Surface Proteins of *Lactobacillus crispatus*: Adhesive Properties and Cell Wall Anchoring

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Cover Figure: A schematic figure of the lactobacillar cell wall

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To Markku and my family,

PREFACE

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Helsinki, March 2007

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LIST OF ORIGINAL ARTICLES

This thesis is based on the following published articles and manuscripts, which in the text are referred to by their roman numerals. The original publications are reprinted with the kind permission of the copyright holders.

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II **Antikainen J, Anton L, Sillanpää J, Korhonen TK.** 2002. Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. *Mol Microbiol*. 46(2):381-94.

III **Antikainen J, Hurmalainen V, Lähteenmäki K, Korhonen TK.** pH-dependent association of enolase and GAPDH of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids. Submitted to Journal of Bacteriology.

IV **Antikainen J, Hurmalainen V, Lähteenmäki K, Korhonen TK.** Enolases from pathogenic bacteria and commensal lactobacilli share functional similarity in virulence-associated traits. Submitted to FEMS Immunology and Medical Microbiology.

SUMMARY

Bacterial surface-associated proteins are important in communication with the environment and bacteria-host interactions. In this thesis work, surface molecules of *Lactobacillus crispatus* important in host interaction were studied. The *L. crispatus* strains of the study were known from previous studies to be efficient in adhesion to intestinal tract and ECM. *L. crispatus* JCM 5810 possess an adhesive surface layer (S-layer) protein, whose functions and domain structure was characterized. We cloned two S-layer protein genes (*cbsA*; collagen-binding S-layer protein A and silent *cbsB*) and identified the protein region in CbsA important for adhesion to host tissues, for polymerization into a periodic layer as well as for attachment to the bacterial cell surface. The analysis was done by extensive mutation analysis and by testing His_6-tagged fusion proteins from recombinant *Escherichia coli* as well as by expressing truncated CbsA peptides on the surface of *Lactobacillus casei*. The N-terminal region (31-274) of CbsA showed efficient and specific binding to collagens, laminin and extracellular matrix on tissue sections of chicken intestine. The N-terminal region also contained the information for formation of periodic S-layer polymer. This region is bordered at both ends by a conserved short region rich in valines, whose substitution to leucines drastically affected the periodic polymer structure. The mutated CbsA proteins that failed to form a periodic polymer, did not bind collagens, which indicates that the polymerized structure of CbsA is needed for collagen-binding ability. The C-terminal region, which is highly identical in S-layer proteins of *L. crispatus*, *Lactobacillus acidophilus* and *Lactobacillus helveticus*, was shown to anchor the protein to the bacterial cell wall. The C-terminal CbsA peptide specifically bound to bacterial teichoic acid and lipoteichoic acids. In conclusion, the N-terminal domain of the S-layer protein of *L. crispatus* is important for polymerization and adhesion to host tissues, whereas the C-terminal domain anchors the protein to bacterial cell-wall teichoic acids.

Lactobacilli are fermentative organisms that effectively lower the surrounding pH. While this study was in progress, plasminogen-binding proteins enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were identified in the extracellular proteome of *L. crispatus* ST1. In this work, the cell-wall association of enolase and GAPDH were shown to rely on pH-reversible binding to the cell-wall lipoteichoic acids. Enolase from *L. crispatus* was functionally compared with enolase from *L. johnsonii* as well as from pathogenic streptococci (*Streptococcus pneumoniae, Streptococcus pyogenes*) and *Staphylococcus aureus*. His_6-enolases from commensal lactobacilli bound

human plasminogen and enhanced its activation by human plasminogen activators similarly to, or even better than, the enolases from pathogens. Similarly, the His_6-enolases from lactobacilli exhibited adhesive characteristics previously assigned to pathogens. The results call for more detailed analyses of the role of the host plasminogen system in bacterial pathogenesis and commensalism as well of the biological role and potential health risk of the extracellular proteome in lactobacilli.

1 INTRODUCTION

Species of *Lactobacillus* form the most numerous genus in the heterogeneous group of Lactic Acid Bacteria. Lactobacilli are Gram-positive, non-spore-forming, and strictly fermentative organisms producing lactic acid as the primary end product (Salminen and von Wright, 1998). The genus contains about one hundred described species, which are subdivided by 16S rRNA analysis, DNA-DNA hybridization and other phylogenetic methods, into eight major groups: *Lactobacillus buchneri*, *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus sakei*, *Lactobacillus salivarius*, and *Lactobacillus brevis* group (Salminen and von Wright, 1998; Dellaglio and Felis, 2005). The *L. delbrueckii* group includes the main species investigated in my PhD work, *Lactobacillus crispatus*, as well as dozens of other species, such as *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii*.

Lactobacilli belong to the normal flora of humans and animals in the oral cavity, the vagina and the gastrointestinal tract. They are widely utilized in production of various food products, in e.g. fermentation of milk, meat, beverages and vegetables, and therefore exploitation of lactobacilli has a huge economic impact. Because of their proposed health promoting properties, *Lactobacillus* species are widely used as probiotics (Ouwehand *et al.*, 2002). Probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on health or well-being (Salminen *et al.*, 1999). An important property proposed for a probiotic bacterium is the ability to adhere and colonize host tissues, which enhances multiplication and survival of bacteria in the host and prevents colonization by pathogenic bacteria. Suppression of the growth of pathogens can also be achieved through competition for nutrients as well as by production of bactericidal components, such as bacteriocins, lactic acid or hydrogen peroxide (Salminen and von Wright, 1998; Blum *et al.*, 1999a; Reid and Burton, 2002). Several studies have indicated potential of lactobacilli in modulation of mammalian immune system. *Lactobacillus* species affect cytokine expression in human monocytes, macrophages, or dendritic cells (Vaarala, 2003; Merk *et al.*, 2005).

In clinical trials, probiotics have been shown to prevent and promote recovery from acute rotavirus infection (Isolauri *et al.*, 1991; Limdi *et al.*, 2006) and their role in antibiotic-induced diarrhoea, irritant bowel disease and food allergy has been suggested (Boyle and Tang, 2006; Limdi *et al.*, 2006). However,

lactobacilli are occasionally associated with endocarditis, bacteraemia and several other localized infections, such as pulmonary infection, abscesses or peritonitis. These infections are usually opportunistic and polymicrobial, and patients have often underlying immunosuppressive conditions, and they may be receiving broad spectrum antibiotic therapy or have other underlying conditions, such as dental infection or heart disease (de Vrese and Schrezenmeir, 2002; Cannon *et al.*, 2005; Salvana and Frank, 2006).

In my Ph.D. work, I have studied the surface proteins of *L. crispatus* and molecular basis of these proteins in host interaction, such as in adhesion to host tissue components and in interaction with the human proteolytic plasminogen (Plg) system.

2 SURFACE PROTEINS OF *LACTOBACILLUS* INVOLVED IN HOST INTERACTION

Lactobacilli interact with the host via several distinct surface components. Adhesion to host tissues is considered to be the first step in bacterial colonization. The role of proteinaceous surface molecules in adhesion has been proposed in several studies (Conway and Kjelleberg, 1989; Tuomola *et al.*, 2000; Lorca *et al.*, 2002), although non-proteinaceous lipoteichoic acids (LTA) have been reported to mediate adhesion (Granato *et al.*, 1999). Several lactobacillar surface proteins, including the surface layer (S-layer) proteins, have been shown to bind to epithelial cells, mucus layer or other host tissue structures (Table 1). S-layers are periodic crystalline arrays that are composed of protein or glycoprotein subunits, which form a solid layer to cover the whole cell surface (Sára and Sleytr, 2000). They are found in both Archaea and Bacteria, including *Lactobacillus* species, especially in the *L. delbrueckii* group, but also in *L. brevis*, *L. buchneri* and in *L. casei* groups. The functional and structural details of lactobacillar S-layers are discussed in Chapter 2.1. Also, several non-S-layer proteins have been characterized to mediate lactobacillar adhesion to host tissues (Table 1). Many of these proteins are anchored covalently to peptidoglycan (PG) by the so-called LPXTG-motif. The protein anchoring mechanisms onto Gram-positive cell wall are discussed more in Chapter 2.3.

One class of lactobacillar proteins important in survival within the host is the bacteriocins, which are produced by several lactobacillar species and are antimicrobial against other microbes. These bacteriocins have a role in food industry, where they prevent spoilage, and promote quality of the products, but they are also proposed to suppress the growth of harmful bacterial species in the gastro-intestinal tract and thus may have potential in clinical applications (Cotter *et al.*, 2005). Bacteriocins of *Lactobacillus* are inhibitory against several pathogens, such as *Campylobacter jejuni* (Stern *et al.*, 2006), *Porphyromonas gingivalis* (Pangsomboon *et al.*, 2006), *Helicobacter pylori* (Kim *et al.*, 2003) and *Listeria monocytogens* (Loessner *et al.*, 2003; Ghalfi *et al.*, 2006) but also against heterologous *Lactobacillus* species (Ouwehand, 1998).

Another class of important lactobacillar surface proteins are proteases, in particular those degrading casein, the most abundant protein in milk. Casein provides essential amino acids for bacterial growth in milk (reviewed in Savijoki

Table 1. Proposed or identified adhesive surface proteins of *Lactobacillus*.

Adhesin	Target	Species/Strain	Reference
Surface layer proteins			
S-layer protein	Avian intestinal epithelial cells	*Lactobacillus acidophilus spp.*	Schneitz *et al.*, 1993
CbsA	Collagens, laminin	*Lactobacillus crispatus* JCM 5810	Toba *et al.*, 1995
SlpA	Fibronectin, human epithelial cell line	*Lactobacillus brevis* ATCC8287	Hynönen *et al.*, 2002
S-layer protein	Red blood cells	*Lactobacillus kefir* CIDCA 8321, *Lactobacillus parakefir* CIDCA 8328	Garrote *et al.*, 2004
SlpA	Murine ileal epithelial cells	*Lactobacillus acidophilus* M92	Frece *et al.*, 2005
LPXTG-motif proteins			
Mub	Hen intestinal mucus, pig mucin	*Lactobacillus reuteri* 1063	Roos and Jonsson, 2002
Mub (LBA1392)	Human intestinal epithelial cell line	*Lactobacillus acidophilus* NCFM	Buck *et al.*, 2005
Lsp	Murine gut epithelium	*Lactobacillus reuteri* 100-23	Walter *et al.*, 2005
Msa (LP1229)	Mannosides	*Lactobacillus plantarum* WCFS1	Pretzer *et al.*, 2005
LspA	Human intestinal epithelial cell line	*Lactobacillus salivarius* UCC118	van Pijkeren *et al.*, 2006b
Anchorless housekeeping proteins			
EF-Tu	Human intestinal epithelial cell line, mucin	*Lactobacillus johnsonii* NCC533	Granato *et al.*, 2004
GroEL	Human intestinal epithelial cell line, mucin	*Lactobacillus johnsonii* NCC533	Bergonzelli *et al.*, 2006
Others			
Cna	Type I collagen	*Lactobacillus reuteri* NCIB 11951	Roos *et al.*, 1996
FbpA	Human intestinal epithelial cell line	*Lactobacillus acidophilus* NCFM	Buck *et al.*, 2005
MapA	Porcine intestinal mucus, human intestinal epithelial cell line	*Lactobacillus reuteri*104R	Rojas et al., 2002; Miyoshi *et al.*, 2006

et al., 2006). Cell-envelope proteases (CEPs), which perform the first step in casein degradation, have been characterized from *Lactobacillus paracasei* (Holck and Naes, 1992), *Lactobacillus bulgaricus* (Gilbert *et al.*, 1996), *L. helveticus* (Pederson *et al.*, 1999), and from *Lactobacillus rhamnosus* (Pastar *et al.*, 2003). These proteases are typically large in molecular size (approximately 2000 amino acids) and comprised of several domains with distinct functions, such as prepro-domain, catalytic domain, spacer domain, and cell wall attachment domain (the LPXTG-motif) (Siezen, 1999; Savijoki *et al.*, 2006). The second phase in casein utilization, the transportation of peptides into the cell, is mediated by the Opp transporter system as well as by DtpT and Dpp systems (Doeven *et al.*, 2005). In *Lactococcus lactis*, the PrtP protease and Opp transporter system are crucial for growth in milk (Tynkkynen *et al.*, 1993; Siezen, 1999; Savijoki *et al.*, 2006), whereas the individual intracellular

peptidases responsible for further degradation of casein are not essential (Christensen *et al.*, 1999; Savijoki *et al.*, 2006). Importance of lactobacillar protease systems in host interaction is poorly known. Liberation of bioactive peptides from casein has been proposed to promote human health by e.g. stimulating the immune system (Pihlanto and Korhonen, 2003; Meisel, 2004). Several reports of lactobacillar interaction with human immune system has recently been published (reviewed in Vaarala, 2003), however, only a few proteins have been shown to be involved in immunological processes, e.g. GroEL and the elongation factor Tu (EF-Tu) discussed in Chapter 2.4.

