# Tissue-Adherence in Lactic Acid Bacteria: Identification and Characterization of the Collagen-Binding S-Layer Protein of *Lactobacillus crispatus*

**Jouko Sillanpää**

Department of Biosciences,
Division of General Microbiology,
University of Helsinki

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Supervisor Professor Timo Korhonen

Department of Biosciences,
Division of General Microbiology,
University of Helsinki

Reviewers Professor Airi Palva

Department of Basic Veterinary Sciences,
Division of Microbiology,
University of Helsinki

Professor Per Saris

Department of Applied Chemistry and Microbiology,
Division of Microbiology,
University of Helsinki

Opponent Professor Tapani Alatossava

Research and Development Centre of Kajaani / Biotechnology Laboratory,
University of Oulu

*Cover figure: Adherence of* Lactobacillus crispatus *strain JCM5810 to immobilized proteins of the extracellular matrix, adherence to fetuin and bovine serum albumin (BSA) is shown for control. Bar 5 mm.*

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# Contents

# ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which in the text are referred to by their Roman numerals.

I **Sillanpää, J., J. Antikainen, P. Sigvart, M. Mannerström, R. Virkola, and T. Toba.** 2001. Cell- and matrix-binding by isolates in the *Lactobacillus acidophilus* homology groups A1-B2. (Manuscript)

II **Toba, T., R. Virkola, B. Westerlund, Y. Björkman, J. Sillanpää, T. Vartio, N. Kalkkinen, and T. K. Korhonen.** 1995. A collagen-binding S-layer protein in *Lactobacillus crispatus*. Appl. Environ. Microbiol. **61:**2467-2471.

III **Sillanpää, J., B. Martínez, J. Antikainen, T. Toba, N. Kalkkinen, S. Tankka, K. Lounatmaa, J. Keränen, M. Höök, B. Westerlund-Wikström, P. H. Pouwels, and T. K. Korhonen.** 2000. Characterization of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus*. J. Bacteriol. **182:**6440-6450.

IV **Martínez, B., S. Sillanpää, E. Smit, T. K. Korhonen, and P. H. Pouwels.** 2000. Expression of *cbsA* encoding the collagen-binding S-protein of *Lactobacillus crispatus* JCM5810 in *Lactobacillus casei* ATCC393. J. Bacteriol. **182:** 6857-6861.

# SUMMARY

Lactic acid bacteria are major members of the commensal flora in the gastrointestinal and urogenital tracts of humans and animals and thought to exert beneficial health effects to their host. Adhesion to host tissue is considered important for colonization and survival of lactic acid bacteria (LAB) on the epithelial surfaces. An analysis of the molecular adhesion mechanisms of LAB was initiated by assessing the adhesiveness to tissue components by 12 isolates of the *Lactobacillus acidophilus* group. The collection of strains consisted of two strains from each *L. acidophilus* DNA homology groups A1-A4, B1 and B2. The majority of the twelve strains expressed adherence to the human intestinal cell lines Caco-2 (9/12 strains) and Intestine 407 (7/12 strains). LAB adhesiveness to the ECM was also common in the strains, binding was observed to the extracellular matrix (ECM) prepared from Intestine 407 cells (8/12 strains) and the mouse basement membrane (BM) preparation Matrigel (5/12 strains) as well as to the major individual matrix components fibronectin (8/12 strains), type I collagen (6/12 strains), type IV collagen (3/12 strains) and laminin (4/12 strains). The adhesion levels and target specificities were largely variable indicating multiple bacterial binding strategies that recognize different tissue receptors. We identified an S-layer protein CbsA of *Lactobacillus crispatus* strain JCM5810 that mediates bacterial binding to collagens and to connective-tissue sites in the chicken colon. The cloned and sequenced *cbsA* gene showed significant homology to other *L. acidophilus* group S-protein sequences deposited in the data banks. Highest homology was found in the signal sequences and in the C-terminal one-third of the molecules, whereas the N-terminal and middle parts were variable with local regions of high conservation. CbsA was expressed in *Escherichia coli* as an N-terminal 6xHis-tag fusion protein, which showed efficient collagen-binding and polymerized into a regular S-layer sheet. The regions in CbsA important for binding to collagen and for the formation of the paracrystalline layer were characterized further by mutation analysis. Amino acids 1-287 in the variable region were found to contain the information for both collagen-binding and, rather surprisingly, also for formation of S-layer sheets. Mutated CbsA molecules that failed to polymerize into an S-layer sheet did not bind collagens, suggesting that the polymerized structure is optimal for collagen binding. CbsA was expressed in *Lactobacillus casei* as a fusion protein with an LPXTG cell wall anchor sequence, the fusion protein was surface-located and the recombinant bacteria exhibited adhesion to collagen. Another S-layer gene, *cbsB*, was identified in *L. crispatus* JCM5810 and was found to share an overall identity of 44% with *cbsA*. By mRNA analysis, *cbsB* was shown to be a silent gene. The functions of lactobacillar S-layer proteins have remained unknown. In this study we demonstrated that the S-layers form a class of LAB adhesins. No *cbsA* homolog was found in the other collagen-binding *L. acidophilus* isolates indicating that other types of collagen adhesin also exist among the *L. acidophilus* group strains.

# 1. INTRODUCTION

Lactic acid bacteria (LAB) form a phylogenetically diverse group and are defined as Gram-positive, non-sporing, catalase-negative, devoid of cytochromes, of anaerobic habit but aerotolerant, fastidious, acid-tolerant, and strictly fermentative bacteria that secrete lactic acid as the major end product of sugar fermentation (Orla-Jensen, 1919; Mitsuoka, 1992; Axelsson, 1998). LAB have a wide distribution in nature and are frequently isolated from environments rich in organic nutrients, such as decaying plant material or the intestinal or urogenital tracts of mammals. They have a long history in industrial use in the fermentation of milk, vegetables and meat, and as an industrial microbe, the importance of LAB is next only to that of the yeast *Saccharomyces cerevisiae*. Interest in LAB as health-promoting bacteria was raised early last century when Elie Metchnikoff suggested that proteolytic ("putrefactive") bacteria of the intestinal normal flora are harmful to human health and that modification of the intestinal flora by the consumption of LAB may contribute to prolonging of life, as exemplified by the apparently long life span of yoghurt-eating Bulgarian peasants (reviewed in Bibel, 1988). Since then, several probiotic products have been marketed for human or animal use. LAB are major members of the complex microbial flora in the mammalian intestine and are gaining growing interest in basic and applied research, *i.e.* as probiotics as well as delivery vehicles for pharmaceutically important compounds.

## 1.1. Lactic acid bacteria: classification and physiological characteristics

There has traditionally been an agreement among scientists that LAB form a uniform bacterial group, which in early times was referred to as "milk-souring organisms" (Orla-Jensen, 1919). Essentially, this still holds true, even though LAB now are known to comprise a phylogenetically heterogeneous group of bacteria (Axelsson, 1998). The "classical" classification schemes of LAB mainly relied on investigation of phenotypic characters. The introduction of modern molecular biology methods, in particular the comparison of ribosomal DNA sequences, has resulted in major revisions in LAB taxonomy and led to the introduction of several new genera into this group. In the 1986 edition of Bergey's Manual, the genera that best fulfill the description of "a typical LAB" are *Aerococcus, Lactobacillus, Leuconostoc, Pediococcus*, and *Streptococcus*. The genus *Streptococcus* is now divided into four new genera: *Enterococcus* (fecal streptococci, group D in the serological Lancefield grouping (Lancefield, 1933)), *Lactococcus* (lactic streptococci, Lancefield group N), *Streptococcus sensu stricto* and *Vagococcus* (motile cocci, Lancefield group N) (reviewed in Stiles and Holzapfel, 1997; Axelsson, 1998). The genus *Carnobacterium* has been created from a group of meat-associated lactobacilli (Collins *et al.*, 1987) and *P. halophilus* has been removed from *Pediococcus* to form a new genus *Tetragenococcus* (Collins *et al.*, 1990). A separate genus, *Weissella*, has been suggested for a distinct phylogenetic cluster of heterofermentative LAB, consisting of species previously assigned to *Leuconostoc* or *Lactobacillus* (Collins *et al.*, 1993). Further, a rapidly evolving *Leuconostoc* species *L. oeni* is now considered to warrant a separate

genus, and *Oenococcus oeni* has been proposed (Dicks *et al.*, 1995). The current taxonomical schemes for the heterogeneous genera of *Lactobacillus* and *Pediococcus* are not in good agreement with the phylogenetic relationships revealed by 16S ribosomal DNA sequences, and hence, further changes are likely in the future (Stiles and Holzapfel, 1997; Axelsson, 1998). *Lactobacillus* is the largest of the LAB genera and currently comprises more than 50 species. The definition of the genus *Lactobacillus* includes essentially rod-shaped lactic acid bacteria and covers isolates with varying phenotypic, biochemical and physiological properties. Identification of many species of LAB, including lactobacilli, is difficult by simple phenotypic criteria, *e.g.* biochemical tests. Other methods are increasingly used, such as the determination of the mol% G+C of DNA, electrophoretic analysis of lactate dehydrogenase and soluble cellular proteins, ribosomal DNA sequencing, DNA-DNA hybridisation of genomic DNA etc. (Kandler and Weiss, 1986; Dicks and Vuuren, 1987; Mitsuoka, 1992; Klein *et al.*, 1995; Vandamme *et al.*, 1996; Tannock *et al.*, 1999).

On the basis of ribosomal DNA sequences, Gram-positive bacteria are divided into the *Clostridium* and the *Actinomycetes* subdivisions. All LAB are included in the *Clostridium* subdivision and form a cluster which phylogenetically lies in between the strictly anaerobic species (*e.g.* clostridia) and facultatively or strictly aerobic species (*e.g.* staphylococci and bacilli) (Kandler, 1984; Kandler and Weiss, 1986; Schleifer and Ludvig, 1995). The genus *Bifidobacterium*, earlier included in LAB but later discovered evolutionarily distant, is presently included in the *Actinomycetes* subdivision (Woese, 1987; Stackebrandt and Teuber, 1988; Vandamme *et al.*, 1996). However, particularly in the field of applied research, the term lactic acid bacteria continues to cover bifidobacteria (Klein *et al.*, 1998), and this understanding of LAB in the broad sense is also used in this study.

LAB have complex nutritional requirements. In addition to carbohydrates, they need amino acids, peptides, fatty acids or fatty acid esters, salts, nucleic acid derivatives, and vitamins for growth (Sharpe, 1981). A key feature in LAB metabolism is the ability to efficiently ferment a large array of carbohydrates to produce energy by substrate-level phosphorylation. LAB are unable to synthesize porphyrin molecules (*e.g.* heme), lack a true catalase as well as cytochromes and an electron transport chain and thus are unable to generate energy through oxidative phosphorylation (Axelsson, 1998). Few strains, however, apparently have a capacity to express a functional respiratory chain if heme is supplied in the growth medium (Whittenbury, 1964; Bryan-Jones and Whittenbury, 1969; Ritchey and Seeley 1976; Wolf *et al.*, 1991; Meisel *et al.* 1994). Two main carbohydrate fermentation pathways are utilized by LAB. Glycolysis via the Embden-Meyerhof pathway is used by the homofermentative LAB and produces almost exclusively lactic acid that is secreted by the cells. Heterofermentative LAB utilize the 6-phosphogluconate/phospho-ketolase pathway and secrete equal molar amounts of ethanol, acetate, $CO_2$ and lactic acid (Kandler and Weiss, 1986). The pattern of secreted end-products is significantly affected by environmental conditions which *e.g.* change pyruvate metabolism or provide external electron acceptors (Axelsson, 1998).

LAB have a wide distribution in nature. They grow in large numbers in nutrient-rich habitats containing carbohydrates, vitamins, peptides, and oligonucleotides (Kandler and Weiss, 1986). Typical features of environments rich in LAB are anaerobiosis, or low oxygen tension as well as acidicity (Kandler and Weiss, 1986). Such environments exist in habitats with decaying organic material, such as rotting plants, sewage or manure. In low numbers, LAB can be isolated from the surfaces of living plants (Keddie, 1959; Mundt and Hammer, 1968). LAB are used in fermentation of vegetables, *e.g.* in the production of sauerkraut or silage, where the low pH resulting from the secreted lactic acid prevents growth of other microbes. Milk and dairy products, meat and beverages can maintain the growth of large numbers of LAB, which can be inoculated for fermentative purposes or are

present as spoilage bacteria, *e.g.* lactobacilli in beer or slime-producing LAB in milk and meat products (Sharpe, 1981; Vandamme *et al.*, 1996; Stiles and Holzapfel, 1997).

## 1.2. LAB as constituents of the intestinal microflora

Major natural habitats of LAB are the gastrointestinal and urogenital tracts of humans and animals, which provide stable conditions and a continuous supply of nutrients in the form of ingested food and secretions of the host. In the intestinal tracts of mammals and avians, species of the genera *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium* are the dominating indigenous lactic microflora. Commonly recovered *Lactobacillus* isolates from the human gastrointestinal tract include *L. acidophilus*, *L. salivarius, L. casei, L. plantarum, L. fermentum, L. brevis* and *L. reuteri* (reviewed in Mikelsaar *et al.*, 1998). The frequency of *L. acidophilus* in the mammalian or avian gastrointestinal tracts and vagina has probably been overestimated, since there has been a tendency to group all isolates of homofermentative lactobacilli as *L. acidophilus* (Johnson *et al.*, 1980; Mitsuoka, 1992; Tannock, 1997), which on the basis of DNA-DNA hybridisation is presently divided into six homology groups A1-B2, or species: *L. acidophilus* (A1), *L. crispatus* (A2), *L. amylovorus* (A3), *L. gallinarum* (A4), *L. gasseri* (B1) and *L. johnsonii* (B2) (Johnson *et al.*, 1980; Lauer *et al.*, 1980; Fujisawa *et al.*, 1992). According to the current identification criteria, *L. crispatus, L. gasseri* and *L. johnsonii* are the most common species of the *L. acidophilus* group in the human intestine (Mitsuoka *et al.*, 1992; Song *et al.*, 1999; 2000). Most of the intestinal strains formerly identified by fermentation patterns as *L. fermentum* are now classified as *L. reuteri*, which forms a distinct group with a low chromosomal G+C content (Kandler and Weiss, 1986). *L. reuteri* is now regarded as the most prevalent heterofermentative *Lactobacillus* species in the intestinal tract of humans and other animals (Mitsuoka, 1992).

