Characterization and applications of *Lactobacillus brevis* S-layer proteins and evaluation of *Lactococcus lactis* as a porcine cytokine producer

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Helsinki 2005
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ACADEMIC DISSERTATION

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Helsinki 2005
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Cover Figure:

Immunofluorescence microscopy of *Lactobacillus brevis* strain GRL1046 cells treated with anti-Myc antibodies and FITC-conjugated secondary antibody.
To Juha and Joel
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ABSTRACT

The members of the genus *Lactobacillus* belong to the heterogeneous group of lactic acid bacteria (LAB), Gram-positive bacteria that have been widely utilized for centuries in the food and feed industry. Like many other bacteria, several species of *Lactobacillus* have a surface (S-) layer as the outermost component of the cell. The thus far characterized functions of *Lactobacillus* S-layers are involved in mediating adhesion to different host tissues.

In this study, the S-layer proteins of two *Lactobacillus brevis* strains, neotype strain ATCC 14869 and ATCC 8287, were investigated with respect to their gene expression and applications related to more efficient mucosal antigen delivery, respectively. The S-layer proteins of *L. brevis* could be utilized as putative antigen carriers and the SlpA protein of *L. brevis* ATCC 8287 was studied in this respect. Two new S-layer proteins (SlpB and SlpD), with potential to be tested as antigen carriers, were characterized and three *slp* genes (*slpB, slpC* and *slpD*) were isolated, sequenced and their expression examined from *Lactobacillus brevis* ATCC 14869. Under different growth conditions, *L. brevis* ATCC 14869 was found to form two colony types, smooth (S) and rough (R), and by a mechanism not involving DNA rearrangements to differently express the S-proteins. In the *L. brevis* ATCC 8287 SlpA protein, a poliovirus epitope VP1 and a c-Myc epitope from the human c-*myc* proto-oncogene were surface displayed in a chimeric form. One of the four insertion sites, allowing the best surface expression determined with the VP1 constructs, was used for the construction of an integration vector carrying the gene region encoding the c-Myc epitopes. As a result of successful gene replacement, an *L. brevis* integrant was obtained that displayed the c-Myc epitope in all of the S-layer protein subunits without any effect on the S-layer lattice structure, demonstrating that at least small epitopes can be successfully surface-displayed as part of the S-layer protein of *L. brevis*.

To study whether a naturally nonadhesive lactic acid bacterium can be rendered adhesive, the receptor-binding region of the *L. brevis* ATCC 8287 SlpA was surface displayed in *Lactococcus lactis* with a cassette additionally encoding a proteinase spacer and an autolysin anchor. The lactococcal transformatants were indeed able to bind to a human intestinal epithelial cell line, Intestine 407, and also to fibronectin, demonstrating the functionality of the receptor-binding region of the SlpA in a heterologous LAB host.

In addition to antigen carriers, the expression of a putative vaccine adjuvant in *L. lactis* was studied. As cytokines are currently being considered as adjuvants, porcine interleukin-2 was chosen for this study. Two secretion cassettes were constructed in which the secretion was achieved by gene fusion between the lactococcal *usp45* secretion signal, a synthetic propeptide and the sequence encoding the mature IL-2. In addition, one of the two secretion cassettes contained the H-domains of *L. lactis* PrtP. Both of the constructed recombinant IL-2 proteins were found to be secreted in the same quantities and the specific biological activities of both purified rIL-2 proteins were found to be of similar levels. The expression system for porcine IL-2 in *L. lactis* developed in this study can thus be utilized for the production of biologically-active porcine IL-2, suitable for adjuvant use in future immunization studies.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxyterminus</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GRAS</td>
<td>generally recognized as safe</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>i.g.</td>
<td>intragastric</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>i.n.</td>
<td>intranasal</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravaginal</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LAB</td>
<td>lactic acid bacteria</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTB</td>
<td>B subunit of <em>Escherichia coli</em> heat-labile toxin</td>
</tr>
<tr>
<td>N-terminal</td>
<td>aminoterminal</td>
</tr>
<tr>
<td>M cell</td>
<td>microfold cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>orf</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>RBS</td>
<td>ribosome binding site</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal DNA</td>
</tr>
<tr>
<td>rIL-2</td>
<td>recombinant interleukin-2</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SCWP</td>
<td>secondary cell wall polymers</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>S-layer</td>
<td>surface layer</td>
</tr>
<tr>
<td>SLH</td>
<td>S-layer homologous</td>
</tr>
<tr>
<td>SP</td>
<td>signal peptide</td>
</tr>
<tr>
<td>spp.</td>
<td>species</td>
</tr>
<tr>
<td>TTFC</td>
<td>tetanus toxin fragment C</td>
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LIST OF ORIGINAL PUBLICATIONS:

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III Åvall-Jääskeläinen S., A. Lindholm and A. Palva. 2003. Surface display of the receptor-binding region of the *Lactobacillus brevis* S-layer protein in *Lactococcus lactis* provides nonadhesive lactococci with the ability to adhere to intestinal epithelial cells. Applied and Environmental Microbiology. 69:2230-2236.

1. INTRODUCTION

The lactic acid bacteria (LAB) family is composed of a heterogeneous group of Gram-positive, nontsporing, catalase and cytochrome negative, anaerobic or aerotolent bacteria (Axelsson, 1998). LAB can be divided into homofermentative, heterofermentative and facultatively heterofermentative according to the end products of their sugar metabolism (Kandler and Weiss, 1986). In nature, LAB have several different habitats including plant surfaces, decaying plant material and the mammalian intestine, vagina and oral cavity, which provide the multiple nutrients required by these fastidious bacteria (Axelsson, 1998). The food and feed industry widely utilizes LAB in the fermentation of vegetables, silage, and dairy and meat products. Certain LAB can also act as spoilage organisms in foods such as meat (Borch et al., 1996), fish (Lyhs et al., 2001) and beverages (Sakamoto and Konings, 2003). Due to their long-standing industrial use and lack of pathogenicity, LAB are generally recognized as safe (GRAS) organisms. Several bacteria belonging to the LAB family have been shown to possess beneficial health-promoting effects to their host and have thus been named as probiotic bacteria (Fuller, 1989), which are currently used in several products intended for both human and animal consumption. Attention has recently also focused on the development of LAB, especially lactobacilli, lactococci and streptococci, as antigen delivery vehicles (Lee, 2003; Seegers, 2002; Xin et al., 2003).

Crystalline bacterial surface layers (S-layers) composed of protein or glycoprotein subunits are present in almost all archaea and all major phylogenetic groups of bacteria as the outermost structure of the cell envelope (Sleytr and Messner, 1983). Thus far, lactobacilli are the only LAB from which S-layers have been identified. S-layer proteins represent 10 to 15% of the total protein of the bacterial cell and are thus the most abundant of all bacterial cellular proteins (Boot and Pouwels 1996; Messner and Sleytr, 1992). The S-layer subunits are non-covalently linked to each other and to the supporting cell envelope (Sára, 2001). Diverse functions have been proposed for S-layers, such as acting as protective coats, cell shape determinants, adhesion sites for exoenzymes, adhesins and virulence factors in pathogenic organisms; however, a general function for all S-layers has not been determined (Sára and Sleytr, 2000).

Mucosal surfaces provide the principle portals of entry for most of the viral, bacterial and parasitic agents (Erikson and Holmgren et al., 2002). Mucosal immunity thus has an essential role in the prevention of initial infections at the mucosal surfaces. This is reflected in the fact that the majority of the lymphoid tissues are distributed along mucous membranes, the gastrointestinal tissues occupying over 80% of them (Takahashi and Kiyono, 1999). Mucosal surfaces are protected by both innate and adaptive immune defense mechanisms. Adaptive immune responses are to a large extent mediated by immunoglobulin A (IgA), which is the predominant antibody in the mucosal secretions (Lamm, 1997).
The development of new mucosal vaccines for the prevention of mucosal infections has gained much interest recently in modern vaccinology. Live bacterial vaccine vectors studied for the delivery of antigens to mucosal surfaces include several species of LAB. The use of LAB as vaccine vectors offers many potential advantages over the use of delivery systems based on attenuated variants of pathogens, which have also been widely studied (Mercenier et al., 2000). From the encouraging results obtained from immunization studies conducted with LAB vaccine carriers it can be concluded that commercial LAB vaccines will eventually also be on the market, although many areas of the vaccine development still require optimization as well as a deeper understanding of mucosal immunology comprising the gut immune system as the major component.
2. REVIEW OF THE LITERATURE

2.1. Surface-layer proteins

2.1.1. Structure of S-layer proteins

S-layers are monomolecular crystalline arrays identified in hundreds of different species from the domains of Bacteria and Archaea as the outermost structure of the cell envelope (Messner and Sleytr, 1992; Sára and Sleytr, 2000). Most S-layers consist of single proteinaceous subunits with molecular weights of 40 to 200 kDa (Sleytr and Messner, 1983). A few organisms, including *Clostridium difficile* and *Bacillus anthracis*, have an S-layer protein consisting of two types of S-layer subunits (Etienne-Toumelin et al., 1995; Mesnage et al., 1997; Takeoka et al., 1999). Some Gram-positive and Gram-negative bacteria produce two superimposed S-layer lattices consisting of different subunit species (Kist and Murray, 1984; Yamada et al., 1981).