Sequencing of lactobacillar genomes has produced new insights into putative surface proteins with a possible role in host interaction. For instance, the genome of *L. plantarum* encodes 223 putative surface proteins identified by domain compositions and homology to characterized surface proteins in other bacterial species. From those surface proteins, 12 were predicted to be involved in adhesion, 69 in enzyme reactions, 30 as transporters and the rest were predicted to function as regulators, phage receptors or possess an unknown function (Boekhorst *et al.*, 2006b). This prediction suggests presence of a biologically important secretome in lactobacilli, and extensive efforts will be needed to confirm and to characterize the possible role of these putative surface proteins in lactobacillar-host interaction. Indeed, several reports on identification of the secretome of *Lactobacillus* have been reported during last years (Wall *et al.*, 2003; van Pijkeren *et al.*, 2006; Hurmalainen *et al.*, 2007). Proteins with an essential physiological function in intracellular processes have been found on the bacterial cell wall and in the extracellular proteome. These proteins are called anchorless since no typical signal sequence or anchoring motif has been detected in their sequence. In *Lactobacillus*, these proteins have been shown to modulate the immune system and to interact with the human proteolytic Plg system (see Chapter 2.4).

2.1 Surface layer proteins

No general function has been identified for S-layer proteins, but several lactobacillar S-layers have been identified as putative adhesins with affinity for various tissue compartments or molecules (Table 1). Treatment of *Lactobacillus kefir* and *Lactobacillus parakefir* cells with lithium chloride (LiCl), which is the routine method to extract the S-layer from the bacterial surface, abolished the hemagglutination ability of these cells (Garrote *et al.*, 2004). However, hemagglutination is apparently not a common characteristic of *Lactobacillus*

(Ocaña *et al.*, 1999; Colloca *et al.*, 2000). Schneitz *et al.*, (1993) proposed that S-layer of *L. acidophilus* mediates binding to intestinal epithelial cells and Frece *et al.*, (2005) showed that treatment of *L. acidophilus* M92 cells with LiCl abolished the bacterial adhesiveness to mouse ileal epithelial cells. However, removal of S-layer with LiCl or other chemical extraction method may simultaneously remove other cell-wall proteins important in adhesion, and these observations remain suggestive. The deletion of the S-layer gene *slpA* in *L. acidophilus* NCFM abolished the bacterial adherence to a human intestinal epithelial cell line, but the authors suggested that phenotype of the mutation likely resulted from loss of other surface proteins bound onto the S-layer (Buck *et al.*, 2005). *L. crispatus* JCM 5810 adheres efficiently to collagens and laminin, which are major components of mammalian extracellular matrix (ECM) and the extracted S-layer protein bound to collagen IV (Toba *et al.*, 1995). Only in one case the adhesive function of a lactobacillar S-layer has been confirmed by genetic means. Treatment of *L. brevis* ATCC 8287 cell with GnHCl abolished binding of this strain to intestinal epithelial cell line and suggested the role of S-layer. Expression of fragments of the *L. brevis* S-layer protein SlpA as a genetic fusion in flagellar FliC subunits in *Escherichia coli* conferred binding of chimeric flagella to human epithelial cells and fibronectin confirming the adhesive characteristics of the *L. brevis* SlpA. The receptor-binding region responsible for binding to fibronectin was mapped to 81 amino acids in the N-terminal part of the protein (Hynönen *et al.*, 2002).

Adhesive S-layers have a role in inhibition of adhesiveness of pathogenic bacteria and thus can contribute to probiotic effects of lactobacilli. The removal of S-layer with GnHCl from *L. crispatus* JCM 5810 diminished the ability of *L. crispatus* cells to inhibit adhesion of pathogenic *E. coli* to a basement membrane (BM) preparation (Horie *et al.*, 2002). The adhesion of enterohaemorrhagic *E. coli* O157:H7 to human epithelial cell line was inhibited in the presence of the S-layer protein extract of *L. helveticus* (Johnson-Henry *et al.*, 2007).

In addition to lactobacillar S-layer, S-layer proteins from other bacterial genera mediate adhesion to host tissues. *Bacillus cereus* binds to laminin and the S-layer protein was identified as a laminin-binding protein by inhibition assays using antiserum against the S-layer (Kotiranta *et al.*, 1998). The native and recombinant S-layer protein of *Clostridium difficile* bind to human and murine gastrointestinal epithelium and lamina propria (Calabi *et al.*, 2002). Also in Gram-negative bacteria, S-layer proteins have been characterized as adhesins and also as virulence factors. In *Aeromonas*, the S-layer functions as an adhesin to fish cell lines as well as to BM and ECM components laminin and fibronectin

(Ishiguro *et al.*, 1981; Doig *et al.*, 1992; Noonan and Trust, 1997). The purified S-layer protein of *Bacteroides forsythus* has hemagglutination ability, and based on antibody inhibition assays, the S-layer is involved in adhesion and invasion to human oral epithelial cell line (Sabet *et al.*, 2003).

Other functions for S-layers have also been identified. The S-protein of *L. helveticus* CNRZ 892 functions as a receptor for a phage (Beveridge *et al.*, 1997). In *Bacillus anthracis*, the two S-layer proteins exhibit murein hydrolase activity (Ahn *et al.*, 2006). The S-layer of *G. stereothermophilus* functions as a molecular sieve by trapping high molecular weight solutes (Sára and Sleytr, 1987) and as an adhesion site for exoenzyme amylase (Egelseer *et al.*, 1995; Jarosch *et al.*, 2001). The S-layer of *Bacillus thuringiensis* is involved in toxicity against an insect host (Peña *et al.*, 2006). S-layers have been proposed to have a role in cell shape determination and cell wall stabilization (Sleytr and Beveridge, 1999). Indeed, the extraction of S-layer protein reduced the viability of *L. acidophilus* at low pH, suggesting a protective role for the S-layer (Frece *et al.*, 2005).

The S-layer represents the outermost surface layer in hundreds of species in Archaea and in both Gram-positive and Gram-negative Eubacteria (Sára and Sleytr, 2000). So-far, the S-layer has been detected in a few species of the genus *Lactobacillus* (Table 2), whereas the presence of S-layer in other species of *Lactobacillus* has been poorly examined. The S-layer genes and proteins have been cloned and characterized from *L. acidophilus* (Boot *et al.*, 1993), *L. gallinarum* (Hagen *et al.*, 2005), *L. helveticus* (Callegari *et al.*, 1998; Gatti *et al.*, 2005) and from *L. brevis* (Vidgren *et al.*, 1992; Jakava-Viljanen *et al.*, 2002). Formerly, *L. johnsonii* and *L. gasseri* were proposed to lack an S-layer (Boot *et al.*, 1996b), but recently, Ventura *et al.*, (2002) identified the protein called aggregation-promoting factor from these species as an S-layer-like protein, having amino acid composition and physical properties similar to lactobacillar S-layers. Despite their similar amino acid composition, such as a low content of cysteine and methionine as well as a high content of hydrophobic amino acids and hydroxyl amino acids, the S-protein primary sequences are conserved only in closely-related species (Åvall-Jääskeläinen and Palva, 2005). Lactobacillar S-layers have a relatively high isoelectric point (pI), a characteristic also of *Methanothermus fervidus* S-layer (Bröckl *et al.*, 1991), whereas other characterized bacterial S-layers are weakly acidic (Sára and Sleytr, 2000). Lactobacillar S-layers are relatively small, 25 kDa to 71 kDa in size (Åvall-Jääskeläinen and Palva, 2005), whereas the molecular masses of S-layers in other bacterial species range up to 200 kDa (Sára and Sleytr, 2000).

Table 2. *Lactobacillus* species reported to possess an S-layer.

Species	Reference
Lactobacillus acidophilus	Boot *et al.*, 1993; Boot *et al.*, 1995
Lactobacillus amylovorus	Boot *et al.*, 1996b
Lactobacillus brevis	Masuda and Kawata, 1979; Vidgren *et al.*, 1992; Jakava-Viljanen et al., 2002
Lactobacillus buchneri	Masuda and Kawata, 1981
Lactobacullus casei	Barker and Thorne, 1970
Lactobacillus crispatus	Toba *et al.*, 1995
Lactobacillus fermentum	Masuda and Kawata, 1983
Lactobacillus gallinarum	Boot *et al.*, 1996b; Hagen *et al.*, 2005
Lactobacillus gasseri	Ventura *et al.*, 2002 *
Lactobacillus helveticus	Lortal et al., 1992; Callegari *et al.*, 1998; Gatti *et al.*, 2005
Lactobacillus johnsonii	Ventura *et al.*, 2002 *
Lactobacillus kefir	Garrote *et al.*, 2004
Lactobacillus parakefir	Garrote *et al.*, 2004

* proposed S-layer like surface protein

Multiple S-layer genes have been identified in the genomes of *L. acidophilus, Lactobacillus amylovorus, L. gallinarum, L. crispatus, L. brevis, L. gasseri and L. johnsonii* (Boot *et al.*, 1996b; Jakava-Viljanen *et al.*, 2002; Ventura *et al.*, 2002) as well as in several bacteria belonging to other genera (Dworkin and Blaser, 1997; Kuen *et al.*, 1997; Mesnage *et al.*, 1997). Boot *et al.*, (1996b) identified two S-layer protein encoding genes, one silent and one actively transcribed, in *L. acidophilus* ATCC 4356 and in the related species, *L. crispatus, L. amylovorus,* and *L. gallinarum*. In the genome of *L. acidophilus,* the active and silent genes are located in opposite orientations on a 6 kb chromosomal segment. The inversion of the *slp* segment causes an interchange of the active and the silent S-layer genes (Boot *et al.*, 1996c), which resembles a mechanism of phase variation in bacterial surface antigen expression. Four S-layer genes are present in *L. brevis* ATCC 14869, and their expression is influenced by growth conditions. In cells grown in aerobic conditions, the *L. brevis* S-layer is composed of two S-layer proteins, in contrast to cells from anaerobic conditions, where only one S-layer protein is synthesized (Jakava-Viljanen *et al.*, 2002). Transcription of *L. brevis* S-layer genes was controlled by an unidentified soluble factor and involved activation of transcription rather than occurring by chromosomal DNA rearrangement (Jakava-Viljanen *et al.*, 2002). Variation in S-layer gene expression as a response to environmental changes has also been described in *G. stereothermophilus* (Scholz *et al.*, 2001), *B. anthracis* (Mignot *et al.*, 2002), and *Campylobacter fetus* (Dworkin and Blaser, 1997).

As S-layer proteins represent 10-15% of the total amount of proteins in *Lactobacillus* cells (Boot and Pouwels, 1996), their transcription and secretion mechanisms must be efficient and tightly regulated. Multiple promoters precede several S-layer genes (Boot and Pouwels, 1996), including S-layer genes of *L. acidophilus* (Boot *et al.*, 1996a) and *L. brevis* (Vidgren *et al.*, 1992; Kahala *et al.*, 1997) and are likely to ensure efficient transcription of these genes. Also, the half-lives of mRNA encoding lactobacillar S-layer proteins are relatively high, approximately 15 min, which enables efficient protein translation (Boot *et al.*, 1996a; Kahala *et al.*, 1997). The predicted lactobacillar S-layer proteins contain a conserved N-terminal signal sequence of 25-30 amino acids (Åvall-Jääskeläinen and Palva, 2005), which indicates that their secretion occurs via the general Sec-pathway. The highly efficient lactobacillar promoter regions and signal sequences have been utilized in various heterologous proteins expression systems (Savijoki *et al.*, 1997; Kahala and Palva, 1999; Åvall-Jääskeläinen *et al.*, 2003), for instance, in expression of the adhesive S-layer protein of *L. crispatus* JCM 5810 (Martinez *et al.*, 2000).

S-layers self-assemble to cover up to 70% of the bacterial cell surface. The S-layer is not impermeable and has pores between the identical lattice units (Sára and Sleytr, 2000). Based on electron microscopy using negative staining or freeze-etching, the S-layer subunits are composed of lattices with oblique, square or hexagonal symmetry (Sára and Sleytr, 2000). The oblique lattice type was identified in the S-layers of *L. acidophilus* (Smit *et al.*, 2001), *L. brevis* (Jakava-Viljanen *et al.*, 2002) and *L. helveticus* (Lortal *et al.*, 1992) and the hexagonal lattice type in *L. casei* and *L. buschneri* (Masuda and Kawata, 1981).

Two types of post-translational modification are known in S-layer proteins. Phosphorylation has been described only in the S-layer protein of *Aeromonas hydrophila*, where the tyrosine residues are post-translationally modified (Thomas and Trust, 1995), whereas glycosylation has been reported for S-layers from Archaea and from Gram-positive bacteria (Claus *et al.*, 2005), including *Geobacillus* (Schäffer *et al.*, 2002; Steiner *et al.*, 2006), and *Clostridium* (Calabi *et al.*, 2001). In Gram-positive bacteria, linear or branched homo- or heterosaccharides have been identified (reviewed in Schäffer and Messner, 2004). The glycan structure has been reported from *L. buchneri* (Upreti *et al.*, 2003), whereas most lactobacillar S-layers apparently are non-glycosylated (Masuda and Kawata, 1983).

