Moonlighting Proteins of *Lactobacillus crispatus* : Extracellular Localization, Cell Wall Anchoring and Interactions with the Host

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ACADEMIC DISSERTATION

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TO PASI AND KAMILLA

PREFACE

This study was carried out at the Department of Biosciences, Faculty of Biological and Environmental Sciences, in the University of Helsinki. I am grateful to Professor Timo Korhonen, Head of the division and Professor Kielo Haahtela, Head of the department, for the opportunity to perform this work and providing excellent working and educational facilities. I want to express my deepest gratitude to Professor Timo Korhonen for supervising this work. His knowledge, advices and visions were priceless.

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Veera, September 2011

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications. The original publications are reprinted with the kind permission of the copyright holders.

I **Veera Hurmalainen***, Sanna Edelman*, Jenni Antikainen, Marc Baumann, Kaarina Lähteenmäki, Timo K. Korhonen. Extracellular proteins of *Lactobacillus crispatus* enhance activation of human plasminogen. *Microbiology,* 2007, 153: 1112-1122

II Jenni Antikainen, **Veera Kuparinen**, Kaarina Lähteenmäki, Timo K. Korhonen. pH-dependent association of enolase and glyceraldehydes-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoid acids. *Journal of Bacteriology*, 2007, 189: 4539-4543

III Jenni Antikainen, **Veera Kuparinen**, Kaarina Lähteenmäki, Timo K. Korhonen. Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunology & Medical Microbiology*, 2007, 51: 526-534

IV **Veera Kainulainen**, Vuokko Loimaranta, Anna Pekkala, Sanna Edelman, Jenni Antikainen, Riikka Kylväjä, Maiju Laaksonen, Jukka Finne, Liisa Laakkonen, Timo K. Korhonen. Glutamine synthetase and Glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37. Manuscript submitted for publication.

The publications are referred to in the text by their roman numerals.

Veera Kainulainen´s contribution to the articles:

I Laboratory experiments except IEM pictures and cloning of GAPDH, participated in planning and writing

II Initial observations about release of proteins to neutral buffer, participated in cell wall association and LTA- binding experiments and planning

III Cloning of lactobacillar genes, protein purification and plasminogen binding and activation experiments, enzyme activity measurements, participated in ECM binding experiments, participated in planning

IV Laboratory experiments except mutanolysin extraction and fractioning the extract, participated in planning and writing

SUMMARY

Moonlighting functions have been described for several proteins previously thought to localize exclusively in the cytoplasm of bacterial or eukaryotic cells. Moonlighting proteins usually perform conserved functions, e. g. in glycolysis or as chaperonins, and their traditional and moonlighting function(s) usually localize to different cell compartments. The most characterized moonlighting proteins in Gram-positive bacteria are the glycolytic enzymes enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which function in bacteria-host interactions, e. g. as adhesins or plasminogen receptors. Research on bacterial moonlighting proteins has focused on Gram-positive bacterial pathogens, where many of their functions have been associated with bacterial virulence. In this thesis work I show that also species of the genus *Lactobacillus* have moonlighting proteins that carry out functions earlier associated with bacterial virulence only. I identified enolase, GAPDH, glutamine synthetase (GS), and glucose-6-phosphate isomerase (GPI) as moonlighting proteins of *Lactobacillus crispatus* strain ST1 and demonstrated that they are associated with cell surface and easily released from the cell surface into incubation buffer. I also showed that these lactobacillar proteins moonlight either as adhesins with affinity for basement membrane and extracellular matrix proteins or as plasminogen receptors.

The mechanisms of surface translocation and anchoring of bacterial moonlighting proteins have remained enigmatic. In this work, the surface localization of enolase, GAPDH, GS and GPI was shown to depend on environmental factors. The members of the genus *Lactobacillus* are fermentative organisms that lower the ambient pH by producing lactic acid. At acidic pH enolase, GAPDH, GS and GPI were associated with the cell surface, whereas at neutral pH they were released into the buffer. The release did not involve *de novo* protein synthesis. I showed that purified recombinant His_6-enolase, His_6-GAPDH, His_6-GS and His_6-GPI reassociate with cell wall and bind *in vitro* to lipoteichoic acids at acidic pH. The *in-vitro* binding of these proteins localizes to cell division septa and cell poles. I also show that the release of moonlighting proteins is enhanced in the presence of cathelicidin LL-37, which is an antimicrobial peptide and a central part of the innate immunity defence. I found that the LL-37-induced detachment of moonlighting proteins from cell surface is associated with cell wall permeabilization by LL-37.

The results in this thesis work are compatible with the hypothesis that the moonlighting proteins of *L. crispatus* associate to the cell wall via electrostatic or ionic interactions and that they are released into surroundings in stress conditions. Their surface translocation is, at least in part, a result from their release from dead or permeabilized cells and subsequent reassociation onto the cell wall. The results of this thesis show that lactobacillar cells rapidly change their surface architecture in response to environmental factors and that these changes influence bacterial interactions with the host.

1 INTRODUCTION

The genus *Lactobacillus* belongs to the heterogenous group of lactic acid bacteria (LAB), which are Gram-positive, catalase-negative, non-sporulating, aerotolerant, chemo-organotrophic bacteria that grow in nutritionally rich media. The genus *Lactobacillus* contains high phylogenetic and functional diversity, and the present classification is mainly based on 16S rRNA sequence analysis (Dellaglio & Felis, 2005, Pot & Tsakalidou, 2009). Lactobacilli are strictly fermentative and secrete lactic acid as the main end-product of sugar fermentation. Species of *Lactobacillus* can be divided into three subgroups; obligate homofermentitives, facultative heterofermentatives and obligate heterofermentatives. The division is based on the presence or absence of fructose-1,6-diphosphate aldolase and phosphoketolase (Axelsson, 1998, Johnson *et al*., 1980). Homofermentatives include *Lactobacillus acidophilus* group, also termed *Lactobacillus delbrueckii* group, which is further divided into six homology groups based on results of DNA-DNA hybridization: *Lactobacillus acidophilus* (A1), *Lactobacillus crispatus* (A2), *Lactobacillus amylovorus* (A3), *Lactobacillus gallinarum* (A4), *Lactobacillus gasseri* (B1) ja *Lactobacillus johnsonii* (B2) (Fujisawa *et al*., 1992, Johnson *et al*., 1980, Schleifer & Ludwig, 1995). The *L. acidophilus* group also contains other species (Pot & Tsakalidou, 2009) that include industrially interesting species and probiotics.

The natural habitat of lactobacilli ranges from dairy, meat and plant material fermentations to the oral cavity and the genital and gastrointestinal tracts of humans and animals (Vaughan *et al*., 2002). The pH of human intestinal tract rapidly changes from highly acid in the stomach to pH 6 in the duodenum. The pH gradually increases in the small intestine to pH 7.4 in the terminal ileum but drops again to pH 5.7 in the caecum. The pH increases again reaching pH 6.7 in the rectum (Fallingborg, 1999). In addition to pH, several other factors such as peristalsis, redox potential, bacterial adhesion, microbial interactions, mucus and bile secretion, immunoglobulins, intestinal enzymes, exfoliated epithelial cells, nutrient availability, diet and bacterial antagonism (Holzapfel *et al*., 1998, Savage, 1977) as well as antimicrobial peptides (Weinberg *et al*., 1998) affect the prevalence of bacteria in different parts of gastrointestinal tract. The human gut microbiota is dominated by anaerobic bacteria. Total number of microbes is estimated to be from 10^2-10^4 /g at proximal small intestine and 10^{11}-10^{12} /g in colon (Kleerebezem & Vaughan, 2009). *L. crispatus*, *L. gasseri* and *L. acidophilus* were found to be prominent intestinal lactobacilli in adult fecal samples (Reuter, 2001, Walter *et al*., 2001).

The healthy microbiota of the lower genital tract in women predominantly consists of *Lactobacillus* spp. with *L. crispatus*, *Lactobacillus jensenii*, *Lactobacillus iners* as well as *L. gasseri* and *L. acidophilus* being the most prevalent species (Donati *et al*., 2010, Pavlova *et al*., 2002, Reid, 2009, Zhou *et al*., 2004). Vaginal lactobacilli metabolize glycogen secreted by the vaginal epithelia and produce lactic acid, which is largely responsible for the normal vaginal acidity (<pH 4.5) (Donati *et al*., 2010). The acidic environment of a healthy vagina is not permissive for growth of potential pathogens (Aroutcheva *et al*., 2001, Donati *et al*., 2010). Additionally, vaginal lactobacilli are thought to displace pathogens through competitive exclusion via the possible formation of biofilms (Domingue *et al*., 1991) as well as through the production of antimicrobials such as hydrogen peroxide and bacteriocin-like substances (Aroutcheva *et al*., 2001).

Lactobacilli have been recognized as potentially health-beneficial organisms in the human gastrointestinal tract. The esseys written by Élie Metchnikoff (1907) are regarded as the birth of probiotics (Ljungh & Wadström, 2009). Fuller (1989) defined probiotics "as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Nowadays, the definition of probiotics formulated by the World Health Organization (WHO) states that probiotics are "live microorganism which when administrated in adequate amounts confer a health benefit on the host". The proposed functions of probiotics include modulation of immune system and weakening of diseases such as cardiovascular diseases and inflammary bowel diseases. Probiotics are also suggested to offer protection against pathogens by enhancing the intestinal barrier function, by competitive exclusion or by producing antibacterial metabolites such as lactic acid, hydrogen peroxide, bacteriocins, acetic acid and nitric oxide. The health benefits associated with probiotics include carbohydrate degradation, degradation of antinutritional factors, production of growth factors, mineral absorbtion and B-vitamin synthesis (Kleerebezem *et al*., 2010, Sanchez *et al*., 2010, Turpin *et al*., 2010). It has also been suggested that probiotics do not necessarily need to be viable and that microbial cell preparations or components of microbial cells showing a beneficial effect on the health and well-being of the host should definied as probiotics (Salminen *et al*., 1999, Taverniti & Guglielmetti, 2011).

Typically, the lactobacilli selected for probiotic application are chosen for their ability to survive transit through the stomach and small intestine and to colonize the human gastrointestinal tract (Kleerebezem & Vaughan, 2009, Tuomola *et al*., 2001). A probiotic strain should tolerate acidic conditions, survive and grow in the presence of bile as well as adhere to intestinal cells (Tuomola *et al*., 2001). Several reports describe that lactobacilli are also efficient in adherence to components of extracellular matrix (ECM) (reviewed by Velez *et al*., 2007). The ECM forms a complex structure surrounding epithelial cells, but molecules from the ECM or their fragments can be shed from the epithelium to the mucus. Moreover, if the mucosa is damaged, the ECM can be exposed, allowing microbial colonization and infection (Westerlund & Korhonen, 1993). For this reason, binding to ECM proteins by lactobacilli can play a role in competition and displacement of pathogens.

The mechanisms underlying the health benefits of probiotic lactobacilli are poorly understood. Probiotic effector molecules, including cell surface associated as well as secreted or selectively released molecules, may have role in probiotic functions, such as inhibition of pathogens, reinforcing mucosal barriers as well as modulation of immune system (Kleerebezem & Vaughan, 2009, Kleerebezem *et al*., 2010).

Lactic acid bacteria have rarely been associated with opportunistic diseases such as infective endocarditis and bacteremia as well as localized infections (Cannon *et al*., 2005, Griffiths *et al*., 1992, Husni *et al*., 1997, Salvana & Frank, 2006). *Lactobacillus casei, Lactobacillus rhamnosus* and *L. acidophilus* have been reported in opportunistic infections (Cannon *et al*., 2005, Husni *et al*., 1997, Salvana & Frank, 2006). These infections are usually polymicrobial and no direct evidence of a primary role for lactobacilli in the infection has been reported (Cannon *et al*., 2005). The patients have underlying immunosuppressive conditions, broad spectrum antibiotic therapy, surgery, cancer or diabetes mellitus (Harty *et al*., 1994, Husni *et al*., 1997, Salminen *et al*., 2004). The molecular mechanisms that contribute to potential pathogenecity of lactobacilli are not known.

In this thesis work I have studied proteins released from the surface of *L. crispatus* into their surroundings, and their anchoring and release mechanisms. Also functions of moonlighting proteins and their possible beneficial or pathogenic role in bacteria-host interactions were investigated.

2 MOONLIGHTING PROTEINS IN GRAM-POSITIVE BACTERIA

2.1 DEFINITION OF A MOONLIGHTING PROTEIN

The term moonlighting protein was introduced to describe the ability of proteins or peptides to have more than one function (Jeffery, 2003a, Jeffery, 2003b, Jeffery, 2009). The origin of the concept was in neuropeptide research (Campbell & Scanes, 1995). Moonlighting proteins are usually highly conserved cytoplasmic proteins of eukaryotes or prokaryotes such as glycolytic enzymes or chaperones and the moonlight activities usually take place in another cellular location, in most cases the cell surface, than does the conventional function (Henderson & Martin, 2011). The moonlighting proteins perform autonomous functions without partitioning these functions into different protein domains, hence they have not evolved through gene fusions (Huberts & van der Klei, 2010). Also, the functions of moonlighting proteins are independent, i. e. the inactivation of one function should not affect the second function and vice versa. The moonlighting proteins perform autonomous functions without partitioning these functions into different protein domains, which suggests that they have not evolved through gene fusions (Huberts & van der Klei, 2010). Structure determination of moonlighting proteins has shown thst they utilize separate protein surfaces for their multiple functions (Jeffery, 2004). The proteins that are described to moonlight may have different moonlighting activities in different organisms. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the first identified bacterial moonlighting protein, already before the term "moonlighting" was adapted, and has become the prototype bacterial moonlighting enzyme. GAPDH was detected on the surface of *Streptococcus pyogenes,* where it exhibits functions unrelated to glycolysis (Pancholi & Fischetti, 1992). The research on bacterial moonlighting enzymes is so far strongly biased for infectious diseases caused by Gram-positive bacteria and virulence-associated functions of the proteins (Henderson & Martin, 2011). Table 1. gives a summary of moonlighting proteins identified in Gram-positive pathogens.

Table 1. Moonlighting proteins in Gram-positive bacteria

Extracellular moonlighting protein	Species	Reference
Metabolic enzymes		
Alcohol acetaldehyde dehydrogenase	*Listeria monocytogenes*	Kim *et al.,* 2007
Aldolase	*Mycobacterium tuberculosis*	Xolalpa *et al.*, 2007
	Streptococcus pneumoniae	Blau *et al.*, 2007, Ling *et al.*, 2004
Aminopeptidase C	*Streptococcus pneumoniae*	Ling *et al.*, 2004
Aspartate carbamoyltransferase	*Streptococcus pneumoniae*	Ling *et al.*, 2004
Carbamoyl-phosphate synthase	*Streptococcus pneumoniae*	Ling *et al.*, 2004
Dihydrolipoamide dehydrogenase	*Mycobacterium tuberculosis*	Xolalpa *et al.*, 2007
Enolase	see chapter 2.3	
Glucose-6-phosphate isomerase	*Streptococcus agalactiae*	Hughes *et al.*, 2002
	Streptococcus pneumoniae	Ling *et al.*, 2004
Glutamine synthetase	*Mycobacterium tuberculosis*	Xolalpa *et al.*, 2007
	Streptococcus agalatiae	Suvorov *et al.*, 1997
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	see chapter 2.2	
Isocitrate lyase	*Mycobacterium tuberculosis*	Xolalpa *et al.*, 2007
L-lactate dehydrogenase	*Streptococcus pneumoniae*	Ling *et al.*, 2004
Malate synthase	*Mycobacterium tuberculosis*	Kinhikar *et al.*, 2006
NADP-spesific glutamate dehydrogenase	*Streptococcus pyogenes*	Ling *et al.,* 2004
Nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase	*Streptococcus agalactiae*	Hughes *et al.,* 2002
Ornithine carbamoyltransferase	*Streptococcus agalactiae*	Hughes *et al.*, 2002
	Streptococcus suis	Winterhoff *et al.,* 2002
Phosphofructokinase	*Streptococcus oralis*	Kinnby *et al.*, 2008
6-phosphogluconate dehydrogenase	*Streptococcus pneumoniae*	Ling *et al.*, 2004

