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Identification and characterization of domains responsible for self-assembly and cell wall binding of the surface layer protein of *Lactobacillus brevis* ATCC 8287

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Abstract

Background: *Lactobacillus brevis* ATCC 8287 is covered by a regular surface (S-) layer consisting of a 435 amino acid protein SlpA. This protein is completely unrelated in sequence to the previously characterized S-layer proteins of *Lactobacillus acidophilus* group.

Results: In this work, the self-assembly and cell wall binding domains of SlpA were characterized. The C-terminal self-assembly domain encompassed residues 179–435 of mature SlpA, as demonstrated by the ability of N-terminally truncated recombinant SlpA to form a periodic structure indistinguishable from that formed by full length SlpA. Furthermore, a trypsin degradation analysis indicated the existence of a protease resistant C-terminal domain of 214 amino acids. By producing a set of C-terminally truncated recombinant SlpA (rSlpA) proteins the cell wall binding region was mapped to the N-terminal part of SlpA, where the first 145 amino acids of mature SlpA alone were sufficient for binding to isolated cell wall fragments of *L. brevis* ATCC 8287. The binding of full length rSlpA to the cell walls was not affected by the treatment of the walls with 5% trichloroacetic acid (TCA), indicating that cell wall structures other than teichoic acids are involved, a feature not shared by the *Lactobacillus acidophilus* group S-layer proteins characterized so far. Conserved carbohydrate binding motifs were identified in the positively charged N-terminal regions of six *Lactobacillus brevis* S-layer proteins.

Conclusion: This study identifies SlpA as a two-domain protein in which the order of the functional domains is reversed compared to other characterized *Lactobacillus* S-layer proteins, and emphasizes the diversity of potential cell wall receptors despite similar carbohydrate binding sequence motifs in *Lactobacillus* S-layer proteins.

Background

Surface layers (S-layers) are cell envelope structures ubiquitously found in Gram-positive and Gram-negative bac-

terial species as well as in *Archaea*. They are composed of numerous identical (glyco)protein subunits, 40–200 kDa in molecular weight, which completely cover the cell sur-

face forming a two-dimensional, regular array having either oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetry. The subunits are held together and attached to the underlying cell surface by noncovalent interactions, and they have an intrinsic ability to spontaneously form regular layers either in solution or on a solid support under suitable conditions [1]. Functions of S-layers are poorly known thus far. They include the determination and maintenance of cell shape, action as a protective coat, molecular sieve or ion trap or as a mediator of adhesion or surface recognition. The contribution of S-layers to virulence has been reported [1,2].

In general, S-layer proteins have two structural regions in which two essential functions reside: a region involved in the attachment of the S-layer subunit to the cell envelope and a region involved in S-layer assembly. These regions have been characterized in several Gram-positive and some Gram-negative bacteria. In many Gram-positive bacilli and in *Thermus thermophilus* so called SLH (S-layer homology) motifs [3], 55–60 amino acids long and often located in the N-terminal part of the protein, are responsible for the attachment of the subunit proteins to the cell wall through a pyruvylated polysaccharide receptor in the cell wall [4]. In S-layers of Gram-positive bacteria not having SLH-motifs the attachment to the cell wall has been proposed to be mediated by an interaction between basic amino acids in the cell wall binding region and negatively charged secondary cell wall polymers. The cell wall receptors of such S-layers in *Geobacillus* species characterized so far contain mannuronic acid and can be classified as acidic oligosaccharides other than teichoic or teichuronic acids, while teichoic and lipoteichoic acids have been shown to be the cell wall receptors of the S-layer proteins of *Lactobacillus acidophilus* and *Lactobacillus crispatus*. However, some cell wall polysaccharides of Gram-positive bacteria proposed to be involved in S-layer binding have a net neutral charge [1,5-7].

Among Gram-positive bacteria, the self-assembly regions of S-layer proteins have so far been studied in the S-layers of lactobacilli (see below), and in the S-layers of *Bacillus anthracis*, *Lysinibacillus sphaericus* and *Geobacillus stearothermophilus*. These studies rely on electron microscopy of recombinant S-layer protein fragments, and the self-assembly region has been shown to be of either central or C-terminal location [8-11].

In addition to *L. brevis*, S-layers have also been found in *Lactobacillus helveticus* as well as in several *Lactobacillus acidophilus* group bacteria [12] including *L. acidophilus*, *L. crispatus* and *L. gallinarum*. The overall sequence similarity between characterized *Lactobacillus* S-layer protein genes is low and similarity is usually found only between related species. The presence of multiple S-layer protein genes in

a single strain is common in lactobacilli. For example, *L. brevis* ATCC 14869 has three S-layer protein genes, two of which are expressed under different environmental conditions and one is silent under laboratory conditions [13]. Other typical features of *Lactobacillus* S-layer proteins include their relatively small size and a high predicted overall pI [7]. Self-assembly and cell wall binding regions have been characterized in the S-layer protein S_A of *Lactobacillus acidophilus* ATCC 4356 [14] and CbsA of *L. crispatus* JCM 5810 [15]. The sequences of S_A and CbsA are homologous especially in the C-terminal region, which mediates the attachment to the cell wall, and the more variable N-terminal part is responsible for the self-assembly of the S-layer subunits.

The S-layer protein of *Lactobacillus brevis* ATCC 8287, SlpA [16], is a 435 amino acid, 46 kDa protein, which assembles on the bacterial cell forming an oblique lattice [17] and for which a fibronectin-binding function has been described [18]. *L. brevis* is phylogenetically distant from *L. acidophilus* group [19], and this is reflected in the unique amino acid sequence of SlpA compared to *L. acidophilus* group S-layer proteins [7]. Foreign epitopes up to 11 amino acids long have been expressed in SlpA in order to develop tools for mucosal immunization [17]. *L. brevis* ATCC 8287 would be a suitable strain to be used as a live oral vaccine, as it has a GRAS (Generally Recognized As Safe) status and it has been shown to possess probiotic properties [20]. For vaccine development, as well as for nanobiotechnological applications, for which regularly arranged S-layers are especially well-suited [21,22], knowledge about the structure-function relationships of SlpA, presented in this study, is essential.

In this work, we have characterized the two-domain structure of the S-layer protein SlpA of *L. brevis* ATCC 8287 with its C-terminal self-assembly and N-terminal cell wall binding domains. Conserved carbohydrate binding motifs were detected in the N-terminal, positively charged regions of six *L. brevis* S-layer proteins; however, the cell wall receptor of SlpA was found to be different from the receptors of previously characterized *Lactobacillus* S-layer proteins.

Methods

Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in Table 1. *Lactobacillus brevis* ATCC 8287 and *Lactobacillus acidophilus* ATCC 4356 were grown in MRS (Difco, Detroit, MI, USA) at 37°C. *E. coli* strains were grown in Luria-Bertani medium or M9ZB-medium [23] at 37°C under aeration. When appropriate, kanamycin, 30 µg/ml, was used for *E. coli*.

Table 1: Strains and plasmids used in this study

Strain or plasmid	Relevant properties ^a	Reference or source
Strains		
<i>Lactobacillus brevis</i> ATCC 8287		ATCC
<i>Lactobacillus acidophilus</i> ATCC 4356		ATCC
<i>Escherichia coli</i> DH5 α F [']	F ['] <i>endA1 hsdI 7 (r_k⁻ m_k⁺) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ (lacZYA-argF) U169 deoR [p80 d lacΔ(lacZ) M15]</i>	58
<i>Escherichia coli</i> BL21(DE3)	F ['] ompT hsdS _B (r _B ⁻ m _B ⁻) gal dcm (DE3)	Novagen
Plasmids		
pET-28a(+)	Km ^r , <i>E. coli</i> expression vector	Novagen
pET-28b(+)	Km ^r , <i>E. coli</i> expression vector	Novagen
pKTH5198	Km ^r , pET-28b(+)(NcoI/XhoI::SlpA ₁₋₄₃₅ -linker _{thrombin} -Tag _{his6})	This study
pKTH5199	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₋₄₃₅)	This study
pKTH5200	Km ^r , pET-28b(+)(NcoI/XhoI::SlpA ₁₄₆₋₄₃₅ -linker _{thrombin} -Tag _{his6})	This study
pKTH5201	Km ^r , pET-28b(+)(NcoI/XhoI::SlpA ₂₉₁₋₄₃₅ -linker _{thrombin} -Tag _{his6})	This study
pKTH5203	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₋₁₄₅)	This study
pKTH5204	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₋₂₉₀)	This study
pKTH5258	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₉₀₋₄₂₃)	This study
pKTH5259	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₂₁₀₋₄₂₃)	This study
pKTH5260	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₋₁₈₉)	This study
pKTH5261	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₉₀₋₄₃₅)	This study
pKTH5262	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₂₁₀₋₄₃₅)	This study
pKTH5264	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₆₇₋₄₃₅)	This study
pKTH5325	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₇₉₋₄₃₅)	This study
pKTH5333	Km ^r , pET-28a(+)(NheI::Tag _{his6} -linker _{thrombin} -SlpA ₁₄₉₋₄₃₅)	This study

^a Km^r, resistance to kanamycin

DNA manipulations and transformation

Routine molecular biology techniques were used essentially as described previously [24]. Plasmid DNA of *E. coli* clones was isolated by using the Wizard Minipreps kit (Promega, Madison, WI, USA). Chromosomal DNA of *L. brevis* was isolated essentially as described before [16]. PCR products were purified with the QIAquick PCR puri-

fication kit (Qiagen). DNA restriction and modification enzymes were used as recommended by the manufacturers (New England Biolabs Inc., Beverly, MA, USA; Promega). PCR was carried out with DyNAzyme II DNA polymerase as recommended by the manufacturer (Finnzymes, Helsinki, Finland). *E. coli* cells were transformed by standard methods [24].

