Structural and functional characterization of the surface layer protein of *Lactobacillus brevis* ATCC 8287

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SUMMARY

Bacterial surface (S) layers are proteinaceous arrays found on the surface of hundreds of bacterial species, including several species of lactobacilli. They are composed of numerous identical, non-covalently bound subunits, which completely cover the cell surface forming a symmetric, porous, lattice-like structure. Several functions for S-layers have been found, but no common one probably exists. S-layer proteins have a wide application potential in nanobiotechnology as well as in health-related applications such as vaccine design.

In this work, the structure and function of the S-layer protein SlpA of *Lactobacillus brevis* ATCC 8287 and the expression of the *slpA* gene were studied. SlpA was identified as a two-domain protein, in which the N-terminal domain is responsible for binding to the cell wall and the C-terminal domain for forming the regular polymer. The domain organization is thus reversed compared with other hitherto characterized *Lactobacillus* S-layer proteins. Conserved carbohydrate binding motifs were identified in the N-terminal, positively charged amino acid sequences of SlpA and five other *Lactobacillus brevis* S-layer proteins. The component in the cell wall interacting with SlpA was shown to be something other than teichoic or lipoteichoic acid, in contrast to the cell wall receptors of S-layer proteins previously characterized in lactobacilli. The structure of the C-terminal self-assembly domain was studied in more detail using cysteine scanning mutagenesis and targeted chemical modification. Importantly for the potential future applications of SlpA as a display vehicle of foreign peptides, four amino acid segments with high surface accessibility in the assembled form of SlpA were detected. The 46 mutated residues could be grouped according to their location in the lattice: in the protein interior, on the inner surface of the lattice, on the outer surface of the lattice and on the subunit interface or the pore region of the lattice.

L. brevis ATCC 8287 very efficiently adheres to cultured human epithelial cells representing the human gut, bladder and blood vessels, while the removal of the S-layer abolishes the binding. This binding was shown to be mediated by SlpA by using flagellum display. Hybrid flagella carrying fragments from the N-terminal part of SlpA bound to epithelial cells and to fibronectin, while flagella carrying the C-terminal part were unable to bind. The smallest fragment conferring binding to Int 407 cells comprised amino acids 66-215 in mature SlpA.

The gene encoding SlpA is preceded by two promoters. By separating them on reporter plasmids, both of the promoters were shown to be used in *L. brevis* in all growth phases. More upstream region was needed for the full activity of the upstream promoter than for the downstream promoter. The promoter activities seen at the reporter enzyme level were also seen at the mRNA level, suggesting transcriptional rather than translational regulation of *slpA*. Three potential regulatory motifs were identified in the upstream region of *slpA*. Both promoters retained their activities under selected conditions mimicking the intestinal environment *in vitro*.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals. These original articles are reprinted with the kind permission of their copyright holders.

I **Hynönen, U., B. Westerlund-Wikström, A. Palva, and T. K. Korhonen.** 2002. Identification by flagellum display of an epithelial cell- and fibronectin-binding function in the SlpA surface protein of *Lactobacillus brevis*. J. Bacteriol. **184:**3360-3367.

II **Åvall-Jääskeläinen, S., U. Hynönen, N. Ilk, D. Pum, U. B. Sleytr, and A. Palva.** 2008. Identification and characterization of domains responsible for self-assembly and cell wall binding of the surface layer protein of *Lactobacillus brevis* ATCC 8287. BMC Microbiol. **8:**165.

III **Vilen, H., U. Hynönen, H. Badelt-Lichtblau, N. Ilk, P. Jääskeläinen, M. Torkkeli, and A. Palva.** 2009. Surface location of individual residues of SlpA provides insight into *Lactobacillus brevis* S-layer. J. Bacteriol. **191**:3339-3349.

IV **Hynönen, U., S. Åvall-Jääskeläinen, and A. Palva.** 2009. Characterization and separate activities of the two promoters of the *Lactobacillus brevis* S-layer protein gene. Submitted manuscript.

ABBREVIATIONS

Aa	amino acid
AAD	antibiotic-associated diarrhoea
AFM	atomic force microscopy
Alexa	AlexaFluor488 C_5-maleimide
APC	antigen presenting cell
ATCC	American Type Culture Collection
BMP	bacterial magnetic particle
bp	base pair
BSA	bovine serum albumin
CRE	catabolite response element
Cryo-EM	cryoelectron microscopy
CSM	cysteine scanning mutagenesis
C-terminus	carboxyterminus
CW, CWF	cell wall fragment
Da	dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
EM	electron microscopy
FTIR	Fourier transform infrared
GFP	Green Fluorescent Protein
GnHCl, GHCl	guanidine hydrochloride
GRAS	generally recognized as safe
Hepes	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid
IEM	immunoelectron microscopy
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pair
LTA	lipoteichoic acid
Mono	monomeric
mPEG-maleimide	methyl-capped polyethylene glycol maleimide
mRNA	messenger ribonucleic acid
MW	molecular weight
NEM	N-ethyl maleimide
NEXAFS	near-edge X-ray absorption fine structure
NMR	nuclear magnetic resonance
N-terminus	aminoterminus
OMP	outer membrane protein
ORF	open reading frame
P	promoter

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	photoemission
PEG	polyethylene glycol
pI	isolelectric point
PIPES	1,4-piperazinediethanesulphonic acid
RBS	ribosome binding sequence
RNA	ribonucleic acid
RNAase	ribonuclease
rRNA	ribosomal ribonucleic acid
rSlpA	recombinant SlpA
SAXS	small angle X-ray scattering
SCWP	secondary cell wall polymer
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELDI-TOF	surface-enhanced laser desorption/ionization-time of flight
SFM	scanning force microscopy
S-layer	surface layer
SLH	S-layer homology
SS	signal sequence
t	transcription terminator sequence
TCA	trichloroacetic acid
TEM	transmission electron microscopy
$TMM(PEG)_{12}$	trimethyl maleimide polyethylene glycol, $(methyl\text{-}PEG_{12})_3\text{-}PEG_4$-maleimide
TRAC	transcript analysis with aid of affinity capture
TREC	topography and recognition imaging
UTR	untranslated region
vol	volume
wt	weight, wild type
Å	ångström, 0.1 nm

1. REVIEW OF THE LITERATURE

1.1 Introduction

1.1.1 Structure of the Gram-positive cell wall

The bacterial cell envelope consists of the cytoplasmic membrane and the overlying cell wall. The cell walls of Gram-negative and Gram-positive bacteria differ fundamentally in several respects: while the cell walls of Gram-negative bacteria are composed of a thin peptidoglycan layer covered by the outer membrane, the Gram-positive cell wall has no outer membrane and is characterized by a very thick peptidoglycan layer and abundant Gram-positive specific cell wall carbohydrates. Peptidoglycan is composed of glycan strands of variable length with alternating N-acetyl-muramic acid and N-acetyl-glucosamine molecules, which are interconnected by short peptides. According to the conventional model, this mesh-like structure lies horizontally to the cell surface, and in Gram-positive cell walls multiple layers are present, interconnected also in the vertical orientation. This mechanically very strong three-dimensional network, the basic function of which is to provide protection and maintain the shape of the cell, is decorated by other cell wall constituents, including proteins and teichoic and lipoteichoic acids, lipoglycans, teichuronic acids and other acidic or neutral polysaccharides (Delcour *et al.*, 1999; Navarre & Schneewind, 1999; Ton-That *et al.*, 2004; Holst & Müller-Loennies, 2007). In addition, capsular polysaccharides, forming a thick outermost polysaccharide layer, as well as exopolysaccharides are present in many Gram-positive species (Holst & Müller-Loennies, 2007). Polyglutamate capsules are also sometimes present

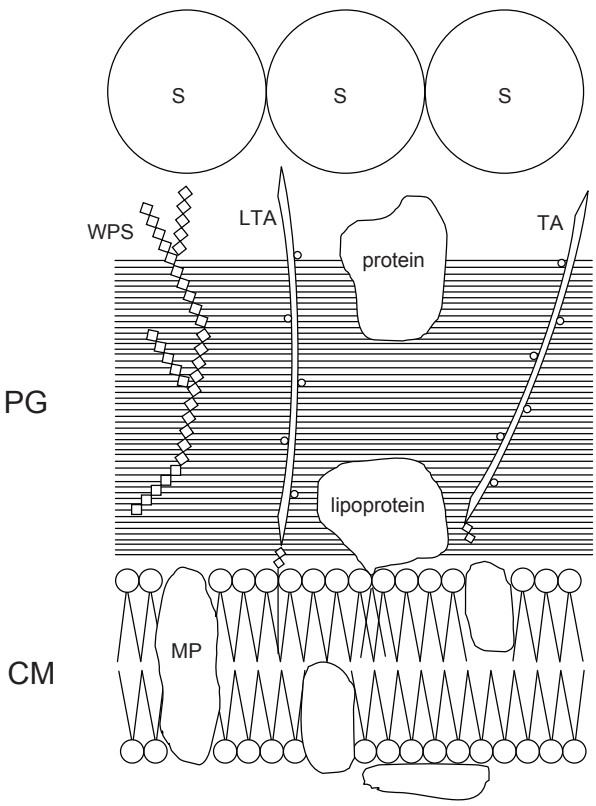

Figure 1. A schematic picture showing the typical constituents of the cell envelope of an S-layered Gram-positive bacterium. The figure is not drawn to scale.
CM, cytoplasmic membrane; PG, peptidoglycan; S, S-layer protein; WPS, wall polysaccharide; LTA, lipoteichoic acid; TA, teichoic acid; MP, membrane protein. Teichuronic acids, lipoglycans and capsular or exopolysaccharides are not depicted.

(Candela & Fouet, 2006). Teichoic acids, teichuronic acids and polysaccharides are collectively referred to as secondary cell wall polymers (SCWPs), and teichoic acids and teichuronic acids have been called "classical" SCWPs (Schäffer & Messner, 2005). Often also the membrane-linked components, lipoglycans, present mostly in high-GC% Gram-positive bacteria such as bifidobacteria (Fischer, 1994), and lipoteichoic acids have been included in SCWPs (Sara, 2001; Desvaux *et al.*, 2006; Dramsi *et al.*, 2008). A schematic presentation of the typical Gram-positive cell wall is shown in Fig.1.

The proteins anchored to the Gram-positive cell wall have been reviewed elsewhere (Desvaux *et al.*, 2006; Scott & Barnett, 2006), and the outermost protein layer frequently present in the cell envelope, the surface (S) layer, will be discussed in detail in Sections 1.1.2 and 1.2. The most often occurring teichoic acids are polyol (usually glycerol or ribitol) phosphate or glycosylpolyol phosphate polymers, typically substituted by glucose and/or esterified by alanine and covalently attached to the muramic acid molecules of peptidoglycan through a glycosidic linkage unit. Lipoteichoic acids essentially differ from teichoic acids only in that they are attached to the cytoplasmic membrane via a glycolipid anchor, which is a diacylglycerol molecule bound to a di- or trisaccharide. Due to the abundance of phosphate groups, both types of molecules are highly negatively charged, the charge being regulated by the level of D-alanylation (Delcour *et al.*, 1999; Navarre & Schneewind, 1999; Naumova *et al.*, 2001; Holst & Müller-Loennies, 2007). Owing to the covalent linkage to peptidoglycan, teichoic and teichuronic acids are sometimes collectively called wall teichoic acids. Teichuronic acids are, however, completely different in structure, as they are composed of sugar monomers directly linked by glycosidic bonds and usually no linkage unit is present (Araki & Ito, 1989; Delcour *et al.*, 1999). They are devoid of phosphate groups; instead, in the teichuronic acids studied thus far, the negative charges are provided by the carboxyl groups of uronic, usually glucuronic or mannosamine uronic, acid residues. Teichuronic acids have been described in *Bacillus*, *Micrococcus* and *Streptomyces* species (Hase & Matsushima, 1972; Ward, 1981; Shashkov *et al.*, 2002), and according to some classifications (Sara, 2001), also in *Geobacillus* (Schäffer *et al.*, 1999); in *B. subtilis*, they replace teichoic acids under phosphate-deprivation conditions (Lang *et al.*, 1982). They are, however, likely to occur also in lactobacilli (Delcour *et al.*, 1999), although until now they have not been described in lactic acid bacteria.

The "non-classical SCWPs" or "wall polysaccharides" are distinguished from capsular polysaccharides, which form an outermost, thick, hydrated shell covalently or non-covalently bound to the cell surface, and from exopolysaccharides (slimes), which are released to the medium (Delcour *et al.*, 1999; Holst & Müller-Loennies, 2007). Generally, they include glycosyl phosphate polymers (according to Araki and Ito, 1989, however, classified as teichoic acids) and anionic or neutral sugar polymers and are covalently attached to peptidoglycan. The sugar polymers are composed of repeated sugar units, where the negative charge very often present arises from acidic substituents such as sulphate or glycerol-phosphate groups or organic acids (Hancock & Poxton, 1988; Schäffer & Messner, 2005). The "non-classical" SCWPs of some members of the family *Bacillaceae* having S-layers have been studied in detail

(Schäffer & Messner, 2005). The polysaccharides characterized were acidic or neutral heteropolysaccharides composed of 2-15 repeating units with 2-5 sugars in each unit. Six different monosaccharide constituents and both linear and branched chains were detected, and the polysaccharides could be classified into three groups according to the sugar backbone structures. The non-carbohydrate modifications decorating the sugar backbone were pyruvate, phosphate or acetate.

1.1.2 Surface layer proteins

1.1.2.1 Occurrence, general features and study methods

Surface (S) layers are cell envelope structures ubiquitously found in Gram-positive and Gram-negative bacterial species as well as in *Archaea* (Sara & Sleytr, 2000). They form the outermost proteinaceous layer on the cell and are sometimes 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 completely cover the cell surface, forming a crystalline, two-dimensional, regular and porous array with oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetry. The subunits of bacterial S-layers are held together and attached to the underlying cell surface by non-covalent interactions, and they have an intrinsic ability to spontaneously form regular layers either in solution or on a solid support after the removal of the disintegrating agent (Sara & Sleytr, 2000). The recrystallization of *Bacillus sphaericus* S-layer protein subunits on hydrophobic silicon surfaces has been studied in real time by atomic force microscopy; the subunits are initially randomly adsorbed to the surface, assembled into small crystalline patches and the number of the patches increases until finally a monolayer is formed (Györvary *et al.*, 2003). The reassembly of S-layer proteins in solution (Teixeira *et al.*, 2009) and on lipid membranes and polyelectrolyte layers has also been studied in detail by biophysical methods (Weygand *et al.*, 1999; Weygand *et al.*, 2000; Weygand *et al.*, 2002; Delcea *et al.*, 2008). However, the incorporation of subunits into the growing S-layer on bacterial cells is largely unexplored. S-layer proteins may be modified by phosphorylation or glycosylation (Sara & Sleytr, 2000). Glycosylated S-layer proteins are very common among *Archaea*, but they are also found in Gram-positive bacteria and have recently been detected in some Gram-negative species. The bacterial S-layer glycan chains characterized to date are O-glycosidically linked, linear or branched homo- or heterosaccharides of 50-150 glycoses, organized into 15-50 repeating units. The general 1-10 % (wt/wt) degree of glycosylation of bacterial S-layer glycoproteins may be dependent on growth conditions (Messner *et al.*, 2008).

