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Lactobacillus surface layers and their applications

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Abstract

Surface (S-) layers are crystalline arrays of proteinaceous subunits present as the outermost component of cell wall in several species of the genus *Lactobacillus*, as well as in many other bacteria and Archaea. Despite the high similarity of the amino acid composition of all known S-layer proteins, the overall sequence similarity is, however, surprisingly small even between the *Lactobacillus* S-layer proteins. In addition, the typical characteristics of *Lactobacillus* S-layer proteins, distinguishing them from other S-layer proteins, are small size and high-predicted p*I* value. Several lactobacilli possess multiple S-layer protein genes, which can be differentially or simultaneously expressed. To date, the characterized functions of *Lactobacillus* S-layers are involved in mediating adhesion to different host tissues. A few applications for the S-layer proteins of lactobacilli already exist, including their use as antigen delivery vehicles.

Keywords: S-layer; S-layer protein; S-layer gene; *Lactobacillus*; Adhesion

Contents

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1. Introduction

Surface layers (S-layers) are monomolecular crystalline arrays composed of protein or glycoprotein subunits with molecular masses ranging from 40 to 200 kDa [1,2]. S-layers have been identified as the outermost structure of cell envelope in numerous organisms from the domains Bacteria and Archaea [3,4]. S-layer proteins represent 10–15% of the total protein of the bacterial cell indicating efficient gene expression, S-layer protein synthesis and secretion [3,5]. The S-layer lattice can exhibit oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetry and is formed by an intrinsic self-assembly process [1,6]. S-layers are normally 5–15 nm thick possessing a smoother outer surface compared with a more structured inner surface [7]. Some organisms have two superimposed S-layers, which are composed of different subunit proteins [8,9]. Each S-layer forms a highly porous structure with pores of an identical size and morphology. The pores comprise up to 70% of the lattice surface area [2].

To date, two types of post-translational modifications of S-layer subunits have been identified. Glycosylated S-layer proteins have been characterized in Gram-positive bacteria and in Archaea [10]. The glycan chains are typically composed of two to six monosaccharides repeated up to 50 units consisting of a wide range of sugars and, in some cases, of non-carbohydrate substituents [10–12]. The sugar residues of S-layer glycoproteins are attached to the protein moiety via *O*-glycosidic or *N*-glycosidic linkages [12]. In addition to glycosylation, post-translational modification of S-layers involving phosphorylation of tyrosine residues has been reported [13].

The S-layer protein subunits are non-covalently linked to each other as well as to the supporting cell wall, and can be disintegrated into monomers by denaturing agents such as urea or guanidine hydrochloride, by metal-chelating agents or by cation substitution [1]. Isolated S-layer subunits can recrystallize into regular arrays on solid supports, liquid-surface interfaces, lipid films and liposomes or in suspension once the disrupting agent used for their isolation has been removed [3,6].

The current knowledge considering the biological functions of S-layers is relatively limited, compared to information that has accumulated on their ultrastructure, biosynthesis, molecular biology and genetics. Many of the functions described to S-layers are still hypothetical and need further experimental data to be revealed. S-layers have been considered to function as protective coats, cell shape determinants, molecule and ion traps, adhesion sites for exoenzymes as well as structures involved in cell adhesion and surface recognition (reviewed in [1,2]). In some pathogenic organisms such as *Campylobacter fetus* [14], *Aeromonas salmonicida* [15] and *Bacteroides forsythus* [16], S-layers have been shown to contribute to virulence. However, a general functional principle for all S-layers has not been determined and will likely not exist, due to the widespread occurrence of S-layer-possessing organisms and their differences in the overall cell surface structure. Due to their highly periodic and regularly arranged porous ultrastructure, S-layers possess great potential for various applications in biotechnology, nanotechnology, nanobiotechnology and biomedical applications [6,17,18].

Lactobacilli are members of the lactic acid bacteria, a phylogenetically diverse group of Gram-positive bacteria characterized by the formation of lactic acid as a sole or main end product of their sugar fermentation [19]. The genus *Lactobacillus* is the largest of the genera included in lactic acid bacteria with over hundred species recognized at present. The genus *Lactobacillus* is very heterogeneous containing species with substantial differences in their phenotypic, biochemical, physiological and genotypic characteristics. Members of the genus *Lactobacillus* are commonly found in nature and they are associated with a number of different habitats rich in carbohydrate or protein such as plants or spoiled food. Moreover, several *Lactobacillus* species are also members of the normal microbiota of human and animal gastrointestinal and genitourinary tracts [19,20]. The food and feed industry utilizes lactobacilli widely in the fermentation of vegetables, silage, sourdough bread, and several dairy and meat products, although some species can be associated with food spoilage [21]. Due to their long history of use in food fermentations and in the food industry as well as lack of pathogenicity, lactobacilli are generally recognized as safe (GRAS) organisms. Several lactobacilli are claimed to be health-benefiting to their host [22] and are therefore currently used as probiotic supplements in several products, intended for either human or animal consumption. Attention has recently also focused on the development of lactobacilli as live antigen delivery vehicles [23].

2. Structure and molecular characterization of *Lactobacillus* S-layers

2.1. S-layer protein genes of Lactobacillus

Many species of the genus *Lactobacillus* possess an S-layer. S-layer protein encoding genes have been cloned and sequenced from two *Lactobacillus brevis* strains [24,25], one *L. acidophilus* strain [26], one *L. helveticus* strain [27], and one *L. crispatus* strain [28]. Deposited in GeneBank (National Center for Biotechnology Information, Bethesda, MD, USA) are also several S-layer protein encoding gene sequences, which are either unpublished or have not been described in detail in publications. These sequences include five from *L. crispatus* (GenBank, accession numbers AF253043, AF253044,

AB110091, AB110090, AJ007839), ten from *L. helveticus* (GenBank, accession numbers AB061776, AB061775, AJ388561, AJ388560, AJ388559, AJ388558, X92752, AJ388564, AJ388563, AJ388562) and one from *L. acidophilus* (GenBank, accession number AF250229). Additionally, strains of *L. amylovorus*, *L. buchneri*, *L. gallinarum*, *L. kefir* and *L. parakefir* have also been shown to possess an S-layer [29–32], but their S-layer protein genes have not yet been sequenced. The study by Boot and colleagues [29] implicated the lack of S-layer protein encoding genes in strains of *L. gasseri* and *L. johnsonii*. In a later study by Ventura et al. [33], several different strains of these species were shown to possess two genes encoding surface proteins with the typical S-layer protein characteristics of lactobacilli. The presence of multiple S-layer protein genes seems to be quite common for lactobacilli, since also *L. brevis* ATCC 14869 has been shown to possess three S-layer protein genes [25] and *L. crispatus* JCM5810 [28] and *L. acidophilus* ATCC 4356 [34] two S-layer protein genes. Furthermore, several *Lactobacillus* isolates have also been described to contain two S-layer protein genes deduced by Southern blot analysis [29]. The presence of several genes coding for these proteins in one strain is not a unique feature of lactobacilli, since also certain other bacteria have several S-layer protein genes [35,36].

The genetic arrangement of the multiple S-layer protein genes in lactobacilli is strain-dependent, and no general consensus structure of their genetic organization can be deduced. In *L. brevis* ATCC 14869, two out of the three S-layer protein genes, *slpB* and *slpC*, are located adjacent to each other in parallel orientation whereas the third gene, *slpD*, is not closely linked to the *slpB–slpC* gene region [25]. In *L. acidophilus* ATCC 4356, the two S-layer protein genes, *slpA* and *slpB*, are in opposite orientation to each other and interspaced with a 3 kb DNA-region [37]. The putative S-layer protein genes *apf1* and *apf2* of *L. gasseri* and *L. johnsonii* are also separated by a short intergenic DNA-region but these genes are in parallel orientation [33]. The spacing and orientation of the *L. crispatus* S-layer protein genes, *cbsA* and *cbsB*, have not yet been deduced [28]. Based on the current data of the genetic organization of the *Lactobacillus* S-layer protein genes, they thus generally seem to be located in a rather close proximity to each other.

Sequence similarity between the S-layer protein genes can only be found between genes of related species [5]. This has also been demonstrated for the S-layer protein genes of lactobacilli by DNA–DNA hybridizations. The S-layer protein gene *slpA* of *L. brevis* ATCC 8287 has been shown to hybridize strongly to the chromosomal DNA of *L. buchneri* DSM 20057, indicating that *L. buchneri* contains homologous sequences to *slpA* (Palva et al., unpublished data). According to Southern blot analysis performed by Boot et al. [29], strains of *L. crispatus*, *L. amylovorus* and *L. gallinarum* possess two S-layer protein-encoding genes, which show in some regions high sequence identity to the *L. acidophilus slp* genes, other *slp* regions being less conserved. The dissimilarity of the S-layer protein genes of those lactobacilli, which are not closely related, is reflected to the lack of high sequence identities at the protein levels as well, as will be discussed in the Section 2.3.