Table 2: Oligonucleotides used in this study.

Oligonucleotide	Nucleotide sequence (5'→3') ^a
1594	GTCAT <u>CCATGGG</u> CAAGTCATACGCTACTGCAGG
1595	TCGCA <u>CTCGAGG</u> GCTGCCGCGCGCACCAAGGCCGCTGCTGTTGAACCAAGTAGTACCGT
1596	TCGTA <u>TCTAGAA</u> AGTCATACGCTACTGCAGG
1597	TCGCAT <u>CTAGATT</u> ATTATTAGTTGAACCAAGTAGTAC
1602	GTCAT <u>CCATGGG</u> CCTTTATGGTGTGCTAAGGAC
1603	GTCAT <u>CCATGGG</u> GCTCCCAAGCAGCTACTTCTAAG
1604	TCGCA <u>CTCGAGG</u> GCTGCCGCGC
1628	GTCAT <u>GCTAGCA</u> AGTCATACGCTACTGCAGG
1629	ATTCC <u>GCTAGCT</u> TTATTAACAGTAGCGTAAACTGTGTT
1630	TGATAG <u>GCTAGCT</u> TTATTAGCTAACTTTACTTGCCTTGAT
1635	ATTCC <u>GCTAGCGG</u> CTTCAGTACTACTGCTACT
1636	TCGCA <u>GCTAGCT</u> TTATTAGTTGAACCAAGTAGTAC
1637	ATTCC <u>GCTAGCG</u> TTCACGCAACCAACGATAAC
1638	TGATAG <u>GCTAGCT</u> TTATTACTTACCAGCGTAAATCC
1639	TGATAG <u>GCTAGCT</u> TTATTACTTACCCATAACAAGGGT
1644	ACTAC <u>GCTAGCG</u> GTTTATTATACTATCACGTAAC
1776	GCGGG <u>GCTAGC</u> AGTGGTATTAGTGGTTGGATTT
1777	GCGGG <u>GCTAGCG</u> TTTGCTAAGGACACCAAGTTT

^a recognition sites of restriction enzymes are underlined

Oligonucleotides and DNA sequencing

Oligonucleotides (Oligomer, Helsinki, Finland) used in this work are listed in Table 2. Nucleotide sequencing was performed by the dideoxy chain termination method of Sanger *et al.* [25] by using an ABI Prism 310 Genetic analyzer (Applied biosystems, Foster City, CA, USA) in combination with the DNA sequencing kit for BigDye Terminator cycle sequencing (Applied Biosystems).

Protein analysis

Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [26] and stained with Coomassie brilliant blue.

Construction of plasmid vectors

For the expression of the mature SlpA protein, SlpA₁₋₄₃₅, the gene was amplified by PCR from the chromosomal DNA of *L. brevis* ATCC 8287 using primer pairs 1594/1595 or 1596/1597 (Table 2). The PCR fragment obtained with primer pair 1594/1595 was digested with *Nco*I and *Xho*I and ligated with *Nco*I-*Xho*I digested pET-28b(+), resulting in plasmid pKTH5198 encoding rSlpA with a C-terminal His-tag (Table 1). The PCR product amplified with primers 1596 and 1597 was digested with *Xba*I and cloned into the *Nhe*I site of plasmid pET-28a(+). The resulting plasmid, encoding rSlpA with an N-terminal His-tag, was named pKTH5199.

Three C-terminal truncations, seven N-terminal truncations and two N- and C-terminal truncations of SlpA were constructed, each with a His-tag sequence at either N- or C-terminus. For a summary of the plasmid constructs, see Table 1. For cloning the C-terminal truncations, primer pairs 1628/1630 (for SlpA₁₋₁₄₅), 1628/1629 (for SlpA₁₋₂₉₀) and 1628/1638 (for SlpA₁₋₁₈₉) (Table 2) were used to amplify the *slpA* sequences with plasmid pKTH5199 as a template. PCR fragments obtained were digested with *Nhe*I and cloned into *Nhe*I-digested pET-28a(+). The resulting plasmids were named pKTH5203, pKTH5204 and pKTH5260, respectively (Table 1).

Cloning of the N-terminal truncations of SlpA with the His-tag sequence at the 3'-terminus was carried out with primer pairs 1602/1604 (for SlpA₁₄₆₋₄₃₅) and 1603/1604 (for SlpA₂₉₁₋₄₃₅) (Table 2) and pKTH5198 as a template. The resulting PCR fragments were cloned as *Nco*I-*Xho*I fragments into pET-28b(+), giving plasmids pKTH5200 and pKTH5201, respectively (Table 1). For cloning the N-terminal truncations with the His-tag sequence at the 5'-terminus, primer pairs 1777/1636 (for SlpA₁₄₉₋₄₃₅), 1644/1636 (for SlpA₁₆₇₋₄₃₅), 1776/1636 (for SlpA₁₇₉₋₄₃₅), 1635/1636 (for SlpA₁₉₀₋₄₃₅), or 1637/1636 (for SlpA₂₁₀₋

435) were used to amplify the *slpA* sequences with plasmid pKTH5199 as a template. The PCR fragments obtained were digested with *Nhe*I and cloned into *Nhe*I-digested pET-28a(+) resulting in plasmids pKTH5333, pKTH5264, pKTH5325, pKTH5261 and pKTH5262, respectively (Table 1).

Sequences encoding N- and C-terminally truncated SlpA were PCR amplified with primers 1635/1639 (for SlpA₁₉₀₋₄₂₃) and 1637/1639 (for SlpA₂₁₀₋₄₂₃), using plasmid pKTH5199 as a template. The resulting PCR fragments were cloned as *Nhe*I-fragments into pET-28a(+), giving plasmids pKTH5258 and pKTH5259, respectively (Table 1). All constructs were sequenced to verify the correct open reading frames.

Heterologous expression of the sequences encoding mature SlpA and its truncated forms

Gene expression was carried out as described in the pET System Manual (Novagen, Madison, WI, USA) by using *Escherichia coli* strain BL21(DE3). Briefly, expression of recombinant SlpA proteins was induced by adding isopropylthiogalactoside (IPTG) at a concentration of 0.5 to 1.0 mM to the medium of exponentially growing *E. coli* strains harboring one of the expression plasmids listed in Table 1. After IPTG was added, the incubation was continued for one to five hours, depending on the protein to be purified. Recombinant SlpA proteins were purified in the presence of 4 M guanidine hydrochloride (GHC1) or 6 M urea with a His Trap HP column according to the instructions given by Amersham Biosciences (Uppsala, Sweden). After purification the fractions containing the recombinant SlpA protein were dialyzed overnight at +4°C against distilled water. Purity of the recombinant proteins, present as a precipitate and/or as soluble proteins after dialysis, was checked by SDS-PAGE.

Isolation of SlpA protein from *L. brevis* ATCC 8287

The S-layer protein was extracted from *L. brevis* cells grown to an OD_{600 nm} of 1.0 in MRS broth. Cells from 1 l of culture were harvested and washed twice with distilled water. The pellet was resuspended in 15 ml of 2 M GHC1 and incubated for 30 min at +4°C followed by centrifugation (15,000 × g for 20 min). The supernatant was concentrated with Centricon Plus-20 centrifugal filter (Millipore, Bedford, MA, USA) before dialysis against distilled water supplemented with 5 mM CaCl₂ overnight at +4°C, followed by dialysis against distilled water overnight at 4°C. Before dialysis the SlpA protein concentration was adjusted to 1 mg/ml. After dialysis a centrifugation step (20,000 × g for 20 min) was performed. The pellet containing the S-layer self-assembly products was resuspended in 25 mM Tris-HCl buffer (pH 8.0).

Proteolytic degradation of SlpA with trypsin and peptide mapping

Isolated SlpA at a concentration of 1 mg/ml was dialyzed against distilled water supplemented with 5 mM CaCl₂ overnight followed by a second overnight dialysis against distilled water. The dialysis was followed by a centrifugation step (16,000 × g for 30 min). The S-layer monomers, present in the supernatant, were digested with trypsin under the following conditions: 300 ng SlpA protein and 3 µg trypsin (Sigma-Aldrich, St. Louis, MO, USA) in 300 µl of 25 mM Tris-HCl (pH 8.0) for 10 to 30 min at 37°C. The reaction was stopped by heating the samples for 10 min at 100°C and the samples were subjected to SDS-PAGE. N-terminal sequencing was performed by a gas-pulsed liquid sequencer as described previously [27] and peptide mapping by a Biflex matrix-assisted laser desorption ionization-time of flight mass spectrometer (Bruker-Franzen Analytic, Bremen, Germany) as described by [28].

Investigation of the self-assembly properties of purified truncated S-layer proteins

To assess the ability of the recombinant S-layer proteins to self-assemble, affinity purified proteins were dissolved in 5 M GHCl at a concentration of 1 mg/ml and the solutions were dialyzed against phosphate-buffered saline (PBS) in Slide-A-Lyzer Mini Dialysis Units (Pierce, Rockford, IL, USA) for two hours at 4°C. Dialysis was followed by a centrifugation step (16,000 × g for 30 min) and the formation of a precipitate was checked by SDS-PAGE. For transmission electron microscopy, 1 mg of the purified, lyophilized proteins were dissolved in 1 ml 5 M GHCl in 50 mM Tris-HCl buffer (pH 7.2) and the solution was dialyzed against 10 mM CaCl₂ in distilled water for 18 h. Samples were transferred onto carbon-coated electron microscope grids rendered hydrophilic by glow discharge, negative stained with 2.5% uranyl acetate as described previously [29], and electron micrographs were taken with Philips CM 12 transmission electron microscope (Philips Eindhoven, the Netherlands) operated at 80 kV in a low-dose mode. Freeze-etched preparations of *L. brevis* ATCC 8287 cells were prepared as previously described [30], and lattice constants of the S-layer formed by SlpA were determined as described by [31].