Only a few lactobacillar S-layer proteins have been characterized in detail, these include the S-layer proteins from *L. acidophilus* ATCC 4356 (Smit *et al.*, 2001) and from *L. brevis* ATCC 8287 as well as the S-layer protein CbsA of *L. crispatus* JCM 5810 characterized in my PhD work. The S-layer protein from *L. acidophilus* has a two-domain structure. A fragment containing the N-terminal two-thirds of the protein (SAN) crystallized into a layer and was proposed to be composed of two sub-domains with a surface-exposed loop (Smit *et al.*, 2002). The C-terminal part (SAC) was responsible for cell wall anchoring (see Chapter 2.3.1). In the adhesive S-layer protein of *L. brevis*, an N-terminal domain is responsible for adhesiveness (Hynönen *et al.*, 2002). However, the predicted amino acid sequences of *L. brevis* and *L. acidophilus* S-layers are not identical and, hence, the domain structure of *L. acidophilus* cannot be extended to *L. brevis*. The successful surface expression of a foreign peptide epitope in the C-terminal part of *L. brevis* S-layer protein suggests that the cell-wall binding domain may not be C-terminal in this S-layer protein (Åvall-Jääskeläinen *et al.*, 2002). Separate crystallization and cell-wall binding domains have also been characterized in S-layer proteins from *B. anthracis* (Candela *et al.*, 2005), *G. stearothermophilus* (Jarosch *et al.*, 2001; Pavkov *et al.*, 2003) and *Clostridium cellulovorans* (Kosugi *et al.*, 2002). Only a few attempts to determine crystal structures of an S-layer protein has been reported (Claus *et al.*, 2002) and these structures are resolved only from subdomains of S-layer from *G. stearothermophilus* and archaeal *Methanosarcina* (Jing *et al.*, 2002; Pavkov *et al.*, 2003).

2.2 Non-S-layer adhesion proteins

In addition to S-layer proteins, a few adhesive surface proteins in lactobacilli have been characterized to bind to epithelial cells, intestinal mucus or components of the ECM (Table 1). Several species of *Lactobacillus* adhere *in vitro* to the mucus preparations isolated from intestine (Rojas and Conway, 1996; Edelman *et al.*, 2002; Gusils *et al.*, 2003) as well as to mucus isolated from human faeces (Kirjavainen *et al.*, 1998; Tuomola *et al.*, 2000; Ouwehand *et al.*, 2001). The binding to mucus has generally been considered to reflect bacterial adherence to tissues, but the binding might also facilitate the removal of the bacteria and mucus can inhibit bacterial adherence to enterocytes (Salminen and von Wright, 1998). A few mucus-binding proteins have been identified, such as Mub and MapA from *L. reuteri* (Roos and Jonsson, 2002; Miyoshi *et al.*, 2006) as well as GroEL and EF-Tu from *L. johnsonii* (Granato *et al.*, 2004; Bergonzelli *et al.*, 2006) discussed further in Chapter 2.4.

The high-molecular-weight (358 kDa) protein Mub of *L. reuteri* contains 14 repeats of approximately 200 amino acids and has features typical of a surface protein, including a signal sequence, an LPXTG anchor motif (see Chapter 2.3) and a membrane-spanning region. Mub extracted from cell surface as well as recombinant Mub protein bind pig gastric mucin (Roos and Jonsson, 2002). Anti-Mub antibodies inhibited the mucus-adhesiveness of *L. reuteri*, which supported the role of Mub in bacterial adherence. Recent analysis of genome sequences of *Lactobacillus* predicted the presence of multiple putative mucus-binding proteins in lactobacilli originating from the gastrointestinal tract (Boekhorst *et al.*, 2006a). These putative mucus-binding proteins have a domain structure; the domains range from 100 to over 200 amino acids in size and in number from 1 to 15. Mutants defective in putative mucin-binding proteins Mub (LBA1392) of *L. acidophilus* (Buck *et al.*, 2005) and LspA of *L. salivarius* (van Pijkeren *et al.*, 2006) showed significantly reduced adherence to human epithelial cell lines. Three mucus-binding domains are present in the mannose-binding protein Msa (LP1229) of *L. plantarum* (Boekhorst *et al.*, 2006a). Deletion of Msa gene from *L. plantarum* abolished agglutination ability of yeast cells, which are covered by α-mannoside oligosaccharides (Pretzer *et al.*, 2005).

A 29 kDa protein from *L. reuteri* 104R (formerly *Lactobacillus fermentum*) binds porcine intestinal mucus and gastric mucin (Rojas *et al.*, 2002). Miyoshi *et al.*, (2006) showed that this protein also binds to human intestinal cells and named the protein MapA (Mucus adhesion promoting factor). Further, this protein shows 94% sequence identity with the characterized collagen-binding protein (Cnb) of *L. reuteri* NCIB 11951, which has sequence similarity to a solute binding component of ABC transporters (Roos *et al.*, 1996). Further, the 29 kDa protein from *L. fermentum*, which has identical N-terminal sequence with Cnb, is released from the cell surface and can inhibit the adhesion of *Enterococcus faecalis* (Heinemann *et al.*, 2000).

Recently, a homolog of the fibronectin-binding protein of *Streptococcus gordonii* and *Streptococcus mutans* was identified in *L. acidophilus* and shown to mediate bacterial adhesion to human intestinal epithelial cells (Buck *et al.*, 2005). *L. reuteri* expresses a high-molecular-mass surface protein (Lsp), which is similar to other surface proteins involved in adherence and biofilm formation by Gram-positive bacteria. Insertion mutagenesis of *lsp* impaired the adherence and initial colonization ability by *L. reuteri* in murine gut (Walter *et al.*, 2005). Also, mutants defective in methionine sulfoxide reductase B (MsrB) of *L. reuteri* showed altered colonization ability in murine gut. Msr proteins protect the

bacterial cell from oxidation, suggesting that tolerance to nitric oxide produced by epithelial cells might be important in colonization (Walter *et al.*, 2005).

Recent analysis of the genome sequence of *L. plantarum* identified 12 proteins with known adhesive domain structures; three collagen-binding, one chitin-binding, one fibronectin-binding and seven mucus-binding proteins (Boekhorst *et al.*, 2006a). However, the expression of these proteins as well as their possible roles in bacterial adhesion and host interaction remain open.

2.3 Cell wall anchoring of lactobacillar surface proteins

The cell envelope of Gram-positive bacteria is composed of a cell membrane covered with a PG layer and secondary cell wall polymers. PG is comprised of glycan strands, which in all bacteria consist of repeated disaccharide units, N-acetylglucosamine-(β1-4)-N-acetylmuramic acid (GlcNAc-MurNAc). These glycan strands are cross-linked by short cell-wall peptides, whose composition varies between bacterial species. PG network forms a huge macromolecular structure completely surrounding the cell (Navarre and Schneewind, 1999; Ton-That *et al.*, 2004). Detailed structure of PG has been determined from several *Lactobacillus* species (Hungerer *et al.*, 1969; Wallinder and Neujahr, 1971). The PG layer is abundantly decorated with secondary cell-wall polymers classified as teichoic acids, teichuronic acids and other neutral or acidic polysaccharides (Schäffer and Messner, 2005). Teichoic acids, which are composed of glycerol-phosphate, ribitol-phosphate or glucosyl-phosphate, are covalently attached to PG, whereas LTA are anchored to cytoplasmic membrane via a lipid moiety and are mostly composed of polymerized glycerol-phosphate. Under phosphate-limited conditions, the synthesis of teichuronic acid, where phosphate is substituted to uronic acid, is enhanced rapidly (Seltman and Holst, 2002). The cell wall has many critical functions, such as protection against the environment and cell lysis, but it also provides an attachment site for the surface proteins interacting with the host.

A variety of distinct mechanisms for anchoring proteins to the Gram-positive cell envelope are currently identified (Figure 1). A common mechanism is the sortase-dependent anchoring via the LPXTG-motif to PG. The proteins with this anchoring mechanism contain a carboxyl terminal LPXTG sequence, a hydrophobic region and a tail of charged amino acids. The LPXTG sequence is

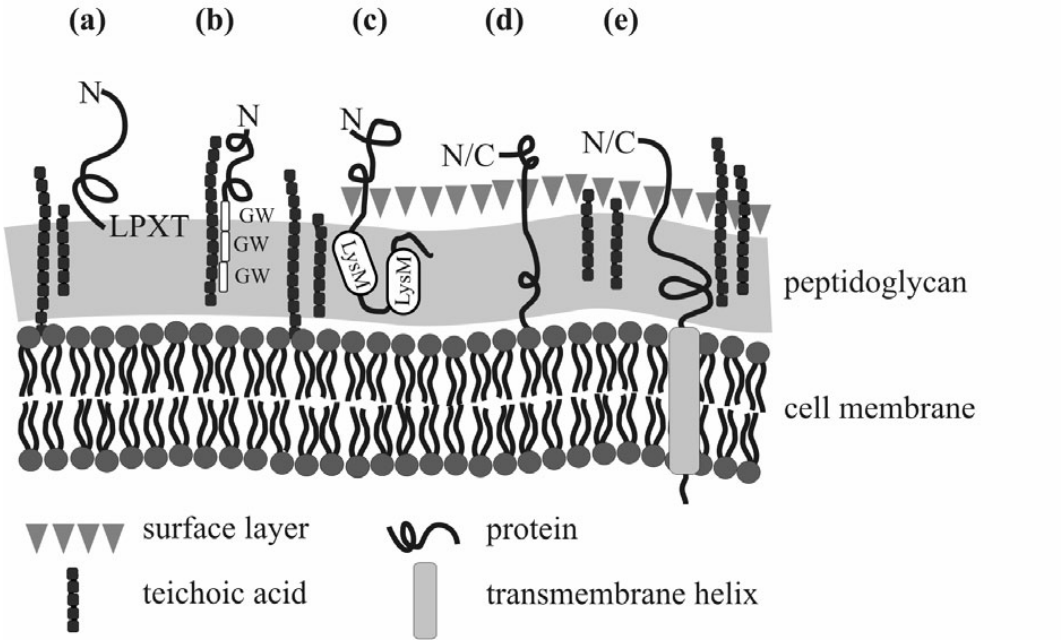

Figure 1. Mechanisms of protein anchoring in the proteins to the Gram-positive cell surface. a) LPXTG-motif covalently anchors surface proteins to peptidoglycan b) Protein anchored to teichoic acids via GW-motif c) LysM protein anchored to peptidoglycan d) Lipoprotein linked to cell membrane e) Trans-membrane protein. N- and C-termini of proteins are indicated (N, C). GW, protein having GW-motif; LysM, proteins with LysM domain.

recognized by a membrane-associated sortase enzyme, which covalently links the protein to peptide cross-bridge of PG (Paterson and Mitchell, 2004; Ton-That *et al.*, 2004). In the published whole genome sequences of *Lactobacillus* species, 4 to 25 LPXTG-proteins are found (Kleerebezem *et al.*, 2003; Pridmore *et al.*, 2004; Altermann *et al.*, 2005; van Pijkeren *et al.*, 2006). Lactobacillar proteins which contain this motif include the adhesins, Mub (Roos and Jonsson, 2002) and Lsp of *L. reuteri* (Walter *et al.*, 2005) and other putative mucus-binding proteins (Boekhorst *et al.*, 2006a), as well as cell-envelope proteases (Savijoki *et al.*, 2006), and other exoenzymes such as fructosyltransferase (van Hijum *et al.*, 2002).

The genomes of *Lactobacillus* species also encode proteins having the LysM domain. For instance, in the genome of *L. reuteri* seven LysM proteins are predicted, and four of those are putative hydrolases (Båth *et al.*, 2005). This domain is widespread in several bacterial genomes, and mediates protein binding to PG (Bateman and Bycroft, 2000). Steen *et al.*, (2003) showed that the C-terminal LysM domain of lactococcal autolysin binds to PG extracted from

many different bacterial species and to distinct PG types suggesting that this domain binds to a component common in PG such as the glycan strands. Autolysin is localized in the cell septum in *L. lactis*, probably as a consequence of steric hindrance of PG-binding by unevenly positioned LTAs.

Several mechanisms for protein anchoring to teichoic acids and other secondary cell polymers are known. *Streptococcus pneumoniae* has choline in the teichoic acids and LTAs and several choline-binding proteins have been identified, which function in cell adhesion, invasion, or colonization, as well as in immunological processes (Bergmann and Hammerschmidt, 2006). These proteins bind to choline moieties of teichoic acids via a C-terminal repeated domain (Yother and White, 1994; García *et al.*, 1998). Limited data is available on choline-binding proteins in other species. However, a choline-binding domain has been identified in *Clostridium beijerinckii* (Sánchez-Beato and García, 1996) and three proteins with the choline-binding domain were detected in the genome of *L. plantarum* (Kleerebezem *et al.*, 2003). The GW-motif was first identified in *Listeria monocytogenes* InlB (Braun *et al.*, 1997). The carboxy terminus, which anchors the GW motif to the cell-wall teichoic acids, contains a repeat region starting with glycine and tryptophan (Jonquières *et al.*, 1999). This motif is also present in several other proteins of *L. monocytogenes* (Cabanes *et al.*, 2002), in other Gram-positive bacteria and also in *Lactobacillus* species. The functions of GW-proteins in *Lactobacillus* remain open.

In addition, a number of proteins bind directly to plasma membrane via a common cysteine-containing lipobox (Sutcliffe and Russell, 1995; Sutcliffe and Harrington, 2002) or an alpha-helical transmembrane anchor (Desvaux *et al.*, 2006). Recently, anchorless multifunctional proteins, which lack established signal sequences or anchoring domains, were identified on the cell surface in pathogenic bacteria, but also in lactobacilli (Chhatwal, 2002; Pancholi and Chhatwal, 2003; Hurmalainen *et al.*, 2007). These proteins are known to contribute to the virulence of pathogenic bacteria by interacting with host components, such as glycoproteins of the ECM and circulating Plg (Chapter 2.4).