The primary structures of bacterial S-layer proteins are similar in that they are generally rich in acidic, hydrophobic and hydroxyl-containing amino acids, and cysteines are very rarely found. The predicted pI values are usually in a weakly acidic range. Sequence similarity between S-layer protein genes, if any, is typically found only between the S-layer protein genes of closely related species (Boot & Pouwels, 1996; Sara & Sleytr, 2000). An exception are the so-called SLH (S-layer homology) motifs (Lupas *et al.*, 1994), which are, however, not present in all S-layer proteins and are found in other proteins as well (see Section 1.1.2.3). Since a universal

"signature" for identifying a protein as an S-layer protein is lacking, the unambiguous identification of surface-expressed S-layers still relies on transmission electron microscopy (TEM) of whole bacterial cells or cell wall fragments. Several electron microscopic techniques have been used, of which freeze-etching and freeze-drying in combination with heavy metal shadowing are the most feasible for this purpose (Schuster *et al.*, 2006); thin sectioning (Jakava-Viljanen *et al.*, 2002; Ventura *et al.*, 2002) and immunogold-labelling (Vidgren *et al.*, 1992) have been used as well. For further structural analysis of S-layer lattices or modified S-layer lattices, other microscopical and biophysical methods have been applied, including cryo-electron microscopy (Lembcke *et al.*, 1993) and cryo-electron tomography (Trachtenberg *et al.*, 2000), cryoelectron microscopy of lipid monolayer crystals (Norville *et al.*, 2007), scanning force microscopy (SFM) (also known as atomic force microscopy (AFM) (Müller *et al.*, 1996; 1999; Scheuring *et al.*, 2002; Györvary *et al.*, 2003; Schär-Zammaretti & Ubbink, 2003; Toca-Herrera *et al.*, 2004; Martin-Molina *et al.*, 2006; Anselmetti *et al.*, 2007; Verbelen *et al.*, 2007; Dupres *et al.*, 2009; Tang *et al.*, 2009) or its application TREC (topography and recognition imaging) (Tang *et al.*, 2008), electron microscopy of non-stained S-layer samples combined with electron holography (Simon *et al.*, 2004), photoemission (PE) and near-edge X-ray absorption fine structure (NEXAFS) spectroscopy (Vyalikh *et al.*, 2005; Kade *et al.*, 2007) and small-angle X-ray spectroscopy (SAXS) of S-layered bacterial cells or self-assembly products (Aichmayer *et al.*, 2006; P. Jääskeläinen, personal communication).

Secondary structures of S-layer proteins are difficult to predict because the prediction algorithms are based on the available structures of very dissimilar types of proteins. Circular dichroism (CD) measurements have been performed mainly for the S-layer proteins of *Bacillus* species (Sara & Sleytr, 2000; Rüntzler *et al.*, 2004). These studies have revealed an α-helix content of approximately 20%, a β-sheet content of 40%, and 5-45% aperiodic folding and β-turns in these proteins. The α-helices were mostly predicted to reside in the N-terminal parts of the proteins (Sara & Sleytr, 2000). Elucidation of the tertiary structure of S-layer proteins has been hindered by their molecular weights not being in the suitable range (<40 kDa) for nuclear magnetic resonance (NMR) studies, and by their low solubility; more specifically, their tendency to form two-dimensional lattices rather than three-dimensional crystals in solution. Therefore, only two structures of bacterial S-layer protein fragments obtained by X-ray crystallization have thus far been available (Pavkov *et al.*, 2008; Fagan *et al.*, 2009). Therefore, physical methods, such as fluorescence spectroscopy (Rüntzler *et al.*, 2004), SAXS (Pavkov *et al.*, 2008; Fagan *et al.*, 2009), combined genetic and biochemical approaches, such as cysteine scanning mutagenesis and chemical cross-linking (Howorka *et al.*, 2000; Kinns & Howorka, 2008), structure prediction by the mean force method (Horejs *et al.*, 2008) and electron microscopy (Norville *et al.*, 2007) have been applied to gain insight into the three-dimensional structures of S-layer proteins. By calculating projection maps from electron micrographs of lipid monolayer crystals, structural information down to a resolution of 7 Å has been obtained (Norville *et al.*, 2007).

1.1.2.2 Expression of S-layer protein genes

Many bacterial species have more than one S-layer gene, but not all of them are necessarily expressed at the same time: both silent genes, antigenic variation based on S-layer gene expression (reviewed by Boot & Pouwels, 1996; Sara & Sleytr, 2000), 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 (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) have been described. As S-layer proteins typically account for 10-25% of total cellular protein (Boot & Pouwels, 1996; Smit, 2008), the expression of S-layer protein genes and the secretion of the proteins must be very efficient. With the exception of the S-layer proteins of *Caulobacter crescentus* (Awram & Smit, 1998), *Serratia marcescens* (Kawai *et al.*, 1998) and *Campylobacter fetus* (Thompson *et al.*, 1998), which are secreted through the ATP-dependent type I machinery, and the S-layer protein of *Campylobacter rectus*, which does not have an N-terminal signal peptide either (Wang *et al.*, 1998), bacterial S-layer proteins are secreted by the Sec-dependent, general secretory pathway (Sara & Sleytr, 2000). The very efficient expression of S-layer protein genes is contributed by their efficient promoters, a biased codon usage typical of efficiently transcribed genes and by the long half-lives of S-layer gene transcripts, which, in some cases, may be due to their long untranslated leader sequences (Boot & Pouwels, 1996; Boot *et al.*, 1996b). Furthermore, many S-layer protein genes are preceded by more than one promoter, which may not only increase the transcription efficiency but also offers a way to regulate the S-layer gene expression in response to, for instance, growth stage (Adachi *et al.*, 1989) or environmental conditions (Novotny *et al.*, 2008). Chromosomal rearrangements cause variation in S-layer gene expression in *Campylobacter fetus* (Dworkin & Blaser, 1996; 1997), *Geobacillus stearothermophilus* (Egelseer *et al.*, 2001; Scholz *et al.*, 2001) and lactobacilli (see Section 1.2.2). However, excluding the thoroughly studied regulation of the S-layer protein genes of *Bacillus anthracis* (reviewed by Mignot *et al.*, 2004; Fouet, 2009), the modulation of bacterial S-layer gene expression by soluble factors is poorly known. Carbon source regulates the S-layer protein production in *Corynebacterium* strains (Soual-Hoebeke *et al.*, 1999), and molecular investigations have revealed the presence of a transcriptional activator of the S-layer protein gene in this species (Soual-Hoebeke *et al.*, 1999; Hansmeier *et al.*, 2006) as well as in *Aeromonas salmonicida* (Noonan & Trust, 1995). In *Thermus thermophilus*, in addition to transcriptional regulation, translational autoregulation of S-layer protein gene expression has been suggested, as the C-terminal fragment of the S-layer protein SlpA specifically binds to the 5' UTR of the *slpA* mRNA *in vitro* (Fernandez-Herrero *et al.*, 1997). Later, the 5' UTR of the *T. thermophilus* S-layer protein gene has been shown to be responsible for the growth phase-dependent repression of the S-layer protein (Castan *et al.*, 2001). The temperature-regulation of *sgsE* in *Geobacillus stearothermophilus* NRS 2004/3a has been suggested to occur at the transcriptional level (Novotny *et al.*, 2004; Novotny *et al.*, 2008). In *Clostridium difficile*, the exposure to high osmolarity or antibiotics increases S-layer gene expression, but the mechanism is not known (Deneve *et al.*, 2008).

1.1.2.3 Cell wall binding and self-assembly regions

According to present knowledge, bacterial S-layer proteins in general have two structural and functional regions: a region involved in the attachment of the S-layer subunit to the cell envelope and a region involved in S-layer assembly. The S-layer proteins of Gram-negative bacteria bind to the O-polysaccharide part of lipopolysaccharides of the outer membrane (Griffiths & Lynch, 1990; Kokka *et al.*, 1990; Walker *et al.*, 1994; Ford *et al.*, 2007), and the S-layer protein of the Gram-positive *Corynebacterium glutamicum*, which has an unusual mycolic acid-containing cell wall, binds to the hydrophobic layer above the cytoplasmic membrane (Chami *et al.*, 1997; Bayan *et al.*, 2003). The Hpi protein of the Gram-positive *Deinococcus radiodurans* is also in contact with the underlying lipid-rich layer of the cell wall, but the contributions of Hpi and the additional S-layer protein SlpA to cell wall anchoring of the S-layer are not clear (Rothfuss *et al.*, 2006). The known interactions between the S-layer protein and the cell wall in Gram-positive bacteria are summarized in Table 1. In many Gram-positive bacilli (Mesnage *et al.*, 2000) and in the ancient thermophile *Thermus thermophilus* (Olabarria *et al.*, 1996; Cava *et al.*, 2004), SLH motifs (Lupas *et al.*, 1994), 55-60 amino acids long and often located in the N-terminal part of the S-layer protein, are responsible for the attachment of the subunit proteins to the cell wall. SLH motifs are not restricted to S-layer proteins, but are found in hundreds of bacterial (Gram-positive and -negative), archaeal, eukaryotic and even viral proteins (http://pfam.sanger.ac.uk//family/slh, cited Aug 31, 2009). In most of the studied *Bacillus*, *Lysinibacillus*, *Geobacillus* and *Thermus* species, the binding of the SLH motifs of the S-layer protein has been shown to occur through a pyruvate-containing polysaccharide receptor in the cell wall, while in *Clostridium thermocellum* F1 the binding of SLH motifs to peptidoglycan has been demonstrated. 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 of the S-layer protein and negatively charged cell wall carbohydrates. For example, the cell wall receptors of such S-layers in *Geobacillus* species characterized so far contain mannuronic acid, and teichoic and lipoteichoic acids have been shown to be the cell wall receptors of the S-layer proteins of *Lactobacillus acidophilus* and *L. crispatus* (see Section 1.2.3). However, some cell wall polysaccharides of Gram-positive bacteria proposed to be involved in S-layer binding have a net neutral charge (Steindl *et al.*, 2002; Schäffer & Messner, 2005). In any case, most interactions characterized thus far between S-layer proteins and underlying cell wall polymers can be considered lectin-like and a degree of specificity is recognized (Sara & Sleytr, 2000). At present, structures of secondary cell wall polymers are also available for Gram-positive bacteria with structurally and/or genetically uncharacterized S-layer proteins (Schäffer *et al.*, 2000; Schäffer & Messner, 2005; Steindl *et al.*, 2005).

Among Gram-positive bacteria, the self-assembly regions of S-layer proteins have thus far been investigated in the S-layers of lactobacilli (see Section 1.2.3) and in the S-layers of *Bacillus anthracis*, *Lysinibacillus sphaericus* and *Geobacillus stearothermophilus*. These studies mainly rely on electron microscopy of recombinant S-layer protein fragments, and the self-assembly region has been shown to be located centrally or at the C- or N-terminus. The experimentally verified self-assembly regions

Table 1. Interactions of S-layer proteins of Gram-positive bacteria with the cell wall.

Strain	S-layer protein	Interaction site in S-layer protein	Cell wall receptor	Reference
Bacillus anthracis	Sap, EA1	N-terminal, three SLH motifs	Pyruvic acid-containing polysaccharide	Mesnage *et al.*, 1999a; 2000
Lysinibacillus sphaericus CCM 2177	SbpA	N-terminal, three SLH motifs, one SLH-like motif	Pyruvic acid-containing polysaccharide	Ilk *et al.*, 1999; Huber *et al.*, 2005
Lysinibacillus sphaericus C3-41	SlpC	N-terminal, three SLH motifs, third essential	Polysaccharide	Li *et al.*, 2009
Bacillus thuringiensis ssp. *galleriae* NRRL 4045	SlpA	N-terminal, three SLH motifs	Pyruvic acid-containing polysaccharide	Mesnage *et al.*, 2001
Geobacillus stearothermophilus PV72/ p2	SbsB	N-terminal, three SLH motifs	Pyruvic acid-containing polysaccharide	Ries *et al.*, 1997; Sara *et al.*, 1998; Mader *et al.*, 2004; Rüntzler *et al.*, 2004
Thermus thermophilus HB8	SlpA	One N-terminal SLH motif	Pyruvic acid-containing polysaccharide	Olabarria *et al.*, 1996; Cava *et al.*, 2004
Thermoanaerobacterium thermosulfurigenes EM1	S-layer protein	N-terminal, at least one SLH motif	Pyruvic acid-containing polysaccharide	Brechtel & Bahl, 1999; May *et al.*, 2006
Clostridium thermocellum NCIMB 10682	SlpA	N-terminal, three SLH motifs	Not determined	Lemaire *et al.*, 1998
Clostridium thermocellum F1	Slp1, Slp2	C-terminal, three SLH motifs	Peptidoglycan	Zhao *et al.*, 2006
Geobacillus stearothermophilus ATCC 12980	SbsC	N-terminal	Mannuronic acid-containing polysaccharide	Egelseer *et al.*, 1998; Schäffer *et al.*, 1999; Ferner-Ortner *et al.*, 2007
Geobacillus stearothermophilus ATCC 12980/G+	SbsD	N-terminal (postulated)	Mannuronic acid-containing polysaccharide	Egelseer *et al.*, 2001; Schäffer *et al.*, 1999
Geobacillus stearothermophilus PV72/ p6	SbsA	N-terminal	Mannuronic acid-containing polysaccharide	Egelseer *et al.*, 1998; Schäffer *et al.*, 1999
Geobacillus stearothermophilus NRS 2004/3a	SgsE	N-terminal (based on sequence similarity)	Mannuronic acid-containing polysaccharide	Schäffer *et al.*, 1999; Schäffer *et al.*, 2002; Schäffer *et al.*, 2007
Aneurinibacillus thermoaerophilus DSM 10155	SatB	Not known	Neutral polysaccharide	Steindl *et al.*, 2002; Schäffer & Messner, 2005
Corynebacterium glutamicum	PS2	C-terminal, hydrophobic	Mycomembrane containing mycolic acids (suggested)	Chami *et al.*, 1997
Lactobacillus acidophilus ATCC 4356	SA	C-terminal	Teichoic acids	Smit & Pouwels, 2002
Lactobacillus crispatus JCM 5810	CbsA	C-terminal	Teichoic acids	Antikainen *et al.*, 2002

of S-layer proteins of Gram-positive bacteria are summarized in Table 2.

Table 2. Self-assembly regions in S-layer proteins of Gram-positive bacteria.

Strain	S-layer protein	Location of crystallization region (residues / total residues)	Reference
Bacillus anthracis	Sap	C-terminal (211-814 / 814)	Candela *et al.*, 2005
Lysinibacillus sphaericus CCM 2177	SbpA	Central (203-1031 /1268)*#	Huber *et al.*, 2005
Geobacillus stearothermophilus PV72/p2	SbsB	C-terminal (177-889 / 889)	Rüntzler *et al.*, 2004
Geobacillus stearothermophilus ATCC 12980	SbsC	Central (258-920 / 1099)*	Jarosch *et al.*, 2001
Lactobacillus acidophilus ATCC 4356	SA	N-terminal (32-321 / 413)	Smit *et al.*, 2001
Lactobacillus crispatus JCM 5810	CbsA	N-terminal (32-271 / 410)	Antikainen *et al.*, 2002

* Signal sequence included in the numbering.

Conclusions drawn from separate N- and C-terminal truncations.