2.2. *Properties of* Lactobacillus *S-layer proteins*

The S-layer proteins characterized from the genus *Lactobacillus* range from 25 to 71 kDa in size (Table 1), being among the smallest known for the S-layer proteins, which can be up to 200 kDa in size [1]. The S-layer proteins of lactobacilli are highly basic proteins with calculated p*I* values ranging from 9.35 to 10.4 (Table 1). In addition to the S-layer proteins of lactobacilli, only that of *Methanothermus fervidus* has a basic p*I* [43]. All the other S-layer proteins characterized are weakly acidic [1]. Although glycan structures have been identified from the S-layer proteins of several Gram-positive bacteria [11], most of those of lactobacilli appear to be non-glycosylated [31]. To date, a detailed glycan structure has been reported only from *L. buchneri* [44].

An N-terminal secretion signal (the signal peptide; SP), typical for the general Sec-pathway, is found in almost all prokaryotic S-layer proteins [1]. Until now, all characterized S-layer proteins of lactobacilli possess the SP consisting of 25–30 amino acids (Table 1). The length of the SP has most often been predicted by the rules of von Heijne [45]. Electrospray ionization mass spectroscopy of the SlpA protein of *L. acidophilus*, computed later for the exact determination of the molecular mass, revealed that actually the SP of this SlpA protein is cleaved six amino acid residues downstream of the predicted cleavage site [26,34]. The failure in the prediction of the SP of *L. acidophilus* was a result of the imperfection at that time of the prediction rules of Von Heijne, since current predictions can identify the correct cleavage site. In the *L. brevis* ATCC 8287 SlpA protein, the SP cleavage has been determined to take place at the predicted site [24,40]. Although the S-layer proteins have been generally assumed to be translocated via the general Sec II pathway, in a few cases also secretion components specific to S-layer proteins have been found [46,47]. For defining putative S-layer protein specific secretion components in lactobacilli/Gram-positive bacteria, extensive mutation analyses and sophisticated genetic tools would be required.

The amino acid composition of S-layer proteins in lactobacilli resembles in most parts the composition of other S-layer proteins, although some *Lactobacillus*-specific characteristics can be found. The S-layer proteins of lactobacilli have a high content of hydrophobic amino

Table 1
Characteristics of lactobacillar S-layer proteins

Species	Strain	Molecular mass (kDa)	Number of amino acid residues including the signal peptide	Length of the signal peptide in amino acids	Lattice symmetry[a], lattice constants *a* and *b* (nm)	Isoelectric point	Reference
L. acidophilus	ATCC 4356	SlpA: 43,6 SlpB: 44,9	SlpA: 444 SlpB: 456	SlpA: 30 SlpB: 30	P	SlpA: 10.4 SlpB: 10.3	[31,26,34]
	Seven strains	41–49	–	–	P	–	[38]
	M92	45	–	–	–	–	[39]
L. amylovorus	LMG 9496	45	–	–	–	–	[29]
L. brevis	ATCC 8287	46	465	30	O	9.88	[24,40]
	ATCC 14869	SlpB: 48 SlpC: 46 SlpD: 42	SlpB: 483 SlpC: 461 SlpD: 413	SlpB:30 SlpC: 30 SlpD: 30	O, *a* = 8.5, *b* = 5.0[b]; *a* = 8.4, *b* = 5.0[c]	SlpB: 9.99 SlpC: 10.12 SlpD: 10.08	[25]
L. buchneri	ATCC 4005	55	–	–	H	–	[32]
	YIT 0040	–	–	–	P	–	[31]
L. casei	ATCC 7469	–	–	–	H	–	[41]
L. crispatus	JCM 5810	CbsA: 43.9	CbsA: 440 CbsB: 452	CbsA: 30 CbsB: 23	P	– –	[28]
L. fermentum	NCIB 6991	–	–	–	P	–	[31]
	NCTC 7230	51.5	–	–	P	–	[31]
L. gallinarum	LMG 9435	45	–	–	–	–	[29]
L. gasseri	VPI 11759	Apf1: 29.8 Apf2: 29.8	Apf1: 302 Apf2: 297	Apf1: 25 Apf2: 25	–	Apf1: 9.98 Apf2: 9.98	[33]
	ATCC 19992	Apf1: 26.3 Apf2: 26.3	Apf1: 289 Apf2: 289	Apf1: 25 Apf2: 25	– –	Apf1: 9.35 Apf2: 9.35	[33]
L. helveticus	CNRZ 892	43.1	439	30	–	10.19	[27]
	ATCC 10797	51.5	–	–	P	–	[31]
	ATCC 12046	52	–	–	O, *a* = 4.5, *b* = 9.6	–	[42]
	ATCC 521	–	–	–	P	–	[31]
L. johnsonii	Five strains	Apf1:25.4–28.7 Apf2: 29.8–32.4	Apf1: 258–265 Apf2: 315–326	Apf1: 25–28 Apf2: 25	– –	Apf1: 9.35–9.72 Apf2: 9.60–9.80	[33]
L. kefir	20 isolates /strains	66–71	–	–	–	–	[30]
	CIDCA 8321	66	–	–	P	–	[30]
L. parakefir	Three isolates/strains	66–69	–	–	–	–	[30]

[a] O; oblique; H; hexagonal; P, periodic structure not further characterized.
[b] Rough colony morphology.
[c] Smooth colony morphology.

acid residues (Table 2), ranging from 31.9% to 38.7%, which is a typical feature for all S-layer proteins [7]. As observed for other S-layer proteins [48], also the *Lactobacillus* S-layer proteins have a low content of sulfur-containing amino acids, the maximum being 2% (Table 2) of which the majority is derived from methionine residues. To date, only one S-layer protein with a single cysteine residue has been found, i.e., the *L. gasseri* VPI 11759 Apf1 protein (Table 2). A characteristic feature for all S-layer proteins, including the ones derived from *Lactobacillus*, is a high percentage of amino acid residues with hydroxyl groups, the abundance being 23–33% in lactobacilli and over 15% in other bacteria [4,26,49] (Table 2). From the basic amino acids, the lysine content of S-layer proteins is usually relatively high, 10% [7], and this can also be noted for some of the *Lactobacillus* S-layer proteins (Table 2). The number of positively charged amino acid residues can be up to 12.5% and is always higher than the number of negatively charged residues in the S-layer proteins of lactobacilli (Table 2), leading to the high p*I* values calculated for these proteins (Table 1). The higher number of positively charged residues compared to the number of negatively charged residues is a *Lactobacillus*-specific characteristics since for most other S-layer proteins a low p*I* is typical.

Table 2
Amino acid composition of mature *Lactobacillus* S-layer proteins

Amino acid composition	Strain									
	L. acidophilus ATCC 4356/ SlpA	*L. acidophilus* ATCC 4356/ SlpB	*L. brevis* ATCC 8287/SlpA	*L. brevis* ATCC 14869/SlpB	*L. brevis* ATCC 14869/SlpC	*L. brevis* ATCC 14869/SlpD	*L. crispatus* JCM 5810/ CbsA	*L. crispatus* JCM 5810/ CbsB	*L. gasseri* VPI 11759/ Apf1	*L. gasseri* VPI 11759/ Apf2
Amino acids with hydroxyl groups (%)	25.9	25.1	33.8	25.2	32.3	24.8	23.2	24	25.7	22.7
Hydrophobic amino acids (%)[a]	36.4	35.9	32.4	34.6	33.8	36.8	36.8	33.7	32.9	32.6
Amino acids with sulfur (%)	0.2	0.5	0.9	0.2	1.6	1.8	2.0	1.2	1.1	0.7
Number of cysteine residues[b]	–	–	–	–	–	–	–	–	1	–
Lysine residues (%)	8.7	7.0	8.3	8.4	10.7	10.7	9.7	10.0	5.8	5.5
Arginine residues (%)	1.7	2.6	1.8	2	1.8	1.6	1.5	1.4	2.9	2.6
Negatively/Positively charged residues (%)	6.8/10.4	7.0/9.6	6.9/10.1	7.1/10.4	7.2/12.5	7.0/12.3	7.1/11.2	8.4/11.4	5.1/8.7	4.8/8.1
	L. gasseri ATCC 19992/ Apf1 and Apf2	*L. helveticus* CNRZ 892/ SlpH	*L. johnsonii* ATCC 332/ Apf1	*L. johnsonii* ATCC 332/ Apf2	*L. johnsonii* NCC 533 and DSM 20553/Apf1	*L. johnsonii* NCC 533 and DSM 20553/ Apf2	*L. johnsonii* ATCC 33200/ Apf1	*L. johnsonii* ATCC 33200/Apf2	*L. johnsonii* ATCC 11506/Apf1	*L. johnsonii* ATCC 11506/Apf2
Amino acids with hydroxyl groups (%)	26.4	25.6	24.6	22.8	27.8	22.6	27.8	24.0	28.3	23.2
Hydrophobic amino acids (%)[a]	31.9	35.9	35.4	37.4	33.0	38.7	33.6	34.9	32.5	36.7
Amino acids with sulfur (%)	0.8	0.7	0.9	0.7	0.8	0.9	0.9	0.7	0.8	0.7
Number of cysteine residues[b]	–	–	–	–	–	–	–	–	–	–
Lysine residues (%)	5.3	10.0	4.7	4.8	5.1	4.9	5.2	4.7	5.1	4.7
Arginine residues (%)	2.7	1.2	2.2	2.4	2.5	2.2	2.6	2.6	2.5	2.3
Negatively/positively charged residues (%)	4.9/8.0	8.8/11.2	6.0/6.9	4.5/7.2	5.9/7.6	4.0/7.1	6.1/7.8	5.5/7.3	5.9/7.6	4.3/7.0

[a] alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, valine.
[b] not detected.