2.3.1 Attachment of the surface layer proteins to the bacterial cell wall

The subunits in the S-layer proteins are non-covalently bound to each other and to the cell wall. Therefore, S-layer proteins can be extracted from the cell surface with chaotropic agents, such as GnHCl and urea, or with high concentration of salts, such as LiCl (Sleytr and Sára, 1997) and from Gram-negative bacteria with metal-chelating agents, such as EDTA (Bingle *et al.*, 1987). Removal of these agents for example by dialysis, enable the S-layer peptides to self-assemble and to form a periodic layer (Sleytr and Sára, 1997).

No general mechanism of anchoring the S-layer proteins to cell wall has been found. A conserved S-layer homology (SLH) motif present in several S-layer proteins of Gram-positive bacteria was first identified by Lupas *et al.*, (1994). The SLH domain is located at the N-terminus of S-layer proteins and, typically, this motif comprises three repeats of 50-60 amino acids each (Engelhardt and Peters, 1998). The SLH domain can be found in several Gram-positive S-layers proteins, including *B. anthracis*, *Bacillus sphaericus*, *B. thuringiensis*, *C. thermocellum*, *G. stearothermophilus* PV72/p2, and *Thermoanaerobacterium thermosulfurigenes*, in which this motif anchors the S-layer protein to the secondary cell wall polymers (Ries *et al.*, 1997; Lemaire *et al.*, 1998; Brechtel and Bahl, 1999; Chauvaux *et al.*, 1999; Ilk *et al.*, 1999; Mesnage *et al.*, 2001; Mader *et al.*, 2004). Mesnage *et al.*, (2000) showed that pyruvulation of PG-associated polysaccharide is needed for anchoring the S-layer protein of *B. anthracis* to the cell wall and this mechanism, which is mediated by the *csaAB* operon, was proposed to be common in bacteria. Recently, evidence for a direct anchoring of a protein via the SLH-domain to PG has been provided (Zhao *et al.*, 2006). In addition to the S-layer proteins, SLH motif is also present in the C-termini of exoenzymes and other exoproteins in Gram-positive bacteria (Engelhardt and Peters, 1998; Chitlaru *et al.*, 2004) as well as in outer membrane proteins (Omps) of Gram-negative bacteria (Kalmokoff *et al.*, 2000). Anchoring of these proteins to the cell wall via the SLH-motif has been demonstrated (Lemaire *et al.*, 1995; Kosugi *et al.*, 2002).

The SLH-motifs is not present in all characterized S-layer proteins, e.g. in the sequences of the S-layer proteins of *Corynebacterium glutamicum*, *G. stearothermophilus* wild-type strain or from lactobacillar S-layer proteins. Chami *et al.*, (1997) proposed that the C-terminal hydrophobic region of the S-layer protein of *C. glutamicum* anchors the protein to cell wall. The S-layer proteins of *G. stearothermophilus* wild-type strains attach to secondary cell wall

polymers via their identical N-terminal regions (Egelseer *et al.*, 1998; Jarosch *et al.*, 2000). The S-layer proteins from *L. buchneri* and *L. brevis* were proposed to bind to a neutral polysaccharide moiety in the cell wall, but not to PG or teichoic acids (Masuda and Kawata, 1980; Masuda and Kawata, 1981). The C-terminal one-third of the S-layer protein from *L. acidophilus* (SAC) was shown to bind to the cell surface after chemical removal of the S-layer. Similarly, the SAC binds to LiCl-extracted cell surface of *L. crispatus* and *L. helveticus*, which have a closely related S-layer protein (Smit *et al.*, 2001). Further, the cell-wall binding site was localized to the N-terminal region of 65 amino acids in the SAC domain, and based on a preliminary analysis of cell wall by selective extraction, Smit and Pouwels, (2002) suggested that SAC binds to the cell wall teichoic acids.

2.4 Anchorless multifunctional proteins

Recently, several proteins with essential intracellular roles in bacterial growth and metabolism have also been found on the bacterial surface or in the extracellular proteome. They enhance virulence of pathogenic bacteria by mediating adhesion, or have proteolytic or immuno-stimulating activities (Chhatwal, 2002; Pancholi and Chhatwal, 2003; Bergmann *et al.*, 2005). These proteins are called anchorless, since no established signal sequence or anchoring motif is present in their predicted sequences. Recently, the anchorless proteins have also been identified in lactobacilli, where they include GroEL and EF-Tu, as well as the glycolytic enzymes enolase and GAPDH.

The GroEL, which is an essential intracellular protein functioning in protein folding, was identified both on the cell surface and in the culture medium of *L. johnsonii* La1 (NCC 533). GroEL binds to mucin and human epithelial cells at acidic pH. In addition, recombinant GroEL stimulates interleukin-8 secretion in macrophages and aggregates cells of the gastric pathogen *Helicobacter pylori*, but not *Salmonella enterica* or *E. coli* cells (Bergonzelli *et al.*, 2006). EF-Tu, which has a role in intracellular protein synthesis as a guanosine binding protein, was found on the surface of *L. johnsonii* La1 and recombinant EF-Tu bound to mucin and human intestinal epithelial cells, and the binding was more efficient in pH 5 than in pH 7.2 (Granato *et al.*, 2004). Similarly, binding of *L. johnsonii* La1 to mucus is promoted at pH 5 (Blum *et al.*, 1999b). The EF-TU of this strain also induced a proinflammatory immune response in the presence of soluble CD14 (Granato *et al.*, 2004).

Enolase and GAPDH are essential intracellular glycolytic enzymes. GAPDH catalyzes oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate, whereas enolase catalyzes dehydration of 2-phosphoglycerate (2-PGE) to phosphoenolpuryvate. Enolase also catalyzes reverse reaction in gluconeogenesis. These enzymes are also present on the surface in several Gram-positive bacterial species (Table 3), in Gram-negative bacteria (Kenny and Finlay, 1995; Hara *et al.*, 2000; Grifantini *et al.*, 2002; Sha *et al.*, 2003) as well as in fungi and other eukaryotic organisms (reviewed in Pancholi, 2001; Pancholi and Chhatwal, 2003).

Eukaryotic α-enolase is a dimer (Pancholi, 2001), whereas some bacterial enolases form octameric structure (Pawluk *et al.*, 1986; Schurig *et al.*, 1995; Brown *et al.*, 1998; Ehinger *et al.*, 2004). The surface-exposed enolases of Gram-positive pathogenic *Listeria monocytogens*, *S. pneumoniae*, *Staphylococcus aureus*, *S. mutans*, and *Streptococcus pyogenes* bind Plg and/or plasmin (Pancholi and Fischetti, 1998; Bergmann *et al.*, 2001; Mölkänen *et al.*, 2002; Ge *et al.*, 2004; Schaumburg *et al.*, 2004). Plg is a precursor of plasmin, a serine protease involved in several physiological processes, such as fibrinolysis, degradation of ECM, enhancement of cell migration and activation of prohormones and growth factors (Mignatti and Rifkin, 1993; Lijnen and Collen, 1995; Plow *et al.*, 1999; Myöhänen and Vaheri, 2004). A number of bacterial species activate Plg to plasmin or bind Plg and by this way enhance Plg activation by human Plg activators tissue-type Plg activator (tPA) or urokinase (uPA) (Lähteenmäki *et al.*, 2001). A few bacterial species express their own Plg activators, which include the streptokinase of *Streptococcus* and staphylokinase of *Staphylococcus* (Lähteenmäki *et al.*, 2001; Walker *et al.*, 2005; Bokarewa *et al.*, 2006), but no evidence of such activity has reported from *Lactobacillus*. Bacteria utilize the human Plg system to degrade ECM and to migrate across tissue barriers (Lähteenmäki *et al.*, 2005), as well as in release of peptides for nutrition (Kitt and Leigh, 1997) and in inactivation of protease inhibitors (Darenfed *et al.*, 1999).

In both eukaryotic and prokaryotic cells, Plg/plasmin binds typically to lysine rich domains, which are often located in the C-terminus of a receptor protein (Redlitz and Plow, 1995). However, importance of arginine and histidine residues in Plg-binding has been reported in the Plg-binding M-like protein (PAM) and from the PAM-related protein Prp of *S. pyogenes* (Sanderson-Smith *et al.*, 2006; Sanderson-Smith *et al.*, 2007). In the enolase of *S. pyogenes*, the C-terminal lysine residues are important in Plg binding, and a mutant strain

Table 3. Gram-positive bacteria reported to express extracellularly localized enolase or GAPDH

Species	Reference
Enolase	
Bacillus anthracis	Lamonica *et al.*, 2005
Group B, C, E, G, H, L streptococci	Pancholi and Fischetti, 1992
Listeria monocytogens	Schaumburg *et al.*, 2004
Lactobacillus acidophilus	Hurmalainen *et al.*, 2007
Lactobacillus amylovorus	Hurmalainen *et al.*, 2007
Lactobacillus crispatus	Hurmalainen *et al.*, 2007
Lactobacillus gallinarum	Hurmalainen *et al.*, 2007
Lactobacillus gasseri	Hurmalainen *et al.*, 2007
Lactobacillus johnsonii	Hurmalainen *et al.*, 2007
Leuconostoc mesenteroides	Lee *et al.*, 2006
Staphylococcus aureus	Mölkänen *et al.*, 2002; Carneiro *et al.*, 2004
Streptococcus agalactiae	Hughes et al., 2002; Fluegge *et al.*, 2004
Streptococcus mutans	Ge *et al.*, 2004
Streptococcus pneumoniae	Bergmann *et al.*, 2001
Streptococcus pyogenes	Pancholi and Fischetti, 1992
GAPDH	
Bacillus anthracis	Lamonica *et al.*, 2005
Group B, C, E, G, H, L streptococci	Pancholi and Fischetti, 1992
Listeria monocytogens	Schaumburg *et al.*, 2004
Lactobacillus acidophilus	Hurmalainen *et al.*, 2007
Lactobacillus crispatus	Hurmalainen *et al.*, 2007
Lactobacillus gallinarum	Hurmalainen *et al.*, 2007
Lactobacillus gasseri	Hurmalainen *et al.*, 2007
Lactobacillus johnsonii	Hurmalainen *et al.*, 2007
Lactobacillus paracasei	Hurmalainen *et al.*, 2007
Lactobacillus rhamnosus	Hurmalainen *et al.*, 2007
Lactococcus lactis	Hurmalainen *et al.*, 2007
Mycobacterium avium	Bermudez *et al.*, 1996
Mycobacterium tuberculosis	Bermudez *et al.*, 1996
Oenococcus oeni	Carreté *et al.*, 2005
Staphylococcus aureus	Modun and Williams, 1999
Staphylococcus epidermidis	Modun and Williams, 1999
Streptococcus agalactiae	Hughes *et al.*, 2002; Seifert *et al.*, 2003
Streptococcus equisimilis	Gase *et al.*, 1996
Streptococcus gordonii	Nelson *et al.*, 2001
Streptococcus oralis	Maeda *et al.*, 2004
Streptococcus pneumoniae	Bergmann *et al.*, 2004
Streptococcus pyogenes	Lottenberg *et al.*, 1992; Pancholi and Fischetti, 1992
Streptococcus suis	Brassard *et al.*, 2004

expressing an enolase, where the C-terminal lysines were substituted with leucines, showed reduced ability in Plg binding and penetration ECM (Derbise *et al.*, 2004). However, the corresponding mutation in *S. pneumoniae* showed similar Plg binding ability as did the parental strain, but the virulence of the mutant strain was attenuated in a mouse model of intranasal infection (Bergmann *et al.*, 2003). The binding activities of the C-terminally mutated enolase proteins of pneumococci suggested presence of another Plg binding site in the molecule (Pancholi and Fischetti, 1998; Bergmann *et al.*, 2003). Assays with synthetic peptides, which covered the whole enolase sequence, revealed a nine-amino-acids long internal sequence (^{248}FYDKERKVYD) capable to bind Plg and inhibit binding of Plg to pneumococcal cells. The crystal structure of pneumococcal enolase reveals that this internal sequence is surface-exposed in the octameric molecule, whereas the C-terminus of the protein is located in a groove between two dimers and is inaccessible for Plg-binding (Ehinger *et al.*, 2004). Substitution of the lysine and glutamic acid residues in the internal sequence significantly reduced Plg-binding by the parental strain and diminished plasmin-dependent degradation of ECM, as well as attenuated pneumococcal infection in a mouse model of intranasal infection (Bergmann *et al.*, 2003; Bergmann *et al.*, 2005). Besides functioning as a Plg-binding molecule, enolases of *S. mutans* (Ge *et al.*, 2004) and *S. aureus* (Carneiro *et al.*, 2004) bind to salivary mucin and to laminin, respectively, and thus may contribute to bacterial adhesiveness. Also, evidence for the role of streptococcal enolase as an immunosuppressive protein has been provided (Veiga-Malta *et al.*, 2004).

The GAPDH proteins from several Gram-positive bacteria, such as *L. monocytogens*, *S. aureus*, *Streptococcus epidermidis*, *Streptococcus equisimilis*, *S. pyogenes*, and *S. pneumoniae*, have been shown to bind Plg or plasmin (Pancholi and Fischetti, 1992; Gase *et al.*, 1996; Modun and Williams, 1999; Bergmann *et al.*, 2004; Schaumburg *et al.*, 2004). In *S. pyogenes*, the substitution of the C-terminal lysine in recombinant GAPDH protein reduced Plg-binding, whereas the mutant strain expressing the C-terminal substitutions bound Plg as efficiently as did the parental strain (Winram and Lottenberg, 1998).