Extracellular moonlighting protein	Species	Reference
Metabolic enzymes		
Phosphoglycerate kinase	group B streptococci	Burnham et al., 2005
	Streptococcus agalactiae	Hughes *et al*., 2002
	Streptococcus anginosus	Kinnby *et al*., 2008
	Streptococcus oralis	Kinnby *et al*., 2008
	Streptococcus pneumoniae	Ling *et al*., 2004
	Streptococcus pyogenes	Pancholi & Fischetti, 2003
Phosphoglycerate mutase	*Streptococcus anginosus*	Kinnby *et al*., 2008
	Streptococcus mutans	Kinnby *et al*., 2008
	Streptococcus pyogenes	Pancholi & Chhatwal., 2003
Purine nucleoside phosphorylase	*Streptococcus agalactiae*	Hughes *et al*., 2002
Pyruvate dehydrogenase subunits PdhB and PdhD	*Bacillus subtilis*	Yang *et al.,* 2011
Pyruvate kinase	*Streptococcus gordonii*	Kesimer *et al*., 2009
Pyruvate oxidase	*Streptococcus pneumoniae*	Ling *et al*., 2004
S-Adenosyl-L-homocysteine hidrolase	*Mycobacterium tuberculosis*	Xolalpa *et al*., 2007
SarA; oligopeptide-binding lipoprotein	*Streptococcus gordonii*	Kesimer *et al*., 2009
Superoxide dismutease SodA	*Bacillus subtilis*	Yang *et al*., 2011
Triosephosphate isomerase	*Staphylococcus aureus*	Furuya & Ikeda, 2009, Ikeda *et al*., 2007, Yamaguchi *et al.,* 2010
	Streptococcus pyogenes	Pancholi & Fischetti, 2003
UDP-glucose 4-epimerase	*Streptococcus pneumoniae*	Ling *et al*., 2004

Extracellular moonlighting protein	Species	Reference
Molecular Chaperones		
Cpn60.2 = GroEL2	*Mycobacterium tuberculosis*	Hickey *et al.,* 2009, Hickey *et al.,* 2010
Cpn60.1 = GroEL1	*Mycobacterium tuberculosis*	Cehovin *et al.*, 2010
GroEL	*Bacillus subtilis*	Yang *et al.*, 2011
GroES	*Mycobacterium tuberculosis*	Xolalpa *et al.*, 2007
DnaK	*Streptococcus gordonii*	Kesimer *et al.*, 2009
	Bacillus subtilis	Yang *et al.*, 2011
	Streptococcus pneumoniae	Ling *et al.*, 2004
	Mycobacterium tuberculosis	Hickey *et al.*, 2009, Xolalpa *et al.*, 2007
	Listeria monocytogenes	Schaumburg *et al.*, 2004
Heat shock protein, chaperonin	*Streptococcus gordonii*	Kesimer *et al.*, 2009
Others		
DNA-directed RNA polymerase, beta' subunit	*Streptococcus gordonii*	Kesimer *et al.,* 2009
EF-Tu	*Streptococcus gordonii*	Kesimer *et al.*, 2009
	Listeria monocytogenes	Schaumburg *et al.*, 2004
	Mycobacterium tuberculosis	Xolalpa *et al.*, 2007
EF-G	*Streptococcus gordonii*	Kesimer *et al.*, 2009
Glutamyl-tRNA amidotranferase subunit A	*Streptococcus pneumoniae*	Ling *et al.*, 2004
Glutamyl-tRNA synthetase	*Streptococcus pneumoniae*	Ling *et al.*, 2004
SecA	*Streptococcus gordonii*	Kesimer *et al.,* 2009

2.2 GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)

GAPDH is an essential glycolytic enzyme, which catalyzes oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate. GAPDH has been found intracellularily, on the cell surfaces, and in extracellular buffer or culture media of several Gram-positive bacterial species (Table 2). The distribution of GAPDH between different cell compartments may vary according to environment. In *Streptococcus gordonii* more GAPDH was found in culture media when pH of the medium was raised from 6.5 to 7.5 (Nelson *et al*., 2001).

Also Gram-negative enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) express GAPDH on the cell surface as well as secrete it to the growth medium (Egea *et al*., 2007, Kenny & Finlay, 1995). GAPDH has been found to be present on the cell surface of *Neisseria meningitidis* and *Neisseria lactamica* (Grifantini *et al*., 2002) and in the periplasm of *Aeromonas hydrophila* (Villamon *et al*., 2003). The smallest self-replicating cells, mycoplasmas that lack a cell wall, were reported to have GAPDH on the cell surface (Alvarez *et al*., 2003). The expression of the extracellular GAPDH is not restricted to bacteria. Also the eukaryotic microorganisms *Candida albicans* (Delgado *et al*., 2001), *Saccharomyces cerevisiae* (Gil-Navarro *et al*., 1997), *Paracoccidioides brasiliensis* (Barbosa *et al*., 2006) were described to have GAPDH bound on the cell surface.

Pancholi and Fischetti (1992) reported GAPDH as a common surface molecule in the genus *Streptococcus.* It was found in all streptococcal groups exept D, F and N (Pancholi & Fischetti, 1992). Streptococcal surface GAPDH (SDH) was later reported as a plasminogen receptor (Winram & Lottenberg, 1996) as well as to bind to the urokinase plasminogen activator receptor (uPAR) present on pharyngeal cells (Jin *et al*., 2005). These findings associated surface-GAPDH with bacterial ability to engage host proteolytic system to advance growth and disseminiation in the host.

Table 2. Pathogenic Gram-positive bacteria reported to express extracellular or cell surface bound GAPDH

Species	Reference
Bacillus anthracis	Lamonica *et al.*, 2005
Group B, C, E, G, H, L streptococci	Pancholi & Fischetti, 1992
Listeria monocytogens	Schaumburg *et al.*, 2004
Mycobacterium avium	Bermudez *et al.*, 1996
Mycobacterium tuberculosis	Bermudez *et al.*, 1996
Oenococcus oeni	Carreté *et al.*, 2005
Propionibacterium acnes	Holland *et al.*, 2010
Staphylococcus aureus	Modun & Williams, 1999
Staphylococcus epidermidis	Modun & Williams, 1999
Streptococcus agalactiae	Seifert *et al.*, 2003
Streptococcus anginosus	Kinnby *et al.*, 2008
Streptococcus equisimilis	Gase *et al.*, 1996
Streptococcus gordonii	Nelson *et al.*, 2001
Streptococcus oralis	Maeda *et al.*, 2004
Streptococcus pneumoniae	Bergmann *et al.*, 2004a
Streptococcus pyogenes	Pancholi & Fischetti, 1992
Streptococcus suis	Brassard *et al.*, 2004

2.2.1 BACTERIA AND THE MAMMALIAN PLASMINOGEN SYSTEM

Plasminogen is the proenzyme of the broad-spectrum serine protease plasmin. Plasmin participates in degradation of ECM proteins as well as activation of pro-matrix metalloproteinases (pro-MMPs) to MMPs (Lijnen & Collen, 1995, Myöhänen & Vaheri, 2004). These activities associate with increased cell migration. Plasminogen is activated by two physiological activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Lijnen & Collen, 1995). tPA has an important role in fibrinolysis, and in the absence of fibrin, tPA shows only low activity towards plasminogen (Redlitz & Plow, 1995, Rijken & Lijnen, 2009). uPA binds to the specific cellular receptor uPAR and uPA-catalyzed plasminogen activation is involved in cell migration, tissue remodeling and repair, macrophage function, ovulation, embryo implantation and tumor invasion (Myöhänen & Vaheri, 2004, Rijken & Lijnen, 2009, Smith & Marshall, 2010).

Several pathogenic bacteria interact with the plasminogen system (Bergmann & Hammerschmidt, 2007, Lähteenmäki *et al*., 2001, Lähteenmäki *et al*., 2005). The binding of plasminogen on the cell surface of *S. pyogenes* was first reported by Lottenberg *et al*. (1987). Bacterial plasminogen receptors immobilize plasminogen on the bacterial surface and thus enhance its activation into plasmin by host tPA. Also, bacteria express plasminogen activators, which are either proteolytic activators, such as the omptin surface proteases Pla of *Yersinia pestis* and PgtE of *Salmonella enterica* (Haiko *et al*., 2009) or change the conformation of plasminogen to advance self-activation. These include streptokinase of *Streptococcus* and staphylokinase of *Staphylococcus* (Lähteenmäki *et al*., 2005). Cell-surface bound plasmin is protected from inactivation by α2-antiplasmin, the primary physiological inhibitor of plasmin (Lijnen & Collen, 1995, Lähteenmäki *et al*., 2001, Lähteenmäki *et al*., 2005, Myöhänen & Vaheri, 2004, Plow *et al*., 1995, Rijken & Lijnen, 2009). The plasminogen molecule contains five disulphide-bonded kringle structures which recognize lysine-rich domains typically located in the carboxyterminus of the receptor molecule (Redlitz *et al*., 1995). Carboxyterminal lysines of GAPDH were described to be important for plasminogen binding and their substitution to leusines in *S. pyogenes* diminished the plasminogen binding. However, the recombinant bacteria expressing the mutated GAPDH showed binding capacity equal to that of the bacteria with the wild type GAPDH (Winram & Lottenberg, 1998), suggesting importance of internal positively charged protein regions in plasminogen binding. The internal arginine and histidine residues are important in plasminogen binding in *S. pyogenes* M-like protein (PAM) (Sanderson-Smith *et al*., 2006) and in PAM-related protein Prp (Sanderson-Smith *et al*., 2007).

The function as a plasminogen or plasmin receptor is also reported for GAPDH of *Streptococcus pneumoniae* (Bergmann *et al*., 2004a) , *Streptococcus agalactiae* (Seifert *et al*., 2003), *Streptococcus equsimilis* (Gase *et al*., 1996), *Streptococcus suis* (Jobin *et al*., 2004), *Listeria monocytogenes* (Schaumburg *et al*., 2004), *Staphylococcus aureus* and *Staphylococcus epidermis* (Modun & Williams, 1999) as well as EHEC and EPEC strains of *E. coli* (Egea *et al*., 2007). Plasminogen-binding may also contribute to bacterial adhesiveness and invasiveness by nonproteolytic mechanisms. Plasminogen binds to bacterial cell surface receptors, such as GAPDH, as well as to receptors on eukaryotic cells and may thus function as a bridge between the bacteria and the epithelium. In accordance, plasminogen, but not active plasmin, was observed to mediate adherence of *S. pneumoniae* to pulmonary epithelial and vascular endothelial cells. The transmigration of *S. pneumoniae* across endothelial and epithelial cell layers is due to degradation of intracellular junctions by active plasmin (Attali *et al*., 2008).

2.2.2 ADHESIVE AND VIRULENCE FUNCTIONS OF GAPDH

Several reports have shown that surface GAPDH is involved in adhesion to host components. SDH of *S. pyogenes* was shown to bind lysozyme, actin, myosin and fibronectin (Pancholi & Fischetti, 1992), *S. agalactiae* GAPDH to actin and fibrinogen (Seifert *et al*., 2003), and GAPDH of EPEC and EHEC to fibrinogen (Egea *et al*., 2007). GAPDH of the swine pathogen *S. suis* serotype 2 was observed to be involved in adhesion to porcine tracheal rings. Incubation of the rings with His_6-GAPDH diminished adhesion of *S. suis* cells and *S. suis* cells defective in expression of GAPDH had dimished binding capacity (Brassard *et al*., 2001, Brassard *et al*., 2004). GAPDH of *S. suis* has been described also as an albumin-binding protein (Quessy *et al*., 1997). GAPDH of *Streptococcus oralis* was shown to act as a coadhesin for fimbria-mediated adhesion of *Porphyromonas gingivalis* in periodontal sites (Maeda *et al*., 2004). An antiserum against GAPDH was observed to block binding of *Mycoplasma genitalium* to mucin, which suggests a role for GAPDH in the adhesion (Alvarez *et al*., 2003).

SDH of *S. pyogenes* has also been described as an ADP-ribosylating enzyme whose activity was enhanced by nitric oxide (Pancholi & Fischetti, 1993). ADP-ribosylation of host proteins is common for bacterial toxins (Henkel *et al*., 2010). *S. pyogenes* may use SDH molecule for signalling with the host (Pancholi & Fischetti, 1993). Streptococcal cells and purified SDH were reported to induce fosforylation of the histone H3 protein in the membrane of pharyngeal cells (Pancholi & Fischetti, 1997). *S. pyogenes* evades phagocytosis by degrading C5a of complement system (Wexler & Cleary, 1985) and SDH binds C5a and inhibits C5a-activated chemotaxis and H_2O_2 production (Terao *et al*., 2006). Recently it was shown that export of SDH is essential for *S.pyogenes* virulence. Genetic fusion of a hydrophobic tail into the C-terminus of SDH prevented the surface translocation, diminished plasminogen binding to the bacteria and bacterial adhesion to pharyngeal cells (Boël *et al*., 2005). Majority of genes involved in *S. pyogenes* virulence were downregulated when SDH was not translocated. Also several phenotypic and physiological changes were observed (Jin *et al*., 2011).

GAPDH of *S. agalactiae* is an immunomodulatory protein. Recombinant GAPDH activates T and B cells. GAPDH-overexpressing strain exhibits an increased *S. agalactiae* colonization in murine liver (Madureira *et al*., 2007). Iron is an essential nutrient for bacteria, but in mammalian hosts, iron is tightly bound to proteins such as hemoglobin, transferrin, lactoferrin and ferritin. Staphylococci have evolved an iron-scavenging system which is based on GAPDH as a cell-wall receptor (Modun *et al*., 1998, Modun & Williams, 1999). Epdiermial growh factor (EGF) enhances growth of intracellular *Mycobacterium avinum* and *Mycobacterium tuberculosis* macrophages. The receptor for EGF was found to be GAPDH (Bermudez *et al*., 1996). The GAPDH of *S. pneumoniae* was reported to be antigenic in humans as well as to elicit protective immune responses in the mouse (Ling *et al*., 2004).

2.3 ENOLASE

α-enolase is a metalloenzyme that catalyzes the dehydration of 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP). In gluconeogenesis, enolase catalyzes hydration of PEP to PGA (Pancholi, 2001). Enolase has been described as a prototypic moonlighting protein (Henderson & Martin, 2011). It has been reported on the cell surface of human cells where it has several functions. It can act as a hypoxic stress protein (Aaronson *et al*., 1995), *myc*-binding protein (Subramanian & Miller, 2000) and plasminogen receptor (Dudani *et al*., 1993, Miles *et al*., 1991, Nakajima *et al*., 1994, Redlitz *et al*., 1995). Enolase has been proposed to have a role in bacterial, fungal and neurological diseases, fungal allergies, cancer and autoimmunity diseases (Pancholi, 2001). Enolase can be located on the cell surface of eukaryotic microorganisms, such as *C. albicans*, where it is an immunodominant antigen (Angiolella *et al*., 1996). Also protists *Trichomonas vaginalis* (Mundodi *et al*., 2008), *Plasmodium falciparum* (Bhowmick *et al*., 2009), *Plasmodium yoelii* (Pal-Bhowmick *et al*., 2007), opportunistic pathogen yeast-like fungus *Pneumocystis carinii* (Fox & Smulian, 2001), pathogenic fungus *P. brasiliensis* (Donofrio *et al*., 2009) as well as parasites *Leishmania Mexicana* (Quinones *et al*., 2007), *Schistosoma bovis* (de la Torre-Escudero *et al*., 2010), *Onchocerca volvulus* (Jolodar *et al*., 2003) and *Echinococcus granulosus* (Gan *et al*., 2010) express surface-localized enolase.

The presence of enolases on the surface of prokaryotes was first reported for group A streptococci (Pancholi & Fischetti, 1998). Subsequently, extracellular enolase has been described in several Gram-positive pathogenic bacteria (Table 3.) as well as in a few Gram-negative bacteria, such as *Aeromonas salmonicida* (Ebanks *et al*., 2005), *A. hydrophila* (Sha *et al*., 2003), *N. meningitidis* (Knaust *et al*., 2007) and *Actinobacillus actinomycetemcomitans* (Hara *et al*., 2000) and in *Mycoplasma fermentas* (Yavlovich *et al*., 2007), which lacks a cell wall. Enolase of *S. pyogenes* was identified as a plasminogen receptor (Pancholi & Fischetti, 1998). Enolase of *S. pneumoniae* was also observed to be important for plasminogen binding (Bergmann *et al*., 2001, Kolberg *et al*., 2006). Plasminogen receptor function has been described also for enolases of *Bacillus anthracis* (Agarwal *et al*., 2008), *L. monocytogenes* (Schaumburg *et al*., 2004), *S. aureus* (Mölkänen *et al*., 2002), *Streptococcus mutans* (Ge *et al*., 2004) and *S. oralis* (Itzek *et al*., 2010). Invasion of pneumococci through basement membranes is tought to be important in meningitis, and plasminogen was found to potentiate pneumococcal penetration through reconstituted basement membrane gel (matrigel) (Eberhard *et al*., 1999). Soluble recombinant enolase binds cell surface of *S. pneumoniae* when associated with plasminogen (Bergmann *et al*., 2001), and pneumococcal enolase-coated microspheres degraded radiolabeled ECM and matrigel (Bergmann *et al*., 2005). Plasminogen binding to enolase of *B. anthracis* is also thought to increase invasive potential of this organism, since bacteria incubated with plasminogen were capable of degrading fibronectin (Agarwal *et al*., 2008).