Currently, two types of post-translational modifications of S-layer subunits have been identified. Glycosylated S-layer proteins have been characterized in Gram-positive bacteria and in Archaea (Schäffer and Messner, 2001). The glycan chains are typically composed of two to six monosaccharides repeated up to 50 units consisting of neutral hexoses, deoxy sugars, amino sugars and in some cases of non-carbohydrate substituents (Schäffer et al., 1996; Schäffer and Messner, 2001; 2004). The sugar residues of the S-layer glycoproteins are attached to the protein moiety via O-glycosidic or N-glycosidic linkages (Schäffer and Messner, 2004). So far, only one report of a phosphorylated S-layer protein exists. The S-layer protein AhsA of *Aeromonas hydrophila* is phosphorylated at its tyrosine residues and preliminary results suggest that S-layer phosphorylation is a common feature of other motile aeromonads with S-layers (Thomas and Trust, 1995a).

The S-layer is usually 5 to 25 nm thick consisting of subunits aligned in lattices with oblique, square or hexagonal symmetry (Sleytr and Beveridge, 1999; Sára and Sleytr, 2000). The centre-to-centre spacings of the morphological units vary from 3 to 30 nm (Sleytr et al., 1994). Due to the composition of identical subunits, the pores in the S-layer proteins also exhibit morphological identity, although the pore size in an individual lattice can show some variability (Sleytr and Messner, 1988). The porosity of the S-layer protein surface area can be up to 70% (Sára and Sleytr, 2000).

The S-layer proteins are among the most abundant bacterial proteins, representing 10 to 15% of the total cellular protein of the bacterial cell (Boot and Pouwels, 1996). The S-layer lattices have been shown to cover the cell surface completely during all stages of growth (Sleytr and Messner, 1983). Amino acid analyses of S-layer proteins has revealed some general features in amino acid composition, but the sequence identities among S-layer proteins of different species or strains within a species are usually very low. The S-layer proteins have a high content of acidic and hydrophobic amino acids (aa) and few or no sulphur-containing aa (Messner and Sleytr, 1988; Sleytr and Beveridge, 1999). The theoretical isoelectric points (pI) of S-layer proteins are usually weakly acidic (Sára and Sleytr, 2000); *Methanothermus fervidus* and lactobacilli, however, possess S-layer proteins with a basic pI (Boot and Pouwels, 1996; Bröckel et al., 1991). Secondary structure
measurements indicate that in most S-layers 40% of the amino acids occur as \( \beta \)-sheets and 10-20% as \( \alpha \)-helices (Sleytr et al., 2001).

### 2.1.2. Expression of S-layer protein genes

The large number of S-layer protein subunits, \( 5 \times 10^5 \), needed to cover the bacterial cell indicates efficient gene expression, S-layer protein synthesis and secretion (Boot and Pouwels, 1996; Sleytr, 1997). In most studied organisms the quantity of S-layer proteins detected in growth medium is miniscule, indicating a strict control of S-layer protein synthesis (Messner and Sleytr, 1992). Only a few organisms produce an excess of S-layer proteins shed into the growth medium, but in *Bacillus* spp. this seems to occur commonly (Sidhu and Olsen, 1997). In *Bacillus thuringiensis*, free S-layer fragments have only been detected in cell cultures in late-exponential and stationary growth phases (Luckevich and Beveridge, 1989).

The S-layer protein genes are preceded by single or multiple promoters. The use of multiple promoters in transcription may lead to higher messenger ribonucleic acid (mRNA) levels compared to the use of a single promoter and provides the bacterium with the opportunity to regulate S-protein gene expression by activating the promoters differentially according to the prevailing physiological conditions (Boot and Pouwels, 1996). In all bacteria studied thus far, the half-lives of mRNAs encoding the S-layer proteins have been relatively long, 10 – 22 min, this stability probably contributing to the efficient production of S-layer protein subunits needed by the bacterium (Boot et al., 1996; Chu et al., 1993; Fisher et al., 1988; Kahala et al., 1997).

### 2.1.3. S-layer proteins of lactic acid bacteria

Among the lactic acid bacteria, S-layers have been found from numerous species of the genus *Lactobacillus* (Masuda, 1992; Masuda and Kawata, 1983; Yasui et al., 1995). Thus far the S-layer protein encoding genes have been cloned and sequenced from *L. brevis* (Vidgrén et al., 1992), *Lactobacillus acidophilus* (Boot et al., 1993), *Lactobacillus helveticus* (Callegari et al., 1998), and *Lactobacillus crispatus* (Sillanpää et al., 2000). Strains of *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus kefir* and *Lactobacillus parakefir* have also been shown to possess an S-layer (Boot et al., 1996b; Garrote et al., 2004), but their S-layer protein genes have not yet been sequenced. Although the study by Boot et al. (1996b) implicated the lack of an S-layer and S-layer protein encoding genes in strains of *Lactobacillus gasseri* and *Lactobacillus johnsonii*, in a recent study by Ventura et al. (2002) several different strains of these species were shown to possess two genes encoding surface proteins with typical S-protein characteristics. Two copies of the S-layer protein gene have also been described for *L. crispatus* JCM5810 (Sillanpää et al., 2000) and *L. acidophilus* ATCC 4356 (Boot et al., 1995). In *L. crispatus*, the additional S-layer protein gene, \( cbsB \), was not expressed under the tested growth conditions, whereas in *L. acidophilus* the silent S-layer protein gene can be expressed after deoxyribonucleic acid (DNA) rearrangements (Boot et al., 1996c) discussed in detail in Chapter 2.1.7. Southern blot analyses by Boot et al. (1996b) also suggested the presence of two S-layer protein genes in *L. amylovorus* and *L. gallinarum*. 

13
The S-layer protein genes of *L. brevis* and *L. acidophilus* are preceded by two promoter sequences (Boot et al., 1996a; Vidgén et al., 1992). The putative S-layer protein gene *apf1* of *L. johnsonii* and *L. gasseri* has also been shown to have two transcription start sites (Ventura et al., 2002). Of the two S-layer promoters in *L. acidophilus*, only the most downstream one has been shown to be used to direct the synthesis of S-layer protein mRNA (Boot et al., 1996a), whereas in *L. brevis* both promoters are active in all phases of growth, albeit the transcripts from the most downstream promoter were found to be predominant in all growth stages (Kahala et al., 1997). The half-lives of the transcripts encoding the S-layer proteins have been determined for *L. acidophilus* and *L. brevis* and were shown to be 15 min and 14 min, respectively (Boot et al., 1996a; Vidgrén et al., 1992). From the untranslated leader sequence of the *L. acidophilus* S-protein mRNA a hair-pin like secondary structure was identified by computer analysis and was found by reporter gene analysis to contribute to the efficient production of S-protein (Boot et al., 1996a). With the aid of the expression and secretion signals of the S-layer protein gene (*slpA*) of *L. brevis* ATCC 8287, high-level heterologous protein production in various *Lactococcus* and *Lactobacillus* hosts has been achieved (Savijoki et al., 1997; Kahala and Palva, 1999).

The molecular masses of lactobacillar S-layer proteins vary from 43 kDa (Sillanpää et al., 2000) to 55 kDa (Masuda and Kawata, 1981; 1983), being among the smallest known for the S-layer proteins. Compared to most other S-layer proteins with acidic nature, the S-layer proteins of *Lactobacillus* species differ in having high calculated pI values (Boot and Pouwels, 1996). The S-layer proteins of some lactobacilli convey hydrophobicity to the *Lactobacillus* cell surface (van der Mei et al., 2003) and for one *Lactobacillus* strain, *L. acidophilus* ATCC 4356, the cell surface hydrophobicity has been shown to change in response to changes in the environmental ionic strength (Vadillo-Rodríguez et al., 2004).

Sequence alignment studies have revealed that the S-layer proteins of *L. acidophilus*, *L. crispatus* and *L. helveticus* show the highest homology in the C-terminal one-third of the proteins (Sillanpää et al., 2000; Smit et al., 2001), whereas no significant homologies between the S-layer protein of *L. brevis* and other lactobacillar S-layer proteins have been described. In *L. acidophilus* and *L. crispatus*, the region carrying the information for the self-assembly of S-layer protein subunits into a regular layer has been shown to be carried by the N-terminal two-thirds of the protein (Sillanpää et al., 2002; Smit et al., 2001). The structural organization of the crystallization domain of *L. acidophilus* S-layer protein has been studied by Smit et al. (2002) and was concluded to consist of two subdomains linked by a surface-exposed loop.

The thus far characterized functions of the S-layer proteins in lactobacilli are involved in mediating adhesion to various extracellular matrix (ECM) proteins and epithelial cells of both human and animal origin (Table 1). Genetic truncation and heterologous surface expression studies with *Lactobacillus casei* have shown that in *L. crispatus* the collagen- and laminin-binding domain is located at the N-terminal two thirds of the protein with almost the same minimal amino acids required for binding to collagen and laminin and self-assembly (Antikainen et al., 2002; Sillanpää et al., 2000), showing that a single domain can be multifunctional. In *L. brevis*, the receptor-binding region was found by in vitro flagellar display experiments in *E. coli*, demonstrating that an N-terminal region of
SlpA, comprising aa residues 96 through 176, mediates the adhesion to human epithelial cells (Hynönen et al., 2002). Moreover, the binding to fibronectin was also shown to be mediated by an N-terminal region of SlpA, comprising aa 96 through 245 (Hynönen et al., 2002).