In humans, the numbers of LAB vary greatly in different sections of the digestive tract and rise gradually when descending down the alimentary canal towards the colon. Important host-mediated factors that affect the growth of bacteria in the GI tract include acidicity, secretions such as bile, salts, immunoglobulins, enzymes, exfoliated cells, mucins and tissue exudate as well as the peristaltic movement (Holzapfel *et al.*, 1998; Tannock, 1999). To resist peristalsis, bacteria either have to adhere to intestinal surfaces or multiply at a fast rate (Fuller, 1989). Since the peristaltic movement may be too rapid for significant bacterial multiplication (Drasar and Barrow, 1985), adherence to intestinal surfaces is probably an important bacterial factor contributing to succesful colonization at the upper intestinal regions (Savage, 1977; Fuller, 1989; Tannock, 1992). In the highly acidic conditions of the stomach, LAB most likely are transients and enter in the saliva and food (Savage, 1977; Lidbeck and Nord, 1993). In duodenum and jejunum, the bacterial densities are still relatively low, typically not exceeding $10^4$ cells/ml contents (Gorbach *et al.*, 1967). As aciduric bacteria (Tannock, 1992), lactobacilli as well streptococci are among the dominating species (Mitsuoka, 1992). In the distal ileum, the lumen contents are less acidic and the peristaltic movement slower and the bacterial densities are higher, $10^5$-$10^7$/ml contents have been reported (Gorbach *et al.*, 1967). Lactobacilli belong to the dominating flora with $10^3$-$10^7$ bacteria/g contents (Lidbeck and Nord, 1993). In the distal ileum, the composition of the flora gradually begins to resemble the complex flora of the colon (Gorbach *et al.*, 1967; Drasar *et al.*, 1969). In humans, the colon is the largest bacterial reservoir of the body. Movement of the contents is slow and travel through the colon takes 18-68 h (Mitsuoka, 1992), which allows the bacteria to multiply in the luminal contents. The bacterial communities in the colon are one of the most diverse in nature

(O'Sullivan, 2000), with over 400 described species (Moore and Holdeman, 1974; Fooks *et al.*, 1999). Bacterial numbers typically range in the region of $10^{11}$-$10^{12}$/g of contents and constitute approximately 30-50% of the faecal mass (Cummings and MacFarlane, 1991; Mitsuoka, 1992; Lidbeck and Nord, 1993). The majority of the flora is apparently represented by only 30–40 bacterial species (Finegold *et al.*, 1974; Moore and Holdeman, 1974; Drasar and Barrow, 1985). While culture-based methods for the enumeration and identification of intestinal bacteria are still commonly used, they easily give a biased view of bacterial composition as a large fraction, estimates ranging from 15 to 85%, of the microbial population cannot be cultivated by standard culture techniques (Langendijk *et al.*, 1995; Wilson and Blitchington, 1996; Suau *et al.*, 1999). Analysis of 16S ribosomal DNA sequences of fecal bacteria has revealed that a large number of the recovered sequences originate from novel bacterial species (Wilson and Blitchington, 1996; Zoetendal *et al.*, 1998; Suau *et al.*, 1999). Ribotyping combined with pulsed-field gel electrophoresis of restriction fragment length polymorphism of DNA, suggested that each human subject harbors their own unique collections of *Lactobacillus* or *Bifidobacterium* strains (McCartney *et al.*, 1996; Kimura *et al.*, 1997; Tannock, 1997). The most prevalent culturable bacteria in the colon are the obligately anaerobic Gram-negative bacteria *Bacteroides, Eubacterium, Bifidobacterium, Fusobacterium,* and *Peptostreptococcus* (Savage, 1977; Holzapfel, *et al.*, 1998; Vaughan *et al.*, 2000). Lactobacilli are recovered in moderate numbers $10^{4}$-$10^{8}$/g wet weight (Lidbeck and Nord, 1993), while bifidobacteria average $10^{10}$ cells/g and constitute 5-10 % of the bacterial flora (Mitsuoka, 1992). Another major reservoir of LAB is in the urogenital tract, especially the vagina, where lactobacilli are often numerically dominant bacteria (McGroarty, 1993; Andreu *et al.*, 1995).

In other monogastric mammals (*e.g.* pigs and rodents), the microbial flora and distribution of LAB in the gastrointestinal tract resemble those of humans in many aspects (Tannock, 1992). An exception is the *pars oesophagea* in pigs and the forestomach in rodents, both lined by a squamous, ceratinized non-secreting epithelium, heavily colonized by LAB (Tannock, 1992). In the chicken, the crop is composed of a squamous, ceratinized epithelium and heavily colonized by lactobacilli (Fuller and Turvey, 1971; Fuller, 1973; Sarra *et al.*, 1992), while bifidobacteria belong to the major flora of the caecum (Sarra, *et al.*, 1992).

## 1.3. LAB as probiotics

Lactic acid bacteria have a long history in biotechnology, especially in the manufacture and storage of food ingredients by fermentation processes. Several health-promoting effects by LAB in humans have been proposed (Table 1) (reviewed in Fernandes *et al.*, 1987; Fuller, 1989; Havenaar and Huis in't Veld, 1992; Lidbeck and Nord, 1993; McGroarty, 1993; Berg, 1998; Kasper, 1998; Salminen *et al.*, 1998; Atlas, 1999; Fooks *et al.*, 1999; Reid, 1999; Rowland, 1999; Burns and Rowland, 2000; Isolauri *et al.*, 2001; Perdigón *et al.*, 2001). Some of the probiotic effects have been documented in clinical tests, *e.g.* the successful treatment of rotavirus diarrhea in small children by LAB administration (Isolauri *et al.*, 1991).

The mechanisms by which LAB exert beneficial health effects are not well understood. The ability to adhere to and colonize the intestinal or the urogenital tracts, even if transiently, are probably important factors that contribute to the survival of LAB and thus help them to induce positive health effects. For this reason, adhesive properties have been proposed by many authors as one of the criteria for the selection of new strains for probiotic use (Table 2) (Goldin and Gorbach, 1992; Havenaar *et al.*, 1992; Salminen *et al.*, 1998; Reid, 1999; Dunne *et al.*, 1999; Morelli, 2000).

TABLE 1. Proposed health-promoting effects of LAB.

| Probiotic effect | Selected reference(s) |
|---|---|
| Prevention of intestinal infections | Isolauri *et al.*, 1991; Colombel *et al.*, 1987; Surawicz *et al.*, 1989; Saavedra *et al.*, 1994; McFarland *et al.*, 1995 |
| Immune stimulation | Halpern *et al.*, 1991; Kaila *et al.*, 1992 |
| Alleviation of allergy | Kalliomäki *et al.*, 2001 |
| Lowering of serum cholesterol | Gilliland *et al.*, 1985; Schaafsma *et al.*, 1998 |
| Anticarcinogenic effects | Goldin and Gorbach, 1984; Reddy, 1998; Rowland *et al.*, 1998 |
| Alleviation of lactose intolerance | Savaiano *et al.*, 1984; Marteau *et al.*, 1990 |

TABLE 2. Selection criteria for new probiotic strains (Reid, 1999)

| |
|---|
| Adherence to epithelial cells |
| Exclusion or reduction of pathogenic adherence |
| Ability to persist and multiply in intestinal or urogenital tracts |
| Production of acids, hydrogen peroxide, and bacteriocins antagonistic to pathogens |
| Resistance to vaginal microbicides, including spermicides |
| Safe and non-invasive, non-carcinogenic, and non-pathogenic organism |
| Ability to coaggregate and form normal, balanced flora |

## 2. ADHESIVE PROPERTIES IN LACTIC ACID BACTERIA

The importance of bacterial adhesion in infectious diseases is well established, and several molecular adhesion mechanisms of bacterial pathogens are known (reviewed in Karlsson, 1989; Korhonen *et al.*, 1990; Hultgren *et al.*, 1991; Patti and Höök, 1994). Adhesin-receptor interactions of commensal bacteria remain less well characterized, in particular, this holds for LAB and the role of adhesion in the colonization of commensal LAB has remained unclear. The intestinal microflora in adults is considered stable and prevents colonization by exogenous bacteria, a phenomenon referred to as "colonization resistance" (Waaij *et al.*, 1971) or "competitive exclusion" (Lloyd *et al.*, 1977). This phenomenon by the indigenous bacteria involves several mechanisms, such as occupation of available niches, secretion of growth-inhibitory factors (organic acids, hydrogen peroxide, bacteriocins) and non-specific activation of the immune system (Havenaar and Huis in't Veld, 1992). For indigenous or exogenous probiotic bacteria, direct evidence for the importance of adhesion as a colonization factor is lacking, although adhesiveness is considered as a criterium to choose a probiotic strain (Table 2).

Various tissue targets, ranging from tissue pieces and isolated epithelial cells to mucus and isolated ECM proteins, have been tested for LAB adherence (Table 3). The level of adhesion has been enumerated either by microscopic visualization or by using metabolically radiolabelled bacteria. While there are numerous studies published on the adhesive properties of LAB, the information remains rather descriptive, *i.e.* the adhesion

mechanisms are poorly known, and the studies mostly analyze a limited set of adhesion targets.Two approaches to prepare the epithelium for LAB adherence studies have been used. Either small pieces of intestinal epithelium have been dissected and used directly for adhesion and colonization assays (Sarem-Damerdji *et al*., 1995) or more frequently, individual epithelial cells have been isolated from tissue surfaces by mechanical scraping (Fuller, 1973), brushing (Barrow *et al.*, 1980) or by freezing the tissue followed by a rapid thawing (Spencer and Chesson, 1994).

The studies by Fuller (1973), Barrow (1980), Mäyrä-Mäkinen *et al.* (1983) and Yuki *et al.* (2000) suggested species specificity in LAB adherence (Table 3). However, Kotarsky and Savage (1979), Lin and Savage (1984), Conway *et al.* (1987), Jacobsen *et al* (1999) and Todoriki *et al.* (2001) reported binding of lactobacilli to non-host tissue targets, leaving the question of host species-specificity open. In a few studies, dairy strains or LAB isolates from environmental samples, such as plants, have shown adhesiveness to mammalian epithelial cells (Sarem-Damerdji *et al.*, 1995; Adlerberth *et al*., 1996; Sarem *et al.*, 1996; Lehto and Salminen, 1997a; Jacobsen *et al.*, 1999) (Table 3). Collectively, LAB seem to express preference for adhesion to epithelial cells of their own isolation hosts but a strict host-species specificity of the adhesion seems unlikely.

## 2.1. Adhesion to epithelial cell lines

Caco-2 cell line isolated from a human colonic adenocarcinoma (Fogh *et al*., 1977) has been commonly used in adhesion studies with LAB (Table 3). This confluent cell line differentiates to a polarized cell layer with an apical and a basolateral membrane separated by tight junctions (Pinto *et al.*, 1982; 1983; Simon and Fuller, 1985). The apical surface faces the growth medium and developes into a functional brush border expressing intestinal hydrolases, while the basolateral surface attaches to the culture plate and expresses peptide receptors involved in the control of intestinal hydroelectrolytic secretion (Laburthe and Aminaroff, 1992). The Caco-2 cell layer structurally resembles differentiated enterocytes at the intestinal epithelium (Pinto *et al.*, 1983). HT-29 is another cell line isolated from human adenocarcinoma (Fogh *et al*., 1977) and resembles Caco-2 cell line in the ability to differentiate to a polarized cell layer (Fogh *et al*., 1977; Pinto *et al.*, 1982; Pinto *et al.*, 1983). Intestine 407 cell line has been used in bacterial adhesion studies and is derived from a malignant small intestine of a 2-month old human embryo (Henle and Deinhardt, 1957). It does not differentiate to a polarized cell layer and secretes a complex extracellular matrix visible in the electron microscope (Favre-Bonte *et al*., 1995). As seen in Table 3, adhesion to these cell lines is commonly expressed by LAB isolates. The mechanisms involved are not known, in one report mannosylconjugates were proposed as adhesion targets (Adlerberth *et al*., 1996).

## 2.2. Adhesion to extracellular matrix

The extracellular matrix (ECM) is a relatively stable structure that underlies epithelia and surrounds connective tissue cells. The ECM is involved in cellular development and function, growth and differentiation, cell adhesion as well as migration. The main components of ECM belong to four major classes of molecules: the collagens, proteoglycans, structural glycoproteins (laminin, fibronectin, vitronectin, entactin) and elastin (Hay, 1991; Haralson and Hassel, 1995). Basement membranes (BM) are a class of

TABLE 3. Adhesive properties of LAB

| Adhesion target | Organism | Source | Comments | Reference |
|---|---|---|---|---|
| Tissue pieces | | | | |
| Mouse or rat stomach | *L. fermentum* | Mouse and rat stomach | Adhesion correlated with host of isolation | Conway and Adams, 1989; Conway and Kjelleberg, 1989 |
| Pig stomach | *L. fermentum* | Pig stomach | The test strain was adhesive | Henriksson *et al.*, 1991 |
| Horse and rat stomach | Lactobacilli, mainly *L. salivarius* and *L. reuteri* | Horse stomach | 65% of isolates adhesive, no adhesion to rat stomach | Yuki *et al.*, 2000 |
| Human colon | *L. acidophilus, L. casei, L. plantarum, L. paracasei* | Human colon and feces, dairy strains | Variable adhesiveness, *L. rhamnosus* GG moderately adhesive | Sarem-Damerdji *et al.*, 1995 |
| Isolated epithelial cells | | | | |
| Chicken crop | Lactobacilli, not identified | Avian crop and feces, mammalian feces | Avian isolates adhesive, other isolates negative | Fuller, 1973 |
| | Lactobacilli, *e.g. L. salivarius* | Chicken crop and intestine | 45% of isolates adhesive | Garriga *et al.*, 1998 |
| Chicken ileum | *L. acidophilus* group, *L. fermentum, L. brevis* | Chicken intestine | Isolates exhibited variable adhesiveness | Jin *et al.*, 1996 |
| Pig *pars esophagea* | *L. fermentum, L. salivarius, L. acidophilus*, other lactobacilli and streptococci | Pig *pars esophagea* | All isolates were adhesive | Fuller *et al.*, 1978 |
| | Lactobacilli and streptococci, not identified | Pig and calf intestine; feces and intestinal contents of other mammals and chicken, dairy strain | Pig and chicken isolates exhibited adhesiveness | Barrow *et al.*, 1980 |
| Pig small intestine | *L. delbrueckii, L. acidophilus, L. fermentum*, other lactobacilli and streptococci | Pig and calf intestine and feces, a plant isolate, dairy strain | Pig isolates exhibited adhesiveness, calf isolates weakly adhesive, dairy and plant isolates negative | Mäyrä-Mäkinen *et al.*, 1983 |

| | | | | |
|---|---|---|---|---|
| Pig jejunum | *L. fermentum, L. salivarius, L. acidophilus*, other lactobacilli | Piglet stomach and small intestine | Adhesiveness commonly exhibited | Spencer and Chesson, 1994 |
| Pig ileum | *L. acidophilus*, other LAB | Human, dairy strains | Human isolates adhesive | Conway *et al.*, 1987 |
| Calf small intestine | *L. fermentum*, other lactobacilli and streptococci | Calf intestine and feces, a plant isolate, dairy strain | Calf isolates exhibited adhesivess, dairy and plant isolates negative | Mäyrä-Mäkinen *et al.*, 1983 |
| Mouse stomach | Lactobacilli, not identified | Pig and mouse | Adhesion did not correlate with host of isolation | Kotarsky and Savage, 1979 |
| Human ileum and colon | *L. plantarum* | Human intestine and colon, fermented drink | Human isolates adhesive, isolates from fermented drink weakly adhesive | Adlerberth *et al.*, 1996 |
| Human ileum | *L. acidophilus*, other LAB | Human, dairy strains | Human isolate was adhesive | Conway *et al.*, 1987 |
| Human vagina | Lactobacilli, not identified | Human vagina | Large variability, most isolates poorly adhesive | Andreu *et al.*, 1995 |
| Epithelial cell lines | | | | |
| Caco-2 | *L. rhamnosus* GG, bifidobacteria | Human feces, dairy strain | *L rhamnosus* GG adhesive, other strains negative | Elo *et al.*, 1991 |
| | Various lactobacilli | Human, pig, chicken, plant origin and dairy strains | 30% of strains adhesive, no species specificity | Chauvière *et al.*, 1992b |
| | *L. acidophilus* | Human feces | The strain was adhesive, a secreted proteinaceous adhesion factor was suggested | Coconnier *et al.*, 1992 |
| | Bifidobacteria | Human feces | 50% of strains adhesive | Bernet *et al.*, 1993 |
| | *L. acidophilus, L. gasseri, L. delbrueckii* | Human feces, a dairy strain | Isolates from feces were adhesive | Greene and Klaenhammer, 1994 |
| | Bifidobacteria | Undefined | Majority of strains adhesive | Crociani *et al.*, 1995 |
| | *L. acidophilus, L. delbrueckii, L. leichmannii* | Human feces, a dairy strain | Variable adhesiveness, dairy strain adhesive | Sarem *et al.*, 1996 |
| | *Lactobacillus, Lactococcus, Propionobacterium* | Human feces, a dairy strain | All strains were adhesive | Lehto and Salminen, 1997a |