Table3. Pathogenic Gram-positive bacteria reported to express extracellular or cell surface bound enolase

Species	Reference
Bacillus anthracis	Lamonica *et al.*, 2005
Group B, C, E, G, H, L streptococci	Pancholi & Fischetti, 1998
Listeria monocytogenes	Schaumburg *et al.*, 2004
Paenibacillus larvae	Antunez *et al.*, 2011
Staphylococcus aureus	Mölkänen *et al.*, 2002
Streptococcus anginosus	Kinnby *et al.*, 2008
Streptococcus gordonii	Kesimer *et al.*, 2009
Streptococcus mutans	Ge *et al.*, 2004
Streptococcus oralis	Itzek *et al.*, 2010, Kinnby *et al.*, 2008
Streptococcus pneumoniae	Bergmann *et al.*, 2001
Streptococcus pyogenes	Pancholi & Fischetti, 1998
Streptococcus suis	Feng *et al.*, 2009

C-terminal lysines of eukaryotic an prokaryotic enolases were identified to be important for plasminogen binding (Bergmann *et al.*, 2001, Derbise *et al.*, 2004, Miles *et al.*, 1991, Redlitz *et al.*, 1995). Substitution of C-terminal lysines with leusines in enolase of *S. pyogenes* reduced plasminogen binding as well as bacterial penetration through reconstituted basement membranes (Derbise *et al.*, 2004). Carboxypeptidase treatment and mutation of C-terminal lysines reduced plasminogen binding capacity also in pneumococcal enolase (Bergmann *et al.*, 2001). Analysis of plasminogen binding to the mutated enolase lacking the C-terminal lysine residues suggested presence of another binding site for plasminogen (Bergmann *et al.*, 2004b). By using short synthetic peptides covering whole enolase sequence, a nine-residue long internal sequence (^{248}FYDKERKVYD) was discovered to mediate binding to plasminogen. The acidic amino acids, aspartic acid (position 3) and glutamic acid (position 5), and the lysine residues at positions 4 and 7 were found to be important for the binding. The same peptide inhibited plasminogen binding to pneumococcal cells (Bergmann *et al.*, 2003). Substitutions in the internal plasminogen binding sequence diminished plasminogen binding to pneumococcal cells, attenuated the virulence of the bacterium in a mouse model of intranasal infection (Bergmann *et al.*, 2003) and reduced degradation of ECM or matrigel (Bergmann *et al.*, 2005). *S. pyogenes* has also an internal sequence in enolase, which is similar to internal sequence in *S. pneumoniae* enolase. Structural analysis of interaction between plasminogen and enolase of *S. pyogenes* suggested importance of internal lysines 252 and 255 as well as of the C-terminal lysines (Cork *et al.*, 2009). A high prevalence of the FYDKERKVY sequence in enolases from clinical isolates of oral streptococci was observed. However, the ability to enhance plasminogen activation did not require full conservation of internal plasminogen binding sequence (Itzek *et al.*, 2010).

The enzymatically active enolase is dimeric. An octameric structure has also been described in prokaryotic enolases (Brown *et al*., 1998, Cork *et al*., 2009, Ehinger *et al*., 2004, Kaufmann & Bartholmes, 1992, Schurig *et al*., 1995). The crystal structure of pneumococcal enolase revealed that the internal plasminogen binding motif is exposed on the surface of the molecule whereas C-terminal lysines are buried in a groove between two dimers and seem to be involved in stabilizing the tertiary structure of the molecule (Ehinger *et al*., 2004). The enolase of *S. pyogenes* is also an octamer, and C-terminal lysines were detected spatially close to the internal plasminogen binding sequence (Cork *et al*., 2009). Together these lysine residues could be responsible for plasminogen binding by *S. pyogenes* enolase.

Enolase has been reported to bind several host components. Enolase of *B. anthracis* (Agarwal *et al*., 2008) and *S. aureus* (Mölkänen *et al*., 2002) were identified as laminin binders, and enolase of *S. suis* was reported to bind fibronectin (Esgleas *et al*., 2008). Enolase binds salivary mucin in *S. gordonii* and *S. mutans* and mediates adhesion of *S. suis* to Hep-2 cells (Feng *et al*., 2009).

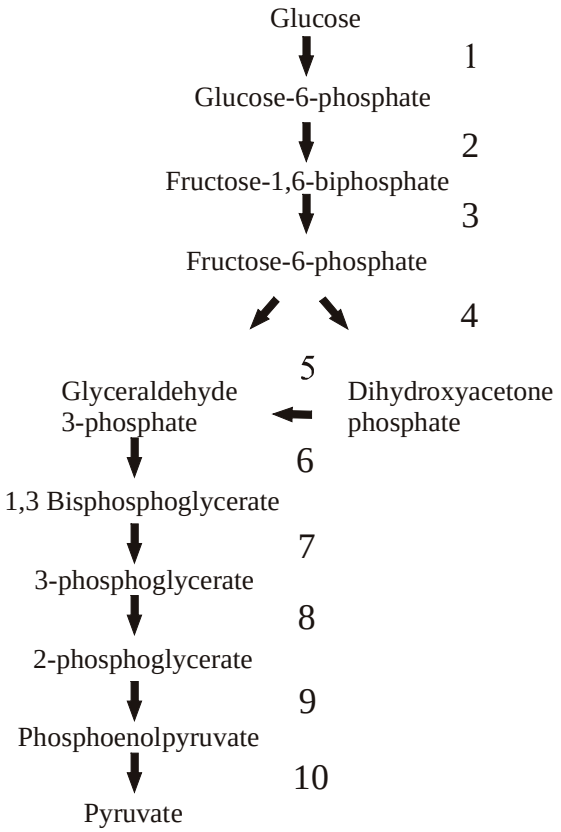
Only a few reports have demonstrated a direct role of enolase in bacterial pathogenesis. Pneumococci expressing enolase lacking the internal plasminogen binding site show reduced virulence in mice (Bergmann *et al*., 2003). It has been reported that recombinant enolase from *Streptococcus sorbinus* is an immunosuppressive protein which can be used to protect against dental caries in the rat (Dinis *et al*., 2009). Immunization with enolase of *S. suis* protects against *S. suis* infections in the mouse (Feng *et al*., 2009).

2.4 OTHER GLYCOLYTIC ENZYMES

Various metabolic proteins exhibit moonlighting functions in Gram-positive bacteria (Table 1.). Fructose-1, 6-bisphosphate aldolase is the fourth enzyme in glycolysis and catalyses the reversible cleavage of fructose-1, 6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Aldolase was found in mutanolysin extract of *S. pneumoniae*. Antibodies against aldolase protected against respiratory challenge with *S. pneumoniae* (Ling *et al*., 2004). Aldolase has also been reported as a lectin which binds to flamingo cadherin receptor (FCR) (Blau *et al*., 2007).

All enzymes of glycolytic pathway except hexokinase have been identified on the surface of Gram-positive bacteria (Figure 1). The diverse moonlighting activities described for these enzymes are related to their surface localization. They may have intracellular moonlighting functions as well but such functions have not been studied or reported yet. However, phosphofructokinase and enolase of *Bacillus subtilis* have been described to form complexes with proteins involved in mRNA processing and forming RNA degrasomes (Commichau *et al*., 2009).

Figure 1. Reported moonlighting activities of glycolytic enzymes in Gram-positive pathogens. The enzymes are discussed in more details in the text.



1 Hexokinase

2 Glucose-6-phosphate isomerase:
Antigenic in humans

3 Phosphofructokinase:
Plasminogen binding

4 Aldolase:
Plasminogen binding,
Enhancement of plasminogen activation,
Lectin,
Antigenic in humans
Protective antigen against *S. pneumoniae*

5 Triosephosphate isomerase:
Plasminogen binding
Adhesin for *C. neoformans*

6 Glyceraldehyde-3-phosphate dehydrogenase:
See chapter 2.2

7 Phosphoglycerate kinase:
Protective antigen against *S. agalactiae* in neonatal animals,
Antigenic in humans,
Plasminogen binding,
Actin binding,
Inhibits epithelial cell invasion by Group B streptococci

8 Phosphoglycerate mutase:
Plasminogen binding

9 Enolase:
See chapter 2.3

10 Pyruvate kinase:
Salivary mucin binding

Group B streptococci are opportunistic human pathogens that cause invasive infections in newborns and elderly people (Paoletti *et al.*, 2000). Phosphoglycerate kinase and ornithine carbamoyltransferase are major surface proteins of *S. agalactiae* (group B streptococci). Hyperimmune sera against phosphoglycerate kinase and ornithine carbamoyltransferase protect neonatal animals from *S. agalactiae* infection (Hughes *et al.*, 2002). Phosphoglycerate kinase was also shown to bind actin and this interaction may have a role in group B streptococcal invasion into epithelial cells (Burnham *et al.*, 2005). Binding of phosphoglycerate kinase of group B streptococci to actin disrupts the actin cytoskeleton of host cells (Boone *et al.*, 2011). Other reported surface proteins of group B streptococci include glutamine synthetase (Suvorov *et al.*, 1997), glucose-6-phosphate isomerase, purine nucleoside phosphorylase oligopeptide-binding lipoprotein and nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (Hughes *et al.*, 2002).

Phosphoglycerate kinase of group B streptococci as well as of *S. oralis* and *Streptococcus anginosus* have been reported to bind plasminogen (Boone *et al.*, 2011, Kinnby *et al.*, 2008). Also phosphoglycerate mutase and triosephosphate isomerase were identified as plasminogen binding proteins of *S. anginosus* and *S. mutans.* 6-phosphofructokinase of *S. oralis* was identified to bind plasminogen as well (Kinnby *et al.*, 2008). Phosphoglycerate kinase, phosphoglycerate mutase and triosephosphate isomerase were also

detected on the surface of *S. pyogenes* (Pancholi & Chhatwal, 2003). Trypsin digest of proteins from *S. pyogenes* surface revealed several putative metabolic enzymes, in addition to GAPDH and enolase. Pyruvate kinase, NAPD-dependent GAPDH, aldolase, and phosphoglycerate kinase were identified as new proteins (Severin *et al*., 2007).

A number of microbial species compete for colonization and space on mucosal surfaces and moonlighting proteins function in competition for niche. *S. aureus* was discovered to kill *Cryptococcus neoformans,* which is an encapsulated yeast that causes fatal meningitis. The staphylococcal protein interacting with the fungal carbohydrate was identified as triosephosphate isomerase (Furuya & Ikeda, 2009, Ikeda *et al*., 2007, Yamaguchi *et al*., 2010). Streptococci are primary colonizers of oral cavity and dental surfaces. Proteins capable of binding to salivary mucin were analyzed in *S. gordonii*, and pyruvate kinase and oligopeptide-binding protein were found to bind mucin (Kesimer *et al*., 2009). Listeria adhesion protein (LAP) has been described as a key adhesin with affinity for intestinal epithelial cells (Santiago *et al*., 1999). LAP was later identified as an alcohol acetaldehyde dehydrogenase (Kim *et al*., 2006).

2.5 NONGLYCOLYTIC MOONLIGHTING ENZYMES

Glutamine synthetase (GS) is an enzyme that plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine. GS of *Mycobacterium tuberculosis* binds plasminogen effiently. In addition, GS binds fibronectin and enhances plasminogen activation. Also S-adenosyl-L-homocysteine hydrolase, dihydrolipoamide dehydrogenase, isocitrate lyase and aldolase were among the plasminogen binding proteins in *M. tuberculosis* (Xolalpa *et al*., 2007).

Malate synthase enzyme belongs to the family of transferases and participates in pyruvate metabolism as well as glyoxylate and dicarboxylate metabolism. This enzyme was found on *M. tuberculosis* cell surface and to bind laminin and fibronectin (Kinhikar *et al*., 2006).

Glutamate racemase (Murl), which generates D-glutamate for peptidoglycan synthesis, functions also as an inhibitor of DNA gyrase in *M. tuberculosis* and *Mycobacterium smegmatis* (Sengupta *et al*., 2008). The phosphodiesterase of *M. tuberculosis,* that was capable of degrading mycobacterial cAMP, functions also as a moonlighting protein that controls cell wall permeability to hydrophobic cytotoxic compounds (Podobnik *et al*., 2009). The aconitase of *M. tuberculosis* has been found to function as an iron-responsive protein involved in iron homeostasis (Banerjee *et al*., 2007). Superoxide dismutase of *M. avinum* has been reported as an adhesin (Reddy & Suleman, 2004).

2.6 MOLECULAR CHAPERONES

Molecular chaperones are a major class of bacterial moonlighting proteins. The bacterial Hsp70 protein, DnaK, was first identified in the cell wall proteome of *L. monocytogens* (Schaumburg *et al*., 2004). The plasminogen binding property of DnaK was reported in *N. meningitidis* (Knaust *et al*., 2007) and in *M. tuberculosis* (Xolalpa *et al*., 2007). Other moonlighting functions described for DnaK of *M. tuberculosis* are the stimulation of chemokine production from CD8 lymphocytes (Lehner *et al*., 2000) and monocytes, stimulation of dendritic cell maturation by binding to the CD40 receptor (Wang *et al*., 2001), as well as the binding to HIV co-receptor CCR5 and competition with HIV for CCR5 (Babaahmady *et al*., 2007, Floto *et al*., 2006)

The genome of *M. tuberculosis* contains two genes encoding chaperonin GroEL that express immunomodulatory functions (Fleischmann *et al*., 2002). Both GroEL proteins of *M. tuberculosis* are released into extracellular space (Cehovin *et al*., 2010) and GroEL2 has been found also on cell surface (Hickey *et al*., 2009). GroEL2 was found to be involved in *M. tuberculosis* adhesion to macrophages (Hickey *et al*., 2009) and CD43 was later determined to be a receptor for GroEL2 (Hickey *et al*., 2010). Both GroEL proteins have been reported to stimulate cytokine release from monocytes (Friedland *et al*., 1993, Lewthwaite *et al*., 2001), and GroEL2 induced synthesis of tumor necrosis factor alpha in human monocytes (Cehovin *et al*., 2010).

GroEL proteins seem to have great impact on virulence of *M. tuberculosis.* GroEL2 was described as essential for survival of the bacterium, and GroEL1 has an important role in stimulating proinflammatory cytokine production needed for granulomatous response and establishment of the tuberculosis disease (Hu *et al*., 2008).

To function properly as a chaperonin, GroEL requires the co-chaperonin protein complex GroES. GroES of *M. tuberculosis* has been suggested to be an important virulence factor. GroES is secreted and it promotes the recuirement of osteoclasts. This is tought to account for the pathological features of spinal tuberculosis (Meghji *et al*., 1997). GroES of *M. tuberculosis* was also found to bind plasminogen. GroES, DnaK and elongation factor Tu (EF-Tu) proteins contain C-terminal lysines which could mediate the interaction with plasminogen (Xolalpa *et al*., 2007).

2.7 OTHER PROTEINS

Elongation factors are a set of proteins that facilitate the events of translational elongation. EF-Tu mediates the entry of the aminoacyl tRNA into a free site in the ribosome. Elongation factor Ts (EF-Ts) serves as the guanine nucleotide exchange factor for EF-Tu, catalyzing the release of GDP from EF-Tu. Elongation factor G (EF-G) catalyzes the translocation of the tRNA and mRNA down the ribosome at the end of each round of polypeptide elongation. Extracellular EF-Tu was described as plasminogen binding protein of *L. monocytogenes* and *M. tuberculosis* (Schaumburg *et al*., 2004, Xolalpa *et al*., 2007). EF-Tu and EF-G were found to mediate binding of *S. gordonii* to salivary mucin. SecA, an ATP-binding protein in the bacterial translocase pathway also binds mucin (Kesimer *et al*., 2009).

HPr is a component of the phosphoenolpyruvate-dependent sugar phosphotransferase system and an abundant surface-associated protein in *S. oralis* cells that are cultured at pH 7 or at pH 5.2. Other putative moonlighting proteins could include adenylate kinase, ribosome recycling factor, ribosomal proteins S6 and L7/L12, transcription elongation factor GreA, a lipoprotein, the electron transport protein thioredoxin, and the glycolytic enzymes phosphoglucomutase, phosphoglycerate kinase, enolase, fructose bisphosphate aldolase, and triosephosphate isomerase. Change in pH did not affect the expression of these proteins but EF-G, EF-Tu, EF-Ts, EF-P, and GAPDH were down-regulated at acidic pH (Wilkins *et al*., 2003). Moonlighting functions of these proteins or their significance to bacterial survival or pathogenicity has not been analyzed further.