2.1.4. Secretion of the S-layer proteins

An N-terminal Sec-type secretion signal seems to be typical for S-layer proteins, since thus far only the S-layer proteins of Campylobacter fetus, Caulobacter crescentus and Serratia marcescens have been found to be devoid of the signal peptide (SP) (Kawai et al., 1998; Sára and Sleytr, 2000). The S-layer proteins with N-terminal SPs are secreted via the general secretory pathway (type II secretion system) (Boot and Pouwels, 1996). The signal sequences of S-layer proteins typically consist of 20 to 30 amino acids which are cleaved off after translocation through the plasma membrane yielding the mature S-layer protein (Sára and Sleytr, 2000). Data concerning the secretion of S-layer proteins are currently limited, since the reported studies have been carried out with Gram-negative organisms. The study by Houssin et al. (2002) demonstrated that the translocation of the Clostridium glutamicum S-layer protein subunits across the plasma membrane is dependent on proton motive force and ATP (adenosine triphosphate), indicating the involvement of the Sec apparatus. In A. hydrophila and Aeromonas salmonicida, the S-layer subunits are translocated across the outer membrane by substrate specific terminal branches of the general secretory pathway (Noonan and Trust, 1995; Thomas and Trust, 1995b).

The thus far characterized S-layer proteins devoid of an N-terminal SP are secreted by the Sec-independent type I secretion apparatus (Awram and Smit, 1998; Kawai et al., 1998; Thompson et al., 1998). In Gram-negative bacteria, the type I pathway most often recognizes an uncleaved C-terminal secretion signal (Fernández and Berenguer, 2000), and this is also the case for the S-layer proteins of C. fetus and C. crescentus (Bingle et al., 1997a; Thompson et al., 1998). From the S-layer protein of S. marcescens, the location of the S-layer protein secretion signal has not yet been determined (Kawai et al., 1998). In C. fetus and S. marcescens the genes encoding the components of the secretion system are located within a single cluster in the vicinity of the S-layer protein-encoding gene (Kawai et al., Thompson et al., 1998). In C. crescentus, the genes encoding the ABC transporter and the membrane fusion protein are located immediately downstream of the S-layer protein encoding gene (Awram and Smit, 1998). Toporowski et al. (2004) have identified two outer membrane proteins from C. crescentus, RsaFα and RsaFβ, which are both required for full-level secretion of the S-layer protein. Both of these outer membrane proteins can, however, function alone, leading to decreased secretion levels (Toporowski et al., 2004). The S-layer protein of S. marcescens is secreted by a LipBCD type I exporter, which is also involved in secreting other proteins in addition to S-layer proteins (Kawai et al., 1998).

2.1.5. Attachment of the S-layer protein to the underlying cell envelope

The interactions between S-layer subunits and with the underlying cell envelope components involve non-covalent linkages that are stronger between the individual
subunits than those connecting the S-layer lattice to the supporting cell envelope (Sleytr and Messner, 1983). The S-layer lattices can be disintegrated into their constituent subunits by different methods. These include the use of chaotropic agents such as urea and guanidine hydrochloride (Masuda and Kawata, 1980; Takeoka et al., 1991), metal-chelating agents such as EDTA (Bingle et al., 1987) or high concentrations of salt such as LiCl (Lortal et al., 1992). Once the disrupting agent used for the isolation of S-layer protein subunits is removed, the subunits have the ability to spontaneously assemble into regular arrays in the presence or absence of supporting layers (Sleytr et al., 1993).

Due to the different structural organization of the cell envelope in Gram-positive and Gram-negative bacteria, the cell envelope part to which the S-layer protein subunits can attach also differs according to species. However, no general mechanism of attachment of the S-layer subunit to the cell envelope that would depend on the classification of the bacterium by Gram staining has been found. Gram-negative bacteria ubiquitously express a lipopolysaccharide (LPS) component consisting of a lipid A-moiety, core section and an O-polysaccharide region (Erridge et al., 2002) as a part of the outer membrane. In several Gram-negative bacteria such as C. fetus, C. crescentus, A. hydrophila and A. salmonicida, the S-layer protein has been shown to be attached to the LPS (Dooley and Trust, 1988; Yang et al., 1992; Walker et al., 1994; Garduno et al., 1995). Studies with A. hydrophila mutant strains have shown that the LPS core oligosaccharide is involved in S-layer anchoring, whereas studies with A. salmonicida mutant strains have shown that the anchoring requires the presence of homogeneous-chain-length O-polysaccharide (Belland and Trust, 1984; Dooley and Trust, 1988). The region responsible for S-layer attachment to LPS has been shown to reside in the N-terminal part of the S-layer protein in C. fetus and C. crescentus (Dworkin et al., 1995; Bingle et al., 1997a), whereas in A. hydrophila the S-layer anchoring domain resides in the C-terminal part of the S-layer protein (Thomas et al., 1992). From the S-layer protein amino acid sequence of Rickettsia prowazekii a hypothetical C-terminal hydrophobic anchor has been found (Carl et al., 1990). Hydrophobic anchor sequences have also been found in some archaeal S-layer proteins where the S-layer protein is the only cell-wall component and is thus in close contact with the plasma membrane (Engelhardt and Peters, 1998).

The rigid cell envelope of almost all Gram-positive bacteria is composed of secondary cell wall polymers (SCWP) such as teichoic acid, lipoteichoic acids, lipoglycans or teichuronic acids in addition to peptidoglycan (Navarre and Schneewind, 1999; Neuhaus and Baddiley, 2003). When present in Gram-positive bacteria, the SCWP have been shown to be responsible for the anchoring of the S-layer protein to the cell envelope through different mechanisms. The S-layer homologous (SLH) motifs first identified by Lupas et al. (1994) are present at the N-terminal part of S-layer proteins in several Gram-positive bacteria and have been found to be responsible for the anchoring of S-layer protein to SCWP (Brechtle and Bahl, 1999; Chauvaux et al., 1999, Ilk et al., 1999; Mader et al., 2004; Mesnage et al., 2000; Mesnage et al., 2001). The S-layer proteins usually possess three repeats of SLH domains, each consisting of about 55 amino acids (Engelhardt and Peters, 1998). Mesnage et al. (2000) have reported that in B. anthracis the anchoring of SLH domains to the cell surface involves the addition of a pyruvyl group to a peptidoglycan-associated polysaccharide fraction and they also suggested that this type of anchoring could occur in other species of the Bacillus cereus group. However, a single
SLH domain present in the S-layer protein of *Thermus thermophilus*, a bacterium of Gram-negative character having a similar subcellular architecture to Gram-positive bacteria (Quintela et al., 1995), seems to bind directly to the peptidoglycan (Olabarría et al., 1996). Structural analyses of the envelope of *T. thermophilus* have shown that the peptidoglycan of this bacterium does not contain any associated macromolecules (Quintela et al., 1995). Several cell-associated exoenzymes and other exoproteins of Gram-positive bacteria as well as outer membrane proteins of Gram-negative bacteria have also been shown to possess SLH domains (Engelhardt and Peters, 1998) that seem to have a cell-surface-anchoring role similar to S-layer protein SLH-domains (Lemaire et al., 1995; Kosugi et al., 2002).

The S-layer proteins of lactobacilli, *Corynebacterium glutamicum* and *Geobacillus stearothermophilus* wild-type strains belong to a group of S-layer proteins from which no SLH-domains have been detected (Chami et al., 1997; Engelhardt and Peters, 1998; Jarosch et al., 2000), but the binding of these S-layer proteins to the cell wall has some similarities to SLH-domain mediated binding. Masuda and Kawata (1980, 1981, 1985) examined the attachment of S-layer proteins of *L. brevis* and *Lactobacillus buchneri* to the cell wall and concluded that the binding site for the S-layer proteins in these bacteria is not peptidoglycan or teichoic acid but a neutral polysaccharide moiety of the cell wall. The C-terminal regions of the S-layer proteins of *L. acidophilus* ATCC 4356 and *L. crispatus* JCM 5810 are almost identical in sequence, and the cell wall binding domain in these bacteria has been shown to reside in the C-terminal region (Antikainen et al., 2002; Smit et al., 2001). Smit and Pouwels (2002) showed that in *L. acidophilus* an N-terminal repeat in the C-terminal SAC domain is most likely responsible for the anchoring of S-layer protein to cell wall fragments. For the S-layer proteins of *L. acidophilus* and *L. crispatus*, teichoic acids have been suggested to be involved in the binding (Antikainen et al., 2002; Smit and Pouwels, 2002). From the S-layer protein of *C. glutamicum* a C-terminal hydrophobic segment involved in the anchoring of the S-layer to the cell wall has also been found (Chami et al., 1997). However, in *C. glutamicum* this hydrophobic domain anchors the S-layer protein to an outer membrane of hydrophobic nature (Bayan et al., 2003; Chami et al., 1997). Wild-type strains *G. stearothermophilus* ATCC12980 and PV72/p6 (formerly *Bacillus*; Nazina et al., 2001) have S-layer proteins with nearly identical N-terminal regions which in these bacteria are responsible for the anchoring of the S-layer protein subunits to the secondary cell wall polymer of the cell envelope (Egelseer et al., 1998; Jarosch et al., 2000). The secondary cell wall polymer, to which the S-layer protein subunits attach, is of identical chemical composition in both of the *G. stearothermophilus* wild-type strains consisting of glucose and N-acetylglucosamine (Egelseer et al., 1998; Sára et al., 1996).