| | | | | |
|---|---|---|---|---|
| | Lactobacilli | Probiotic or dairy strains | Variable adhesiveness | Tuomola and Salminen, 1998 |
| | Lactobacilli, *e.g. L. fermentum, L. plantarum, L. acidophilus* group, *L. reuteri, L. rhamnosus* | Human, pig, rat, fermented maize, sourdough | Adhesiveness of human isolates common; a pig, sourdough and dairy isolates also adhesive | Jacobsen *et al.*, 1999 |
| | *L. casei, L. paracasei, L. rhamnosus* | Human bacteremia | Majority of strains (90%) were adhesive | Kirjavainen *et al.*, 1999 |
| | *Lactococcus lactis* ssp. *lactis* | Undefined | 70 % of strains were adhesive | Kimoto *et al.*, 1999 |
| | *L. acidophilus* group, *L. fermentum* | Human, chicken, hog, mouse, calf, fermented molass | Variable adhesiveness, no species specificity | Todoriki *et al.*, 2001 |
| HT-29 | *L. acidophilus* | Human feces | The strain was adhesive | Coconnier *et al.*, 1992 |
| | *L. plantarum* | Human intestine and colon; fermented drink | Adhesiveness mannose-sensitive | Adlerberth *et al.*, 1996 |
| HT-29 MTX | *B. breve* | Human feces | The strain was adhesive | Bernet, *et al.*, 1993 |
| Intestine 407 | *L. acidophilus, L. delbrueckii, L. leihmannii* | Human feces, a dairy strain | Variable adhesiveness, dairy strain adhesive | Sarem *et al.*, 1996 |
| Hs0074 | *L. acidophlus,* other lactobacilli | Human, chicken, dairy strains | Calcium dependent adhesion observed | Kleeman and Klaenhammer, 1982 |
| Mucus | | | | |
| Piglet | *L. fermentum* | Pig stomach | The strain was adhesive | Rojas and Conway, 1996 |
| Rat | *L. acidophilus* group | Human, chicken, pig, dairy strains | The binding of extracted surface proteins was studied. Homology group B1 showed highest binding activity | Matsumura *et al.*, 1999 |
| Human | *L. rhamnosus, L. acidophilus* group, *L. salivarius, L. paracasei, Bifidobacterium* | Human feces | Strains exhibited variable adhesiveness | Kirjavainen *et al.*, 1998 |
| | Various LAB | Probiotic products, dairy strains | Lactobacilli were adhesive | Tuomola *et al.*, 1999 |
| | Bifidobacteria | Human feces, dairy strains | All strains were adhesive | Ouwehand *et al.*, 1999 |

| | | | | |
|---|---|---|---|---|
| | *L. casei, L. paracasei, L. rhamnosus* | Human bacteremia | Strains were adhesive | Kirjavainen *et al.*, 1999 |
| Purified ECM proteins | Various lactobacilli | Human, animal, plant and dairy sources | 75% bound soluble CnI[a] | Aleljung *et al.*, 1991 |
| | Lactobacilli | Human vagina | Binding of soluble Fn[a] common | Nagy *et al.* 1992 |
| | *L. rhamnosus, L. paracasei* and other lactobacilli | Human IE and mouth | Fn and Fb[a] binding at low pH, CnI and V binding higher than CnIII and IV | Harty *et al.*, 1994 |
| | Various lactobacilli | Human mouth | Majority of strains bound solubilized CnI and CnII | McGrady *et al.*, 1995 |
| | Bifidobacteria | Human, pig, chicken feces; human intestine | 15% adhered to immobilized CnI and V, no adhesion to Lam[a], Fn, CnIII and CnIV | Mukai *et al.*, 1997 |
| Platelets | *L. rhamnosus, L. paracasei,* other lactobacilli | Human IE and mouth | Aggregation commonly exhibited in IE and other isolates | Harty *et al.*, 1993; Harty *et al.*, 1994 |
| | *L. rhamnosus* GG | Human feces | Aggregation not observed | Korpela *et al.*, 1997 |
| | *L. casei, L. paracasei, L. rhamnosus* | Human bacteremia | 40% of strains aggregated platelets | Kirjavainen *et al.*, 1999 |
| Erythrocytes | *L acidophilus* group | Human, rat, pig, calf, chicken | Most strains weakly hemagglutinative | Yamada *et al.*, 1994 |
| | Lactobacilli, not identified | Human vagina | Majority of isolates hemagglutinated | Andreu *et al.*, 1995 |
| | *L. plantarum* | Human and environment | Half of strains hemagglutinated weakly | Adlerberth *et al.*, 1996 |
| Agglutination of yeast | *L. plantarum* | Human and environment | Half of strains agglutinated yeast cells weakly | Adlerberth *et al.*, 1996 |

[a] Cn, collagen; Fn, fibronectin; Fb, fibrinogen; Lam, laminin.

specialized extracellular matrices that appear as amorphous sheet-like structures between a cell layer and a thick collagenous stroma, *e.g.* between the intestinal epithelium and the underlying connective tissue. Type IV collagen and laminin are the major components of BM which interact to form a network structure.

Adhesion to extracellular matrix proteins is expressed by several pathogenic bacteria and thought to contribute to their invasiveness (Westerlund and Korhonen, 1993). Among LAB strains, adhesiveness to ECM proteins has also been reported (Table 3). Aleljung *et al.* (1991) demonstrated that binding to solubilized collagen is frequently expressed among *Lactobacillus* strains of different origins; 75% of their LAB isolates bound solubilized type I collagen. Lactobacilli isolated from dental caries lesions showed similar levels of binding to solubilized type I collagen (McGrady *et al.*, 1995). Binding of LAB to another major ECM protein, fibronectin, was expressed by 17 % of human vaginal *Lactobacillus* isolates (Nagy *et al.*, 1992). In bifidobacteria, adhesion to ECM proteins seems less frequent. Two isolates from a collection of 13 *Bifidobacterium* strains adhered to immobilized type I and type V collagens, but none to laminin, fibronectin, or type III and IV collagens (Mukai, *et al.*, 1997). Biological function(s) of ECM-binding by LAB have remained open. Harty *et al.* (1993; 1994) suggested a connection between the binding of ECM proteins by oral lactobacilli and their ability to cause infective endocarditis (IE) in humans. Soluble fibronectin in saliva might coat the bacterial cells, which invade into the bloodstream through damaged tissue sites, and fibronectin could form a bridge between bacteria and the damaged endothelium of the heart valve. Harty *et al.* (1994) also reported efficient binding to immobilized collagen types I and V by lactobacilli associated with IE, type V collagen in particular has been demonstrated at sites of endothelial damage (Kerényi *et al.*, 1985). Infectious diseases caused by *Lactobacillus* are rare and the role of ECM binding in IE remains speculative. Many pathogens adhere to ECM (Westerlund and Korhonen, 1993; Patti and Höök, 1994), and adherence of LAB to subintestinal ECM can, on the other hand, be a probiotic characteristic. Such an adherence may protect the host against bacterial invasion at damaged epithelia where the ECM has become exposed.

## 2.3. Adhesion to mucus

Mucus is a gel-like structure secreted by the goblet cells and mucosal glands, and covers the intestinal epithelium. The main structural components of mucus are large molecules (>$2x10^6$ Da) called mucins that are polymers of a highly glycosylated protein monomer and held together by disulfide bonds (Mantle *et al.*, 1984; Bell *et al.* 1985). Other components of mucus include protein, lipid, DNA and membrane fragments from epithelial cells (Mantle and Husar, 1994). Mucins give physical protection to epithelia and provide lubrication for smooth transit of ingested food material (Tannock, 1999). The mucus layer diminishes the access of harmful bacteria to intestinal tissue surfaces by providing a physical barrier that pathogens must penetrate before invading the intestinal epithelium (Cover and Aber, 1989; Mantle and Husar, 1994). Degradation of mucus by mechanical forces or enzymes releases partially degraded or denatured mucins into the intestinal lumen, which may further enhance mucosal protection by binding to bacterial adhesins (Mantle and Husar, 1994). As the outermost lumenal layer, mucus is the first intestinal component or surface that LAB are likely to contact before they reach epithelial cells. Hence, it can have a substantial role in the colonization of intestinal surfaces. There are a number of studies that report binding of LAB strains to mucus from animals and humans (Table 3).

# 3. Adhesins of lactic acid bacteria

Proteinase treatment of LAB cells has decreased adhesion in a number of studies, suggesting a proteinaceous adhesion molecule (Fuller, 1975; Conway and Kjelleberg 1989; Henriksson *et al.*, 1991; Chauvière *et al.*, 1992b; Reid *et al.*, 1993; Bernet *et al.*, 1994; Greene and Klaenhammer, 1994; Adlerberth *et al.*, 1996). The adherence of *L. fermentum* strain 737 to mouse stomach squamous epithelium and *L. fermentum* 104 to pig small intestinal mucus were suggested to be promoted by small secreted proteins, but they remain uncharacterized (Conway and Kjelleberg, 1989; Rojas and Conway, 1996). A proteinaceous bridging molecule was proposed to mediate *L. acidophilus* strain BG2FO4 adhesion to Caco-2 cells (Coconnier *et al.*, 1992), but this was not confirmed in a more detailed study by Greene and Klaenhammer (1994). The S-layer of an *L. acidophilus* isolate may contribute to bacterial adhesion to chicken epithelium, since changes in the S-layer, as seen in the electron microscope, affected LAB adhesiveness (Schneitz *et al.*, 1993).

A well-characterized LAB adhesin is the cell-surface protein Cnb of *L. reuteri* NCIB 11951 which binds collagen type I (Aleljung *et al.*, 1994). Cnb is 29 kDa in molecular size and represents 0.5 % of total proteins of the cell. The predicted amino acid sequence of Cnb shows two motifs typical of extracellular solute-binding receptors of bacteria (Roos *et al.*, 1996). Highest homology was found with a subunit of a putative ABC transport protein of *Bacillus subtilis*. An open reading frame upstream of *cnb* is homologous to an ATP-binding component of these systems, which strengthens the argument that Cnb is part of an ABC transporter operon. Another collagen-binding protein, 31 kDa, was isolated from the strain *L. reuteri* NCIB 11951 (Aleljung *et al.*, 1994). The two proteins cross-react immunologically, indicating that they are related proteins, but the N-terminal amino acid sequences show low homology (Aleljung, *et al.*, 1994). The *cnb* gene has been expressed in *E. coli* and the resulting recombinant protein was shown to bind solubilized type I collagen (Roos *et al.*, 1996). Twenty N-terminal amino acids of a 29 kDa surface protein from *L. fermentum* RC-14 share 100% identity with the N-terminus of Cnb, similarity for the rest of their sequences is not yet known. The protein was suggested to inhibit the adhesion of *Enterococcus faecalis* to polystyrene (Heinemann *et al.*, 2000) and to bind collagen (Howard *et al.*, 2000) and could be a related adhesin molecule of *L. fermentum*. A large (358 kDa) surface protein, Mub, from a pig intestinal isolate of *L. reuteri* 1063 mediates bacterial binding to pig and hen intestinal mucus. The deduced amino acid sequence of the *mub* gene contains two types of large amino acid repeats (Roos *et al.*, 2000).

Adhesion involving carbohydrates in LAB has been observed in many studies, but the adhesive structures have not been identified further (Fuller, 1975; Henriksson *et al.*, 1991; Coconnier *et al.*, 1992; Greene and Klaenhammer, 1994). A major cell-surface molecule of LAB, the lipoteichoic acid, is possibly an adhesion factor in the attachment of *Lactobacillus johnsonii* strain La1 to human Caco-2 cells (Granato *et al.*, 1999). Adhesion was reduced to 60% in the presence of 150 µg/ml lipoteichoic acid extracted from this strain, but direct interaction of lipoteichoic acid with epithelial cells was not demonstrated.

## 4. Effect of lactic acid bacteria on the colonization, adhesion and invasion of pathogenic bacteria

One of the health-promoting effects of LAB is the prevention of microbial infections in the gastrointestinal and urogenital tracts (Table 1). Possible mechanisms include immune modulation of the host and strengthening of the gut mucosal barrier against pathogens (Holzapfel *et al.*, 1998; Kasper, 1998). LAB are known to inhibit the growth of pathogenic bacteria *in vitro* (Gilliland and Speck, 1977; Chateau *et al.*, 1993; Drago *et al.*, 1997; Hudault *et al.*, 1997; Dunne *et al.*, 1999) and secrete antimicrobial compounds, such as lactic acid, fatty acids, hydrogen peroxide or bacteriocins (Havenaar *et al.*, 1992; McGroarty, 1993). Adherence of LAB to intestinal epithelium could prevent pathogen colonization by steric hindrance or competition for epithelial receptors (McGroarty, 1993). In animal feeding experiments, the ingestion of LAB has decreased the colonization ability of enteric pathogens in most of the studies (Table 4). In humans, clinical studies have aimed at the prophylaxis or treatment of microbial infections with varying efficiency. Many of the studies suffer from lack of a sufficient number of subjects, proper controls, and statistical analysis of the results (Kasper, 1998). Most convincing effects have been obtained in the decrease of rotavirus infections by LAB as well as in the prevention of pathogen overgrowth during antibiotic therapy (Table 4).

Adhesion to the intestinal epithelium is important for invading pathogens and a determinant in host as well as tissue tropism of the bacteria (Finlay and Cossart, 1997; Finlay and Falkow, 1997; Klemm and Schembri, 2000). Several *in vitro* models have been used to assess the effect of LAB on the adhesion and invasion of pathogenic bacteria. The epithelial cell line Caco-2 has often been used, and inhibitory effects against the adhesion and invasion of pathogens, such as *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, *Listeria monocytogenes* and *E. coli*, have been reported (Table 5). However, some of the results were obtained by including the acidic growth medium of the LAB strains in the assays (Chauvière *et al.*, 1992b; Bernet *et al.*, 1993; Coconnier *et al.*, 1993). It was later suggested that the low pH is deleterious to Caco-2 cells and leads to cell death (Greene and Klaenhammer, 1994; Lehto and Salminen, 1997b).

# 5. S-LAYER PROTEINS

Surface-layer (S-layer) proteins are found in more than 300 species in Bacteria and Archaea (Messner and Sleytr, 1992). S-layers are two-dimensional paracrystalline sheets that completely cover the bacterial cell surface. They mostly consist of a single protein subunit, ranging 40-200 kDa in size, that assembles into the two-dimensional S-layer sheet (Sleytr *et al.*, 1993). The S-layer sheet is attached to the underlying cell wall non-covalently and can usually be dissociated and solubilized into protein monomers by hydrogen bond-breaking agents (Pum and Sleytr, 1999; Sleytr and Beveridge, 1999).