2.8 MOONLIGHTING PROTEINS IN COMMENSAL BACTERIA

Several anchorless multifunctional proteins described above are virulence factors of Gram-positive pathogens. Homologs have been also identified either cell-surface-associated and/or in extracellular space of commensal bacteria including lactobacilli. Their number and moonlighting functions have significantly increased during the last five years (Table 4). *L. plantarum* efficiently adheres to colonic mucin (Kinoshita *et al.*, 2007), and the adhesion drastically decreased after washing. GAPDH extracted from washing buffer was identified as the molecule mediating adhesion (Kinoshita *et al.*, 2008b). GAPDH of *L. plantarum* was shown to bind also to A and B blood group antigens which are present in intestinal mucin. The catalytic site in the GAPDH protein was suggested to be involved in the binding to colonic mucin since the binding was inhibited by NAD (Kinoshita *et al.*, 2008a). Extracellular GAPDH of *L. plantarum* also binds fibronectin (Sanchez *et al.*, 2009a). GAPDH and phosphoglycerate kinase were found to be secreted to culture media by *L. rhamnosus* GG. However, neither of the proteins bound mucin or fibronection (Sanchez *et al.*, 2009b).

Cell surface GAPDH as well as EF-Tu and triosephosphate isomerase mediate adhesion of *L. plantarum* to intestinal epithelial cells. Removal of surface proteins by guanidine-HCl decreased adhesivness (Ramiah *et al.*, 2008). EF-Tu has been found also on the surface of *L. johnsonii* La1 (NCC533). Recombinant EF-Tu bound to mucin and human intestinal epithelial cells. The binding was shown to be more efficient at pH 5 than at pH 7.2. GroEL was also identified on the cell surface and in the culture medium of *L. johnsonii* La1. This protein binds to mucin and human epithelial cells at acidic pH. Recombinant GroEL was found to stimulate interleukin-8 secretion in macrophages and to aggregate cells of the gastric pathogen *Helicobacter pylori* (Bergonzelli *et al.*, 2006).

Bifidobacteria are important health-promoting bacterial group in the human intestinal microbiota. Information about the specific mechanisms of interaction with the host is limited. A function possibly involved in the *Bifidobacterium*-host interaction is plasminogen binding (Candela *et al.*, 2007, Candela *et al.*, 2008a). *Bifidobacterium animalis* subsp. *lactis* was shown to recruite plasminogen to the cell surface, where it can be converted to plasmin by host-derived plasminogen activators. With a surface-associated plasmin, *B. animalis* subsp. *lactis* could degrade physiological substrates such as extracellular matrix components, fibronectin and fibrinogen (Candela *et al.*, 2008b). *B. animalis* subsp. *lactis* was also shown to recruit plasminogen to the cell surface from human fecal extracts (Candela *et al.*, 2011). The ability of bifidobacteria to intervene with the host plasminogen/plasmin system may have a role in bifidobacterial colonization of the host gastrointestinal tract (Candela *et al.*, 2008b). The putative plasminogen binding proteins of *B. animalis* subsp. *lactis* include enolase, DnaK, GS, bile salt hydrolase and phosphoglycerate mutase (Candela *et al.*, 2007). Enolase was reported as a surface-localized plasminogen binding protein also in *Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium breve* (Candela *et al.*, 2009). The presence of bile salts in the growth medium increased the amounts of enolase and DnaK as well as the ability of bacteria to interact with the host plasminogen system (Candela *et al.*, 2010). The mechanisms behind the increase in bile salts were not analyzed.

The genome of *L. plantarum* contains two genes for enolase, and enolase 1 was identified as fibronectin binding protein (Castaldo et al., 2009). The cell surface enolase of a vaginal bacterium, *L. jensenii* is a potent inhibitor of the adherence of *Neisseria gonorrhoeae* to epithelial cells (Spurbeck & Arvidson, 2010).

Table 4. Moonlighting proteins in commensal bacteria

Moonlighting protein	Species	Reference
Aldolase	*Lactococcus lactis*	Katakura *et al.*, 2010
Bile salt hydrolase	*Bifidobacterium animalis* subspecies *lactis*	Candela *et al.*, 2007
Cysteine synthase	*Lactococcus lactis*	Katakura *et al.*, 2010
D-alanyl transfer protein	*Lactobacillus plantarum*	Castaldo *et al.*, 2009
DnaK	*Bifidobacterium animalis* subspecies *lactis*	Candela *et al.*,2010
	Lactococcus lactis	Katakura *et al.*, 2010
EF-Ts	*Lactococcus lactis*	Katakura *et al.*, 2010
EF-Tu	*Lactobacillus johnsonii*	Granato *et al.*, 2004
	Lactobacillus plantarum	Ramiah *et al.*, 2008
enolase	*Bacteroides fragilis*	Sijbrandi *et al.*, 2005
	Bifidobacterium bifidum	Candela *et al.*, 2007
	Bifidobacterium breve	Candela *et al.*, 2007
	Bifidobacterium animalis subspecies *lactis*	Candela *et al.* 2007
	Bifidobacterium longum	Candela *et al.*, 2007
	Lactobacillus jensenii	Spurbeck & Arvidson, 2010
	Lactobacillus plantarum	Castaldo *et al.*, 2009
	Leuconostoc mesenteroides	Lee *et al.*, 2006
Fructose-6-phosphate phosphoketolase	*Bifidobacterium bifidum*	Sanchez *et al.*, 2004
GAPDH	*Lactobacillus plantarum*	Kinoshita*et al.*, 2008a, Kinoshita *et al.*, 2008b, Sanchez *et al.*, 2009a
	Lactococcus lactis	Katakura *et al.*, 2010
Glutamine synthetase	*Bifidobacterium animalis* subspecies *lactis*	Candela *et al.*, 2007
Glutamyl-tRNA synthetase	*Lactococcus lactis*	Katakura *et al.*, 2010
Glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	*Lactobacillus plantarum*	Castaldo *et al.*, 2009

Moonlighting protein	**Species**	**Reference**
GroEL	*Lactobacillus johnsonii*	Bergonzelli *et al*., 2006
	Lactococcus lactis	Katakura *et al*., 2010
GrpE	*Lactococcus lactis*	Katakura *et al*., 2010
GTP cyclohydrolase II	*Lactobacillus plantarum*	Castaldo *et al*., 2009
Malate oxidoreductase	*Lactococcus lactis*	Katakura *et al*., 2010
Mannose-6-phosphate isomerase	*Lactococcus lactis*	Katakura *et al*., 2010
Oligopeptide ABC transporter, ATP-binding protein	*Lactobacillus plantarum*	Castaldo *et al*., 2009
Phosphoglycerate kinase	*Lactococcus lactis*	Katakura *et al*., 2010
Pyruvate dehydrogenase complex, E1 component, alfa subunit	*Lactobacillus plantarum*	Castaldo *et al*., 2009
Pyruvate kinase	*Lactococcus lactis*	Katakura *et al*., 2010
6-phosphofructokinase	*Lactococcus lactis*	Katakura *et al*., 2010
Phosphoglycerate kinase	*Lactobacillus rhamnosus*	Sanchez *et al*., 2009b
Phosphoglycerate mutase	*Bifidobacterium animalis* subspecies *lactis*	Candela *et al*., 2007
	Lactococcus lactis	Katakura *et al*., 2010
30S ribosomal protein S1	*Lactococcus lactis*	Katakura *et al*., 2010
Triosephosphate isomerase	*Lactobacillus plantarum*	Ramiah *et al*., 2008

3 SECRETION AND CELL WALL ANCHORING OF LACTOBACILLAR SURFACE PROTEINS

3.1 THE CELL WALL AND ANCHORING OF SURFACE PROTEINS

The cell wall of Gram-positive bacteria has a thick peptidoglycan (PG), which provides a physical barrier against the environment and an attachment site for other cell wall polymers (Navarre & Schneewind, 1999). The glycan strands consist of repeating disaccharide *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine but in lactobacilli small variations in the composition and modifications of peptidoglycan have been found (Kleerebezem *et al.*, 2010). The cell wall of Gram-positive bacteria contains also teichoid acids, which are anionic polymers covalently attached to the peptidoglycan or associated with the cytoplasmic membrane. It contains also teichuronic acid and other neutral or acidic polysaccharides (Navarre & Schneewind, 1999, Schaffer & Messner, 2005).

Several lactobacillar proteins are predicted to be anchored to the cell by single N- or C-terminal transmembrane anchors. N-terminally anchored proteins constitute the largest group of membrane-anchored proteins whereas variable numbers of C-terminally anchored proteins are predicted in the published genomic sequences of *Lactobacillus* (Kleerebezem *et al.*, 2010). Sortases are often used to anchor proteins covalently to peptidoglycan. The proteins anchored sortase-dependently typically contain N-terminal signal sequence, C-terminal LPXTG-motif followed by a C-terminal membrane anchor domain, consisting of a stretch of hydrophobic residues and a positively charged tail (Boekhorst *et al.*, 2006, Leenhouts *et al.*, 1999, Marraffini *et al.*, 2006, Ton-That *et al.*, 2004). In genomes of *Lactobacillus* the number of genes encoding LPXTG-containing proteins varies from 2 to 27 proteins (Kleerebezem *et al.*, 2010).

Lipoproteins are the second largest membrane-anchored group of proteins in predicted *Lactobacillus* exoproteomes (Kleerebezem *et al.*, 2010). These proteins contain cysteine-rich lipobox within the lipoprotein signal sequence. Cysteine in a lipobox is modified by diaglycerol moiety, which binds to the plasma membrane (Desvaux *et al.*, 2006, Sutcliffe & Harrington, 2002).

In addition to covalently bound proteins, lactobacillar proteins are associated with the cell wall by non-covalent interactions. The extracellular proteomes of *Lactobacillus* contain proteins possessing lysin motif (LysM) domain (Kleerebezem *et al.*, 2010). LysM-containing proteins are typically involved in bacterial cell wall metabolism and N-acetylglucosamine is a general constituent of LysM substrates (Buist *et al.*, 2008). The best-characterized LysM-containing protein is AcmA of *Lactococcus lactis* (Buist *et al.*, 1995). The binding of AcmA was shown to be localized in cell septum areas of *L. lactis* cells. The binding was hindered by other cell wall constituents, propably lipoteichoid acids (LTA; Steen *et al.*, 2003).

The mechanisms for anchoring proteins to teichoic acids have been described in Gram-positive bacteria. Choline-binding proteins (CBPs) are well-studied in *S. pneumoniae*. The cholin-binding domains in *S. pneumoniae* CBPs are in a stretch of 20 amino acids that include multiple conserved tandem repeats in their C-termini (Bergmann & Hammerschmidt, 2006). Choline-binding domains have been found in *L. plantarum* WCFS1 (Kleerebezem *et al.*, 2003) as well as in *Lactobacillus fermentum* IFO3956, *Lactobacillus reuteri* DSM20016 and *Lactobacillus salivarius* UCC118 (Kleerebezem *et al.*, 2010). Also

GW-motifs anchor proteins to LTA (Jonquières *et al*., 1999). The GW-motif was first identified in InlB of *L. monocytogenes* (Braun *et al*., 1997) but proteins are anchored by GW motifs also in *L. reuteri* ATCC55730 (Bateman & Bycroft, 2000). Interestingly, GW domains of InlB resemble eukaryotic SH3 domains (Marino *et al*., 2002).

The C-terminal cell wall binding domain WxL was first identified in *Lactobacillus* based on *in silico* analysis (Boekhorst *et al*., 2006, Chaillou *et al*., 2005, Kleerebezem *et al*., 2003). This domain has been shown to be responsible for non-covalent interactions of internalin-like proteins and the bacterial cell wall in *Enterococcus faecalis* (Brinster *et al*., 2007).

3.2 PROTEIN SECRETION MECHANISMS IN LACTOBACILLI

The secretion mechanisms characterized in Gram-positive bacteria include the Sec-pathway, twin-arginine translocation (Tat), flagella export apparatus, fimbrillin-protein exporter (FPE), holin (pore forming), peptide-efflux ABC and Wss (WXG100 secretion system) pathways (Desvaux *et al*., 2009, Kleerebezem *et al*., 2010). The secretion mechanisms of *Lactobacillus* are not characterized and most of the knowledge in this area is based on genomic predictions. By applying sequence homology and protein-domain searches, the presence of components of different secretion pathways was evaluated in genomes of *L. plantarum* WCFS1, *L. johnsonii* NCC 533, *L. acidophilus* NCFM, *L. gasseri* ATCC33323, *L. fermentum* IFO3956, *L. delbrueckii* subsp. *bulgaricus* ATCC11842 and ATCC BAA-365, *L. salivarius* UCC118, *L. reuteri* DSM20016, *Lactobacillus helveticus* DPC4571 *Lactobacillus brevis* ATCC 367 *Lactobecillus sakei* 23K, *Lactobacillus casei* ATCC 334. These species are deficient in factors involved in the Tat-translocation, the flagella export apparatus, or theWss pathway, wheras genes encoding the Sec, FPE, peptide-efflux ABC and holin systems were found to be present (Kleerebezem *et al*., 2010).

The Sec translocase is the main system that mediates protein transfer across the cytoplasmic membrane in bacteria. The system consist of membrane-embedded highly conserved protein-conduction channel (SecYEG) and a component, which can be either an ATP-driven motor protein Sec A or a translating ribosome, that delivers energy for the transport process (Driessen & Nouwen, 2008). SecA was found in the all 13 LAB genomes described above (Kleerebezem *et al*., 2010).

Holins are small integral membrane proteins frequently coded by bacteriophages and are involved in the secretion of muralytic enzymes (Wang *et al*., 2000). Holins have also been found in genomes of *Lactobacillus* as part of the cell lysis system (Kleerebezem *et al*., 2010).

The FPE pathway allows exogenous DNA uptake across the bacterial cytoplasmic membrane. It has been proposed that a pseudopilus participates in the transport of DNA during transformation. In *B. subtilis*, the FPE system consists of *comG* genes (*comGA-GG*) and a genetically unlinked *comC* gene (Chen & Dubnau, 2004). These genes are present in several *Lactobacillus* genomes suggesting that the major components of the FPE pathway are present at least in some lactobacilli (Kleerebezem *et al*., 2010).

Peptide efflux transporters that are predominantly involved in export of antimicrobial peptides have been described to be responsible for bacteriocin secretion at least in *L. acidophilus* (Dobson *et al*., 2007) and *L. plantarum* (Diep *et al*., 1996). Genes encoding predicted bacteriocins are found to be genetically linked with genes encoding ABC exporters in genomes of several *Lactobacillus* species suggesting that peptide transport via ABC exporters is common in lactobacilli.

3.2.1 HYPOTHETIZED MECHANISMS FOR SECRETION OF MOONLIGHTING PROTEINS

The number of moonlighting proteins in Gram-positive bacteria is rapidly growing. Currently there is no identified mechanism for their secretion. These proteins lack signal sequences or cell wall anchoring motives. Several of these proteins are essential for growth so knock-out mutants are not achievable. Boël *et al.* (2005) used an insertion mutagenesis strategy. A nucleotide sequence encoding a hydrophobic tail was added to the C-terminus of GAPDH of *S. pyogenes.* In this mutant strain GAPDH was not secreated but retained in cytoplasm and the strain was used to study the role of GAPDH in pathogenesis of *S. pyogenes* (Boël *et al.*, 2005).

Interestingly, *E. coli* has two forms of the GAPDH protein with different isoelectric points. Only more basic form seems to be exported out of the cells (Egea *et al.*, 2007). *Listeria* was reported to have a *secA2* gene which is involved in secretion of several peptides including enolase (Lenz *et al.*, 2003). It has also been suggested that automodification of enolase by its substrate 2-PGE is associated with its secretion. Enolase was shown to bind 2-PGE via lysine 341, which is located at the active site of the enzyme. Mutation in this reidue in the enolase of *E. coli* prevented its export (Boël *et al.*, 2004). A heat shock-induced serine protease, HtrA, is needed for maintaining protein homeostasis in extracytosolic compartments. HtrA has been proposed to be involved in secretion of moonlighting metabolic enzymes. Deletion of *htrA* gene in *S. mutans* increased surface expression of enolase and GAPDH (Biswas & Biswas, 2005). In addition, enolase and other moonlightning proteins such as GroEL and DnaK, have been suggested to be secreted from *B. subtilis* by an unknown mechanism. This was inferred from the finding that deletion of an internal hydrophopic α-helical domain blocked the export (Yang *et al.*, 2011). On the other hand, some results also favor more passive leakage to cell exterior. Double labeling of bacterial cells with anti-GAPDH antibodies and propidium iodide suggested that increased plasma membrane permeability is critical for the suface location of GAPDH (Saad *et al.*, 2009). Several hypothesis have been presented about secretion of moonlighting proteins. Moonlighting proteins are very variable and it seems that none of the specific secretion mechanisms will fit all moonlighting proteins described so far.