### 2.1.6. Functions of S-layer proteins

Diverse functions have been described for the S-layers (Table 1), but no general function for all S-layers has been found (Sleytr and Beveridge, 1999). In some organisms such as *C. fetus* and *A. salmonicida* S-layers are multifunctional (Table 1), acting as virulence factors in these pathogens. S-layers act as adhesins in several bacteria such as lactobacilli and *B. cereus*, mediating the adherence of these bacteria to epithelial cells and/or ECM.
(Hynönen et al., 2002; Kotiranta et al., 1998; Schneitz et al., 1993; Toba et al., 1995). S-layers have also been shown to act as adhesion sites for exo-enzymes in *G. stearothermophilus* spp. (Egelseer et al., 1995; 1996; Jarosch et al., 2001), protect the cells from predation by *Bdellovibrio bacteriovorus* (Koval and Hynes, 1991), function as cell shape determinants (Messner et al., 1986; Pum et al., 1991), templates for fine-grain mineral formation (Schultze-Lam et al., 1992) or molecular sieves (Sára and Sleytr, 1987).

### 2.1.7. S-layer phase variation

Several bacteria, including both pathogens and nonpathogens, have been shown to be capable of varying their S-layer proteins based on DNA rearrangements. S-layer variation

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>S-layer protein</th>
<th>Function</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Geobacillus. stearothermophilus</em> ATCC 12980</td>
<td>SbcC</td>
<td>Exoamylase-binding site</td>
<td>Jarosch et al., 2001</td>
</tr>
<tr>
<td><em>G. stearothermophilus</em> DSM 2358</td>
<td></td>
<td>High-molecular weight amylase binding site</td>
<td>Egelseer et al., 1995; 1996</td>
</tr>
<tr>
<td><em>G. stearothermophilus</em> spp.</td>
<td></td>
<td>Molecular sieve</td>
<td>Sára and Sleytr, 1987</td>
</tr>
<tr>
<td><em>Rickettsia prowazekii</em> and <em>Rickettsia typhi</em></td>
<td></td>
<td>Responsible for humoral and cell-mediated immunity</td>
<td>Carl and Dasch, 1989</td>
</tr>
<tr>
<td><em>Campylobacter fetus</em></td>
<td>SapA</td>
<td>Resistance to serum-mediated killing and phagocytosis</td>
<td>Blaser et al., 1987; 1988</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td>Virulence factor in infection</td>
<td>Blaser and Pei, 1993; Grogono-Thomas et al., 2000; Pei and Blaser, 1990</td>
</tr>
<tr>
<td><em>Synechococcus</em></td>
<td></td>
<td>Template for fine-grain mineral formation</td>
<td>Schultze-Lam et al., 1992</td>
</tr>
<tr>
<td><em>Aeromonas salmonicida</em></td>
<td>VapA</td>
<td>Virulence factor</td>
<td>Ishiguro et al., 1981</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td>Adhesion to ECM proteins, macrophages and non-phagocytic cells</td>
<td>Doig et al., 1992, Garduno et al., 1992; 2000</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td>Responsible for humoral and cell-mediated killing</td>
<td>Munn et al., 1982</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td>Prevents predation by <em>Bdellovibrio bacteriovorus</em></td>
<td>Koval and Hynes, 1991</td>
</tr>
<tr>
<td><em>Aquaspirillum</em> spp.</td>
<td></td>
<td>Prevents predation by <em>Bdellovibrio bacteriovorus</em></td>
<td>Koval and Hynes, 1991</td>
</tr>
<tr>
<td><em>Bacteroides forsythus</em></td>
<td></td>
<td>Virulence factor</td>
<td>Sabet et al., 2003</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td></td>
<td>Adhesion to laminin</td>
<td>Kotiranta et al., 1998</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td></td>
<td>Adhesion to avian epithelial cells</td>
<td>Schnetz et al., 1993</td>
</tr>
<tr>
<td><em>Lactobacillus brevis</em></td>
<td>Sla</td>
<td>Adhesion to human epithelial cells and fibronectin</td>
<td>Hynönen et al., 2002</td>
</tr>
<tr>
<td><em>Lactobacillus crispatus</em></td>
<td>CbsA</td>
<td>Adhesion to ECM proteins</td>
<td>Toba et al., 1995</td>
</tr>
<tr>
<td><em>Thermoproteus</em> spp.</td>
<td></td>
<td>Determination of cell shape</td>
<td>Messner et al., 1986</td>
</tr>
<tr>
<td><em>Methanocorpusculum sinense</em></td>
<td></td>
<td>Determination of cell shape and cell division</td>
<td>Pum et al., 1991</td>
</tr>
</tbody>
</table>
most often occurs as a response to environmental factors. An example of S-layer variation thus far only detected at the DNA level is that of *L. acidophilus* ATCC 4356 (Boot et al., 1996c). In addition to an actively transcribed S-layer protein gene *slpA*, *L. acidophilus* has a silent *slpB* gene (Boot et al., 1993; Boot et al., 1995). A 6-kb (kilobase) fragment contains *slpA* and *slpB* genes in opposite orientations and an inversion of this fragment places the silent *slpB* gene behind the *slpA* promoter (Boot et al., 1996). The inversion most likely occurs by site-specific recombination at a 5’-identity region present in *slpA* and *slpB*, but the inducer for this change has not been determined. In the growth conditions used only a small minority (0.3%) of the chromosomes had the *slpB* gene behind the *slpA* promoter and the formation of an altered S-protein could not be detected.

The S-layer phase variation detected in *G. stearothermophilus* sp. is induced by environmental factors such as oxidative stress and elevated growth temperatures. Under oxygen-limiting conditions *G. stearothermophilus* produces S-layer protein SbsA, which in the presence of an oxygen supply is irreversibly replaced by SbsB resulting in an S-layer lattice with a different symmetry to that of SlpA (Sára and Sleytr, 1994; Kuen et al., 1994; 1997). The *sbsB* gene is located on a natural megaplasmid of strain PV72/p6 and for the expression of *sbsB*, the coding region integrates into a chromosomally located expression site (Scholz et al., 2001). During the switch the *sbsA* coding region is removed from the chromosome, leaving only the upstream regulatory region of *sbsA* in the chromosome. The switch from *sbsA* expression to *sbsB* seems to be irreversible, since no reversion of the process could be detected. In *G. stearothermophilus* ATCC 12980 elevated growth temperatures have been shown to lead to the production of an altered S-layer, which was found to be glycosylated and encoded by a new gene, *slpD*, absent from the chromosome and megaplasmids of the wild-type strain (Egelseer et al., 2001). The S-layer variation induced by an elevated growth temperature also seems to be an irreversible process, since no revertants could be detected when the growth temperature of the variant was reduced to the normal level.

For *B. anthracis*, having S-layer protein types Sap and EA1, the modification of the S-layer protein content of the cell is dependent on the growth phase and conditions. In a rich medium the *B. anthracis* cells are covered with a Sap S-layer in the exponential growth phase, which is is replaced by an EA1 layer in the stationary phase (Mignot et al., 2002). This S-layer regulation appears to involve Sap and EA1 proteins, which may both act as transcriptional repressors of the *eag* gene (Mignot et al., 2002). Sequential expression of the S-layer genes is not observed when the *B. anthracis* cells grow in a defined medium designed to mimic the in vivo conditions that the cells encounter during infection (Mignot et al., 2003). Under these growth conditions the regulation of the S-layer genes was found to be controlled by plasmid encoded genes (Mignot et al., 2003).

Wild-type strains of *C. fetus* have been shown to express S-layer proteins with different molecular weights with one form predominating for a single strain (Pei et al., 1988). Each S-layer protein is encoded by promoterless *sapA* homologue (Dworkin and Blaser, 1996). From the *sapA* locus only a single promoter can be detected upstream of the *sapA* homologue (Tu et al., 2003), and for the expression of *sapA* homologs only this single *sapA* promoter is used (Dworkin and Blaser 1996). Variation in S-layer protein expression occurs by a single DNA inversion event in which the *sapA* promoter alone or together with one or more of the flanking *sapA* homologs inverts (Dworkin and Blaser, 1996;
The S-layer variation in \textit{C. fetus} results in antigenic variation of the S-layer (Garcia et al., 1995; Wang et al., 1993), thus providing the pathogen a means to delay the host antibody response, as has been observed to occur in experimentally challenged sheep (Grogono-Thomas et al., 2003).

### 2.1.8. S-layer applications and fusion proteins

Due to the highly ordered and regular structure of S-layers and the capability of isolated S-layer subunits to assemble into regular arrays in suspension, on suitable surfaces or liquid-surface interfaces, S-layers have a broad spectrum of applications in biotechnology and nanotechnology (Sleytr et al., 2001; Pum and Sleytr, 1999). These include their use as isoporous ultrafiltration membranes (Sára et al., 1992) and matrices for immobilization of functional macromolecules such as antibodies (Breitwieser et al., 1996), allergens (Bohle et al., 2004) or oligosaccharide haptens (Messner et al., 1992). S-layers can also be utilized as matrices for the development of dipstick-style immunoassays (Völkel et al., 2003), templates for the formation of regularly arranged nanoparticles (Mertig et al., 2001) or as stabilizing structures for solid-supported lipid membranes (Pum and Sleytr, 1999). S-layers also have potential for vaccine development. For the treatment of furunculosis in fish caused by \textit{A. salmonicida}, the S-layer protein preparations of \textit{A. salmonicida} have been tested (Lund et al., 2003). S-layers also have been studied as possible vaccine carriers with encouraging results (Jahn-Scmid et al., 1996).

