Crystalline Surface-Layers of the Genus Lactobacillus

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Chapter 6

CRYSTALLINE SURFACE-LAYERS OF THE GENUS LACTOBACILLUS

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INTRODUCTION

Lactobacilli are fundamental contributors to food technology and agriculture. Moreover, they are thought to induce beneficial effects to public health as valuable inhabitants of the intestinal and urogenital tract of humans. The envelope of these microorganisms could play an important role (i.e., resistance, adhesion, etc.) in all of these applications. The aim of this review is to summarize the available knowledge about S-layer carrying strains of the genus *Lactobacillus*.

BIOTECHNOLOGICAL USES OF LACTOBACILLI

Lactobacilli decrease the pH of their growth environment to below 4.0 by lactic acid production, thus preventing, or at least efficiently delaying, growth of virtually all others competitors except other lactic acid bacteria and yeasts. Since ancient times they have been involved in the manufacture of a variety of food and beverages; these are dairy products, wine, meat products, vegetables and bakery products as illustrated in Table 1. These applications have been well described (Sharpe, 1979; Sugihara, 1985; Daeschel et al., 1987; McKay and Baldwin, 1990). Lactobacilli are also valuable contributors to agriculture. The inoculation of silage by *Lactobacillus* spp. effectively preserves the food stuff and suppresses the growth of unwanted pathogenic bacteria like clostridia (Seale, 1986). Moreover, various *Lactobacillus* species associated with plants are believed to inhibit the growth of plant pathogens such as *Xanthomonas* and *Erwinia* (Visser et al., 1986).

The importance of *Lactobacillus* as a member of the normal flora of the mouth, intestine and urogenital tract of women is also well recognized. Thus a recent and exciting field of research concerns the potential applications of *Lactobacillus* species for public health (Gilliland, 1990). The term "probiotic" is largely used to name preparations containing viable enteric bacteria, such as lactobacilli (mainly *L. acidophilus*), bifidobacteria and enterococci. The hypothetical beneficial effects of the

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Table 1. Biotechnological uses of some *Lactobacillus* species

Species	Products
L. fermentum	Kefir, and bakery products
L. helveticus	Cheeses (Swiss type cheese)
L. brevis	Kefir, fermented vegetables (sauerkraut, olives and silage), and bakery products
L. bulgaricus	Yoghurts
L. acidophilus	Cheeses and probiotics
L. casei	Cheeses, bakery products, and probiotics
L. plantarum	Cheeses, fermented vegetables (sauerkraut, olives, pickles and silage), bakery products, and wines
L. lactis	Cheeses (Swiss type cheese)

ingestion of probiotics include nutritional benefits, prevention of undesirable intestinal infections (Perdigon et al., 1990), an anticholesteremic effect (Fernandez et al., 1987), and an induction of mucosal and systemic immune response against epitopes associated with these organisms (Perdigon et al., 1988 ; Gerritse et al., 1990).

To exert a positive action, the probiotic microorganisms have to colonize or to multiply in the gut. These conditions imply that they are resistant to the lytic enzymes of the mouth and the acidic conditions and bile salts of the stomach. The outermost envelope of the bacterium could contribute to this resistance and to adhesion onto the intestinal mucosa.

SURFACE LAYERS OF THE GENUS *LACTOBACILLUS*

The presence of surface layers in *Lactobacillus* was first shown in 1974 (Table 2). By freeze-etching and negative staining of *L. fermentum,* Kawata et al. (1974) clearly observed a regular surface array. The lattice had an oblique symmetry (p2) with a 9.6/6.2 nm center-to-center spacing. Biochemical information about the S-layer protein was not provided. Interestingly, in previous work about the cell wall of *L. fermentum* (mainly focused on the peptidoglycan; Wallinder and Neujahr, 1970), a large fraction of the crude cell wall was protein (20% of the dry weight). Nevertheless electron microscopy observations of thin sections revealed an homogeneous (40 nm) cell wall without any separate surface layer.

