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MINI-REVIEW

Lactobacillus surface layer proteins: structure, function and applications

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Abstract Bacterial surface (S) layers are the outermost proteinaceous cell envelope structures found on members of nearly all taxonomic groups of bacteria and *Archaea*. They are composed of numerous identical subunits forming a symmetric, porous, lattice-like layer that completely covers the cell surface. The subunits are held together and attached to cell wall carbohydrates by non-covalent interactions, and they spontaneously reassemble in vitro by an entropy-driven process. Due to the low amino acid sequence similarity among S-layer proteins in general, verification of the presence of an S-layer on the bacterial cell surface usually requires electron microscopy. In lactobacilli, S-layer proteins have been detected on many but not all species. *Lactobacillus* S-layer proteins differ from those of other bacteria in their smaller size and high predicted p*I*. The positive charge in *Lactobacillus* S-layer proteins is concentrated in the more conserved cell wall binding domain, which can be either N- or C-terminal depending on the species. The more variable domain is responsible for the self-assembly of the monomers to a periodic structure. The biological functions of *Lactobacillus* S-layer proteins are poorly understood, but in some species S-layer proteins mediate bacterial adherence to host cells or extracellular matrix proteins or have protective or enzymatic functions. *Lactobacillus* S-layer proteins show potential for use as antigen carriers in live oral vaccine design because of their adhesive and immunomodulatory properties and the general non-pathogenicity of the species.

Keywords Surface layer protein · S-layer protein · *Lactobacillus* · Adhesion

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Introduction

Bacterial surface (S) layers are proteinaceous cell envelope structures ubiquitously found in Gram-positive and Gram-negative bacterial species and in *Archaea* (Sára and Sleytr 2000). When present, they form the outermost layer of the cell, being occasionally covered only by capsules (Fouet et al. 1999). S-layers are composed of numerous identical (glyco)protein subunits, 40–200 kDa in molecular weight, which form a two-dimensional, regular and highly porous array with oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetry. The subunits are held together and attached to the underlying cell surface by non-covalent interactions and have an intrinsic, entropy-driven tendency to form regular structures either in solution or on a solid support in vitro. The subunit proteins are typically rich in acidic and hydrophobic amino acids but low in sulphur-containing amino acids and have a low predicted overall p*I* value (Sára and Sleytr 2000). S-layer protein genes are highly expressed. Several S-layer protein genes in the genome of a single strain have been described, but all of the genes are not necessarily expressed at the same time; silent genes, antigenic variation based on S-layer gene expression (reviewed by Boot and Pouwels 1996; Sára and Sleytr 2000; Thompson 2002), alternative expression of S-layer protein genes in or ex vivo (reviewed by Fouet 2009), sequential expression during growth (Mignot et al. 2004) and, rarely, superimposed S-layers (Stewart and Murray 1982; Cerquetti et al. 2000) or S-layers composed of two different S-layer proteins (Rothfuss et al. 2006; Fagan et al. 2009; Goh et al. 2009; Sekot et al. 2012) have been described. Due to the low overall sequence similarity among S-layer protein genes and the lack of a universal signature sequence, confirmation of the presence of an S-layer still relies largely on electron microscopy.

In recent decades, information about the biological functions of S-layer proteins has accumulated, but no common function for all S-layers has emerged. The functions

characterized thus far include, e.g., the determination or maintenance of cell shape (Mescher and Strominger 1976; Engelhardt 2007a) and functions as a molecular sieve (Sára and Sleytr 1987; Sára et al. 1990), as a binding site for large molecules (Kay et al. 1985; Phipps and Kay 1988; Matuschek et al. 1994; Egelseer et al. 1995, 1996; Peters et al. 1995), ions (Schultze-Lam et al. 1992; Pollmann et al. 2006; Klingl et al. 2011) or phages (Howard and Tipper 1973; Ishiguro et al. 1984; Fouet 2009) and as a mediator of bacterial adhesion (Doig et al. 1992; Toba et al. 1995; Noonan and Trust 1997; Hynönen et al. 2002; Buck et al. 2005; Sakakibara et al. 2007; Poppinga et al. 2012). In pathogenic bacteria, S-layers may contribute to virulence by several mechanisms, including adhesion, coaggregation (Shimotahira et al. 2013), antigenic variation (Thompson 2002; Spigaglia et al. 2011), protection from complement or from phagocytosis (Doig et al. 1992; Thompson 2002; Shimotahira et al. 2013) or modulation of T-cell or cytokine responses (Wang et al. 2000; Ausiello et al. 2006; Sekot et al. 2011; Settem et al. 2013). Further, S-layer proteins may protect the bacterial cell from various environmental factors such as mechanical and osmotic stresses (Engelhardt 2007a, b), antimicrobial peptides (de la Fuente-Núñez et al. 2012), radiation (Kotiranta et al. 1999), changes in environmental pH (Gilmour et al. 2000), bacteriophages (Howard and Tipper 1973), bacterial or eukaryotic microbial predators (Koval and Hynes 1991; Tarao et al. 2009) or bacteriolytic enzymes (Lortal et al. 1992). Some S-layer proteins have the potential to act as degradative enzymes (Calabi et al. 2001; Ahn et al. 2006; Prado Acosta et al. 2008), and the S-layer protein of a marine *Synechococcus* strain is involved in motility (Brahamsha 1996; McCarren et al. 2005).

Due to the self-assembly properties and the highly ordered, regular structure down to the nanometer scale, S-layers have a vast application potential in (nano)biotechnology. Applications of S-layers can be roughly divided into two groups. The first comprises applications utilizing (genetically engineered) S-layered bacterial cells, S-layer (fusion) proteins or only the expression and/or secretion signals of S-layer protein genes in various biological systems, including vaccine development, heterologous protein production and surface display. The second group utilizes isolated, usually recombinant, S-layer proteins for non-life (nano) technological applications (see the comprehensive recent reviews by Schuster et al. 2006; Sleytr et al. 2007; Schuster and Sleytr 2009; Ilk et al. 2011; Pum et al. 2013).

Lactic acid bacteria are Gram-positive, non-pathogenic micro-organisms characterized by the production of lactic acid as the main end-product of carbohydrate metabolism. Within lactic acid bacteria, the genus *Lactobacillus* forms a large, heterogeneous group consisting of non-sporulating, anaerobic or microaerophilic, catalase-negative, fermentative organisms with a low G+C content (32–53 %) and complex nutritional requirements. Lactobacilli have been isolated from various environments, including plants, foodstuffs, silage and sewage, and they have been found in the gastrointestinal and genital tracts of humans and animals, where they form part of the normal flora (Kandler and Weiss 1986; Axelsson 1998; Hayashi et al. 2005; Felis and Dellaglio 2007). Besides having a long history of use in food and feed fermentations, lactic acid bacteria have aroused interest owing to the health beneficial (probiotic) properties of some strains. They have proved promising also as potential vehicles for the delivery of therapeutic and prophylactic molecules, such as vaccine antigens in humans (Seegers 2002; Wells and Mercenier 2008).

Occurrence and general properties of *Lactobacillus* S-layer proteins

In the genus *Lactobacillus*, S-layers have been found in several but not all species. Biochemical or functional data have been published about the S-layer proteins of *Lactobacillus brevis, Lactobacillus buchneri*, *Lactobacillus helveticus* and *Lactobacillus hilgardii* and organisms of the former *Lactobacillus acidophilus* group (Johnson et al. 1980), including *L. acidophilus, Lactobacillus amylovorus, Lactobacillus crispatus* and *Lactobacillus gallinarum*. In addition, strains of *Lactobacillus amylolyticus*, *Lactobacillus gigeriorum, Lactobacillus kefiranofaciens*, *Lactobacillus pasteurii* and *Lactobacillus ultunensis* carry predicted S-layer protein genes in their completely or partially sequenced genomes (see Table 1). *Lactobacillus kefir* and *Lactobacillus parakefir* have been shown to possess an S-layer (Garrote et al. 2004), although the genes have not been sequenced. In earlier studies, S-layers have been demonstrated by electron microscopy on *Lactobacillus fermentum* and *Lactobacillus delbrueckii* subspecies *bulgaricus* (Kawata et al. 1974; Masuda and Kawata 1983), but species identification of these strains has subsequently been questioned (Boot et al. 1996c). Supporting this is the fact that no *L. fermentum* S-layer protein gene sequence can be found in public databases, and the whole genome sequencing of *L. delbrueckii* subspecies *bulgaricus* did not reveal any S-layer protein gene (Hao et al. 2011; Makarova et al. 2006); thus, at present these species can be considered as non-S-layer producers. Likewise, in an earlier study, a regular layer was observed on *Lactobacillus casei* (Barker and Thorne 1970), but according to Boot et al. (1996b), no S-layer protein-encoding gene is present in this species, and the isolate probably would now be reclassified to another species. Moreover, as the demonstration of S-layer proteins on the surface of *L. casei*, *Lactobacillus paracasei* subspecies *paracasei* and *Lactobacillus rhamnosus* by Zhang et al. (2010b) and Guo et al. (2012) is not yet confirmed, *L. casei* is also currently considered as a non-S-layer producer.