The GAPDH protein of *S. pyogenes* binds fibronectin and lysozyme (Pancholi and Fischetti, 1992) as well as to human pharyngeal cells (Jin *et al.*, 2005). Further, the interaction of GAPDH with pharyngeal cells involved phosphorylation of cellular proteins (Pancholi and Fischetti, 1997) and the urokinase Plg activator receptor (uPAR/CD87) was identified as the epithelial receptor for GAPDH (Jin *et al.*, 2005). In addition, a mutant strain of *S.*

pyogenes unable to secrete GAPDH after a genetic fusion of a C-terminal hydrophobic peptide bound less Plg and adhered poorly to human pharyngeal cells suggesting that extracellular localization of GAPDH has a role in streptococcal infection (Boël *et al.*, 2005). The mutant strain had lost the antiphagocytic activity, but the direct role of GAPDH in this process remained open. The GAPDH of *S. pyogenes* captures C5a, thus inhibiting chemotaxis and H_2O_2 production by neutrophils and enabling the escape of *Streptococcus* from immune defence (Terao *et al.*, 2006). Similarly, Madureira *et al.*, (2007) suggested that the GAPDH of *Streptococcus agalactiae* interferes with immune system. Recombinant GAPDH induced B cell and T cell activation and a strain overexpressing GAPDH showed increased virulence in a mouse model. The GAPDH of *S. agalactiae* binds actin and fibrinogen (Seifert *et al.*, 2003) and Brassard *et al.*, (2004) showed that the GAPDH of *Streptococcus suis* binds porcine tracheal rings.

Recently, interaction of commensal lactobacilli with the human Plg system was reported (Hurmalainen *et al.*, 2007). *L. crispatus* ST1 and several other species of the genus *Lactobacillus* were shown to enhance both tPA- and uPA- mediated formation of plasmin. Enolase and GAPDH were identified in the extracellular proteome and shown to bind Plg and enhance its activation by tPA and uPA (Hurmalainen *et al.*, 2007). In contrast to Gram-positive pathogens, which bind Plg onto the cell surface, only limited binding of Plg to the lactobacillar cell surface was detected, whereas the lactobacillar extracellular proteome obtained at neutral pH efficiently enhanced activator mediated plasmin formation (Hurmalainen *et al.*, 2007). The commensal *Bacteroides fragilis* also immobilizes Plg on its surface (Sijbrandi *et al.*, 2005). These findings demonstrate that commensal bacteria interact with the human Plg system and that among bacteria such interactions are more common than what have been expected. Enolase and GAPDH of *L. crispatus* lack the C-terminal lysine residues that in many Plg receptors have been shown to interact with Plg. However, sequence of the enolase of *L. crispatus* contains a similar internal Plg-binding sequence, FYNKDDHKY, in the same position as in the pneumococcal enolase (Hurmalainen *et al.*, 2007). No other nonenzymatic function has so far been identified for lactobacillar enolase or GAPDH.

Besides enolase and GAPDH, several other proteins are released to extracellular proteome of *Lactobacillus* (Hurmalainen *et al.*, 2007). Recently, the cell-free culture medium of *L. rhamnosus* GG was shown to inhibit pro-inflammatory cytokine expression, induce heat-shock protein expression, and modulate signal transduction pathways in murine macrophages (Peña and Versalovic, 2003; Tao

et al., 2006). Further, Tao *et al.*, (2006) showed that the factor responsible of heat-shock protein induction is a small-molecular-weight peptide. However, further characterization of this peptide, including expression analyses and study of effects of different environmental conditions in its release, remain to be performed.

Secretion mechanisms of these anchorless proteins remain poorly known. Boël *et al.*, (2004) suggested that automodification of enolase by its substrate 2-PGE is associated with its secretion. By mass spectrometry analysis with proteolytic peptides, enolase was shown to bind 2-PGE via lysine 341, which is located in the active site. Mutation in this point in the enolase of *E. coli* prevented the export of the enolase. On the other hand, deletion of *htrA*, which encodes a surface protease known to be involved e.g. in the folding and maturation of extracellular proteins, increased expression of enolase and GAPDH in the culture medium of *S. mutans* (Biswas and Biswas, 2005). In *S. gordonii*, more GAPDH protein was found in culture media when the pH of the medium was raised from 6.5 to 7.5 (Nelson *et al.*, 2001). However, no mechanistic explanations have been reported for the observations described above.

3 AIMS OF THE STUDY

This study was aimed to characterize lactobacillar surface proteins, their role in adhesion, their structures, as well as their anchoring mechanisms on the bacterial cell wall. Collagen-binding by the S-layer protein of *L. crispatus* JCM 5810 had been reported (Toba *et al.*, 1995). Adhesiveness of lactobacilli to host tissues is considered important for lactobacillar colonization and therefore characterization of the domain structure of the adhesive S-layer protein CbsA of *L. crispatus* JCM 5810 and regions important for adhesion and crystallization were the first topics in my PhD thesis work. In particular, identification of the regions and possible domains important for tissue-adhesion and self-crystallization was a topic in my thesis. Identification of the domain structure in CbsA led to the study on its anchoring mechanisms onto the cell wall. During my thesis work, it became evident that glycolytic enzymes, enolase and GAPDH are found on the surface of lactobacilli, and their surface-association as well as functions became a second major topic in my thesis. Surface-associated enolases function as Plg receptors or activation cofactors in Gram-positive pathogens, and this study was the first step in comparing the human Plg system in bacterial pathogenesis and commensalism.

4 MATERIALS AND METHODS

The bacterial strains and plasmids used in this study are listed in Table 4. The methods are described in detail in the original publications and are summarized in Table 5.

Table 4. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Origin/relevant property	Article	Reference and/or source
Bacterial strains			
Lactobacillus acidophilus ATCC 4356 (JCM 1132)	human pharynx	I	Johnson *et al.*, 1980, JCM
Lactobacillus acidophilus JCM 1023	rat faeces	I	Johnson *et al.*, 1980, JCM
Lactobacillus amylovorus F81	calf feces	I, II	Fujisawa *et al.*, 1992, JCM
Lactobacillus amylovorus JCM5807	pig intestine	I	Mitsuoka, 1969, JCM
Lactobacillus brevis ATCC 8287	green fermenting olives	II	Vidgren *et al.*, 1992, ATCC
Lactobacillus casei ATCC 393	cheese	II	Martinez *et al.*, 2000
Lactobacillus crispatus A269-21	human feces	I	Fujisawa *et al.*, 1992, JCM
Lactobacillus crispatus JCM 5810	chicken feces	I, II	Mitsuoka, 1969, JCM
Lactobacillus crispatus LMG 12003	human feces	I, II	BCCM
Lactobacillus crispatus LMG 9479	eye	II	BCCM
Lactobacillus crispatus ST1	chicken feces	III, IV	Edelman *et al.*, 2002
Lactobacillus gallinarum F41	chicken feces	I, II	Fujisawa *et al.*, 1992, JCM
Lactobacillus gallinarum T-50	chicken feces	I	Fujisawa *et al.*, 1992, JCM
Lactobacillus gasseri JCM 1130	human feces	I, II	Lerche and Reuter, 1962, JCM
Lactobacillus gasseri JCM 5813	human feces	I	Mitsuoka, 1969, JCM
Lactobacillus johnsonii 5 F49	mouse feces	I, II	Fujisawa *et al.*, 1992, JCM
Lactobacillus johnsonii F133	calf feces	I, IV	Fujisawa *et al.*, 1992, JCM
Escherichia coli XL1 Blue MRF'	cloning host	I	Stratagene Inc.
Escherichia coli M15(pREP4)	host for pQE-30 vector	I, II, IV	Qiagen
Streptococcus pneumoniae TIGR4		IV	Tettelin *et al.*, 2001
Streptococcus pyogenes serotype T1 IH32030		IV	Miettinen *et al.*, 1998
Staphylococcus aureus 8325-4		IV	Novick, 1967
Plasmids			
pBluescript KS	cloning plasmid	I	Stratagene Inc.
pQE-30	His-tag expression vector	I, II, IV	Qiagen
pLPMSSA3	lactobacillar expression vector	II	This study
pLPMSSA4	lactobacillar expression vector	II	This study

ATCC, American Type Culture Collection

BCCM, Belgian Co-ordinated Collections of Micro-organisms

JCM, Japan Collection of Microorganisms

Table 5. Methods used in this study

Method	Described and used in
Genetic methods	
Isolation of chromosomal DNA	I, IV
Cloning to pQE-30 vector	I, II, IV
Cloning to *L. casei* expression system	II
PCR mutagenesis	I, II
RNA analysis	I, III, IV
Southern hybridization	I, III
DNA sequencing	I, II, IV
Adhesion assays	
Binding of soluble ^{125}I-labelled glycoproteins	I, II
Adherence of bacterial cells to immobilized glycoproteins	II
Adherence of bacterial strains to frozen section of chicken intestine	I, II
Adherence of peptides to glycoproteins by ELISA	I, II, IV
Binding of peptides to cell surfaces and cell wall material	II, III
Interaction of proteins with LTA	II, III
Immunological methods	
ELISA	I, II, IV
Whole-cell ELISA	II
Indirect immunofluorescence	II, III
Western blotting	III
Protein assays	
SDS-PAGE	I, II, III, IV
Expression and purification of His-peptides	I, II, III, IV
Transmission electron microscopy	I, II
Polymerization assay (cross-linking with glutaraldehyde)	II
Mass spectrometry	I, II
Protein and peptide sequencing	I
Extraction of cell surface components	II, III
Enolase enzyme activity measurement	IV
Enhancement of plasminogen activation	III, IV
Plasminogen binding	III, IV

5 RESULTS AND DISCUSSION

5.1 Characterization of the S-layer protein of *Lactobacillus crispatus* (I, II)

When this study was initiated, *L. crispatus* JCM 5810 had been shown to strongly adhere to mammalian ECM via its collagen-binding S-layer protein (Toba *et al.*, 1995). In this thesis work, the gene encoding the S-layer protein of JCM 5810, *cbsA* (collagen-binding S-layer protein A), was cloned and expressed as a recombinant protein, and the domains responsible for crystallization, binding to host tissues and attachment to cell wall were characterized. The overview of the domain structure of CbsA identified in this thesis work is presented in Figure 2.

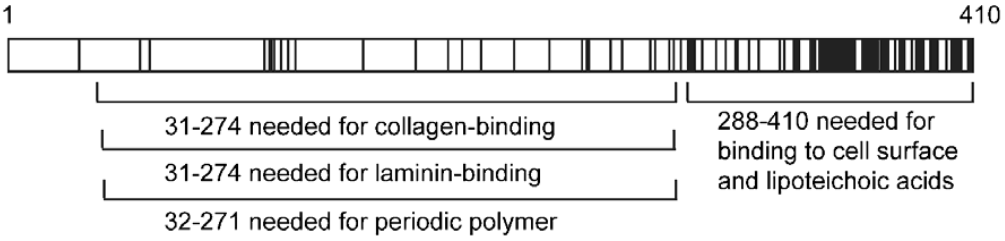

Figure 2. Overview of domains of S-layer protein of *L. crispatus* CbsA. The bar represents CbsA with 410 amino acid residues, and residues conserved in eight S-layer proteins of *L. crispatus*, *L. acidophilus* and *L. helveticus* were identified by the CLUSTALW program and are represented by a line. Functional regions are indicated below the bar. Modified from Figure 1 of article II with permission of Blackwell Publishing Ltd.

5.1.1 Cloning of S-layer genes *cbsA* and *cbsB* of *L. crispatus* JCM 5810 (I)

To characterize the S-layer protein CbsA of *L. crispatus* JCM 5810, the *cbsA* gene was cloned and sequenced. N-terminal and internal peptide sequences were determined from the S-layer protein extracted from cell surface and DNA probes were designed to localize *cbsA* by Southern hybridization of genomic DNA from JCM 5810. The chromosomal fragment encoding CbsA was cloned and sequenced. The predicted open reading frame of *cbsA* encodes a 440 amino-acids long peptide with a 30-amino-acids long N-terminal signal sequence. It was known that isolates of *L. crispatus* commonly possess two S-layer genes, a

transcribed one and a silent one, which have highly conserved 5´ and 3´ regions and may be subjected to phase variation (Boot *et al.*, 1996b). To analyze whether this also is the case in JCM 58190, the S-layer *cbs* fragment from JCM 5810 was amplified, isolated and digested with *Xho*I to cut and eliminate the *cbsA*, which contains a single *Xho*I site, and the remaining DNA fragment was cloned and sequenced. The cloned region encoded a predicted protein of 453 amino acids where the first 23 amino acids were identical with CbsA. The overall predicted amino acid sequence of the peptide was 46% identical to the CbsA sequence. Therefore this gene was considered to encode another S-layer protein which was named CbsB. To analyze which of the proteins, CbsA or CbsB, is expressed, total RNA was analyzed by specific *cbsA* and *cbsB* probes. The *cbsA*-specific probe detected a transcript of predicted size of *cbsA*, whereas no signal was obtained from the *cbsB* probe. Also, the peptide sequencing of 16 proteolytic fragments of the S-layer protein extracted from cell surface completely matched the amino acid sequence of CbsA but not of CbsB, and it was concluded that CbsA was the expressed S-layer peptide.