S-layer fusion proteins can be used to study the secretion, cell wall anchoring and self-assembly of S-layer proteins. They also have several potential applications, including use as vaccine carriers and functional monomolecular lattices required for applications in nanobiotechnology. For the construction of the S-layer fusion proteins, the whole S-layer protein (Bingle et al., 1997b; Umelo-Njaka et al., 2001), a C- or N-terminally truncated S-layer protein (Breitwieser et al., 2002; Moll et al., 2002) or only the cell wall-targeting domains (Mesnage et al., 1999b; Smit et al., 2001) can be utilized. The expression of the S-layer fusion proteins can be plasmid-derived in a heterologous host (Riedmann et al., 2003; Moll et al., 2002) or a homologous null-mutant host lacking the wild-type S-protein gene (Bingle et al., 1997b). The S-layer fusion protein can also be chromosomally encoded (Smit et al., 2002).

The domains involved in secretion and cell wall anchoring of S-layer proteins in various Gram-negative and Gram-positive bacteria and the adhesion domains of several lactobacilli have been localized with use of S-layer fusion proteins. The ability of the SLH motifs of the \textit{B. anthracis} S-layer proteins EA1 and Sap to mediate cell surface anchoring has been demonstrated in a study by Mesnage et al. (1999a) in which the SLH domains were fused with the levansucrase of \textit{B. subtilis}. A fusion protein consisting of green fluorescent protein and the SAC domain of the S-layer protein of \textit{L. acidophilus} has been shown to bind to \textit{Lactobacillus} cells stripped of their S-layers, thus demonstrating the role of the SAC domain in cell wall anchoring of the S-layer protein (Smit et al., 2001). Linker insertion mutagenesis, resulting in the insertion of four to six amino acids at different positions in the S-layer protein, RsaA, of \textit{C. crescentus}, has shown that the N-terminus is involved in the cell surface anchoring of the S-layer protein and suggested the presence of a C-terminal secretion signal (Bingle et al., 1997a).
constructs consisting of a 443-aa exoglucanase enzyme linked to the RsaA C-terminus of different lengths, the secretion signal could be localized to the C-terminal 82 amino acids of the RsaA (Bingle et al., 2000). In studying the adhesion of lactobacilli to various ECM proteins and epithelial cells, whole or truncated S-layer protein genes have been fused with fliC (Hynönen et al., 2002), the cell wall anchoring sequence of the prtP gene (Martínez et al., 2000) or histidine tags (Antikainen et al., 2002; Sillanpää et al., 2000).

The self-assembly and lattice formation of S-layer proteins can be studied with S-layer fusion proteins. By means of linker insertion mutagenesis, inserting a c-myc epitope at four sites in the S-layer protein, and with his-tagged, truncated S-layer protein fragments, Smit et al. (2002) have studied the crystallization of the S-layer protein of L. acidophilus. Insertions in conserved regions or in regions with predicted secondary structural elements resulted in the absence of crystalline sheets and none of the histidine-tagged, N- or C-terminally truncated peptides could form regular arrays. The fusion of the major birch pollen allergen to a C-terminally truncated S-layer protein of G. stearothermophilus has been shown to result in a fusion protein with the ability to form self-assembly products with an oblique lattice in vitro and also to recrystallize on native wall sacculi with the allergen portion located on the outer surface (Breitwieser et al., 2002). A C-terminally truncated S-layer protein of B. sphaericus has also been used in the construction of S-layer fusion proteins, and these fusion proteins have been observed to recrystallize into a regularly structured lattice on cell wall fragments (Ilk et al., 2002; 2004; Pleschberger et al., 2003), on solid supports coated with SCWP (Pleschberger et al., 2003; Völlenkle et al., 2004) or on positively charged liposomes (Ilk et al., 2004).

Since the S-layer proteins have been shown to be capable of surface-displaying even large proteins and different epitopes and they have been shown to possess some intrinsic adjuvant properties (Jahn-Schmid et al., 1996), S-layers may be utilized as vaccine vectors. Two immunization studies conducted with B. anthracis strains surface-expressing either TTFC (tetanus toxin fragment C) or levansucrase with the SLH-domains (Mesnáger et al., 1999b; 1999c) have resulted in a humoral response to the antigen and protection against tetanus toxin challenge in the TTFC immunized animals. In a study by Riedmann et al. (2003), immunization with lysed E. coli cells harboring the SbsA protein of G. stearothermophilus fused with the outer membrane protein (Omp) 26 of Haemophilus influenzae resulted in a humoral response to the Omp26. Recombinant vaccine candidates utilizing the S-layer protein, RsaA, of C. crescentus have also been tested. A humoral response could be obtained against the pilus tip epitope of P. aeruginosa when different RsaA-pilus tip epitope constructs were used for immunization; however, no significant protection against a P. aeruginosa infection was obtained (Umelo-Njaka et al., 2001). Simon et al. (2001) immunized rainbow trout fry with several RsaA-IHNV surface glycoprotein fusion proteins, but only a limited level of protection was induced against IHNV challenge.

2.2. The mucosal immune system

Mucosal surfaces are protected against environmental pathogens by both innate and adaptive immune defense mechanisms. Innate, nonadaptive mechanisms form the early lines of defence, including physical, chemical, cellular and molecular factors such as the
movement of mucus by epithelial cells, enzymes secreted by specialized epithelial cells, the complement system, tissue macrophages and cytokines secreted by activated macrophages (Janeway et al., 2001). Adaptive immune responses generate antigen-specific effector cells and eventually an immunological memory against the encountered pathogen. Adaptive immune responses are mainly achieved by immunoglobulin A (IgA), the predominant antibody at mucosal surfaces (Lamm, 1997). The sampling of luminal antigens occurs at the inductive sites of the mucosal immune system, consisting of organized aggregates of lymphoid cells known as the mucosa-associated lymphoid tissue, and the immune response operates at effector sites where the lymphoid tissue tends to be more diffuse (Hathaway and Kraehenbuhl, 2000). The mucosal immune system is distinct from the systemic immune system, but they are not totally segregated (Hathaway and Kraehenbuhl, 2000; Takahashi et al., 1999)

2.2.1. Gut immune system

The majority of the body’s mucosal surface area is occupied by the gastrointestinal tissues (Takahashi et al., 1999), which encounter a vast array of antigens including invasive organisms and harmless antigens such as dietary proteins and commensal microbiota (Stokes and Bailey, 2000). The gut-associated lymphoid tissue (GALT) consists of inductive sites including Peyer’s patches in the small intestine, isolated lymphoid follicles scattered throughout the gut lamina propria and mesenteric lymph nodes, and of effector sites including the lamina propria surrounding Peyer’s patches and lymphocytes scattered throughout the epithelium (Fagarasan and Honjo, 2003; Mowat, 2003). Only a single epithelial cell layer lines the entire intestinal mucosal surface separating the gut lumen from the underlying lymphoid tissue (Spahn and Kucharzik, 2004).

The antigens may gain access to the intestinal immune system by several different routes (Figure 1). The specialized follicle-associated epithelium overlying Peyer’s patches and also isolated lymphoid follicles (Fagarasan and Honjo, 2003) contain microfold (also called membranous; M) cells, which lack the surface microvilli characteristic of small intestinal epithelial cells and also the surface glycocalyx (Mowat, 2003). The apical surface of M cells has broad membrane microdomains from where the endocytosis of foreign antigens or particles occurs; the uptake of antigens may also occur by phagocytosis (Neutra et al., 1996). The uptaken antigen is transported through the interior of the cell in vesicles to the basolateral surface, where it is released to the underlying lymphoid tissue. The basolateral surface of M cells is deeply invaginated, thus facilitating the contact between the underlying cells of the immune system and the newly transported antigens (Hathaway and Kraehenbuhl, 2000). Antigens and micro-organisms are usually transported undegraded and alive across M cells (Neutra et al., 1996), but the exact role of M cells in the processing and presentation of antigens is not known (Neutra et al., 2001). Several pathogens such as Salmonella typhi (Pascopella et al., 1995) and reoviruses (Wolf et al., 1981) preferentially target M cells to gain entry to the body. Reoviruses have been shown to attach to specific M-cell carbohydrate structures (Helander et al., 2003), but because the glycosylation patterns of M cells vary in different intestinal regions and also in different species (Neutra et al., 2001), the M cells are able to transport a wide variety of microorganisms (Neutra et al., 1996).
Transepithelial transport of antigens may also involve dendritic cells and intestinal epithelial cells (Figure 1). Dendritic cells have been shown to extend their processes between epithelial tight junctions and transport both pathogenic and nonpathogenic bacteria to the lamina propria (Rescigno et al., 2001). The integrity of the epithelial barrier remains intact during this process because the dendritic cells are capable of reforming the tight junctions sealing the apical epithelium, as shown by the regulated expression of several tight junction proteins (Rescigno et al., 2001). The intestinal epithelial cells take up antigens by endocytosis, which may be receptor-mediated (Hershberg and Mayer, 2000). Antigens may also gain entry to the basolateral surface of intestinal epithelial cells by disrupting the tight junction structure (Nusrat et al., 2001).