4 AIMS OF THE STUDY

When this study was initiated, cell surface associated glycolytic moonlighting enzymes had been described in a few pathogenic Gram-positive bacteria. Little was known about their functions or about the mechanisms of surface association and secretion. The first described bacterial moonlighting enzymes were enolase and GAPDH of group A *Streptococcus*, and their multifunctional nature as adhesins as well as plasminogen receptors had been characterized and found to be involved in streptococcal virulence. Activation of plasminogen is an established virulence function in several highly invasive bacterial infections. To our surprise, extracellular proteins from the commensal *L. crispatus* strain ST1 exhibited an exceptionally high advancement of plasminogen activation by tPA. This was the basic observation in my thesis work, which aimed at characterizing the phenomenon as well as the extracellular proteins released from *L. crispatus* cell surface.

The goals of this thesis work were:

- to identify plasminogen binding proteins and other moonlighting proteins released from *L. crispatus* ST1 into the buffer extract
- to characterize mechanisms of the cell-wall anchoring and conditions of release of these proteins
- to identify moonlighting functions of these proteins
- to compare moonlighting proteins from commensal lactobacilli to corresponding proteins from Gram-positive pathogens

5 MATERIALS AND METHODS

Table 5. Bacterial strains and plasmids used in this thesis study

Bactrial strain/ plasmid	Origin/ relevant property	Article	Reference
Bacterial strain			
Lactobacillus acidophilus E507	dairy	I, II	Miettinen *et al*., 1996
Lactobacillus amylovorus JCM5807	pig intestine	I, II	Mitsuoka, 1969, JCM
Lactobacillus crispatus ST1	chicken feces	I, II, III, IV	Edelman *et al*., 2002
Lactobacillus gallinarum T-50	chicken feces	I, II	Fujisawa *et al*., 1992, JCM
Lactobacillus gasseri JCM 1130/ ATCC19992	human feces	I, II	Lerche & Reuter, 1962, JCM, ATCC
Lactobacillus johnsonii F133	calf feces	I, II, III	Fujisawa *et al*., 1992, JCM
Lactobacillus rhamnosus GG (ATCC 53103)	human feces	I, IV	Kankainen *et al*., 2009
Lactobacillus paracasei E506	dairy	I	Miettinen *et al*., 1996
Lactococcus lactis subsp. *cremoris* E523	fermented dairy	I	Miettinen *et al*., 1996
Streptococcus pneumoniae TIGR4	human	III	Tettelin *et al*., 2001
Streptococcus pyogenes serotype T1 IH32030	human	III	Miettinen *et al*., 1998
Staphylococcus aureus 8325-4	human	III	Novick., 1967
Escherichia coli M15(pREP4)	host for pQE-30 vector	I, III, IV	Qiagen®
Plasmids			
pQE-30	His_6-tag expression vector	I, III, IV	Qiagen®

ATCC, American Type Culture Collection
JCM, Japan Collection of Microorganisms

Table 6. Methods used in this thesis study

Method	Article
Genetic methods	
Isolation of chromosomal DNA	I, III, IV
Cloning to pQE-30 vector	I, III, IV
Isolation of mRNA	II, III
RT-PCR	II, III
Southern hybridization	III
DNA sequencing	I, III, IV
Adhesion assays	
Biacore analysis of adherence to ECM proteins	IV
Binding of ^{125}I-labelled plasminogen, plasmin and tPA	I
Binding of peptides to glycoproteins by ELISA	III
Adherence of intact cells to immobilized Matrigel	IV
Binding of peptides to cell surfaces and cell wall material	II, IV
Interaction of proteins with LTA	I
Immunological methods	
Immunoelectron microscopy	I
Indirect immunofluorescence	II, IV
Western blotting	I, II, IV
Protein assays	
Expression and purification of His-peptides	I, II, III, IV
GAPDH enzyme activity measurement	I
GPI enzyme activity measurements	IV

Protein assays	
Extraction of cell surface components	I, II, IV
Enolase enzyme activity measurement	I, III
Enhancement of plasminogen activation	I, III, IV
Plasminogen binding	I, II, III, IV
Plasmin and tPA binding	I
SDS-PAGE	I, II, III, IV
Time-resolved fluorometry	I, IV

Others	
Propidium iodide staining of bacteria	IV
Viable counting	IV

6 RESULTS AND DISCUSSION

6.1 IDENTIFICATION OF ENOLASE, GAPDH, GS AND GPI IN BUFFER EXTRACT AND CELL WALL OF L. CRISPATUS (I, IV)

When this study was initiated, characterization of lactobacillar adhesion molecules was ongoing in our laboratory, and the strain ST1 of *L. crispatus* had been isolated from the crop of newborn chicken and shown to adhere to epithelial tissues (Edelman *et al.*, 2002). Also, during this study the genomic sequence of *L. crispatus* ST1 (Ojala *et al.*, 2010) became available and could be utilized in gene identification and cloning. The search for *L. crispatus* ST1 plasminogen-binding proteins and adhesins was initiated by treating bacterial cells with mutanolysin, a peptidoglycan-cleaving autolysin, which has commonly been used in detachment of adhesive sortase-dependent proteins from the surface of Gram-positive bacteria (Yokogawa *et al.*, 1974). Mutanolysin treatment indeed abolished the adhesion of *L. crispatus* ST1 to tissue sections of chicken intestinal tract (data not shown). It became evident later in this work that the moonlighting proteins studied in this thesis are not covalently bound to peptidoglycan. They were released from cell surface by the Tris-HCl (pH 6.8) buffer used in the mutanolysin treatment. We first fractionated the mutanolysin-released surface proteins by gel filtration and ionic exchange chromatography. Subsequent purifications were performed with proteins released with PBS or Tris-HCl buffer. We analyzed peptide contents of the fractions by SDS-PAGE, and selected peptides were subjected to N-terminal sequencing for identification.

We first analyzed two major peptides that were released from *L. crispatus* ST1 cells into PBS (Fig. IA of I). A peptide of 38 kDa in apparent molecular mass contained N-terminal sequence TVKIGINGFGRIGRLAFRRI. This sequnece has 85-100 % identity with GAPDH N-terminal sequences from *L. plantarum* (Kleerebezem *et al.*, 2003), *L. johnsonii* (Pridmore *et al.*, 2004) and *L. lactis* (Bolotin *et al.*, 2001). The N-terminal sequence is identical to the predicted GAPDH translated from the genomic sequence of *L. crispatus* ST1, and the peptide reacted with the antiserum raised against the His_6-GAPDH of *L. crispatus* ST1 (see below).

Enolase is another glycolytic enzyme characterized on the surface of pathogenic streptococci (Bergmann *et al.*, 2001, Esgleas *et al.*, 2008, Ge *et al.*, 2004, Hughes *et al.*, 2002, Kesimer *et al.*, 2009, Pancholi & Fischetti, 1998). Peptide of 47 kDa in apparent size in the buffer extract from *L. crispatus* ST1 (Fig. 1A of I) reacted with an antiserum raised against *S. pneumoniae* enolase (Bergmann *et al.*, 2001). We thus concluded that the 38 kDa peptide was GAPDH and the 47 kDa peptide was enolase of *L. crispatus* ST1.

Fractionation of the buffer gave two peptides with apparent molecular masses of about 50 kDa (Fig. 1A of IV). Gel filtration, anion or cation chromatography did not separate the peptides. N-terminal sequencing of the peptides gave the sequences RCQYTAEEIKQEV(G/N)D(R/D)KV(T/V)RF and SLIKFDSSKLTPFVHENLS. By comparing these sequences with the genome sequence of *L.* crispatus ST1 (Ojala *et al.*, 2010), the former was identified as glutamine synthetase (GS) and the latter as glucose-6-phosphate isomerase (GPI). The predicted masses of these proteins were 50 kDa for GS and 49.5 kDa for GPI.

To confirm the presence of enolase, GAPDH, GS and GPI in the "extracellular buffersome" of *L. crispatus* ST1, their enzymatic activities were assessed. The incubation buffer exhibited enolase, GAPDH and

GPI activities but GS activity was not detected (data not shown). The absence of GS activity might be due to low-level of secreted proteins or insensitivity of the activity assay.

After identification, the genes encoding each protein were cloned and the amplicons were ligated into pQE30 vector for expression and isolation as N-terminal His_6-fusion proteins. The genes encoding *L. crispatus* ST1 enolase and GAPDH were first amplified from the chromosome by using primers designed according to the sequences of the corresponding genes in *L. johnsonii* (Pridmore *et al.*, 2004), *L. plantarum* (Kleerebezem *et al.*, 2003) and *L. lactis* (Bolotin *et al.*, 2001) and the 5´and 3´termini of the genes were sequenced by direct chromosomal walking. The primers for amplification of GS encoding gene *glnA* and GPI encoding gene *pgi* were based on their sequence in the genome of *L. crispatus* ST1 (Ojala *et al.*, 2010). The antisera against the His_6-proteins were produced and IgGs were separated from hyperimmune and preimmune sera by using Protein A affinity chromatography.

We used immunoelectron microscopy (IEM) to demonstrate enolase and GAPDH on the surface of *L. crispatus* ST1. In the postcutting IEM detection using IgGs against enolase and GAPDH the proteins were detected on bacterial cell surface as well as in the cytoplasm (Fig. 1C of I). The IEM procedures are routinely done at neutral pH, which was later found to detach proteins from ST1 cell surface (article II). Therefore, the IEM shown in Fig. 1C of I most likely is an underrated view of the occurrence of these proteins in the cell wall.

By indirect immunofluorescence enolase, GAPDH, GS and GPI were detected on the *L. crispatus* ST1 cell surface at acidic pH, but the cells incubated at pH 8 showed only weak fluorescence (Fig. 1A of II, Fig. 2A of IV). In contrast, the S-layer protein of *L. crispatus* ST1 was detected cell-surface bound at both pHs (Fig. 1A of II). We observed that at pH 4 the cells which were strongly stained with the antibodies shared an appearance of damaged or dying cells. We therefore doublestained cells with IgGs produced against His_6-enolase, His_6-GAPDH, His_6-GS and His_6-GPI and propidium iodide (PI; Fig. 2 of IV). PI penetrates the cell membranes of cells with increased permeablity and stains the cell by binding to DNA. The general view was that those bacterial cells which were strongly stained by PI were also positive for the IgGs i. e. the IgG molecules stained a cell subpopulation that was permeabilized. This was observed for all four moonlighting proteins of *L. crispatus* ST1. Most reactive pH-4 cells were uniformly stained by IgGs, indicating that the binding was not exclusively to the cell surface. In a small fraction, i. e. below 1 % of the cell population, staining for enolase, GAPDH, GS and GPI were not distributed evenly around the cells but rather seemed to be concentrated to cell poles (Fig. 2B of IV). Also, Western blotting with the antisera against enolase, GAPDH, GS and GPI *L. crispatus* ST1 cells from low-pH buffer indicated the presence of these proteins on cell surface (Fig. 1B of II and Fig. 1B of IV).

Our results described above demonstrate that these traditionally cytoplasmic proteins enolase, GAPDH, GS and GPI are on the cell surface as well as in the cytoplasm of *L. crispatus* ST1. The extracellular location of enolase and GAPDH has been reported on surface of several Gram-positive pathogens (Bergmann *et al.*, 2001, Bergmann *et al.*, 2004, Bermudez *et al.*, 1996, Brassard *et al.*, 2004, Carreté *et al.*, 2005, Feng *et al.*, 2009, Gase *et al.*, 1996, Ge *et al.*, 2004, Holland *et al.*, 2010, Itzek *et al.*, 2010, Kesimer *et al.*, 2009, Lamonica *et al.*, 2005, Maeda *et al.*, 2004, Modun & Williams, 1999, Mölkänen *et al.*, 2002, Nelson *et al.*, 2001, Pancholi & Fischetti, 1998, Schaumburg *et al.*, 2004, Seifert *et al.*, 2003) where they have been described as potential virulence factors that function e. g. as plasminogen and/or plasmin receptors on the bacteria (Agarwal *et al.*, 2008, Bergmann *et al.*, 2001, Bergmann *et al.*, 2004, Gase *et al.*, 1996, Ge *et al.*, 2004, Jones & Holt, 2007, Matta *et al.*, 2010, Pancholi & Fischetti,

1998, Schaumburg *et al*., 2004, Seifert *et al*., 2003,Winram & Lottenberg, 1996) or adhesins (Brassard *et al*., 2004, Carneiro *et al*., 2004, Esgleas *et al*., 2008, Feng *et al*., 2009, Ge *et al*., 2004, Jin *et al*., 2005, Kesimer *et al*., 2009, Maeda *et al*., 2004, Modun & Williams, 1999, Pancholi & Fischetti, 1992, Seifert *et al*., 2003). Thus, there are plenty of reports describing surface localization of enolase and GAPDH, whereas reports on GS and GPI are few.The surface localization of GS in *S. agalactiae* has been hypotetized since antisera against streptococcal GS recombinant protein recognized a peptide in the mutanolysin extract from Group B streptococci (Suvorov *et al*., 1997). Extracellularly associated GS has been identified in *M. tuberculosis* and (Xolalpa *et al*., 2007) *B. animalis* subsp. *lactis* (Candela *et al*., 2007). The GPI has been detected in the culture supernatants of *S. agalactiae* (Hughes *et al*., 2002) but its possible association to the cell wall has not been analyzed earlier. Enolase and GAPDH are major peptides in extracellular buffersome of *L. crispatus* ST1 as well as in several other members of *Lactobacillus* (Fig. 5C of I).

6.1.1 PROPERTIES OF ENOLASE, GAPDH, GS AND GPI (I, II, III, IV)

S. pneumoniae, S. pyogenes and *S. aureus* possess only one gene encoding enolase (Ferretti *et al*., 2001, Iandolo, 2000, Tettelin *et al*., 2001). The number of enolase genes varies in the genus *Lactobacillus.* Three enolase genes (*eno 1-3)* are present in *L. johnsonii* NCC533 genome (Pridmore *et al*., 2004) and two in *L. plantarum* (Kleerebezem *et al*., 2003) and *Lactobacillus kefiranofaciens* ZW3 (Wang *et al*., 2011) genome. The genome of *L. gasseri* contains two or three *eno* genes depending on strain (Makarova *et al*., 2006). The *L. crispatus* ST1 genome contains one *eno* gene as well as one gene for GAPDH (Ojala *et al*., 2010). The GAPDH encoding gene exists usually as one copy both in *Streptococcus* and *Lactobacillus.* The number of genes encoding GS and GPI is also variable in lactobacilli. The genome of *L. casei* (Makarova *et al*., 2006) as well as *L. rhamnosus* GG (Kankainen *et al*., 2009) contain two copies GS encoding genes and the genome of *L. fermentum* (Morita *et al*., 2008) contains two genes for GPI. However, *L. crispatus* ST1 genome contains only one gene for GS and GPI (Ojala *et al*., 2010).

Mutagenesis of genes encoding enolase is often difficult since enolase is essential for bacterial growth. However, the presence of two eno genes (*enoA1* and *enoA2*) expressed under standard growth conditions has allowed the isolation of *L. plantarum* strain carrying a mutation in the one of the *eno* gene. The moonlighting functions were shown to be performed by only the enolase A1 (Castaldo *et al*., 2009). The *enoA1* gene belongs to the so-called central glycolytic genes operon (*cggR*) (Kleerebezem *et al*., 2003). Similarly, one of the *eno* genes of *L. johnsonii* NCC533 is linked to the genes encoding for glycolytic enzymes in the chromosome while the two other *eno* genes are located close to genes encoing for hypothetical proteins and prophage proteins (Pridmore *et al*., 2004). The gene encoding for *L. crispatus* ST1 enolase belongs to *cggR*.

To compare properties of the enolases from Gram-positive pathogens to those in lactobacilli, we cloned genes encoding enolases from *L. johnsonii* F133, *S. pneumoniae* TIGR4, *S. pyogenes* IH32030 and *S. aureus* 8325-4 using available genomic DNA sequences (Ferretti *et al*., 2001, Iandolo, 2000, Pridmore *et al*., 2004, Tettelin *et al*., 2001). The amplified *eno* genes from strains *L. johnsonii* F133 and *S. pyogenes* IH32030 were sequenced in this study. The genes encoding *L. rhamnosus* GG enolase, GAPDH, GS and GPI were cloned utilizing the whole genome sequence of *L. rhamnosus* GG (Kankainen *et al*., 2009). The amino acid sequences of enolase, GAPDH, GS and GPI of *L. rhamnosus* GG, *L. johnsonii, S. pyogenes, S. pneumoniae* and *S. aureus* compared to those of *L. crispatus* ST1 (Table 7.). The functional comparison of the enzymes will be described in Chapter 6.6.