In 1979 and 1980, Masuda and Kawata characterized a surface layer in *L. brevis*. A tetragonal lattice with a center-to-center spacing of 7.0/4.5 nm was observed using freeze-etching and negative staining. The S-layer protein (M_r = 51000) was extracted by H-bond disrupting agents like urea or guanidine hydrochloride (GHCl) and by ionic detergents such as sodium dodecyl sulphate (SDS). The protein on isolated cell walls was resistant to proteases with the exception of pepsin. Moreover the GHCl-extracted subunits formed regular self-assembly products on dialysis, even in the absence of specific metal cations. The reformation of the S-layer on GHCl-treated cell walls of *L. brevis* was also effective. Some experiments of heterologous re-attachment were successful with *L. fermentum* cell walls and unsuccessful with *L. plantarum* and *L. casei* cell walls.

Table 2. Crystalline surface layers on *Lactobacillus* species

Species	No of Strains		Characterization of S-layer			References
	S^+	S^-	M_r in kDa	Glycosylation	Lattice Type	
L. helveticus	2		51.5	--	ND	Masuda and Kawata, 1983
	15		51.0-58.0	--	p2	M. Sára, unpublished data
	10		52.0	ND	ND	S. Lortal, unpublished data
	1		52.0	--	p2	S. Lortal et al., 1992
L. acidophilus	4+/-		41.0	--	ND	Masuda and Kawata, 1983
	2+/-		50.0	--	p2	S. Lortal, unpublished data
	4+/-		46.0	+	ND	Bhowmik et al., 1985
	30+/-		46.0-59.0	ND	ND	Johnson et al., 1987
L. bulgaricus	1		51.5	--	ND	Masuda and Kawata, 1983
		3	ND	ND	ND	S. Lortal, unpublished data
L. fermentum	1		ND	ND	p2	Kawata, 1974
	2		51.5	--	ND	Masuda and Kawata, 1983
L. brevis	1		51.0	ND	p4	Masuda and Kawata, 1979
	2		51.0	--	ND	Masuda and Kawata, 1980
						Masuda and Kawata, 1983
L. casei	1		ND	ND	p6	Backer and Thorne, 1970
		3	ND	ND	ND	Masuda and Kawata, 1983
L. buchneri	1		55.0	ND	p6	Masuda and Kawata, 1981
	2		55.0	--	ND	Masuda and Kawata, 1983

+/-, for *L. acidophilus* the results between S^+ and S^- are variable and strain dependent.
ND, not determined.

The S-layer of *L. buchneri* was also studied by Masuda and Kawata (1981, 1985). The protein contained no carbohydrate and had a M_r = 55000. The hexagonal periodicity of the lattice was seen by freeze-etching and the center-to-center spacing was 6 nm. In order to identify the nature of the binding of the protein to the other cell wall components, the re-attachment of isolated subunits on chemically modified cell walls was attempted. The authors suggested a linkage with the neutral polysaccharide moiety of the cell wall. Both the positive charges (amino groups) and the negative charges (carboxyl groups) of the S-layer protein were required for the morphogenesis of the regular array and its binding to the underlying neutral polysaccharide.

In 1983, the presence of a surface layer on 13 different species of lactobacilli was investigated more systematically using SDS-PAGE electrophoresis and negative staining (Masuda and Kawata, 1983). *L. fermentum, L. brevis* and *L. buchneri* exhibited a surface layer as well as three species not previously described, *L. helveticus, L. bulgaricus,* and *L. acidophilus.* The M_rs of the S-layer proteins were in the range of 51000 to 55000 except for the *L. acidophilus* strain (M_r=41000), and all were considered to be not glycosylated. The amino acid composition of these S-layers was provided for the first time and, as for other bacterial surface layers, hydrophobic amino acids were in the range of 40-50% and the cysteine and methionine contents were low (Messner and Sleytr, 1992). Using an immunodiffusion assay these authors concluded that there were no common antigenic determinants among the S-layer proteins of the different *Lactobacillus* species.