![Figure 1. Antigen uptake and recognition by CD4+ T-cells in the intestine.](image)

Antigen might enter through M cells in the follicle-associated epithelium overlying Peyer’s patches (a), and after transfer to local dendritic cells might be presented directly to T-cells in the Peyer’s patch (b), or alternatively the antigen or antigen-carrying dendritic cells migrate through afferent lymphatics to the mesenteric lymph node, where T-cell recognition occurs (c). If the antigen enters through the epithelium covering the villus lamina propria, a similar process of antigen or antigen-presenting cell dissemination to mesenteric lymph nodes might occur (d), but it is also possible that the enterocytes act as local antigen presenting cells (e). Antigen might also gain direct access to the bloodstream from the intestine (f) and interact with T-cells in the peripheral lymphoid tissues (g). From the mesenteric lymph nodes the antigen-responsive T cells enter the bloodstream through the thoracic duct and exit into the mucosa through vessels in the lamina propria or gain access to systemic distribution. DC; dendritic cell, GC; germinal centre. Adapted from: Mowat, 2003.
The various possible routes for antigen presentation to naive CD4+ T cells are depicted in Figure 1. Antigen presenting cells (APC) in the gut can be dendritic cells, macrophages, B-cells or enterocytes (Makala et al., 2004; Mowat, 2003). Exosome-like vesicles, secreted by intestinal epithelial cells and bearing high amounts of MHC class I and class II molecules, have also been suggested to present luminal antigens to immature dendritic cells (Van Niel et al., 2003). Intestinal epithelial cells have been shown to express major histocompatibility complex (MHC) class I, II and CD1d molecules, through which the T-cell interactions may occur (Hershberg and Mayer, 2000). The importance of these interactions has not yet been established (Makala et al., 2004), but they are probably limited to pathological conditions such as inflammation (Hershberg and Mayer, 2000). Dendritic cells are most likely the APC involved in the interactions of naïve lymphocytes in Peyer’s patches (Mowat 2003). IgA-secreting B cells can also be activated by a T-cell-independent and follicularly organized lymphoid tissue-independent mechanism, which has been shown to occur in response to commensal intestinal bacteria (Macpherson et al., 2000).

The structure of Peyer’s patches (Figure 1) favours strong interactions between B cells, APC and local CD4+ T cells facilitating B-cell proliferation, class-switch recombination and somatic hypermutation, which occur in the germinal centres of Peyer’s patches (Fagarasan and Honjo, 2003) and lead to the preferential generation of IgA+ lymphoblasts (Makala et al., 2002-2003). The IgA+ B cells exit through the draining lymphatics to the mesenteric lymph nodes, where they proliferate and differentiate into plasmablasts (Fagarasan and Honjo, 2003). In addition to IgA+ B cells, activated antigen-specific CD4+ T cells and CD8+ T cells leave the inductive site and are carried via draining lymph nodes before migration into the bloodstream through the thoracic duct (Hathaway and Kraehenbuhl, 2000; Takahashi and Kiyono, 1999). Activated lymphocytes home to the mucosal site from which they originated or to distant mucosal sites (Brandtzaeg et al., 1999). The homing is mediated by tissue-specific adhesion molecules and chemokines (Kunkel and Butcher, 2002; Brandtzaeg et al., 1999). The upregulated expression of adhesion molecule α4β7 integrin in the absence of L-selectin is believed to be the main determinant for the homing of GALT-derived B and T cells to gut mucosa (Braentzaeg et al., 1999). The ligand for α4β7 integrin is mucosal addressin cell-adhesion molecule 1, which is constitutively expressed to endothelium of venules of intestinal lamina propria (Briskin et al., 1997).

The differentiation of B cell plasmablasts into antibody-secreting plasma cells occurs in lamina propria (Corthesy and Kraehenbuhl, 1999). IgA+ B cells might also be produced from IgM+ B cells either in the lamina propria or in the isolated lymphoid follicles scattered throughout the lamina propria (Fagarasan and Honjo, 2003). The plasma cells of lamina propria produce enormous amounts of IgA at mucosal surfaces daily (5-10 g), making IgA the predominant antibody responsible for the humoral immune response in gut (Corthesy and Kraehenbuhl, 1999; Takahashi and Kiyono, 1999).

Mucosal IgA is typically secreted as a dimeric molecule associated with an intersubunit J chain (Lamm, 1997), whereas systemic IgA circulates as a monomer (Nagler-Anderson, 2001). After binding to the polymeric immunoglobulin receptor present on the basolateral surface of enterocytes, the IgA is transported by transcytosis to the apical surface (Rojas and Apodaca, 2002). At the surface, the poly-Ig receptor is enzymatically
cleaved, separating the external domain from the membrane spanning domain and leaving
the external portion of the receptor bound to the dimeric IgA molecule as the secretory
component (Lamm, 1997). Once transported to the mucosal surfaces, secretory IgA (s-
IgA) antibodies protect the epithelial surfaces from infectious agents by cross-linking
them and thus preventing their attachment to and invasion of the mucosal surface; this
mechanism is termed immune exclusion (Corthesy and Kraehenbuhl, 1999). IgA antibodies
have also been shown to neutralize several viruses intracellularly during transcytosis
(Feng et al., 2000; Mazanek et al., 1995; Yan et al., 2002). An additional defence
mechanism of dimeric IgA includes the transport of IgA-antigen complexes out of the
lamina propria by the same route as free dimeric IgA, limiting the amounts of antigen
reaching the circulation (Kaetzel et al., 1991; Robinson et al., 2001). Pentameric IgM,
the primordial mucosal antibody, can also be secreted at mucosal surfaces by the polymeric
immunoglobulin receptor with the same mechanism as dimeric IgA (Rojas and Apodoca,
2002). IgM is a more potent activator of the complement system than IgA (Lamm, 1997).
Plasma cells also produce IgE antibodies, which have a major role in activating local
mast cells (Corthesy and Kraehenbuhl, 1999). The transcytosis of IgG from the lamina
propria to mucosal secretions, where IgG constitutes a minority of immunoglobulins
(Lamm, 1997), has been suggested to be mediated by the FcRn receptor, capable of
transcytosizing IgG also in the apical-to-basolateral direction (Dickinson et al., 1999).

Oral tolerance is defined as a mechanism of tolerance induction in which prior
administration of the antigen by the oral route renders the mature lymphocytes in the
local and peripheral lymphoid tissues into a state of antigen-specific and active
unresponsiveness (Strobel, 2002). Oral tolerance most likely serves as a mechanism for
the prevention of adverse immune reactions against luminal antigens derived from food
and the commensal gut microbiota (Spahn and Kucharzik, 2004). The precise mechanisms
involved in oral tolerance are still partly unknown (Nagler-Anderson, 2001), and several
mutually non-exclusive mechanisms are likely to occur, depending on the nature and
dose of the antigen, the frequency of antigen administration and several host factors such
as the genetic background, age and the commensal microbiota (Strobel and Mowat, 1998).
The primary mechanisms by which tolerance may be mediated are clonal anergy
characterized by the inability of antigen-specific T cells to proliferate (Sun et al., 2003)
and by the reduction in Th1 specific cytokines (Melamed and Friedman, 1994), deletion
of antigen-specific T cells via apoptosis (Chen et al., 1995) and active suppression by
special regulatory T cells secreting down-regulating cytokines (Chen et al., 1994). The
precise location of intestinal antigen presentation for induction of oral tolerance has not
been clearly determined (Spahn and Kucharzik, 2004), but enterocytes and dendritic
cells have been suggested to be antigen presenting cells involved in the induction of oral
tolerance (Strobel, 2002).

2.3. Lactic acid bacteria as vaccine delivery vectors

Vaccination represents one of the most efficient tools for the prevention and even
eradication of infectious diseases. Since the majority of infections occur at or through
the mucosal surfaces, the use of a mucosal route of vaccination instead of a parenteral
route would be preferable (Holmgren et al., 2003). Compared with the parenteral route
of vaccination, mucosal vaccination offers several advantages, such as prevention of the initial infection and replication of the pathogen at the site of entry, stimulation of both local and systemic immune responses, easy administration and low delivery costs (Medina and Guzmán, 2001). The key factor in inducing an active immune response at mucosal sites is the delivery system of the antigen, the development of which is under active research.

Several strategies have been employed for the mucosal delivery of antigens, including the use of synthetic (non-living) delivery systems such as liposomes (Ninomya et al., 2002) or immune stimulating complexes (van Pinxteren et al., 1999), virus-like particles (Takamura et al., 2004), bacterial vectors (Mielcarek et al., 2001), and genetically engineered plants (Sala et al., 2003). Widely studied micro-organisms as vaccine carriers are attenuated variants of pathogens including *Salmonella*, *Shigella* and *Mycobacterium* (Mielcarek et al., 2001), but the potential risks associated with the use of attenuated pathogens as vaccine carriers necessitate the development of alternative antigen delivery systems. Lactic acid bacteria have in recent years attracted considerable attention in the field of mucosal vaccine research. Several benefits associated with LAB favour their use as vaccine delivery vehicles. These benefits include the GRAS status of LAB (Adams and Marteau, 1995), the long-term experience of their production in the food industry, the capacity of numerous strains to adhere and colonize to mucosal surfaces, beneficial health-effects for the hosts of several strains, intrinsic immunogenicity, resistance to bile acid, lack of lipopolysaccharides in their cell wall eliminating the risk of an endotoxic shock, and the capability to modulate the immune responses obtained by inducing cytokine production of the host (Mercenier et al., 2000; Pouwels et al., 1998; Seegers et al., 2002). LAB have been utilized in targeting antigens to the gut, the oro-nasal cavity and the vagina for the induction of local as well as systemic immune responses (Mercenier et al., 2000). The LAB strains currently studied and evaluated as antigen delivery vehicles include *L. lactis*, *Streptococcus gordonii* and several *Lactobacillus* species.