Both of these proteins have typical features of a lactobacillar S-layer protein, such as a high content of hydrophobic amino acids and a high pI (Åvall-Jääskeläinen and Palva, 2005). CbsA and CbsB sequences show high homology to lactobacillar S-layer proteins from *L. acidophilus* ATCC 4356 (SlpA and SlpB; Boot *et al.*, 1993; Boot *et al.*, 1995), *L. helveticus* strains, as well as from *L. crispatus* LMG 12003 (SlpnA and SlpnB; Pouwels and Martínez, unpublished). Especially, the C-termini of these proteins exhibit a high identity (64-98 %), whereas the N-termini share 30-71% identity (Figure 2 and Figure 1 of article I), and contain short conserved peptide stretches. Further, CbsA and CbsB show only very low identity with S-layer proteins of *L. brevis* (7-14%) or with sequenced S-layer proteins of other bacteria or Archaea. Thus, the predicted S-layer proteins in lactobacilli exhibit considerable sequence variability, in particular in their N-terminal halves, and an emerging question was whether this is related to the reported functional differences.

5.1.2 Expression of the S-layer proteins as His-tag fusion proteins (I, II)

In order to initiate the analysis of possible domain structure in CbsA, it was expressed and purified as an N-terminal His_6-fusion protein from *E. coli*. In addition, His_6-fusions were generated from *cbsB*, the S-layer gene *slpA* of *L. acidophilus* ATCC 4356, as well as from *slpnB*, a silent S-layer gene of *L.*

crispatus LMG 12003, which possesses a very high sequence identity (73%) with CbsA. The proteins were purified in denaturating conditions in presence of 6 M urea and after extensive dialysis against phosphate-buffer saline (PBS), the His_6-S-layer proteins auto-assembled to periodic crystalline layers similar to the S-layers extracted from the lactobacillar surface (Figure 3 of I; Smit *et al.*, 2001). *L. crispatus* JCM 5810 adheres efficiently to collagens and laminin as well as ECM and the S-layer protein extracted from bacterial surface was identified to bind solubilized ^{125}I-labelled type IV collagen (Toba *et al.*, 1995). However, *L. acidophilus* ATCC 4356 and *L. crispatus* LMG 12003 do not adhere to collagens or laminin (Sillanpää, 2001; J. Antikainen, unpublished). The His_6-CbsA bound efficiently radiolabelled type I and IV collagens, whereas no collagen-binding to His_6-SlpA, His_6-CbsB or His_6-SlpnB were detected (Table 1 of I). Binding of radiolabelled collagens to His_6-CbsA was inhibited (by >99%) by excess of unlabelled collagens, but not by fibronectin (I) as previously observed with JCM 5810 cells (Toba *et al.*, 1995). We concluded that the expression and purification of S-layer proteins as a His-tag fusion protein in *E. coli* did not affect crystallization into periodic S-layer or the ability to bind collagens, which indicates that histidine residues do not affect to polymerization ability *in vitro*. Formation of regular S-layers in recombinant *E. coli* has been reported for S-layer proteins from several species, such as *G. stearothermophilus* (Kuen *et al.*, 1996; Jarosch *et al.*, 2000), *B. anthracis* (Candela *et al.*, 2005) and *L. acidophilus* (Smit *et al.*, 2001).

5.1.3 N-terminal domain of CbsA is responsible for crystallization (I, II)

To characterize the domain structure of CbsA, more than 50 truncated peptides, hybrid fusions, and specific substitution mutations were created in this work (I, II). As a first step, the His_6-peptides and mutated His_6-proteins (Table 1 of I) were analyzed by transmission electron microscopy for crystalline layer formation. Only the peptide with first N-terminal 287 amino acids formed a periodic S-layer like structure (Table 1 of I, Fig. 3 of I), whereas no regularly arranged structures were recognized in shorter peptides (1-250, 1-212, 42-287). Also, the C-terminal peptide CbsA288-410 failed to polymerize into a crystalline layer.

After observing that the first 287 amino acids formed a periodic structure and that the first 41 N-terminal amino acids are important in this process, we mapped the borders of the crystallization domain in more detail by generating amino acid

deletions at the N- and C-termini of this domain region (Figure 2 of II). The C-terminal truncations revealed that 275 or more of the N-terminal amino acids are needed for polymerization to sheet-like structures, which, however, were mixed with regularly arranged, large cylindrical polymers (Figure 2 of II). The sheet layer was observed only in CbsA1-410 suggesting that the C-terminal region stabilizes the sheet-like structure. Further deletions at the C-terminus changed the polymer structure first to small tubular polymers (1-274, 1-271) and further to non-regular aggregates (1-269 and shorter). The truncation of N-terminus revealed that the region 30-34 is important in polymer formation, since the peptides with a deletion more of than 32 amino acids from the N-terminus (33-287, 34-287, 29-287) failed to polymerize.

These regions important for polymerization, i.e. the residues 30-32 and 269-273 (Figure 1 of II), are highly conserved in the S-layer proteins of closely related lactobacillar species and rich in valines. Substitution of valines at these two regions (VNV30TNT, VTVNV269TTTNT) changed the morphology type to a mixture of large cylindrical polymers and small tubular polymers (Table 2 of II). In article I, two short N-terminal deletions and 11 amino acid substitutions were constructed at sites where the lactobacillar S-layer protein sequences differ (Table 1 of I) with an aim to identify sites and residues important for collagen binding by CbsA. These substitutions did not have dramatic effects on polymer formation (Table 1 of I). The His_6-CbsA protein with the substitutions KSDV257TANN and V260N formed large cylinder-like structures and not sheet-like layers observed in the other mutant His_6-CbsA proteins. The KSDV257TANN mutation is situated in a conserved region in lactobacillar S-layer proteins (Figure 1 of I), and this conserved region might be important in for correct assembly of the S-layer.

The multimeric structure of selected CbsA peptides was also analyzed by cross-linking the peptides with glutaraldehyde (Figure 3 of II) and was detected to correlate with the transmission electron microscopy results. The peptides (1-271, 1-275, 1-287, and 32-287) observed to crystallize to periodic polymer by electron microscopy, formed multimers in the cross-linking study. The smaller peptides (1-269, 33-287) that failed to form a periodic structure, formed dimers and tetramers in the cross-linking study (Figure 3 of II) but also by mass spectrometric analysis tested with non-fixed peptides. Formation of these smaller multimers is likely to assist in the assembly to S-layers by advancing correct folding of protein domains.

We concluded that the amino acids 32-271 of CbsA are needed for formation of a periodic polymer and that the two conserved hydrophobic regions 30–34 and 269–274 are extremely important for polymerization. The stepwise truncation of CbsA probably causes destabilization of the S-layer three-dimensional polymer, and the conserved regions are essential in forming intramolecular interaction. Domain structure in an S-layer protein has also been studied by deletion analysis or substitutions in the SbsC and SbsB of *G. stearothermophilus.* Requirements for self-assembly of SbsC were studied by generating N- and C-terminal truncations and their expression and purification as recombinant proteins from *E. coli*. The N-terminal part of SbsC is responsible for anchoring to cell surface and needed for formation of sheet-like layers, whereas further deletion of SbsC changed the structure to cylinder-like structure or peptides failed to form periodic polymer (Jarosch *et al.*, 2000; Jarosch *et al.*, 2001). This is in congruent with CbsA, where truncation of the C-terminal part of the protein, which is responsible for anchoring to cell wall (Chapter 5.1.5), changed the polymeric structure from sheet-like layers to cylinders (Chapter 5.1.3). Howorka *et al.*, (2000) substituted 75 residues in SbsB of *G. stearothermophilus* with cysteine, which is not present in the native protein, and analyzed surface-accessible residues of these mutants. They concluded that functional domains are dispersed in SbsB sequence. Only few crystal structures of S-layer proteins have been reported; i.e. the S-layer protein of archaeal *Methanosarcina* (Jing *et al.*, 2002) and SbsC of *G. stearothermophilus* (Pavkov *et al.*, 2003). However, sequences of these proteins are not related to lactobacillar S-layer protein sequences, therefore, it is impossible to estimate properties of lactobacillar S-layer domain structures on basis of these published structures.

5.1.4 N-terminal domain is responsible for binding to collagen-containing tissue sites (I, II)

L. crispatus JCM 5810 adheres to proteins of ECM, such as collagen I and IV as well as laminin (Toba *et al.*, 1995) and the S-layer protein was shown to bind collagen IV. To further study the structure/function relationships in CbsA, we assessed the binding of radiolabelled, solubilized collagen I and IV to His_6-constructs. First, we generated hybrid S-layer proteins, where the N- or the C-terminus was exchanged between CbsA and non-adhesive SlpA or SlpnB (Table 1 of I). Investigation of protein functions using hybrid molecules between closely related but functionally distinct proteins is a commonly used method to localize functional domains (Koulich *et al.*, 1997; Kukkonen *et al.*, 2001). The hybrids His-CbsA1-287/SlpA290-413 and His-CbsA1-287/SlpnB287-409

efficiently bound collagen I and IV, whereas no binding was detected with the counter-wise hybrids containing N-terminus from SlpA or SlpnB (Table 1 of I). These findings indicate the importance of the N-terminus in collagen-binding. Also, the hybrids SlpnB1-19/CbsA29-287 and SlpnB1-72/CbsA82-410 efficiently bound collagens indicating that the extreme N-terminus of CbsA can be substituted without loss in collagen-binding efficiency. As a detailed structure of collagen-CbsA interaction has not been resolved, it is not possible to infer whether structural features in the N-terminus of SlpnB compensate for CbsA structure in collagen-binding. The hybrids with 194, 212 or 250 N-terminal amino acids from CbsA did not bind collagens (Table 1 of I).

To further characterize the role of N-terminus of CbsA in collagen-binding, we assessed the collagen-binding by the N or C-terminally truncated peptides (Table 1 of I; Figure 2 of II). Surprisingly, binding of collagens by the C-terminally truncated peptides 1-287, 1-279, 1-277, 1-275 and by the N-terminally truncated peptides 29-287, 30-287 were two or three fold higher than the binding to the entire His_6-CbsA 1-410. This phenomenon has several possible explanations. First, the assembly domain in entire CbsA might shield the collagen-binding sites. Second, the distinct polymer types may have unequal coating efficiency on the membrane used for measurement or, third, it is possible that the sheet-like structures are immobilized in an upside-down orientation. Peptides 1-274 and 32-287 showed reduced level binding and shorter peptides (1-274, 1-273, 1-271, 1-269, 1-250, 33-287, 34-297, 39-287, 42-287) did not bind collagens at all, but at the same time they lost the ability to form periodic polymers. No binding of collagens to C-terminal peptides 250-410 and 288-410 were observed. We concluded that amino acids 32-274 are needed for collagen-binding and that the binding was best exhibited by sheet- or cylinder-like structures.

We also made several point mutations in the regions where the CbsA sequence differs from those of the non-collagen binding proteins SlpA and SlpnB. The N-terminal deletion amino acids 22-26 or 91-96 reduced collagen binding by more than 70%. In addition, substitutions of D130N, N226A, TA264SK, and P269A reduced binding by 40% to 70%, whereas NNN14INL and F19S had less effect. A complete loss of collagen-binding was observed after the substitution KSDV257TANN (Table 1 of I). Further mapping of this site showed that S258A and V260N had a significant effect to collagen-binding, whereas K257T and D259N had less effect (Table 1 of I). By electron microscopy, all constructs were observed to form periodic structure; however, KSDV257TANN and V260N formed cylinder-like structure, instead of sheet-like structure observed by other substitutions.

Because collagens occur in insoluble, immobilized networks in tissues, and to ascertain the biological function of the CbsA-collagen interaction, we assessed the binding of S-layer peptides to immobilized collagen by enzyme-linked immunosorbent assay (ELISA). The entire CbsA and the peptide 1-287 efficiently bound to immobilized collagen I and IV and the binding was considered specific since it was dependent on the amount of added CbsA peptide (Figure 4 of I). The shorter peptides (1-212, 1-250, 42-287) or the C-terminal peptide 288-410 did not bind immobilized collagens, which is in agreement with earlier results observed with soluble collagen. Also, no binding was detected with SlpnB or the mutant KSDV257TANN of CbsA. No binding to laminin, fibronectin or bovine serum albumin (BSA) were detected (Figure 4 of I).

The *L. crispatus* JCM 5810 was originally isolated from the chicken, and *L. crispatus* strains colonize efficiently chicken and human intestine (Lan *et al.*, 2002; Vásquez *et al.*, 2002; Guan *et al.*, 2003; Antonio *et al.*, 2005). We tested the adherence of this strain onto frozen sections of chicken colon. JCM 5810 showed adherence to connective tissue sites, which were rich in type III collagen detected by antibody staining (Figure 5 of I). Removal of the S-layer with GnHCl completely abolished the adherence (Figure 5 of I), which is in agreement with CbsA binding to collagen.