1.1.2.4 Functions

Considering the wide occurrence of S-layers in the microbial world, information about their functions is still insufficient, and no common function for all S-layers appears to exist. The functions characterized thus far include the determination and maintenance of cell shape, various protective functions and actions as a molecular sieve, as a binding site for large molecules or ions and as a mediator of bacterial adhesion; the contribution to virulence reported for the S-layers of many pathogens may result from many of these functions (Sara & Sleytr, 2000). Furthermore, one S-layer protein thus far, SwmA of a marine *Synechococcus* strain, has been shown to be involved in motility (Brahamsha, 1996; McCarren *et al.*, 2005), and for three S-layer proteins, those of *Clostridium difficile*, *Bacillus anthracis* and *Lactobacillus acidophilus*, a degradative enzymatic function has been demonstrated (Calabi *et al.*, 2001; Ahn *et al.*, 2006; Prado Acosta *et al.*, 2008). More specifically, S-layers may offer protection against mechanical and osmotic stress (Engelhardt, 2007a; b), radiation (Kotiranta *et al.*, 1999), changes in the environmental pH (Gilmour *et al.*, 2000), bacteriophages (Howard & Tipper, 1973), bacterial or eukaryotic microbial predators (Koval & Hynes, 1991; Tarao *et al.*, 2009) or enzymes (Lortal *et al.*, 1992). They may act as binding sites for exoenzymes (Matuschek *et al.*, 1994; Egelseer *et al.*, 1995; Peters *et al.*, 1995; Egelseer *et al.*, 1996), immunoglobulins (Phipps & Kay, 1988), porphyrins (Kay *et al.*, 1985) or phages (Howard & Tipper, 1973; Ishiguro *et al.*, 1984; Fouet, 2009), or catch toxic metals (Pollmann *et al.*, 2006) or calcium leading to mineral formation (Schultze-Lam *et al.*, 1992). In pathogenic bacteria, S-layers may contribute to virulence by several mechanisms, including adhesion to host tissues or cells, antigenic variation, protection from phagocytosis or complement (reviewed by Kotiranta *et al.*, 2000; Sara & Sleytr, 2000) or by suppression (Wang *et*

al., 2000) or induction (Ausiello *et al.*, 2006) of cytokine secretion. On the other hand, also the S-layer protein of a health-promoting *Lactobacillus* strain has been shown to interact specifically with immune cells, regulating their function through cytokine induction (Konstantinov *et al.*, 2008).

1.1.2.5 Applications

Applications of S-layers can be divided into two groups. The first comprises applications utilizing (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 (nano)biotechnological applications. In vaccine development, the high antigen amount provided by the S-layer (carrier) as well as the intrinsic adjuvant and immunostimulatory properties of the S-layer arrays (Smith *et al.*, 1993) may be advantageous (Seegers, 2002; Wells & Mercenier, 2008). As a few examples, S-layer protein preparations purified from pathogens have been tested as vaccines in fish (Lund *et al.*, 2003) or in animal models for AAD (antibiotic-associated diarrhoea) (Ni Eidhin *et al.*, 2008), and S-layers chemically coupled with polysaccharide antigens have shown potential as therapeutic cancer vaccines or as traditional vaccines in animal models (reviewed by Sleytr *et al.*, 1999). S-layer-antigen fusion proteins, either on/in bacterial cells or as isolated proteins, have produced humoral responses and/or protection against challenge in animals (Mesnage *et al.*, 1999b; Umelo-Njaka *et al.*, 2001; Riedmann *et al.*, 2003; Liu *et al.*, 2008), and an S-layer-allergen fusion protein has proved effective in modulating the immune response to a more favourable one in experiments utilizing immune cells of allergic humans *in vitro* (Bohle *et al.*, 2004). As further examples of the first application group, tools for immunoassays or for bioremediation have been generated by the display of the immunoglobulin binding domain of Protein G (Nomellini *et al.*, 2007) or a hexahistidine tag (Wang *et al.*, 2004; Patel *et al.*, 2009), respectively, in the S-layer protein on bacterial cells. Further, an immunogenic mycobacterial peptide has been efficiently produced in a biologically active form using S-layer gene expression signals (Salim *et al.*, 1997), and a plasmid-based secretion system utilizing the secretion signal of the S-layer protein of *Caulobacter crescentus* has been developed and commercialized (Bingle *et al.*, 1997a; 2000; Duncan *et al.*, 2005). The second group of applications is largely based on the ability of S-layer proteins to spontaneously form periodic, porous structures on various supports with identical physicochemical properties on each molecular unit down to the nanometer scale, and this application field is expanded further by the use of fusion proteins. Several excellent reviews on nanobiotechnological applications of S-layer proteins are available (Pum & Sleytr, 1999; Sleytr *et al.*, 1999; Schuster *et al.*, 2006; Sleytr *et al.*, 2007; Schuster & Sleytr, 2009). Some of the conventional applications in this field include the use of S-layers as ultrafiltration membranes and as matrices for the covalent attachment of molecules (enzymes, antibodies, protein A, biotin, avidin, fluorophores) for use in affinity membranes, amperometric or optical biosensors or solid-phase immunoassays (Pum & Sleytr, 1999; Sleytr *et al.*, 1999; Sleytr & Beveridge, 1999; Scheicher *et al.*, 2009). More recently, S-layers proteins have been

genetically fused to e.g. enzymes, streptavidin, specific antibody fragments, green fluorescent protein (GFP) and a protein A immunoglobulin-binding domain analogue (Z domain); these fusion proteins, which retain the ability to recrystallize, may find numerous applications varying from biosensors and label-free detection systems to blood detoxification (Schuster *et al.*, 2006; Schäffer *et al.*, 2007; Sleytr *et al.*, 2007; Tschiggerl *et al.*, 2008). S-layer proteins can also be recrystallized on lipid films and liposomes, which causes a remarkable stabilization of these structures. S-liposomes have a broad application potential as drug or plasmid delivery and targeting systems with a possibility for specific receptor-mediated intake. As S-layer-supported lipid membranes on porous or solid supports maintain their functionality and allow even single membrane protein (pore) recordings, they are valuable tools in drug discovery and protein-ligand screening and have potential as membrane biosensors and in the development of electronic and optical devices (Schuster *et al.*, 2006; Sleytr *et al.*, 2007; Schuster & Sleytr, 2009). S-layers have been used in microlithographic procedures in which patterns are formed on S-layers on solid supports by ultraviolet irradiation (Pum *et al.*, 1997a;b; Sleytr *et al.*, 1999); the patterned S-layers are currently used as resistors in electronics (Pum & Sleytr, 1999). Finally, S-layers have been exploited in the formation of regularly arranged nanoparticles for applications in molecular electronics and non-linear optics. These applications include wet chemical processes, *i.e.* the formation of nanoparticle superlattices on the S-layers in metal-salt solutions, including processes in which the binding nanoparticles are preformed and thus of defined size, and systems in which S-layers act as etching masks before the deposition of the particle-forming metal (Pum & Sleytr, 1999; Sleytr *et al.*, 1999; 2007; Badelt-Lichtblau *et al.*, 2009). At present, most of the above mentioned applications are, however, at the stage of invention and development rather than in commercial use.

1.2. Lactobacilli and their S-layer proteins

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. Besides having a long history of use in food and feed fermentations, lactic acid bacteria have aroused interest because of the health beneficial (probiotic) properties of some strains. Probiotic preparations have been shown to be effective in, for example, the treatment or prevention of rotavirus or antibiotic associated diarrhea, relief of the symptoms of irritable bowel syndrome, treatment of inflammatory bowel disease or pouchitis, prevention and treatment of atopic disease and prevention of recurrent urinary tract infections in women (Pham *et al.*, 2008). Other beneficial effects, such as beneficial influences on malignancies, on plasma lipid levels or on lactose maldigestion in humans, have also been suggested (Ouwehand *et al.*, 2002; Ljungh & Wadström, 2006). Furthermore, lactic acid bacteria are attractive candidates for biotechnological health-related applications currently under investigation, such as oral vaccination, passive immunization, tolerance induction or the development of strains producing pharmaceutically important proteins (enzymes, microbicides, cytokines) *in vivo* (Seegers, 2002; Steidler, 2003; Wells & Mercenier, 2008).

The genus *Lactobacillus* forms a large, very heterogeneous group among lactic acid bacteria, the one most often used in probiotic preparations. It consists of non-sporulating, anaerobic or microaerophilic, catalase-negative, fermentative organisms with a low G+C percent (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 & Weiss, 1986; Axelsson, 1998; Hayashi *et al.*, 2005; Felis & Dellaglio, 2007). According to recent culture-independent enumerating methods utilizing either tissues or contents of the gastrointestinal canal of humans, they seem, however, to represent a minor proportion (0.01-4.9%) of the total microbial flora and part of this may comprise transients. In contrast, in the human oral cavity, lactobacilli may attain considerable populations (Walter, 2008), and in the human female urogenital tract they usually dominate the healthy microbiota (Redondo-Lopez *et al.*, 1990; Zhou *et al.*, 2007). In animals, lactobacilli are found in the crops (Fuller & Brooker, 1974; Guan *et al.*, 2003) and ceca (Zhu *et al.*, 2002) of chickens and in the gastrointestinal tracts of pigs (Fuller *et al.*, 1978; Pedersen & Tannock, 1989; Pryde *et al.*, 1999; Leser *et al.*, 2002; Konstantinov *et al.*, 2006), horses (Yuki *et al.*, 2000; Bailey *et al.*, 2003; Al Jassim *et al.*, 2005), ruminants (Krause *et al.*, 2003; Collado & Sanz, 2007; Busconi *et al.*, 2008) and rodents (Savage *et al.*, 1968; Morotomi *et al.*, 1975; Diaz *et al.*, 2004; Lesniewska *et al.*, 2006). The *Lactobacillus brevis* strain ATCC 8287 used in this study has originally been isolated from green fermented olives. *L. brevis* is often detected in the oral cavity and faeces of humans (Walter, 2008), and the strain ATCC 8287 has been shown to survive passage through the human gastrointestinal tract (Rönkä *et al.*, 2003).

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

In the genus *Lactobacillus*, S-layers have been found in several, but not all, species. In public databases, sequences of S-layer protein genes from strains of *L. brevis*, *L. helveticus*, *L. suntoryeus* and organisms of the former *L. acidophilus* group (Johnson *et al.*, 1980), including *L. acidophilus*, *L. crispatus* and *L. gallinarum*, are available. Furthermore, the Apf1 and Apf2 proteins of *L. gasseri* and *L. johnsonii* of the same group, the gene sequences of which are available, have been described as S-layer-like (Ventura *et al.*, 2002). In addition, strains of *L. amylovorus* (Boot *et al.*, 1996a), *L. buchneri* (Masuda & Kawata, 1981; 1983), *L. kefir* and *L. parakefir* (Garrote *et al.*, 2004) have been shown to possess an S-layer, although the genes have not been sequenced. S-layers have been demonstrated by electron microscopy also on *L. fermentum* and *L. delbrueckii* subspecies *bulgaricus* (Kawata et al., 1974; Masuda & Kawata, 1983), but the species identification of these strains has subsequently been questioned (Boot *et al.*, 1996a), and at present these species can be considered non-S-layered. Likewise, in an early study, a regular layer was seen on *L. casei* (Barker & Thorne, 1970), but according to Boot *et al.* (1996b), no S-protein encoding genes are present in this species, and the isolate probably would now be reclassified to another species.

All of the *Lactobacillus* S-layer proteins characterized thus far are preceded by a 25-30 amino acid signal peptide indicating secretion through the general secretory pathway. The deduced amino acid sequences of mature *Lactobacillus* S-layer proteins vary considerably, the range of identical amino acids varying from 7% to 100% (Åvall-Jääskeläinen & Palva, 2005), and even the S-layer proteins of the same strain may be markedly different in sequence (Jakava-Viljanen *et al.*, 2002; Hagen *et al.*, 2005). As in the case of S-layers in general, similarity between the deduced amino acid sequences, when present, can be found only between related species, *e.g.* between the S-layer proteins of the former *L. acidophilus* group organisms and *L. helveticus* (Antikainen *et al.*, 2002; Hagen *et al.*, 2005). However, when the phylogenetic trees constructed on the basis of 16S rRNA or *tuf* gene sequences of a set of *L. acidophilus*-related organisms, including strains of the novel *L. suntoryeus* species, were compared with those constructed on the basis of S-layer protein genes of the same species, the novel strains no longer grouped together, indicating strong selective pressure forcing the diversification of S-layer protein genes within *L. acidophilus*-related organisms as well (Cachat & Priest, 2005). In a similar analysis, however, the comparison of phylogenetic trees based on 22 deduced *Lactobacillus* S-layer protein sequences and 16S rRNA sequences of corresponding *Lactobacillus* species available revealed a similar overall clustering of strains (Åvall-Jääskeläinen & Palva, 2005).

S-layer proteins of lactobacilli differ from S-layer proteins in general in their smaller size (25-71 kDa) and a high predicted overall pI value (9.4-10.4). The lattices formed by *Lactobacillus* S-layer proteins characterized thus far are of oblique or hexagonal type (Åvall-Jääskeläinen & Palva, 2005). An electron micrograph of the S-layer lattice of *Lactobacillus brevis* is shown in Fig. 2. A glycan structure on a

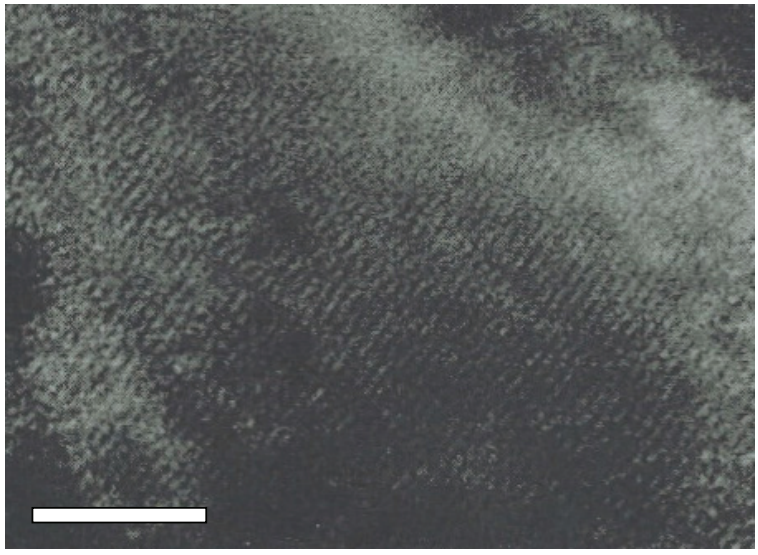

Figure 2. The self-assembly product of the recombinant S-layer protein of *Lactobacillus brevis* ATCC 8287 observed by negative staining and transmission electron microscopy. Bar, 100 nm. Figure by Ulla Hynönen.

Lactobacillus S-layer protein has to date been described only for *L. buchneri* (Messner *et al.*, 2008), but glycosylated S-layer proteins have been described also in *L. kefir* (Mobili *et al.*, 2009a). As mentioned earlier, secondary structure predictions for S-layer proteins are of limited value thus far (see Section 1.1). 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 coil in these proteins (Åvall-Jääskeläinen & Palva, 2005). In the S-layer-like proteins Apf1 and Apf2 of *L. gasseri* and *L. johnsonii*, the β-sheet content was predicted to be 26-31% and the overall folding of the proteins was suggested to be irregular (Ventura *et al.*, 2002). Physical measurements revealing secondary structures have been performed for a few *Lactobacillus* species. A Fourier transform infrared (FTIR) 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 coil, of 37-63 % in these proteins. Interestingly, the proportions of α-helix, β-sheet and other structures in SlpA of *L. brevis* ATCC 8287 studied in this thesis work, were 0%, 50% and 50%, respectively (Mobili *et al.*, 2009b). Atomic force microscopy (AFM) 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). Until now, no three-dimensional structures of *Lactobacillus* S-layer proteins on atomic resolution have been available.