Unambiguous identification of a S-layer to be a component of the bacterial cell wall/cell envelope structure relies on the use of electron microscopy. Ultrastructure of S-layer proteins has been studied by the following electron microscopy techniques: thin-sectioning, freeze-etching, negative-staining, shadow-casting and freeze-drying, the best methods being freeze-etching and negative staining [50]. The most frequently employed electron microscopy techniques for studying the S-layer proteins of lactobacilli are negative staining [28,31,38,40,51], thin-sectioning [25,33,52] and freeze-etching [25,42,52]. Immunogold labelling has been

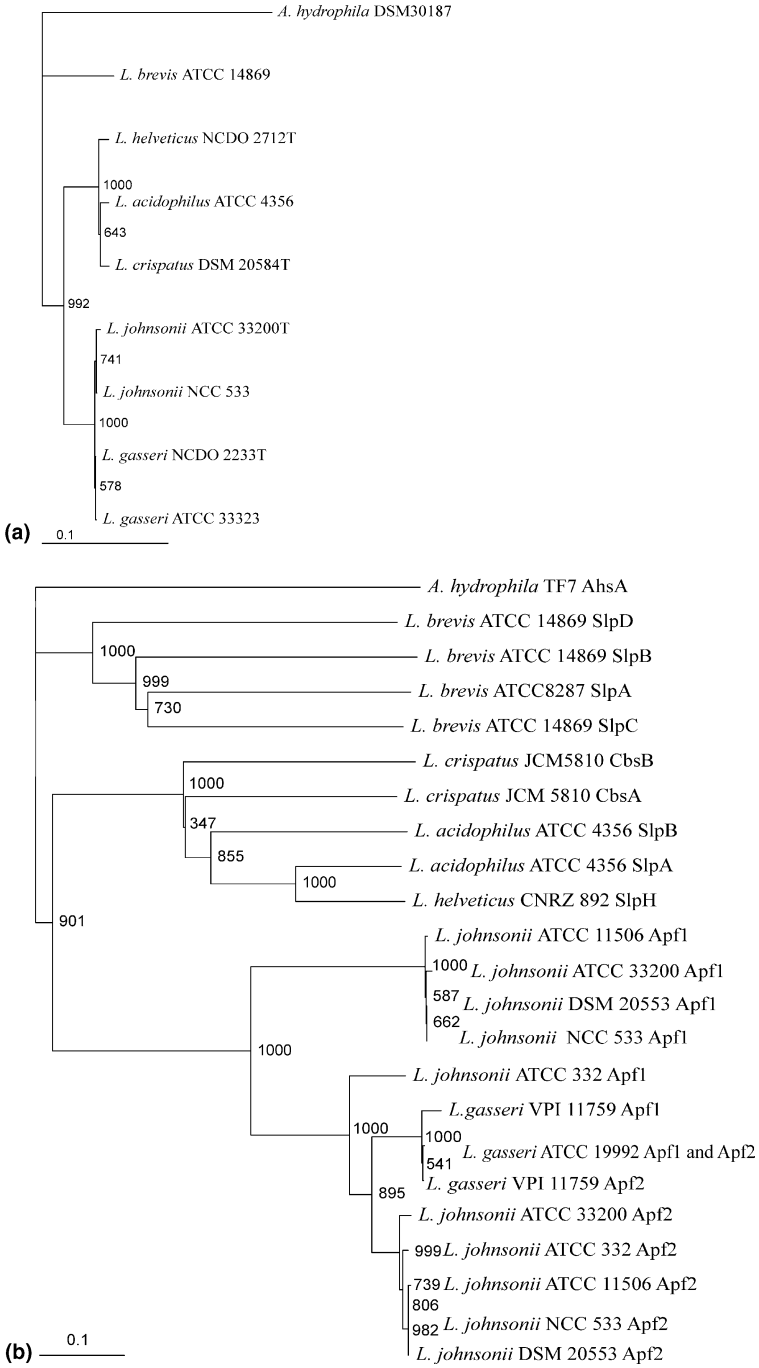

Fig. 1. Neighbour-joining phylogenetic trees obtained using the 16S rRNA genes (a) and the S-layer proteins (b) of lactobacilli. The scale bars indicate the phylogenetic distances. Bootstrap values are indicated at the nodes for a total of 1000 replicates. For the phylogenetic tree in (a) the 16S rRNA genes of lactobacilli and *A. hydrophila*, included as an outgroup, corresponding *E. coli* 16S rRNA numbering 107–1433 were used. Sequence alignments were performed using ClustalW [57]. Phylogenetic trees were constructed using the neighbour-joining program from the ClustalW (1.8) software package and the trees were visualized with the TreeView program.

applied to study the S-layer protein SlpA from *L. brevis* [24] and SlpH from *L. helveticus* [27]. From the *Lactobacillus* S-layer proteins, only oblique and hexagonal lattice types have been characterized but in most cases, only a periodic structure, without further characterization, has been observed using electron microscopy analysis (Table 1). Problems related to electron microscopy of *Lactobacillus* S-layers have been reported [40], and these problems may be one of the reasons why the lattice type and lattice constants have not been determined for most of the S-layer proteins of these bacteria.

Self-assembly process of S-layer protein subunits has been studied for the *L. acidophilus* ATCC 4356 SlpA and *L. crispatus* JCM 5810 CbsA proteins [28,53,54]. Despite high sequence variability of the N-terminal two thirds of the *L. acidophilus* SlpA and *L. crispatus* CbsA, in both of these S-layer proteins this region carries the information required for the self-assembly of the protein subunits into a regular S-layer array [28,53]. Smit et al. [53] named the crystallization domains of the *L. acidophilus* SlpA and *L. crispatus* CbsA as SAN. They suggested, on the basis of sequence comparisons that also in *L. helveticus* the crystallization domain is located in the corresponding region of the S-layer protein SlpH, although no experimental evidence for this yet exists. Further characterization of the *L. crispatus* CbsA by genetic truncation studies has shown that amino acids from 32 to 281 are essential for the self-assembly process [54]. A more detailed experimental data of the structural organization of SAN has been derived only from the *L. acidophilus* SlpA protein, suggesting that the SAN region consists of an N- and C-terminal subdomain interspaced by a surface exposed loop [51]. C-terminal truncation studies performed with the S-layer proteins of *Bacillus sphaericus* and *Geobacillus stearothermophilus* indicate that the outmost C-terminal amino acids do not participate in the self-assembly process [55,56]. However, based on the substantial dissimilarity of the amino acid sequences between the known S-layer proteins, the conclusions of the N-terminal crystallization domain of the *L. acidophilus* SlpA cannot be necessarily extended to other *Lactobacillus* S-layer proteins.

2.3. Phylogenetic analysis of the Lactobacillus S-layer proteins

We performed a phylogenetic analysis based on the S-layer protein sequences (including the signal peptides) of lactobacilli (Fig. 1(b)). A phylogenetic tree was also generated using the 16S ribosomal RNA-encoding gene sequences (rRNA) from the same set of *Lactobacillus* species that were used for the S-layer protein-based trees (Fig. 1(a)). When the 16S rRNA gene sequence was not available for a specific strain included in the S-layer protein or gene-based trees, the 16S rRNA gene sequence of a type strain for that species was used. The reliability of the trees generated was supported by bootstrap analysis. According to the trees obtained (Fig. 1(a) and (b)), the main clusters of the studied S-layer proteins are the same as obtained with the 16S rRNA based-tree. In the S-layer protein-based tree, the closely related strains with nearly identical 16S rRNA gene sequences, including *L. acidophilus* B group (*L. gasseri* and *L. johnsonii*) and *L. acidophilus* A group (*L. acidophilus* and *L. crispatus*) with *L. helveticus,* clearly branch separately. The 16S rRNA gene sequence of *L. brevis* clusters separately from the other lactobacilli included in the phylogenetic tree, and this separation can also be observed in the S-layer protein-based tree. Two main clusters for the Apf1 and Apf2 proteins can be observed in the phylogenetic trees, although some are located either in a mixed branch with both Apf1/Apf2 sequences or in a clearly separate branch as observed for the Apf1 of *L. johnsonii* ATCC 332.