Table 1 *Lactobacillus* strains carrying S-layer protein genes with sequences in public databases

	Strain	Slp reference[a]	Genome	
			Status	Reference
L. acidophilus	ATCC 4356	Boot et al. (1995)		
	JCM 1038	AAF65561	–	
	Unspecified	AEW12794	–	
	NCFM	Buck et al. (2005)	Completed	Altermann et al. (2005)
	30SC	Annotation	Completed	Oh et al. (2011)
	ATCC 4796	Annotation	Ongoing	
L. amylolyticus	DSM 11664	Annotation	Ongoing	
L. amylovorus	GRL 1112	Jakava-Viljanen and Palva (2007)	Completed	Kant et al. (2011a)
	GRL 1118	Jakava-Viljanen and Palva (2007)	Completed	Kant et al. (2011b)
	GRL 1115	Jakava-Viljanen and Palva (2007)	Ongoing	
	DSM 16698	Palva et al. unpublished	Ongoing	
L. brevis	ATCC 8287	Vidgren et al. (1992)	Ongoing	
	ATCC 14869	Jakava-Viljanen et al. (2002)	–	
	KB290	BAK78870	–	
	M8	AFD33419	–	
	ATCC 367	Åvall-Jääskeläinen et al. (2008)	Completed	Makarova et al. (2006)
	DSM 20054 ABBC45	Annotation	Ongoing	
L. brevis ssp *gravesensis*	ATCC 27305	Annotation	Ongoing	
L. buchneri	CD034	Annotation	Completed	Heinl et al. (2012)
	ATCC 11577	Annotation	Ongoing	
L. crispatus	JCM 5810	Sillanpää et al. (2000)	–	
	LMG 12003	Sillanpää et al. (2000)	–	
	F5.7	Mota et al. (2006)	–	
	ZJ001	Chen et al. (2009)	–	
	K2-4-3	Hu et al. (2011)	–	
	K313	Sun et al. (2012)	–	
	M247	AJ007839	–	
	MH315	AB110090, AB11091	–	
	Unspecified	AAY41912	–	
	Unspecified	AAY41916	–	
	ST1	Hurmalainen et al. 2007	Completed	Ojala et al. (2010)
	125-2-CHN CTV-05 MV-1A-US MV-3A-US 214–1 JV-V01	Annotation	Ongoing	
L. gallinarum	D109, D195-2, D256, D109, D255, ATCC 33199, D42 D44-2	Hagen et al. (2005)	–	
	DSMZ 10532	AAS83409	–	
L. gigeriorum	CRBIP 24.85	Annotation	Ongoing	
L. helveticus /suntoryeus	CNRZ 892	Callegari et al. (1998)	–	
	CNRZ 1269	CAA63409	–	
	ATCC 12046	Lortal et al. (1992) CAB46984	–	
	GCL 1001[b]	BAB72065	–	
	JCM 1003[b]	BAB72066	–	
	JCM 1007	BAB72067	–	
	JCM 1008	BAB72068	–	
	IMPC M696 CNRZ 35 IMPC HLM1 IMPC I60 CNRZ 303 ATCC 15009	Ventura et al. (2000)	–	
	K1/R0052	ADK74769	–	
	Y10 M4	Cachat and Priest (2005)	–	

Table 1 (continued)

	Strain	Slp reference[a]	Genome	
			Status	Reference
	Slh02	AGD98690	–	
	MIMLh5	Taverniti et al. (2012)	–	
	Unspecified	AAL36968	–	
	DPC4571	Annotation	Completed	Callanan et al. (2008)
	H10	Annotation	Completed	Zhao et al. (2011)
	MTCC5463	Annotation	Completed	Prajapati et al. (2011)
	R0052	Annotation	Completed	Tompkins et al. (2012)
	DSM20075	Annotation	Ongoing	
L. hilgardii	B706	Dohm et al. (2011)	–	
	ATCC 8290	Annotation	Completed	–
L. kefiranofaciens	ZW3	Annotation	Completed	Wang et al. (2011)
L. pasteurii	CRBIP 24.76	Annotation	Ongoing	
L. ultunensis	DSM 16047	Annotation	Ongoing	

[a] GenBank reference number is indicated if no publication about the S-layer protein is available. Annotation; identification based on genomic annotation only

[b] Proteinase (PrtY)-like (Gatti et al. 2005; Yamamoto et al. 2000); see text

All of the *Lactobacillus* S-layer proteins characterized thus far are preceded by a 25–32-amino-acid signal peptide, indicating secretion through the general secretory pathway. The deduced amino acid sequences of mature *Lactobacillus* S-layer proteins vary considerably (Åvall-Jääskeläinen and Palva 2005), and even the S-layer proteins of the same strain, when present, may be markedly different in sequence (Jakava-Viljanen et al. 2002; Hagen et al. 2005). As in the case of S-layers in general, a remarkable similarity between the deduced amino acid sequences can only be found between related species, e.g. between the S-layer proteins of the former *L. acidophilus* group organisms and some *L. helveticus* strains (Antikainen et al. 2002; Hagen et al. 2005). Methods for the identification of *L. crispatus* (Horie et al. 2002b) and *L. helveticus* (Ventura et al. 2000) based on the presence of the S-layer protein genes have been developed, but the applicability for the latter species has subsequently been questioned due to the observed heterogeneity within *L. helveticus* S-layer protein gene sequences. Moreover, the relationship between *L. helveticus* cell surface proteinase and surface layer proteins is currently not clear (Gatti et al. 2005). The comparison of phylogenetic trees based on 22 deduced *Lactobacillus* S-layer protein (or the S-layer-like Apf protein) sequences and the 16S rRNA gene sequences of the corresponding *Lactobacillus* species revealed a similar overall clustering of strains (Åvall-Jääskeläinen and Palva 2005). However, when the phylogenetic trees constructed on the basis of the S-layer protein genes of a set of *L. acidophilus*-related organisms, including strains of the novel *Lactobacillus suntoryeus* species [later reclassified as *L. helveticus* (Naser et al. 2006)], were compared with trees constructed on the basis of 16S rRNA or elongation factor Tu (*tuf*) gene sequences of the same species, the novel strains grouped together in the latter tree, but not in the former tree based on the S-layer protein genes. This indicates a strong selective pressure driving the diversification of S-layer protein genes within at least some *L. acidophilus*-related organisms as well (Cachat and Priest 2005). Nevertheless, the remarkable similarities on the amino acid level between the S-layer proteins of *L. acidophilus*-related organisms have led to the proposal of using LC-MS/MS analysis of S-layer proteins for typing strains within this group (Podlesny et al. 2011). A phylogenetic tree based on those *Lactobacillus* S-layer protein sequences for which gene expression data are currently available is shown in Fig. 1. It clearly separates the S-layer proteins of *L. brevis* and *L. hilgardii* from the S-layer proteins of *L. helveticus* and the *L. acidophilus* group organisms but indicates great variability within the S-layer proteins of *L. acidophilus* group as, e.g., the S-layer proteins of different strains of *L. gallinarum* may be very distantly related (see, e.g., LgsF of *L. gallinarum* D109).