1.2.2 Expression of *Lactobacillus* S-layer protein genes

The very efficient synthesis of S-layer proteins in lactobacilli is achieved by several means: i) The half lives of the S-layer protein gene transcripts of *L. brevis* (Kahala *et al.*, 1997) and *L. acidophilus* (Boot *et al.*, 1996b) have been determined to be exceptionally long (14 and 15 min, respectively). In the case of *L. acidophilus*, this is supposed to be due to the long 5´ untranslated region (UTR) of the transcript forming a stabilizing secondary structure (Boot *et al.*, 1996b), while the 5´ UTR of *L. brevis slpA* transcript is not exceptionally long (Vidgren *et al.*, 1992). ii) A biased codon usage, correlating with efficient gene expression in lactobacilli (Pouwels & Leunissen, 1994), has been observed for the S-layer protein genes of *L. brevis* (Vidgren *et al.*, 1992) and *L. acidophilus* (Boot *et al.*, 1995) as well as for the S-layer-like protein genes *apf1* and *apf2* of *L. gasseri* and *L. johnsonii* (Ventura *et al.*, 2002). iii) 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 Section 1.2.5). In the promoter regions of the *apf1* and *apf2* genes of *L. johnsonii* encoding S-layer-like proteins, a TG motif upstream of the -10 box, responsible for increased transcriptional activity, has been identified (Ventura *et al.*, 2002). iv) Two promoters, offering a possibility to enhance and/or regulate gene expression, have been identified upstream of the *slpA* gene of *L. brevis* ATCC 8287 (Vidgren *et al.*, 1992), *slpB* and *slpD* of *L. brevis* ATCC 14869 (Jakava-Viljanen *et al.*, 2002), *slpA* of *L. acidophilus* ATCC 4356 (Boot *et al.*, 1996b) and the S-layer-like gene *apf1* of *L. johnsonii* (Ventura *et al.*, 2002). Of these, data about the use of the promoters are available for *L. brevis* ATCC 8287 (Kahala *et al.*, 1997) and *L. johnsonii* (Ventura *et al.*, 2002), in which both of the promoters are used,

and for *L. acidophilus*, in which 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.*, 1996a; Hagen *et al.*, 2005). In fact, excluding *L. helveticus*, for all *Lactobacillus* species with genetically characterized S-layer proteins and sequences publicly available, two or more complete S-layer protein genes in the same strain have been described. Of these, only the S-layer protein genes *slpB* and *slpD* of *L. brevis* ATCC 14869 (Jakava-Viljanen *et al.*, 2002), *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) as well as the S-layer-like protein genes *apf1* and *apf2* of *L. johnsonii* and *L. gasseri* (Ventura *et al.*, 2002) have been shown to be expressed simultaneously. Thus, silent S-layer protein genes, under the conditions tested, are common and represented by the *slpB* genes of *L. acidophilus* ATCC 4356, NCIMB 8607, LMG 11428, LMG 11469 (Boot *et al.*, 1995) and NCFM (Buck *et al.*, 2005), *cbsB* of *L. crispatus* JCM 5810 (Sillanpää *et al.*, 2000), *SlpNB* of *L. crispatus* LMG 12003 (unpublished, GenBank AF253044), s*lpC* of *L. brevis* ATCC 14869 (Jakava-Viljanen *et al.*, 2002), by 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 hybridization (Boot *et al.*, 1996a), 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 in the rest only one S-layer protein was present (Jakava-Viljanen & 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 recently sequenced genome of *L. brevis* ATCC 367 (Makarova *et al.*, 2006), two complete genes and one truncated S-layer protein gene have been identified by homology, but nothing is known about the expression of these genes.

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 S-promoter. 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 conditions favouring the expression of the silent gene have thus far been characterized (Boot *et al.*, 1996). 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 leads 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 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 or mechanism of action of the soluble regulator nor the reason for the silence of the *slpC* gene in this strain is known (Jakava-Viljanen & Palva, 2007). Stress-mediated regulation was also suggested for the expression of the S-layer protein gene of *L. acidophilus* NCC2628, which was induced when the strain was cultivated under conditions of limited protein supply (Schär-Zammaretti *et al.*, 2005).

Although the S-layer protein genes seem to be essential for lactobacilli, as S-layer-negative mutants are difficult or impossible to create (Boot *et al.*, 1996; Martinez *et al.*, 2000; Buck *et al.*, 2005), and expression of S-layer protein genes thus could be anticipated to be constitutive, the examples above indicate that variation and regulation at the transcriptional and/or transcriptional level also exists. 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). However, currently the transcriptional and translational regulation mechanisms of *Lactobacillus* S-layer protein genes on a molecular level are almost totally unexplored.

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

Before this thesis work, the two structural regions of S-layer proteins, the region involved in the attachment of the S-layer subunit to the cell envelope and the region involved in S-layer assembly, were characterized in the S-layer proteins of only two *Lactobacillus* strains: in the SA protein of *L. acidophilus* ATCC 4356 (Smit *et al.*, 2001) and in the CbsA protein of *L. crispatus* JCM 5810 (Antikainen *et al.*, 2002) (see also Table 1). Both of these organisms belong to the former *L. acidophilus* group (Johnson *et al.*, 1980), and the amino acid sequences of their S-layer proteins show extensive similarity, especially in the C-terminal parts (Smit *et al.*, 2001), suggesting a conserved function for the C-terminal region. Extending the alignment to the amino acid sequences of eight mature S-layer proteins of *L. acidophilus* group organisms and the closely related *L. helveticus* (Collins *et al.*, 1991; Felis & Dellaglio, 2007) also indicates a remarkable conservation of the C-terminal parts (Antikainen *et al.*, 2002).

In both SA of *L. acidophilus* ATCC 4356 (Smit *et al.*, 2001) and CbsA of *L. crispatus* JCM 5810 (Antikainen *et al.*, 2002), the conserved C-terminal part of the S-layer protein, approximately 125 amino acids or one-third of the mature amino acid sequence, is responsible for binding to the cell envelope, and the more variable N-terminal part for the self-assembly of the S-layer protein monomers to a periodic S-layer lattice. Both of these proteins have a similar charge distribution with a high predicted pI in the C-terminal part rich in lysines. Overall, the C-terminal parts of these proteins consist mainly of hydrophilic amino acid residues and are predicted to contain β-strands (Smit *et al.*, 2001; Antikainen *et al.*, 2002).

Lactobacillus S-layer proteins do not possess SLH domains. 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 SA and CbsA, in the C-terminal parts of the silent S-layer protein SB of *L. acidophilus* ATCC 4356 and the S-layer protein of *L. helveticus* CNRZ 892, as well as in non-S-layer proteins of lactic acid bacteria known to be associated with the cell envelope or to agglutinate red blood cells (Smit *et al.*, 2001). The cell wall receptors for the C-terminal parts of SA and CbsA have been shown to be teichoic acids (Antikainen *et al.*, 2002; Smit & Pouwels, 2002), and CbsA binds also to lipoteichoic acids (LTA) isolated from *Staphylococcus aureus* and *Streptococcus faecalis*, but not to the teichuronic acid/polysaccharide fraction of the cell wall of *L. crispatus* JCM 5810. Based on the lack of amino acid sequence similarity of CbsA with other positively charged LTA binding proteins, and binding studies performed after the oxidation of carbohydrates in LTA showing no effect on binding, the LTA binding of the C-terminal part of CbsA was suggested to be mediated by electrostatic interactions involving the lysine residues in the CbsA C-terminal part (Antikainen *et al.*, 2002). Participation of such electrostatic interactions was not excluded in the case of the cell wall binding of SA either (Smit *et al.*, 2001). For 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 & Pouwels, 2002). In the case of CbsA, no further dissection of the C-terminal part for cell wall binding studies has been performed.

The self-assembly regions of SA and CbsA have been mapped by studying the self-assembly properties of truncated recombinant proteins by transmission electron microscopy (Sillanpää *et al.*, 2000; Smit *et al.*, 2001; Antikainen *et al.*, 2002; Smit *et al.*, 2002). The fragments comprising the C-terminal two-thirds of SA (residues 1-290 in the mature protein) form a lattice with p2 symmetry, identical to that formed by SA extracted from *L. acidophilus* ATCC 4356 cells (Smit *et al.*, 2001). The lattice parameters or symmetry type of the lattice formed by full-length recombinant CbsA and its N-terminal self-assembly part (residues 32-271 in the mature protein) (Antikainen *et al.*, 2002) have not been determined.

Both SA and CbsA can be viewed as two-domain proteins with an N-terminal domain facing the environment and a non- or less-exposed C-terminal domain; in SA this view was supported by proteolytic and chemical breakdown experiments (Smit *et al.*, 2001). 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 (residues 30-32 and 269-273 in mature CbsA) have been shown to be 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 are 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. Although not necessary for self-assembly, the C-terminal cell wall binding domain has a stabilizing role in the recrystallization of CbsA monomers by allowing a more efficient sheet formation. The region in CbsA responsible for self-assembly also binds collagen (see Section 1.2.4), and the binding correlates with the ability of recombinant CbsA fragments to form a regular lattice structure (Antikainen *et al.*, 2002).

In addition to the two well-characterized proteins described above, only fragmentary data are available about the cell wall binding or self-assembly of other *Lactobacillus* S-layer proteins. In early studies, the cell walls of *L. buchneri* (Masuda & Kawata, 1981) and *L. brevis* ATCC 8287 (Shimohashi *et al.*, 1976) 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 (Masuda & Kawata, 1980; 1981; 1985). In comparison with the well-characterized exopolysaccharides of lactic bacteria (De Vuyst & Degeest, 1999; Welman & 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. Regarding organisms related to *L. acidophilus*, the S-layer protein of *L. helveticus* CNRZ 892 can, based on amino acid sequence similarity, be anticipated to be composed of similar functional domains as SA and CbsA, although the detailed mechanisms of cell wall binding and, especially, self-assembly are more likely to vary.

1.2.4 Functions of *Lactobacillus* S-layer proteins

Until now, only a couple of functions 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) and the susceptibility of *L. acidophilus* M92 to gastric and pancreatic juice (Frece *et al.*, 2005), and a role as a phage receptor has been suggested for the S-layer protein of *L. helveticus* CNRZ 892 (Callegari *et al.*, 1998). 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). Recently, the C-terminal part of the S-layer protein SA of *L. acidophilus* ATCC 4356 was 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.

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 & 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 supposed to be linked to their S-layer proteins, but the involvement of other guanidine hydrochloride-extractable components of the cell wall in this lectin-like activity could not be excluded, and no attention was paid to the aggregation of the S-layer proteins possibly causing unspecific effects (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 non-soluble LiCl extracts of *L. kefir* were used in the aggregation assays. The study of Uchida *et al.* (2006), in which the dialysed guanidine hydrochloride extract containing the S-layer protein of *L. brevis* OLL2772 was shown to bind to the human blood group A antigen in a surface plasmon resonance assay, can especially be criticized for the use of an aggregated solution as an analyte. Inconclusive and indirect evidence of S-layer protein binding to epithelial cells is also available from studies where dialysed guanidine hydrochloride- or lithium chloride extracts of *L. helveticus* (Johnson-Henry *et al.*, 2007) or *L. crispatus* (Chen *et al.*, 2007), containing aggregates of the S-layer proteins of the strains, inhibit the binding of pathogenic *E. coli* or *Salmonella* strains to epithelial cells. In a related study, the lithium chloride-extracted, aggregated S-layer protein of a *L. kefir* strain was shown to bind to *Salmonella* cells, and a role for the S-layer protein in the inhibition of Caco-2/TC-7 cell association and invasion of *Salmonella* by *L. kefir* was suggested (Golowczyc *et al.*, 2007).

Before this work, the role of a *Lactobacillus* S-layer protein in bacterial adherence had been unequivocally shown in two cases, where recombinant S-layer proteins or S-layer-negative mutants had been used (Toba *et al.*, 1995; Buck *et al.*, 2005; Konstantinov *et al.*, 2008). First, CbsA of *L. crispatus* JCM 5810 binds collagen types I and IV (Toba *et al.*, 1995; 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 acids 31-274 of mature CbsA are needed for collagen binding, and practically 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). However, the display of CbsA on the surface of a non-S-layered *L. casei* strain through a PrtP cell wall anchor renders 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). In contrast, the recombinant form of the non-expressed SlpB protein of *L. crispatus* JCM 5810 does not bind collagen types I or IV (Sillanpää *et al.*, 2000). The second well-characterized adhesive *Lactobacillus* S-layer protein is SlpA on *L. acidophilus* NCFM cells, which binds to the 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 is significantly reduced in binding to DC-SIGN, and the interaction leads to the induction of different cytokines (Konstantinov *et al.*, 2008). A role for SlpA of *L. acidophilus* NCFM has also been demonstrated in binding to Caco-2 cells, as the binding of the knock-out

mutant is decreased by 84% compared with the wild type (Buck *et al.*, 2005). The region in *L. acidophilus* SlpA responsible for binding to DC-SIGN or Caco-2 cells has not been determined.

While this thesis work was in progress, a direct interaction between the chromatographically purified, monomeric form of the S-layer protein SlpA of *L. brevis* ATCC 8287 and soluble fibronectin or laminin was demonstrated 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 (de Leeuw *et al.*, 2006). Later, in the study of Khang *et al.* (2009) SlpA-GFP fusion proteins were shown to bind to undifferentiated human HT-29 cells, although appropriate controls were lacking and no attention was paid to the solubility of the protein, making the specificity questionable.

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, as they are generally hydrophobic (see Section 1.1.2.1)(van der Mei *et al.*, 2003). 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 N-terminal domain (see Section 1.2.3) (Vadillo-Rodriguez *et al.*, 2004).

1.2.5. Applications of *Lactobacillus* S-layer proteins

Until now, rather few applications have been developed for the S-layer proteins of lactobacilli. The field currently most 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 the hybrid proteins on their surface is of interest, as such strains have potential for use as live mucosal vaccines. 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 homologous recombination. Similarly, surface display of green fluorescent protein (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). Furthermore, 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 a very attractive tool for oral vaccine design. A system utilizing *L. acidophilus* NCFM (but not its S-layer) as a carrier for an antigen with a small dendritic cell targeting peptide has already been developed (Mohamadzadeh *et al.*, 2009). Preliminary experiments have also been performed in the field of passive immunization by utilizing the 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).

Another potential application is the use of S-layers or S-layered lactobacilli as anti-adhesion agents for the prevention of infectious diseases. The collagen, laminin and fibronectin binding *L. crispatus* strain JCM 5810 (Toba *et al.*, 1995) and its well-characterized collagen adhesin CbsA (see Section 1.2.4) inhibit the binding of enterotoxigenic *E. coli* to a reconstituted basement membrane preparation as well as to its main component laminin *in vitro* (Horie *et al.*, 2002). The observed anti-adhesive effects of lactobacilli or their surface protein extracts mentioned in Section 1.2.4., including those of *L. crispatus* (Chen *et al.*, 2007), *L. helveticus* (Sherman *et al.*, 2005; Johnson-Henry *et al.*, 2007) and *L. kefir* (Golowczyc *et al.*, 2007) have been presented as useful, probiotic traits, although the extracts used in the inhibition studies apparently contained aggregates of the S-layer protein, and thus, the specificities of the inhibitions were compromised. In addition, the experiments have thus far only been performed *in vitro*, and their application potential in human or animal medicine is obscure.

The biochemical modification studies of *Lactobacillus* S-layer proteins for technological applications have recently been initiated, with small molecular probes like biotin or FITC 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 & Gilchrist, 2004).

The expression and/or secretion signals of *Lactobacillus* S-layer protein genes have also been utilized in biotechnological applications (Savijoki *et al.*, 1997; Kahala & Palva, 1999; Novotny *et al.*, 2005). The signals of the *slpA* gene of *L. brevis* ATCC 8287 have been used in *Lactobacillus* and *Lactococcus* hosts for intracellular (Kahala & Palva, 1999) and extracellular (Savijoki *et al.*, 1997) 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 protein production with *slpA* signals is achieved in *Lactococcus lactis* and *Lactobacillus plantarum* and moderate in *Lactobacillus gasseri*, while in *Lactobacillus casei* the expression signals are not recognized (Savijoki *et al.*, 1997). On the other hand, the promoter region of *Lactobacillus acidophilus* ATCC 4356 S-layer protein gene is highly functional in *L. casei* (Boot *et al.*, 1996b).