The rates of evolutionary substitution in the 16S RNA genes have been somewhat lower compared to the level of substitution taken place in the S-layer proteins, since the phylogenetic distances of the nearly identical strains in the 16S RNA gene-based tree are more considerable in the S-layer protein-based trees. Notably the *L. crispatus* S-layer proteins branch separately from the *L. acidophilus* and *L. helveticus* S-layer proteins, although the 16S rRNA genes for these three species are nearly identical (Fig. 1(a) and (b)). In the S-layer proteins of the two *L. brevis* strains included in the phylogenetic analysis, a clear differentiation has occurred at the strain level, as can also be observed for the different S-layer proteins of the same *L. gasseri*/*L. johnsonii* strain. Thus, the phylogenetic analysis performed showed that the level of species/strain identity obtained by the 16S rRNA gene sequences largely determines the level of identity between the S-layer proteins of lactobacilli. Selection pressure of the *Lactobacillus* habitat could be one of the factors contributing to the more rapid differentiation of the S-layer proteins compared to the evolution of the 16S rRNA genes of these bacteria. The strains included in the phylogenetic analysis in Fig. 1 are of different origin, including human (*L. acidophilus* ATCC 4356), animal (*L. crispatus* JCM 5810), plant (*L. brevis* ATCC 8287) and a dairy product (*L. johnsonii* DSM 20553). To make a reliable conclusion about the role of a habitat of a *Lactobacillus* strain in the diversification of its S-layer protein(s), several S-layer proteins characterized from a single *Lactobacillus* species derived from different types of ecological niches would be needed. Thus, the S-layer proteins identified to date do not offer sufficiently data to make such an analysis.

2.4. Sequence comparisons and secondary structure analysis of Lactobacillus S-layer proteins

The primary structures of the *Lactobacillus* S-layer proteins show extensive variability, even between the S-layer proteins of the same strain. The number of identical amino acids varies from 7.2% to 100% between different S-layer proteins (Fig. 2). The highest identity can be found in *L. johnsonii* strains NCC 533 and DSM 20553, which possess two identical S-layer proteins, and in *L. gasseri* ATCC 19992 carrying the Apf1 and Apf2 proteins, which are also identical (Fig. 2). Furthermore, in *L. gasseri*, the two S-layer proteins are almost identical in the two strains included in Fig. 2, whereas in *L. brevis*, the S-layer proteins are relatively dissimilar (<40% identity) between the strains ATCC 8287 and ATCC 14869. The three different S-layer proteins of *L. brevis* ATCC 14869 are surprisingly different from each other (<36% identity). The identity between the two S-layer proteins in *L. acidophilus* and *L. crispatus* instead is clearly higher being 55.3% and 43.2%, respectively. The S-layer proteins of *L. gasseri* and *L. johnsonii* differ significantly (<17% identity) from the other *Lactobacillus* S-layer proteins listed in Table 2. The overall lack of identity between the S-layer proteins of different species is a common characteristic of other S-layer proteins [5]. Therefore, lactobacilli make no exception in this respect.

To locate the regions with putative high sequence similarities in the *Lactobacillus* S-layer proteins, known to be synthesized under the conditions studied, the S-layer protein sequences of *L. brevis*, *L. acidophilus*, *L. crispatus* and *L. helveticus* were aligned with each other (Fig. 3(a)). In accordance with sequence alignments reported earlier [28,53], the S-layer proteins of *L. acidophilus*, *L. crispatus* and *L. helveticus* show the highest similarity in the C-terminal region, whereas the N-terminal region of these S-layer proteins is more variable. However, the *L. acidophilus* and *L. helveticus* S-layer proteins share high similarity throughout their entire sequences (Fig. 3(a)). The signal sequences of *L. acidophilus*, *L. crispatus* and *L. helveticus* share a high level of sequence similarity (Fig. 3(a)). In contrast, in the different S-layer proteins of *L. brevis*, the highest similarity resides in the signal peptide and in the N-terminal parts of the mature proteins, whereas the C-terminal regions are rather divergent (Fig. 3(a)). The largest similarity region was found between the N-terminal parts of the *L. brevis* SlpA and SlpB (Fig. 3(a)). The sequence alignments clearly show the dissimilarity between the *L. brevis* SlpA protein in comparison to the *L. acidophilus*, *L. crispatus* and *L. helveticus* S-layer proteins, since in these alignments only few scattered regions of similarity can be found (Fig. 3(a)). Clear distinction between the conserved regions of the *L. brevis* Slp proteins compared

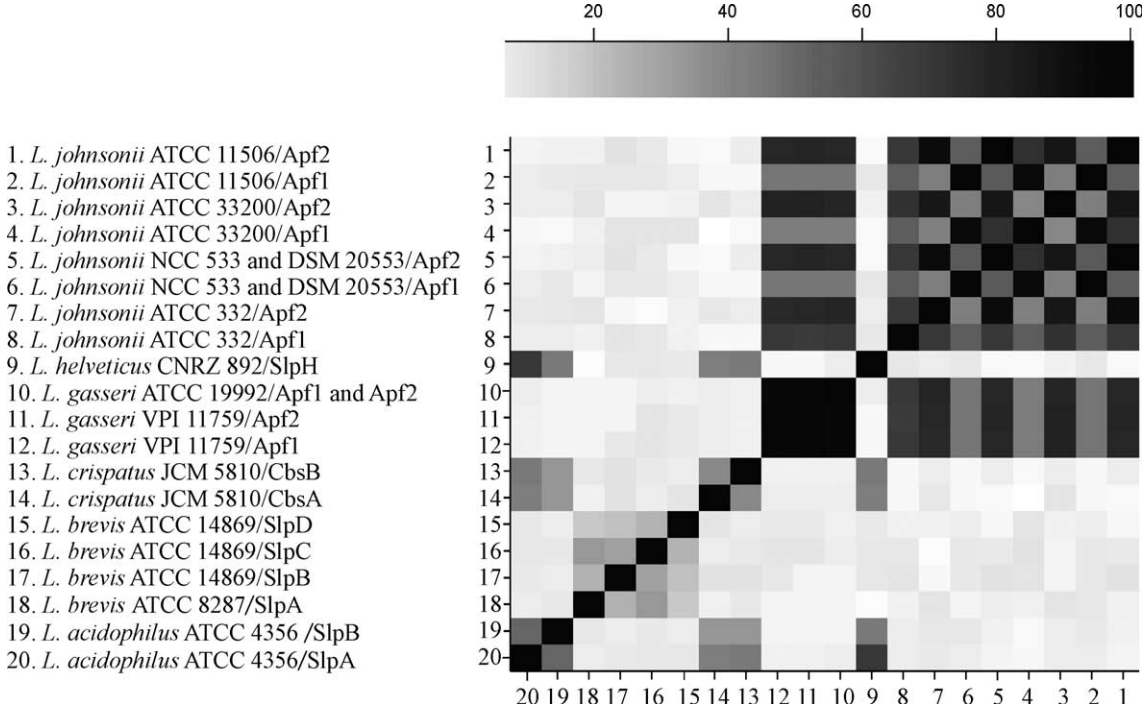

Fig. 2. Matrix comparison of homology levels between the deduced amino acid sequences of the *Lactobacillus* S-layer proteins in their unprocessed forms. Alignment studies were performed on the ExPASy server using the SIM program. The comparison matrix used in the alignments was PAM400 (gap open penalty, 12; gap extension penalty, 4). The % of identical amino acids between different proteins is indicated by the gray-color scale bar. The figure output was constructed using the IgorPro program (WaveMetrics).