The first extensive work concerning the S-layer of *L. acidophilus* was done by Bhowmik et al. (1985) and Johnson et al.(1987). The presence of a surface protein in this species has a particular importance because of its use in probiotic products. These authors confirmed that the presence of an S-layer was strain dependent. Chemical extraction suggested that H-bonds were important for S-layer attachment to the cell wall. The M_r (46000) and amino acid composition were slightly different from the previous results of Masuda and Kawata (1983). Moreover, this time, the S-layer of *L. acidophilus* was found to be glycosylated. The growth conditions (temperature, carbon source, atmosphere, and threonine and calcium supplementation) did not significantly influence the amount of S-layer synthesized. As shown by our own unpublished observation (Fig. 1), the lattice has an oblique symmetry with irregularities in some places. Surprisingly, freeze-drying of *L. acidophilus* whole cells induced a significant removal of the S-layer protein and should be done under the protective effect of glycerol (5%; v/v) (Ray and Johnson, 1986). The S-layer protein on whole cells was resistant to proteolytic enzymes with the exception of pronase. It was also sensitive to pepsin when isolated wall fragments were treated with these enzymes. In addition, the removal of the S-layer protein by pronase decreased the surface hydrophobicity to nearly zero (Fig. 2). From these last experiments the authors suggested a protective function for the S-layer as well as a role for binding the bacterium to the intestinal mucosa.

Several recent studies based on DNA-DNA hybridization revealed great genetic heterogeneity between strains that are presently designated as *L. acidophilus* (i.e., the six homology groups are A1 to A4, B1 and B2; Johnson et al., 1980). Since none of the strains in the B1 and B2 groups possess an S-layer protein, this feature could be used to differentiate them from the strains in the groups A1 to A4. All the strains in the A1 homology group showed a M_r = 46000 S-layer protein. The M_rs of the S-layer proteins in the groups A2, A3, A4 seem to be different from this. Future studies (e.g., N-terminal sequence, amino acid composition and antigenic specificity)

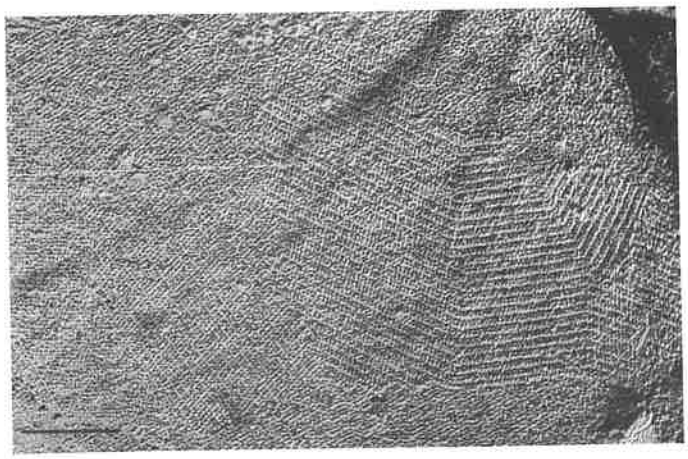

Figure 1. S-layer of *L. acidophilus* CNRZ 55 observed by freeze-etching. Bar = 100 nm. (unpublished data).

on the S-layer protein could offer a phenotypical way to differentiate the strains in the four homology groups (A1 to A4) without DNA homology data. This is important because "acidophilus preparations" for use as dietary adjuncts should contain bone fide *L. acidophilus* strains belonging to the A1 homology group (Johnson et al., 1987). Indeed, these strains have the desirable characteristics of rapid hydrolysis of lactose with a concomitant high lactic acid production.

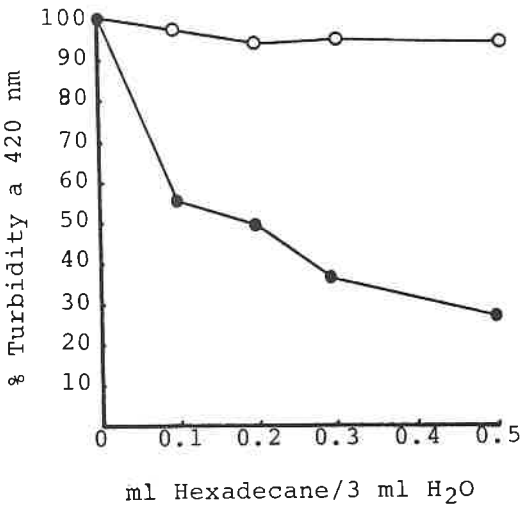

Figure 2. The hydrolysis of the S-layer protein of *L. acidophilus* by pronase drastically decreased the hydrophobicity of the cells. ●, percentage of untreated cells remaining in the aqueous phase ; ○, the same for cells digested with pronase. (From Bhowmik et al., 1985 with permission).