TABLE 4. Effect of LAB strains on the colonization of enteric pathogens

| Host | LAB | Pathogen/infection | Comments | Reference(s) |
|---|---|---|---|---|
| Mouse | *L. rhamnosus* GG | *S. typhimurium* | Carriage of *Salmonella* shortened | Hudault *et al.*, 1997 |
| | Various LAB | *S. typhimurium* | Protection of pathogen invasion observed | Perdigón *et al.*, 2001 |
| Rat | *L. salivarius* | *E. coli* | Suppression of pathogen growth | Cole and Fuller, 1984 |
| | Undefined | *S. enteritidis* | Mortality rate reduced | Hitchins *et al.*, 1985 |
| | Undefined | *S. typhimurium* | Number of *Salmonella* reduced | Bovee-Oudenhoven, 1996 |
| Chicken | *Lactobacillus, Enterococcus* | *S. kedougou* | Number of *Salmonella* in caeca not reduced | Hinton and Mead, 1991 |
| | Undefined | *S. typhimurium* | Persistence of *Salmonella* in feces shortened | Salvat *et al.*, 1992 |
| | *L. salivarius* | *S. enteritidis* | Prevention of colonization by *Salmonella* | Pascual *et al.*, 1999 |
| | Lactobacilli | *S. infantis* and *E. coli* | No significant effect | Adler and DaMassa, 1980 |
| | Lactobacilli and bifidobacteria | *Salmonella* | No significant effect | Stavric *et al.*, 1992 |
| Rabbit | *Ent. faecium* | Diarrhea | Prophylactic effect | Wadström, 1984 |
| Pig | *L. acidophilus* | Diarrhea | Prophylactic effect | Kohler and Bohl, 1964 |
| | *Ent. faecium* | " | Prophylactic effect | Underdahl *et al.*, 1982 |
| Calf | Undefined | *E. coli* O157:H7 | Carriage of pathogen reduced | Zhao *et al.*, 1998 |
| Human | Lactobacilli | Traveller's diarrhea | No significant effect | Pozo-Olano *et al.*, 1978 |
| | Lactobacilli | " | No significant effect | Pearce and Hamilton, 1974 |
| | Mixture of LAB | " | Prophylactic effect | Black *et al.*, 1989 |
| | *L. rhamnosus* GG | " | Variable results | Oksanen *et al.*, 1990 |
| | Lactobacilli | " | No significant effect | Katelaris *et al.*, 1995 |
| | *L. rhamnosus* GG | " | Prophylactic effect | Hilton *et al.*, 1997 |
| | *E. faecium* SF68 | Diarrhea in children | Promotion of recovery | Bellomo *et al.*, 1982 |
| | *L. rhamnosus* GG | Rota-virus diarrhea in children | Promotion of recovery | Isolauri *et al.*, 1991; Kaila *et al.*, 1992;Isolauri *et al.*, 1994; Majamaa *et al.*, 1995 |
| | *B. bifidum, S. thermophilus* | " | Prophylactic effect | Saavedra, *et al.*, 1994 |
| | *L. rhamnosus* GG | Antibiotic-associated diarrhea (AAD) | Promotion of recovery | Biller *et al.*, 1995; Gorbach *et al.*, 1987 |
| | *B. longum* | AAD | Prophylactic effect observed | Colombel *et al.*, 1987 |
| | *L. rhamnosus* GG | AAD | Prophylactic effect observed | Siitonen *et al.*, 1990 |
| | *L. rhamnosus* GG | AAD in children | Reduction in the frequency of diarrhea | Arvola *et al.*, 1999 |
| | *L. rhamnosus GG* | *Klebsiella oxytoca* | No significant effect | Grönlund *et al.*, 1997 |

TABLE 5. Effect of LAB strains on the adhesion and invasion of pathogens

| Adhesion/ invasion target | LAB | Pathogen | Comments | Reference(s) |
|---|---|---|---|---|
| Human uroepithelium | Lactobacilli | Uropathogens | Inhibition of adhesion | Chan *et al.*, 1985 |
| Pig jejunum | *L. fermentum*, other lactobacilli | *E. coli* (ETEC) | No inhibition of adhesion | Spencer and Chesson, 1994 |
| Chicken ileum | *L. acidophilus, L. fermentum* | *S. pullorum, S. typhimurium, S. enteritidis* | Adhesion reduced with variable efficiencies | Jin *et al.*, 1996 |
| | *L. acidophilus, L. fermentum* | *E. coli* | No inhibition of adhesion | Jin *et al.*, 1998 |
| Caco-2 | *L. rhamnosus* GG | *S. typhimurium* | Inhibition of invasion only in acidic conditions | Hudault *et al.*, 1997 |
| | *L. acidophilus* LB | *E. coli* (EPEC), *Y. pseudotuberculosis, L. monocytogenes, S. typhimurium* | Inhibition of adhesion and invasion | Coconnier *et al.*, 1993; Coconnier *et al.*, 1997; Coconnier *et al.*, 2000 |
| | Bifidobacteria | *E. coli, Y. pseudotuberculosis, S. typhimurium* | Inhibition of adhesion and invasion | Bernet *et al.*, 1993 |
| | Lactobacilli | *E. coli* (ETEC) | Inhibition of adhesion | Chauvière *et al.*, 1992a |
| | *L. crispatus, L. reuteri* | *E. coli* (ETEC), *S. typhimurium, E. faecalis* | Both strains inhibited pathogen adhesion | Todoriki *et al.*, 2001 |
| Mucus | *L. fermentum* | *E. coli* K88 | Inhibition of adhesion to piglet ileal mucus | Blomberg *et al.*, 1992; Ouwehand and Conway, 1996 |
| Gangliotetraosyl-ceramide | *B. longum* | *E. coli* (ETEC) | Adhesion inhibited by a secreted protein | Fujiwara *et al.*, 1997; 1999 |
| Polystyrene | *L. fermentum* | *E. faecalis* | Adhesion inhibited by a 29 kDa surface protein | Heinemann *et al.*, 2000 |
| Plastic and glass | *L. casei* and *Str. hyointestinalis* | *E. faecalis* | Inhibition of adhesion | Millsap *et al.*, 1994 |
| Glass | *L. rhamnosus, L. fermentum, L. acidophilus* | *E. faecalis* | Adhesion inhibited by secreted compounds | Velraeds *et al.*, 1996 |

S-proteins are major proteins in the bacterial cell and constitute 10-15 % of the total protein in the cell (Boot and Pouwels, 1996). It has been estimated that $5x10^5$ protein monomers are needed to cover the entire cell surface (Sleytr and Messner, 1988). This necessitates an efficient expression and secretion machinery for the S-protein, since approximately 500 copies of the monomer per second need to be synthesized in exponentially growing bacteria (Sleytr and Beveridge, 1999). The primary amino acid sequences of S-proteins are not well conserved in different bacterial species, but their overall amino acid compositions are similar. They contain a relatively high number of threonine, serine and hydrophobic residues, but no or only a few cysteines or methionines (Boot and Pouwels, 1996). Most S-proteins are weakly acidic with isoelectric points ranging from 3 to 6 (Sleytr and Sára, 1997). In LAB however, the pI values range between 9 and 10 (Vidgrén *et al.*, 1992; Boot *et al.*, 1995; Boot and Pouwels, 1996; Callegari *et al.*, 1998). Many S-proteins are glycosylated or phosphorylated (reviewed in Sleytr and Messner, 1988; Sleytr and Sára, 1997; Sleytr and Beveridge, 1999; Fernandez and Berenguer, 2000). Several S-layer proteins, extracellular enzymes and outer membrane proteins have an N- or C-terminal S-layer homology domain (SLH) (Lupas *et al.*, 1994). These conserved motifs are ca. 55 amino acids long and mediate binding of surface proteins to peptidoglycan (Lemaire *et al.*, 1995; Olabarría *et al.*, 1996; Egelseer *et al.*, 1998) but are lacking in the sequences of LAB S-layer proteins (Engelhardt and Peters, 1998).

Various functions have been described for the S-layers (Table 6). The S-layer of the fish pathogen *Aeromonas salmonicida* mediates bacterial adhesion to the basement membrane proteins laminin and type IV collagen as well as to fibronectin and vitronectin (Noonan and Trust, 1997). The human enteric pathogen *Yersinia enterocolitica* expresses an S-layer-like surface protein, YadA, which forms fibrillar structures on the bacterial surface that mediate adherence to laminin, fibronectin and several types of collagen (Emödy *et al.*, 1989; Schulze-Koops *et al.*, 1992, Tertti *et al.*, 1992; Tamm *et al.*, 1993).

S-layers are attractive candidates for use in nanotechnological applications, since they form a regular paracrystalline array with high periodicity (reviewed in Sleytr and Sára, 1997; Sleytr and Beveridge, 1999; Pum and Sleytr, 1999). They are being developed as alternatives for synthetic membranes, for example as supporting matrices for enzymes, antibodies or other functional molecules. S-layers have a high porosity, ca. 70 % of surface area is made of pores of diameters suitable for *e.g.* ultrafiltration purposes (Sleytr and Messner, 1988; Sleytr and Sára, 1997; Pum and Sleytr, 1999). By genetic techniques, foreign peptides can be fused with S-proteins and be presented on the bacterial cell surface. The SLH domain of the S-protein of *Bacillus anthracis* was used as a cell wall anchor to express tetanus toxin fragment C on the bacterial cell surface (Mesnage *et al.*, 1999a; 1999b) and a pilin epitope of 12 amino acids was surface-displayed as an internal fusion with the *Caulobacter crescentus* S-protein (Bingle *et al.*, 1997; Hahn *et al.*, 1997). The S-layers of LAB as carriers of foreign molecules are discussed in chapter 6.

## 5.1. S-layer proteins in lactic acid bacteria

S-layer proteins are frequently found in the *L. acidophilus* group. They are expressed by isolates of the DNA homology groups A1-A4 (*L. acidophilus, L. crispatus, L. amylovorus* and *L. gallinarum*), but are absent in the homology groups B1 and B2 (*L. gasseri* and *L. johnsonii*) (Masuda and Kawata, 1983). The gene sequences of one *L. brevis* (Vidgrén *et al.*, 1992), two *L. acidophilus* (Boot *et al.*, 1993; 1995), one *L. crispatus* (Ventura *et al.*, GenBank, accession number AJ007839), and eight *L. helveticus* (Callegari *et al.*, 1998; Ventura *et al.*, 2000) S-proteins are known and are deposited in Genbank (National Center for Biotechnology Information, Bethesda, Md., USA). The strains of the homology groups

A1-A4 possess two S-layer genes, of which only one is expressed under laboratory conditions (Boot *et al.*, 1996a). *L. acidophilus* group strains have the capacity to change the S-layer expression through an inversion of a 6 kb chromosomal segment (Boot *et al.*, 1996b). The inversion takes place between conserved regions in the 5´ upstream sequences of the S-layer genes. In *L. acidophilus* strain ATCC 4356, the genes *slpA* and *slpB* are in an opposite orientation on a 6 kb chromosomal segment. During inversion, this segment changes orientation and the promoterless *slpB* replaces *slpA* behind the S-promoter in the expression cassette. Two promoter sequences are present in front of the expression site, but only the most downstream promoter is active at all growth phases (Boot *et al.*, 1996a). Transcription of *slpA* gives a relatively stable mRNA with an estimated half-life of 15 min. An untranslated leader sequence of ca. 200 nucleotides forms a stable hairpin-loop structure, which was suggested to protect the mRNA from degradation and thereby increase its stability (Boot, *et al.*, 1996).

TABLE 6. S-layers with defined biological functions

| Organism | S-layer protein | Function | Reference(s) |
|---|---|---|---|
| *Aeromonas salmonicida* | VapA | Binding to ECM proteins, virulence factor in fish, protection against bactericidal activity of serum | Ishiguro *et al.*, 1981; Munn *et al.*, 1982; Doig *et al.*, 1992; Trust *et al.*, 1993 |
| *Campylobacter fetus* | SapA | Virulence factor in humans and ovine abortion, protection against bactericidal activity of serum, resistance to phagocytosis | Blaser *et al.*, 1987; 1988; Pei and Blaser, 1990; Blaser and Pei, 1993; Grogono-Thomas *et al.*, 2000 |
| *Bacillus stearothermophilus* | | Attachment site for extracellular amylase | Egelseer *et al.*, 1995 |
| *Thermoanaerobacterium thermosulfurigenes* | | Attachment site for extracellular pullulanase | Matuschek *et al.*, 1994 |
| *Methanocorpusculum sinense* | | Maintenance of cell shape | Pum *et al.*, 1991 |

## 6. LACTIC ACID BACTERIA AS CARRIERS OF FOREIGN MOLECULES

Lactic acid bacteria are attractive candidates as carriers of foreign proteins, *e.g.* as live vaccines. They enable localized delivery of antigens to stimulate the local immune system, especially the secretory IgA type immune response. A systemic immune response can also be elicited through interaction with the mucosal immune system. In recent years interest in LAB as vaccine carriers has increased, since they have a GRAS status (generally regarded as safe) and are commensals of humans, LAB have adjuvant activity, are easily administered by the oral route, and can be cheaply produced in large-scale industrial fermentation processes (reviewed in Mercenier *et al.*, 2000). Further, LAB are resistant to acidic conditions and can survive the passage through the stomach. As normal residents of the intestinal tract, certain LAB *e.g.* lactobacilli, have the potential for colonization and thus, can persist in the intestine to induce the immune system more efficiently (Mercenier *et al.*, 2000).

The foreign molecules have been expressed in LAB in three compartments: intracellularly in the cytoplasm, on the cell surface, or as a molecule secreted into the growth medium. Intracellular expression has mostly been done in *L. lactis*. This bacterium is not a member of the human normal flora in the intestinal tract and is unable to colonize the human intestine. Apparently, *L. lactis* passes through the gut

in three days after oral administration (Klijn *et al.*, 1995). Several antigenic epitopes, such as tetanus toxin fragment C (TTFC), diphtheria toxin fragment B, the 28 kDa immunogen of *Schistosoma mansoni*, and several TTFC fusion proteins, have been expressed in the cytoplasm of *L. lactis* (reviewed in Wells *et al.*, 1995; 1996; Chamberlain *et al.*, 1997; Medaglini *et al.*, 1997a). Steidler *et al.* (1998) co-expressed cytokines IL-2 or IL-6 together with the TTFC antigen. After intranasal immunization of mice, the cytokine-secreting *L. lactis* cells induced 10 to 15 fold higher α-TTFC IgG response than the strain expressing TTFC only. An *L. lactis* strain that secreted a down-regulator of the immune response, IL-10, was succesfully used to treat colitis in the mouse intestine (Steidler *et al.*, 2000). A recombinant strain of *Streptococcus gordonii* secreting or displaying a microbicidal single-chain antibody provided efficient therapy for mucosal candidiasis in rat vagina (Beninati *et al.*, 2000). In lactobacilli, the strong promoter of the *L. brevis* S-layer protein has shown potential for high level production of heterologous proteins (Savijoki *et al.*, 1997; Kahala and Palva, 1999).

For expression on the cell surface of LAB, the foreign peptides have been fused to a cell-wall anchoring domain of a surface protein or inserted in an S-layer protein (Table 6). The anchor domains have been derived from membrane spanning regions of membrane proteins, lipoproteins, the repeat region of a surface enzyme, the LPXTG motif of Gram-positive surface proteins (reviewed in Leenhouts *et al.*, 1999). The transmembrane domains from membrane proteins have been used for surface-anchoring of an antigenic epitope from human immunodeficiency virus (HIV) and human cytomegalovirus (hCMV) as well as a staphylococcal nuclease reporter enzyme in *L. lactis*. A large insert might be needed to facilitate surface exposure of the epitope, since at least 100 amino acids are needed to cross the thick peptidoglycan layer of LAB cells (Fischetti *et al.*, 1990). Anchoring domains of lipoproteins bind covalently to the lipid bilayer via a cysteine residue which is located immediately C-terminal to the signal sequence cleavage site (Pugsley, 1993). The lactococcal cell wall hydrolase AcmA contains three repeat regions in the cell binding domain. One of the repeats is sufficient for cell wall binding (Buist *et al.*, 1995). Lactococcal lipoprotein and AcmA anchor domains have been used to immobilize reporter enzymes and a surface antigen of the parasite *Plasmodium falciparum* on the cell envelope of *Lactococcus*. In Gram-positive bacteria, the attachment of several surface proteins to the cell wall is mediated by a protein anchor which includes a carboxy-terminal LPXTG motif, a hydrophobic core of approximately 30 amino acid residues, and a positively charged (Arg- or Lys- rich) tail that remains in the cytoplasm (Fischetti *et al.*, 1990; Schneewind *et al.*, 1992; Schneewind *et al.*, 1993). The LPXTG polypeptide motif is proteolytically cleaved after the threonine residue and covalently linked to the pentaglycine peptide in the peptidoglycan layer by a putative sortase (Navarre and Schneewind, 1994; Schneewind *et al.*, 1995). The LPXTG anchors used include Protein A anchor from *Staphylococcus aureus*, the M6 protein anchor of *Streptococcus pyogenes* and the anchoring signals of the cell wall proteinases (PrtP) from *L. lactis* and *L. paracasei* for the display of several antigenic epitopes (Table 6). The S-layer sheet covers the entire bacterial cell wall and is made of approximately $5x10^5$ identical protein monomers (Sleytr and Messner, 1988). This makes it suitable for surface display to present high numbers of foreign molecules per cell and thus potentially provides multivalency.