The S-layer protein SlpA of *L. acidophilus* NCFM is identical in sequence with SA of *L. acidophilus* ATCC 4356, although the strains are clearly distinquishable by pulse field gel electrophoresis of chromosomal DNA (Sanders and Klaenhammer 2001). *L. acidophilus* NCFM harbours a larger diversity of mobile genetic elements than other probiotic lactic acid bacteria (Altermann et al. 2005). Although the elements do not show similarity to currently known integrative and conjugative elements (Wozniak and Waldor 2010), the possibility of horizontal gene transfer in

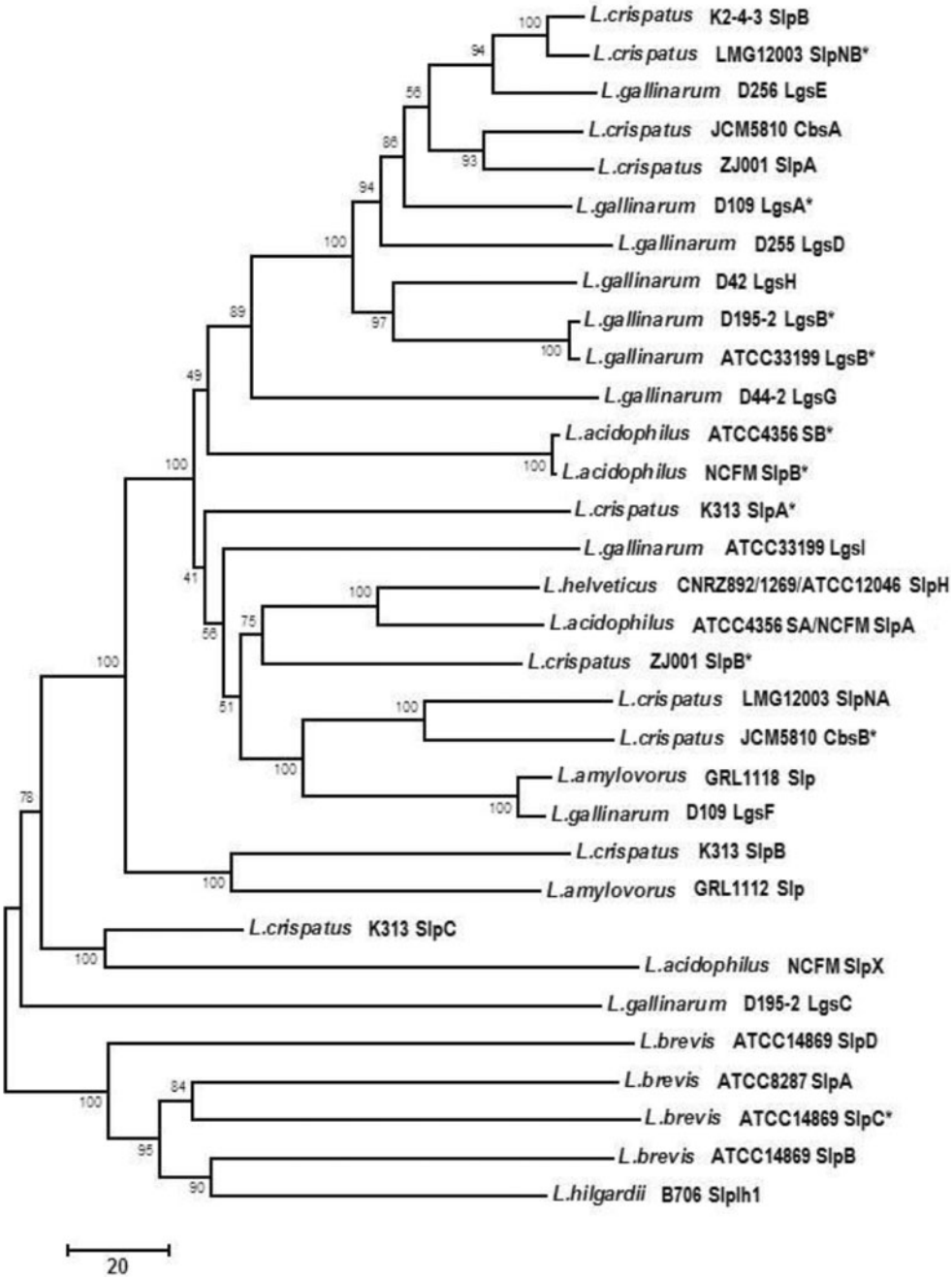

Fig. 1 A neighbour-joining phylogenetic tree based on *Lactobacillus* S-layer protein sequences for which gene expression data are currently available. The *scale bar* indicates the phylogenetic distances expressed as the number of amino acid substitutions per sequence. Bootstrap values are indicated at the nodes for 500 replicates. The partial S-layer protein sequence of *L. helveticus* MIMLh5 was excluded from the analysis. *Asterisk* corresponding gene is known to be silent

the acquisition of *slpA* gene in *L. acidophilus* cannot be ruled out. A more likely explanation for the presence of an identical *slp* gene in two genetically different strains is, however, that the strains have a common origin as their genomic organization is predominantly the same (Sanders and Klaenhammer 2001).

S-layer proteins of lactobacilli differ from S-layer proteins in general in their smaller size (25–71 kDa) and high predicted overall p*I* value (9.4–10.4). The lattice symmetry of *Lactobacillus* S-layer proteins, when known, is of oblique or hexagonal type (reviewed by Åvall-Jääskeläinen and Palva 2005). A glycan structure of a *Lactobacillus* S-layer protein has to date been determined only for *L. buchneri* (Messner et al. 2008), whereas glycosylated S-layer proteins have been described in *L. kefir* (Mobili et al. 2009a). Secondary structure predictions for S-layer proteins are of limited value thus far as the prediction algorithms are based on the available structures of very dissimilar types of proteins. A prediction performed for the amino acid sequences of the unprocessed forms of six *Lactobacillus* S-layer proteins suggested on average 14 % α-helices, 39 % extended strands and 47 % random coils in these proteins (Åvall-Jääskeläinen and Palva 2005). Physical measurements revealing secondary structures have been conducted for a few *Lactobacillus* species. A Fourier

transform infrared spectroscopy study performed for the S-layer proteins of *L. kefir* and *L. brevis* indicated α-helix contents of 0–21 %, β-sheet contents of 23–50 % and other structure contents, including β-turns and random coils, of 37–63 % in these proteins. For example, the proportions of α-helix, β-sheet, and other structures in SlpA of *L. brevis* ATCC 8287 were 0, 50 and 50 %, respectively (Mobili et al. 2009b). Atomic force microscopy studies of the S-layer protein CbsA of *L. crispatus* and its N- and C-terminal fragments suggested the presence of at least four α-helical structures of variable sizes, rather than β-sheets, in the N-terminal part of CbsA (Verbelen et al. 2007). Elucidation of the tertiary structure of S-layer proteins has been hindered by their molecular weights mostly not being in the suitable range (<40 kDa) for nuclear magnetic resonance studies and by their low solubility, i.e., their tendency to form two-dimensional lattices rather than three-dimensional crystals in solution. Consequently, no three-dimensional structures of *Lactobacillus* S-layer proteins at atomic resolution are currently available.