The S-layer of *L. helveticus* has recently been studied more extensively (Lortal et al., 1992). The oblique lattice has a center-to-center spacing of 4.5/9.6 nm. A new procedure using lithium chloride was shown to extract the S-layer protein from intact cells efficiently and selectively without a drastic loss of viability. Interestingly, the S-layer reappeared when treated cells were allowed to grow in new medium (Fig. 3). The reappearance of the S-layer subunits was random. Self-assembly products are formed on dialysis of the LiCl crude extract against distilled water. The amino acid composition, M_r (52000) and N-terminal sequence (ATTINADSAINANTNAKYDVDVT) of the purified protein were determined. This surface protein accounts for 14% of the total protein content of *L. helveticus* as estimated from previous work (Lortal et al., 1991). More recently we attempted to obtain the exact molecular weight by mass spectrometry. This was 43533 and will be confirmed in the near future.

The presence of a surface layer on *L. casei* is still uncertain. Barker and Thorne (1970) observed whole cells of *L. casei* ATCC 7469 by negative staining and described an outer layer which was "irregular, amorphous, and having hexagonal subunits". Nevertheless, Masuda and Kawata (1983) did not find a regular array on *L. casei subsp. casei* ATCC 393.

The case of *L. bulgaricus* is also doubtful. The presence of a surface layer was assumed in this species (Masuda and Kawata, 1983). However, we have recently assayed three strains of *L. bulgaricus* without finding any regular array.

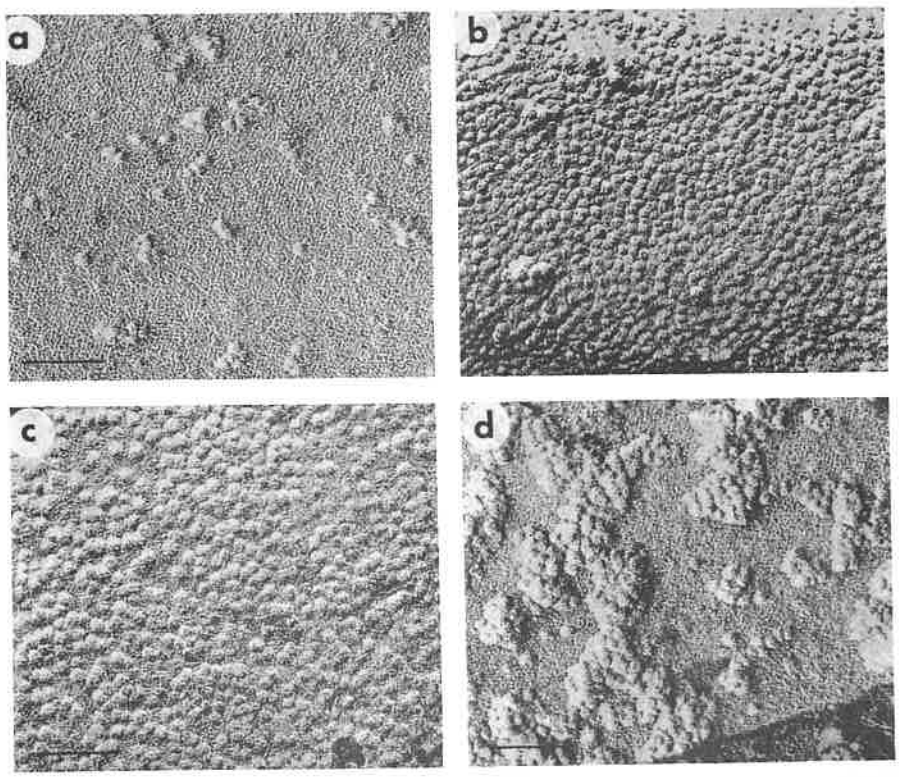

Figure 3. Freeze-dried preparations of *L. helveticus* labelled with polycationic ferritin (PCF), (a) untreated cells, (b) LiCl-treated cells, and (c) and (d) LiCl-treated cells after 8 h and 24 h in a new growth medium respectively. The reappearance of the S-layer is shown by the decrease in the number of bound PCF particles. Bar = 100 nm. (From Lortal et al., 1992).