By comparing amino acid sequences, enolases could be divided into at least two main subfamilies (Fig. 1 of III), one composed of the *L. crispatus* ST1 enolase and enolases 1 and 2 of *L. johnsonii* and the other of the enolase of *S. pneumoniae, S. aureus* and enolase 3 of *L. johnsonii* F133. Enolase of *L. rhamnosus* GG is most similar to enolase 3 of *L. johnsonii*. *L. crispatus* belongs to DNA homology group A2 in the *acidophilus* group of lactobacilli (Johnson *et al.*, 1980). Similarly, the predicted GAPDH, GS and GPI sequences of *L. crispatus* ST1 are closer to those of *L. johnsonii* than to the other enzymes. This was expected as *L. crispatus* and *L. johnsonii* are evolutionarily related (Johnson *et al.*, 1980). Enolase proteins in lactobacilli are more variable than the GAPDH, GS and GPI proteins.

The four moonlighting proteins of *L. crispatus* ST1, their orthologs from *L. rhamnosus* GG, as well as enolases described above were purified as His_6-recombinant proteins under non-denaturing conditions. All His_6-proteins were enzymaticly active suggesting that the His_6-tag did not disturb the protein folding needed for enzymatic reactions. By comparing enolase proteins in SDS-PAGE gel, the apparent molecular weights of enolases varied from 46.6 kDa to 47.3 kDa (Fig. 2A of III). All His_6-enolases reacted similarly with monoclonal anti-His_6 IgGs indicating that His_6-tag was exposed to protein surface.

Table 7. Predicted sequence identity of *L. crispatus* ST1 proteins with the homologs from *L. rhamnosus* GG, *L. johnsonii* F133/NCC533, *S. pyogenes* M1/IH32030 , *S. pneumoniae* TIGR 4 and *S. aureus* 8325-4.

	L. crispatus ST1 **enolase**
L. rhamnosus GG **enolase**	**51%**
L. johnsonii NCC533/F133 **enolase 1**	**93%**
L. johnsonii NCC533/F133 **enolase 2**	**72%(F133),71%(NCC533)**
L. johnsonii NCC533/F133 **enolase 3**	**54%**
S. pyogenes M1/IH32030 **enolase**	**49%**
S. pneumoniae T4 **enolase**	**49%**
S. aureus 8325-4 **enolase**	**49%**

	L. crispatus ST1 **GAPDH**
L. rhamnosus GG **GAPDH**	**77%**
L. johnsonii NCC533/F133 **GAPDH**	**88%**
S. pyogenes M1/IH32030 **GAPDH**	**56%**
S. pneumoniae T4 **GAPDH**	**57%**
S. aureus 8325-4 **GAPDH**	**58%**

	L. crispatus ST1 **GS**
L. rhamnosus GG **GS1**	**61%**
L. rhamnosus GG **GS2**	**29%**
L. johnsonii NCC533/F133 **GS**	**84%**
S. pyogenes M1/IH32030 **GS**	**57%**
S. pneumoniae T4 **GS**	**57%**
S. aureus 8325-4 **GS**	**55%**

	L. crispatus ST1 **GPI**
L. rhamnosus GG **GPI**	**70%**
L. johnsonii NCC533/F133 **GPI**	**83%**
S. pyogenes M1/IH32030 **GPI**	**63%**
S. pneumoniae T4 **GPI**	**64%**
S. aureus 8325-4 **GPI**	**61%**

We analyzed *L. crispatus* ST1 His_6-enolase by analytical gel-filtration. The size of the eluted protein was 415 kDa. The octameric structure of enolases have been reported in several bacterial species (Brown *et al.*, 1998, Cork *et al.*, 2009, Ehinger *et al.*, 2004, Kaufmann & Bartholmes, 1992, Schurig *et al.*, 1995). For a control, the well-characterized His_6-enolase of *S. pneumoniae* with an octameric structure (Ehinger *et al.*, 2004) was analyzed and gave the same apparent size of 415 kDa. The size of the natural extracellular enolase from *L. crispatus* ST1 was estimated to be 370 kDa. These results suggest the multimeric state of *L. crispatus* ST1 enolase.

We were interested in resolving whether all three *eno* genes in *L. johnsonii* F133 are transcribed. The reverse transcription PCR (RT-PCR) analysis detected transcripts of *eno1* and *eno3* in actively growing cells but no transcription of *eno2* gene was detected (Fig. 2B of III). The *eno 2* could be a pseudogene in *L. johnsonii* F133, or it might be expressed in different growth conditions. Molecular differences in the intra-species variants of enolase are not known, and also here we could not specify whether the observed peptide in the buffersome of *L. johnsonii* represents enolase 1 or 3 or both (Fig. 5B of I).

In Western blotting analysis using specific antibodies against GS1 and GS2 proteins of *L. rhamnosus* GG, only GS 1 was detected in the buffer after incubation in 50 mM Tris-HCl pH 8.0 for 1 to 5 hours (data not shown). We could not detect GS2 in lysed cells either.We also analyzed transcription of *L. rhamnosus* GG genes encoding GS (*glnA)* by RT-PCR using complementary primers to 5´and 3´ ends of both *glnA* open reading frames (ORF). The *glnAI* gene was transcribed in cells from logarithmic and

stationary growth phase whereas no transcription of *glnAII* genes was detected (data not shown). The results indicate that GS2, similarly to *L. johnsonii eno 2*, is not expressed in cells cultivated in MRS to stationary growth phase.

The predicted pIs for *L. crispatus* ST1 proteins are 4.9 for enolase, 5.7 for GAPDH, 5.3 for GS and 4.9 for GPI. The pIs for *L. rhamnosus* GG proteins are 4.8 for enolase, 5.8 for GAPDH, 5.9 for GS1, 5.1 for GS2 and 5.4 for GPI. Similarly to other surface-expressed glycolytic enzymes, the protein sequences of lactobacillar enolases, GAPDH, GS or GPI showed no putative anchoring motifs, membrane-spanning hydrophobic regions or signal sequences. The mechanism of possible secretion of these proteins remains open. The presence of *SecA2* gene was reported in *Listeria* where it is involved in secretion of various peptides including enolase (Lenz *et al.*, 2003). Two *sec* genes are also present in *L. johnsonii* (Pridmore *et al.*, 2004) and *secA2* may be involved in secretion of one or more enolase of the bacterium. However, the genome of *L. crispatus* ST1 contains only one copy of *sec* (Ojala *et al.*, 2010).

6.2 ENOLASE, GAPDH, GS AND GPI ARE RELEASED FROM CELL SURFACE AT NEUTRAL PH AND BY HIGH IONIC STRENGTH (I, II, IV)

Western blotting with specific antibodies against enolase, GAPDH, GS and GPI confirmed that these proteins were present in the extracellular extract of *L. crispatus* ST1 (Fig. 1B of I and Fig. 1B of IV). We first analyzed conditions in which the proteins are released to the buffer. *L. crispatus* ST1 cells were incubated from 0 to 5 hours in PBS or in 50 mM Tris-HCl at pH 8.0. The cells were then separated by centrifugation, and the supernatant was filtered to remove any remaining cells. Enolase and GAPDH were observed in the pH-8 buffer immediately after suspension of *L. crispatus* ST1 cells into the buffer, and their amounts increased considerably only after 24 h (Fig. 1C of II), which probably reflected cell lysis as enolase and GAPDH were released from pH-5 cells. In PBS, with pH 7.1, the release of enolase and GAPDH was somewhat slow and increased until 2-3 hour of incubation. After 5 h incubation, the amount of enolase and GAPDH in PBS was 20-22% of their amounts in corresponding lysed cell samples (Fig. 1B of I). Western bloting of enolase and GAPDH (Fig. 1B of II) and of GS, GPI, enolase and GAPDH (Fig. 1B of IV) showed that these proteins were mostly on cell surface at acidic pH but in the supernatant fraction at pH 7.1 or pH 8. Immunofluorescence staining also showed that pH-5 cells were positive for anti-enolase and anti-GAPDH IgGs, whereas pH-8 cells failed to react (Fig. 1A of II).

The release of enolase and GAPDH from *L. crispatus* ST1 cells became detectable at pH 5.2, i. e. above or close to the pI of the proteins (Fig. 1D of II). Presence of high salt, i. e. choline or sodium chloride, at acidic pH also released enolase and GAPDH into the buffer. This is a further indication that these proteins are bound to the cell surface by ionic interactions. This hypothesis is supported by the finding that surface location of the S-layer protein was not altered by the pH change from 4 to 8 (Fig. 1 of II). Lactobacillar S-layer proteins have pIs ranging from 8 to 10 (Sleytr & Beveridge, 1999) and hence their net charge is not reversed in the pH shift from 4 to 8.

Enolase, GAPDH, GS and GPI were also detected in culture media of *L. crispatus* ST1. When bacteria were inoculated to sterile MRS medium, at pH 6.5, enolase, GAPDH, GS and GPI were detected by Western blotting in the medium (data not shown). When the pH of the medium lowered as a consequence of bacterial growth, these proteins were not detected in the medium, but they were cell bound (data not shown).

We next tranfered over night cultured *L. crispatus* ST1 cells to pH-8 or pH-4 buffer and stained the cell population with PI to determine effect of the transfer to the cell wall permeability. Most cells from pH 4 were nonreactive and appeared healthy under light microscopy, whereas ca. 1-2 % of cells were very strongly stained by PI and appeared by light microscopy translucent and probably dead (Fig. 1C of IV). Instead after transfer to pH 8 the portion of PI-stained cells was higher than at pH 4, albeit the positive cells were less brightly stained by PI. Immediately after the transfer, 17 % of cells were permeabilized to PI, and their frequency in cell population reduced to 5 % after a 2-h incubation (Fig. 1D of IV). These results suggest that suspension of stationary-growth-phase cells of *L. crispatus* ST1 to pH 8 is an alkaline stress situation and leads to transient increase in cell wall permeability. This is very similar to the recovery of *L. plantarum* from acid stress, where the bacterial population displays phenotypic, morphological heterogeneity after a rapid pH downshift and then recovers over a period of few hours (Ingham *et al.*, 2008).

We found that enolase, GAPDH, GS and GPI were released into PBS and the pH-8 buffer in approximately same ratio as they occurred in lysed cell samples of *L. crispatus* ST1. We compared their release to that of RNA polymerase β1-subunit which had been used as a marker for lack of cell lysis in mycoplasmas (Alvarez *et al.*, 2003). No polymerase was detected in PBS-extracted proteins of *L. crispatus* ST1 (Fig. 1A of I; 1 B and C of II). The polymerase was a problematic control as the amount in cells is significantly lower than those of enolase and GAPDH (Fig. 1A of I). In addition, RNA polymerase was recently reported on the surface *S. gordonii* (Kesimer *et al.*, 2009). As only ca. 20% of the cellular moonlighting proteins were released into the buffers, our failure to detect released polymerase may have resulted from its low amount in *L. crispatus* ST1 cells. Therefore lack of the polymerase in the PBS-extract does not convincingly indicate lack of cytoplasmic leakage.

The pH sift did not affect the expression level of enolase and GAPDH. Equal amount of the proteins were detected by Westren blotting when cells fom pH 5 and pH 8 were lysed (Fig. 1B of II). Chloramphenicol had no effect to release of enolase or GAPDH (Fig. 2A and 2B of II), and the transcription levels of enolase and GAPDH were similar in logarithmic-growth phase cells from pH 5 and pH 8 (Fig. 2C of II). The results suggest that the pH-induced release of enolase and GAPDH from *L. crispatus* ST1 represents detachment of existing proteins.

To analyze whether the release of enolase and GAPDH is a phenomenom restricted to *L. crispatus*, the extracellular buffersome of five strains of *Lactobacillus* belonging to homology group A1 (*L. acidophilus* E507), A3 (*L. amylovorus* JCM 5807), A4 (*L. gallinarum* T-50), B1 (*L. gasseri* JCM 1130/ATCC 19992) and B2 (*L. johnsonii* F133) as well as three probiotic or dairy strains (*L. rhamnosus* GG, *L. paracasei* E506 and *Lactococcus lactis* E523) were screened by SDS-PAGE and Western blotting with antibodies raised against enolase and GAPDH of *L. crispatus* ST1. The extracellular GAPDH was detected in all strains except *L. amylovorus* JCM 5807 (Fig. 5C of I), which released less protein than *L. crispatus* ST1 (compare to Fig. 1A of I). Release of enolase was detected in strains of the *acidophilus* group but not in the dairy strains, which however released a peptide of the apparent size of enolase. Hence the lack of reactivity probably resulted from poor serological reactivity to *L. crispatus* ST1 anti-enolase IgG.

Lactic acid bacteria are strictly fermentative and secrete lactic acid as a primary fermentation product, thus rapidly lowering the surrounding pH. The results above suggest that enolase, GAPDH, GS and GP associate with the cell wall of *L. crispatus* ST1 through ionic interactions at acidic pH, which prevails in the natural niches of *L. crispatus*, i. e. the intestine and the vagina, and are released to environment under alkaline stress conditions. The release does not involve change in transcription or in synthesis of

novel proteins. Thus, *L. crispatus* ST1 alters its surface architecture during growth and in response to pH or salt concentration in the growth medium. Such extracellular localization and release of anchorless proteins seem rather common in lactic acid bacteria as identified here and subsequently in *L. plantarum* (Castaldo *et al*., 2009, Kinoshita *et al*., 2008b, Ramiah *et al*., 2008) and *Bifidobacterium* (Candela *et al*., 2009). The pH-dependent surface-association of moonlighting proteins seems to be common in lactic acid bacteria. The release of enolase from the surface of *S. gordonii* is affected by changes at pH (Nelson *et al*., 2001), and presence of GAPDH in the culture supernatant of *S. oralis* has been described to be affected by pH (Wilkins *et al*., 2003). Further, release of moonlighting proteins seems associated with also other type of stress situations. Iron starvation affects the release of GAPDH from cell surface of *S. pyogenes* (Eichenbaum *et al*., 1996). Altought the phenomenon seems to be common, it is obvious that species-related differences exist, as exemplified in this work (Fig. 5 of I). The mechanistic reason(s) for this difference remains open, our hypothesis is that it reflects structure differences in cell wall components of bacteria. Also, too few strains have been analyzed to allow conclusions on possible strain-spesific variation. A practical conclusion from these observations is that great care should be taken in interpretation of studies where *in-vitro* cultivated lactic acid bacteria are washed or functionally tested in neutral buffers.

6.3 ENHANCEMENT OF THE RELEASE BY EPITHELIAL CATHELIDICIN LL-37 (IV)

Antimicrobial peptides are ancient and potent effectors of the innate immune system in all life forms (Hancock, 2001), and they selectively target and permeabilize the negatively charged bacterial cell membranes (Sochacki *et al*., 2011, Tossi *et al*., 2000). One of the antimicrobial peptides is LL-37, a 37-residue, amphipathic, helical peptide found throughout the human body (Durr *et al*., 2006, Sochacki *et al*., 2011). It is primarily produced by phagocytic leucocytes and epithelial cells, and it is up-regulated at sites of infection (Bowdish *et al*., 2005, Durr *et al*., 2006, Nijnik & Hancock, 2009, Scott *et al*., 2002). LL-37 has been shown to exhibit a broad spectrum of antimicrobial activity (Johansson *et al*., 1998, Turner *et al*., 1998) as well as to have additional defensive roles such as immunomodulatory activity (Bowdish *et al*., 2005, Scott *et al*., 2002) and chemo-attracting cells of the adaptive immune system to infection sites (Chertov *et al*., 1996, Chertov *et al*., 1997, Niyonsaba *et al*., 2002, Niyonsaba *et al*., 2003, Scott *et al*., 2002), binding and neutralizing LPS (Larrick *et al*., 1994), promoting wound closure (Borregaard *et al*., 2005, Carretero *et al*., 2008, Tokumaru *et al*., 2005) and angiogenesis (Bucki *et al*., 2010, Durr *et al*., 2006). LL-37 binds the outer membrane (OM) and translocates the OM by forming small pores. This is followed by penetration through the cytoplasmic membrane. The growth is interrupted before LL-37 enters the cytoplasm (Sochacki *et al*., 2011). LL-37 concentrations in secretions vary from 1-2 µg/ml in saliva, up to 20 µg/ml in tracheal aspirates and 85 µg/ml (19.7 µM) in seminal plasma.The concentration may increase two- to threefold during infection or inflammation (Bucki *et al*., 2010). LL-37 is active against Gram-negative and Gram-positive bacterial species and it is lethal especially against dividing cells. The minimal inhibitory concentration of LL-37 varies from 0.2 µg/ml (0.05 µM) to 450 µg/ml (104.3 µM) depending on bacterial species (Durr *et al*., 2006).

Our results described above suggested that stress due to rapid pH upshift increases release of enolase, GAPDH, GS and GPI to the buffersome. Our hypothesis was that LL-37 potentiates the release of the moonlighting proteins from lactobacilli due to its cell-wall permeabilizing effect, thus giving a possible biologically relevant alternative to the alkaline stress described above. A complex stress response in *B. subtilis* has been reported when the organism was subjected to subinhibitory concentrations of LL-37

(Pietiainen *et al.*, 2005). We treated *L. crispatus* ST1 cells with LL-37 concentrations from 0 µM to 16 µM; the MIC value for *L. acidophilus* has been described as 19 µM (Zhao *et al.*, 2001), and in our assays the highest concentration of LL-37 (16 µM) was the MIC_{50}-value for *L. crispatus* ST1 (Fig. 4A of IV).