TABLE 6. Surface-display of foreign peptides in LAB. Adapted from Leenhouts *et al.* (1999)

| Host bacterium | Anchor | Origin | Displayed peptide | Origin | Reference(s) |
|---|---|---|---|---|---|
| *S. gordonii* | M6[d] | *S. pyogenes* | M6 | *S. pyogenes* | Pozzi *et al*, 1992b; Oggioni and Pozzi, 1996 |
| | | | E7 | HPV | Pozzi *et al.*, 1992a; Oggioni *et al.*, 1995; Medaglini *et al.*, 1997b |
| | | | Peptide epitope of gp120 | HIV | Pozzi *et al.*, 1994 |
| | | | Peptide epitopes of gp120/E7 | HIV/HPV | Di Fabio *et al.*, 1998; Medaglini, 1998 |
| | | | Allergen Ag5.2 | Hornet venom | Medaglini *et al.*, 1995 |
| | | | HA | Measles virus | Medaglini *et al.*, 1998; Pozzi and Wells, 1997 |
| | | | LTB | *E. coli* | Pozzi and Wells, 1997 |
| | | | Peptide epitopes of gp120/LTB | HIV/*E. coli* | Medaglini, 1998; Pozzi and Wells, 1997 |
| | | | scFvH6 | *Pichia anomala* | Beninati *et al*., 2000 |
| *L. lactis* | Holin[a] | Phage rlt | Peptide epitope of gp41 | HIV | Leenhouts *et al.*, 1999 |
| | LcnD[a] | *L. lactis* | Peptide epitope of pp65 | hCMV | Franke, 1998 |
| | Tmp1-7[a] | *L. lactis* | Nuclease | *S. aureus* | Poquet *et al.*, 1998 |
| | Nlp1-4[b] | *L. lactis* | Nuclease | *S. aureus* | Poquet *et al.*, 1998 |
| | OppA[b] | *L. lactis* | MSA2 | *Plasmodium falciparum* | Leenhouts *et al.*, 1999. |
| | ProtA[d] | *S. aureus* | Streptavidin | *Streptomyces avidinii* | Steidler *et al.*, 1998 |
| | M6[d] | *S. pyogenes* | Nuclease | *S. aureus* | Piard *et al.*, 1997b |
| | PrtP[d] | *L. lactis* | TTFC | *C. tetanii* | Norton *et al.*, 1995, 1996 |
| | | | MSA2 | *P. falciparum* | Leenhouts *et al.*, 1999 |
| | AcmA[c] | *L. lactis* | β-lactamase | *E. coli* | Buist, 1997 |
| | | | α-amylase | *B. licheniformis* | Buist, 1997 |
| | | | MSA2 | *P. falciparum* | Leenhouts *et al.*, 1999 |
| *Lactobacillus* sp. | M6[d] | *S. pyogenes* | LTB | *E. coli* | Rush *et al.*, 1997 |
| | | | Peptide epitope of gp41 | HIV | Mercenier *et al.*, 1996 |
| | PrtP[d] | *L. paracasei* | GusA | *E. coli* | Pouwels *et al.*, 1996; 1998 |
| | | | HA | Influenza virus | Pouwels *et al.*, 1996 |
| | | | TTFC | *C. tetanii* | Maassen *et al.*, 1999 |
| | | | VP7 and 8 proteins | Rotavirus | Pouwels *et al.*, 1998 |
| | | | Urease A and B | *H. pylori* | Pouwels *et al.*, 1998 |
| | AcmA[c] | *L. lactis* | β-lactamase | *E. coli* | Leenhouts *et al.*, 1999. |
| | SlpA[e] | *L. brevis* | Capsid protein VP1 | Enterovirus | Palva *et al.*, unp. |

[a]transmembrane anchor, [b]lipoprotein membrane anchor, [c]AcmA repeats cell-wall anchor, [d]LPXTG-type cell-wall anchor, [e]S-layer protein; HA, hemagglutinin; LTB, heat labile toxin B; scFvH6, anti-idiotype antibody that reproduces microbicidal activity of killer toxin; MSA2, surface antigen; TTFC, tetanus toxin fragment C; HPV, human papilloma virus; hCMV, human cytomegalovirus; HIV, human immunodeficiency virus

# 7. AIMS OF THE STUDY

A primary aim of this study was to initiate molecular characterization of LAB adhesion proteins. For this purpose, the adhesiveness of LAB isolates to human epithelial cell lines and ECM preparations was first characterized. This was done to obtain a view of the tissue-adherence properties expressed by LAB isolates. An S-layer protein from *L. crispatus* strongly binding to subintestinal ECM, basement membranes as well as collagens was identified and characterized. Structure-function relationships of this protein were the main aim of the second part of my thesis work.

# 8. MATERIALS AND METHODS

Bacterial strains, human cell lines and plasmids used in this study are listed in Table 7. The methods are described in detail in the original articles and summarized in Table 8.

TABLE 7

| Bacterial strain, plasmid or cell line | | Designations in other culture collections | Origin | Reference |
|---|---|---|---|---|
| *Lactobacillus acidophilus* group | | | | |
| *L. acidophilus* (A1) | JCM1132 | ATCC4356, CECT903, CIP76.13, CCRC10695, CCUG5917, DSM20079, IFO13951, KCTC3164, LMG9433, NCDO1748, NCFB1748, NCIB8690, NRIC1547 | human pharynx | Johnson *et al.*, 1980 |
| | JCM1023 | ATCC832, CIP103597, KCTC3142, LMG11428, NCFB1, NCTC1723, NCIB1723, VPI11760-B | rat feces | Johnson *et al.*, 1980 |
| *L. crispatus* (A2) | JCM5810 | KCTC3178, LMG18191, CIP105002 | chicken feces | Mitsuoka, 1969 |
| | A269-21 | | human feces | Fujisawa *et al.*, 1992 |
| *L. amylovorus* (A3) | F81 | | calf feces | Fujisawa *et al.*, 1992 |
| | JCM5807 | KCTC3175, LMG18188 | pig intestine | Mitsuoka, 1969 |
| *L. gallinarum* (A4) | T-50 | | chicken feces | Fujisawa *et al.*, 1992 |
| | F41 | | chicken feces | Fujisawa *et al.*, 1992 |
| *L. gasseri* (B1) | JCM1130 | ATCC19992, CIP103699, DSM20077, KCTC3162, VPI6033 | human feces | Lerche and Reuter, 1962 |
| | JCM5813 | KCTC3181, LMG18194 | human feces | Mitsuoka, 1969 |
| *L. johnsonii* (B2) | 5F49 | | mouse feces | Fujisawa *et al.*, 1992 |
| | F133 | | calf feces | Fujisawa *et al.*, 1992 |
| *Lactobacillus casei* | ATCC393 | | cheese | Hansen and Lessel, 1971 |
| *E. coli* | XL1BlueMRF' M15(pREP4) | | Stratagene Inc. Qiagen GmbH | |
| Plasmid pBluescriptII KS | | | Stratagene Inc. | |

| | | |
|---|---|---|
| pQE-30 | Qiagen GmbH | |
| pLPM11 | | Pouwels *et al.*, 1996 |
| pTUAT, pTUT | | Maassen *et al.*, 1999 |
| Human cell line | | |
| Caco-2 | ATCC HTB37 | Fogh *et al.*, 1977 |
| Intestine 407 | ATCC CCL6 | Henle and Deinhardt, 1957 |

TABLE 8. Methods used in this study

| Method | Used and described in |
|---|---|
| Adherence tests with bacterial cells | |
| Chicken tissue | III |
| Epithelial cells | I |
| ECM, basement membrane, immobilized ECM proteins | I, II |
| Binding to solubilized ECM proteins | I, II |
| Effect of lactobacilli on *Salmonella* invasion and adhesion | I |
| Genetic methods | |
| Isolation of chromosomal DNA | III |
| Cloning and sequencing of CbsA and CbsB | III |
| PCR mutagenesis of CbsA | III |
| Dot blot hybridization | III |
| RNA analysis | III |
| Construction of expression cassette for expression in lactobacilli | IV |
| Protein work | |
| Extraction of S-layer proteins from lactobacilli | II |
| Expression and purification of the S-layer proteins in *E. coli* | III |
| Binding of S-proteins to immobilized and solubilized ECM proteins | II, III |
| Protein digestion and mass spectrometry | III |
| Protein and peptide sequencing | III |
| Western blotting | IV |
| Protein concentration determination by ELISA assay | IV |
| Electron microscopy | III |

# 9. RESULTS AND DISCUSSION

## 9.1. Expression of tissue-adhesiveness in the *L. acidophilus group* (I, II, III)

We first analyzed the adherence of 12 strains representing the DNA homology groups of *L. acidophilus* to human intestinal cell lines Caco-2 and Intestine 407, ECM and BM preparations, and their individual components. The collection of strains consisted of two strains from each *L. acidophilus* DNA homology groups A1-A4, B1 and B2 (Table 7).

Most strains (9/12) expressed adhesiveness to Caco-2 cells, while seven strains showed efficient adhesion to Intestine 407 (Table 9, Fig. 2 of I). Large differences between the adhesion levels to the two epithelial cell lines were observed. Some strains exhibited epithelial cell type-specific adhesion: strain JCM1132 and T-50 adhered strongly to Caco-2 but no adhesion was observed to Intestine 407 cells and a reversed pattern was seen with 5F49. Two binding patterns were observed on the non-confluent Intestine 407 cells (Fig. 1 of I). Some strains, *e.g.* F41, bound exclusively to the cell surface of Intestine 407, while other strains, exemplified by JCM5810 bound to the area surrounding epithelial cells. Some strains, *e.g.* JCM5813, expressed both adhesion types. To test whether binding to ECM indeed is involved in the adhesion type represented by JCM5810, we assessed the adhesiveness to two ECM preparations: an ECM prepared by detergent extraction from Intestine 407 cell culture (Hedman *et al.*, 1982) and Matrigel, a commercially available reconstituted BM preparation from mouse sarcoma cells. The latter is widely used as an *in vitro* model for the basement membrane and composed mainly of a network of laminin and type IV collagen (Kleinman *et al.*, 1986). We also tested the adhesiveness to the individual major glycoproteins of the ECM and BM: type I and IV collagens, laminin and plasma and cellular fibronectin. Adherence to the ECM prepared from Intestine 407 cells was expressed by eight strains (Fig. 3 of I). Five of the six strains that bound immobilized type I collagen were also adhesive to the ECM from Intestine 407. The strong adhesion to the ECM from Intestine 407 by the strain T-50, which showed no adhesion to type I collagen, could be mediated by the efficient recognition of cellular fibronectin (Table 9, Fig. 3 of I). The basement membrane preparation Matrigel was recognized by five of the *L. acidophilus* group strains, and this adhesion is likely to be based on binding to laminin and type IV collagen, as four of the five Matrigel-adhesive strains were able to recognize these proteins (Table 9, Fig. 3 of I). Strain F81 showed efficient adhesion to Matrigel, whereas no adhesion was observed to type IV collagen and laminin (Table 9, Fig. 3 of I). Possibly F81 recognizes one of the minor components of Matrigel. Basement membranes are complex structures composed of type IV collagen and laminin networks that are connected to entactin and heparan sulphate proteoglycan, and of several other minor components (Timpl, 1989; Yurchenko and Schittny, 1990; Timpl. 1996). Fibronectins can be divided in two major forms: plasma (pFn) and cellular (cFn) fibronectin. They have different spatial conformations, which result in part from different slicing patterns of the fibronectin gene (Petersen *et al.*, 1989; Schwarzbauer, 1991). pFn is produced in the liver by hepatocytes and released to blood circulation (Tamkun and Hynes, 1983). Cellular Fn is formed locally in tissues by fibroblasts (Hedman *et al.*, 1982; McKeown-Longo and Mosher, 1989) and endothelial cells (Peters *et al.*, 1990), and is mainly bound to cell

TABLE 9. Adherence of the lactic acid bacteria to human intestinal cell lines and components of the extracellular matrix (ECM), adherence to bovine serum albumin (BSA) is shown for control.

| Strain | | Bacterial adherence to | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Caco-2 | Intestine 407 | | Immobilized ECM protein | | | Solubilized ECM protein | | Immobilized basement membrane material | | | BSA[b] |
| | | | cell surface | matrix | pFn[b] | cFn[b] | CnI[b] | $^{125}$I-pFn (%)[c] | $^{125}$I-CnI (%)[c] | Matrigel | Lam[b] | CnIV[b] | |
| *L. acidophilus* | JCM1132 | +++[a] | - | - | - | - | - | 3 | 4 | -[b] | - | - | - |
| (A1) | JCM1023 | + | + | + | ++ | ++ | ++ | 4 | 3 | - | - | - | - |
| *L. crispatus* | JCM5810 | ++ | - | ++ | - | - | +++ | 6 | 41 | +++ | +++ | +++ | - |
| (A2) | A269-21 | - | - | +++ | +++ | +++ | +++ | 9 | 7 | +++ | +++ | - | - |
| *L. amylovorus* | F81 | - | - | + | - | - | - | 2 | 23 | ++ | - | - | - |
| (A3) | JCM5807 | + | + | - | - | - | - | 4 | 21 | - | - | + | - |
| *L. gallinarum* | T-50 | +++ | - | +++ | ++ | +++ | - | 3 | 19 | - | - | - | - |
| (A4) | F41 | ++ | + | +++ | +++ | +++ | + | 4 | 21 | +++ | +++ | +++ | - |
| *L. gasseri* | JCM1130 | +++ | + | - | +++ | +++ | + | <2 | 3 | - | - | - | - |
| (B1) | JCM5813 | +++ | + | +++ | +++ | +++ | +++ | 5 | 4 | + | + | ++ | - |
| *L. johnsonii* | 5F49 | - | + | - | - | + | - | 4 | 21 | - | - | - | - |
| (B2) | F133 | +++ | + | + | ++ | ++ | - | <2 | 10 | - | - | - | - |

[a] +++, >200 bacteria/field; ++, >100 bacteria/field; +, >50 bacteria/field.

[b] pFn, plasma fibronectin; cFn, cellular fibronectin; CnI, type I collagen; CnIV, type IV collagen; Lam, laminin; BSA, bovine serum albumin.

[c] For the assay, 100 µl bacterial suspension of 5 x $10^9$ cells was mixed with 100 µl 0, 10, 100 or 500 ng of $^{125}$I-labelled protein. Binding efficiency was determined from 500 ng of the protein.

surfaces or assembled in an insoluble multimer in extracellular matrices (Hedman and Vaheri, 1989; McKeown-Longo and Mosher, 1989). Seven of the twelve *L. acidophilus* group strains bound to both pFn and cFn (Table 10, Fig. 3 of I). Strain F41 exhibited strong adhesion to pFn, otherwise no major differences in adhesion levels to pFn and cFn were seen (Table 9, Fig. 3 of I). Many bacterial species express differential binding to immobilized and solubilized forms of ECM proteins (Kuusela *et al.*, 1985; Lowrance *et al.*, 1988; Tarkkanen *et al.*, 1990). Therefore, we also tested adhesiveness to solubilized type I collagen and pFn. The binding of solubilized pFn was above 4 % by only three strains (Table 9, Table 2 of I) and the adhesion levels to the immobilized and soluble pFn did not correlate. Eight strains bound solubilized type I collagen with a binding percentage above 4 %, and seven strains exhibited efficient binding with a value above 10 %. Recognition of immobilized and solubilized collagen was not parallelled in 8/12 of the strains.