Isolation of native cell wall fragments (CWF) from *L. brevis* ATCC 8287

L. brevis ATCC 8287 cells were cultivated overnight in 1 l of MRS broth, collected and washed three times with distilled water. Cells were suspended in 30 ml of 2 M GHCl, incubated shaking for 30 minutes at +4°C, collected and washed once with 50 mM Tris-HCl (pH 7.4). Cells were disrupted by French Pressure Cell Press (SLM Instruments Inc, IL, USA) in 50 mM Tris-HCl (pH 7.4), and the lysate was centrifuged at 3000 g for 5 minutes at +4°C. Cell wall fragments were collected, washed five times with 50 mM

Tris-HCl (pH 7.4) and treated with DNAase I (25 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) and RNAase I (25 µg/ml, Roche Diagnostics GmbH, Mannheim, Germany) in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂ for 30 minutes at 37°C. Cell wall fragments were collected, treated with 1% SDS for 30 minutes at 100°C, washed extensively with distilled water at room temperature and lyophilized.

Treatment of native cell wall fragments with TCA

0.5 mg or 0.25 mg of isolated CWF in water were incubated in the presence of 5% (V/V) TCA either at +4°C or at +37°C for 24 h in a rotary shaker. The cell walls were collected, washed three times with distilled water at +4°C and suspended in distilled water. The treatment at +4°C was performed twice. Organic phosphorous was measured from native and treated CWF and from the supernatant obtained in the extraction by the method described by [32].

Binding of the truncated S-layer proteins to bacterial cells and isolated cell wall fragments

Binding assays of recombinant S-layer proteins to LiCl-extracted *L. brevis* ATCC 8287 and *L. acidophilus* ATCC 4356 cells were performed essentially as described previously [14]. The amount of recombinant S-layer protein used in one binding reaction was 50 µg and the buffer was 50 mM Tris-HCl (pH 7.5) with 150 mM NaCl. The presence of cell-bound S-layer protein in the samples was verified by SDS-PAGE. In binding assays with isolated cell wall fragments, monomeric truncated S-layer proteins, present in the supernatant after centrifugation (20 minutes at 16 000 g at +4°C), were used. 20 µg CWF and 10 µg full length recombinant SlpA or an equimolar amount of truncated S-layer proteins were combined in 50 µl of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl. After incubation (1 hour at room temperature) cell wall fragments were collected, washed once with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and analyzed by SDS-PAGE.

Analysis of primary amino acid sequences

The isoelectric point (pI) values of the *L. brevis* S-layer proteins as well as those of the constructed rSlpA proteins were obtained by ProtParam [33], and the analyses of the hydrophobicity patterns of S-layer proteins of *L. brevis* were performed by the Kyte-Doolittle method [34] with ProtScale [35] on the ExpASY server. Repeat structures from S-layer proteins were localized by REPRO [36,37]. The comparison matrix used in the protein repeat analysis was blosum62 (gap open penalty, 12; gap extension penalty, 1). Sequence alignment analyses of *L. brevis* S-layer proteins were performed by ClustalW [38] using gonnet as a comparison matrix (gap open penalty, 10; gap extension penalty, 0.2). Pairwise comparison analyses were performed by SIM [39,40] using blosum62 as a comparison matrix (gap open penalty, 12; gap extension penalty, 4).

From the complete genome sequence of ATCC 367, deposited under GenBank accession number [CP000416](#), the hypothetical S-layer proteins of *L. brevis* ATCC 367 were identified by BLAST [39] using complete SlpA, SlpB, SlpC and SlpD sequences. The identified hypothetical S-layer proteins of ATCC 367 have been deposited in Swiss-Prot under accession numbers Q03P39 and Q03NT3.

Results

Primary amino acid sequence analysis of the S-layer proteins of *L. brevis*

The only thus far characterized S-layer proteins in *L. brevis* are the SlpA protein of *L. brevis* ATCC 8287 [16] and the SlpB, SlpC and SlpD proteins of *L. brevis* ATCC 14869 [13]. By performing a homology search for the recently sequenced genome of *L. brevis* ATCC 367 [41] with the BLAST program, two new putative S-layer proteins, Q03P39 and Q03NT3, were identified in the genome.

The amino acid sequences encoding the mature S-layer proteins of *L. brevis* ATCC 8287 (SlpA) and ATCC 14869 (SlpB, C and D) and the putative mature S-layer proteins of ATCC 367 (Q03NT3 and Q03P39) were subjected to a number of analyses. A multiple alignment of SlpA, SlpB, SlpC, SlpD, Q03NT3 and Q03P39 amino acid sequences revealed significant conservation in the N-terminal regions (see Fig. 1a and additional file 1: Multiple amino acid sequence alignment of the *L. brevis* S-layer proteins). Analysis of the distribution of the isoelectric point values in *L. brevis* S-layer proteins revealed a distinction between the N-terminal region with a high predicted pI and a C-terminal region with a low predicted pI in each of the proteins (Fig. 1c). In SlpA and SlpB the region of a high predicted pI comprises approximately two fifths of the protein, while in SlpD and its homolog, Q03P39, as well as in SlpC and its homolog, Q03NT3, the region of an overall high pI extends further towards the C-terminus and the most distinct boundary between the differently charged regions is located around residue 260 in SlpC and Q03NT3 and around residue 290 in SlpD and Q03P39. Hydrophobicity analysis performed for the S-layer protein sequences of *L. brevis* showed a similar distribution of hydrophilic and hydrophobic amino acid residues along the mature proteins with evenly alternating hydrophobic and hydrophilic residues, as exemplified by the hydrophobicity plot of SlpA in Fig. 1b.

To predict the domain organization of SlpA, the analyses performed were compared with similar analyses of the S-layer proteins of *L. acidophilus* group organisms [14]. In each of the *L. acidophilus* S-layer proteins, one region is found which is conserved, located at the C-terminus and contains positively charged and hydrophilic sequences. In S_A of *L. acidophilus* ATCC 4356 and CbsA of *L. crispatus* JCM 5810 these domains mediate the binding to the cell

wall, while the variable N-terminal regions are responsible for the assembly of the S-layer [14,15]. In CbsA the variable N-terminal domain is also responsible for collagen binding [42]. The only function characterized for SlpA so far, binding to fibronectin and human epithelial cells [18], resides in the conserved N-terminal region. However, the pattern of sequence conservation and the distribution of charge in the six *L. brevis* S-layer proteins strongly suggested a domain organization similar to that found in *L. acidophilus*-group S-layer proteins with the functional domains in a reverse order.

Cloning and expression of gene sequences encoding the mature or truncated forms of the S-layer protein SlpA and purification of the recombinant proteins

PCR products encoding the mature SlpA and the various N- or C-terminal SlpA truncations were cloned in *E. coli* DH5 α F' and expressed in *E. coli* BL21(DE3). The proteins produced are summarized in Fig 2. After induction of expression by the addition of IPTG, samples of cultures from *E. coli* BL21(DE3) were harvested and analyzed by SDS-PAGE. On SDS-gels, each of the recombinant SlpA proteins became visible as an additional protein band, corresponding approximately to the calculated molecular mass of the rSlpA proteins (data not shown). The proteins were purified in a large scale, and total protein yields varied from 5 to 25 mg per a batch cultivation of 200 ml.

Proteolytic degradation of SlpA protein with trypsin and peptide mapping

To gain insight to the domain structure of SlpA, wild type SlpA was digested with trypsin. This revealed two protease resistant fragments with apparent molecular masses of 25 kDa and 23 kDa (Fig. 3). The N-terminal sequences of these peptides were determined to be GFSTTAG (larger peptide) and SVTATND (smaller peptide). These correspond to amino acids starting from 190 and 209 of mature SlpA, respectively. Peptide mass mapping of the protease resistant fragments obtained after trypsin digestion revealed that the peptide encompassing the last 12 amino acids of full length SlpA is lacking from these fragments (data not shown). The protease resistance of the regions 190 to 423 and 209 to 423 in mature SlpA strongly suggested the existence of a compact domain structure most likely representing a region exposed on the outer surface of the S-layer.