The effect of LL-37 on *L. crispatus* ST1 cell permeability was estimated by PI-staining. A gradual increase in PI-stainable cells from 1% to 20% upon rising LL-37 concentration was observed (Fig. 4A of IV), which thus resembled the permeability change in response to alkaline stress. We first estimated protein release by measuring enzymatic activity of GPI at different LL-37 concentrations. A gradual increase in the enzymatic activity was observed (Fig. 4B of IV). We assessed possible release of the four moonlighting proteins by Western blotting of the buffer extract samples. Also this assay showed increase of the proteins in the buffers containing LL-37 (Fig. 4C of IV).

The results above suggest that plasma membrane permeability is related to the efflux or the detachment of enolase, GAPDH, GS and GPI. Since LL-37 is produced by epithelial cells and is present in secretions as well, lactobacilli will encounter the peptide in their natural niches i. e. in gastrointestinal and urogenital tracts. Our results agree with those of Saad *et al.* (2009) who found that *L. plantarum* cells with increased membrane permeability have higher amount of GAPDH in the cell wall. Saad *et al.* did not detect GAPDH in the growth medium, which, on the other hand, was acidic and between 3.8 and 5.9 during bacterial growth. While our results describe how the moonlighting proteins are released to the surroundings as a stress response, they do not describe how the proteins are translocated to the cell surface, this could take place by active transport or by diffusion at specific cell sites, e. g. cell division sites.

6.4 INTERACTION OF ENOLASE AND GAPDH WITH LIPOTEICHOID ACIDS (II)

Enolase and GAPDH are positively charged at acidic pH-values and thus capable to bind negatively charged cell wall components, such as LTA. We used *L. crispatus* ST1 His_6-enolase and His_6-GAPDH purified from extracellular proteome of *L. crispatus* ST1 in a mobility shift assay to detect their binding to LTA from *S. aureus* and *Streptococcus faecalis* and to PG of *S.aureus.* At pH 4, enolase and GAPDH migrated towards the negative pole, whereas addition of LTAs abolished the motility indicating binding of enolase and GAPDH to the LTAs. No mobility shifts were observed at pH 5.6 and addition of PG did not affect the mobility of the proteins at either pH (Fig. 3A of II). The pH-dependent binding to LTA was confirmed by testing binding of enolase- and GAPDH-coated fluorescent beads on the LTAs, PG and BSA coated coated on glass (Fig. 3B of II). Enhanced binding of the proteins to LTAs at low pH was observed, whereas the LTA-binding peptide of the S-layer of *L. crispatus* bound to LTAs at both pH values.

Jonquières *et al.* (1999) described the LTA-binding as a novel mechanism of protein association on the surface of Gram-positive bacteria. The association of InlB of *L. monocytogenes* to the cell surface was based on glycyl-tryptophan (GW) modules of the proteins (Jonquières *et al.*, 1999). Other bacterial LTA-binding proteins include choline-binding proteins (García *et al.*, 1998) and CbsA, the S-layer protein of *L. crispatus* (Antikainen *et al.*, 2002). For comparison, LTA-binding fragment of *L. crispatus* S-layer protein CbsA (Antikainen *et al.*, 2002) was here included in the LTA and PG binding tests. The binding of His_6-CbsA 288-410 to LTA was not dependent on pH and no binding of enolase, GAPDH or CbsA to PG was detected. The PI-value of CbsA 251-410 fragment is 9.9 and therefore it is positively charged at

pH-values tested here and capable to bind LTA. HlpA, surface protein of *S. pneumoniae* which has pI 9.8, forms complexes with LTA by ionic interactions similarly to CbsA (Stinson *et al*., 1998).

These results suggest that enolase and GAPDH are anchored to LTA at low pH-values. GS and GPI are as well capable to bind cell surface at pH-values below the isoelectric point of the proteins. However, the cell surface of Gram-positive bacteria contains also other negatively charged compounds such as teichuronic acids and covalently cell wall associated proteins. Their possible role in anchoring of moonlighting proteins remains open.

6.5 REASSOCIATION OF ENOLASE, GAPDH, GS AND GPI TO CELL SURFACE AT LOW PH (II, IV)

The results above suggested that stress conditions increase release of moonlighting proteins from *L. crispatus* surface and that they might under suitable conditions reassociate back onto the cell surface. To assess whether these proteins bind *in-vitro* to the bacterial surface, we tested the binding of His_6-proteins onto *L. crispatus* ST1. Release and reassociation of enolase on the cell surface of *S. pneumoniae* has been earlier observed (Bergmann *et al*., 2001). The cells were first incubated at pH 8, buffersome was collected, and the released proteins were allowed to reassociate onto cells at pH 4.4 or at pH 7. The binding of enolase and GAPDH to the cells was evident at pH 4.4.but only weak association was detected at pH 7 (Fig. 4 of II). The binding was dimished when LTA was added, which further supports the anchoring role of LTA.

We also tested the binding of His_6-recombinant proteins to the cell *L. crispatus* ST1 surface. For comparison, we included the probiotic *L. rhamnosus* GG in the assay. His_6-enolase, His_6-GAPDH, His_6-GS and His_6-GPI bound to the cell surface of *L. crispatus* ST1 at pH 4 but not at pH 8 (Fig. 3 of IV). The binding was not evenly distributed around the cells but was concentrated to cell division areas as well as to the ends of the cells. Somewhat surprisingly, His_6-enolase, His_6-GAPDH, His_6-GS and His_6-GPI of *L. rhamnosus* GG bound *L. crispatus* ST1 cells but the *L. crispatus* ST1 and *L. rhamnosus* GG fusion proteins failed to bind onto *L. rhamnosus* GG cell surface (Fig. 3 of IV). Similarly, we found that the *L. crispatus* ST1 and *L. rhamnosus* GG buffersomes bound onto *L. crispatus* ST1 cells but not to *L. rhamnosus* GG cells (data not shown).

The results described above suggest that enolase, GAPDH, GS and GPI are not evenly localized on the cell surface but rather concentrate to cell poles and septum area. Asymmetric localization of surface proteins in Gram-positive bacteria has been described earlier. Autolysins such as LytE and LytF of *B. subtilis* (Yamamoto *et al*., 2003) and Atl of *S. aureus* (Yamada *et al*., 1996) have been reported to localize to the cell division areas. Some of penicillin-binding proteins (PBPs) of *B. subtilis*, one of the four PBPs of *S. aureus* and the PBP of *S. pneumoniae* have been reported to show a disperse localization within the membrane (Scheffers & Pinho, 2005). Septation also involves changes in the synthesis of capsule of *S. pneumoniae* (Henriques *et al*., 2011) and the division septum of *S. aureus* is a preferred target for binding by telavancin, a bactericidal peptide that inhibits cell wall synthesis and disrupts membrane barrier function by binding to peptidoglycan precursor (Lunde *et al*., 2010).Localization to spesific sites on the Gram-positive bacterial surface has been observed also for some proteins that are not involved in cell division. ActA of *L. monocytogenes* serves as the receptor on which actin filaments polymerize in the host cell. ActA is shown to be present only in the one pole of the cell (Rafelski & Theriot, 2006) and in *B. subtilis* chemotaxis proteins McpB and TlpA has been reported to localize to

the poles of the cells (Kirby *et al*., 2000, Meile *et al.*, 2006). It has been speculated that cell wall passage of proteins is restricted to limited number of sites, to maintain cell wall rigidity and withstanding turgor pressure (Buist *et al*., 2006).

His_6-proteins of *L. crispatus* ST1 and *L. rhamnosus* GG as well as extracellularly released proteins were found to associate with the cell surface of *L. crispatus* ST1. The results suggest that lactobacilli are capable to immobilize moonlighting proteins from other lactobacillar origin to their surfaces. The typical property of moonlighting proteins is that they may have different moonlighting activities in different organisms, which raises the possibility that bacteria could modify their surface and perhaps expand their competence in host interactions without encoding all proteins by themselves. The failure of the recombinant moonlighting proteins from *L. crispatus* ST1 or *L. rhamnosus* GG to bind onto *L. rhamnosus* GG surface indicates that mechanisms of surface association of moonlighting proteins vary in bacterial species. The biochemical explanation for the difference remains open. It has been reported that the exopolly saccharides of *L. rhamnosus* GG protects the bacterium against LL-37 (Lebeer *et al*., 2011).

6.6 FUNCTIONS OF LACTOBACILLAR ENOLASE, GAPDH, GS AND GPI (I, II, III, IV)

6.6.1 INTERACTION OF LACTOBACILLI WITH THE HUMAN PLASMINOGEN SYSTEM (I, II, III, IV)

Several pathogenic bacteria have been described to bind plasminogen and plasmin on their cell surface (Bergmann & Hammerschmidt., 2007, Boyle & Lottenberg, 1997, Lähteenmäki *et al*., 2001, Lähteenmäki *et al*., 2005). Binding of plasminogen to the cell surface receptor enhances plasminogen activation by the host PAs and the bacteria turn themselves into proteolytic organisms (Lähteenmäki *et al*., 2005). To analyze whether interactions with the host plasminogen system are restricted to pathogens only, we assessed the plasminogen receptor function in lactic acid bacteria.We also analyzed material released into the incubation buffer. Washed *L. crispatus* ST1 cells as well as cell-free incubation buffer enhanced activation of human and bovine plasminogen by physiological plasminogen activators, tPA and uPA (Fig. 2A of I, Fig. 3B of I and Figure 2.).

Next, we tested the capacity of *L. crispatus* ST1 to bind ^{125}I-plasminogen, ^{125}I-plasmin and ^{125}I-tPA. We also analyzed the conversion of the one-chain plasminogen into two-chain plasmin in the presence of *L. crispatus* ST1 cells and determined the distribution of plasmin activity between *L. crispatus* ST1 cells and incubation buffer. *L. crispatus* ST1 cells bound 1-3% of added amount of ^{125}I-plasminogen whereas plasmin binding was 2- or 3-fold higher (Fig. 2B and 2C of I). The level of plasminogen and plasmin binding onto *L. crispatus* ST1 cells was low compared to pathogens in similar assays (Bergmann *et al*., 2001, Kukkonen *et al*., 1998, Kuusela & Saksela, 1990, Lähteenmäki *et al*., 1995, Pancholi & Fischetti, 1992, Ullberg *et al*., 1990, Ullberg *et al*., 1992). No binding of tPA was detected (Fig. 2D of I). All bindings were inhibited by lysine analog ε-aminocaproic acid, EACA, indicating that kringle mediated binding of plasminogen and plasmin to their receptors on the cell surface. A similar result was obtained when *L. crispatus* ST1 cells were incubated with plasminogen or plasmin and cells and the supernatant fraction were separated and analyzed by Western blotting (Fig. 2E of I). No

plasminogen was detected on the cell surface but a small amount of plasmin was immobilized on the cell surface. Most of the plasmin and all plasminogen were present in the cell-free supernatant. When tPA was added to plasminogen and *L. crispatus* ST1 cells, rapid generation of plasmin was observed and 92% of the activity was in the buffersome (Fig. 2E of I). The plasmin activity was almost completely inhibited by α2-antiplasmin (Fig. 2F of I), that is primary circulating inhibitor of plasmin. It binds to the kringle domains, and efficiently inactivates soluble plasmin, whereas binding of plasminogen on the receptors takes place via the kringle domains which then are resisant to α2-antiplasmin binding and inactivation. An important property of plasminogen receptors is to protect plasmin from inhibition of α2-antiplasmin. At neutral pH, plasminogen/plasmin remains bound on the pathogen surface and thus inaccessible to the protease inhibitor (Lähtenmäki *et al*., 2001). In contrast, plasminogen activation by tPA is enhanced by the cell-free buffer extract from *L. crispatus* ST1 cells, and the formed plasmin is not protected from α_2-antiplasmin (Fig. 3C of I) and thus will be rapidly inactivated *in vivo.*

We tested whether interaction between lactobacilli and plasminogen system is also affected by pH. *L. crispatus* ST1 cells were incubated with plasminogen at pH 5 and pH 8 followed by separation of cells and supernatant. As analyzed by Western blotting, the plasminogen molecule was detected in the supernatant fraction at pH 8, whereas most of plasminogen was cell-surface bound at pH 5 (Fig. 5A of II). Cells incubated at pH 5 and the supernatant of pH 8 incubated cells enhanced the tPA-catalyzed plasminogen activation (Fig. 5B of II).

The results described here demonstrate that lactobacilli are not only capable of to bind plasminogen-binding proteins on the cell surface, but also release plasminogen receptors to the environment. We have observed that plasmin does not degrade its choromogenic substrate, H-D-Val-Leu-Lys-p-nitroaniline dihydrochloride at low pH-values (data not shown). Similar observations about effects of pH to plasmin activity have been published earlier (Christensen, 1975). *Lactobacillus* strains tested here have capacity to bind plasminogen molecules mostly in acidic environments, whereas several bacterial pathogens bind plasminogen and enhance formation of plasmin to cell surface at neutral pH (Bergmann *et al*., 2001, Kukkonen *et al*., 1998, Kuusela & Saksela, 1990, Lähteenmäki *et al*., 1995, Pancholi & Fischetti, 1992, Ullberg *et al*., 1990, Ullberg *et al*., 1992). For example, enolase of *S. pneumoniae* has been described to function in plasmin-mediated degradation of ECM, laminin, matrigel and fibrinogen (Bergmann *et al*., 2005). The fermentative metabolism of lactic acid bacteria creates acidic microenvironment, which makes cell-bound plasmin unable to proteolysis. At neutral pH, lactobacilli release plasminogen-binding enolase into the surrounding media. A similar effect has not been described for Gram-positive pathogens. This is a major difference in interaction of pathogens and lactobacilli with the plasminogen system, and in theory this should prevent lactobacilli to turn themselves into proteolytic organisms using plasminogen system.

Also extracellular material of other members of *acidophilus* group, *L. acidophilus* E507, *L. amylovorus* JCM 5807, *L. gallinarum* T-50, *L. gasseri* JCM 1130/ATCC 19992, *L. johnsonii* F133, and of tested dairy and probiotic strains, *L. rhamnosus* GG, *L. paracasei* E506 and *L. lactis* E523, enhanced tPA- and uPA-mediated plasminogen activation suggesting that plasminogen activation cofactor function is common in lactic acid bacteria (Fig. 5A of I). The extracellular material released from tested lactic acid bacteria strains contained variable amounts of proteins as visualized in the SDS-PAGE gel. The capacity to enhance Plasminogen activation seemed to be proportional to the amount of extracellular proteins (Fig. 5 of I).

We compared enolases from lactobacilli and pathogenic Gram-positive cocci and found that the proteins from lactobacilli were equally, or in some cases even better, effective in plasminogen binding and activation as were enolases from pathogens (Fig. 3 of II). GS, but not GPI, bound plasminogen and enhanced its activation by tPA (Fig. 5D of IV). The same was found for enolase and GAPDH (Fig. 4 of I). Thus, *L. crispatus* ST1 expresses several plasminogen-binding proteins on the surface. Comparison of enhancement of tPA mediated plasminogen activation by *L. crispatus* ST1, *L. johnsonii* F133, and by *S. pyogenes* IH32030 and *S. aureus* 8325-4 is shown in Figure 2. The results show that the lactobacillar strains are equal or more efficient than the pathogens. Thus, lactobacilli have a high potential to enhance plasminogen activation in humans and it is likely that activation in the buffer is more rapid because the reagents are more easily ccessible than on the cell surface.

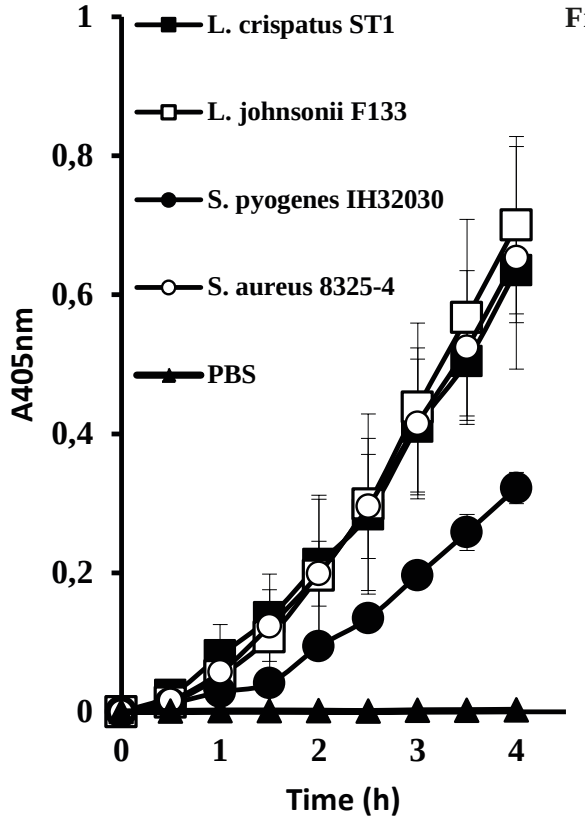


Figure 2. Plasminogen activation enhanced by commensal lactobacilli and Pathogenic Gram-positive cocci. Plasminogen and tPA were incubated in absence (▲) or presence of *L. crispatus* ST1 (■), *L. johnsonii* F133 (□), *S. pyogenes* IH32030 (●) and *S. aureus* (○) for four hours and formation of plasmin was measured using a chromogenic substrate of plasmin and measuring absorbance at 405 nm. The means are average of two independent assays with duplicate samples.