### 2.3.1. Expression and cellular targeting of heterologous antigens in LAB

Several systems have been developed and tested in LAB for the differential expression of antigens resulting in cytoplasmic, extracellular medium or cell surface targeting of antigens. Replicative plasmids (Bermúdez-Humaran et al., 2002; Oliveira et al., 2003) or chromosomal integration (Oggioni et al., 1999; Smit et al., 2002; Turner and Giffard, 1999) can be utilized for the expression of antigens in LAB. Plasmid-based expression systems are easier to manipulate than integration systems and are thus more widely used, although integration systems allow a more stable expression of antigens (Seegers, 2002). When replicative plasmids are used for antigen expression, one of the key factors in determining the level of heterologous protein production is the promoter used in controlling the antigen expression. Promoters used for the expression of heterologous antigens in LAB can allow either constitutive or inducible expression. Constitutive promoters used in LAB for the expression of antigens or medically interesting proteins include *L. lactis* derived promoters P$_{59}$ (Dieye et al., 2003), P$_{23}$ (Chang et al., 2003), and P1 (Schotte et al., 2000), P$_{25}$ of *Streptococcus thermophilus* (Hols et al., 1997), and P$_{idb}$ of *L. casei* (Zegers et al., 1999). To avoid possible harmful effects to the cell resulting from continuous high...
level production of antigens, several inducible expression systems have been developed. The most widely used inducible expression system in LAB is the nisin-inducible system, which has been used both in lactococci (Bermúdez-Humarán et al., 2003a; Cortez-Perez et al., 2003; Enouf et al., 2001; Ribeiro et al., 2002) and in lactobasilli (Pavan et al., 2000). The nisin-inducible system was originally developed for \textit{L. lactis} and is based on the autoregulatory properties of the nisin gene cluster (de Ruyter et al., 1996a; 1996b). Transcription of the genes under the \textit{nisA} promoter can be induced by nisin, mediated by a two-component regulatory system consisting of the histidine kinase NisK and response regulator NisR (Kuipers et al., 1995; van der Meer et al., 1993). Other controllable expression systems utilized for antigen expression in LAB include the \textit{P}_{\text{lac}} of \textit{L. casei} (Oliveira et al., 2003), \textit{P}_{\text{amy}} of \textit{L. amylovorus} (Maassen et al., 2003) and \textit{P}_{170} of \textit{L. lactis} (Theisen et al., 2004).

When cytoplasmic production of antigens is desired, in addition to a promoter sequence, a ribosome binding site and the start of an open reading frame are required for protein synthesis to occur. Extracellular and cell-surface located production of antigens additionally requires a SP for the translocation to occur. Signal peptides used in LAB for the translocation of heterologous proteins and antigens across the plasma membrane include the SP Usp45 from the main secreted protein in \textit{L. lactis} (Dieye et al., 2001; Lindholm et al., 2004; Ribeiro et al., 2002), the SP of the fibrillar surface protein M6 of \textit{Streptococcus pyogenes} (Hols et al., 1997; Piard et al., 1997), the SP of the $\alpha$-amylase of \textit{L. casei} (Shaw et al., 2000), SPs of the cell surface proteinases of \textit{L. lactis} (Slos et al., 1998), and \textit{Lactobacillus} spp. (Bernasconi et al., 2002; Maassen et al., 1999), SPs from different S-layer proteins (Chang et al., 2003; Lindholm et al., 2004), SP310 of \textit{L. lactis} and its derivatives (Ravn et al., 2003; Theisen et al., 2004), and the SP of \textit{Staphylococcus aureus} Nuc protein (Chatel et al., 2003), which all utilize the sec-dependent secretion machinery of the host bacterium. The secretion efficiency of several heterologous proteins produced by lactococci has been improved by inserting a synthetic propeptide LEISSTCDA between the SP cleavage site and the mature moiety of the heterologous protein to be exported (Bermúdez-Humarán et al., 2003a; Langella and Le Loir, 1999; Ribeiro et al., 2002). The synthetic propeptide LEISSTCDA alters the N terminus of the mature protein by introducing negative charges on the protein at positions +2 and +8. The LEISSTCDA propeptide can potentially optimize the charge balance between the N-termini of the precursor and the mature protein and/or affect precursor conformation, thus leading to the enhancement of precursor translocation and processing (Le Loir et al., 1998). The expression of heterologous proteins in \textit{L. lactis} has also been stabilized by using a host strain defective in HtrA (Miyoshi et al., 2002; Poquet et al., 2000), an extracellular protease degrading abnormal exported proteins (Poquet et al., 2000).

The cell-surface located production of antigens by LAB can involve several strategies. The most widely exploited method for targeting heterologous proteins to the cell wall in LAB is the utilization of a C-terminal cell wall anchor domain, consisting of a conserved LPXTG motif, a transmembrane fragment and a charged C terminus (Leenhouts et al., 1999). A transpeptidation reaction between the LPXTG motif and peptidoglycan attaches the exported protein to the cell wall by the action of sortase machinery (Navarre and Schneewind et al., 1999). Anchoring of several antigens and other heterologous proteins in diverse LAB species using the LPXTG-motif anchor of protein M6 of \textit{S. pyogenes} Review of the Literature
(Byrd et al., 2002; Cortes-Perez et al., 2003; Reveneau et al., 2002), PrtP proteins of *L. paracasei* (Maassen et al., 1999) and *L. lactis* (Lindholm et al., 2004), Rlp protein of *Lactobacillus fermentum* (Turner et al., 2003) and protein A of *S. aureus* (Steidler et al., 1998) has been achieved. Another type of anchor domain utilized thus far only in lactococci for the surface display of heterologous proteins includes the use of the C-terminal region of cell wall hydrolase AcmA of *L. lactis* (Lindholm et al., 2004). The AcmA anchor domain, consisting of three highly homologous repeats of 45 aa, binds to the peptidoglycan by an unidentified mechanism (Steen et al., 2003), suggested to be of a non-covalent nature (Leenhouts et al., 1999). Other modes of anchoring antigens or other heterologous proteins to the surface of LAB include the use the S-layer proteins of lactobacilli (discussed in chapter 2.1.7.), the use of a non-covalently anchored BspA protein of *L. fermentum* (Turner and Giffard, 1999; Turner et al., 2003) and the use of a lipoprotein or transmembrane anchors (Leenhouts et al., 1999).

2.3.2. *Lactococcus lactis* as a vaccine vector

A LAB species intensively studied as a possible vaccine delivery vehicle is *L. lactis*, a bacterium which does not colonize the oral or intestinal cavities of humans or other animals (Wells et al., 1996). In mice (Kimoto et al., 2003) and humans (Klijn et al., 1995) the passage of lactococci through the enteric tract is transitory, the survival rate being only 1% in humans (Klijn et al., 1995). Lactococci administered together with food have been shown to be relatively resistant to gastric acidity, viable cells retaining their metabolic activity all the way through the digestive tract of rats (Drouault et al., 1999). Due to the limited capacity of lactococci to produce antigens in vivo in the gut, preloading of the bacteria with antigen before delivery would be desirable. This necessitates the use of expression systems leading to high-level expression of the antigen. Indeed, intracellular antigen production levels representing up to 22% of total soluble cell proteins (Wells et al., 1993) have been achieved in lactococci.

Several antigens of bacterial (Iwaki et al., 1990), viral (Dieye et al., 2003), and eukaryotic (Chatel et al., 2003) origin have been produced in *L. lactis*. Tetanus toxin fragment C (TTFC), a model antigen with high immunogenicity, consisting of a monomeric, non-toxic subunit of the tetanus toxin of *Clostridium tetani* (Mercenier et al., 2000), is the most widely studied antigen in the context of *Lactococcus* vaccine delivery systems. The use of *Lactococcus* as a cytokine delivery vehicle has recently also gained considerable attention. Steidler et al. (1995) first described the secretion of a biologically active cytokine in *L. lactis*, murine interleukin-2, and to date several additional cytokines of murine (Bermúdez-Humarán et al., 2003c; Schotte et al., 2000; Steidler et al., 1998a), ovine (Bermúdez-Humarán et al., 2003b) and human (Steidler et al., 2003) origin have been produced intra- or extracellularly by *Lactococcus*. The *in situ* delivery of cytokines by *Lactococcus* is warranted for two main strategies: stimulation of the onset and the maintenance of immune reactions to the co-expressed antigen or as for interleukin-10, to be used as a therapeutic agent in chronic intestinal inflammation (Steidler, 2002; Steidler et al., 2000).
2.3.3. Immune responses to *Lactococcus* vaccines

Immunization studies conducted with using *Lactococcus* as a vaccine and/or cytokine delivery vehicle are listed in Table 2. Several routes of immunization have been investigated (Table 2), including mucosal and parenteral routes, the mucosal route of delivery being the most commonly used. Antigens delivered by the mucosal route have been shown to elicit antigen-specific mucosal as well as systemic antibody responses (Table 2), although both responses have not always been obtained simultaneously (Chatel et al., 2001; 2003; Grangette et al., 2002; Lee et al., 2001; Mannam et al., 2004; Pontes et al., 2003). Few studies have also reported a failure to elicit a local antibody response after a mucosal administration of the antigen (Grangette et al., 2002; Lee et al., 2001). Several studies have shown that mucosal immunization can lead to antigen specific immune responses at a distant mucosal site in addition to the induction site (Chatel et al., 2001; Norton et al., 1997; Robinson et al., 1997; 2004).