Investigation of the self-assembly properties of the truncated S-layer protein forms

As a preliminary test for the putative self-assembly properties of the truncated S-layer proteins, the formation of a precipitate after dialysis from GHCl was inspected. As shown in Fig. 2, C-terminally truncated proteins rSlpA₁₋₁₄₅, rSlpA₁₋₁₈₉ and rSlpA₁₋₂₉₀ had lost the ability to form precipitates. N-terminally truncated forms rSlpA₁₇₉₋₄₃₅

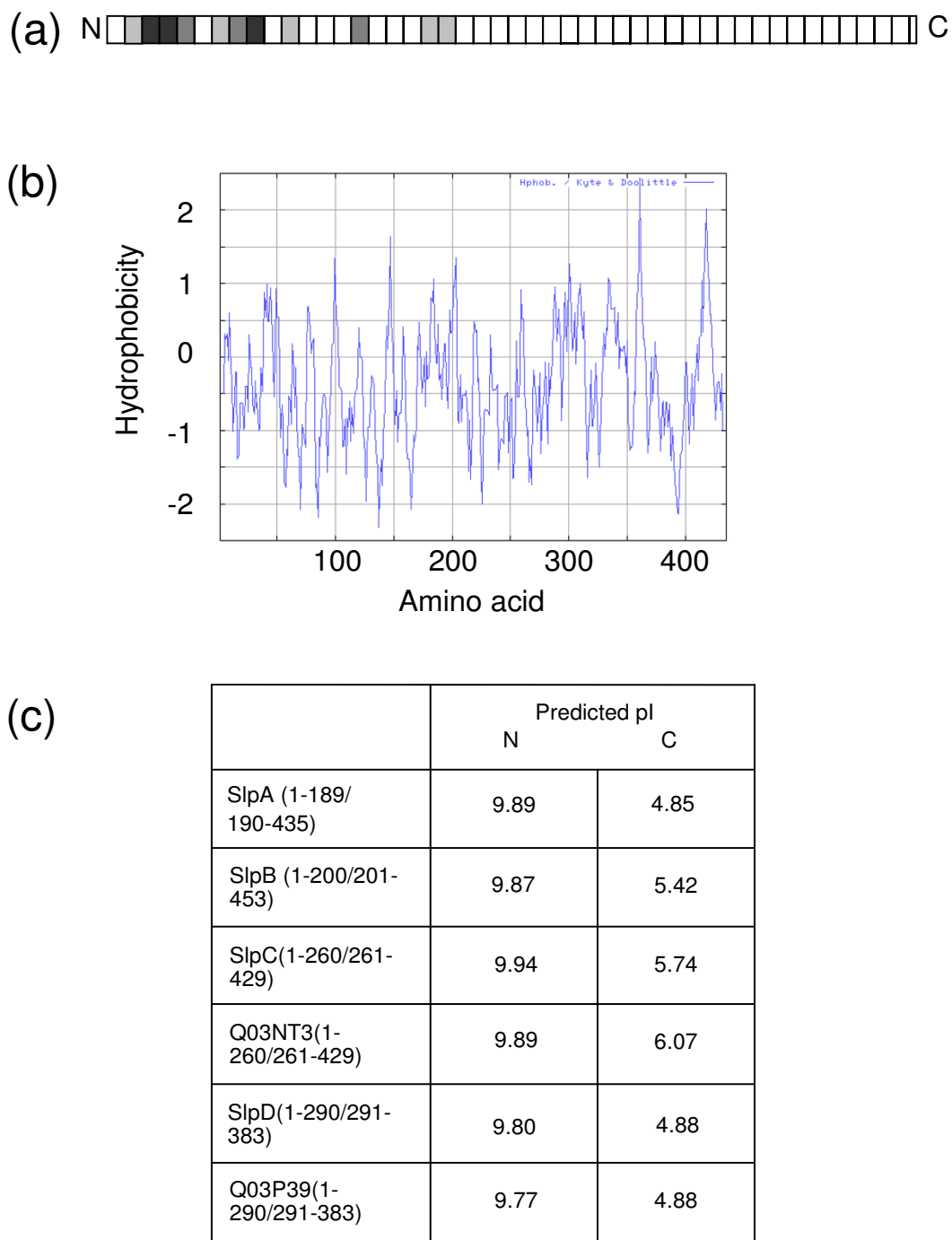


Figure 1

1(a) – Alignment of *L. brevis* S-layer protein sequences. The mature forms of S-layer proteins were aligned by 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: white, 0–20%, light gray, 21–40%, medium gray, 41–60%, dark gray, 61–80%. 1(b) Hydrophobicity of mature SlpA. The hydrophobicity was calculated according to Kyte and Doolittle [34] with a window of seven amino acids. 1(c) Predicted pI values of the N- and C-terminal regions of *L. brevis* S-layer proteins. The lengths of the N- and C-terminal regions as well as the full lengths of the mature forms of the proteins are indicated in brackets.

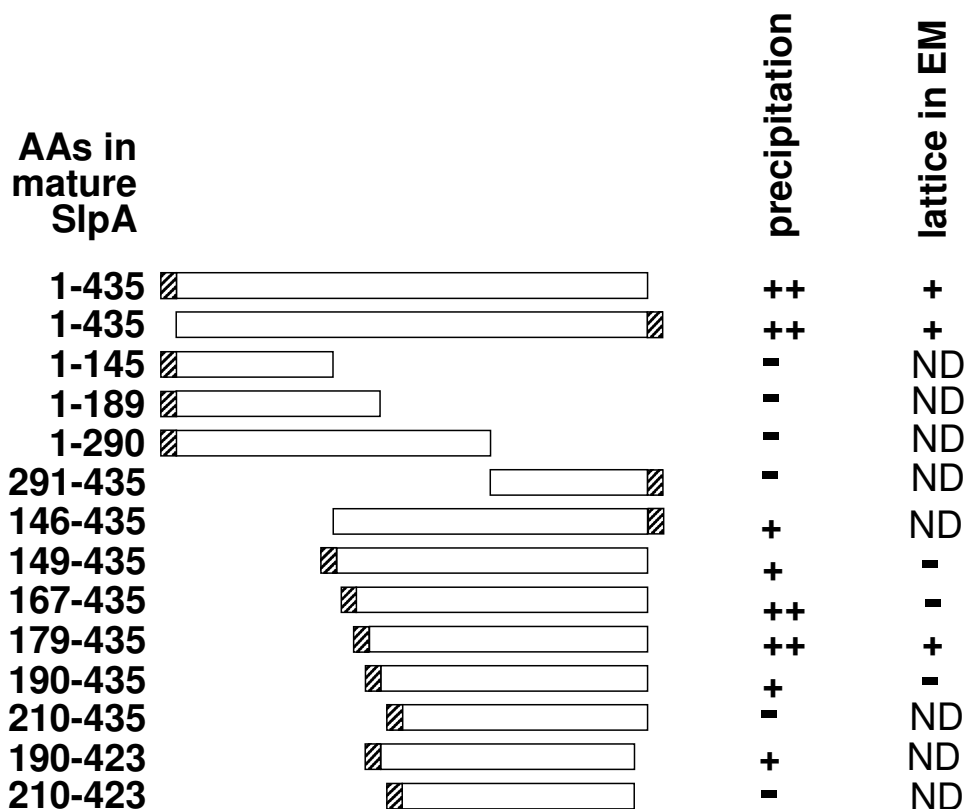


Figure 2
Schematic presentation of the recombinant SlpA proteins expressed and their self-assembly properties. Shaded bars, tags consisting of a 13 amino acid linker and six histidine residues.

and rSlpA₁₆₇₋₄₃₅ precipitated efficiently after dialysis, while rSlpA₁₄₆₋₄₃₅, rSlpA₁₄₉₋₄₃₅ as well as rSlpA₁₉₀₋₄₃₅ and rSlpA₁₉₀₋₄₂₃ showed a reduced precipitation. The last twelve residues in the C-terminus of SlpA had no effect on the precipitation, as rSlpA₁₉₀₋₄₂₃ showed a precipitation similar to rSlpA₁₉₀₋₄₃₅. The removal of 209 residues or more from the N-terminus of SlpA abolished the ability of the truncated proteins to form precipitates.

Precipitate-forming N-terminally truncated SlpA proteins were chosen for transmission electron microscopical analysis of lattice formation. In these studies, rSlpA formed an oblique lattice that was identical with that formed by SlpA isolated from wild type *L. brevis* ATCC 8287 cells (compare Figures 4a and 4c), as well as with the lattice seen on *L. brevis* ATCC 8287 cells in the freeze-etched preparation (Fig. 4d), proving the native conformation of recombinant SlpA. In accordance, lattice constants for the self-assembly products of rSlpA (a = 10.38, b = 6.36 and 72.7°) and for the self-assembly products of SlpA isolated from *L. brevis* ATCC 8287 cells (a = 9.39, b = 6.10 and 79.8°) were practically identical. The recombinant pro-

tein SlpA₁₇₉₋₄₃₅ (Fig. 4b) was found to form a regular, oblique lattice indistinguishable from that formed by full length rSlpA, but the removal of eleven residues more from the N-terminus resulting in rSlpA₁₉₀₋₄₃₅ prevented lattice formation (Fig. 2). Surprisingly, the two larger N-terminally truncated proteins, rSlpA₁₆₇₋₄₃₅ and rSlpA₁₄₉₋₄₃₅, were unable to form regular lattice structures. Thus, residues 179-435 in mature SlpA define the region responsible for the crystallization of SlpA monomers.