1.2.6. Tools to study S-layer structure and function: cysteine scanning mutagenesis and bacterial surface display

The typical features of S-layer proteins, the rather large molecular weight and poor solubility (see Section 1.1.2.1) pose problems for the structural and adhesion studies of also *Lactobacillus* S-layer proteins, necessitating the use of methods overcoming these difficulties, like cysteine scanning mutagenesis or flagellar display.

1.2.6.1. Cysteine scanning mutagenesis and sulfhydryl chemistry

CSM is based on the targeted mutagenesis of selected amino acid residues in the protein of interest to cysteines, which are then amenable to chemical modification owing to their sulfhydryl groups. The stability and functionality of the mutant proteins generated

are first tested. Several application modes can then be used. In chemical reactivity scanning, only one residue in each mutant protein is changed, resulting in a series of mutant proteins each having a single modifiable residue at a different location. Several sulfhydryl-reactive reagents can be used for the modification of these residues, and the modified proteins can be distinguished from the unmodified ones by, for instance, fluorescence or a change in molecular weight. The extent of modification is a measure of the surface accessibility of the mutated residue. In disulphide-based applications, one or a pair of cysteine residues is introduced to the protein, and the engineered sulfhydryl groups at different locations in the generated pool of mutant proteins are allowed to react with each other. The presence of disulphide bonds is detected based on migration in a polyacrylamide gel, and the observed contacts between pairs of positions elucidate the tertiary or quaternary structure of the protein (Bass *et al.*, 2007). Disulphide mapping as a method of investigating the proximity of two residues in a protein is thus based on the same principle as chemical cross-linking, also used in structural and interaction studies (Kluger & Alagic, 2004; Sinz, 2006), although the distance constraints between the reacting residues may be different. A limitation of CSM is that the wild-type protein is not allowed to contain cysteines, but in S-layer proteins this does not generally pose a problem, as cysteines in bacterial S-layer proteins are usually absent (see Section 1.1.2.1). Major advantages of the method are that it can be applied to an entire protein or to a specific domain or subdomain, and it can be carried out in the native environment of the protein, *e.g.* in a lipid bilayer or a multiprotein complex, making it a method of choice for many proteins inaccessible to high-resolution methods like X-ray crystallography. Furthermore, it is a versatile system that is able to map out secondary, tertiary and quaternary structures, analyse conformational changes and structure-function relationships and reveal thermal motions within a protein structure by a method called disulphide trapping (Frillingos *et al.*, 1998; Bass *et al.*, 2007). However, CSM-based methods are all rather laborious.

CSM has been widely used in the study of membrane proteins and large heteromeric multiprotein complexes of both bacterial and eukaryotic proteins (Bass *et al.*, 2007). Especially, the structure and function of bacteriorhodopsin (Altenbach *et al.*, 1990), microbial pore-forming toxins (Merzlyak *et al.*, 2005; Iacovache *et al.*, 2006; Girard *et al.*, 2008), microbial transporters of drugs, antibiotics, nutrients or ions (Sobczak & Lolkema, 2005; Hassan *et al.*, 2006; Kuwabara *et al.*, 2006; Xu *et al.*, 2006; Greene *et al.*, 2007; Papakostas *et al.*, 2008; Wang *et al.*, 2008) and microbial membrane components involved in environmental sensing (Goldberg *et al.*, 2008), chemo- and aerotaxis (Winston *et al.*, 2005; Bass *et al.*, 2007; Taylor *et al.*, 2007) and protein secretion (Mori *et al.*, 2004) have been studied by CSM. Before this thesis work, the only CSM study of a bacterial S-layer protein had been performed with the SbsB protein of *Geobacillus stearothermophilus* PV72/p2. In that work, the spatial locations of 75 residues out of 920 were studied, and 23 of them were found to be located on the surface of SbsB monomers (Howorka *et al.*, 2000; Kinns & Howorka, 2008). In a subsequent study, these selected mutant proteins with surface-accessible cysteines were analysed by chemical cross-linking to find the residues located at the protein/protein interface or on the inner surface of the S-layer lattice. Specific modified sites were also found to be subject to intramolecular cross-linking, indicating conformational changes upon self-assembly (Kinns & Howorka, 2008).

1.2.6.2 Flagellar display

Bacterial surface display means the presentation of foreign peptides on the bacterial surface, usually through recombinant DNA technology. Both Gram-positive and Gram-negative bacterial species have been used as display hosts (Samuelson *et al.*, 2002). In Gram-positive bacteria, the most common anchoring mechanism of the foreign peptide to the cell envelope is a covalent linkage to peptidoglycan through the LPXTG sequence of the foreign peptide, but other anchoring peptide sequences derived from Gram-positive bacteria (e.g. SLH domains, AcmA repeats) and transmembrane and lipoprotein membrane anchors have also been used (reviewed by Sillanpää, 2001; Samuelson *et al.*, 2002; Lee *et al.*, 2003). Studies aiming at the utilization of whole S-layer proteins (Smit *et al.*, 2002; Åvall-Jääskeläinen *et al.*, 2002; Mota *et al.*, 2006) (see Section 1.2.5), spores (reviewed by Kim & Schumann, 2009) or flagella (Crampton *et al.*, 2007) of Gram-positive bacteria as display vehicles have also been initiated. In Gram-negative bacteria, surface display is based on S-layer proteins (Bingle *et al.*, 1997b; Nomellini *et al.*, 2007), outer membrane proteins (OMPs) or OMP-lipoprotein fusions (reviewed by Georgiou *et al.*, 1997; Lång, 2000; Samuelson *et al.*, 2002), outer membrane proteins modified by circular permutation (Rice *et al.*, 2006), autotransporters (reviewed by Jose, 2006; Jose & Meyer, 2007), secreted proteins (Kornacker & Pugsley, 1990), purified intracellular structures like bacterial magnetic particles (BMPs)(reviewed by Wu *et al.*, 2008) or surface organelles, *i.e.* fimbriae (reviewed by Klemm & Schembri, 2000), flagella (reviewed by Westerlund-Wikström, 2000) or other filaments (Crepin *et al.*, 2005). Bacterial surface display techniques not involving gene fusions have also been developed for both Gram-positive and Gram-negative bacteria (Sadamoto *et al.*, 2004; Tanaka *et al.*, 2004; Bosma *et al.*, 2006).

In the flagellar display system of Gram-negative bacteria, the sequence encoding the foreign peptide is inserted into the flagellin gene (*fliC*) encoding the main subunit of the flagellar filament. Methods have been developed for the flagella of both *E. coli* and *Salmonella*, the flagellar structures of which are very similar (see Fig. 3 for a schematic presentation). In addition to the helical filament consisting of several thousand copies of the main subunit FliC, the flagellum is composed of a pentameric cap protein (FliD) at the tip of the filament, and the hook (FlgE) and the junction proteins (FlgK, FlgL) between the filament and the basal body structure, which connects the filament to the outer membrane (Fernandez & Berenguer, 2000; Westerlund-Wikström, 2000). FliC is composed of four domains: two conserved ones, inner and outer, important for the polymerization of flagellins and the stability of the flagellum, and a variable surface-exposed region, composed of two domains, responsible for the antigenic variation of flagella (Westerlund-Wikström, 2000; Yonekura *et al.*, 2003; Beatson *et al.*, 2006). The variable region is utilized in the flagellar display system, as it allows the deletion of up to 187 residues without a loss of flagellar integrity or function (Kuwajima, 1988; Lu *et al.*, 1995). After the replacement of a part of this non-essential sequence of *fliC* by the desired sequence, the plasmid construct is used to complement a Δ*fliC* host strain, resulting in flagella composed of only the hybrid flagellins (Westerlund-Wikström *et al.*, 1997; Westerlund-Wikström, 2000).

Flagellar display has been used e.g. for the study of adhesive peptides (Westerlund-Wikström *et al.*, 1997), for the selection of metal-binding peptides for

use as bioadsorbents (Dong *et al.*, 2006) and for vaccination trials (Chauhan *et al.*, 2005; reviewed by Westerlund-Wikström, 2000; Ben-Yedidia & Arnon, 2005). An application utilizing FliC- thioredoxin fusions, with random peptides displayed in the thioredoxin part of the fusion, has also been developed as an alternative to phage display (Lu *et al.*, 1995). Peptides up to 302 amino acids have been successfully expressed using flagellar display (Westerlund-Wikström *et al.*, 1997). A limitation of the method is that the type III system used for the secretion of flagellins bypasses the periplasmic space, where disulphide bonds are formed (Macnab, 2004), and thus, disulphide bonds cannot be formed in the peptide to be displayed. In the case of S-layer proteins this, however, does not usually pose a problem (see Section 1.1.2.1). Flagellar display has several significant advantages. Hybrid flagella can be used either attached to the bacterial cell or in purified form, and purification is easily accomplished on a large scale (Westerlund-Wikström, 2000). In vaccine design, the presence of the antigenic epitope as multiple copies, the possible adjuvant effects of other accompanying bacterial components and the capacity of the conserved regions of flagellin to interact with TLR5 leading to immunostimulation (Eaves-Pyles *et al.*, 2001; Hayashi *et al.*, 2001) are advantageous (Seegers, 2002; Wells & Mercenier, 2008). The flagellin part in antigen-flagellin fusion proteins has been shown to induce APC (antigen presenting cell) maturation, cytokine secretion and the development of strong and specific immune responses towards the antigen (Cuadros *et al.*, 2004). Flagellins have also been demonstrated to have a probiotic effect by inducing the synthesis of an antimicrobial peptide in epithelial cells (Ogushi *et al.*, 2001; Schlee *et al.*, 2007). The multivalency of the hybrid flagella facilitates their use also in diagnostics and as tools to identify and isolate adhesins and their receptors (Westerlund-Wikström, 2000). Further versatility is provided by the possibility to present two or more epitopes simultaneously on the same flagellar filament by utilizing FliD as well as FliC as fusion partners for the foreign peptides (Tanskanen *et al.*, 2000; Majander *et al.*, 2005).

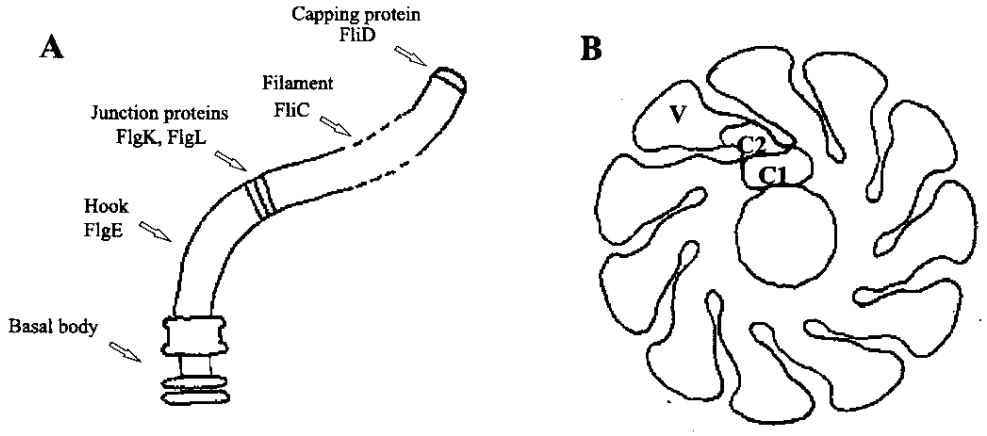

Figure 3. Schematic presentation of (A) the flagellar filament and (B) the cross-section of a *Salmonella* flagellar filament. C1 and C2, conserved domains; V, variable region composed of two domains. This figure is a reprint from Westerlund-Wikström (2000), with permission granted by Elsevier.

1.2.7 Non-S-layer adhesive surface proteins of lactobacilli

Despite some contradictory opinions about the importance of *Lactobacillus* adherence for persistence in the human gastrointestinal tract (Walter, 2008), the conventional view is that a good adherence capacity of a strain to mucus or epithelial cells promotes its colonization or residence time in the gut. As such, this is usually also considered a desirable trait for a probiotic organism, as it may facilitate pathogen exclusion, immune modulation and epithelium protection effects possibly exerted by the strain (Lebeer *et al.*, 2008). Adhesion of lactobacilli has mostly been studied by *in vitro* experiments using immortalized cell lines, isolated epithelial cells or tissue fragments, purified proteins or other components of the extracellular matrix, and mucus or mucus components as binding targets. Red blood cell, yeast cell and platelet agglutination assays have also been used. The molecular mechanisms mediating the adherence remain rather poorly known. A role for carbohydrates on the bacterial surface in adherence has been proposed in many studies, but only the binding of one non-protein molecule, LTA, to epithelial cells has been directly demonstrated (Chan *et al.*, 1985) as well as suggested by inhibition studies (Granato *et al.*, 1999; Kapczynski *et al.*, 2000). Although not completely excluding the involvement of bacterial carbohydrates, in many cases *Lactobacillus* surface proteins seem to mediate adherence.

Considering the large number of studies, in which protein-mediated adhesion of lactobacilli to various targets, based on the effect of protease treatment, has been described, relatively few adhesive surface proteins have been functionally and genetically characterized. On the other hand, in August 2009, 16 complete chromosome sequences representing 12 *Lactobacillus* species were publicly available, and these and ongoing sequencing projects of *Lactobacillus* genomes are continuously providing sequence homology-based information about genes potentially involved in adhesion. For example, the genome of *L. plantarum* WCFS1 is predicted to encode 12 adhesive proteins with domains with affinity to mucus, collagen, fibronectin or chitin (Boekhorst *et al.*, 2006b), and the genome of *L. gasseri* ATCC 33323, according to an *in silico* analysis, encodes as many as 14 proteins with mucus-binding domains as well as proteins with similarity to bacterial adhesins binding to fibronectin, human epithelial cells or saliva (Azcarate-Peril *et al.*, 2008). In this section, however, the emphasis will be on genetically and functionally well-characterized adhesive *Lactobacillus* surface proteins.

The role of *Lactobacillus* S-layer proteins as adhesins has been discussed in Section 1.2.4. Grouping of the rest of the adhesive proteins according to species or binding target is difficult since strains of the same species, or even a single strain, may carry different adhesive proteins and several targets may have been identified for the same adhesin. In the following, adhesins with amino acid sequence similarity are presented as groups and an approximate chronological order of identification is followed. A summary of *Lactobacillus* non-S-layer adhesins is presented in Table 3.

The first *Lactobacillus* adhesin characterized at a genetic level was the collagen-binding protein CnBP of *L. reuteri* NCIB 11951, which shares amino acid sequence similarity with the solute binding components of bacterial ABC transporters (Aleljung *et al.*, 1994; Roos *et al.*, 1996). A homologue of CnBP in *L. reuteri* 104R, with 94% amino acid identity (Miyoshi *et al.*, 2006), was originally characterized as a mucus-

Table 3. Genetically and functionally characterized non-S-layer adhesive surface proteins of lactobacilli. FnBp, fibronectin-binding protein; MBp, mucin-binding protein.