Adherence to both epithelial cells and the ECM seem common characteristics of LAB, since the majority of the *L. acidophilus* group strains adhered to either of the epithelial cell lines, Caco-2 or Intestine 407 (10/12 strains), and to the ECM material (10/12 strains). Large variability was evident in the adhesion. Binding to both surfaces was exhibited by half of the strains, JCM1023, T-50, F41, JCM1130, JCM5813 and 5F49, whereas the strains JCM1132, JCM5807 and 5F49 were adhesive to epithelial cells and three strains, JCM5810, A269-21 and F81 showed adhesiveness to ECM. *L. crispatus* (homology group A2) and *L. gasseri* (B1) belong to the dominant *L. acidophilus* group species in the human intestine (Mitsuoka, 1992; Song *et al*., 1999; 2000). Both of the homology-group-A2 strains of this study were strongly adhesive to ECM material, while the B1-group strains exhibited both adhesion types. Matsumura *et al.* (1999) observed that surface proteins from strains of the B1 group bind more efficiently to rat colonic mucin than the other groups of *L. acidophilus*. The surface proteins of the strains JCM1132 (A1) and T-50 (A4), also included in our study, exhibited intermediate binding to rat colonic mucus. Both of the strains were strongly adhesive to Caco-2 cells, but only T-50 exhibited adherence to ECM (Table 9; Figs. 2 and 3 of I). Together, these results indicate that the adhesion of *L. acidophilus* group strains is strain-specific and not dependent on the homology group. Assesment of adhesion in several A2-group strains is needed to clarify whether the group A2 is associated with ECM-adherence.

Highest adhesion levels to epithelial cells were observed with the human isolates, JCM1132, JCM1130 and JCM5813. On the other hand, the chicken isolates T-50 and F41 as well as the mouse isolate 5F49 and the calf isolate F133 all exhibited adhesiveness to human epithelial cells, which indicates lack of strict host-specificity in adhesion. Similar observation was made with adhesion to the ECM material: the chicken isolates JCM5810, A269-21 and F41 showed efficient adhesion to human fibronectins and type IV collagen as well as to the mouse basement membrane preparation Matrigel and laminin. From these studies, it can be concluded that LAB have the capacity to recognize tissue components of non-host mammals.

Recognition by LAB of different receptors on the epithelial cell surfaces is suggested by the lack of a clear correlation in the adhesion to Caco-2 and Intestine 407 by the *L. acidophilus* group isolates. *E. coli* and *S. typhimurium* express several adhesins that recognize different structures on epithelial cells (van der Velden *et al.*, 1998; Klemm and Schembri, 2000), and it remains to be seen whether this applies to LAB also.

The ability of LAB to bind immobilized fibronectins is shared by a number of pathogenic bacteria (reviewed in Westerlund and Korhonen, 1993; Patti *et al.*, 1994) and fibronectin-binding is thought to be important in the adhesion and colonization of *S. aureus* (Kuyers and Proctor, 1989) and *S. sanguis* (Lowrance *et al.*, 1990) at damaged heart valves. Fibronectin acts as a molecular bridge in the binding of *S. aureus* to the epithelial cell-surface receptor receptor $\alpha_5\beta_1$ integrin (Sinha *et al.*, 1999). This may apply to lactobacilli also, since the majority of strains in this study adhered to both epithelial cells and fibronectin (Fig. 3 of I). We observed no major differences in the recognition of plasma and cellular fibronectins by *L. acidophilus* group strains. This is different to the YadA protein of *Y. enterocolitica* (Schulze-Koops *et al.*, 1993) and the S-fimbrial adhesin of meningitis-associated *E. coli* (Sarén *et al.*, 1999) which adhere to immobilized cFn but not to pFn. The selective recognition of cFn may increase the colonization potential of S-fimbriated *E. coli* at sites of tissue trauma or inflammation (Sarén *et al.*, 1999), where cFn is accumulated (Peters *et al.*, 1989). The low level binding of solubilized fibronectin may relate to recognition of conformation dependent epitopes or to the high density of ECM proteins in solid-phase

assays. In solution, fibronectin has a globular conformation which unfolds as the protein is immobilized in the ECM (Williams *et al.*, 1982; Akiyama and Yamada, 1987). Group A streptococci bind strongly to soluble Fn and weakly to the immobilized form (Kuusela *et al.*, 1985), whereas a reversed situation is known for *S. sanguis* (Lowrance *et al.*, 1988).

Specific recognition of different collagen types was indicated by the adhesion of strains A269-21 and JCM5810 to the fibril-forming type I collagen and by strain F41 to the network-forming type IV collagen (Table 9, Fig. 3 of I). These collagens have a different tissue distribution and therefore selective binding to collagen types can affect tissue tropism of bacteria. Several adhesion mechanisms that mediate binding to collagen have been described in pathogenic bacteria (reviewed in Westerlund and Korhonen, 1993; Patti and Höök, 1994), and their role in tissue colonization is established in the pathogenesis of *S. aureus* (Holderbaum *et al.*, 1987; Switalski *et al.*, 1993; Rhem *et al.*, 2000) and *Y. enterocolitica* (Kapperud *et al.*, 1987; Tamm *et al.*, 1993; Roggenkamp *et al.*, 1995). The ability of the majority of the strains (67%) of this study to bind solubilized type I collagen is in good agreement with the frequency of 75% detected on lactobacilli of different taxonomic groups and a variety of sources (Aleljung *et al.*, 1991) (Table 3). The majority of the *L. acidophilus* group strains (8/12) recognized with different efficiency the immobilized and solubilized forms of type I collagen. Selective binding to the immobilized form of type V collagen was demonstrated by the type-3-fimbria of *Klebsiella pneumoniae* (Tarkkanen *et al.*, 1990).

The biological significance of bacterial binding to solubilized ECM proteins can only be speculated. As targets for bacterial tissue adhesion, the immobilized forms of ECM proteins are probably relevant, whereas soluble ECM proteins in body fluids may act as adhesion inhibitors or, on the other hand, as bridging molecules between bacterial cells and epithelial receptors, *e.g.* fibronectin to $\alpha_5\beta_1$ integrin (Sinha *et al.*, 1999). Lactobacilli are regarded non-invasive organisms and the biological rationale for the expression of adhesiveness to ECM by LAB is not known. Since adhesion to ECM and BM is an initial step in the invasion into epithelial cells or from intestinal lumen to circulation by bacterial pathogens (Westerlund and Korhonen, 1993; Finlay and Cossart, 1997; Finlay and Falkow, 1997; Klemm and Schembri, 2000), adherence of lactobacilli to ECM structures can have a direct probiotic effect by excluding invading pathogens from damaged intestinal epithelia.

### *9.1.1. Adherence of* Lactobacillus crispatus *JCM5810 to human and chicken ECM*

One of the twelve *L. acidophilus* group strains, *L. crispatus* JCM5810, expressed efficient adhesiveness to the matrix material surrounding the Intestine 407 cells (Fig. 1 of I). Strain JCM5810 also adhered efficiently to the ECM prepared from Intestine 407 and to the BM preparation Matrigel (Fig. 3 of I and Fig. 3 of II). JCM5810 also adhered strongly to several purified ECM proteins: immobilized type I and IV collagens as well as laminin, and with a lower affinity, type V collagen and fibronectin (Fig. 3 of I and Fig. 1 of II). To localize the receptor region on the fibronectin molecule, we assessed the adhesiveness of JCM5810 to proteolytic fragments of human pFn. Efficient adhesion was observed to the C-terminal 120 kDa cell-binding domain, but no adhesion to the N-terminal 30 kDa fragment or the gelatin-binding 40 kDa fragment (Fig. 2 of III). *L. crispatus* JCM5810 bound solubilized, $^{125}$I-labelled type IV collagen efficiently; 64 % of the radiolabel was bound by JCM5810 cells. This binding could be inhibited with unlabelled type IV and I collagens in a dose dependant manner. Inhibition with type V collagen was weaker, laminin and fibronectin had no inhibitory effect. JCM5810 adhered to the immobilized forms of laminin and fibronectin, in contrast, only weak binding with the solubilized forms of these proteins were detected, 3% of $^{125}$I-labelled laminin and 7% of $^{125}$I-fibronectin were bound to the bacterial cells.

These results show that the matrix-adhesive *L. crispatus* strain JCM 5810 exhibits a wide capacity to bind several major proteins of the ECM: immobilized laminin and fibronectin, as well as the immobilized and solubilized forms of collagens. The binding of JCM5810 to the cell-binding 120 kDa domain on the fibronectin molecule is similar to that of *S. sanguis*, whereas most of the fibronectin binding bacteria so far identified recognize the N-terminal 30 kDa fragment (Westerlund and Korhonen, 1993). *S. sanguis* also binds solubilized pFn weakly which may reflect a similar binding epitope on the fibronectin molecule for JCM5810 and *S. sanguis*.

*L. crispatus* belongs to the numerically major *Lactobacillus* species in the human or the chicken intestine and feces (Mitsuoka, 1992; Song *et al.*, 1999; Song *et al.*, 2000), and the strain JCM5810 had

originally been isolated from chicken feces (Table 7). The adhesion tests were performed with human collagens as they were commercially available. To explore whether JCM5810 is able to recognize chicken tissue sites rich in collagen, we used a double-staining procedure to visualize the possible adhesion of JCM5810 to collagen-containing regions in tissue sections of chicken colon. As seen in Fig. 5 of III, JCM5810 did not adhere to the luminal surface of the colon epithelium but adhered to the connective tissue areas which were strongly positive with a monoclonal antibody specific for chicken type III collagen. Bacteria also adhered to basolateral aspects of the epithelial cells, which were not stained by the anti-type III collagen antibody. Adherence of JCM5810 to regions of chicken colon rich in ECM material gives support for the observed *in vitro* binding to collagens as a true tissue-binding property.

## 9.2. A collagen-binding S-layer protein in *Lactobacillus crispatus* (II, III)

### *9.2.1. Identification of the S-layer protein as the collagen-binding adhesin*

Strains of the *L. acidophilus* DNA homology groups A1-A4 express an S-layer sheet on the surface of the cell wall (Masuda and Kawata, 1983). As an outermost proteinaceous structure facing the environment, the S-layer is an obvious candidate for an adhesin. When the S-layer proteins were removed from the surface of JCM5810 cells by guanidine hydrochloride (GHCl), adhesiveness to sections of chicken colon was sharply decreased (Fig. 5 of III). A similar effect was also seen on the adhesiveness of JCM5810 to collagens (not shown). The GHCl extract of JCM5810 contained the 43 kDa S-layer protein as the major polypeptide as revealed by SDS-PAGE, and no S-layer protein was detected remaining on the cell surface (not shown). The S-layer protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. In a ligand blotting procedure, solubilized $^{125}$I type IV collagen bound efficiently to this 43 kDa peptide (Fig. 5A of II), while there was no binding to the S-layer protein of the non-adhesive strain JCM1132. The specificity of the reaction was demonstrated by a nearly complete inhibition of binding by unlabelled type IV and I collagens, as well as partial inhibition by type V collagen. Fibronectin had no inhibitory effect. The weak inhibition by laminin can be due to competition of same or related binding epitopes on CbsA or to the direct interaction of laminin with type IV collagen. JCM5810 cells bound solubilized laminin weakly which gives support for the latter hypothesis.

S-layers are frequently expressed by lactobacilli, but their biological function(s) has remained unknown. Our results show that the S-protein of *L. crispatus* JCM5810 is an adhesive molecule which recognizes structurally different collagen types: the fibril forming type I and V as well as the network-forming type IV, and is able to recognize both the immobilized and solubilized forms of the collagens. Further, the results indicate that the S-protein mediates adhesion of JCM5810 to collagen-rich regions in the chicken colon. Pathogenic bacteria express several collagen adhesins that have the capacity to recognize different collagen types. The S-layer protein VapA of *Aeromonas salmonicida* binds to type I and IV collagens (Trust *et al.*, 1993). The S-layer-like protein YadA of *Y. enterocolitica* is a multifunctional adhesin that recognizes several ECM proteins including collagen types I, II, III, IV, V and XI (Emödy *et al.*, 1989; Schulze-Koops *et al.*, 1992; Tamm *et al.*, 1993). The Cna protein of *S. aureus* binds type I and II collagens (Patti *et al.* 1995; Rich *et al.*, 1999a) and the Ace protein of *Enterococcus faecalis* (Rich *et al.*, 1999b) as well as the Cnb protein of *L. reuteri* type I collagen (Roos *et al.*, 1996). The fimbrial adhesins MrkD of *Klebsiella pneumoniae*, Dr fimbria of uropathogenic *E. coli* and FimH of meningitis-associated *E. coli* distinguish between collagen types. MrkD and Dr fimbria recognize only one collagen type, type V and IV, respectively (Westerlund *et al.*, 1989; Tarkkanen *et al.*, 1990), whereas FimH binds to type I and IV collagens but not to type III (Pouttu *et al.*, 1999).

### *9.2.2. Cloning of the genes encoding CbsA and CbsB*

The S-layer protein of JCM5810 was named as CbsA (collagen-binding S-layer protein A). To further characterize the protein and to obtain the gene, the DNA region coding for CbsA was cloned and

sequenced. The *cbsA* gene was located on the genome of JCM5810 by Southern hybridization using degenerate probes, 5´-ATGAA(C/T)AT(A/C/T)GA(C/T)-GTIGA(C/T)GC-3´ and 5´-TA(C/T)AA(C/T)TCIGCIACIGTIGCIATG-3´, designed on the basis of two of the four internal amino acid sequences of CbsA that were obtained by peptide sequencing. The cloned DNA region of 1,584 bp revealed an ORF encoding 440 amino acids. The ORF encodes a signal sequence of 30 amino acids preceded by a putative ribosome binding site AGGAGG ten nucleotides upstream of the ATG start codon. The predicted molecular mass of the mature protein is 43,910, which is in good agreement with 43 kDa that was earlier estimated by SDS-PAGE and suggests that no extensive post-translational modifications occur. The predicted amino acid composition of CbsA is typical of S-layer proteins; it has a high content of hydrophobic amino acids (37%), as well as serine (6%) and threonine (13%) and lacks cysteine residues. No significant stretches of accumulated hydophobicity were identified. As other *Lactobacillus* S-proteins characterized so far (Vidgrén *et al.*, 1992; Boot *et al.*, 1993; Boot *et al.*, 1995; Callegari *et al.*, 1998), CbsA is strongly basic with a predicted pI of 9.6.

The S-protein genes of the *L. acidophilus* group have highly conserved 5´ and 3´ regions. Specific primers complementary to the start of the signal sequence and the C-terminal end of CbsA were used in PCR to analyze whether there exists a silent S-layer gene in the genome of JCM5810. A DNA fragment of 1,4 kb was isolated, digested with *Xho*I to avoid contamination by *cbsA*, and cloned for sequencing. The ORF encoded a protein of 453 amino acids of which the 23 first amino acids were identical to the signal sequence of CbsA. The protein was named CbsB. The amino acid sequences of CbsA and CbsB are highly homologous, the overall sequence identity is 43 %. The signal sequences are almost perfectly conserved. The C-terminal one-third of the molecules (corresponding to amino acids 288-410 in CbsA) exhibit high identity, 75%, while the N-terminal and central regions (1-287 in CbsA) share only 31% sequence identity.

Amino acid sequencing of four peptides and the molecular sizes of 16 proteolytic fragments of the S-layer protein that was isolated from the surface of JCM5810 perfectly matched the deduced amino acid sequence of CbsA, which gives proof that CbsA is the actively expressed S-layer protein in JCM5810. To confirm this, RNA from logarithmically growing cells was isolated and subjected to Northern blot analysis with *cbsA*- and *cbsB*-specific probes that were derived from the variable regions of the genes. The *cbsA*-specific probe detected a transcript of 1.5 kb which is close to the expected size of monocistronic *cbsA* mRNA, whereas no mRNA transcript was detected with the probe specific for *cbsB* (data not shown). The detection of the *cbsA*-transcript gives further evidence that CbsA is expressed on the surface of the cell, whereas *cbsB* is a silent gene under the growth conditions we used. JCM5810 harbours a plasmid of 2.9 kb (data not shown) and it is presently not certain whether CbsA and CbsB are chromosomally or plasmid encoded. The former is likely, since the S-protein encoding genes *slpA* and *slpB* of the closely related strain *L. acidophilus* JCM1132 are located on 6 kb chromosomal fragment (Boot *et al.*, 1996a).