Isolation of native cell wall fragments and binding of the truncated S-layer proteins to cell wall fragments

Cell wall fragments were purified from a stationary phase culture of *L. brevis* cells by differential centrifugation of GdCl₃-treated, mechanically disrupted cells followed by treatments with nucleases and boiling SDS as described in Materials and methods. This method efficiently removes membrane fragments and noncovalently bound cell wall components like lipoteichoic acids (LTAs), but leaves covalently bound secondary cell wall polymers, like wall teichoic acids and other carbohydrates, essentially intact. The purity of the cell wall preparation was checked by

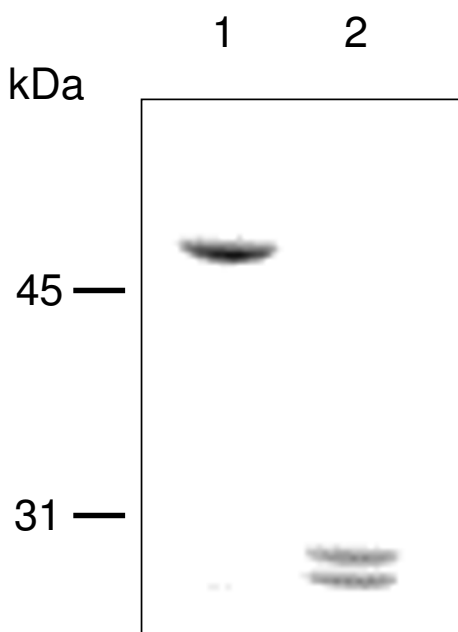


Figure 3
SDS-PAGE analysis of SlpA fragments obtained after trypsin digestion. Lane 1, undigested SlpA. Lane 2, SlpA after digestion with trypsin. Numbers on the left indicate molecular weights in kilodaltons.

light and transmission electron microscopy (Fig. 5). From 1.6 g of wet cells approximately 28 mg of cell wall fragments (dry weight) were recovered.

To test the hypothesis that the N-terminal, positively charged region of SlpA is responsible for anchoring the S-layer to the cell wall, truncated recombinant SlpA-proteins encompassing the N- and C-terminal regions of SlpA were tested for binding to isolated *L. brevis* cell wall fragments. As shown in Fig. 6a, full length rSlpA, rSlpA₁₋₁₄₅ and rSlpA₁₋₁₈₉ localized in the pellet fraction after incubation with the cell wall fragments, while rSlpA₁₉₀₋₄₃₅ and rSlpA₁₆₇₋₄₃₅ were unable to bind to CWF and were found in the supernatant. Truncated S-layer proteins incubated alone mainly localized to the supernatant, although small amounts were found in the pellets due to the inherent tendency of S-layer proteins to aggregate. These results are in good accordance with our results from similar experiments with whole, LiCl-treated *L. brevis* ATCC 8287 and *L. acidophilus* ATCC 4356 cells. In these experiments, full length rSlpA, rSlpA₁₋₁₄₅ and rSlpA₁₋₁₈₉ as well as rSlpA₁₋₂₉₀ bound to *L. brevis* cells, while rSlpA₁₉₀₋₄₃₅, rSlpA₁₆₇₋₄₃₅ and rSlpA₂₁₀₋₄₃₅ were unable to bind. Full-length rSlpA and its cell wall binding fragment rSlpA₁₋₁₄₅ also bound to LiCl-treated *L. acidophilus* ATCC 4356 cells (data not shown).

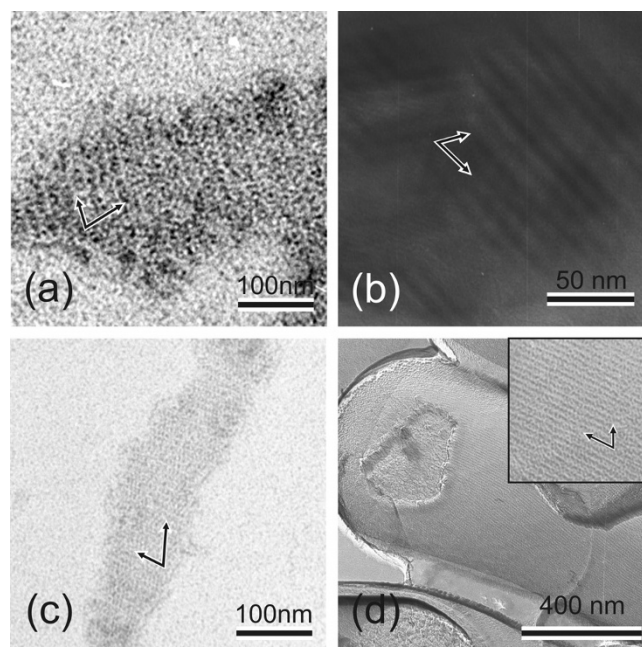


Figure 4
Self-assembly of SlpA, rSlpA and rSlpA C-terminal domain. (a-c) Transmission electron micrographs showing self-assembly products of (a) nontruncated rSlpA, (b) rSlpA₁₇₉₋₄₃₅, and (c) wild type SlpA isolated from *L. brevis* ATCC 8287 cells. (d) Transmission electron micrograph showing a freeze-etched preparation of *L. brevis* ATCC 8287 cells completely covered with the oblique S-layer lattice formed by SlpA. Arrows indicate the base vectors of the oblique lattice.

To get preliminary information about the cell wall component interacting with the N-terminal region of SlpA, binding tests with full length rSlpA and CWF treated with TCA at +4 °C or at +37 °C were performed. rSlpA bound to CWF treated with TCA at +4 °C as efficiently as to native CWF, while the treatment of CWF with TCA at +37 °C substantially reduced the binding of rSlpA (Fig. 6b). Repeated TCA-extraction at +4 °C and a change in the rSlpA: CWF-ratio from 1:2 to 1:1 did not change the result (data not shown). TCA extracts carbohydrate polymers, and the treatment at +4 °C has been reported to selectively remove teichoic acids, while the treatment at +37 °C removes teichuronic acids and polysaccharides [43]. The efficiency of the extraction was confirmed by the measurement of organic phosphorous from native and TCA-treated CWF as well as from the supernatant obtained in the extraction, which indicated the loss of approximately 75% of the organic phosphorous from the CWF by the treatment at +4 °C (data not shown). These results suggest that the binding component in the *L. brevis* ATCC 8287 cell wall is other than teichoic acid, and that cell wall components

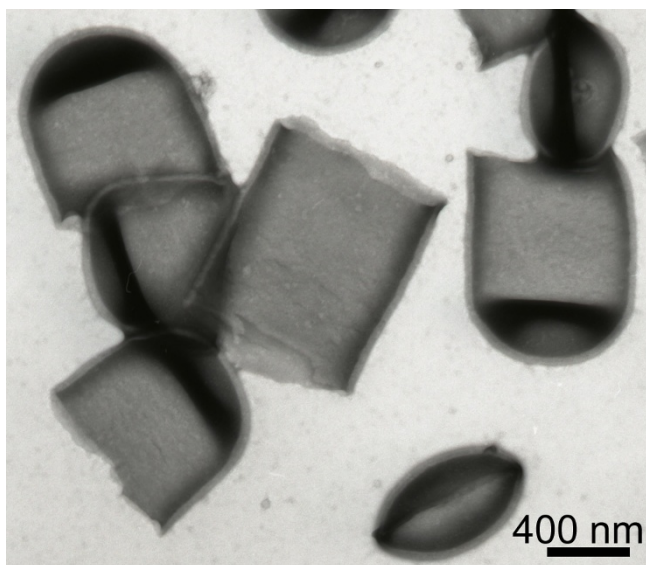


Figure 5
Transmission electron micrograph of isolated native cell walls of *L. brevis* ATCC 8287.

extractable by TCA at +37°C, presumably polysaccharides, participate in the binding.

The amino acid sequence analysis of SlpA and other *Lactobacillus brevis* S-layer proteins revealed sequences in the N-terminal regions with apparent similarity to the repetitive carbohydrate binding motifs of clostridial toxins and streptococcal glucosyltransferases [44,45]. These regions were found within amino acids 60–90 and 165–192 in each of the mature proteins (Fig. 7). In the N-terminal parts of SlpC and Q03NT3 as well as SlpD and Q03P39 additional regions with less obvious similarity were detected as well. Similar motifs have also been detected in the C-terminal cell wall binding regions of *L. acidophilus* ATCC 4356 S_A protein and *L. crispatus* JCM 5810 CbsA protein as well as in the S-layer protein of *L. helveticus* CNRZ 892 and in several other bacterial cell surface-associated proteins, in which they were located in two tandemly repeated sequences of 65–72 amino acids [14]. A protein repeat analysis of the *L. brevis* S-layer proteins did not indicate the presence of the carbohydrate binding sequences in obvious repeat sequences, and despite the similar carbohydrate binding motifs in SlpA and in *L. acidophilus* group S-layer proteins, the cell wall receptor of SlpA apparently is dissimilar.

Discussion

In this study, we have identified the cell wall binding and self-assembly domains in the S-layer protein SlpA of *L. brevis* ATCC 8287, a strain phylogenetically distant from *L. acidophilus* group organisms, the S-layer proteins of

which have previously been functionally characterized. Two new putative S-layer proteins, Q03P39 and Q03NT3, were identified in the recently sequenced genome of *L. brevis* ATCC 367 [41] and compared with SlpA and other *L. brevis* S-layer proteins characterized thus far. Q03P39 is almost identical (99% identity) with SlpD of *L. brevis* ATCC 14869 and relatively dissimilar (<40% identity) from the SlpA, SlpB and SlpC sequences. Q03NT3 is highly similar with SlpC of *L. brevis* ATCC 14869 (87% identity) whereas not that similar with the other characterized *L. brevis* S-layer proteins (<44% identity). Similarity of the mature forms of the new putative S-layer proteins or the mature forms of SlpA, SlpB, SlpC or SlpD proteins with *L. acidophilus* group S-layer proteins is negligible.

Analysis of the six *L. brevis* S-layer protein sequences deposited in databanks indicated the subdivision of each sequence into two regions: a conserved N-terminal region characterized by a high predicted pI and potential carbohydrate binding motifs, and a more variable C-terminal region with an acidic predicted pI, with the N-terminal region corresponding for 40–75% of the sequence lengths. The observed high predicted overall pI values of *Lactobacillus* S-layer proteins [7] thus seem to be due to the concentration of basic amino acids to a defined region, as is also the case in the S-layer proteins of *L. acidophilus* group, which have cationic, cell wall binding C-terminal regions.