The role of CbsA 1-287 in collagen- and tissue-binding was ascertained by expressing CbsA and truncated peptides on the surface of recombinant *L. casei*. In this display system, CbsA peptides were fused to an LPXTG motif to anchor the peptide to the cell wall (Martinez *et al.*, 2000). The strong promoter and signal sequence of CbsA were utilized to ensure efficient transcription and to direct the protein onto the cell wall. Surface expression was confirmed with anti-CbsA antibodies detected both by immunofluorescence and ELISA (Figure 4A of II). CbsA of the strain JCM 5810 adheres to immobilized laminin and collagens (Toba *et al.*, 1995; Table 1 of I). In accordance, the strain JCM 5810 adhered efficiently to collagen IV and laminin immobilized on glass, whereas the surface-display vector strain (pLPMSSA3) did not adhere (Figure 4 of II). *L. casei* derivatives expressing CbsA 1-287, 1-274, 28-287, or 31-287 adhered to both laminin and collagen IV. In contrast, the CbsA peptides 1-269, 34-287, 39-287 failed to confer adherence. Reduced adhesiveness was seen with *L. casei* expressing CbsA 251-410, accordant with the results obtained with recombinant His_6-CbsA 251-410. None of the constructs bound to BSA. *L. casei* expressing the entire CbsA 1-410 showed only low level of adherence to laminin or type IV collagen (Figure 4B of II). A probable reason for the failure to adhere might be simultaneous presence of two anchoring motives, i.e. the LPXTG-motif and the

C-terminus of CbsA (Chapter 5.1.5), which may cause conformational distortion in the CbsA molecule on bacterial surface. The adhesive *L. casei* derivatives and the strain JCM 5810 adhered to laminin- and collagen-containing BM areas in chicken colon and ileum, whereas no binding of the vector strain or the non-adhesive derivatives were detected (Figure5 of I, Figure 4C of II).

Our results show that *L. crispatus* JCM 5810 adheres to collagens and laminin as well as to connective tissue sites on chicken intestine and confirm the adhesive function of CbsA. A few lactobacillar S-layers have been proposed as adhesins binding to different tissue targets (see Table 1), but CbsA is so far the only collagen-binding lactobacillar S-layer protein that has been identified. In article I, genomic DNA from 11 strains representing closely related species of *L. crispatus* were hybridized with the 819-bp *Hpa*I fragment that encodes the N-terminal collagen-binding domain. Five of the isolates were adhesive to collagen, but a hybridization signal was detected only with JCM 5810 DNA. This suggests that CbsA-like S-layers are not common in lactobacilli, which, however, commonly express adhesiveness to collagens and/or laminin (McGrady *et al.*, 1995; Styriak *et al.*, 2001; Horie *et al.*, 2005). It seems that Cnb-like ABC transporter proteins (Roos *et al.*, 1996) or other non-S-layer collagen-binders (Boekhorst *et al.*, 2006b) are more common in lactobacilli. Collagen-binding ability of *Lactobacillus* was suggested to mediate colonization at tooth surfaces, and thus affect to pathogenesis of dental diseases (McGrady *et al.*, 1995). Allen *et al.*, (2002) suggested that laminin-binding proteins of Gram-positive pathogens mediate bacterial adherence to heart valves and play a role in endocarditis, a disease also associated with lactobacilli. However, adhesiveness of *Lactobacillus* in the intestinal tract is associated with probiotic, health promoting property. Adhesion to collagens is likely to promote persistent colonization at tissues, as reported with *E. coli* expressing collagen-binding Dr fimbriae (Selvarangan *et al.*, 2004) and with collagen-binding outer membrane protein of *Haemophilus* (Fulcher *et al.* 2006). Adhesion to tissue sites also inhibits adhesion of pathogenic bacteria (Tuomola *et al.*, 1999; Vaughan *et al.*, 2002; Edelman *et al.*, 2003; Lee *et al.*, 2003) and Horie *et al.*, (2002) suggested that CbsA has a role in inhibition of pathogenic *E. coli*.

The extensive analysis of His_6-constracts revealed that the collagen-binding epitope is comprised of a large N-terminal domain, which exhibited a regularly polymerized structure. The N-terminal sequence of CbsA shows no significant sequence similarity to the known bacterial collagen-binding proteins, but large collagen-binding regions are typical also for several other collagen-binding adhesin. In adherence to collagens and laminin, CbsA resembles the YadA

adhesin of *Yersinia enterocolitica*, which forms a regular layer composed of lollipop-shaped molecules on the bacterial surface; the collagen-binding region is large and conformational (El Tahir and Skurnik, 2001; Nummelin *et al.*, 2004). A collagen-binding region is also large in the structurally related proteins Cna of *S. aureus*, Acm of *Enterococcus faecium*, and Ace of *Enterococcus faecalis* (Patti *et al.*, 1992; Rich *et al.*, 1999; Nallapareddy *et al.*, 2003; Zong *et al.*, 2005). However, these domains do not show significant sequence homology to CbsA.

5.1.5 C-terminal domain of CbsA binds to cell wall and teichoic acids (II)

Lactobacillar S-layer proteins lack the SLH-motif, which in several other bacteria anchors the S-layer protein to the bacterial cell wall (Engelhardt and Peters, 1998). To characterize the domain in CbsA responsible for cell-wall binding, we assessed the binding of N-terminal (1-269) and C-terminal peptides (251-410, 288-410) of CbsA to the surface of native cells as well as of cells extracted with GnHCl and LiCl. These chaotropic agents remove the S-layer, which is non-covalently linked to the cell wall (Sleytr and Beveridge, 1999). Binding of peptides was assessed by ELISA (Figure 5B of II) and by indirect immunofluorescence (Figure 5B of II). None of peptides (1-269, 251-410, 288-410) bind to native cells, however, binding of CbsA 1-287 to native cells was observed (Figure 4 of I) indicating that polymerized molecules are able to attach to cell surface covered with S-layer and that this interaction is mediated by polymeric structure. Efficiently binding of C-terminal peptides to JCM 5810 cells from where the S-layer had been removed was observed, whereas the N-terminal peptide failed to bind. Further treatment of GnHCl-treated JCM 5810 cells with mutanolysin, which cleaves N-acetylmuramyl bonds in peptidoglycan (Hancock and Poxton, 1988) and is commonly used in isolation of proteins from Gram-positive cell surface (Granato *et al.*, 2004; Maeda *et al.*, 2004; Walter *et al.*, 2005), decreased the binding of the C-terminal peptides. We also assessed the binding of CbsA peptides to other strains of *Lactobacillus*. The C-terminal peptides bound to GnHCl-extracted cells of *L. crispatus* LMG 9479 and LMG 12003 and of *L. brevis* ATCC 8287 but showed only poor binding to cell surface of *L. amylovorus* F81, *L. gallinarum* F41, *L. gasseri* JCM 1130 and *L. johnsonii* 5F49 (Figure 6 of II).

As the further treatment of GnHCl-extracted cells with mutanolysin decreased the binding of peptides, we assessed their binding to the material released by the

mutanolysin treatment. The two C-terminal peptides bound to the mutanolysin extract, whereas no binding of the N-terminal peptide was detected (Figure 7 of II). To identify the cell-wall components responsible for S-layer attachment, we purified PG, teichoic acid as well as teichuronic acid and polysaccharides from *L. crispatus* JCM 5810 and assessed them for peptide binding. The C-terminal peptides bound to the teichoic acid fraction, whereas no binding was detected to other fractions. In addition, the C-terminal peptides bound to the commercially available LTAs from *Streptococcus faecalis* and *S. aureus* as assessed both by ELISA and a mobility shift assay in nondenaturating PAGE (Figure 7 of II). In conclusion, the C-terminal domain of CbsA is responsible for anchoring the S-layer protein of *L. crispatus* JCM 5810 to the cell wall and to teichoic acids. This is not surprising as the C-terminus of CbsA is highly basic (10.00), in contrast to the N-terminus, which has neutral pI (6.81). Similarly, the anchoring domains of several well-known teichoic acid or LTA-binding proteins are highly basic (Rigden *et al.*, 2003). The lactobacillar cell wall is not characterized in sufficient details for us to understand why the C-terminus of CbsA only binds to some of the tested lactobacillar strains.

The anchoring mechanism of CbsA shares features with the anchoring mechanisms seen with the S-layer protein of *L. acidophilus* ATCC 4356 (SlpA). The C-terminal region of SlpA (SAC) is 76% identical with C-terminus of CbsA. SAC binds to cell wall of *L. acidophilus*, from which S-layer had been removed. Further, the binding of SAC was abolished after treatment of the cells with hydrofluoric acid, which extracts the PG-associated components, such as teichoic acids (Smit and Pouwels, 2002). This suggests that lactobacillar surface proteins, which have a similar C-terminal sequence, interact via similar mechanisms with the negatively charged teichoic acid on bacterial surface.

5.2 Enolase and GAPDH are associated with the lactobacillar cell surface (III)

5.2.1 pH-dependency of association (III)

While this PhD study was in progress, it became evident in our laboratory that enolase and GAPDH, which are well-characterized surface-associated Plg-binding proteins in streptococci and staphylococci (Pancholi and Fischetti, 1992; Pancholi and Fischetti, 1998; Bergmann *et al.*, 2001; Mölkänen *et al.*, 2002; Bergmann *et al.*, 2004; Derbise *et al.*, 2004; Bergmann *et al.*, 2005), are major

components in the cell-free, extracellular proteome obtained from *L. crispatus* and other Acidophilus group lactobacilli at neutral pH (Hurmalainen *et al.*, 2007). Lactobacilli are strictly fermentative organisms and produce lactic acid as an end product of their carbohydrate metabolism, which rapidly lowers the pH of the environment below pH 5. This prompted us to assess the distribution of enolase and GAPDH as well as of the S-layer protein on the cell surface and in the extracellular proteome at two pH values, pH 5 and pH 8. During the assays, the pH dropped further to 4.5 and 7.5. This assay was done with the strain ST1 of *L. crispatus*, which is characterized for its adhesins and in which the extracellular proteome was identified (Edelman, 2005; Hurmalainen *et al.*, 2007). Using indirect immunofluorescence assay and Western blotting, we found that enolase and GAPDH are attached to the cell surface at pH 5, whereas at pH 8 enolase and GAPDH are found mainly in the supernatant from where the cells had been removed by filtration (Figure 1 of III). Further, stepwise increase of pH from 4.4 to 7.0 revealed that the release of enolase and GAPDH becomes detectable at pH 5.2, which are are close to the pI values of enolase and GAPDH (4.8 and 5.2, respectively). The release of enolase and GAPDH was instant at pH 8, whereas at pH 5 no release was detected until 24 hours. Further, the enolase and GAPDH were also released at pH 5 by 0.25 M sodium chloride, which indicate the role of ionic interactions in the cell wall anchoring. In contrast, the surface association of the S-layer protein was not dependent on pH, and it was detected on cells from both pHs.

Chloramphenicol had no effect on release of enolase and GAPDH, which indicates that protein synthesis is not needed for the release (Figure 2 of III). Further, we did not see any significant differences in the transcription of *eno* or *gap* in cells from the two pHs (Figure 2 of III). We concluded that the release of enolase and GAPDH is not related to intracellular expression, but is exclusively distributed between the cell surface and the extracellular proteome.

5.2.2 Binding of enolase and GAPDH to lipoteichoic acids (III)

Enolase and GAPDH of *L. crispatus* have isoelectric points of 4.8 and 5.4, respectively, and thus they have a positive charge at lower pH values and could bind to negatively charged cell-wall components, such as LTA. Indeed, using mobility shift assay, we were able to show that both enolase and GAPDH bind to LTAs, but not to PG in a pH-dependent manner (Figure 3 of III). At low pH, LTA clearly retarded the movement of enolase and GAPDH in nondenaturating polyacrylamide gel electrophoresis, whereas at pH 5.6, where enolase and

GAPDH have a negative charge, no association with the negatively charged LTA was detected. Further, enolase and GAPDH –coated fluorescent beads bound efficiently to LTA at pH 4.4, whereas only a low-level-binding was detected at neutral pH. No binding to PG was observed at either of the pHs (Figure 3 of III).

Pneumococcal enolase reassociates to the cell wall (Bergmann *et al*., 2001). We assessed the reassociation of enolase and GAPDH of *L. crispatus* to the cell wall at pH 4.4 and pH 7.0. Both enolase and GAPDH were recovered on the cell wall at pH 4.4, whereas at pH 7.0 only a low-level-binding was detectable. LTA inhibited efficiently the reassociation of enolase and GAPDH at pH 4.4 (Figure 4 of III). Mechanisms for surface association by bacterial enolases and GAPDHs have not been previously reported, and a very interesting feature of the present anchoring model is that lactobacilli rapidly change their surface properties in response to pH that changes during their growth.

Our results suggest that enolase and GAPDH are anchored to LTA at low pH by ionic interactions. Several other bacterial proteins that bind to LTA have been identified. These include glycyl-tryptophan (GW) module proteins, such as InlB of *L. monocytogens* (Jonquières *et al.*, 1999), choline-binding proteins (García *et al.*, 1998) and the S-layer protein (CbsA) of *L. crispatus* characterized in my thesis (Chapter 5.1.5). The pI of the cell-wall-binding domains of these proteins are above nine, thus they are positively charged at pH values lower 9, and can bind to LTAs and to cell wall also at neutral pH, which indeed was demonstrated with CbsA in articles II (Figure 7) and III (Figure 1).

