{PG}} + C_{\text{TA}} + C_{\text{HC}} + C_{\text{Gl}} \quad (9)$$

where xps denotes the ratios obtained by XPS analysis, C_{Pr}, C_{PG}, etc. are the proportions of the total surface carbon associated with each molecular constituent mentioned in the subscript and the numerical values (theoretical ratios) from Table 4 take TAa. If, instead of the theoretical TAa values, one introduces the theoretical TAb values, equations 2, 3, 5, 6, 7 and 8 will be slightly different. Equations (1)–(3) are based on the elemental analysis (Table 2); equations (4)–(6) are based on the functional analysis derived from the decomposition of the carbon peak (Table 3); equations (7)–(8) are based on the functional analysis derived from the decomposition of the oxygen peak (Table 3). There are more equations than

unknown variables, so the equations cannot be linearly independent; e.g., equation (5) depends on equations (1) and (2), or equation (2) is the sum of equations (7) and (8). The proportions (C_i) obtained by solving these equations can be converted to weight fractions of the constituents by using their carbon content (last column, Table 4). Equation (9) serves as a control.

The calculations were performed for various combinations of all, or part, of the five constituents using the SAS/IML matrix programming language. A minimum of five equations is required for solving the five unknown variables (C_i). However, the system can treat a higher number of equations and use a least squares routine to provide best fitted solutions. The calculations were therefore carried out with sets of the first five, six or eight equations. A solution was considered acceptable if all the unknown variables (C_i) were positive fractions ($0 < C_i < 1$) and equation (9) was valid ($\Sigma C_i = 1 \pm 0.05$).

The results provided by the different sets of equations were generally the same. Using the theoretical values of TAB instead of TAa resulted in somewhat higher solutions for teichoic acid content. The small differences in experimental XPS data observed between duplicate samples were amplified by the computations. The retained combinations were the following (results expressed as weight percentage, mean values obtained for duplicate samples and for the two TAs):

For intact cells: (a) protein (70 %) + glycosidic residues (30 %);
or (b) protein (66 %) + teichoic acid (34 %);
or (c) protein (51 %) + teichoic acid (42 %) + hydrocarbon-like residue (7 %);
or (d) protein (50 %) + glycosidic residues (17 %) + teichoic acid (25 %) + hydrocarbon-like residue (8 %).

For treated cells: (e) peptidoglycan (70 %) + teichoic acid (17 %) + glycosidic residues (13 %);
or (f) peptidoglycan (50 %) + teichoic acid (21 %) + hydrocarbon-like residue (7 %) + glycosidic residues (22 %).

For extract-pct: (g) protein (100 %);
or (h) protein (97 %) + glycosidic residues (3 %);
or (i) protein (96 %) + teichoic acid (4 %);
or (j) protein (87 %) + teichoic acid (9 %) + hydrocarbon-like residue (4 %).

For the last sample (extract-pct), the ratios derived from the experimental values (Tables 2 and 3) were very close to the theoretical values for protein (Table 4). Therefore, this sample could be considered as consisting of pure protein. Nevertheless, the computation shows that it could contain up to 13 % of other residues.

The choice of the above combinations depends very much on the criteria used. While the criterion of ($0 < C_i < 1$) is undisputable, the second one ($\Sigma C_i = 1 \pm 0.05$) was taken arbitrarily, a more severe or more relaxed criterion could modify the conclusion. Furthermore, some mathematically accepted solutions were rejected since they were regarded as biologically senseless.

The numerical values presented above should be considered only as rough approximations. The accuracy of the surface molecular composition is limited because of the numerous simplifications made. Moreover, there was no attempt to evaluate the variability/reproducibility of the computation in strict statistical terms. Two out of the three types of samples were analysed as true independent duplicates. Although the reproducibility of the experimental data seemed to be quite satisfactory, the computation amplified the small differences between individual samples of the same type.

The modelling could not provide definite solutions; the discriminative power of the system was not strong enough. Taking into account the considerations described at the beginning of the Discussion, the following combinations are considered the most probable ones: (d) for intact cells, (e) for treated cells and (h) for the extract-pct sample.

Conclusion

The use of XPS in combination with biochemical methods has extended our knowledge about the cell wall structure and composition of *L. helveticus* ATCC 12046. In particular, the intermediate layer of the cell wall of this strain, which has been the less well-characterized in previous works, was shown to consist mainly of peptidoglycan, lipoteichoic acids and polysaccharides. XPS also detected the presence of non-proteinaceous components [saccharides and (lipo)teichoic acids] in the outer surface layer, protruding from the regular pattern formed by the S-layer protein. The extracted S-layer-forming-protein was shown to be practically pure protein, with small amounts of glycosidic residues.

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