The presence of a conserved N-terminal region with a high predicted pI in *L. brevis* S-layer proteins strongly suggested an N-terminal cell wall binding domain. This was confirmed for SlpA of *L. brevis* ATCC 8287 by interaction studies performed with truncated rSlpA proteins and LiCl-treated *L. brevis* cells or isolated *L. brevis* cell wall fragments. In these studies, truncated proteins encompassing the whole positively charged region of SlpA bound to the cell wall; however, the first 145 residues in mature SlpA contained sufficient information for cell wall binding. An assay suitable for measuring the binding strength would be needed to detect the putative difference between the cell wall binding affinities of SlpA_{1–145} and SlpA_{1–189}. In S-layer proteins of lactobacilli, no SLH motifs have been detected. Instead, interactions between a positively charged S-layer protein region and negatively charged secondary cell wall polymers have been shown to mediate the cell wall binding in the case of S_A of *L. acidophilus* ATCC 4356 [46] and CbsA of *L. crispatus* JCM 5810 [41]. S_A and CbsA were shown to bind teichoic acids, and CbsA bound also to lipoteichoic acids purified from *Staphylococcus aureus* and *Streptococcus faecalis*, but not to the teichuronic acid/polysaccharide fraction of the cell wall of *L. crispatus* JCM 5810. In contrast, the results of this study suggest the involvement of another cell wall structure

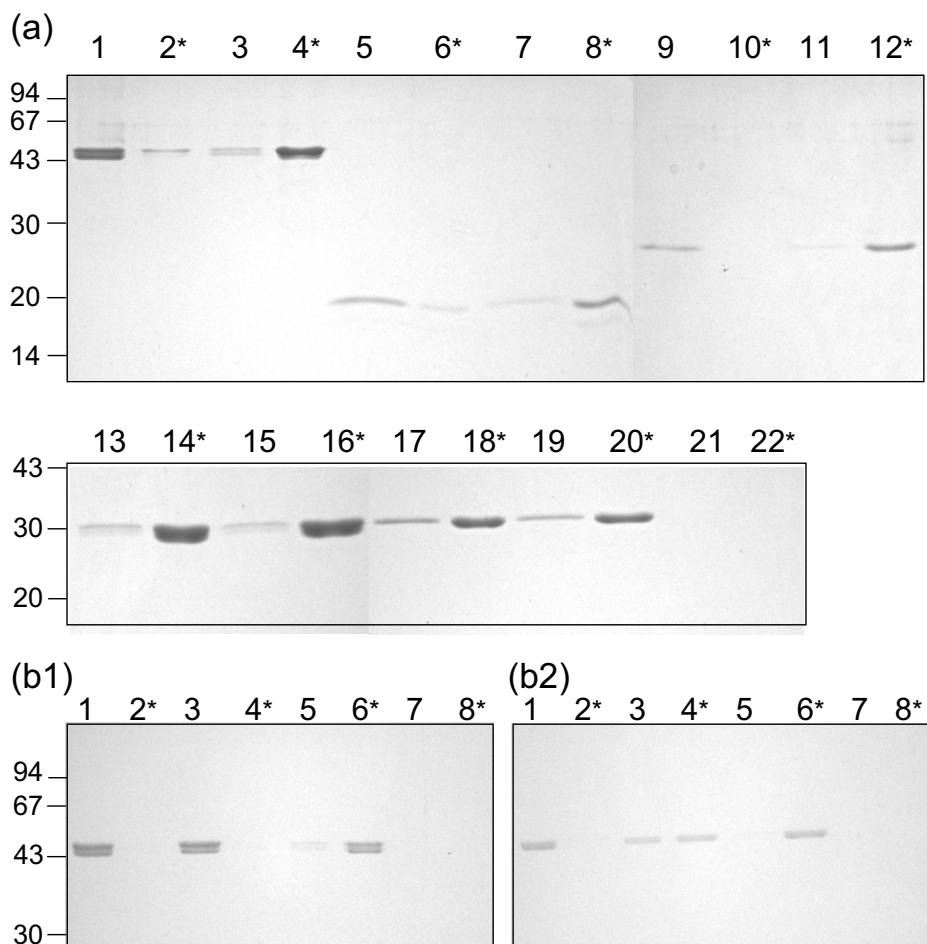


Figure 6

6(a) – Binding of truncated rSlpA proteins to isolated native cell walls of *L. brevis* ATCC 8287. Full-length rSlpA (lanes 1–4), rSlpA_{1–145} (lanes 5–8), rSlpA_{1–189} (lanes 9–12), rSlpA_{190–435} (lanes 13–16) and rSlpA_{167–435} (lanes 17–20) were incubated with (lanes 1–2, 5–6, 9–10, 13–14 and 17–18) or without (lanes 3–4, 7–8, 11–12, 15–16 and 19–20) cell walls (CWF), and the pellets and supernatants (*) recovered by centrifugation were analyzed by SDS-PAGE. Lanes 21–22, CWF incubated alone; *, supernatant. Numbers on the left indicate molecular weights in kilodaltons. **6(b) – Effect of treatment of cell walls with TCA at +4°C (b1) or at +37°C (b2) on the binding of rSlpA.** Full length rSlpA was incubated with native CWF (lanes 1–2) or with CWF treated with 5% TCA (lanes 3–4) and the pellets and supernatants (*) were analyzed by SDS-PAGE. Lanes 5–6, full-length rSlpA incubated alone, lanes 7–8, TCA-treated CWF incubated alone; *, supernatant. Numbers on the left indicate molecular weights in kilodaltons.

than teichoic acid or lipoteichoic acid in the interaction between SlpA and the cell wall, as the purification process of the CWF used efficiently removed LTAs, and the extraction of CWF with TCA at +4°C to remove teichoic acids had no effect on the binding of SlpA.

The chemical nature of the cell wall component interacting with the S-layer protein has been determined in *Geobacillus stearothermophilus* strains [47–49], in *Lysinibacillus sphaericus* [50] and in *Aneurinibacillus thermoaerophilus* [6]. In *G. stearothermophilus* and *L. sphaericus* S-layers, which possess SLH-domains, the component is a pyruvylated

GlcNac and GalNac-containing polysaccharide not groupable as a teichoic or lipoteichoic acid. In other *G. stearothermophilus* strains the component is a negatively charged mannuronic acid-containing cell wall polymer, and in *A. thermoaerophilus* the cell wall receptor is a neutral biantennary oligosaccharide.

The secondary cell wall polymers of lactobacilli are poorly characterized. The detailed structure of a wall polysaccharide of *L. casei* has been determined [51], but no precise structures for polysaccharides of *L. brevis* strains are available at present. In early studies, the cell walls of *L. buchneri*

SlpC	60	YFRAYRVAKT <u>TRGTVVY</u> KMVSMDGKYRGYVYGGRSV-----KAFAGGIKKAAT	107
Q03NT3	60	YFRAYRVAKT <u>TRGTVVY</u> KMVSMDGKYRGYVYGGRSV-----KAFAGGIKKAAT	107
SlpA	60	<u>YFRAYG</u> VKT <u>TNRGSV</u> YVYRVV <u>TMDGKY</u> RGYVYGGKSD-----TAFAGGIKSAET	107
SlpB	60	NFRAYRVAKT <u>TNRGSI</u> YKVVSYNKAYRGWVYGGKSD-----TAFAGGLTSYDT	107
SlpD	53	VLRGYKVAKL <u>LSNKS</u> YYMKVVSFDGRYRGYIYVGKKNPVNNFKKVGGLTYLST	105
Q03P39	53	VLHGYKVAKL <u>LSNKS</u> YYMKVVSFDGRYRGYIYVGKKNPVNNFKKVGGLTYLST	105
		KAVTGWxTIxGxxYYFxxNG	Wren (45)
		KAVTGWQTIDGkwYYFDxNG	von Eichel-Streiber et al (44)
		KAVTGWxTIxGxxYYFxxNG	
		KAVTGWQTIDGkwYYFDxNG	
SlpC	150	TYANDALVVTDAARKTREGSLYYYVT-- <u>DAAHPEVNGWVYAKGLTTTKPTTDN</u>	200
Q03NT3	150	TYANDALVVTDAARKTREGSLYYYVT-- <u>DAAHPEVNGWVYAKGLTTTKPTTD-</u>	199
SlpA	149	VAKDTKFTVDQAATK <u>TREGSLY</u> YHVT--ATNGSG <u>ISGWI</u> YAGKGFSTTATGTQ	199
SlpB	155	AYKDATFSVDKVGTRTREGDTWVHVVNQNTADTKADG <u>WILL</u> SNLTQTNAFNE-	206
SlpD	150	DYKGHKLVAASKAAMNDMDGQKWL <u>YVS</u> -DVTEPTNASG <u>WVLASQL</u> TDYVAPKAP	201
Q03P39	150	DYKGHKLVAASKAAMNDMDGQKWL <u>YVS</u> -DVTEPTNASG <u>WVLASQL</u> TDYVAPKAP	201
		KAVTGWxTIxGxxYYFxxNG	Wren (45)
		KAVTGWQTIDGkwYYFDxNG	von Eichel-Streiber et al (44)
		KAVTGWxTIxGxxYYFxxNG	
		KAVTGWQTIDGkwYYFDxNG	

Figure 7

Similarity of the N-terminal regions of *L. brevis* S-layer proteins with carbohydrate binding motifs. The motifs were determined by Wren [45] and von Eichel-Streiber et al [44]. In the consensus sequences upper case letters indicate highly conserved residues [44] or residues with an identity of 50% or higher [45]. X, variable residue. A broken underline indicates potential carbohydrate-binding motifs at different locations: YFRAYG of SlpA corresponds to YFDxNG of the consensus sequence of Ref [44]; KAYRGW of SlpB corresponds to KAVTGW of the consensus sequences of References [44] and [45]; LSNKSYY of SlpD and Q03P39 corresponds to IDGkwYY of the consensus sequence of Ref [44].