### *9.2.3. Presence of CbsA-related proteins in lactobacilli and other bacteria*

Alignment comparisons revealed high homology of CbsA with seven other *L. acidophilus* group and *L. helveticus* S-proteins with amino acid identity ranging between 48-74% (Fig 1 of III). The highest identity, 74%, was found with SlpnB, a non-expressed S-layer protein from *L. crispatus* strain LMG12003 (Pouwels and Martínez, unpublished). All eight S-protein sequences shared a similar pattern of variable and conserved regions: an almost completely conserved signal sequence (not shown), a highly homologous C-terminal and a variable N-terminal region (Fig. 1 of III). Weaker homology was found with the S-layer protein of *L. brevis* (Vidgrén *et al.*, 1992). In database searches, no significant similarity was found with the S-proteins of other bacteria and archaea. Only weak local homologies with known collagen-binding adhesins of other bacteria were found, sequence identity ranged between 17-20 % with YadA of *Y. enterocolitica*, the S-protein VapA of *A. salmonicida*, MrkD of *K. pneumoniae*, Cna of *S. aureus* or Ace of *E. faecalis*.

To evaluate whether CbsA homologues are common in the *L. acidophilus* group, we searched with a DNA fragment of the variable part of CbsA as the probe for homologous DNA in the other 11 LAB strains used in this PhD study (Table 7; Table 1 of I). The probe covered the predicted CbsA amino acid sequence from residue –2 to +271, where +1 is the first amino acid of mature CbsA. A strong signal was observed from genomic DNA of JCM5810, whereas no hybridization was detected with DNA from the other strains

in low stringency conditions. Lack of hybridization of the *cbsA* variable part fragment to the other collagen-binding strains, JCM1023, A269-21, F81, JCM5807, T-50, F41, JCM1130, JCM5813, 5F49 and F133 (Fig. 3 and Table 2 of I), means that adhesins other than a CbsA homolog must be involved in their adherence to collagens. One possibility is a homolog to the solute-binding component Cnb of the ABC transport system from *L. reuteri*. Expression of several surface-proteins with affinity to collagen by four strains that belong to the species *L. fermentum, L. rhamnosus and L. casei* was recently suggested (Howard *et al.*, 2000). However, the characterization of the collagen-binding was promiscuous and lacked controls and thus, the significance of the observed affinity to collagen remains open.

### *9.2.4. Expression of CbsA and related S-layer proteins as His-tag fusion proteins in E. coli*

To generate tools for further characterization of the collagen-binding region in CbsA, CbsA was expressed in *E. coli* as an N-terminal 6xHis-tag fusion protein. For comparison, we also produced CbsB, SlpA and SlpnB in *E. coli* as His-tag proteins. The expressed and purified N-terminal His-tagged S-proteins formed visible aggregates during purification when they were dialyzed into phosphate buffered saline (PBS) (Fig. 2 of III). A similar aggregation was observed when CbsA and SlpA were extracted from the surface of *Lactobacillus* cells by GHCl and dialyzed to PBS. Lactobacillar S-layer proteins have the capacity to auto-assemble into polymerized S-layer sheets upon dialysis (Masuda and Kawata, 1980; Lortal, 1990). By electron microscopy, the His-tagged S-layer proteins were seen organized into two-dimensional regular crystalline sheets that were highly similar to the endogenous proteins isolated from *Lactobacillus* (Shown for CbsA in Fig. 3 of III). These findings indicated that the fusion of six histidine residues in the N-terminus of the S-protein did not affect the ability of the S-proteins to assemble *in vitro* into a regular S-layer sheet.

### *9.2.5. Binding of collagen by the His-tagged S-layer proteins*

We assessed whether the heterologous expression in *E. coli* and the fusion of the N-terminal His-tag affected the ability of CbsA to bind collagen. Solubilized $^{125}$I-labelled type IV and type I collagens bound strongly to CbsA and His-CbsA immobilized on nitrocellulose membranes, whereas no binding to SlpA, His-SlpA, His-CbsB, His-SlpnB or BSA was observed (Table 1 of III). The amount of bound radiolabel was recorded with Phosphor imager technology (BAS-1500, Fujifilm Medical Systems Inc.) and digitally quantitated by the Tina (version 2.0) image analysis program. The His-tagged CbsA expressed in *E. coli* was reduced by 20% in collagen-binding activity as compared to CbsA extracted from the cell-surface of *L. crispatus* JCM5810. The inhibition by unlabelled type IV and type I collagens of the $^{125}$I-labelled type IV collagen-binding was equally efficient with His-CbsA and CbsA (over 99%), inhibition with unlabelled type V collagen was 95 % and with fibronectin less than 1%. These results indicated that the His-tag had no dramatic effect on the binding specificity of CbsA. His-CbsA was also tested for the binding to immobilized type IV and type I collagens in an ELISA assay. His-CbsA bound efficiently to both collagen types, but not laminin, fibronectin or BSA, while His-SlpnB showed no significant binding activity to any of the surfaces (Fig. 4C of III). Hence, CbsA recognizes the solubilized and the immobilized forms of collagens I and IV, and these results confirm the function of CbsA as a collagen-adhesin. It was concluded that heterologous expression as a fusion protein in the Gram-negative host and the subsequent purification from the *E. coli* cytoplasm did not significantly affect the self-assembly of CbsA into a functional S-layer.

## 9.3. Identification of the collagen-binding region in CbsA (III)

### *9.3.1. Collagen-binding by CbsA-SlpA and CbsA-SlpnB hybrid proteins*

To analyze whether the collagen-binding region is located within the variable N-terminal part of CbsA, we created hybrid His-tag proteins where the N-terminal half of CbsA was fused to the C-terminal part of

either SlpA or SlpnB, and *vice versa* (Fig.1 and Table 1 of III). Four hybrid proteins between CbsA and SlpA were produced, the sites of the peptide switch were at amino acids 212 and 287 in CbsA. The hybrid CbsA1-212/SlpA208-413 did not bind collagens, whereas efficient collagen-binding was observed with CbsA1-287/SlpA290-413. Neither of the two counter hybrids bound collagen. When the more homologous sequence of the non-adhesive SlpnB became available, we created fusions between CbsA and SlpnB at positions 28, 81, 194, 212, 250 and 287 in CbsA. Similarly to the two CbsA-SlpA fusions, only the CbsA-SlpnB hybrid fused at amino acid 287 bound collagens. Two of the counter hybrids, SlpnB1-19/CbsA29-410 and SlpnB1-72/CbsA82-410 bound collagens efficiently (Table 1 of III). Apparently, the N-terminal variable part of CbsA to residue 287 contains information for the collagen-binding epitope. The region 212-287 appears critical for the binding activity, since both CbsA/SlpA and CbsA/SlpnB hybrids N-terminal to the amino acid 287 in CbsA failed to bind collagens. The role of the extreme N-terminus remains less clear. The efficient collagen-binding by the hybrid His-SlpnB1-71/CbsA82-410 demonstrates that the N-termini can be exchanged without loss of binding. This may be due to complementation of the active amino acids in CbsA by a homologous sequence in SlpnB, or this region might be involved only indirectly in the collagen binding.

### *9.3.2. Collagen-binding by truncated His-CbsA polypeptides and mutated His-CbsA*

To characterize the collagen-binding region further, five truncated peptides, 1-212, 1-250, 1-287, 42-287 and 288-410 were created from His-CbsA (Fig. 2 of III). The peptide CbsA1-287 efficiently bound both solubilized and immobilized collagens (Table 1 and Fig. 4C of III). The C-terminal peptide 288-410 showed a low level of binding to immobilized type I collagen, whereas no binding to solubilized collagens was detected. Since truncation beyond amino acids 1-287 resulted in non-functional peptides, we continued the analysis by making several local mutations in this region at positions of CbsA where the sequence differs from those of SlpA or SlpnB (Table 1 of III). Two N-terminal deletions ($\Delta$22-26 and $\Delta$91-96) decreased the binding by 90 and 70 %, respectively; while substitutions D130N, N226A, TA264SK and P268A reduced the binding by 40 to 70%. Other substitutions, NNN14INL and F19S, had less effect on the binding. Complete loss of binding was observed with substitution mutant KSDV257TANN (Table 1 and Fig. 4C, III). Among single amino acid substitutions at this site, V260N completely abolished binding, a large reduction was seen with S258A, whereas the substitutions K257T and D259N had less effect on the binding.

Apparently CbsA contains two regions able to bind collagen. The major region is located between amino acids 1-287 and confers binding to immobilized and soluble type I and IV collagens, a second less efficient region was identified in the C-terminal region between amino acids 288-410. CbsA peptides 1-250 and 42-287 failed to bind solubilized collagen and the results indicate that the major binding epitope encompasses a long primary amino acid sequence and that both ends of the CbsA 1-287 peptide are necessary for the collagen-binding. Large collagen-binding regions are typical for the other bacterial collagen adhesins. In YadA of *Y. enterocolitica*, a region of 300 amino acids contains the binding information and the active binding site is likely to be dispersed along the sequence (Tamm *et al.*, 1993; Westerlund-Wikström *et al.*, 1997). The three-dimensional structure of the 19-kDa collagen-binding domain of the 135-kDa Cna adhesin from *Staphylococcus aureus* has been determined (Symersky *et al.*, 1997). The domain is folded in a "jelly roll" topological pattern composed of two antiparallel $\beta$-sheets and two short $\alpha$-helices. A trench-shaped groove, 1-5 Å deep and 10-15 Å wide, accommodates the collagen triple helix (Symersky *et al.*, 1997). Two amino acid residues within the binding groove, N232 and Y233, were found critical for the collagen binding (Patti *et al.*, 1995). A related collagen-binding domain with similar folding pattern was identified by structural modelling in the Ace protein of *E. faecalis* (Rich *et al.*, 1999b). The $\beta$-sheet content of CbsA is 38 % according to secondary structure predictions and is nearly the same as in Cna and Ace. The primary sequence of CbsA is not related to those of Cna and Ace. Limited local homology are found in amino acid overlaps at 92 to 172 in CbsA and 88 to 166 in Cna, as well as 211 to 282 in CbsA and 35 to 103 in Cna. These regions are in the variable part of CbsA and in the vicinity or partially within the collagen-binding region in Cna. The active amino acids residues identified in the collagen-binding groove of Cna do not have homologs in CbsA, which suggests that the collagen-binding regions in the two proteins are not closely related.

### 9.3.3. In vitro *polymerization of CbsA into an S-layer*

By transmission electron microscopy, we evaluated the capacity of the mutated His-CbsA peptides to polymerize into an S-layer sheet (Table 1 of III). Selected examples are shown in Fig. 3 of III. CbsA, His-CbsA and CbsA1-287 formed similar regular, crystalline, periodic, two-dimensional structures. The shorter peptides, 1-212, 1-250, 42-287 and 288-410 and the five hybrid molecules CbsA1-212/SlpA208-413, SlpA1-207/CbsA213-410, CbsA1-81/SlpnB73-409, CbsA1-250/SlpnB250-409 and SlpnB1-249/CbsA251-410 were not seen to form a regular structure (Table 1 of III). The mutated protein KSDV257TANN, which had lost collagen-binding capacity (Table 1 of III), was seen to assemble into cylinder-like S-layer structures that were morphologically different from the S-layer sheet exhibited by the other S-proteins (Fig. 3 of III). The cylinder diameter was ca. 90 nm. A similar structure was observed by the substitution of V260N in CbsA whereas substitutions K257T, D258A and D259N had no effect on the ability to polymerize into normal sheet-like crystalline layers, which indicates that V260 is an important residue in the assembly of CbsA.

All mutations in CbsA that prevented the S-layer assembly also abolished collagen binding (Table 1 of III), suggesting that a paracrystalline sheet structure is optimal for collagen recognition by CbsA. The region 250-287 in CbsA seems important for the S-layer polymerization process. The hybrid proteins between CbsA and SlpnB at residue 250 failed to form an S-layer sheet, and the peptide 1-250 was unable to form a regular array. Search for sequence motifs in the *L. acidophilus* group S-layer proteins revealed several conserved regions (Fig. 1 of IV), which might be involved in the assembly of the S-layer proteins. The region 250-287 lies in a conserved block and there are smaller and less conserved regions in the N-terminal half of the protein. The inability of 42-287 to polymerize into a layer suggests that also the N-terminus is involved in the S-layer assembly. Our results suggest that the strongly conserved C-terminus is not involved in the *in vitro* assembly of the CbsA S-layer. The functional role of the C-terminus remains open, it could be involved in the secretion of CbsA to the cell surface or in the attachment of the S-layer sheet to the underlying cell wall. The latter is likely, since the higly homologous C-terminal domain of SlpA mediates binding to the cell wall (Smit *et al.*, 2001). The C-terminal CbsA peptide 288-410 was unable to form an S-layer and it is not known whether the observed binding activity to type I collagen represents a cryptic binding epitope that is revealed in the peptide or if it is truly a second binding site with weaker affinity.

The collagen-binding epitope seems sensitive to changes in the morphology of CbsA sheets. The mutation at the site KSDV257TANN abolished collagen-binding activity and directed the polymerization exclusively into a morphology of cylinders with smaller crystal dimensions than in His-CbsA, as suggested by preliminary Fourier transformation analysis. On the other hand, we identified several sites along amino acids 1-287 which affected collagen-binding but had no apparent effect on the S-layer morphology. These amino acids may be directly involved in collagen-binding, and the findings are in agreement with the view that the binding site covers a large region of the primary sequence.

The dependence of collagen-binding on the S-layer sheet formation by CbsA is different to what has been observed with another collagen-binding S-layer protein, the VapA of *Aeromonas salmonicida*. A soluble proteolytic N-terminal fragment of 38 kDa did not polymerize to a tetragonal S-layer sheet but retained the ability to bind collagen (Doig *et al.*, 1992; Thomas *et al.*, 1992). The capacity to polymerize does not seem necessary for the collagen-binding activity of the S-layer-like surface protein YadA of *Y. enterocolitica*. The binding region of YadA was surface-displayed as an internal fusion peptide in the flagella of *E. coli*, where it does not form a visible layer and binds collagen (Westerlund-Wikström *et al.*, 1997).

### 9.3.4. *Interaction of* in vitro *polymerized CbsA with JCM5810 cells*

A saturable level of binding was not reached in binding studies of CbsA1-287 in the ELISA assays (Fig. 4 of III) or plasmon resonance spectroscopy (Biacore, not shown). Furthermore, we detected no inhibition by CbsA1-287 on the binding of $^{125}$I-labelled collagens to JCM5810 cells. To assess if polymerized His-CbsA binds to the surface of CbsA-expressing JCM5810 cells, we incubated JCM5810 cells and S-layer proteins together and visualized the mixture in a light microscope after methylene blue staining. When His-

CbsA or His-CbsA1-287 was mixed with JCM5810 cells, very large aggregates were seen covered with bacteria, this phenomenon was not observed with the peptides 1-212, 1-250 or 42-287, and the bacteria in the absence of peptides did not aggregate (Fig. 6 of III). These results suggest that the polymerized CbsA molecules are able to attach to the CbsA-covered surface of JCM5810 cells and that the interaction depends on the S-layer sheet structure.

## 9.4. Expression of *cbsA* on the surface of heterologous lactobacilli (IV)

Specific silencing of S-layer genes by mutagenesis has proven unsuccesful in the *L. acidophilus* group, which complicates functional studies of the proteins. Therefore, we decided to produce CbsA in a *Lactobacillus* species that does not express an endogenous S-layer gene and is genetically amenable and, furthermore, does not bind collagen.