In addition to the verified S-layer proteins presented earlier, surface proteins described as "S-layer like" have been identified on the surface of several *Lactobacillus gasseri* and *Lactobacillus johnsonii* strains. These aggregation promoting factor (Apf) proteins share several characteristics with *Lactobacillus* S-layer proteins, such as their relative abundance on the cell surface, extractability by lithium chloride (LiCl), amino acid composition, predicted physical properties like high p*I* and indispensability for growth (Ventura et al. 2002). The Apf proteins of *L. gasseri* 4B2 are critical for cell shape maintenance, but they are not the sole components responsible for the aggregative phenotype (Jankovic et al. 2003). Despite the similarities with surface layer proteins, the S-layer nature of Apf proteins has not yet been fully confirmed. Each *L. gasseri* and *L. johnsonii* strain carries two *apf* genes in tandem orientation, both of which are expressed at the same time as monocistronic units (Ventura et al. 2002; Jankovic et al. 2003), a feature not very common among *Lactobacillus slp* genes (see "Expression of *Lactobacillus* S-layer protein genes). A three-layered cell envelope structure similar to that observed on S-layered *Lactobacillus* strains and modifiable by LiCl has been observed in thin-section samples of *L. johnsonii* cells by electron microscopy (Ventura et al. 2002). However, no micrographs obtained by freeze-fracturing techniques or by other methods revealing the symmetric organization of the proteins have been published, and no reports are available about the recrystallization of the proteins in vitro. Proteins designated as Apf, with C-terminal similarity to Apf proteins of *L. gasseri* and *L. johnsonii*, have subsequently been described for several *Lactobacillus* and other bacterial species (Goh and Klaenhammer 2010), like *L. crispatus* M247 (Marcotte et al. 2004) and *L. acidophilus* NCFM (Goh and Klaenhammer 2010). *L. acidophilus* NCFM has also a true S-layer (Konstantinov et al. 2008), and *L. crispatus* M247 at least carries an S-layer gene (Table 1). Thus, based on the current data, the designation of *L. gasseri* and *L. johnsonii* Apf proteins as belonging to an S-layer like family (Ventura et al. 2002), but not as true S-layer proteins, is justifiable.

Expression of *Lactobacillus* S-layer protein genes

The very efficient synthesis of S-layer proteins in lactobacilli is achieved by several means: (1) The half lives of the S-layer protein gene transcripts have been determined to be exceptionally long (14 and 15 min for the S-layer protein genes of *L. brevis* ATCC 8287 and *L. acidophilus* ATCC 4356, respectively) (Boot et al. 1996b; Kahala et al. 1997). At least in the case of *L. acidophilus* ATCC 4356 (Narita et al. 2006) and *L. crispatus* K313 (Sun et al. 2012), this is due to the long 5′ untranslated region (UTR) of the transcript forming a stabilizing secondary structure, as originally suggested by Boot et al. (1996b). However, the 5′ UTR of *L. brevis* ATCC 8287 *slpA* transcript is not exceptionally long (Vidgren et al. 1992); (2) A biased codon usage, correlating with efficient gene expression in lactobacilli (Pouwels and Leunissen 1994), has been observed for the S-layer protein genes of *L. brevis* ATCC 8287 (Vidgren et al. 1992) and *L. acidophilus* ATCC 4356 (Boot et al. 1995); (3) The promoters of S-layer protein genes are efficient, even to the extent that they have been used in heterologous expression and protein production systems (see "Applications of *Lactobacillus* S-layer proteins); (4) The presence of two promoters, which may act to enhance and/or regulate gene expression, has been identified upstream of the *slpA* gene of *L. brevis* ATCC 8287 (Vidgren et al. 1992; Hynönen et al. 2010), *slpB* of *L. brevis* ATCC 14869 (Jakava-Viljanen et al. 2002) and *slpA* of *L. acidophilus* ATCC 4356 (Boot et al. 1996b). In *L. brevis* ATCC 8287, both *slp* promoters were shown to be active (Kahala et al. 1997; Hynönen et al. 2010), whereas in *L. acidophilus* ATCC 4356, only the downstream promoter is functional under the conditions tested (Boot et al. 1996b).

The presence of multiple S-layer protein genes in the same strain is common in lactobacilli (Boot et al. 1996c; Hagen et al. 2005), but so far only the S-layer protein genes *slpB* and *slpD* of *L. brevis* ATCC 14869 (Jakava-Viljanen et al. 2002), *slpB* and *slpC* of *L. crispatus* K313 (Sun et al. 2012) and *slpA* and *slpX* of *L. acidophilus* NCFM (or *slpB* and *slpX* of the *slpA* knock-out mutant of *L. acidophilus* NCFM) (Goh et al. 2009) have been shown to be expressed simultaneously. Thus, silent S-layer protein genes, under the conditions tested, are common and represented, e.g., by the *slpB* genes of *L. acidophilus* ATCC 4356, NCIMB 8607, LMG 11428, LMG 11469 (Boot et al. 1995) and NCFM (Konstantinov et al. 2008), *cbsB* of *L. crispatus* JCM 5810 (Sillanpää et al. 2000) and ZJ001 (Chen et al. 2009), *SlpNB* of *L. crispatus* LMG 12003 (personal communication; Sillanpää et al. 2000), *slpA* of *L. crispatus* K313 (Sun et al. 2012), s*lpC* of *L. brevis*

ATCC 14869 (Jakava-Viljanen et al. 2002), several *lgs* genes of *L. gallinarum* (Hagen et al. 2005), and probably also by one of the two S-layer protein genes identified in *L. amylovorus* by DNA hybridization (Boot et al. 1996c), although the presence of two identical-sized S-layer proteins on the bacterial surface cannot be excluded. According to a preliminary SDS-PAGE analysis of seven porcine *L. amylovorus* isolates, only one isolate was suggested to express two S-layer protein genes at the same time, while for the remaining strains, only one S-layer protein was present (Jakava-Viljanen and Palva 2007). The genomes of *L. gallinarum* strains have two genes encoding S-layer proteins: a common one and a strain-specific one, but each strain produces only a single S-layer protein, which is always encoded by the strain-specific gene (Hagen et al. 2005). In the sequenced genomes of *L. brevis* ATCC 367 (Makarova et al. 2006) and *L. buchneri* CD034 (Heinl et al. 2012), two or several complete genes, respectively, have been identified by sequence homology (Åvall-Jääskeläinen et al. 2008; Heinl et al. 2012), but the expression of these genes is unknown.

The mechanism of the differential expression of *slp* genes has been well documented in *L. acidophilus* 4356, in which an inversion of a chromosomal segment leads to the placement of the silent gene in front of the active *slp* promoter (Boot et al. 1996a). This event seems to be unfavoured under laboratory conditions as the silent gene is at the expression site only in 0.3 % of the chromosomes of a broth culture of *L. acidophilus* 4356. No condition favouring the expression of the silent gene has thus far been characterized (Boot et al. 1996a). A similar chromosomal inversion mechanism has subsequently been shown to operate in *L. acidophilus* NCFM, where the inactivation of the S-layer protein gene *slpA* by homologous recombination led to the appearance of an alternate S-layer protein, SlpB, in the mutant strain NCK1377-CI (Buck et al. 2005; Konstantinov et al. 2008).

Information about adaptive changes in *Lactobacillus* S-layer gene expression, not known to involve chromosomal rearrangements, is scarce. In *L. brevis* ATCC 14869, the differential expression of the *slpB* and *slpD* genes is related to the oxygen content of the growth medium and the growth stage: *slpB* is expressed irrespective of oxygen content and equally in different growth phases, while *slpD* is predominantly expressed in aerated cultures and mainly in the exponential phase. The onset of *slpD* expression is most likely mediated by a soluble cytoplasmic factor, and it was surmised to be part of a stress response; a concomitant change in colony morphology, presumably not directly linked to the S-layer protein type, was also observed. Neither the nature/mechanism of action of the soluble regulator nor the reason for the silence of the *slpC* gene in this strain is known (Jakava-Viljanen et al. 2002). Stress-mediated regulation has been suggested also on other occasions. The expression of the S-layer protein gene of *L. acidophilus* NCC 2628 was induced when the strain was cultivated under conditions of limited protein supply (Schär-Zammaretti et al. 2005). An effect of bile salts was observed in *L. acidophilus* ATCC 4356, where concentrations of 0.01–0.05 % were shown to increase *slpA* gene expression, while the expression was decreased in 0.1 % bile; concomitant changes were observed in colony morphology and cell surface hydrophobicity (Khaleghi et al. 2010). In the same strain, sublethal concentrations of penicillin G were shown to increase the amount of SlpA on the bacterial surface, but the results were not in accordance with *slpA* gene expression (Khaleghi et al. 2011). The expression of the S-layer protein gene of *L. acidophilus* NCFM, in contrast, was not significantly induced during the passage through an in vitro gastrointestinal tract model (Weiss and Jespersen 2010). In *L. brevis* ATCC 8287, a slight repression effect of bile on *slpA* promoter activity was observed, but neither bile, pancreatin nor an uncommon carbon source had an effect on the amount of SlpA expressed on the bacterial cell surface (Hynönen et al. 2010). Similarly, in *L. hilgardii*, wine-related stress factors, like the presence of ethanol, copper sulphate or *p*-coumaric acid, did not affect S-layer protein production measurable by SDS-PAGE (Dohm et al. 2011).