[52] and *L. brevis* ATCC 8287 [53] were shown to contain neutral polysaccharides, which were suggested to be involved in the anchoring of the S-layer protein to the cell wall through hydrogen bonding [54,55]. These results are in agreement with the data presented in this study, which suggest a non-teichoic acid polysaccharide, either neutral or anionic, involved in the cell wall binding of SlpA, but the detailed structure of this polysaccharide remains to be elucidated.

Interestingly, despite the fact that polysaccharides rather than (lipo)teichoic acids of *L. brevis* ATCC 8287 are involved in the cell wall binding of SlpA, the C-terminal cell wall binding region of the S-layer protein CbsA of *L. crispatus* JCM 5810 bound to GHCl-treated *L. brevis* ATCC 8287 cells [15]. Using the same experimental design we showed that rSlpA and its cell wall binding fragment rSlpA₁₋₁₄₅ bind to LiCl-treated *L. acidophilus* ATCC 4356 cells. The interaction between the S-layer protein and the secondary cell wall component is supposed to be lectin-like and highly specific [56], and in artificial experimental

procedures the lack of a specific interaction between two complementary surfaces may be masked by unspecific charge interactions with lower affinity. To obtain information about specific interactions, competition experiments with fragments of S_A, CbsA and SlpA and the corresponding bacterial strains, or direct determinations of the K_D values of the interactions, e. g. by surface plasmon resonance studies, are needed.

Amino acid sequence analysis of the *L. brevis* S-layer proteins revealed motifs with similarity to repeated C-terminal carbohydrate binding sequences detected in clostridial toxins, streptococcal glucosyltransferases and the S-layer proteins of *L. acidophilus* group organisms [14,44,45]. These motifs are supposed to play a general role in protein-carbohydrate interactions by acting as initial attachment sites and thus enabling the specific interactions to occur [44] and may thus be partly responsible for the observed positive cross-binding results between SlpA and *L. acidophilus* ATCC 4356 cells, and between the cell wall binding domain of CbsA and *L. brevis* ATCC 8287 cells

(see above). The sequences of the potential *L. brevis* carbohydrate-binding motifs deviated to some extent from the consensus sequences determined for clostridial toxins and streptococcal transferases [44,45]. The divergence of the sequences in distantly related organisms and different macromolecular structures is apparently allowed as long as the basic function of the motif, bringing the interacting partners to initial contact, is preserved.

The self-assembly domain of SlpA was shown to comprise residues 179–435 in mature SlpA, as truncated proteins encompassing this region were able to form a periodic structure indistinguishable from that formed by full length SlpA, as detected by electron microscopy. The length of the truncated protein was critical, since rSlpA_{167–435} and rSlpA_{149–435} as well as N-terminal truncations shorter than rSlpA_{179–435} were unable to form regular lattices. Apparently, the region, or part of the region, encompassing amino acids 149–178 disturbs the lattice formation of the truncated proteins either by steric hindrance and/or by preventing the acquisition of a native conformation. Trypsin degradation experiments revealed two protease resistant peptides encompassing residues 190–423 and 209–423 in mature SlpA supporting the hypothesis about a morphologically separate, compact C-terminal unit, which most probably corresponds to the round, periodically arranged structures seen in electron microscope pictures of self-assembly products of SlpA (Fig. 4). Similar trypsin degradation experiments with whole *L. brevis* cells resulted in identical fragments but at a very low efficiency (data not shown), indicating poor accessibility of the enzyme to the N-terminal domain through the pores in the S-layer, and further supporting the presumption about a protease sensitive, more flexible N-terminal domain shielded from the environment beneath the C-terminal self-assembly domain. A protease-resistant, surface-located self-assembly domain has also been observed in the N-terminal part of the S-layer protein S_A of *L. acidophilus* ATCC 4356 [14]. The results of the present study are supported by the report of Hynönen *et al* [18], in which an antiserum specific for the recombinant peptide SlpA_{66–215}, originating from the cell wall binding region, was not able to recognize polymerized SlpA on *Lactobacillus brevis* ATCC 8287 cells. In the same report, whole S-layered *L. brevis* ATCC 8287 cells as well as the N-terminal part of SlpA, residues 66–146 of mature SlpA in minimum, were shown to bind to immobilized fibronectin. Fibronectin is highly glycosylated, and the binding of fibronectin to a region of SlpA shielded beneath the C-terminal domain may be explained by an interaction between the protruding oligosaccharide moieties of fibronectin and the identified N-terminal carbohydrate binding sequences of SlpA. In this respect the identification of human blood group A-trisaccharide as the receptor for the S-layer protein of a human *L. brevis* isolate [57] is

of interest, especially considering that the nine N-terminal amino acids of the S-layer protein of this strain were identical with the N-terminus of SlpA.

Conclusion

In this work SlpA of *L. brevis* ATCC 8287 was shown to be a two-modular protein in which the domains responsible for the self-assembly (C-terminal) and cell wall binding (N-terminal) are located in a reverse order compared to those in all other *Lactobacillus* S-layer proteins characterized thus far, reflecting the unrelatedness of SlpA with previously characterized *Lactobacillus* S-layer proteins. The study confirms the role of conserved, repeated, general carbohydrate binding sequences in the cell wall binding domains of *Lactobacillus* S-layer proteins, but in contrast to the *Lactobacillus acidophilus*-group organisms, the specific cell wall component interacting with the S-layer protein in *L. brevis* ATCC 8287 was shown to be other than (lipo)teichoic acid. As SlpA is a potential tool for mucosal immunization, the data presented in this study forms a basis for further studies concerning vaccine development. The mapping of surface exposed residues in the self-assembly domain of SlpA is currently in progress.

Authors' contributions

SÅJ and UH performed the experiments (excluding electron microscopy, N-terminal sequencing and peptide mass mapping), analyzed the results, carried out the sequence analyses and prepared the manuscript. NI carried out electron microscopy, DP determined the lattice constants and performed the statistical analysis, UBS coordinated the EM studies, AP participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Multiple amino acid sequence alignment of the L. brevis S-layer proteins. ClustalW – alignment of the predicted mature forms of SlpA, SlpB, SlpC, SlpD, Q03NT3 and Q03P39 proteins. Asterisks, colons and dots indicate identical, strongly similar and weakly similar amino acids, respectively. A primary consensus sequence is shown below the alignment.