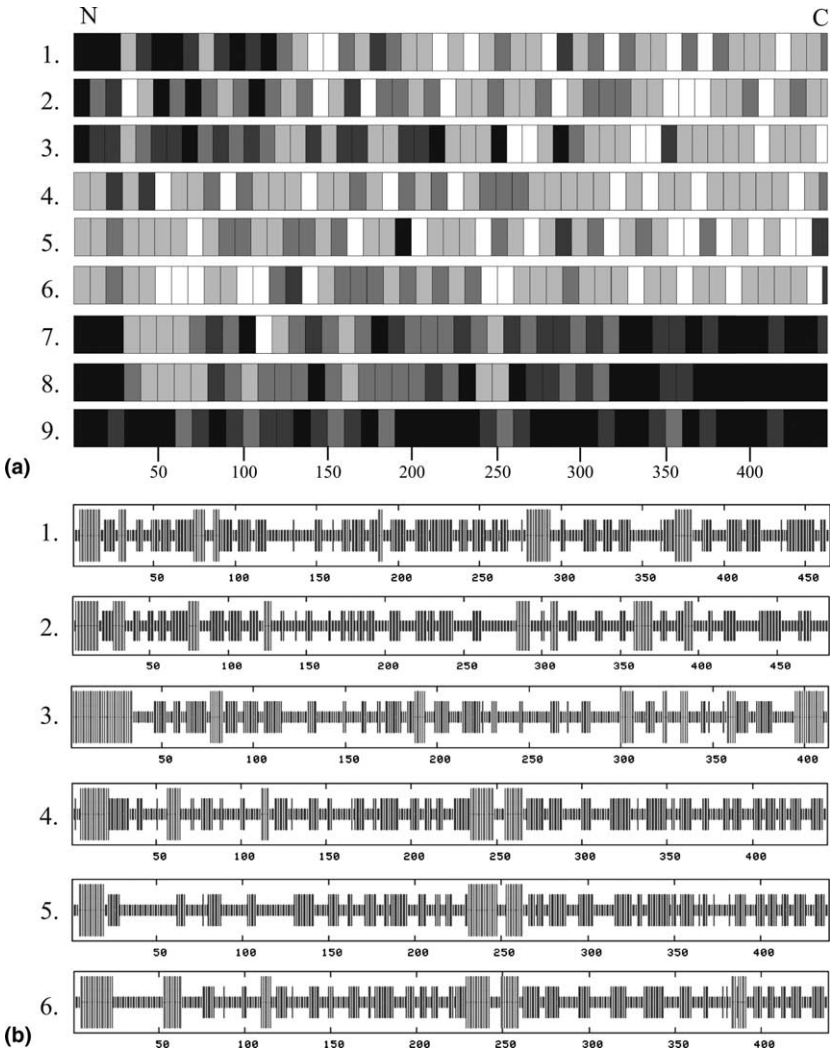

Fig. 3. Protein sequence alignments (a) and secondary structure predictions (b) of the S-layer proteins of lactobacilli. (a) The unprocessed S-layer proteins were aligned by ClustalW program (http://www.ebi.ac.uk/clustalw/#) and the alignments were divided into stretches of 10 amino acids, from which the percentage of identical amino acids and amino acids with conserved substitutions were calculated. The following colours are used to indicate the percentages of identical and similar amino acids in each calculated stretch: □, 0–20%; ▒, 21–40%; ▓, 41–60%; ▓, 61–80%; ■, 81–100%. Sequence alignments between the SlpA of *L. brevis* ATCC 8287 and the SlpB of *L. brevis* ATCC 14869 (1), the SlpA of *L. brevis* ATCC 8287 and the SlpD of *L. brevis* ATCC 14869 (2), the SlpB of *L. brevis* ATCC 14869 and the SlpD of *L. brevis* ATCC 14869 (3), the SlpA of *L. brevis* ATCC 8287 and the *L. acidophilus* ATCC 4356 SlpA (4), the SlpA of *L. brevis* ATCC 8287 and the SlpH of *L. helveticus* CNRZ 892 (5), the SlpA of *L. brevis* ATCC 8287 and the *L. crispatus* JCM 5810 CbsA (6), the *L. crispatus* JCM 5810 CbsA and *L. acidophilus* ATCC 4356 SlpA (7); the *L. crispatus* JCM 5810 CbsA and the SlpH of *L. helveticus* CNRZ 892 (8) and the *L. acidophilus* ATCC 4356 SlpA and the SlpH of *L. helveticus* (9) are shown. (b) Secondary structure prediction of the S-layer proteins SlpA of *L. brevis* ATCC 8287 (1), SlpB of *L. brevis* ATCC 14869 (2), SlpD of *L. brevis* ATCC 14869 (3), SlpA of *L. acidophilus* ATCC 4356 (4), CbsA of *L. crispatus* JCM 5810(5) and the SlpH of *L. helveticus* CNRZ 892 (6). The longest bars represent α-helices, the second longest bars represent extended strands and the shortest bars represent random coils. The secondary structure predictions were made using the PHD program [58].

to the S-layer proteins from the *L. acidophilus*, *L. crispatus* and *L. helveticus* group may suggest dissimilarity also in the location of the functional domains in these proteins.

According to secondary structure predictions, the S-layer proteins comprise an average of 40% α-helical regions and 20% β-sheet domains [4], with most of the α-helices located at the N-terminal part of the protein [1]. For the *Lactobacillus* S-layer proteins, only few secondary structure predictions have been computed [24,28,33]. Since the computer programs applied for these secondary structure analyses are not necessarily comparable, we performed secondary structure predictions of the unprocessed *L. brevis*, *L. acidophilus*, *L. crispatus* and *L. helveticus* S-layer proteins (Fig. 3(b)), which were also analysed earlier for their sequence similarities (Fig. 3(a)). According to these computed secondary structure predictions, the

L. brevis S-layer proteins are quite dissimilar when compared to each other and as well as to the *L. acidophilus*, *L. crispatus* and *L. helveticus* S-layer proteins. All the *L. brevis* S-layer proteins consist of alternating short stretches of α-helices, extended strands and random coils but the locations of these secondary structures are not directly comparable to each other, except for the α-helices at the signal sequences of these proteins. The direct repeats found in the *L. brevis* SlpA have been suggested to be associated with β-turn structures in an earlier analysis [24] but according to the results of the computed secondary structure prediction for this study, no β-turn structures can be found in the SlpA protein. A closer analysis of the location of the direct repeats of SlpA revealed that they are located mostly at the predicted extended strands and at random coils or at regions containing both of these structures (results not shown).

The secondary structures of the *L. acidophilus*, *L. crispatus* and *L. helveticus* S-layer proteins share a high degree of overall similarity (Fig. 3(b)). All of these proteins have a predicted α-helix region of 15–19 amino acids at their signal sequences as well as two α-helix regions located approximately in the middle of the unprocessed protein. In addition, the C-terminal region of the *L. helveticus* SlpH is predicted to have an α-helix structure (Fig. 3(b)/6). However, the prediction probability of this structure is substantially lower than that of the other α-helices, which may further support the high similarity of these three proteins. The most frequently occurring secondary structures in the *L. acidophilus*, *L. crispatus* and *L. helveticus* S-layer proteins are random coils (over 44% in each) and extended strand (over 34% in each) (Fig. 3(b)).

According to the limited data on the S-layer protein secondary structure predictions shown in Fig. 3(b), the *Lactobacillus* S-layer proteins seem to contain on average 14% of α-helices, 39% of extended strands and 47% of random coils. Because this data also includes the signal peptides, the amount of α-helices is higher than that of the mature proteins. These results differ from an earlier observation [4], which indicated that most S-layer proteins possess a high content of α-helices.

According to secondary structure prediction analyses by Ventura et al. [33], the Apf1 proteins of *L. gasseri* and *L. johnsonii* have been suggested to possess a high content of β-sheet (from 28.5% to 32.3%), whereas in the Apf2 proteins of these species, the β-sheet content was predicted to be somewhat lower (from 26.1% to 27%). These secondary structure prediction analyses further suggested that the *L. gasseri* and *L. johnsonii* S-layer proteins seem to form overall irregularly folded structures, with different secondary structures alternating [33], as can be observed also for the S-layer proteins included in Fig. 3(b).

3. Attachment of the S-layer protein to the underlying cell wall

In addition to peptidoglycan, the rigid cell wall of lactobacilli is composed of secondary cell wall polymers (SCWP) such as teichoic acid, lipoteichoic acids, lipoglycans or teichuronic acids [59,60]. Several Gram-positive bacteria possess at the N-terminal part of their S-layer proteins the so called S-layer homologous (SLH) motifs, which are responsible for anchoring the S-layer protein mostly to SCWP [61–65] and at least in one case directly to the peptidoglycan [66]. From the *Lactobacillus* S-layer proteins, no SLH motifs have been found [67], but the attachment of the S-layer protein to the cell wall seems to involve also SCWP in several lactobacilli.