The *cbsA* gene was cloned into the *E. coli-Lactobacillus* shuttle vector pLPM11 under the control of an inducible α-amylase promoter from *L. amylovorus* (Fig. 1 of IV). The cloned *cbsA* sequence contained its own secretion signal and a 128 bp fragment upstream of the ATG codon which is almost completely identical to the corresponding sequence of *slpA* from *L. acidophilus* ATCC4356. This untranslated leader sequence renders the mRNA stable and is involved in efficient S-protein production (Boot *et al.*, 1996a). The resulting plasmid was named pLPCA5´. It is not known whether CbsA secreted by *L. casei* can attach to the cell surface of the bacterium and form an S-layer. Therefore, to facilitate covalent attachment of CbsA to the peptidoglycan, another plasmid construct was created where CbsA was fused at its C-terminus to a sequence encoding an LPXTG motif cell wall anchor of the PrtP proteinase from *L. casei* (Maassen *et al.*, 1999) giving plasmid pLPCA5´A. Both plasmids, pLPCA5´ and pLPCA5´A, were transformed into *L. casei* ATCC393, and the expression of CbsA was induced in the presence of galactose.

Production of CbsA in *L. casei* ATCC393 was detected by immunoblotting with polyclonal rabbit α-His-CbsA antiserum from different culture fractions (Fig. 2 of IV). CbsA cloned without an anchor fusion in pLPCA5´ was secreted into the culture medium, only small amounts could be extracted from the cell surface with LiCl. By electron microscopy, the secreted CbsA was seen to self-assemble into an S-layer sheet. The majority of CbsA fused to the cell wall anchor in pLPCA5´A was found in the whole cell extract and could not be detached by LiCl. Only small amounts were detected in the culture medium, they probably were released after cell lysis. In Western blots (Fig. 2 of IV) anti-CbsA antiserum also recognized peptides with a molecular weight higher than CbsA. These were attributed to the presence of cell wall fragments associated with CbsA, since they were not detected after treatment of cell extracts with mutanolysin. The S-protein SlpH of *L. helveticus* has been expressed in *L. lactis* (Callegari *et al.*, 1998). The *slpH* gene with its own signal sequence was cloned under the control of the lactococcal promoter P32 and without a cell wall anchor fusion. The expressed SlpH was secreted to the growth medium where it formed regularly ordered protein aggregates.

Immunofluorescence microscopy showed that CbsA expressed with the anchor fusion in pLPCA5´A was located on the surface of *L. casei* (not shown). No signal was detected without the anchor fusion (pLPCA5´) or with the vector alone (pLPM11). Surface location was further confirmed with a fluorescence activated cell sorter (FACS scan) (Fig. 3. of IV). The signal from *L. casei* expressing the *cbsA-prtP* fusion was as strong as the signal from *L. crispatus* JCM5810 suggesting similar amount of CbsA molecules on the cell surface. However, Western blots of recombinant cells revealed that the amount of CbsA produced in *L. casei* was substantially lower than on JCM5810 cells. In the same expression system utilizing the same host, expression vector and anchor sequence, Maassen *et al.* (1999) estimated that $1.4x10^3$ tetanus toxin C fragments were displayed on the surface of *L. casei*. In *L. lactis*, $5x10^4$ M6 protein molecules with the endogenous cell wall anchor (Piard *et al.*, 1997a) and $10^4$ lipase molecules fused to the anchor sequence of the FnBPB protein of *S. aureus* (Strauss and Gotz, 1996) have been found surface-located. In contrast, $5x10^5$ S-protein monomers are needed to cover the bacterial cell wall (Sleytr and Messner, 1988). By electron microscopy, we were unable to demonstrate an S-layer sheet structure on the surface of *L. casei*/pLPCA5´A. The PrtP anchor or inefficient surface expression on *L. casei*/pLPCA5´A cells might

prevent proper folding of CbsA and hinder the S-layer assembly. Recognition of cryptic CbsA epitopes on *L. casei*/pLPCA5´A that are hidden on the densely packed S-layer sheet of JCM5810 cells possibly cause the equally strong signals from *L. casei*/pLPCA5´A and JCM5810 in the FACS scan analysis with the anti-CbsA antiserum (Fig. 3 of IV).

*L. casei* expressing CbsA fused to the PrtP anchor adhered to glass-immobilized type I and type IV collagens more efficiently than *L. casei* expressing CbsA without the anchor fusion or *L. casei* without an insert, but the adhesion levels were lower than with JCM5810 (Fig. 4 of IV). This may be caused by the lower density of CbsA molecules on the *L. casei* cell-surface. Our mutation analysis of CbsA suggested that the formation of a paracrystalline array is optimal for the collagen-binding activity by CbsA, and inability to form an S-layer sheet on the *L. casei* cell-surface, as indicated by negative EM analysis (not shown), can contribute to the observed low collagen-binding.

## 9.5. Effect of lactic acid bacteria on the adhesion and invasion of *Salmonella typhimurium* (I)

One of the proposed probiotic functions of LAB is the prevention of epithelial infections by competition of binding sites with invading pathogens. We tested the effect of the LAB strains, an adhesive and a poorly adhesive strain, on the ability of *S. typhimurium* to adhere and invade into Caco-2 cells. Similar effects on *Salmonella* adhesion and invasion were seen (Fig. 4 of I) with both strains, a Caco-2 -adhesive strain JCM1132 and a non-adhering strain F81. Adhesion was inhibited by 90 % and invasion by 50 to 60 %. Other LAB strains behaved similarly regardless of their Caco-2 adhesiveness, indicating that competition for specific binding sites was not responsible for the inhibitory effects (not shown). Several other LAB strains have similar or even higher inhibitory effects on enteric pathogens (Table 5), but the factor(s) that cause this apparent inhibition are not known. Recently, Todoriki *et al.* (2001) reported that two strongly Caco-2 adherent strains, *L. crispatus* JCM8779 and *L. reuteri* JCM1081, inhibited the adhesion of enterotoxigenic *E. coli, S. typhimurium* and *E. faecalis* to Caco-2 cells. The antiadhesive activity was attributed to competition for binding sites with LAB and the pathogens, and in the case of the *L. crispatus* strain, production of an antimicrobial substance was suggested as an antiadhesive factor as well. Similar result was obtained by strongly adherent lactobacilli that did not affect the attachment of enterotoxigenic *E. coli* to isolated porcine (Spencer and Chesson, 1994) or chicken enterocytes (Jin *et al.*, 1998). In our study, a strongly Caco-2 adherent (JCM1132) and non-adherent strain (F81) were equally effective in decreasing *Salmonella* adhesion and invasion to Caco-2 cells, raising the question whether other inhibitory mechanisms than competition for specific receptors are also involved. LAB have an antagonistic effect on the growth of enteric pathogens (Gilliland and Speck, 1977; Chateau *et al.*, 1993; Drago *et al.*, 1997; Hudault *et al.*, 1997; Dunne *et al.*, 1999). Therefore, we tested the effect of LAB on the viability of *Salmonella*. Strain JCM1132 caused a reduction of 40 % in *Salmonella* cell numbers and strain F81 only a 5 % reduction. Similar lack of correlation with growth inhibition and the effect on *Salmonella* adhesion and invasion was seen with the other strains as well. LAB cells did not produce significant amounts of acid either, since the pH remained above 7 during the assay. Thus, neither competition for specific binding sites or antagonistic activity against *Salmonella* viability appear universal factors of LAB to reduce *Salmonella* adhesion and invasion. Further study is needed to find out whether other factors are involved, such as non-specific steric blocking of *Salmonella* receptors by LAB, and whether the results obtained with the Caco-2 assay are also applicable *in vivo*.

# 10. CONCLUSIONS

The results of this study show that adhesiveness of lactobacilli to human tissue targets is common in LAB from different sources and of different *Lactobacillus* species. Adhesiveness to epithelial cells and ECM

was equally common, 10 of the 12 *L. acidophilus* group strains showed adhesiveness to either one of these targets. The *L. acidophilus* group strains expressed different adhesion patterns to intestinal tissue components or cells and exhibited large variability in adhesion efficiencies. JCM1132 bound to Caco-2 cells and is an example of an epithelium-specific strain, whereas strains JCM5810 and A269-21 adhered efficiently to the ECM and BM. Adhesiveness to both the epithelial cells and ECM was also expressed by the strains, *e.g.* F41 which showed capacity to adhere to all tested tissue-components. None of the strains adhered significantly to the control proteins BSA (Table 9, Fig. 3 of I) and fetuin (not shown), which indicates specificity for the adhesion targets. Lack of correlation in the adhesion to the epithelial cell lines Caco-2 and Intestine 407 as well as the large variation in the ability to bind ECM components indicates the presence of multiple adhesive functions in LAB. This notion is supported by the presence of two unrelated collagen-binding adhesins, CbsA and Cnb (Roos *et al*., 1996) in LAB.

The role of host-specificity in LAB adhesion has been a matter of controversy (Fuller, 1973; Kotarsky *et al.*, 1979; Barrow, 1980; Mäyrä-Mäkinen *et al.*, 1983; Lin and Savage, 1984; Conway *et al.*, 1987; Jacobsen *et al.*, 1999; Todoriki *et al.*, 2001). The results of this thesis do not support strict host-species specificity in LAB adherence to epithelial cells or ECM. The highest adhesion levels to Caco-2 cells were exhibited by the human strains JCM1132, JCM1130 and JCM5813, but LAB from chicken, rat, pig or calf isolates were also adhesive to the human epithelial cell lines. Similar absence of strict host-specificity in LAB adhesion was also observed in adhesion to the human or mouse ECM and its components. Most LAB isolates so far tested seem to adhere to isolated epithelial cells or tissue pieces of their own isolation hosts, but several exceptions have been reported (Table 3).

The frequently observed adherence of LAB to collagens may be a tissue-adherence mechanism. The localization of the adherence of *L. crispatus* JCM5810 to collagen-rich connective tissue sites in the chicken colon supports this notion. ECM-adherent LAB may protect the host against invasion of pathogens at sites, such as wounds or cell sloughing, where the ECM is exposed to the intestinal lumen. Furthermore, LAB can apparently be translocated into *lamina propria* across the intestinal epithelium, to mesenteric lymph nodes (Ma *et al.*, 1990) and Peyer's patches (Perdigón *et al.*, 2000). This was proposed to take place when the permeability of the mucosal barrier is increased by local inflammation and by the intestinal antigen sampling mechanisms of the host. Adhesion to collagens could be advantageous for the colonization by LAB once they reach subintestinal regions. On the other hand, bacterial adherence to collagens can lead to long-term colonization (Miettinen *et al.*, 1993). The frequent binding of fibronectin and epithelial cells by the *L. acidophilus* group strains may reflect to fibronectin acting as a bridging molecule between the bacterial cell and the epithelial cell surface. Such molecular bridging via fibronectin to integrin $\alpha_1\beta_5$ is an adhesion and invasion mechanism in *S. aureus* (Sinha *et al.*, 1999). An RGD-motif on the central part of the fibronectin molecule mediates binding to the integrin molecule (Potts and Campbell, 1994).

The DNA homology groups of *L. acidophilus* were not clearly associated with the binding patterns observed with the intestinal tissue components, rather, the adhesion properties in LAB seem to be strain-specific. Both of the homology group A2 strains, JCM5810 and A269-21, expressed efficient adhesiveness to basement membrane material, but more strains need to be tested to see whether adhesiveness to BM is associated with the A2 group.

In this study, we have identified a novel class of *Lactobacillus* adhesins, the S-layer protein. CbsA together with the collagen-binding surface protein Cnb (Aleljung *et al.*, 1994; Roos *et al.*, 1996) and mucin-binding Mub (Roos *et al.*, 2000) of *L. reuteri*, are presently the only *Lactobacillus* adhesins characterized on a genetic level. Collagen-binding was frequent among the twelve *L. acidophilus* group strains, but only JCM5810 expressed an ECM-binding S-layer and hybridized to the *cbsA*-specific DNA probe. This indicates that other collagen-binding mechanisms must exist in the strains and that as a collagen-adhesin, CbsA and S-layers do not represent the most common type in the *L. acidophilus* group of lactobacilli. This is also suggested by the collagen-binding that was seen with the strain JCM5813 which lacks an S-layer. It is possible that the collagen-binding by the other strains is mediated by a Cnb-homolog of an ABC transport system.

As demonstrated by mutation analysis, the large collagen-binding epitope of CbsA has several regions which are involved in the formation of the active site and in the assembly of the S-layer sheet. Dependence of the collagen-binding activity on S-layer polymerization may indicate that the adherence involves several monomers or that the conformation of the binding site is dependent on the folding of CbsA into the S-layer.

In Cna of *S. aureus* and Ace of *E. faecalis*, a binding groove for the collagen triple helix is formed within one adhesin molecule (Symersky *et al.* 1997; Rich *et al.*, 1999b). The binding site(s) on the collagen molecule for CbsA is currently not known. Cna binds at multiple regions within one collagen molecule with varying affinities (Rich *et al.*, 1999a).

Surprisingly, the amino acids 1-287 in the variable region of CbsA were found to contain the information for the S-layer assembly process. The short conserved motifs in the variable N-terminal half of *L. acidophilus* group S-proteins (Fig. 1 of III) might represent evolutionary preserved framework needed for the proper folding of LAB S-layers. Presently, little is known about the incorporation of S-proteins into a growing S-layer (reviewed in Fernández and Berenguer, 2000). Data has been reported on the insertion points of S-protein monomers on the cell wall. In *Bacillus sphaericus* and *Bacillus stearothermophilus*, the highest insertion rates of S-layer monomers was observed in the septation sites of dividing cells (Howard *et al.*, 1982; Gruber and Sleytr, 1988), whereas in *Caulobacter crescentus* a diffuse pattern of S-protein incorporation was observed on the cell surface (Smit and Agabian, 1992). The highly conserved C-terminal domain of CbsA is not essential for the interaction of CbsA monomers and the S-layer assembly. Possibly the C-terminal region has a role in the translocation of the S-protein onto the cell-surface or in the attachment of the S-layer to the underlying cell wall, as was suggested for the C-terminal domain of SlpA (Smit *et al.*, 2001).

It remains to be seen whether CbsA forms a similar trench-like groove on the surface of the molecule as Cna of *S. aureus* and Ace of *E. faecalis*. Structural similarity is feasible despite the weak sequence homology of the primary sequences. To clarify this, the three-dimensional structure of CbsA would be needed. Structure analysis of S-layers by X-ray crystallogpraphy is challenged by their strong preference to form two-dimensional crystals and the difficult production of good-quality protein crystals (Engelhardt and Peters, 1998). Creation of a truncated domain of CbsA that is unable to polymerize but binds collagen could help to circumvent this problem. Our truncated peptides of CbsA, however, lost collagen-binding activity simultaneously with the ability to polymerize.

Heterologous expression of functional CbsA in *L. casei* gives tools to transfer an adhesive phenotype of LAB to another LAB species. This could be advantageous in creation of novel tissue-adherent probiotic strains or targeted vaccine delivery strains of LAB. For the induction of efficient mucosal immunity, the vaccine carrier strain should adhere to tissue structures of the intestine and be recognized by the immune cells.

All of the 12 *L. acidophilus* group strains were effective in the inhibition of *Salmonella* adhesion and invasion to Caco-2 cells regardless of their Caco-2 adhesiveness. Killing of *Salmonella* cells by the production of antagonistic substances such as lactic acid by the LAB strains did not inhibit *Salmonella* adhesion and invasion either. The Caco-2 cell is a simplified model of the intestinal environment, *e.g.* other bacterial residents of the normal flora, which may contribute to colonization resistance, are not present. Preventive effect of LAB administration against intestinal infections is nevertheless well documented (reviewed in Kasper, 1998; Salminen *et al.*, 1998). One of the proposed mechanisms is pathogen exclusion by adherent LAB, which is possible in the intestine *in vivo* even though we were unable to demonstrate inhibitory effect with the Caco-2 cell model. Another possible protective mechanism by LAB is the modulation of the immune responses, which could lead to increased protection against pathogen invasion (reviewed in Isolauri *et al.*, 2001; Perdigón *et al.*, 2001).

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