C-terminal lysines of enolase of *S. pyogenes* and *S. pneumoniae* have been described to play an important role in plasminogen binding (Bergmann *et al*., 2004, Derbise *et al*., 2004, Pancholi & Fischetti, 1998). However, enolases of *L. crispatus* ST1 or *L. johnsonii* do not contain C-terminal lysines (Ojala *et al*., 2010, Pridmore *et al*., 2004). The number of enolase genes and C-terminal sequences are variable in *Lactobacillus.* Of all sequenced lactobacillar genomes only enolase of *L. sakei* subs. *sakei* 23k (Chaillou *et al*., 2005) and one of the enolases of *L. gasseri* JV-V03 (GenBank accession number ZP_07058602) have douple lysines in their C-terminus. Bergman *et al* (2003) have demonstrated that enolase of *S. pneumoniae* has an additional internal plasminogen binding epitope ^{248}FYDKERKVY (amino acid residues needed for plasminogen binding are underlined). This internal motif is crucial for the interaction of the enolase with human plasminogen. A similar sequence ^{248}FYNKDDHKY is present in *L. crispatus* ST1 enolase but the substitution of lysines at position 4 and 8 did not significantly decreased tPA-mediated plasminogen activation (data not shown). The results suggest that cationic residues elsewhere in the *L. crispatus* ST1 enolase protein are involved in interaction with plasminogen.

Plasmin and other components of plasminogen system are present in human and mammalian milk (Bastian & Brown, 1996, Heegaard *et al.*, 1997). Lactobacilli require an exogenous source of amino acids or peptides. Many lactic acid bacteria strains contain a cell-envelope proteinase which degrades the protein into oligopeptides (Savijoki *et al.*, 2006). However, all lactobacilli do not contain such proteinase and may use plasmin for nutritional demands.

Enolase and GAPDH are well-characterized plasminogen receptor proteins cells (Agarwal *et al.*, 2008, Al-Haroni *et al.*, 2008, Barbosa *et al.*, 2006, Bergmann *et al.*, 2001, Bergmann *et al.*, 2004, Candela *et al.*, 2009, de la Torre-Escudero *et al.*, 2010, Donofrio *et al.*, 2009, Dudani *et al.*, 1993, Egea *et al.*, 2007, Gase *et al.*, 1996, Ge *et al.*, 2004, Jones & Holt, 2007, Knaust *et al.*, 2007, Matta *et al.*, 2010, Mundodi *et al.*, 2008, Nogueira *et al.*, 2010, Pancholi & Fischetti, 1998, Pancholi & Chhatwal, 2003, Seifert *et al.*, 2003, Seweryn *et al.*, 2007, Sha *et al.*, 2009, Vanegas *et al.*, 2007, Winram & Lottenberg, 1996, Yavlovich *et al.*, 2007). GPI is known to function as a neuroleukin (Faik *et al.*, 1988), an autocrine motility factor (Watanabe et al., 1996, Yanagawa *et al.*, 2004), a differentiation and maturation mediator for myeloid cells (Xu et al., 1996), an implantation factor (Schulz & Bahr, 2003) and important modulator of tumor progression and a target for cancer therapy (Fairbank *et al.*, 2009). GPI has also been detected on the surface of *S. agalactiae* (Hughes *et al.*, 2002). Interaction between the plasminogen system and GPI has not been reported thus far. GS has been identified as a plasminogen binding protein of *M. tuberculosis* and it enhances plasminogen activation to plasmin and binds to fibronectin (Xolalpa *et al.*, 2007). It was identified as a putative plasminogen binding protein of *B. animalis* subsp. lactis (Candela *et al.*, 2007). We studied functionality of His_6-GS and His_6-GPI as plasminogen receptors. His_6-GS protein bound plasminogen (Fig. 5D of IV) and plasmin (data not shown) as well as enhanced plasminogen activation mediated by tPA (Fig. 5D of IV). In contrast, His_6-GPI showed only weak interaction with the components of the plasminogen system (Fig. 5D of IV). Here, we describe GS as a novel lactobacillar plasminogen receptor.

Our data demonstrate that although *Lactobacillus* and *Lactococcus* were highly efficient in enhancing the tPA-catalyzed plasminogen activation, the binding of plasminogen and plasmin onto the bacterial cells at neutral pH was poor. Lactobacilli were not able to retain cell-bound plasmin activity (Fig. 5 of II) suggesting that they might not be able to take advantage of the plasminogen system for proteolysis in a manner similar to pathogenic bacteria. This suggests that enhancement of plasmin activity by lactobacilli remains local and could function e. g. to contribute to dissolution of fibrin clots or thrombi over a wound site. It has been reported that treatment with *L. casei* of mice suffering from pneumococcal pneumonia lowers fibrin(ogen) deposits in the lung (Aguero *et al.*, 2006, Haro *et al.*, 2009) and the potential of synbiotics to reduce pro-coagulatory factors has been recognized (Bengmark, 2003). The mechanisms of the *in vivo* effects described above obviously are complex, but our results encourage further studies on the role and the mechanisms in the modulation of the fibrinolysis/coagulation cascade by commensal bacteria.

6.6.2 LACTOBACILLAR MOONLIGHTING PROTEINS AS ADHESINS (III, IV)

Lactobacilli bind epithelial cells and tissue samples of intestinal tract of humans and animals (Coconnier *et al*., 1992, Conway *et al*., 1987, Conway & Kjelleberg, 1989, Li *et al*., 2008, Reid *et al*., 1993), human vaginal epithelial cells (Boris *et al*., 1998, Kwok *et al*., 2006, Ocana & Nader-Macias, 2001, Ocana & Nader-Macias, 2004), intestinal, gastric or faecal mucus (Gusils *et al*., 2003, Kinoshita *et al*., 2007, Kirjavainen *et al*., 1998, Kirjavainen *et al*., 1999, Li *et al*., 2008, Rojas & Conway, 1996, Wang *et al*., 2008), cultured human carcinoma intestinal cell lines (Chauviere *et al*., 1992, Coconnier *et al*., 1993, Greene & Klaenhammer, 1994, Kirjavainen *et al*., 1999, Wang *et al*., 2008) and to components of the extracellular matrix (Aleljung *et al*., 1991, de Leeuw *et al*., 2006, Harty *et al*., 1994, Kapczynski *et al*., 2000, McGrady *et al*., 1995, Munoz-Provencio *et al*., 2009, Styriak *et al*., 2001, Toba *et al*., 1995). The studies reporting about adherence of lactobacilli are numerous, but only few adhesive surface proteins in addition of S-layer proteins have been characterized (Antikainen *et al*.,2002, Bergonzelli *et al*., 2006, Buck *et al*., 2005, Granato *et al*., 2004, Kankainen *et al*., 2009, Miyoshi *et al*., 2006, Pretzer *et al*., 2005, Rojas & Conway., 1996, Roos *et al*., 1996, Roos & Jonsson, 2002, van Pijkeren *et al*., 2006, Walter *et al*., 2005).

We compared His_6-enolases from commensal lactobacilli, pathogenic streptococci and staphylococci for binding to extracellular matrix proteins using a routine enzyme-linked immunosorbent assay (ELISA) at neutral pH. Lactobacillar enolases as well as enolase of *S. aureus* bound laminin (Fig. 4 of III). Enolase of *L. crispatus* ST1 and *S. aureus* showed binding to collagen I, whereas streptococcal enolases showed only week binding capacity (Fig. 4 of III). No binding of His_6-enolases to collagen IV, fibronection or BSA was detected. Enolase from extracellular proteome of *L. crispatus* ST1 showed similar binding to laminin and collagen I as His_6-enolase. This indicates that binding is a real property of *L. crispatus* ST1 enolase. Also enolase of *B. anthracis* (Agarwal *et al*., 2008) and *S. aureus* (Carneiro *et al*., 2004) have been identified as laminin-binding proteins and enolase of *L. plantarum* has been described to bind fibronectin (Castaldo *et al*., 2009).

Since we demonstrated that lactobacillar plasminogen-binding was pH-dependent, we next tested effect of pH to adherence. *L. crispatus* ST1 cells bound matrigel dramatically better at the acidic pH than at neutral pH (Fig. 5A of IV).The same was observed in *L. crispatus* ST1 adherence to individual ECM proteins (Figure 3.). His_6-GS and His_6-GPI of *L. crispatus* ST1 were shown by surface plasmon resonance to bind collagen I and laminin (Fig. 5B and 5C of IV). Binding of His_6-GS to fibronectin was also observed (Fig. 5B of IV). Neither of these proteins bound collagen IV suggesting that protein(s) responsible for mediating *L. crispatus* ST1 adherence to collagen IV remains to be identified. The biacore assay allows binding tests at different pH-values. The binding of both His_6-proteins to collagen I and laminin was diminished when pH upshifted from 5.5 to 6.0 and further to 6.5 (Fig. 5B and 5C of IV). It has been shown earlier that the binding of both fibronectin and fibrinogen by lactobacilli is greatly enhanced at acidic pHs (Harty *et al*., 1994) and the binding of GroEL of *L. johnsonii* to mucin was pH-dependent (Bergonzelli *et al*., 2006).

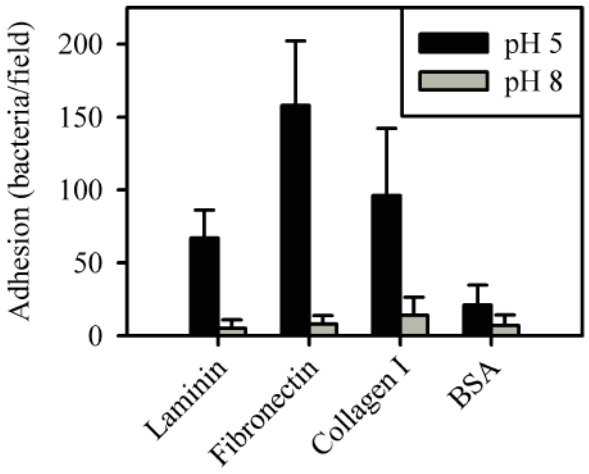


Figure 3. Modification of the adhesiveness of *L. crispatus* ST1 in response to pH. Bacterial adhesiveness to immobilized laminin, fibronectin, type I collagen and bovine serum albumin (BSA) at pH 5 and 8 are shown. (Originates from Antikainen *et al.* (2009) and is published with permission of Caister Academic press).

Lactobacilli colonize several acidic environmental niches such as the oral cavity, the small intestine and the vaginal epithelia, and therefore it seems logical that their adhesion proteins function more efficiently at acidic conditions. Thus, the pH-dependency of lactobacillar adhesiveness is manifested in two mechanisms: presence or detachment of the moonlighting proteins on the cell surface and their pH-dependent affinity.

7 CONCLUSIONS

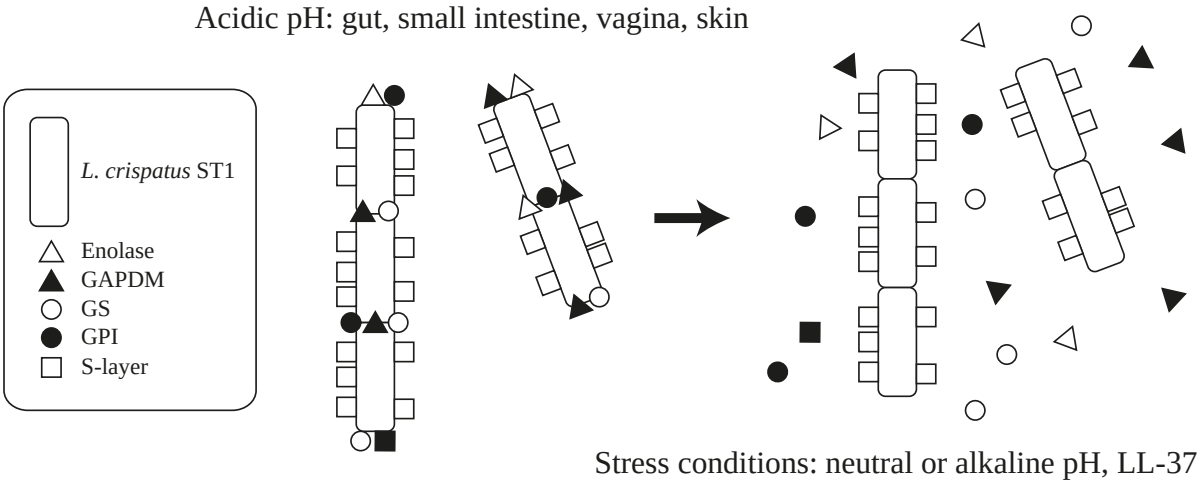
In this work non-covalently surface-associated moonlighting proteins of *L. crispatus* ST1 were studied. When this study was initiated, such proteins had been found on the cell surface of Gram-positive pathogens, and enolase and GAPDH had been described as potential virulence factors of Gram-positive cocci. This work was among the first ones showing that surface association of enolase and GAPDH and plasminogen immobilization are common in the genus *Lactobacillus* which contains commensal and probiotic species and strains. During this thesis work, increasing number of moonlighting proteins has been described, and their surface location seems to be a common property in all Gram-positive bacteria, commensal or pathogenic. A benefit from moonlighting proteins is that their multifunctional nature improves maximal exploitance of the small lactobacillar genomic information.

This study demonstrates for the first time that enolase, GAPDH and GS of *L. crispatus* are cofactors of the human plasminogen system. At the beginning of this study interactions between bacteria and the human plasminogen system had been described mostly in pathogenic bacteria and plasminogen cofactor activity combined to adhesiveness to components of extracellular matrix have been suggested to indicate pathogenic characteristics of the organisms. Suprisingly, lactobacillar cells or extracellular material released from them were even better in enhancing plasminogen activation than pathogenic bacteria or their surface proteins. However, lactobacillar cells were poor in plasminogen binding and they could not retain plasmin activity on their surfaces. A difference between pathogens and commensals is that several pathogens express plasminogen activators of their own whereas lactobacilli do not. Pathogens can retain plasmin on their surfaces at pH values in which plasmin is active, whereas lactobacilli release plasmin into the surrounding environment. The lactobacillar interaction with plasminogen system may have a role in opportunistic infections, or on the other hand, it can be beneficial and e. g. to contribute to dissolution of fibrin clots or thrombi over a wound site or to fragmentation of the plasminogen molecule further into health-promoting peptides such as angiostatins. However, discovering enolase or other glycolytic enzymes in extracellular media does not necessarily indicate plasminogen cofactor activity. Under iron-restricted conditions enolase was found to be localized primary in the cytoplasmic membrane of the Gram-negative anaerobic bacterium *Bacteroides fragilis*, which is a member of the commensal flora of the human intestine but is also frequently found in severe intra-abdominal infections (Sijbrandi *et al.*, 2005). *B. fragilis* cells were found to bind plasminogen but it was not dependent on enolase (Sijbrandi *et al.*, 2005). Likewise, extracellular enolase of *Leuconostoc mesenteroides* was not found to bind plasminogen (Lee *et al.*, 2006).

This work demonstrates that lactobacillar moonlighting proteins also mediate bacterial adhesion to ECM components. It can be concluded that adhesiveness to ECM components as well as interaction with the plasminogen system are rather common properties of Gram-positive bacteria and that plasminogen-bacteria interactions differ mechanistically in lactobacilli and pathogenic Gram-positive cocci.

A major finding in this thesis work is that *L. crispatus* modifies surface-association of moonlighting proteins in response to the environment, and in part as a consequence of its own growth. This is illustrated in Figure 4. The changes will alter surface architecture of the bacteria as well as its interactions with the host. An exiting observation of this work is that the released moonlighting proteins are able to reassociate onto different bacteria species, thus giving a novel mechanism of bacteria-bacteria interactions.

Figure 4. Surface association of enolase, GAPDH, GS and GPI. At acidic pH i. e. pH values below isoelectric point of enolase, GAPDH, GS and GPI, proteins are associated with the cell surface of *L. crispatus* ST1 wheras they are released from the bacterial surface in stress situations such as alkaline PH or or precence of LL-37.



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