Completely S-layer-negative *Lactobacillus* mutants are difficult or impossible to create (Palva et al. unpublished data; Boot et al. 1996a; Martinez et al. 2000; Buck et al. 2005), indicating that at least one functional S-layer protein gene is essential for S-layered lactobacilli, and expression of S-layer protein genes thus could be anticipated to be constitutive. However, some of the examples earlier indicate that variation and regulation at the transcriptional and/or translational level also exist. Recently, genes encoding alternative sigma factors have been identified in the sequenced genomes of several *Lactobacillus* species, and numerous potential transcription factor genes are also present (Azcarate-Peril et al. 2008), indicating potential for the regulated expression of *slp* genes under special conditions in these organisms. However, currently the transcriptional and translational regulation mechanisms of *Lactobacillus* S-layer protein genes on a molecular level are almost completely unexplored.

Cell wall binding and self-assembly regions of *Lactobacillus* S-layer proteins

One or both of the two structural regions generally present in S-layer proteins, i.e., the region involved in the attachment of the S-layer subunit to the cell envelope and the region involved in S-layer assembly, have so far been characterized in the following S-layer proteins: in the SA protein of *L. acidophilus* ATCC 4356 (Smit et al. 2001), in CbsA of *L. crispatus* JCM 5810 (Antikainen et al. 2002), in SlpB of *L. crispatus* strains K313 and K2-4-3 (Hu et al. 2011; Sun et al. 2012), in SlpA of *L. crispatus* ZJ001 (Chen et al. 2009), in SlpA of *L. brevis*

ATCC 8287 (Åvall-Jääskeläinen et al. 2008) and in the S-layer protein of *L. hilgardii* B706 (Dohm et al. 2011).

The first five strains listed above belong to the former *L. acidophilus* group (Johnson et al. 1980), and the amino acid sequences of their S-layer proteins show extensive similarity in the C-terminal region (Hu et al. 2011; Sun et al. 2012). Extending the alignment to the S-layer proteins of organisms of the closely related *L. helveticus* (Collins et al. 1991; Felis and Dellaglio 2007) also indicates a remarkable conservation of the C-terminal region (Antikainen et al. 2002), suggesting a conserved function for this region. Indeed in SA (Smit et al. 2001), CbsA (Antikainen et al. 2002) and in the SlpB proteins of K313 (Sun et al. 2012) and K2-4-3 (Hu et al. 2011), the C-terminal part of the S-layer protein, approximately 125 amino acids in length, is responsible for binding to the cell envelope. In SA, only one of the two 65-amino-acid repeats of the cell wall binding region is necessary for binding, and an enhancing role for the other repeat has been suggested (Smit and Pouwels 2002). In contrast, in the S-layer proteins of *L. brevis* ATCC 8287 and *L. hilgardii* B706, organisms completely unrelated to *L. acidophilus*, the N-terminal parts of the proteins comprise the cell wall binding region (Åvall-Jääskeläinen et al. 2008; Dohm et al. 2011). Nevertheless, all of the S-layer proteins described earlier have a similar charge distribution with a high predicted p*I* in the cell wall binding part (Smit et al. 2001; Antikainen et al. 2002; Åvall-Jääskeläinen et al. 2008; Sun et al. 2012; Dohm et al. 2011). Thus, an electrostatic interaction occurring between the cell wall binding regions and the negatively charged cell wall polymers has been proposed (Antikainen et al. 2002).

Lactobacillus S-layer proteins do not possess surface layer homology domains (Lupas et al. 1994), repeated motifs 50–60 amino acids in length, which are known to be involved in the binding of many S-layer proteins to the cell wall, for instance, those of *Bacillus anthracis* (Mesnage et al. 1999), *Geobacillus stearothermophilus* PV72/p2 (Ries et al. 1997; Sára et al. 1998), *Lysinibacillus sphaericus* CCM 2177 (Ilk et al. 1999) and *Clostridium thermocellum* NCIMB 10682 (Lemaire et al. 1998). Instead two repeated amino acid sequences with homology to the tyrosine/phenylalanine containing carbohydrate-binding motifs of clostridial toxins and streptococcal glucosyltransferases (Wren 1991; von Eichel-Streiber et al. 1992) are present in the cell wall binding regions of the above-mentioned SA, CbsA and SlpA of *L. acidophilus*, *L. crispatus* and *L. brevis*, respectively. These motifs are also found in the C-terminal parts of the silent S-layer protein SB of *L. acidophilus* ATCC 4356, the S-layer protein of *L. helveticus* CNRZ 892 and the non-S-layer proteins of lactic acid bacteria known to be associated with the cell envelope (Smit et al. 2001; Åvall-Jääskeläinen et al. 2008). The cell wall receptors of the S-layer proteins of *L. acidophilus* and *L. crispatus* have indeed been shown to be carbohydrates: SA of *L. acidophilus* ATCC 4356 and CbsA and SlpB of *L. crispatus* JCM 5810 and K313, respectively, interact with teichoic acids (Antikainen et al. 2002; Smit and Pouwels 2002; Sun et al. 2012); CbsA also binds to lipoteichoic acids isolated from *Staphylococcus aureus* and *Streptococcus faecalis*, but not to the teichuronic acid/polysaccharide fraction of the cell wall of *L. crispatus* JCM 5810 (Antikainen et al. 2002). On the contrary, the cell wall components interacting with the S-layer proteins of *L. brevis* ATCC 8287 and *L. hilgardii* apparently are non-teichoic acid polysaccharides as trichloroacetic acid (TCA) treatment of the cell walls had no effect on the interaction (Åvall-Jääskeläinen et al. 2008; Dohm et al. 2011); TCA treatment at +4 °C has been reported to selectively remove teichoic acids (Hancock and Poxton 1988). Supporting this is the fact that, in earlier studies, the cell walls of *L. brevis* and *L. buchneri* were shown to contain neutral polysaccharides that were suggested to be involved in the anchoring of the S-layer protein via hydrogen bonding (Masuda and Kawata 1980, 1985). In contrast to the well-characterized exopolysaccharides of lactic acid bacteria (De Vuyst and Degeest 1999; Welman and Maddox 2003), the cell wall polysaccharides of lactobacilli other than teichoic acids are poorly known. The detailed structure of a neutral wall polysaccharide of *L. casei* has been determined (Nagaoka et al. 1990), but no precise structures for such polysaccharides of *L. buchneri* or *L. brevis* strains are available.

In SA of *L. acidophilus*, CbsA of *L. crispatus* and SlpA of *L. crispatus* ZJ001 and *L. brevis*, the more variable part of the protein (N-terminal in the S-layer proteins of *L. acidophilus* or *L. crispatus*, C-terminal in SlpA of *L. brevis*) is responsible for the self-assembly of the S-layer protein monomers to a periodic S-layer lattice, as shown by the mapping of the self-assembly properties of truncated recombinant S-layer proteins by transmission electron microscopy (Sillanpää et al. 2000; Smit et al. 2001, 2002; Antikainen et al. 2002; Åvall-Jääskeläinen et al. 2008; Chen et al. 2009); these regions thus most likely represent the surface-exposed parts of the proteins. SA, CbsA and SlpA of *L. brevis*, and apparently also the other S-layer proteins described earlier, can thus be viewed as two-domain proteins with a cell wall binding domain and a self-assembly domain facing the extracellular environment, the former being not or less exposed to the environment. In SA, this view is supported by extensive proteolytic and chemical breakdown experiments (Smit et al. 2001); moreover, in the S-layer proteins of *L. brevis* and *L. hilgardii*, the C-terminal parts were found to be trypsin resistant (Åvall-Jääskeläinen et al. 2008; Dohm et al. 2011).