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References

- Sará M, Sleytr UB: **S-layer proteins**. *J Bacteriol* 2000, **182**:859-868.
- Sleytr U, Messner P: **Crystalline surface layers in prokaryotes**. *J Bacteriol* 1988, **170**:2891-2897.
- Lupas A, Engelhardt H, Peters J, Santarius U, Volker S, Baumeister W: **Domain structure of the Acetogenium kivui surface layer revealed by electron crystallography and sequence analysis**. *J Bacteriol* 1994, **176**:1224-1233.
- Messner S, Fontaine T, Mignot T, Delepierre M, Mock M, Fouet A: **Bacterial SLH domain proteins are non-covalently anchored to the cell surface via a conserved mechanism involving wall polysaccharide pyruvylation**. *EMBO J* 2000, **19**:4473-4484.
- Schäffer C, Messner P: **The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together**. *Microbiology* 2005, **151**:643-651.
- Steindl C, Schäffer C, Wugeditsch T, Graninger M, Matecko I, Müller N, Messner P: **The first biantennary bacterial secondary cell wall polymer and its influence on S-layer glycoprotein assembly**. *Biochem J* 2002, **368**:483-494.
- Ávall-Jääskeläinen S, Palva A: **Lactobacillus surface layers and their applications**. *FEMS Microbiol Rev* 2005, **29**(3):511-529.
- Candela T, Mignot T, Hagnerelle X, Haustant M, Fouet A: **Genetic analysis of Bacillus anthracis Sap S-layer protein crystallization domain**. *Microbiology* 2005, **151**:1485-1490.
- Huber C, Ilk N, Rüntzler D, Egelseer EM, Weigert S, Sleytr UB, Sára M: **The three S-layer-like homology motifs of the S-layer protein SbpA of Bacillus sphaericus CCM 2117 are not sufficient for binding to the pyruvylated secondary cell wall polymer**. *Mol Microbiol* 2005, **55**:197-205.
- Jarosch M, Egelseer EM, Huber C, Moll D, Mattanovich D, Sleytr UB, Sára M: **Analysis of the structure-function relationship of the S-layer protein SbsC of Bacillus stearothermophilus ATCC 12980 by producing truncated forms**. *Microbiology* 2001, **147**:1353-1363.
- Rüntzler D, Huber C, Moll D, Köhler G, Sára M: **Biophysical characterization of the entire bacterial surface layer protein SbsB and its two distinct functional domains**. *J Biol Chem* 2004, **279**:5207-5215.
- Johnson JL, Phelps CF, Cummins CS, London J, Gasser F: **Taxonomy of the Lactobacillus acidophilus group**. *Int J Syst Bacteriol* 1980, **30**:53-68.
- Jakava-Viljanen M, Ávall-Jääskeläinen S, Messner P, Sleytr UB, Palva A: **Isolation of three new surface layer protein genes (slp) from Lactobacillus brevis ATCC 14869 and characterization of the change in their expression under aerated and anaerobic conditions**. *J Bacteriol* 2002, **184**:6786-6795.
- Smit E, Oling F, Demel R, Martinez B, Pouwels PH: **The S-layer protein of Lactobacillus acidophilus ATCC 4356: Identification and characterization of domains responsible for S-protein assembly and cell wall binding**. *J Mol Biol* 2001, **305**:245-257.
- Antikainen J, Anton L, Sillanpää J, Korhonen TK: **Domains in the S-layer protein CbsA of Lactobacillus crispatus involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly**. *Mol Microbiol* 2002, **46**(2):381-394.
- Vidgren G, Palva I, Pakkanen R, Lounatmaa K, Palva A: **S-layer protein gene of Lactobacillus brevis: cloning by polymerase chain reaction and determination of the nucleotide sequence**. *J Bacteriol* 1992, **174**:7419-7427.
- Ávall-Jääskeläinen S, Kylä-Nikkilä K, Kahala M, Miikkulainen-Lahti T, Palva A: **Surface display of foreign epitopes on the Lactobacillus brevis S-layer**. *Appl Environ Microbiol* 2002, **68**:5943-5951.
- Hynönen U, Westerlund-Wikström B, Palva A, Korhonen TK: **Identification by flagellum display of an epithelial cell- and fibronectin-binding function in the SlpA surface protein of Lactobacillus brevis**. *J Bacteriol* 2002, **184**:3360-3367.
- Axelsson L: **Lactic acid bacteria: Classification and physiology**. In *Lactic acid bacteria: microbiology and functional aspects* Edited by: Salminen S, von Wright A. New York: Marcel Dekker Inc; 1998:1-73.
- Rönkä E, Malinen E, Saarela M, Rinta-Koski M, Aarnikunnas J, Palva A: **Probiotic and milk technological properties of Lactobacillus brevis**. *Int J Food Microbiol* 2003, **83**:63-74.
- Sleytr UB, Huber C, Ilk N, Pum D, Schuster B, Egelseer EM: **S-layers as a tool kit for nanobiotechnological applications**. *FEMS Microbiol Lett* 2007, **267**:131-144.
- Sleytr UB, Egelseer EM, Ilk N, Pum D, Schuster B: **S-layers as a basic building block in a molecular construction kit**. *FEBS Journal* 2007, **274**:323-334.
- Studier FW: **Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system**. *J Mol Biol* 1991, **219**:37-44.
- Sambrook J, Russell DW: *Molecular cloning: a laboratory manual* 3rd edition. New York: Cold Spring Harbor Laboratory Press; 2001.
- Sanger F, Nicklen S, Coulson AR: **DNA sequencing with chain-terminating inhibitors**. *Proc Natl Acad Sci USA* 1977, **74**:5463-5467.
- Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4**. *Nature* 1970, **227**:680-685.
- Kalkkinen N, Tilgmann C: **A gas-pulsed-liquid-phase sequencer constructed from a Beckman 890D instrument by using Applied Biosystems delivery and cartridge blocks**. *J Protein Chem* 1988, **7**:242-243.
- Nyman TA, Matikainen S, Saraneva T, Julkunen I, Kalkkinen N: **Proteome analysis reveals ubiquitin-conjugating enzymes to be a new family of interferon-alpha-regulated genes**. *Eur J Biochem* 2000, **267**:4011-4019.
- Pum D, Sára M, Sleytr UB: **Structure, surface charge and self-assembly of the S-layer lattice from Bacillus coagulans E38-66**. *J Bacteriol* 1989, **171**:5296-5303.
- Messner P, Pum D, Sleytr UB: **Characterization of the ultrastructure and the self-assembly of the surface layer of Bacillus stearothermophilus strain NRS 2004/3a**. *J Ultrastruct Mol Struct Res* 1986, **97**:73-88.
- Amos LA, Henderson R, Unwin PN: **Three-dimensional structure determination by electron microscopy of two-dimensional crystals**. *Prog Biophys Mol Biol* 1982, **39**:183-231.
- Ames BN, Dubin DT: **The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid**. *J Biol Chem* 1960, **235**:769-775.
- ProtParam [<http://us.expasy.org/tools/protparam.html>]
- Kyte J, Doolittle R: **A simple method for displaying the hydrophobic character of a protein**. *J Mol Biol* 1982, **157**:105-132.
- ProtScale [<http://www.expasy.org/tools/protscale.html>]
- REPRO [<http://bivu.cs.vu.nl/programs/reprowww/>]
- George RA, Heringa J: **The REPRO server: finding protein internal sequence repeats through the web**. *Trends Biochem Sci* 2000, **25**:515-517.
- ClustalW [http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html]
- SIM [<http://au.expasy.org/tools/sim-prot.html>]
- BLAST [<http://www.ncbi.nlm.nih.gov/genomes/Geblast.cgi?gi=19987>]
- Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee JH, Diaz-Muniz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D: **Comparative genomics of the lactic acid bacteria**. *Proc Natl Acad Sci USA* 2006, **103**:15611-15616.
- Sillanpää J, Martínez B, Antikainen J, Toba T, Kalkkinen N, Tankka S, Lounatmaa K, Keränen J, Höök M, Westerlund-Wikström B, Pouwels PH, Korhonen TK: **Characterization of the collagen-binding S-layer protein CbsA of Lactobacillus crispatus**. *J Bacteriol* 2000, **182**:6440-6450.
- Hancock IC, Poxton IR: *Bacterial cell surface techniques* New York: John Wiley & Sons Inc; 1988.
- von Eichel-Streiber C, Sauerborn M, Kuramitsu HK: **Evidence for a modular structure of the homologous repetitive C-terminal carbohydrate-binding sites of Clostridium difficile toxins and Streptococcus mutans glucosyltransferases**. *J Bacteriol* 1992, **174**:6707-6710.
- Wren BW: **A family of clostridial and streptococcal ligand-binding proteins with conserved C-terminal repeat sequences**. *Mol Microbiol* 1991, **5**:797-803.

46. Smit E, Pouwels PH: **One repeat of the cell wall binding domain is sufficient for anchoring the *Lactobacillus acidophilus* surface layer protein.** *J Bacteriol* 2002, **184**:4617-4619.
47. Egelseer EM, Leitner K, Jarosch M, Hotzy C, Zayni S, Sleytr UB, Sára M: **The S-layer proteins of two *Bacillus stearothermophilus* wild-type strains are bound via their N-terminal region to a secondary cell wall polymer of identical chemical composition.** *J Bacteriol* 1998, **180**:1488-1495.
48. Ries W, Hotzy C, Schocher I, Sleytr UB, Sára M: **Evidence that the N-terminal part of the S-layer protein from *Bacillus stearothermophilus* PV72/p2 recognizes a secondary cell wall polymer.** *J Bacteriol* 1997, **179**:3892-3898.
49. Schäffer C, Kählig H, Christian R, Schulz G, Zayni S, Messner P: **The diacetamidodideoxyuronic-acid-containing glycan chain of *Bacillus stearothermophilus* NRS 2004/3a represents the secondary cell-wall polymer of wild-type *B. stearothermophilus* strains.** *Microbiology* 1999, **145**:1575-1583.
50. Ilk N, Kosma P, Puchberger M, Egelseer EM, Mayer HF, Sleytr UB, Sára M: **Structural and functional analyses of the secondary cell wall polymer of *Bacillus sphaericus* CCM 2177 that serves as an S-layer specific anchor.** *J Bacteriol* 1999, **181**:7643-7646.
51. Nagaoka M, Muto M, Nomoto K, Matuzaki T, Watanabe T, Yokokura T: **Structure of polysaccharide-peptidoglycan complex from the cell wall of *Lactobacillus casei* YIT9018.** *J Biochem* 1990, **108**:568-571.
52. Masuda K, Kawata T: **Characterization of a regular array in the wall of *Lactobacillus buchneri* and its reattachment to the other wall components.** *J Gen Microbiol* 1981, **124**:81-90.
53. Shimohashi H, Kodaira S, Suegara N: **Grouping antigens of four *Lactobacillus* species and their characteristics.** *Jpn J Microbiol* 1976, **20**(5):405-413.
54. Masuda K, Kawata T: **Reassembly of a regularly arranged protein in the cell wall of *Lactobacillus buchneri* and its reattachment to cell walls: chemical modification studies.** *Microbiol Immunol* 1985, **29**:927-938.
55. Masuda K, Kawata T: **Reassembly of the regularly arranged subunits in the cell wall of *Lactobacillus brevis* and their reattachment to cell walls.** *Microbiol Immunol* 1980, **24**:299-308.
56. Sára M: **Conserved anchoring mechanisms between crystalline cell surface S-layer proteins and secondary cell wall polymers in Gram-positive bacteria.** *Trends Microbiol* 2001, **9**:47-49.
57. Uchida H, Kinoshita H, Kawai Y, Kitazawa H, Miura K, Shiiba K, Horii A, Kimura K, Taketomo N, Oda M, Yajima T, Saito T: ***Lactobacilli* binding human A-antigen expressed in intestinal mucosa.** *Res Microbiol* 2006, **157**:659-665.
58. Woodcock D, Crowther P, Doherty J, Jefferson S, De Cruz E, Noyer-Weidner M, Smith S, Michael M, Graham M: **Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants.** *Nucleic Acids Res* 1989, **17**:3469-3478.

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