The thus far characterized cell wall ligands, to which the *Lactobacillus* S-layer attach, include teichoic acids, lipoteichoic acids and neutral polysaccharides. The S-layer proteins from *L. brevis* and *L. buchneri* are reported to bind to a neutral polysaccharide moiety of the cell wall [32,68,69], but the location of the cell wall binding domain of these proteins is currently unknown. On the other hand, the location of the cell wall binding domain of the *L. acidophilus* ATCC 4356 SlpA and *L. crispatus* JCM 5810 CbsA has been determined to reside in the C-terminal one third of these proteins [53,54]. Sequence alignment studies, performed between the highly conserved C-terminal sequences of the S-layer proteins of *L. acidophilus*, *L. crispatus* and *L. helveticus*, revealed a putative carbohydrate-binding consensus sequence of repetitive nature in this region, which was then suggested to be involved in cell wall binding [53]. These putative carbohydrate-binding repeats comprise approximately the last 130 C-terminal amino acids, and in Fig. 3(a) this area can indeed be seen as a highly similar region in the sequence alignments between the S-layer proteins of these lactobacilli. The secondary structure predictions of the *L. acidophilus*, *L. crispatus* and *L. helveticus* S-layer proteins are also highly similar in the putative carbohydrate-binding repeats (Fig. 3(b)), consisting of short adjacent β-sheet and random coil regions. The study by Smit and Pouwels [70] showed that in the C-terminal cell wall binding domain (SAC) of the *L. acidophilus* SlpA, an N-terminal repeat, named SAC1, is most likely responsible for the anchoring of SlpA subunits to a cell wall teichoic acids. In *L. crispatus*, lipoteichoic acids and teichoic acids have been shown to be the cell wall ligands of CbsA [54]. Differences in the teichoic acids composition may explain the fact that when expressed in a *L. casei* strain without an S-layer, the CbsA protein of *L. crispatus* is not retained at the cell surface but is secreted into the

growth medium [71]. Due to the conserved nature of the C-terminal ends of the S-layer proteins of *L. acidophilus, L. crispatus* and *L. helveticus*, it is likely that also in *L. helveticus* the cell wall ligands of the S-layer protein are teichoic acids and/or lipoteichoic acids. However, this remains to be experimentally confirmed. A common cell wall binding domain for the S-layer proteins of *L. acidophilus*, *L. crispatus* and *L. helveticus* can be explained by close relatedness of these species both at the 16S rRNA gene level and the S-layer protein level (Figs. 1 and 3). This does not imply that other *Lactobacillus* S-layer proteins would also have C-terminal cell wall binding domains, instead of N-terminal domains, which also occur in other Gram-positive bacteria [72,73].

4. Expression and regulation of S-layer protein genes in lactobacilli

Efficient transcription of the S-layer protein genes is required, since the synthesis of up to 5×10^5 S-layer protein subunits is required during each cell generation. This high number of subunits has been calculated to be required for complete formation of an S-layer on an average-size cell [4]. Lactobacilli have indeed developed several species-characteristic systems in order to cope with the high expression levels required for their S-layer proteins.

A multiple-promoter structure has been described for several S-layer protein genes of lactobacilli. The S-layer protein genes of *L. brevis* ATCC 8287 and *L. acidophilus* ATCC 4356 are preceded by two promoter regions [24,74]. Of the S-layer genes of *L. brevis* ATCC 14869, the *slpB* gene is preceded by two putative promoter regions, whereas for the *slpC* and *slpD* genes only two putative −10 regions for a promoter have been identified [25]. The S-layer *apf1* gene of three *L. johnsonii* strains is also preceded by only two putative −10 regions for a promoter [33]. The multiple-promoter structure, preceding several *Lactobacillus* S-layer genes, is likely to be required for the efficient transcription of these genes. Of the two S-layer promoters in *L. acidophilus*, only the downstream one has been shown to be used to direct the synthesis of *slpA* transcripts during different phases of growth under the conditions tested [74]. Instead, in *L. brevis* ATCC 8287 both *slpA* promoters are active throughout the growth [75]. The level of transcripts derived from the downstream promoter of the *L. brevis slpA* was found to be approximately 10 times higher than that of the upper promoter during all phases of growth [75]. The use of promoters of other *Lactobacillus* S-layer protein genes has not been documented in the literature. However, the use of multiple promoters is likely to be strain and growth condition dependent.

Half-life of the transcripts encoding the S-layer proteins of *L. brevis* and *L. acidophilus* has been determined to be 14 and 15 min, respectively [74,75]. These half-lives can be considered to represent pronounced stability as compared to the typical half-life of 2–3 min of prokaryotic mRNAs [76]. A stable hairpin-like secondary structure, one of the structures known to protect mRNA from degradation [76], is predicted to be formed by a 191-nucleotide-long untranslated leader in the *slpA* mRNA of *L. acidophilus* [74]. A reporter gene analysis in *L. casei* in fact showed that truncation of this leader sequence results in a reduction in protein production. However, since this result is based only on the protein yield, it is difficult to distinguish between the roles of mRNA stability and conformation related frequency of the initiation of translation. A hairpin-like structure has also been predicted to be formed by the untranslated leader sequence of the *apf1* gene in the three *L. johnsonii* strains, but it is not currently known whether this hairpin-like structure has any effect on the half-life of *apf1* transcripts [33]. Instead, the transcripts derived from the main *L. brevis slpA* promoter do not contain any extensive leader sequences. It remains to be tested whether the long half-life of *slpA* mRNA is also dependent on the 5′-end sequence of these transcripts. The stability observed for the *Lactobacillus* S-layer protein gene transcripts is expected to contribute to the high level of translation required for this major extracellular component of the cell wall.

A biased codon usage is strongly correlated with the level of gene expression in *Lactobacillus* genes [77]. For the S-layer protein genes of lactobacilli, a biased codon usage has been observed in *L. brevis* [24] and in both S-layer genes of *L. gasseri* and *L. johnsonii* [33]. In *L. gasseri* and *L. johnsonii*, only seven triplets are used to encode over 50% of the Apf1 and Apf2 proteins [33]. The codon usage of *L. brevis slpA* has been reported to resemble that of highly expressed *B. subtilis* proteins [24] and in *L. acidophilus* ATCC 4356, the codon usages of the *slpA* and *slpB* genes are typical for highly expressed *Lactobacillus* genes [34]. The codon usage in the S-layer protein genes of *L. gasseri*, *L. johnsonii* and *L. brevis* shares some similarities: *L. brevis* uses predominantly the same codons for glutamine, threonine, tyrosine and glycine, which are uniquely used in the *apf1* and *apf2* genes of *L. gasseri* and *L. johnsonii* to encode these amino acids [24,33].

Several bacteria including *Campylobacter fetus* [78] and *G. stearothermophilus* [36] are capable of varying their S-layer proteins by a mechanism involving DNA rearrangements. The only case of S-layer variation detected thus far at the DNA level in lactobacilli is that of *L. acidophilus* ATCC 4356 [37]. In addition to the actively transcribed S-layer protein gene, *slpA L. acidophilus* has also the silent *slpB* gene [26,34]. The *slpA* and *slpB* genes are located on a 6-kb fragment in opposite orientation, and an inversion of this fragment relocates the silent *slpB* gene behind the *slpA* promoter [37]. This

inversion occurs most likely by a site-specific recombination at a 5′-identity region present in *slpA* and *slpB*, but the inducer for this change is unknown. Only a small minority (0.3%) of the chromosomes was found to carry the *slpB* gene in the active form under the growth conditions tested and therefore the synthesis of the SlpB protein could not be observed [37]. Variation of S-layer proteins as a response to environmental factors seems to be rather common in Gram-positive bacteria [79–81]. Hence, it is quite likely that some natural habitats of *L. acidophilus* may induce the formation of the SlpB type S-layer.

The S-layer protein gene expression of *L. brevis* ATCC 14869 takes place by a mechanism not yet described for the regulation of other S-layers. In the study by Jakava-Viljanen et al. [25], this *L. brevis* strain was shown to express the *slp* genes differently under aerated and anaerobic growth conditions. Under aerated conditions, both *slpB* and *slpD* specific transcripts were detected, whereas under anaerobic conditions only *slpB* specific transcripts could be observed. The *slpC* gene was found to be silent under the growth conditions tested. Northern blot analysis revealed that the *slpB* gene is equally expressed during all growth phases, whereas the *slpD* gene is predominantly expressed at the exponential phase of growth under aerated conditions [25]. Although the effect of aeration on the onset of *slpD* transcription was shown in the study, it could not be excluded that other concomitant changes (like stress responses), related to the change from anaerobic to aerated growth conditions, would not have an effect on the transcription of *slpD* [25]. In addition to *L. brevis*, oxygen content has also been shown to affect the S-layer protein gene expression of *G. stearothermophilus* [79,80].