More detailed information is available about the structures of the self-assembly domains of SA of *L. acidophilus* ATCC 4356, CbsA of *L. crispatus* JCM 5810 and SlpA of *L. brevis* ATCC 8287. According to insertion and deletion mutagenesis and proteolytic studies of SA, the N-terminal self-assembly domain is probably organized into two subdomains of approximately 12 and 18 kDa, linked by a surface-exposed loop. The

very N-terminus of SA is not critical for crystallization and is probably buried inside the domain or facing the cell wall or S-layer pore. Conserved regions and regions predicted to form secondary structures in SA are necessary for the formation of a regular lattice (Smit et al. 2002). The lack of necessity of the very N-terminal end and the importance of the conserved regions for self-assembly have also been demonstrated for CbsA, where the conserved, valine-rich flanking regions of the self-assembly domain are especially important for the formation of the S-layer lattice and may have a role in directing the formation of a regular polymer; changes in the morphology of the self-assembly products of CbsA fragments were seen accompanying a mutation of even a single residue in these conserved border regions as well as with the stepwise truncation of the self-assembly region. The C-terminal cell wall binding domain has a stabilizing role in the recrystallization of CbsA monomers by allowing a more efficient sheet formation (Antikainen et al. 2002). The locations of a set of defined amino acids in SlpA of *L. brevis* have been mapped by cysteine-scanning mutagenesis combined with sulfhydryl modification. The analysis, based on measuring the surface accessibilities of the residues when the protein is in a monomeric or self-assembled form, grouped the residues according to their locations within the polymerized S-layer structure: to those located in the interior of the subunit, to those on the outer surface of the polymerized protein layer, to those on the inner surface of the layer and to those likely located in the subunit–subunit interface and pore or inner surface of the layer. The results confirmed the two-domain structure of SlpA and revealed several sites of high surface accessibility (Vilen et al. 2009).

Functions of *Lactobacillus* S-layer proteins

Adhesive functions

The most often proposed function for *Lactobacillus* S-layers is the mediation of bacterial adherence to various targets. In a number of studies, the loss of the S-layer protein from the bacterial surface by chemical means (Kos et al. 2003; Garrote et al. 2004; Frece et al. 2005; Chen et al. 2007; Jakava-Viljanen and Palva 2007; Tallon et al. 2007) or the covering of the layer by other molecules during prolonged cultivation (Schneitz et al. 1993) has been shown to decrease adhesion to different targets, but the role of the S-layer protein in adherence in these studies has not been directly demonstrated. The haemagglutinating activity of *L. acidophilus* JCM 1034 and the mucin binding activities of related strains were shown to be linked to their S-layer proteins, although the involvement of other guanidine hydrochloride-extractable components of the cell wall in this lectin-like activity could not be excluded, and/or the effect of aggregation of the S-layer proteins possibly causing unspecific effects could not be completely ruled out (Yamada et al. 1994; Takahashi et al. 1996). Likewise, in the study of Golowczyc et al. (2009), where the carbohydrate-dependent co-aggregation of *L. kefir* with yeast or red blood cells was suggested to be S-layer-mediated, conclusions were drawn from the effects of LiCl and SDS treatments of *L. kefir* cells, and the solubility of the S-layer proteins in the LiCl extracts of *L. kefir* used in the aggregation assays was not demonstrated. Also, in the study of Uchida et al. (2006), which showed an interaction between the S-layer protein of *L. brevis* OLL 2772 and human blood group A antigen by a surface plasmon resonance assay, a dialysed guanidine hydrochloride extract of bacterial cells was used as an analyte, leaving the effects of the levels of purity and solubility of the protein debatable.

The role of a *Lactobacillus* S-layer protein in bacterial adherence has been unequivocally shown in a few instances, where recombinant S-layer proteins (Toba et al. 1995; Sun et al. 2012), S-layer-negative mutants (Konstantinov et al. 2008), highly purified monomeric proteins (de Leeuw et al. 2006) or a surface display system for the S-layer protein (Hynönen et al. 2002) was used.

Recombinant forms of CbsA of *L. crispatus* JCM 5810 (Toba et al. 1995; Sillanpää et al. 2000) and SlpB of *L. crispatus* K313 (Sun et al. 2012) both bind collagen types I and IV. In contrast, the recombinant form of the non-expressed SlpB protein of *L. crispatus* JCM 5810, which showed 43 % sequence identity to CbsA at the amino acid level, does not bind these collagens (Sillanpää et al. 2000). *L. crispatus* JCM 5810 cells also bind to collagen-rich regions of chicken colon in vitro, while guanidine hydrochloride-treated cells are unable to bind, suggesting biological relevance for the observed collagen binding of CbsA (Sillanpää et al. 2000). The N-terminal amino acid residues at position 31–274 of mature CbsA are needed for collagen binding, and mostly the same residues (32–271) are needed for the reassembly of CbsA monomers to an S-layer, suggesting the dependence of collagen binding on the periodic structure (Sillanpää et al. 2000). The display of CbsA on the surface of a non-S-layered *L. casei* strain through a PrtP cell wall anchor rendered the recombinant cells able to bind collagens, although the anchoring system probably does not allow the monomers to form a true S-layer (Martinez et al. 2000). While the sequence similarity between CbsA and the S-layer protein SlpB of *L. crispatus* K313 is restricted to the C-terminal cell wall binding region, the N-terminal part of SlpB also binds collagen. More than 341 N-terminal amino acid residues are needed for binding (Sun et al. 2012), but no data are available if the collagen binding and polymerization require the same amino acid residues as demonstrated for CbsA. The recombinant protein comprising the N-terminal part of the S-layer protein of *L. crispatus* strain ZJ001, in turn, binds to detached HeLa cells (Chen et al. 2009).

A further well-characterized adhesive *Lactobacillus* S-layer protein is SlpA on *L. acidophilus* NCFM cells, which binds to the dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) receptor on human immature dendritic cells, leading to cytokine production and modulation of the immune response. The *slpA* knock-out mutant expressing SlpB and SlpX is significantly reduced in binding to DC-SIGN, and the interaction leads to the induction of different cytokines (Konstantinov et al. 2008). Initially, a role for SlpA of *L. acidophilus* NCFM was demonstrated in binding to Caco-2 cells as the binding of the knock-out mutant of the gene in locus LBA 1377 was decreased by 84 % compared with the wild type (Buck et al. 2005). However, the gene in locus LBA 1377 was subsequently annotated as a putative mucus binding protein, and SlpA was localized in locus LBA 0169. Nevertheless, SlpA encoded by the gene in LBA 0169 has later been detected on the surface of several Caco-2 cell binding *L. acidophilus* isolates (Ashida et al. 2011). It is identical in sequence with the SA protein of *L. acidophilus* ATCC 4356, suggesting that these strains might have similar adhesive and immunomodulatory properties as well as surface layer-associated murein hydrolase activity (see "Protective, enzymatic and other functions").

Finally, the S-layer protein SlpA of *L. brevis* ATCC 8287 mediates the binding of the bacterial cells to several human epithelial cell lines and fibronectin, as revealed by expressing fragments of *slpA* in a surface display system based on the H7 flagella of *Escherichia coli*. Eighty-one amino acids from the N-terminal part of SlpA were sufficient to confer binding to epithelial cells (Hynönen et al. 2002). The binding functions of SlpA were verified using a non-adhesive *Lactococcus* strain, in which a nicin-inducible surface display system with a cell wall-anchoring peptide from lactococcal AcmA was used to display the binding region of SlpA on the cell surface (Åvall-Jääskeläinen et al. 2003). Khang et al. (2009), in turn, used purified SlpA– green fluorescent protein (GFP) fusion proteins to show the binding of SlpA to undifferentiated human HT-29 cells, although more attention could have been focused on controlling the specificity of the interaction in this study. The binding of SlpA to extracellular matrix proteins has been further confirmed by de Leeuw et al. (2006), who demonstrated a direct interaction between the chromatographically purified, monomeric form of SlpA and soluble fibronectin or laminin by surface plasmon resonance assays. The binding mechanisms to fibronectin and laminin were found to be different and proposed to be mediated by different regions of SlpA.