Adhesin	Organism	Binding target	Reference
CnBP	*L. reuteri* NCIB 11951	Collagen	Aleljung *et al.*, 1994; Roos *et al.*, 1996
MAPP/MapA	*L. reuteri* 104R	Mucus, Caco-2 cells	Rojas *et al.*, 2002; Miyoshi *et al.*, 2006
32-Mmubp	*L. fermentum* BCS87	Mucus, mucin	Macias-Rodriguez *et al.*, 2009
Mub	*L. reuteri* 1063	Mucus	Roos & Jonsson, 2002
LBA 1392 (MBp homologue)	*L. acidophilus* NCFM	Caco-2 cells	Buck *et al.*, 2005
Msa	*L. plantarum* WCFS1	Mannosides	Adlerberth *et al.*, 1996; Pretzer *et al.*, 2005
Lsp	*L. reuteri* 100-23	Mouse forestomach epithelium	Walter *et al.*, 2005
LspA	*L. salivarius* UCC118	Caco-2, HT-29 cells	van Pijkeren *et al.*, 2006
LBA 1148 (FnBp homologue)	*L. acidophilus* NCFM	Caco-2 cells	Buck *et al.*, 2005
EF-Tu	*L. johnsonii* NCC533	Caco-2, HT-29 cells, mucin	Granato *et al.*, 2004
GroEL	*L. johnsonii* NCC533	HT-29 cells, mucin	Bergonzelli *et al.*, 2006
Eno	*L. crispatus* ST1	Laminin, collagen	Antikainen *et al.*, 2007a
Eno1-3	*L. johnsonii* F133	Laminin	Antikainen *et al.*, 2007a
EnoA1	*L. plantarum* LM3	Fibronectin	Castaldo *et al.*, 2009
GAPDH	*L. plantarum* LA318	Mucin	Kinoshita *et al.*, 2008b

adhesion-promoting protein (MAPP) (Rojas *et al.*, 2002), but was subsequently found to bind to Caco-2 cells as well, and was named MapA (Miyoshi *et al.*, 2006). Another homologue of CnBP, with approximately 90% amino acid sequence identity, present as the major constituent of the LiCl extract of *L. fermentum* BR11, was initially defined as a basic surface protein (BspA) (Turner *et al.*, 1997). It was later shown to be an L-cystine binding protein in an ABC transporter system (Turner *et al.*, 1999; Hung *et al.*, 2005), necessary for oxidative defence (Hung *et al.*, 2003), and the designation CyuC was suggested (Hung *et al.*, 2005). For BspA/CyuC, no role in bacterial adhesion has thus far been demonstrated, although it cannot be ruled out, as many bacterial adhesins are multifunctional (Kukkonen & Korhonen, 2004; Leon-Kempis Mdel *et al.*, 2006; Antikainen *et al.*, 2007a; Sanchez *et al.*, 2008) and numerous potential binding targets exist. A third homologue of CnBP (or CnBP itself, deduced from the 20 identical N-terminal amino acids), referred to originally as p29, is present in the pool of secreted biosurfactants of *L. fermentum* RC-14 and has anti-adhesive activity towards *E. faecalis* (Heinemann *et al.*, 2000). The same protein has subsequently been shown by SELDI-TOF (surface-enhanced laser desorption/ionization-time of flight) analysis to bind collagen types III and VI, and its gene has been cloned (Howard *et al.*, 2000), although no report about the gene sequence is available. Recently, in *L. reuteri* JCM 1081, an HT-29 cell- and mucin-binding homolog of CnBP has been identified (Wang *et al.*, 2008), and an ABC transporter component, which binds to pig mucus and mucin, has been detected on the surface of *L. fermentum* BCS87 (Macias-Rodriguez *et al.*, 2009).

The mucus-binding protein Mub of *L. reuteri* 1063 is one of the largest bacterial surface proteins (3269 amino acids, 358 kDa) and the first *Lactobacillus* adhesin shown to have a LPXTG cell wall anchoring motif. Its 14 so-called Mub-repeats, 200 amino acids each, are responsible for the binding of the protein to the carbohydrate structures in pig and hen mucus. Two types of repeats can be distinguished, with slightly different patterns of inhibition of binding by carbohydrates, but both repeat types are important for adhesion (Roos & Jonsson, 2002). Based on colony hybridization, the *mub* gene is frequently present in pig intestinal *Lactobacillus* isolates closely related to *L. reuteri*, *L. fermentum* and *L. pontis*, and these strains have been suggested to constitute a new species, *L. mucosae* (Roos *et al.*, 2000).

Several Mub-like proteins in lactobacilli have been functionally characterized. In *L. fermentum* BR11, a vaginal isolate of a guinea pig, a large Mub-like protein (Mlp) has been identified with 10 repeat sequences and 21% overall amino acid sequence identity with Mub of *L. reuteri*, but the protein does not bind to pig gastric mucin (Turner *et al.*, 2003). A Mub homologue, LBA 1392, with 24% overall amino acid sequence identity has been identified in *L. acidophilus* NCFM, where it has been shown by knock-out mutation of the gene to be involved in adherence to Caco-2 cells (Buck *et al.*, 2005). A Mub-repeat-carrying protein in *L. plantarum* was initially identified as a mannose-specific adhesin capable of agglutinating yeast cells and binding to HT-29 cells (Adlerberth *et al.*, 1996). When the whole genome sequence of *L. plantarum* had become available (Kleerebezem *et al.*, 2003), the corresponding gene was identified and cloned, designated *msa*, and shown by knock-out mutation and complementation to be responsible for the yeast cell agglutination phenotype of *L. plantarum*. However, in addition to Mub-repeats, Msa has a domain with similarity to a ConA-like lectin (Pretzer *et al.*, 2005). In the *in situ* pig small intestinal segment perfusion model, Msa has been shown to contribute to adherence to porcine jejunal epithelium and to induce host responses (Gross *et al.*, 2008). The large surface protein (Lsp) of *L. reuteri* 100-23, which has a role in the adherence of the strain to mouse forestomach epithelium *in vivo* and an effect on the colonization dynamics in mice, has 83% nucleotide sequence identity with a Mub-domain-carrying protein of *L. johnsonii* (Walter *et al.*, 2005), and thus, probably has Mub-repeats, although a detailed analysis of the sequence of Lsp has not been presented. However, in the homologous protein in *L. johnsonii*, other domains with predicted adhesive properties are also present. The Mub-repeat-containing protein LspA of *L. salivarius* UCC 118 mediates adherence to HT-29 and Caco-2 cells, as shown by an isogenic LspA negative mutant strain (van Pijkeren *et al.*, 2006). With the appearance of the whole genome sequences of lactobacilli, several copies of *mub*-homologous genes have been identified in *L. gasseri* (fourteen), *L. johnsonii* (nine), *L. plantarum* (four), *L. acidophilus* (twelve) and *L. brevis*, *L. fermentum* and *L. reuteri* (two in each) (Boekhorst *et al.*, 2006a; Azcarate-Peril *et al.*, 2008). Interestingly, one truncated *mub*-repeat-containing gene is present in the plasmid carried by *L. reuteri* ATCC 55730, raising the question of lateral transfer of *mub* genes in lactobacilli (Bath *et al.*, 2005). The presence of a potential adhesin gene in a *Lactobacillus* plasmid has been confirmed by others as well (Desmond *et al.*, 2005).

The only functionally characterized *Lactobacillus* adhesive protein with the procaryotic fibronectin-binding domain is present in *L. acidophilus* NCFM. The inactivation of the gene encoding this protein (LBA 1148) results in a marked decrease in the adherence of *L. acidophilus* NCFM to Caco-2 cells (Buck *et al.*, 2005), but the binding of the strain to fibronectin has not been studied.

One group of *Lactobacillus* adhesive surface proteins consists of those with a previously characterized cytoplasmic function (so called moonlighting or anchorless multifunctional proteins (Sanchez *et al.*, 2008)). The *L. johnsonii* proteins EF-Tu, a guanosine-nucleotide binding protein participating in protein synthesis, and GroEL, a stress-induced chaperonin (heat shock protein), are both present on the bacterial surface non-covalently bound in addition to their known cytoplasmic locations, and have a pH-dependent adhesive function. EF-Tu binds to Caco-2 cells, to non-differentiated HT29 cells from the human colon and to mucins purified from HT29-MTX cells or from the human colon at low pH, while GroEL has been reported to bind to non-differentiated HT29 cells and to mucins purified from HT29-MTX cells under the same conditions (Granato *et al.*, 2004; Bergonzelli *et al.*, 2006). The binding of EF-Tu to mucus or cells has been proposed to have a role in gut homeostasis (Granato *et al.*, 2004), but for the binding of GroEL a minor role has been suggested; instead, it may exert its action by specifically aggregating *Helicobacter pylori* cells after its liberation from deceased *L. johnsonii* cells in the acidic stomach (Bergonzelli *et al.*, 2006). Another example of a cytoplasmic protein acting as an adhesin on lactobacilli is the glycolytic enzyme enolase, present on the surface of *L. johnsonii* F133, *L. crispatus* ST1 and *L. plantarum* LM3. *L. johnsonii* and *L. crispatus* enolases bind to laminin and/or collagen *in vitro*, and also bind and activate plasminogen, the precursor of the proteolytic enzyme plasmin present in plasma and extracellular fluids (Antikainen *et al.*, 2007a), while the EnoA1 of *L. plantarum* binds fibronectin (Castaldo *et al.*, 2009). Variable laminin/collagen binding and plasminogen activation efficiencies are observed in individual enolases of lactobacilli; in *L. johnsonii* F133, for instance, three enolase genes are present, of which *eno2* is silent under standard laboratory conditions (Antikainen *et al.*, 2007a). The enolase of *L. crispatus* binds electrostatically to LTA and is released from the surface of the bacterium at neutral or basic pH, above its isoelectric point (Antikainen *et al.*, 2007b). The cell surface-bound form of another glycolytic enzyme, GAPDH, has been identified as a mucus binding adhesin on *L. plantarum* (Kinoshita *et al.*, 2008b), and a putative receptor polysaccharide structure has been determined (Kinoshita *et al.*, 2008a).

A very distinct group of adhesins in lactobacilli are fimbriae (pili). Fimbriae are common and well-characterized adhesive organelles in Gram-negative bacteria, but only recently have they been thoroughly studied in Gram-positive bacteria. After the initial observation of fimbriae on *Corynebacterium renale* by electron microscopy (Yanagawa *et al.*, 1968), fimbriae have been identified in several other Gram-positive species, including *Actinomyces*, *Ruminococcus*, *Enterococcus*, *Clostridium*, *Mycobacterium* and several species of *Streptococcus*. A peculiar feature of Gram-positive fimbriae characterized thus far is that they are covalently linked to peptidoglycan and their main subunits also to each other, and the subunits are further stabilized by intramolecular covalent bonds (Proft & Baker, 2009). In the

pilus assembly process, a pilin-specific sortase is involved (Ton-That & Schneewind, 2003). For a long time, the presence of fimbriae in lactobacilli was debatable, and, in August 2009, unequivocal evidence of their role in adherence is still lacking. In early studies, fimbrium-like protruding structures were detected by electron microscopy on the surface of *L. fermentum* (Barrow *et al.*, 1980) and on several human vaginal isolates belonging to species *L. rhamnosus*, *L. acidophilus*, *L. jensenii*, *L. casei* and *L. fermentum* (McGroarty, 1994), and a role in adherence for these fimbrium-like structures was suggested. Recently, in the completely sequenced chromosome of *L. johnsonii* NCC533, two fimbrial operons were identified, which possibly encode fimbriae with a glycosylated fimbrial protein reminiscent of the Fap1 fimbrial adhesin of *Streptococcus parasanguis*, and fimbriae were also detected by electron microscopy on *L. johnsonii* cells (Pridmore *et al.*, 2004), but no functional studies of these structures on *L. johnsonii* have been performed. Very recently, in the context of creating an exopolysaccharide-negative mutant of the probiotic strain *L. rhamnosus* GG, fimbrium-like structures were detected by electron microscopy, and the increased adhesion to mucus and Caco-2 cells of the mutant was postulated to be due to the more efficient exposure of these structures (Lebeer *et al.*, 2009).

In conclusion, increasing numbers of adhesive *Lactobacillus* surface proteins are being characterized as the combined result of comparative genomics and functional studies, and the general view is that in most cases many factors contribute to the adherence of a particular strain. The specific receptors in epithelial cells or mucus are mostly uncharacterized to date. Furthermore, excluding the report of Lsp of *L. reuteri* 100-23 (Walter *et al.*, 2005) and that of Msa of *L. plantarum* (Gross *et al.*, 2008), studies on the biological role of *Lactobacillus* adhesive proteins *in vivo* have not yet been performed. In the future, more adhesive S-layer proteins will potentially be discovered.

2. AIMS OF THE STUDY

As food-grade organisms able to survive, persist and potentially exert health-promoting effects in the gastrointestinal tract, lactobacilli have aroused interest as possible carriers of oral vaccine antigens or other medically important molecules. In addition, the S-layer structure carried by several *Lactobacillus* species offers an effective means of expressing foreign peptides as multiple copies in an ordered manner on the bacterial surface. The goal of this study was to provide comprehensive knowledge about the structure and function of SlpA of *L. brevis* and about the expression of its gene in order to develop SlpA-based tools for live mucosal vaccines or other health-related applications in man or animals as well as for nanobiotechnology.

Specific aims were as follows:

I To specify the role of the S-layer protein in the adherence of *L. brevis* ATCC 8287 to human epithelial cell lines and fibronectin

II To identify and characterize the domains responsible for self-assembly and cell wall binding in the S-layer protein of *L. brevis* ATCC 8287

III To perform a detailed mapping of individual residues in SlpA to gain further insight into the structure of SlpA and the S-layer formed

IV To study the function of the two consecutive promoters of the *slpA* gene

3. MATERIALS AND METHODS

The bacterial strains, cell lines and plasmids used in Studies I-IV are listed in Table 4. The methods are described in detail in the original articles and summarized in Table 5.

Table 4. Bacterial strains, plasmids and cell lines used in Studies I-IV

Strain, plasmid or cell line	Origin / relevant property	Study	Reference or source
Bacterial strains			
Lactobacillus brevis ATCC 8287	Green olives	I, II, III, IV	ATCC
Lactobacillus acidophilus ATCC 4356	Human pharynx	II	ATCC
Lactobacillus helveticus 53/7	Industrial starter strain	IV	Valio, Finland
Lactococcus lactis MG 1363	Plasmid- and prophage-free derivative of wt *S. lactis* NCDO 712	IV	Gasson, 1983
Escherichia coli JT1	*E. coli* C600 *fliC*::Tn10 *fimA*::*cat*	I	Westerlund-Wikström *et al.*, 1997
Escherichia coli M15(pREP4)	Host for pQE-30	I	Stratagene
Escherichia coli DH5αF'	Cloning host for pET-28 vectors	II	Woodcock *et al.*, 1989
Escherichia coli BL21(DE3)	Expression host for pET-28 vectors	II, III	Novagen
Escherichia coli XL-1 Blue	Cloning host in site-directed mutagenesis	III	Stratagene
Plasmids			
$pFliC_{H7\Delta}$	pBluescript II KS(+) with serotype H7 *fliC* gene with 174 bp deletion	I	Westerlund-Wikström *et al.*, 1997
pQE-30	Expression vector for his-tagged constructs	I	Qiagen
pET-28a(+)	Expression vector for his-tagged constructs	II	Novagen
pET-28b(+)	Expression vector for his-tagged constructs	II	Novagen
pKTH5199	pET-28a(+) with *slpA* and N-terminal his-tag	III	II
pKTH2121	pGK12 derivative pKTH2095 with P1-$P2_{slpA}$, SS_{slpA} and *bla*-t_{slpA}	IV	Savijoki *et al.*, 1997
Cell lines			
Intestine 407	Human embryonic intestine	I	ATCC (CCL6)
Caco-2	Human colon carcinoma, differentiatiates to enterocyte-like	I	ATCC (HTB-37)
EA-hy926	Hybrid cell line with characteristics of human endothelial cells	I	Edgell *et al.*, 1983
T24	Human urinary bladder	I	ATCC (HTB-4)

Table 5. Methods used in Studies I-IV

Method	**Used and described in Study**
Genetic methods	
Cloning to pFliC$_{H7\Delta}$ vector	I
Cloning to pQE-30 vector	I
Cloning to pET-28 vectors	II
Cloning to pKTH2121 vector	IV
Site-directed mutagenesis	III
Isolation of RNA	IV
Northern hybridization	IV
TRAC	IV
DNA sequencing	I, II, III, IV
Immunological methods	
Western hybridization	I
Immunoelectron microscopy	I
Immunofluorescence microscopy	I
Adhesion assays with bacteria/flagella	
Adherence of bacterial cells to human cell lines and fibronectin	I
Haemagglutination by bacterial cells	I
Adherence of chimeric flagella to human cell lines and fibronectin	I
Bacterial cell wall-related techniques	
FITC-labelling of bacterial cells	I
Isolation of cell walls of *L. brevis*	II, III
TCA extraction of cell walls	II
Determination of total phosphorous	II
Protein assays	
Expression and purification of his-tagged peptides	I, II, III
Extraction of S-layer proteins	I, II
Binding of recombinant S-layer proteins to bacterial cells and cell walls	II
Reassembly of recombinant S-layer proteins by dialysis	II, III
SDS-PAGE	I, II, III, IV
Proteolytic degradation of SlpA	II
N-terminal sequencing	II
Peptide mapping	II
Testing of solvent accessibility of cysteine residues in mutant proteins	III
SAXS	III
Determination of beta-lactamase activity	IV
Determination of aminopeptidase activity	IV
Electron microscopy	I, II, III
Amino acid and/or nucleotide sequence analyses	I, II, III, IV

4. RESULTS AND DISCUSSION

4.1. Structure and function of SlpA

4.1.1 Adhesive functions (I)

When Study I was initiated, the probiotic effects and potential health-related applications of *Lactobacillus* strains were of special interest, and the adherence to host tissues or cells was rather unanimously considered an important feature of a probiotic strain. The initial finding that the chemical removal of the S-layer protein from the surface of *L. brevis* ATCC 8287 abolishes the binding of the strain to cultured human epithelial cells prompted us to examine the role of the S-layer protein in adherence to human cells. As the 435-amino-acid S-layer protein of *L. brevis* ATCC 8287, SlpA, was genetically well-characterized (Vidgren *et al.*, 1992), the difficulties in creating S-layer-negative *Lactobacillus* mutants (Boot *et al.*, 1996; Martinez *et al.*, 2000; Buck *et al.*, 2005) and those related to the poor solubility of S-layer proteins were overcome by utilizing the previously established flagellar display system (Westerlund-Wikström *et al.*, 1997).