The mechanism by which the *slp* gene expression of *L. brevis* ATCC 14869 is regulated is currently yet uncharacterized [25]. Northern analyses, expression of a PepI reporter protein under the control of the *slpD* promoter and quantitative real-time PCR analysis of the *slpD* gene under aerated and anaerobic conditions indicated that the variation of S-layer protein content in *L. brevis* ATCC 14869 occurs at the transcriptional level, involving activation of *slpD* transcription in the exponential growth phase by a soluble factor as a result of an environmental change rather than with DNA rearrangements. *L. brevis* ATCC 14869 was also observed to change its colony morphology under different growth conditions, producing smooth colonies under anaerobic conditions and preferentially rough colonies under aerobic conditions, this morphology being reversible upon changes in growth conditions. The single S-layer detected on the *L. brevis* cells of both colony types was identical by EM, indicating that the S-layer is not directly involved in the colony morphology, since a chimeric S-layer consisting of both SlpB and SlpD subunits is most likely formed under aerated conditions. Because the regulon responsible for the variation in *slp* gene expression has not been characterized, it cannot be currently stated whether the alteration in colony morphology is linked to the variation of *slp* gene expression taking place in these growth conditions [25].

In *L. crispatus*, the additional S-layer protein gene, *cbsB*, is not expressed under the thus far tested growth conditions [28]. In *L. johnsonii* and *L. gasseri*, both S-layer genes *apf1* and *apf2* are transcribed at all growth phases, although the maximum expression is reached during the exponential growth phase [33]. On the basis of currently available information on the expression of the multiple S-layer protein genes of lactobacilli, it seems that each *Lactobacillus* strain has developed its own unique S-layer protein gene expression system, perhaps as a response to the requirements set by their different and changing habitats.

5. *Lactobacillus* S-layer proteins as adhesins

The functional roles of *Lactobacillus* S-layers are yet poorly characterized; the only function described thus far being involvement in mediating adhesion to different host surfaces. The S-layer protein SlpA of *L. brevis* ATCC 8287 has been confirmed to perform as an adhesin mediating attachment to human intestinal epithelial cell lines and fibronectin [82], a component of the extracellular matrix. Binding of SlpA to erythrocytes of a wide variety of origins could not be demonstrated, indicating that the receptor structure of the *L. brevis* SlpA is not among the known and unknown receptors present on erythrocytes [82]. *L. brevis* ATCC 14869 adheres as well to human and pig intestinal epithelial cells (Jakava-Viljanen, M. and Palva, A., unpublished data) but it is not currently known whether the binding is mediated by the S-layer proteins in this *L. brevis* strain. The S-layer protein CbsA of *L. crispatus* mediates adhesion to collagens and laminin, which are components of the subintestinal extracellular matrix [28,54,83]. In addition to the performance of the CbsA adhesions using immobilized or solubilized extracellular matrix components of human or mouse origin, the CbsA binding assays were also carried out in vitro with chicken tissues, showing that this *L. crispatus* strain of chicken faeces origin can indeed adhere to collagen- and laminin containing regions in chicken intestinal tissues [28,54]. Collagen adhesions of the S-layer proteins encoded by the unexpressed *cbsB* genes of two *L. crispatus* strains have also been studied using CbsB proteins heterologously produced in *Escherichia coli*. In both cases, CbsB proteins were unable to adhere to collagen [28]. The S-layer structures in *L. brevis* and *L. crispatus* thus appear to be critical for the adhesion to intestinal surfaces.

The S-layer protein of *L. acidophilus* ATCC 4356 does not mediate binding to immobilized collagens

[28]. The S-layer protein of *L. acidophilus* isolated from a fowl has, nevertheless, been reported to mediate binding to avian intestinal epithelial cells [84]. The molecular characterization of this binding process is still lacking. A potential S-layer protein of *L. acidophilus* M92 has been proposed to mediate binding to porcine ileal epithelial cells, since removal of the S-layer proteins by LiCl extraction reduced significantly the adhesiveness of this strain to intestinal epithelium [39]. Regarding the binding experiment with negative controls based on the use of chemical extraction methods for removal of S-layer material, it should be taken in to account that also other cell wall components, critical to binding activity, may be simultaneously removed. Removal of the S-layer proteins with LiCl also reduced the autoaggregation ability of *L. acidophilus* M92 and *L. acidophilus* ATCC 4356, the latter of which was used as the control with a characterized S-layer protein [39]. Removal of the S-layer protein by LiCl treatment abolished also the autoaggregation capabilities of *L. kefir* and *L. parakefir*, but had no effect on the adhesion properties of these strains to a human colon adenocarcinoma cell line (Caco-2), suggesting that S-layer proteins are not involved in the Caco-2 cells adhesion of these lactobacilli [30]. In some studies, the auto- and co-aggregation abilities of Gram-positive bacteria have been linked to the adhesion properties of these bacteria [85,86]. However, according to Garrote et al. [30], the aggregation phenotype may not be directly linked with the adhesion process. Instead, autoaggregation may, e.g., promote genetic exchange in *Lactobacillus*, as has been suggested by Roos et al. [87].

Hydrophobicity of the cell surface, conveyed by S-layer to lactobacilli [88], may be a factor required for the adhesion to different surfaces. Vadillo-Rodríguez et al. [89] have shown that changes in the environmental ionic strength lead to variations in the cell surface hydrophobicity of *L. acidophilus* ATCC 4356, but not for two other *Lactobacillus* strains with S-layers. They suggest that in some lactobacilli, an adaptable mechanism lies behind the adhesion process, although not all lactobacilli with S-layers possess such a mechanism [89].

To date, the location of the adhesive domain has been determined from the S-layer proteins of *L. brevis* ATCC 8287 [82] and *L. crispatus* JCM 5810 [28,54]. In *L. brevis*, the receptor-binding region was found by in vitro flagellar display experiments in *E. coli*, showing that an N-terminal region of SlpA, comprising amino acid residues 96 through 176, mediates the adhesion to human epithelial cells [82]. Moreover, the binding to fibronectin was also shown to be mediated by an N-terminal region of SlpA, comprising amino acids 96 through 245 [82]. The collagen- and laminin-binding domain of the *L. crispatus* CbsA protein has been studied by genetic truncation analysis and heterologous expression in *E. coli* and in *L. casei* lacking an S-layer [28,54]. The results of these studies have shown that similar to *L. brevis*, in *L. crispatus* the collagen- and laminin-binding domain is located at the N-terminal part of CbsA, including approximately two thirds of the protein. The amino acids required for the adhesion are nearly the same as are needed for the self-assembly of the *L. crispatus* CbsA into a regular array, showing that a single domain can be multifunctional [54]. As already suggested by the different binding targets of these two S-layer proteins, the overall sequence similarity of these two receptor-binding domains is very low (Fig. 3(a)). This applies also for the secondary structure predictions shown in Fig. 2(b).

6. Application potential of S-layers

The unique features of S-layers, including their high degree of structural regularity and their self-assembly properties in several matrices as well as the substantial body of information already available on their structure, genetics, chemistry, assembly and physicochemical properties, make them as ideal structures with potential to offer a broad spectrum of applications in biotechnology, nanotechnology, nanobiotechnology and biomimetics [6,18]. A wide range of applications for S-layers already exists, including their use as matrices for immobilization of foreign molecules such as antibodies [90,91], allergens [92], oligosaccharide haptens [93] or biochemically and biomedically interesting proteins [94,95]. In addition, S-layers have also been utilized as matrices for the development of dipstick-style immunoassays [96], templates for the formation of regularly arranged metallic nanoparticles [97] or as isoporous ultrafiltration membranes [98]. Due to the intrinsic adjuvanticity of S-layers [99], as well as their capability to surface-display proteins and epitopes [100–102], S-layers have also potential to be used as antigen carriers. In fact, some encouraging results have already been obtained from immunization studies [99,103].

Currently the most extensively studied S-layer proteins of Gram-positive bacteria in an applicative respect are those of *G. stearothermophilus* and *B. sphaericus*. Most of the applications are based on fusions between the desired functional molecule with the S-layer proteins (or part of it) of *G. stearothermophilus* or *B. sphaericus*, the goal being that the specific intrinsic properties of the S-layer proteins are not affected by the fusion partner molecule, while the functional sequences are represented on the outermost surface of the S-layer lattice. The truncated forms of the *G. stearothermophilus* SbsA and SbsB S-layer proteins are capable of carrying amino acid insertions of up to 500 residues without any effect on their self-assembly capacity, when produced heterologously in *E. coli* [104]. Although the *B. sphaericus* S-layer protein, SbpA, has been characterized just recently [55], C-terminally truncated SbpA has already been employed in constructing numerous S-layer fusion proteins

[95,105,106]. These fusion proteins have been observed to recrystallize into a regularly structured lattice on cell wall fragments [55,91], on solid supports [91,95] or on the surfaces of liposomes [106], demonstrating that functional sequences remain available for binding reactions in these SbpA fusion protein constructs.