In addition to the above-mentioned examples of specific binding, the S-layers of lactobacilli may have a non-specific enhancing effect on binding to surfaces, like those encountered in the gastrointestinal or urogenital tract, as they are generally hydrophobic and may thus enhance adhesion to hydrophobic surfaces (van der Mei et al. 2003). This effect is, however, dependent on the ionic strength of the environment (Vadillo-Rodríguez et al. 2005). Some *Lactobacillus* S-layers, but not all, have even been found to change their surface hydrophobicity in response to environmental ionic strength, thus possibly offering different binding capacities. In the case of the SA protein of *L. acidophilus* ATCC 4356, the decrease in hydrophobicity associated with higher environmental ionic strength is hypothesized to be due to the shrinkage of the S-layer and the consequent partial exposure of the inner, more hydrophilic C-terminal domain (Vadillo-Rodriguez et al. 2004).

Protective, enzymatic and other functions

To date, a couple of functions other than adhesion have been shown or proposed for *Lactobacillus* S-layer proteins. The presence of the S-layer protein decreases the susceptibility of *L. helveticus* ATCC 12046 to mutanolysin (Lortal et al. 1992), the susceptibility of *L. acidophilus* M92 to gastric and pancreatic juice (Frece et al. 2005) and the susceptibility of *L. hilgardii* wine isolate B706 to wine-related conditions like the presence of copper sulphate or tannic acid (Dohm et al. 2011). On the other hand, the S-layer proteins of brewery isolates of *L. brevis* were deduced not to act as barriers for the hop bittering substance isohumulone (Yasui et al. 1995). The auxiliary S-layer component SlpX of *L. acidophilus* NCFM probably affects the permeability of the S-layer as the *slpX*-negative mutant is more susceptible to SDS and more resistant to bile than the wild type (Goh et al. 2009). The C-terminal part of the S-layer protein SA of *L. acidophilus* ATCC 4356 has been shown to have murein hydrolase (endopeptidase) activity against the cell wall of, e.g., *Salmonella enterica* (Prado Acosta et al. 2008), but the biological relevance of this finding was not investigated. A role as a phage receptor has been suggested for the S-layer protein of *L. helveticus* CNRZ 892 (Callegari et al. 1998).

Applications of *Lactobacillus* S-layer proteins

Vaccine development

During the recent years, the number of applications developed or suggested for *Lactobacillus* S-layer proteins has gradually increased. One of the fields currently extensively studied is the construction of S-layer fusion proteins for use in immunization in man or animals. Especially the development of live *Lactobacillus* strains carrying S-layers composed of hybrid proteins on their surface is of interest as such strains have potential for use as live mucosal vaccines. Several findings support this approach: (1) The non-pathogenicity of lactobacilli and their ability to survive the passage through the gastrointestinal tract enables a simple, safe and efficient route of oral antigen delivery; (2) A clear relationship exists between antigen expression levels and

immune response (Grangette et al. 2001; Seegers 2002), and surface display with an S-layer protein as a carrier results in the simultaneous expression of the foreign peptide as hundreds of thousands of regularly arranged copies on the cell; (3) *Lactobacillus* cells as well as surface layer arrays have intrinsic adjuvant properties (Smith et al. 1993; Miettinen et al. 1996; Maassen et al. 2000; Seegers 2002; Beganović et al. 2011), and the simultaneous display of immunomodulating molecules in the S-layer could further enhance or direct the immune response; (4) As antigen carrier systems can be significantly improved by the co-display of adhesins (Cano et al. 1999; Liljeqvist et al. 1999), the various binding functions described earlier might prove advantageous in the targeted delivery of antigenic molecules. For instance, the identification of the S-layer protein of *L. acidophilus* NCFM as the binding ligand for the dendritic cell-specific antigen DC-SIGN (Konstantinov et al. 2008) makes this probiotic strain or its S-layer an attractive tool for oral vaccine design. So far, only a system utilizing *L. acidophilus* NCFM cells, not yet its S-layer, as a carrier for an antigen with a small dendritic cell-targeting peptide has been developed (Mohamadzadeh et al. 2009).

The development of *Lactobacillus* vaccine carriers based on hybrid S-layers is at an early stage. Small model peptides have been displayed in each monomer of the S-layer of *L. brevis* ATCC 8287 (Åvall-Jääskeläinen et al. 2002) and *L. acidophilus* ATCC 4356 (Smit et al. 2002) by chromosomal integration based on homologous recombination. Similarly, surface display of GFP in the S-layer proteins on chicken *Lactobacillus* isolates has been achieved by utilizing the gene fragment encompassing the expression and secretion signals and the region encoding the cell wall binding domain of the S-layer protein of *L. crispatus* (Mota et al. 2006). As a prerequisite for hybrid S-layer-based vaccine development, a systematic mapping of surface-accessible amino acids has been performed for the S-layer protein of *L. brevis* ATCC 8287 (Vilen et al. 2009). Apart from hybrid S-layer proteins, non-adhesive antigen delivery vehicles like lactococci have been rendered adhesive by the surface display of adhesive S-layer proteins or S-layer-derived peptides such as those of *L. crispatus* JCM 5810 (Martinez et al. 2000) or *L. brevis* ATCC 8287 (Åvall-Jääskeläinen et al. 2003). Preliminary experiments have also been performed in the field of passive immunization by utilizing the epithelial cell binding S-layer protein of *L. brevis* KCTC 3102 (ATCC 8287) as a purified, immunoglobulin binding fusion protein to target antibodies to the intestinal surfaces of calves in order to prevent neonatal diarrhoea (Khang et al. 2009). In this small field study, a higher recovery of calves from diarrhoea was obtained by administering antiviral and antibacterial antibodies in combination with the fusion protein than by administering the antibodies alone, although the mechanism of protection remained speculative.

Applications based on anti-adhesive and anti-infectious effects

Another potential application is the use of S-layers or S-layered lactobacilli as anti-adhesive agents or as other therapeutic or preventative measures against infectious diseases. In many studies, however, the anti-adhesive effects observed for S-layer proteins against different pathogens, as described, e.g., for the S-layer proteins of *L. crispatus* (Horie et al. 2002a; Chen et al. 2007), *L. helveticus* (Sherman et al. 2005; Johnson-Henry et al. 2007) and *L. kefir* (Golowczyc et al. 2007), cannot unequivocally be attributed to the surface layer proteins per se, as the dialysed extracts used in the inhibition studies apparently contained also other LiCl or guanidium hydrochloride-extractable cell surface components as well as aggregates of the S-layer protein, and thus the specificities of the inhibitions were compromised. The same holds true for the study of Martínez et al. (2012), which demonstrated the inhibition of JUNV infection by the surface protein extract of *L. acidophilus* ATCC 4356 in a DC-SIGN expressing cell culture model, although the interaction between SlpA of ATCC 4356/NCFM and DC-SIGN has previously been demonstrated (Konstantinov et al. 2008). Similarly, the results of the study of Carasi et al. (2011), which showed the potential of *L. kefir* S-layer proteins for decreasing the cytopathic effect of *Clostridium difficile* culture supernatants or toxins to Vero cells, were obtained using unpurified LiCl extracts of *L. kefir* cells and thus cannot be considered as fully conclusive.