Different regions of the *slpA* gene were expressed as internal in-frame fusions in the variable region of the flagellin gene of *E. coli*, and the adhesive properties of the resulting chimeric flagella were examined (Figures 1 and 4 and Table I in I). Chimeric flagella displaying a peptide from the N-terminal part of SlpA ($SlpA_{66-215}$) bound to the same targets as S-layered *L. brevis* cells, *i.e.* to human epithelial cells representing human gut (Int 407, Caco-2), urinary bladder (T24) and blood vessels (EA-hy926) and to the immobilized form of the human extracellular matrix protein fibronectin. Flagella with no inserts and those displaying the C-terminal part of SlpA ($SlpA_{209-417}$) failed to bind, confirming binding specificity and delineating the adhesive property to the N-terminal part of SlpA. The smallest SlpA fragment binding to Int 407 cells was 81 amino acids in size and comprised amino acids 66-146 in the mature SlpA protein. Neither bacterial cells nor flagella agglutinated human erythrocytes.

The binding of *L. brevis* cells to Int 407 cells could not be inhibited either by chimeric flagella or by antibodies from an antiserum raised against whole SlpA or from an antiserum raised against the histidine-tagged N-terminal binding peptide his-$SlpA_{66-215}$. To explain the lack of inhibition by antibodies and to the verify the location of the SlpA inserts in the chimeric flagella, the immunological reactivities of the chimeric flagella, of the N-terminal peptide his-$SlpA_{66-215}$ and of whole *L. brevis* cells with the two antisera were tested using Western hybridization, immunoelectron microscopy or immunofluorescence microscopy (Figures 1 and 2 in I). These experiments led to two major conclusions. First, as the anti-peptide antiserum failed to recognize its antigen on whole *L. brevis* cells in an immunofluorescence assay, the region encompassing residues 66-215 of SlpA was demonstrated to be poorly accessible to antibodies in the polymerized S-layer. Second, the reactivities of the two antisera with the N-terminal part of SlpA were clearly different. The anti-peptide serum was more reactive, as it recognized its antigen both from whole SlpA and the chimeric flagella in Western hybridization and from the flagellar inserts in immunelectron microscopy, while the anti-SlpA serum recognized only large N-terminal inserts in the flagella in Western

hybridization and immunoelectron microscopy. This indicates that the N-terminal region of SlpA is poorly immunogenic when whole SlpA is used as an immunogen. In light of these results, the lack of inhibition by antibodies could be explained by the poor accessibility of the binding region to antibodies from the serums and by the low amount of binding-site-specific antibodies in the anti-SlpA serum. The lack of inhibition by chimeric flagella, in turn, could at least partly be explained by the relatively low concentration of flagella used in the inhibition experiments compared with the very efficient adherence of bacterial cells having the binding epitope as hundreds of thousands of copies on their surface. The intriguing immunological findings of the location of the binding region in SlpA in a rather non-accessible environment lead at this stage of investigation to the hypothesis of the binding site being located in a groove or "pocket" in the three-dimensional structure of SlpA not accessible to antibodies but still able to bind to the receptor on target cells and fibronectin. Further research has shed more light on the structure and structure-function relationships of different regions of SlpA (see Sections 4.1.2-4.1.4).

The binding of SlpA to both epithelial cells and fibronectin raised the question about the role of cell surface fibronectin as a receptor for SlpA. Fibronectin is a large glycoprotein present in plasma and extracellular fluids, in the extracellular matrix and on eukaryotic cells, where it specifically binds to a variety of integrin molecules (Pankov & Yamada, 2002). It has been demonstrated also in the intestine of humans (Korhonen *et al.*, 2000; Groos *et al.*, 2003) and rodents (Quaroni *et al.*, 1978; Laurie *et al.*, 1982; Kolachala *et al.*, 2007) and it is produced by cultured human (Nickerson *et al.*, 2001; Walia *et al.*, 2004; Kolachala *et al.*, 2007) and rodent intestinal epithelial cell lines (Quaroni *et al.*, 1978; Göke *et al.*, 1996). A number of bacterial species have fibronectin binding proteins on their surface mediating attachment or invasion to eukaryotic cells either directly or through a bridging mechanism (Joh *et al.*, 1999; Monteville *et al.*, 2003; Schwarz-Linek *et al.*, 2004; 2006; Graham *et al.*, 2008). Fibronectin binding seems to be rather common also in lactobacilli (Toba *et al.*, 1995; Turner *et al.*, 1999; Kapczynski *et al.*, 2000; Sillanpää, 2001; Styriak *et al.*, 2001; Lorca *et al.*, 2002; Styriak *et al.*, 2003; Jakava-Viljanen & Palva, 2007; Munoz-Provencio *et al.*, 2009) but, with the exception of the surface localized enolase of *L. plantarum* (Castaldo *et al.*, 2009) (see section 1.2.7), the binding molecules have usually not been characterized. Only one *Lactobacillus* protein with a predicted procaryotic fibronectin-binding domain and a role in bacterial adherence to Caco-2 cells has been identified (Buck *et al.*, 2005). As the synthesis of fibronectin in Caco-2 cells decreases during differentiation (Levy *et al.*, 1994), and in the study of Buck *et al.* (2005) the binding of fibronectin of the *Lactobacillus* strain was not verified, the role of fibronectin in the Caco-2 cell binding of this strain remains obscure. Kapczynski *et al.* (2000) demonstrated that the adherence of *Lactobacillus acidophilus* to Int 407 cells correlates with the spatial distribution of fibronectin on the surface of the cells. In the study of Kapczynski *et al* (2000), the participating *Lactobacillus* molecule was, based on inhibition studies, suggested to be a lipoteichoic acid. The binding of *L. brevis* through SlpA to epithelial cells might also be partially mediated by fibronectin. An interaction between SlpA and fibronectin was demonstrated by de Leeuw *et al.* (2006) by surface plasmon resonance studies, although soluble fibronectin with a

conformation different from that of the immobilized form was used. Fibronectin has 4-9% carbohydrate (Pankov & Yamada, 2002), and the carbohydrate chains might in principle extend to the shielded N-terminal region of SlpA on bacterial cells. Furthermore, indications of carbohydrate binding by an S-layered *L. brevis* strain and its guanidine hydrochloride extractable surface material have been presented (Uchida *et al.*, 2006), although aggregates of the S-layer protein were present in the binding assay compromising its specificity. However, Int 407 cells cultivated as monolayers express only small amounts of fibronectin on their surfaces (Nickerson *et al.*, 2001), and the binding of the flagella to fibronectin was very weak (Fig. 4 in I), while the binding of the chimeric flagella to epithelial cells was efficient. Such sparse and weak interactions are therefore not likely, at least alone, to be responsible for the observed very efficient binding of *L. brevis* to epithelial cells (Table 1 in I). The principal receptor for SlpA in epithelial cells thus remains to be investigated.

The binding of *L. brevis* to Caco-2 cells was only moderately inhibited by the chemical removal of SlpA from *L. brevis* cells (Table 1 in I), indicating that in addition to SlpA, *L. brevis* ATCC 8287 probably has other adhesive surface molecules contributing to adherence. The expression of multiple adhesins is common in bacterial pathogens (Holden & Gally, 2004; Wright & Hultgren, 2006; Sillanpää *et al.*, 2008; Flanagan *et al.*, 2009; Jakubovics *et al.*, 2009; Nicholson *et al.*, 2009) and is likely to occur in commensal bacteria as well. The contribution of other adhesive factors in *L. brevis* adherence to the other cell lines can not be completely excluded either, as the extraction by guanidium hydrochloride also removes some other, minor proteins from the cell surface (Fig. 2 in I), and the extraction or loss of SlpA may alter the conformation and/or functionality of the remaining protein or non-protein components of the cell wall.

The molecular mechanism of the binding of SlpA to fibronectin or epithelial cells is currently not known. The demonstration of the binding by flagellar display indicates that the binding is not critically dependent on the presence of a two-dimensional, regular S-layer structure, which is not formed in the flagella. As later shown in Study II, most of the fragments conferring binding in the flagella were unable to form regular self-assembly products as recombinant proteins. The lack of necessity of the S-layer structure in adherence has also been confirmed in studies where the N-terminal part of SlpA, including the adhesive region ($SlpA_{1-217}$), displayed on the surface of a non-adhesive *Lactococcus* strain rendered the strain adhesive to Int 407 cells and fibronectin (Åvall-Jääskeläinen *et al.*, 2003). Similarly, the collagen-binding property of the S-layer protein CbsA of *Lactobacillus crispatus* was preserved when the protein was displayed on the surface of a non-S-layered, non-adhesive *L. casei* strain, where it does not form a regular lattice structure (Martinez *et al.*, 2000). Surface plasmon resonance experiments have shown that SlpA binds soluble fibronectin with a moderate affinity (K_d, 89.8 nM) and that the binding is inhibited by a serine protease inhibitor, benzamidin, while the binding of laminin by SlpA is threefold more efficient and not inhibited by benzamidin, suggesting different mechanisms and possibly different regions of SlpA involved (de Leeuw *et al.*, 2006). In the study of de Leeuw *et al.* (2006), the hydrophobicity and, specifically, the arginines in the N-terminal region of SlpA were suggested to contribute to the inhibition of fibronectin binding by the

polar benzamidine molecule. Not excluding the potential participation of arginine molecules in specific interactions, the overall hydrophobicity of SlpA is higher in the C-terminal than in the N-terminal region (I).

4.1.2 Cell wall binding (II)

Several pieces of evidence suggested the existence of a two-domain organization in SlpA with an N-terminal cell-wall binding region. Trypsin degradation of SlpA and the N-terminal sequencing of the proteolytic fragments revealed a protease-resistant C-terminal part of 246 amino acids and a protease-susceptible N-terminal part in SlpA (Fig. 3 in II). The same pattern of proteolytic fragments, but at a very low intensity, was seen after the trypsin degradation of S-layered *L. brevis* cells. A comparison of the amino acid sequences of SlpA and five other S-layer proteins of *L. brevis* strains revealed that the N-terminal parts were conserved and had a high predicted pI value (Fig. 1 in II); also the (C-terminal) cell wall binding domains in the S-layer proteins of *L. acidophilus* ATCC 4356 and *L. crispatus* JCM5810 are conserved and highly positively charged (Smit *et al.*, 2001; Antikainen *et al.*, 2002). Finally, the N-terminal cell wall binding region in SlpA was confirmed by testing the cell wall binding capabilities of C- and N-terminally truncated recombinant SlpA proteins, and the first 145 amino acids of mature SlpA alone were found to be sufficient for binding to isolated cell wall fragments of *L. brevis* ATCC 8287, while N-terminally truncated proteins were unable to bind (Fig. 6 in II).

Conserved carbohydrate binding motifs were identified in the positively charged N-terminal regions of six *L. brevis* S-layer proteins (Fig. 7 in II). These regions share similarity with the repeated C-terminal carbohydrate binding sequences detected in clostridial toxins, streptococcal glucosyltransferases (Wren, 1991; von Eichel-Streiber *et al.*, 1992) and the S-layer proteins of *L. acidophilus* group organisms (Smit *et al.*, 2001) (see also Section 1.2.3). These motifs are supposed to play a general role in protein-carbohydrate interactions by acting as initial attachment sites between the protein and the carbohydrate, enabling the specific interactions to occur (von Eichel-Streiber *et al.*, 1992). They may thus have a role in the observed binding of SlpA to the glycosylated extracellular matrix protein fibronectin or carbohydrate-containing epithelial cell receptors (see Section 4.1.1). More likely, however, they contribute to the anchoring of SlpA to the cell wall polysaccharides. While the S-layer proteins of *L. acidophilus* ATCC 4356 and *L. crispatus* JCM5810 bind to teichoic acids, and electrostatic interactions were suggested in the binding (Smit *et al.*, 2001; Antikainen *et al.*, 2002; Smit & Pouwels, 2002), the cell wall receptor of SlpA was in this study shown to be one other than teichoic or lipoteichoic acid: a treatment removing teichoic acids had no effect on the ability of the cell walls to bind SlpA, whereas a treatment affecting wall polysaccharides had, and lipoteichoic acids were not present in the cell wall preparations used in the binding experiments. The results of this study are thus in accordance with early studies, in which neutral polysaccharides in the cell walls of *L. brevis* ATCC 8287 were suggested to be involved in the anchoring of the SlpA to the cell wall (Shimohashi *et al.*, 1976; Masuda & Kawata, 1980), but the detailed structure of this polysaccharide remains to be determined. The location of the cell wall binding domain in the N-terminal part of SlpA and the dissimilarity of the cell

wall receptor apparently reflect the phylogenetic non-relatedness of *L. brevis* and its S-layer protein to *L. acidophilus* group organisms and their S-layer proteins.

4.1.3 Self-assembly (II)

The protease resistance of the C-terminal part of SlpA, the variable amino acid sequences of the known *L. brevis* S-layer proteins in this distinct region and the localization of the cell wall binding domain in the N-terminal part (see Section 4.1.2) suggested an external location and a role in self-assembly for the C-terminal part of SlpA. The location of the self-assembly region was confirmed by transmission electron microscopy of the self-assembly products of N-terminally truncated recombinant SlpA proteins. In these studies, the protein encompassing amino acids 179-435 in mature SlpA was able to form a lattice identical to that formed by wild-type SlpA, as recognized by electron microscopy (Fig. 4 in II) and by the determination of lattice constants. The removal of 11 residues more from the N-terminus in $rSlpA_{190-435}$ prevented lattice formation. Surprisingly, also larger N-terminally truncated proteins $rSlpA_{167-435}$ and $rSlpA_{149-435}$ were unable to form regular lattices, indicating that the extension of the peptide further to the cationic N-terminal region interferes with lattice formation when the rest of the protein and the cell wall are not present, probably by steric hindrance and/or by preventing the acquisition of the native conformation of the peptide.