Some applications utilizing the *Lactobacillus* S-layer proteins and genes have already emerged during the last few years as the knowledge of S-layer proteins of these bacteria has accumulated. However, the applications described for *Lactobacillus* S-layers are not yet as versatile as those obtained for other Gram-positive bacteria, e.g., *G. stearothermophilus*, mainly due to the lack of appropriate molecular biology tools available for the species of this heterogenous group of bacteria. The very first applications of the *Lactobacillus* S-layer protein genes are based on the use of their promoters and/or the signal sequence for intra- or extracellular heterologous protein production in lactic acid bacteria. The promoters of the *L. brevis* ATCC 8287 and *L. acidophilus* ATCC 4356 *slpA* genes have been shown to function very well in intracellular protein production in various lactic acid bacteria [74,107], the functionality of the promoters, however, being dependent on the reporter protein and the host used. Moreover, the secretion signal of the *slpA* gene of *L. brevis* ATCC 8287 has been successfully applied to a number of heterologous secretion and export constructs [108,109].

Experimental approaches to apply the receptor binding domains of the *Lactobacillus* S-layer proteins have been demonstrated. Non-adhesive lactic acid bacteria can be turned into adhesive by heterologous surface expression of the S-layer protein or its receptor-binding region [71,110]. The anchoring of the *L. crispatus* CbsA protein to the cell wall of *L. casei*, in conjunction with the *L. casei* cell wall-sorting signal of proteinase PrtP, resulted in transformants being able to bind to immobilized collagens, albeit to a lesser degree than the wild type *L. crispatus*. The low amount of CbsA produced in *L. casei* did not allow the S-layer formation [71]. The receptor-binding region of the *L. brevis* SlpA has been surface displayed in *Lactococcus lactis*, a naturally non-adhesive bacterium, with a cassette encoding additionally a proteinase spacer and an autolysin anchor, rendering the lactococcal transformants able to bind to a human intestinal epithelial cell line Intestine 407 and also to immobilized fibronectin [110]. In this case, similar to the *L. crispatus* study by Martínez et al. [71], the binding capacity of the recombinant lactococci to the human Intestine 407 cells was not as high as that obtained with the wild type *L. brevis*. A result that is also likely to be explained by the lower level of expression of the receptor-binding region in *Lactococcus* compared to the high expression level of SlpA in wild type *L. brevis* [110]. Lactic acid bacteria with an adhesion capability could be utilized, e.g., in the development of mucosal antigen delivery vehicles or in targeting molecules/cells to a receptor of interest. The use of receptor-binding domains of lactobacillus origin may offer a safe alternative for targeted delivery of various effectors on mucosal membranes.

The current key approaches for applications of *Lactobacillus* S-layers are based on construction of live *Lactobacillus* strains carrying modified S-layer subunits. The S-layer proteins of *L. brevis* and *L. acidophilus* have been studied as possible antigen carriers using short heterologous model epitopes [40,51]. Unlike the thus far reported *B. sphaericus* and *G. stearothermophilus* S-layer fusion proteins, which are based on the use of heterologous host expression [94,95,111,105], the currently reported *Lactobacillus* S-layer epitope constructs have been expressed in the original hosts [40,51]. In the *L. brevis* SlpA protein, a poliovirus epitope VP1 and a c-Myc epitope from the human c-*myc* proto-oncogene, have been surface displayed [40,51]. One of the four insertion sites, allowing the best surface expression determined with the VP1 constructs, was used for the construction of an integration vector carrying the gene region encoding the c-Myc epitopes. As a result of a successful gene replacement, an *L. brevis* integrant was obtained, which displayed the c-Myc epitope in all of the S-layer protein subunits without any effect on the S-layer lattice structure [40]. Smit et al. [51] have studied the functional domains of the *L. acidophilus* SlpA with insertion mutagenesis, resulting in the surface expression of the c-Myc epitope in three insertion sites in the *slpA* gene in wild type *L. acidophilus* host. In this study, the ultrastructure of the c-Myc epitope integrant strains was not studied in the *L. acidophilus* host background [51]. The studies by Åvall-Jääskeläinen et al. [40] and Smit et al. [51] have thus shown that at least small epitopes can be successfully surface-displayed as part of the S-layer proteins of lactobacilli, and that an intact S-layer without any changes in the crystalline structure can be formed in spite of the presence of a heterologous epitope in every S-layer subunit [40].

The S-layer protein CbsA of *L. crispatus* has been studied as a possible anti-adhesion agent to prevent the adhesion of pathogens in the intestine [112]. Isolated CbsA proteins were shown to inhibit the adhesion of an enterotoxigenic *E. coli* strain to laminin and Matrigel, a reconstituted basement membrane preparation. The inhibition of adherence of *E. coli* strains to basement membrane could also be observed with intact *L. crispatus* cells and the removal of the S-layer from these *Lactobacillus* cells abolished this inhibitory effect, suggesting the role of the CbsA as the sole adhesin of *L. crispatus* to these matrices. The most presumable mechanism, by which the CbsA inhibits the adhesion of enterotoxigenic *E. coli* is competition for binding sites in the laminin molecule, since both strains were shown to bind to peptide residues in the laminin molecule [112].

The only nanotechnological application with a *Lactobacillus* S-layer to date is the study by Sampathkumar and Gilchrist [113], where the S-layer protein of *L. brevis* was utilized in the formation of bioconjugates. With amine-based coupling chemistry, small molecule probes and polymers were conjugated to the *L. brevis* S-layer and the self-assembly of these bioconjugates was studied by amine-terminated polystyrene beads as the assembly substrate. According to different microscopy analyses, these bioconjugate coated microspheres showed a homogenous distribution of the polymer conjugates. Moreover, it was also demonstrated using this method, that surfaces containing homogenous displays of mixed monolayers can be achieved [113].

7. Concluding remarks

S-layers are common cell surface structures in many *Lactobacillus* species and the primary structures of several lactobacillus S-layer proteins are currently known. Resulting from the heterogeneity of the *Lactobacillus* group, the gene cloning and expression tools are often required to be optimized for each *Lactobacillus* strain used. This has considerably hampered the research on S-layers of these bacteria.

Since only few *Lactobacillus* S-layer proteins have been studied in more detail with respect to their structural and functional domains, further characterization of these domains is needed to fully understand the self-assembly of monomers to crystalline arrays and the regions necessary for the cell wall binding. This data will further facilitate more extensive modifications of the *Lactobacillus* S-layers for various applications. A European Union funded project ''Nano arrayed systems based on self-assembling proteins'' (contract 13523) beginning in 2005 aims at in one part of the project to elucidate the self-assembly domains of S-layers, including one *Lactobacillus* S-layer protein.

The thus far characterized functions of the S-layer proteins of lactobacilli are involved in mediating adhesion to various extracellular matrix proteins and epithelial cells of both human and animal origin. For several *Lactobacillus* S-layers, like those of *L. helveticus, L. gasseri* and *L. kefir*, no specific functions have yet been described. When present, the S-layer is apparently essential for lactobacilli, since the S-layer protein genes, tested so far, have been recalcitrant for inactivation [28,40]. This suggests that in addition to the adhesive functions, also other cellular functions may be attributed to S-layer proteins of lactobacilli, which, however, remain to be elucidated.

Future perspectives on the research on *Lactobacillus* S-layers include their utilization in different biotechnological, nanotechnological and biomedical applications. Since the nanotechnological applications for *Lactobacillus* S-layers are currently more or less lacking, excluding the study by Sampathkumar and Gilchrist [113], further developments in this field are likely to proceed as the knowledge about the ultrastructure of *Lactobacillus* S-layer proteins increases. Molecular nanotechnology with S-layer proteins is the latest field of S-layer research and as the studies with other S-proteins have demonstrated, it offers numerous applications as well to the S-layers of lactobacilli, ranging from the use of S-layers as templates for the formation of inorganic nanocrystalline arrays to the use of S-layers as supports for functional lipid membranes.

One of the most interesting applications for the S-layer proteins of lactobacilli includes their use as live antigen delivery vehicles. By surface displaying epitopes/antigens as part of an S-layer, up to 5×10^5 epitope monomers can be obtained to surround a single *Lactobacillus* cell, making such lactobacilli very attractive candidates for mucosal vaccine delivery vehicles [40]. The application of intrinsic adjuvanticity possessing lactobacilli as hosts, in constructing these vaccine strains, may further lead to the stimulation of immune responses to the co-administered antigen, surface-displayed as a part of the S-layer. Further advantages in employing lactobacilli as surface-display vectors include the claimed health-promoting properties possessed by several strains [114], the GRAS status of lactobacilli [115] and the lack of lipopolysaccharides in the cell wall, which eliminates the risk of an endotoxic shock [116]. Although immunization studies with *Lactobacillus* S layer antigen constructs are yet lacking, several successful mucosal immunization studies have already been performed with *Lactobacillus* hosts, surface-displaying different antigens in non-S-layer protein based surface-display systems [117–119]. These results further encourage the development of *Lactobacillus* S-layer based antigen carriers.

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