REMAINING QUESTIONS AND PERSPECTIVES

Distribution of Surface Layers in the Genus *Lactobacillus*

Lactobacillus is a heterogeneous genus and the 50 species in the genus are not all useful for food technology, agriculture or human health. Only these more useful species have been surveyed for S-layers and it is impossible to accurately understand S-layer distribution throughout lactobacilli without a larger screening.

In the few biotechnologically significant species where S-layers have been described, as summarized previously in this chapter, the screening is only preliminary. Except for *L. acidophilus* and *L. helveticus* the number of strains tested is very limited. In addition several ambiguous cases like *L. casei* and *L. bulgaricus* have to be solved. Moreover, all the strains so far studied have come from national or international collections. Do S-layers exist under industrial conditions (e.g., those species used in food technology)? Are they present on the indigeneous strains of the human intestinal and urogenital tract? These questions have to be answered in order to determine the distribution and the stability of S-layers in this genus.

Ultrastructural and Biochemical Characteristics of *Lactobacillus* S-layer Proteins

Depending on the species, the S-layer lattice can have an oblique, square or hexagonal symmetry. The attachment of the S-layer to the neutral polysaccharide moiety of the cell wall in the case of *L. buchneri* has to be verified for other lactobacilli. The M_r of the S-layer proteins is between 41000-60000 and they appear to be non glycosylated (with the exception of *L. acidophilus*, which is still controversial, Table 2). In some cases the amino acid composition is available but there are no features which distinguishes them from other S-layer proteins. The N-terminal sequence plus the exact molecular weight has been determined for only one strain of *L. helveticus*. The accurate determination of the molecular weight by mass spectrometry could be an interesting way to see the variability of S-layer proteins between strains of the same species as well as providing precious information for further genetical approachs. Indeed, to our knowledge, total sequence analysis has never been attempted in lactobacilli, even though genetic systems have been developed (Chassy, 1987), and could be used to rapidly determine sequences or to obtain S^- mutants. The synthetic control of the S-layer proteins also remains unknown. The only observation shows that environmental conditions do not quantitatively influence this S-layer protein synthesis in *L. acidophilus*.

Surface Properties and Roles

Only two experiments can be reported in this part: (i) the S-layer of *L. helveticus* is not an effective barrier against the lytic action of the mutanolysin (Lortal et al., 1992) and (ii) the S-layer of *L. acidophilus* produces an apparent hydrophobic surface (Fig. 2).

Most of the lactobacilli described in this chapter are used in dairy processes. *L. helveticus* could be an interesting model to study the surface properties determined by the presence of an S-layer. Due to non-lethal extraction conditions using LiCl, the comparison of S^+ cells and S^- cells would be possible in this species. This may answer the following questions. Does the S-layer: (i) mask the underlying strong negatively charged peptidoglycan, (ii) protect the cells against the hydrolytic action of milk proteases and lytic enzymes, (iii) allow ionic interactions with milk proteins (caseins) which are then hydrolysed by the cell wall proteases (the proteolysis of milk is of

great technological importance), (iv) act as a bacteriophage receptor as suggested by M. Callegari (unpublished data) and, (v) play a role in the interactions between cells? For the latter, spontaneous aggregation or conjugation has previously been observed in *L. helveticus* (Thompson and Collins, 1989).

CONCLUSION

Clearly, basic knowledge about the distribution, stability, ultrastructure and biochemical character of the S-layer has to be extended in lactobacilli. The surface properties of S-layer carrying strains should first be studied (eventually by a comparison between S^+ and S^- strains derived by mutagenesis or mild S-layer extraction) in order to answer the most important question: Are S-layers essential for the various biotechnological applications of lactobacilli?

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