In conclusion, SlpA was found to be a two-domain protein in which the N-terminal domain (residues 1-178), including the cell wall binding region, is shielded from the external environment by the protease-resistant C-terminal domain (residues 179-435), responsible for the self-assembly of the SlpA monomers to a regular layer. SlpA thus follows the current view of Gram-positive S-layer proteins having two separate functional domains. However, the order of the functional regions is the opposite of the other thus far characterized *Lactobacillus* S-layer proteins. A schematic presentation of the structure of SlpA is shown in Fig. 4.

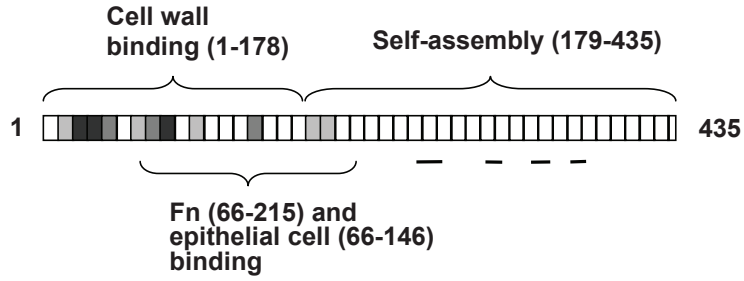

Figure 4. A schematic presentation of the functional regions in mature SlpA. The different colours of the ten amino acid stretches shown by boxes indicate the level of similarity between six *L. brevis* S-layer proteins: white, 0-20%; dark grey, 61-80% identical or similar amino acids in a stretch. Short black bars indicate the regions most accessible in the assembled S-layer, as detected by maleimide modification (III). Adapted from Figure 1 of (II) with permission of the publisher.

4.1.4 Locations of individual residues (III)

Considering potential applications of SlpA as a display vehicle of foreign peptides, a detailed mapping of the locations of individual residues in the self-assembly region of SlpA was performed by cysteine scanning mutagenesis and targeted chemical modification. A series of 46 SlpA mutant proteins was generated with 40 of the single, novel cysteine residues located in the C-terminal self-assembly region. The abilities of the mutant proteins to self-assemble into regular lattices in solution and on cell wall fragments was confirmed by transmission electron microscopy and by SAXS combined with cryoelectron microscopy, respectively (Figures 1 and 2 in III). The surface accessibility of the mutated residues was studied using two sulfhydryl-reactive maleimide reagents of different molecular weights, TMM$(PEG)_{12}$ and AlexaFluor488-maleimide. Their reactions with both monomeric proteins in solution and with proteins assembled on cell wall fragments were investigated.

Using the combined results of the different experimental settings (Figures 4 and 5 in III) and previous data about the domain organization of SlpA (Study II), the mutated residues were assigned to four groups according to their most probable location in the protein monomer and the lattice structure: those on the outer surface of the lattice, those in the protein interior, those on the inner surface of the lattice and those on the subunit interface/pore region of the lattice (Table 2 in III). The latter two groups were characterized by a different extent of modification of the cysteine residue in the monomeric and assembled form, and were differentiated from each other on the basis of the location of the residues, the "inner surface" group consisting of residues of the cell wall binding region and the extreme N-terminal part of the self-assembly region. The assignments to the four groups were supported by several facts. The groups were clearly distinguishable by the characteristic amino acid compositions of the regions surrounding the mutated residues: residues mapped to the outer surface were in a more hydrophilic and negatively charged environment than those mapped to the protein interior; the surroundings of the residues mapped to the inner surface were positively charged, and the environments of the interface/pore group residues were distinguishable by the lack of charge (Table 2 in III). The assignments were also supported by the sites of predicted, but not observed, trypsin cleavage, most of which were located at sites predicted to be in the protein interior or pore region (Fig. 6 in III). In addition, the assignments were in agreement with the PredictProtein secondary structure prediction of SlpA (Rost *et al.*, 2004), chosen on the basis that it most accurately reproduces the secondary structure estimates obtained by physical measurements (Mobili *et al.*, 2009b): residues assigned to the outer surface of the lattice were mostly located in the predicted loops, residues assigned to protein interior within the predicted β-strands and residues assigned to the pore/interface regions within both loops and β-strands (Fig. 6 in III).

The mutated residues that were most accessible in the assembled S-layer were located in four segments of SlpA spanning amino acids 256-273, 303-316, 335-349 and 362-372 (Figures 4 and 5 in III). This finding is in good accordance with a previous study, in which an 11-amino-acid poliovirus VP1 epitope was displayed in SlpA (Åvall-Jääskeläinen *et al.*, 2002): of the three insertion sites in the self-assembly region of SlpA tested, the epitope was accessible to antibodies when inserted between

residues 251 and 252 (near the first surface-accessible segment of Study III) or between residues 365 and 366 (within the last surface-accessible segment of Study III). The inability of antibodies to recognize the epitope inserted between residues 316 and 317, which is an accessible site as measured by maleimide modification in Study III, may be due to the altered conformation of the inserted peptide or SlpA at the insertion site, or to the possibly different interactions in antibody binding compared with those in a cysteine-maleimide reaction.

Overall, the results of the cysteine scanning mutagenesis study confirm the two-domain organization of SlpA. The residues mapped to the outer surface of the layer may have potential in further applications, *e.g.* in the surface display of antigenic or other effector molecules in SlpA. Furthermore, as the amino acid sequences of S-layer proteins usually fit poorly the existing algorithms for the prediction of secondary structures or solvent accessibilities, the relationships found between the primary structure and surface accessibilities of residues in SlpA could prove helpful when developing a program for reliably predicting S-layer protein structures.

4.2. Activities of *slpA* promoters (IV)

The gene encoding SlpA is preceded by two consecutive promoters, P1 and P2 (Vidgren *et al.*, 1992). Previous studies have indicated that both of these promoters are functional during all growth phases in *L. brevis* ATCC 8287, with significantly more transcripts detected from the downstream promoter P2, and the expression of *slpA* has been suggested to be tightly regulated (Kahala *et al.*, 1997). As *Lactobacillus* S-layer proteins are attractive candidates for display vehicles of antigens or other effector molecules in humans or animals and the factors affecting the expression of S-layer protein genes in lactobacilli are poorly known, a study aiming at the characterization of the function of the two promoters in more detail and at the recognition of potential *cis*-acting elements upstream of *L. brevis slpA* was initiated. The strategy was to separate the two promoters from each other on reporter plasmids with different parts of their upstream sequences deleted (Fig. 1 in IV), and to measure the enzyme activities conferred by the different promoter regions (Fig. 2 in IV). Moreover, promoter activities were investigated at the mRNA level (Fig. 3 in IV), effects of selected intestinal conditions on promoter activities were evaluated *in vitro* (Fig. 4 in IV) and sequence analyses of the upstream regions of *slpA* and other *Lactobacillus* S-layer protein genes were performed.

As shown in Fig. 2 in Study IV, in *L. lactis*, only promoter 1 was recognized, and the reporter enzyme activities of the *L. lactis* strains carrying the smallest region upstream of P1, 57 base pairs above the transcription start, were significantly lower than the activities of strains carrying larger promoter regions. In *L. brevis*, both promoters were recognized during all growth phases, as expected, and the wild-type double promoter was more efficient than either P1 or P2 alone. However, no difference was observed between the efficiencies of the two promoters, except that the reporter enzyme activity of the strain carrying the smallest region upstream of P1 was somewhat lower than the very high activities of all of the other strains. This conflicting result compared with a previous study (Kahala *et al.*, 1997) is probably attributed to the different methods used in these two studies. In the study of Kahala *et al.* (1997), the

slpA mRNAs were quantified from *L. brevis* by a method based on the hybridization of two oligonucleotide probes specific for either P1 transcript or both transcripts; this method is prone to errors due to possible differences in the binding efficiencies of the two probes and the relatively poor linearity of the immunological digoxigenin-based detection (unpublished observation). In the present work, two methods, the highly reproducible TRAC (transcript analysis with aid of affinity capture) (Rautio *et al.*, 2008) and conventional Northern blotting, were used to quantify reporter gene transcripts from *L. brevis* clones carrying different *slpA* promoter regions on plasmids. Essentially the same pattern of promoter activities seen in the *L. brevis* clones at a reporter enzyme level was also seen at the mRNA level, suggesting regulation of *slpA* expression at the transcriptional rather than at the translational level (Fig. 3 in IV). The high activities of the promoters were retained when the recombinant *L. brevis* strains were cultivated under conditions mimicking the intestinal environment (Fig. 4 in IV). No indications of an effect of the composition of the growth medium on *slpA* gene expression in *L. brevis* were obtained.

Three of the potential regulatory motifs, originally identified by Wels *et al.* (2006) in the upstream regions of conserved genes in bacilli and lactic acid bacteria, were also detected in the upstream, intergenic region of *slpA* (Fig. 1B in IV). None of the three motifs was, however, located in the region missing from the smallest P1 promoter construct, and the motifs could thus not directly explain the observed patterns of promoter activities. However, a few pieces of evidence suggest that *cis*-acting factors may be involved in the regulation of *slpA* expression in *L. brevis* ATCC 8287 under some, as yet unidentified conditions. All of the three potential regulatory motifs identified were found also in the genome of *L. brevis* ATCC 14869 upstream of the S-layer protein gene *slpB*, but not upstream of *slpC* or *slpD* of the same strain; *slpB* and *slpD* are expressed under different conditions, the differential expression probably being mediated by a soluble cytoplasmic factor, and *slpC* is known to be silent under laboratory conditions (Jakava-Viljanen *et al.*, 2002). The upstream region of *slpA* is well conserved in a subset of *L. brevis* strains, as the sequences upstream of *slpA*, *slpB* and the truncated *slpA* homologue in *L. brevis* ATCC 367 are practically identical, although the similarity between the coding regions of *slpA* and *slpB* is only 30% (Jakava-Viljanen *et al.*, 2002). Finally, upstream of *slpA*, an ORF with similarity to bacterial genes encoding aminotransferases with helix-turn-helix DNA-binding domains (so-called MocR-type GntR-family transcriptional regulators) has been identified (unpublished data), though the possible participation of this gene in the regulation of *slpA* expression is currently highly hypothetical.

In summary, the present information about the function of the two promoters of *slpA* indicates a very efficient function of both promoters during all growth phases in *L. brevis*, but not in *Lactococcus lactis*. More upstream sequence is needed for the full activity of promoter 1 than for promoter 2 in *L. brevis*. The possible regulation of *slpA* expression occurs at the transcriptional level, and *cis*-acting factors are likely to participate. The two promoters retain their activities under experimental intestinal conditions *in vitro*, which is advantageous for the potential use of SlpA as a carrier of foreign molecules in applications in which bacterial replication in the gastrointestinal tract is desired, as in live oral vaccines.

5. CONCLUSIONS

In this work, the structure and function of the S-layer protein SlpA of *Lactobacillus brevis* ATCC 8287 and the use of the promoters of its gene were studied. S-layer proteins have a wide application potential in nanobiotechnology. As food-grade and potentially probiotic organisms, lactobacilli are also excellent candidates for health-related applications, where their abilities to survive in the gastrointestinal tract could be utilized in the design of live oral vaccines, and their S-layer proteins could be used as carriers of antigens or other medically important molecules. A comprehensive study of bacterial S-layers and their expression is thus warranted.

This work supported 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 was also supported. In the S-layer protein SlpA of *Lactobacillus brevis* ATCC 8287, the N-terminal domain was found to bind to the cell wall and the C-terminal domain to be responsible for self-assembly; the domains were thus in a reversed order compared with the other thus far characterized *Lactobacillus* S-layer proteins, those of two phylogenetically distant, *L. acidophilus*-related species (Smit *et al.*, 2001; Antikainen *et al.*, 2002). Also the binding mechanism of SlpA to the cell wall independently of teichoic acids was found to be unique among the *Lactobacillus* S-layer proteins examined to date. The structures of the polysaccharides in the cell wall of *L. brevis* ATCC 8287 are currently being investigated. As at the moment only three *Lactobacillus* S-layer proteins have been structurally and functionally thoroughly studied, different types of structure-function relationships and cell wall binding mechanisms will presumably be revealed as more *Lactobacillus* S-layer proteins are characterized. Biophysical methods are increasingly utilized in the structural studies of S-layers, and together with computer modelling-based methods will probably allow for more high-resolution structures of bacterial S-layer proteins, which currently are scarce owing to difficulties in obtaining high-quality crystals for X-ray crystallography. To investigate the S-layer formed by SlpA in detail, comparative SAXS studies of the SlpA lattice on bacterial cells and of SlpA reassembled in solution and on different biological surfaces have already been performed (P. Jääskeläinen, personal communication). Also the solution structure of the C-terminal part of SlpA has been determined by SAXS (Serimaa *et al.*, 2009), and attempts to crystallize parts of SlpA are under way. SlpA of *L. brevis* ATCC 8287 is currently the only structurally and functionally characterized S-layer protein with a demonstrated fibronectin-binding function. Further studies are needed to determine the biological importance of the epithelial cell and fibronectin binding functions of SlpA, and the elucidation of the structure of SlpA at high resolution would be helpful in understanding the intriguing location of the binding site in the shielded N-terminal domain.

With regard to applications, several findings of this study are important. Surface-exposed regions in the assembled form of SlpA were determined. Surface display utilizing 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 living

cell. Since a clear relationship exists between antigen expression levels and immune response (Grangette *et al.*, 2001; Seegers, 2002), and *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), the identified surface-located residues are especially attractive candidates for the insertion of foreign epitopes for live vaccine design. In the future, the simultaneous display of immunomodulating molecules in the S-layer could be used to further enhance or direct the immune response. The final suitability of the sites for each application, however, remains to be experimentally determined; studies aiming at the display of an F18 fimbrial adhesin fragment in SlpA are currently in progress. The preservation of high activities of the *slpA* promoters under conditions mimicking the intestinal environment is essential in live oral vaccine applications, where bacterial replication *in vivo* is desired. The oral delivery route of vaccines is simple and safe and efficiently induces mucosal immunity, which is ideal for preventing the initial infection of most pathogens (Ryan *et al.*, 2001; Seegers, 2002; Wells & Mercenier, 2008). Furthermore, as antigen carrier systems can be significantly improved by the co-display of adhesins (Cano *et al.*, 1999; Liljeqvist *et al.*, 1999), the epithelial cell and fibronectin binding function of the N-terminal part of SlpA could be utilized in the targeted delivery of antigenic or other effector molecules. Preliminary experiments have been performed utilizing SlpA as a purified immunoglobulin binding fusion protein to target antibodies to the intestinal surfaces of calves to prevent neonatal diarrhoea by passive immunization (Khang *et al.*, 2009). Fusion partners in recombinant SlpA proteins produced in *E. coli* might further include various therapeutics, such as immunoglobulins, enzymes, bacteriocides or anti-adhesive agents, which could prove effective on different mucosal surfaces. Considering other biotechnological applications, the identified surface-located residues could be modified for the immobilization of bacterial cells or the S-layer protein, and the finding that residues on the inner surface of the SlpA lattice are positively charged suggests that immobilization of SlpA on a negatively charged support without further modification is possible. Immobilization of bacterial cells or S-layers 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; in these applications also the thermostability of SlpA (Mobili *et al.*, 2009b) may be advantageous. The results of this study thus form a basis for the development of SlpA to become a tool for a wide range of biotechnological applications.

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