The identification of the S-layer protein of *L. acidophilus* NCFM as the binding ligand for the dendritic cell-specific antigen DC-SIGN and the different cytokine responses elicited by SlpA and the alternative S-layer protein SlpB (Konstantinov et al. 2008) have raised interest in studying the contribution of the S-layer protein of NCFM to its probiotic action. There is an association between DC-SIGN polymorphisms and allergic sensitization, and the colonization of 1-month-old infants by *L. acidophilus* slightly decreases the risk of allergic dermatitis (Penders et al. 2010), but still the role of the SlpA–DC-SIGN interaction in immunological tolerance and its biological significance is far from clear. On the other hand, in the cellular mechanisms of inflammatory bowel disease, lipoteichoic acids of *L. acidophilus* NCFM seem to have a major role, as pre-treatment of mice by LTA-negative *L. acidophilus* NCFM ameliorated dextran sulphate sodium-induced inflammatory colitis (Mohamadzadeh et al. 2011). Interestingly, the presence of SlpA on an *slpB*$^-$*slpX*$^-$ mutant actually increases the pro-inflammatory action of LTA compared with the LTA-expressing parental strain (Zadeh et al. 2012). Thus, a role for SlpB and SlpX in regulating LTA-induced inflammation has been suggested (Lightfoot and Mohamadzadeh 2013). A mutant lacking SlpB and SlpX but carrying SlpA also tends to be cleared from the mouse gastrointestinal tract more rapidly than the wild type, but the

mechanism is not known (Zadeh et al. 2012). Some indications about the contribution of the S-layer protein of *L. acidophilus* ATCC 4356/NCFM to probiotic action were obtained in the study of Li et al. (2011b), in which the chromatographically purified S-layer protein SlpA was shown to counteract a *Salmonella*-induced transepithelial electric resistance decrease and IL-8 secretion as well as to inhibit *Salmonella*-induced F-actin rearrangements and JNK and p38 activation in Caco-2 cells. In another study, the same protein was shown to activate the ERK1/2 signaling pathway and to inhibit caspase-3 activity in *Salmonella*-infected Caco-2 cells, thereby decreasing *Salmonella*-induced Caco-2 cell apoptosis and cell damage (Li et al. 2011a). Interestingly, apart from probiotics, even the S-layer protein of a dairy strain of *L. helveticus* was found to reduce NF-κB activation in Caco-2 cells while triggering the expression of TLR2-mediated pro-inflammatory factors in human and mouse macrophages, thus showing stimulatory effects on innate immunity. In this study, special effort was exerted to demonstrate the purity of the S-layer preparation used (Taverniti et al. 2012).

A novel application has been suggested for the S-layer protein SA of *L. acidophilus* ATCC 4356. The murein hydrolase activity of the C-terminal part of SA, shown by the degradation of cell wall preparations of Gram-negative pathogens (Prado Acosta et al. 2008), acts synergistically with the well-documented antibacterial agent, nisin, against bacterial pathogens. The combination of these two inhibits the growth of both Gram-positive and Gram-negative pathogens, as exemplified by *Salmonella enterica*, *Staphylococcus aureus* and *Bacillus cereus*, through a mechanism that involves the dissipation of the transmembrane proton motive force (Prado-Acosta et al. 2010).

Chemical conjugates and liposomes

Some biochemical and physical studies of isolated *Lactobacillus* S-layer proteins aiming at biotechnological or clinical applications have also been initiated. Small molecular probes like biotin or fluorescein isothiocyanate have been conjugated to purified S-layer proteins of *L. brevis* using amine-based coupling chemistry. The S-layer protein bioconjugates formed, purified by affinity chromatography, were capable of self-assembling into regular layers, where the surface coverage of the conjugated molecules is homogeneous and the density controllable. The method offers a way to display several different and high molecular weight molecules at an interface (Sampathkumar and Gilchrist 2004). Further, positively charged liposomes have been coated by the S-layer proteins of *L. brevis* and *L. kefir* (Hollmann et al. 2007, 2010a). Importantly for future vaccine applications, the S-layer proteins markedly increased the stability of the liposomes under unfavourable conditions, e.g. at low pH, at high temperature or in the presence of bile salts or pancreatic extract, as measured by the release of a fluorescent marker compound, and the effect could be further enhanced by cross-linking the proteins with glutaraldehyde (Hollmann et al. 2007). The stabilizing effect was shown to be based on the neutralization of the charge repulsion between stearylamine molecules in the liposome, leading to increased acyl chain packing and membrane rigidity. The glycosylated S-layer protein of *L. kefir* had higher affinity to the liposomes than the non-glycosylated one of *L. brevis* (Hollmann et al. 2010a), but no striking differences were found between the liposome-stabilizing effects of the two proteins (Hollmann et al. 2007). The kinetics of the interaction between the S-layer protein and a lipid monolayer was found to be dependent on the composition of the membrane and could be modulated by components that modify the hydration state of the lipid interface (Hollmann et al. 2010b).

Expression/secretion signals in heterologous gene expression

The expression and/or secretion signals of *Lactobacillus* S-layer protein genes have also been utilized in biotechnological applications (Savijoki et al. 1997; Kahala and Palva 1999; Novotny et al. 2005; Lizier et al. 2010; Zhang et al. 2010a). The region encompassing the double promoter and the ribosome-binding sequence up to the start of the *slpA* gene of *L. brevis* ATCC 8287 (Kahala and Palva 1999), or the region containing additionally the *slpA* signal peptide gene sequence (Savijoki et al. 1997), have been used in *Lactobacillus* and *Lactococcus* hosts for intracellular or extracellular protein production. Using *slpA* expression and secretion signals, secretion levels of beta-lactamase up to 80 mg/l have been achieved. Differences exist between the recognition efficiency of the signals in different hosts; high-level extracellular protein production with *slpA* signals was achieved in *Lactococcus lactis* and *Lactobacillus plantarum* and moderate production in *L. gasseri*, while in *L. casei* the expression signals were not recognized (Savijoki et al. 1997). On the other hand, the promoter region of *L. acidophilus* ATCC 4356 S-layer protein gene was highly active in *L. casei* (Boot et al. 1996b) but functioned poorly in *L. reuteri* (Lizier et al. 2010). The transcriptional activity in heterologous hosts could be significantly improved or decreased by the modification of native *slp* gene promoter, and both strain- and context-dependent effects of the introduced sequences have been detected (McCracken and Timms 1999). Adding merely the signal peptide encoding sequence of *slpA* from *L. brevis* ATCC 8287 upstream of the 5′ end of the human interferon alpha gene increased the secretion efficiency of interferon alpha in *L. lactis* threefold compared to the signal peptide encoding sequence of lactococcal Usp45, but the total interferon production was lower in the strain with the *slpA* signal peptide encoding sequence (Zhang et al. 2010a). The recent development of a counterselective gene replacement system for the chromosomal integration of genes

in *L. acidophilus* (Goh et al. 2009) has enabled protein production using chromosomally located S-layer protein promoters in lactobacilli. The promoter of the S-layer protein gene *slpA* of *L. acidophilus* NCFM was found to direct the expression of the reporter gene *gusA3*, leading to a higher expression level than that obtained from a plasmid, when the reporter gene was placed between the stop codon and the transcriptional terminator of *slpA* (Douglas and Klaenhammer 2011).

Concluding remarks

Present knowledge about *Lactobacillus* S-layer proteins supports the view of Gram-positive S-layer proteins as two-domain entities, where one domain is responsible for cell wall binding and the other for the self-assembly of the regular surface layer. The common theme of carbohydrates as the binding sites for S-layer proteins in the cell walls of Gram-positive bacteria is also supported, although the detailed anchoring molecules and mechanisms vary among different lactobacilli. Biophysical methods are increasingly utilized in the structural studies of S-layers, and together with computer modelling-based methods they will probably allow for high-resolution structures of *Lactobacillus* S-layer proteins, which currently are scarce owing to difficulties in obtaining high-quality crystals for X-ray crystallography.

As food-grade and potentially probiotic organisms, lactobacilli are excellent candidates for health-related applications like live oral vaccines, where their ability to survive in the gastrointestinal tract could be utilized and their S-layer proteins could be used as carriers of antigens or other medically important molecules, possibly in combination with immunostimulatory or adhesive molecules. In this approach, the polymeric nature and inherent adjuvant properties of S-layers are apparently an advantage. Further, immobilization of recombinant S-layer proteins combined with the display of foreign molecules in the S-layer forms the basis for the development of different solid-phase reagents, such as biocatalysts, diagnostic devices, biosensors and biosorbents, where the typical positive charge of the cell wall-binding domain of *Lactobacillus* S-layer proteins could augment the immobilization. While most of the biotechnological applications of S-layer proteins so far have been designed for the S-layer proteins of thermophilic bacilli, the increasing knowledge about the structure and biology of *Lactobacillus* S-layer proteins, as well as the developing tools to genetically manipulate these organisms, will pave the way to applications utilizing the S-layer proteins of these beneficial and easily cultivable bacteria.

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