5.2.3 Plasminogen-binding by *L. crispatus* at different pHs (III)

The Plg-binding characteristic of enolase and GAPDH was also observed in the proteins of *L. crispatus* (Hurmalainen *et al.*, 2007) and used in article III as a functional assay for studying the pH-dependent surface variation of these enzymes. We showed that Plg binds to lactobacillar cell surface at low pH, but was recovered in the cell-free supernatant from pH 8 (Figure 5 of III). Similarly, Plg binds poorly onto *L. crispatus* cells at neutral pH (Hurmalainen *et al.*, 2007). Further, we tested enhancement of tPA-mediated Plg activation by the cells and the supernatant fractions originating from pH 5 and pH 8. The cells from pH 5, but the supernatant from pH 8 enhanced plasmin formation.

In an analogy to the GAPDH of *L. crispatus*, GAPDH of *S. gordonii* was found primarily on the cell surface at acidic pH, whereas at neutral pH, GAPDH (more than 90%) was in culture medium. With GAPDH of *S. pyogenes*, no release to buffer was detected at neutral pH, or after treatment of the cells with 2% SDS or 2 M sodium chloride. Further, Plg remained bound to *S. pneumoniae* and *S. pyogenes* cells at neutral pH (Derbise *et al.*, 2004; Bergmann *et al.*, 2005), whereas *L. crispatus* cells bound poorly soluble Plg (Hurmalainen *et al.*, 2007). It thus seems likely that pathogenic streptococci and commensal lactobacilli have differing mechanisms in the surface association of "anchorless" Plg-binding surface enzymes.

Our results suggest that lactobacilli response to a change in environmental pH by modifying cell surface and releasing surface proteins. Lactobacilli colonize several acidic tissue sites in humans, such as the vaginal epithelia, the oral cavity, and the small intestine, where lactobacilli reduce the environmental pH by producing lactic acid as a primary end product of metabolism. The pH-dependency of surface protein anchoring here described indicates that pH changes are likely to strongly affect lactobacillar-host interactions.

5.3 Comparison of enolases from commensal lactobacilli and pathogenic streptococci (IV)

5.3.1 Expression of enolases (IV)

Pathogenic streptococci expose Plg-binding enolase on the cell surface, where the activation of Plg is enhanced and leads to degradation of ECM and BM proteins; this promotes transmigration of bacteria in tissues (Derbise *et al.*, 2004; Bergmann *et al.*, 2005). Recently, enolase of commensal *Lactobacillus* was shown to bind Plg and enhance tPA-mediated plasmin formation (Hurmalainen *et al.*, 2007). We compared the enolases from lactobacilli with enolases from severe pathogenic bacteria, *S. pyogenes*, *S. pneumoniae* and *S. aureus*. The enolases were cloned from *S. pneumoniae* TIGR4, *S. pyogenes* IH32030 and *S. aureus* 8325-4 as well as from two lactobacillar strains, *L. crispatus* ST1 and *L. johnsonii* F133. One enolase gene is present in published genomes of *S. pneumoniae, S. pyogenes* and *S. aureus* as well as in *L. crispatus* (Hurmalainen *et al.*, 2007), whereas three enolase genes are present in the genome of *L. johnsonii* NCC 533 (Pridmore *et al.*, 2004) and their sequences were utilized to clone *eno 1-3* from *L. johnsonii* F133. Existence of more than one enolase is

common in the genome of *Lactobacillus*, since *L. plantarum* and *L. gasseri* have also more than one enolase gene in the genome (Kleerebezem *et al.*, 2003; Makarova *et al.*, 2006). Sequence comparison of these enolase sequences revealed two subfamilies. Enolase of *L. crispatus* is highly similar with enolase 1 and 2 of *L. johnsonii*, whereas enolase of *S. pneumoniae*, *S. pyogenes*, *S. aureus* and enolase 3 of *L. johnsonii* form another subfamily (Figure 1 of IV).

The enolases were expressed and purified as His_6-fusion proteins, whose apparent molecular weights corresponded to the predicted molecular sizes (46.6-47.4 KDa; Figure 2A of IV). All His_6-enolases except enolase 2 of *L. johnsonii* were found enzymatically active (Figure 2B of IV), which indicates that His_6-fusion tag does not disturb the enzymatic activity of enolases or cause dramatic disturbances in the structure. By transcription analysis of RNA of *L. johnsonii* F133, we found that only enolases 1 and 3 are expressed in exponentially growing cells, whereas no expression of enolase 2 was detected in the test condition (Figure 2C of IV). These results suggest that enolase 2 could be a cryptic gene or a pseudogene in *L. johnsonii* F133.

Several bacterial enolases have an octameric structure (Pawluk *et al.*, 1986; Schurig *et al.*, 1995; Brown *et al.*, 1998; Ehinger *et al.*, 2004). To define the multimeric state of enolase from *L. crispatus* and compare it with the structures of pneumococcal enolases, whose structure was recently reported (Ehinger *et al.*, 2004), the His_6-enolase of *L. crispatus* and the enolase in extracellular proteome of *L. crispatus* were analysed in an analytical gel filtration column. The enolases from both samples eluted with a size of approximately 370-415 KDa, which suggests an octameric structure. The same size was in this study estimated for the pneumococcal enolase, which has earlier been shown to be an octamer (Ehinger *et al.*, 2004). Our results indicate that the *L. crispatus* enolase forms an octameric structure both in extracellular proteome and as a His_6-fusion protein.

5.3.2 Functional similarity of His_6-enolases (IV)

Binding of Plg is a well-characterized and biologically important function of enolases from several bacterial species (Pancholi and Fischetti, 1998; Bergmann *et al.*, 2001; Ge *et al.*, 2004; Hurmalainen *et al.*, 2007). Therefore, we first compared the Plg-binding by His_6-enolases (Figure 3 of IV). *L. crispatus* enolase, *L. johnsonii* enolases 1 and 2 as well as *S. aureus* enolase efficiently bound to Plg by a lysine-dependent manner, whereas a significantly lower level of Plg binding was detected with the closely related *L. johnsonii* enolase 3 and *S.*

pneumoniae and *S. pyogenes* enolases. A similar pattern in enolases was seen in enhancement of tPA- and uPA-mediated Plg activation (Figure 3 of IV).

Enolase has been identified as a laminin-binding protein on the staphylococcal surface (Carneiro *et al.*, 2004), and therefore we tested the laminin-binding, but also fibronectin, collagen I and BSA binding by the His_6-enolases (Figure 4 of IV). Enolases of *S. aureus*, as well as *L. crispatus* and enolase 1 of *L. johnsonii* bound to laminin and with a lower efficiency to collagen I, whereas other enolases bound significantly less to laminin and collagen I. None of these proteins bound to fibronectin or BSA. Similarly, the enolase from extracellular proteome of *L. crispatus* bound to laminin and collagen I, but no binding to fibronectin or BSA was detected (Figure 4 of IV) suggesting that laminin-binding is a true property of *L. crispatus* enolase and not of the recombinant protein alone.

Laminin- and collagen-binding property might direct the lactobacillar enolase protein in the ECM areas of tissues and facilitate plasmin-mediated degradation of tissue component, but it might also inhibit pathogenic bacteria to adhere to tissue sites via laminin- or collagen-binding proteins. Enolase was shown to associate with the cell surface at acidic pH (Chapter 5.2) and the lactobacillar binding to host tissues is strongly promoted at lower pH values (Harty *et al.*, 1994; Blum *et al.*, 1999b), therefore, enolase might have role in bacterial adhesion to host tissues in acidic environment, such as vagina or oral cavity.

In Plg-binding assay, in particular the enolase of *L. crispatus* and the structurally similar *L. johnsonii* enolase 1 were highly efficient. In general, Plg-binding has been associated with bacterial pathogenesis, and, e.g., the virulence role of streptococcal enolases and its Plg-binding ability have been characterized (Bergmann *et al.*, 2003; Derbise *et al.*, 2004; Bergmann *et al.*, 2005). C-terminal lysines are important in several Plg-binding proteins, including the enolase of *S. pyogenes* (Pancholi and Fischetti, 1998; Derbise *et al.*, 2004). However, the sequences of lactobacillar enolases do not contain C-terminal lysines (Pridmore et al., 2004; Hurmalainen *et al.*, 2007). An internal Plg-binding ^{248}FYDKERKVY site was identified from pneumococcal enolase (Ehinger *et al.*, 2004) and substitution of lysines and glutamic acid reduced the Plg-binding ability of both a recombinant protein and a parental strain. Our ongoing analysis has shown that substitution of the two lysines in the related sequence in *L. crispatus* (^{248}FYNKDDHKY) only marginally reduced enhancement of tPA-mediated plasmin formation. Therefore, it is likely that residues elsewhere in enolase of *L. crispatus* are involved in the interaction with Plg.

At neutral pH, lactobacilli release their enolase into to medium (Hurmalainen *et al*., 2007; Chapter 5.2), which has not been described for streptococcal pathogens. Immobilization of Plg on its receptors, such as the enolase on bacterial surface, is important for enhancement of Plg activation and protection of the plasmin activity against the main circulating plasmin inhibitor, α_2-antiplasmin (Wiman *et al*., 1979; Mangel *et al*., 1990). This is a major difference in Plg immobilization and enhancement of Plg activation by the pathogens and the lactobacillar commensals, and in theory, should *in vivo* severely prevent generation of high level of plasmin proteolysis. The biological role and the risk potential of lactobacillar enolase in opportunistic, e.g. endocarditis and bacteremia, remain open.

6 CONCLUSIONS

In this work, the molecular basis of adhesion and host interaction of *L. crispatus* was studied. This study characterized the domain structure in the S-layer protein CbsA, which is presently the best characterized lactobacillar adhesive surface protein. We showed that the N-terminal part of the molecule is responsible for binding to extracellular matrix in intestinal tissue and also for formation of the paracrystalline structure. Collagen-binding ability was associated with the S-layer polymerization, indicating that the adherence simultaneously involves several CbsA molecules or that the three-dimensional S-layer like structure is optimal for collagen-binding ability. Further characterization of CbsA polymerization and collagen-binding would require structural analysis by X-ray crystallography, but the difficulties in production of crystals of good quality retards the potential of structure determinations (Engelhardt and Peters, 1998). Recently, atomic force microscopy has risen as a technique to solve internal forces between S-layer subunits (Györvary *et al.*, 2003; Vadillo-Rodríguez *et al.*, 2005; Ebner *et al.*, 2006; Martín-Molina *et al.*, 2006), and its use in analyzing the forces involved in the stability and assembly of CbsA has begun (C. Verbelen, J. Antikainen, T.K. Korhonen, Y.F. Dufrêne, submitted).

As a collagen-binding adhesin, CbsA is an exceptional S-layer protein in lactobacilli. We have not identified another collagen-binding S-layer protein in lactobacilli. This is accordant with the sequence variability in the N-terminal regions of lactobacillar S-layer proteins, but, however, rather surprising in regard of how common the collagen-adherence is among lactobacilli (McGrady *et al.*, 1995; Styriak *et al.*, 2001; Harty *et al*., 1994); indeed, this study identified another group of collagen-binding surface proteins in lactobacilli, the enolases. The polymeric S-layer seems an ideal adhesin for binding to the huge collagen molecules and networks, a similar polymeric platform for collagen-binding is formed by the YadA adhesin of *Y. enterocolitica* (El Tahir and Skurnik, 2001), which in adhesive functions resembles CbsA. The biological role of collagen- and ECM-binding by lactobacilli remain to be established, by an analogy to other bacterial systems, one can speculate that collagen-binding promotes long-term colonization in host tissues (Selvarangan *et al*., 2004; Fulcher *et al*. 2006).

The highly conserved C-terminal part of CbsA anchors the protein to the cell wall and binds to teichoic acids. C-terminal part of CbsA has an alkaline pI, and it can bind to the negatively charged teichoic acids. Also, we showed that the multifunctional enzymes enolase and GAPDH bind to the cell wall and LTAs, but the binding is dependent on pH. In contrast to CbsA, these enzymes have an

acidic pI, and they are released from surface-LTAs at neutral and alkaline pH but remained attached to the cell wall at acidic pH. Enolase and GAPDH belong to the so-called anchorless surface proteins, and it will be interesting to learn whether these surface-associated enzymes have a similar cell-wall attachment mechanism in other bacterial species. Lactobacilli change their surrounding pH very efficiently, and a major conclusion from this work is that they have such a simple and rapid mechanism to alter their surface architecture in response to changes in pH.

Enolase and GAPDH are characterized Plg-binding proteins in several organisms, in particular in streptococcal pathogens, where they may increase bacterial infectivity and/or colonization by adhesive characteristics as well. We found that the lactobacillar enolases, as a group, do not drastically differ from enolases from streptococcal or staphylococcal pathogens. Lactobacillar enolases exhibit adhesiveness to collagen and laminin and are efficient Plg-binders. This thesis work can be seen as a first step in comparing the Plg system in bacterial pathogenesis and commensalism. Plg activation is not restricted to tissue damage and cell migration, but is also utilized in release of peptides for nutrition (Kitt and Leigh, 1997). An obvious difference between the pathogenic and commensal bacteria here studied is that the former group expresses Plg activators of their own, i.e. streptokinases and staphylokinase, such activities have not been detected in lactobacilli. The lactobacillar interaction with Plg can in principle be harmful, e.g. in tissue damage associated with opportunistic infections such as infective endocarditis, or beneficial, such as in generation peptide fragments from Plg.

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