The isotypes of IgG antibodies elicited in response to *Lactococcus* vaccines have been determined in numerous immunization studies (Table 2). The mucosal route of immunization with TTFC *Lactococcus* vaccine strains has been documented to lead to the predominance of isotypes IgG1 and IgG2a (Robinson et al., 1997; 2004) or to the predominance of isotypes IgG1 and IgG2b (Grangette et al., 2002; 2004; Norton et al., 1997), indicating that the antigen-specific T-helper subset responses cannot be predicted solely on the basis of the used antigen or route of immunization, but vary according to the used immunization scheme. Intraperitoneal (i.p.) immunizations with *Lactococcus* vaccine strains have resulted in the detection of IgG isotypes IgG1 and IgG3 (Gilbert et al., 2000; Robinson et al., 2004). The route and scheme of immunization thus determine the IgG isotype profile elicited by *Lactococcus* vaccines.

The cellular immune responses occurring in the recipient after encountering *Lactococcus* expressing a heterologous antigen have thus far been studied by both in vitro and in vivo assays only in reports by Bermúdez-Humarán et al. (2003c), Robinson et al. (2004) and Xin et al. (2003). Antigen-specific cellular immune responses have been detected in spleen and in mesenteric lymph nodes following the mucosal delivery of *Lactococcus* vaccine carriers, leading to the secretion of Th1 and Th2 cytokines (Bermúdez-Humarán et al., 2003c; Robinson et al., 2004). By the i.p. route of vaccination with lactococci expressing TTFC intracellularly, the cytokine expression profile of the spleen cells varied depending on the stimulus agent used (Bermúdez-Humarán et al., 2003c).
Table 2. Immunization and cytokine delivery studies with *Lactococcus lactis* as an antigen and/or cytokine delivery vehicle.

<table>
<thead>
<tr>
<th>Antigen or cytokine/origin*</th>
<th>Cellular location in lactococci</th>
<th>Route of immunization (animal)</th>
<th>Immune responses obtained [Studied but not obtained]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAc/<em>Streptococcus mutans</em></td>
<td>Intracellular</td>
<td>i.g. (mouse)</td>
<td>IgG, IgA, IgM</td>
<td>Ibaki et al., 1990</td>
</tr>
<tr>
<td>TTFC/<em>Clostridium tetani</em></td>
<td>Intracellular</td>
<td>s.c. (mouse)</td>
<td>TTFC protection, TTFC specific antibodies</td>
<td>Wells et al., 1993</td>
</tr>
<tr>
<td>TTFC/<em>C. tetani</em></td>
<td>Intracellular</td>
<td>s.c. (mouse)</td>
<td>IgG, TTFC protection [IgA]</td>
<td>Norton et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Bacterial envelope</td>
<td>s.c. (mouse)</td>
<td>IgG, IgM, TTFC protection [IgA]</td>
<td>Norton et al., 1997</td>
</tr>
<tr>
<td>TTFC/<em>C. tetani</em></td>
<td>Intracellular</td>
<td>i.n. (mouse)</td>
<td>IgA, IgG1, IgG2b, TTFC protection, antigen-specific lymphocytes</td>
<td>Norton et al., 1997</td>
</tr>
<tr>
<td>TTFC/<em>C. tetani</em> with IL-2/murine</td>
<td>Intracellular (TTFC), extracellular (IL-2)</td>
<td>i.n. (mouse)</td>
<td>IgA, IgG1, IgG2a [total IgA production]</td>
<td>Steidler et al., 1998a</td>
</tr>
<tr>
<td>TTFC/<em>C. tetani</em> with IL-6/murine</td>
<td>Intracellular (TTFC), extracellular (IL-6)</td>
<td>i.n. (mouse)</td>
<td>IgA, IgG1, IgG2a [total IgA production]</td>
<td>Steidler et al., 1998a</td>
</tr>
<tr>
<td>Type 3 capsular polysaccharide/<em>Streptococcus pneumoniae</em></td>
<td>Extracellular</td>
<td>i.p. (mouse)</td>
<td>IgG1, IgG3, IgM</td>
<td>Gilbert et al., 2000</td>
</tr>
<tr>
<td>Interleukin-10/murine</td>
<td>Extracellular</td>
<td>i.g. (mouse)</td>
<td>Therapeutic effect in chronic colitis</td>
<td>Steidler et al., 2000</td>
</tr>
<tr>
<td>β-lactoglobulin/bovine</td>
<td>Mostly intracellular</td>
<td>i.n., i.g. (mouse)</td>
<td>IgA (i.n., i.g.)[IgE, IgG1, IgG2a, serum IgA]</td>
<td>Chatel et al., 2001</td>
</tr>
<tr>
<td>NSP4/rotavirus</td>
<td>Intracellular</td>
<td>i.m. (rabbit)</td>
<td>Production of NSP4 specific antibodies</td>
<td>Enouf et al., 2001</td>
</tr>
<tr>
<td>UreB/<em>Helicobacter pylori</em></td>
<td>Intracellular</td>
<td>Oral (mouse)</td>
<td>IgG [IgA, protection against <em>H. pylori</em> infection]</td>
<td>Lee et al., 2001</td>
</tr>
<tr>
<td>TTFC/<em>C. tetani</em></td>
<td>Intracellular</td>
<td>i.g. (mouse)</td>
<td>IgG1, IgG2a, IgG2b [IgA, protective humoral response]</td>
<td>Grangette et al., 2002</td>
</tr>
<tr>
<td>Interleukin-12/murine</td>
<td>Surface displayed (E7), Extracellular (IL-12)</td>
<td>i.n. (mouse)</td>
<td>IL-2, IFN-γ production</td>
<td>Bermúdez-Humarán et al., 2003c</td>
</tr>
</tbody>
</table>

*Antigen or cytokine/origin* refers to the antigen or cytokine used in the studies, with *Streptococcus mutans* and *Clostridium tetani* indicating the organism from which the antigen or cytokine was derived.
<table>
<thead>
<tr>
<th><strong>Epitope of β-lactoglobulin/bovine</strong></th>
<th><strong>Extracellular</strong></th>
<th><strong>i.g. (mouse)</strong></th>
<th><strong>IgA [IgG, IgG1, IgG2a]</strong></th>
<th><strong>Chatel et al., 2003</strong></th>
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</thead>
<tbody>
<tr>
<td></td>
<td><strong>s.c. (mouse)</strong></td>
<td><strong>n.d.’ [IgE, IgG1, IgG2a]</strong></td>
<td></td>
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<tr>
<td><strong>HPV-16 E7/papillomavirus</strong></td>
<td><strong>Surface displayed</strong></td>
<td><strong>i.n. (mouse)</strong></td>
<td><strong>Production of HPV-16 E7 specific antibodies</strong></td>
<td><strong>Cortes-Perez et al., 2003</strong></td>
</tr>
<tr>
<td><strong>VP2 or VP3/IBDV</strong></td>
<td><strong>Surface displayed, extracellular</strong></td>
<td><strong>Oral (chicken)</strong></td>
<td><strong>n.d. [IgG]</strong></td>
<td><strong>Dieye et al., 2003</strong></td>
</tr>
<tr>
<td><strong>L7/L12 ribosomal protein/Brucella abortus</strong></td>
<td><strong>Intracellular</strong></td>
<td><strong>i.g. (mouse)</strong></td>
<td><strong>IgA, partial protection against <em>B. abortus</em> infection [IgG]</strong></td>
<td><strong>Pontes et al., 2003</strong></td>
</tr>
<tr>
<td><strong>V2-V4 loop of Env/ HIV</strong></td>
<td><strong>Surface displayed</strong></td>
<td><strong>Oral (mouse)</strong></td>
<td><strong>IgG, IgA, IFN-γ production, reduction of viral load after HIV challenge</strong></td>
<td><strong>Xin et al., 2003</strong></td>
</tr>
<tr>
<td><strong>HPV-16 E7/papillomavirus</strong></td>
<td><strong>Surface displayed, extracellular, intracellular</strong></td>
<td><strong>i.n. (mouse)</strong></td>
<td><strong>IL-2, IFN-γ production</strong></td>
<td><strong>Bermúdez-Humarán et al., 2004</strong></td>
</tr>
<tr>
<td><strong>SpaP/Erysipelothrix rhusiopathiae</strong></td>
<td><strong>Extracellular</strong></td>
<td><strong>i.n., i.g. (mouse)</strong></td>
<td><strong>IgG, IgA, protection against <em>E. rhusiopathiae</em> infection</strong></td>
<td><strong>Cheun et al., 2004</strong></td>
</tr>
<tr>
<td><strong>TTFC/C. tetani</strong></td>
<td><strong>Intracellular</strong></td>
<td><strong>i.g. (mouse)</strong></td>
<td><strong>IgG1, IgG2a, IgG2b, protective humoral response [IgG3, serum IgA]</strong></td>
<td><strong>Grangette et al., 2004</strong></td>
</tr>
<tr>
<td><strong>CRR region of M6/Streptococcus pyogenes</strong></td>
<td><strong>Surface displayed</strong></td>
<td><strong>i.n. (mouse)</strong></td>
<td><strong>IgA, protection against pharyngeal infection, promotion of survival after challenge [IgG]</strong></td>
<td><strong>Mannam et al., 2004</strong></td>
</tr>
<tr>
<td></td>
<td><strong>s.c. (mouse)</strong></td>
<td><strong>IgG, promotion of survival after challenge [IgA, protection against pharyngeal infection]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>i.n. plus s.c. (mouse)</strong></td>
<td><strong>IgA, IgG, protection against pharyngeal infection, promotion of survival after challenge</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>TTFC/C. tetani</strong></td>
<td><strong>Intracellular</strong></td>
<td><strong>i.g. (mouse)</strong></td>
<td><strong>IgG1, IgG2a, IgA, TTFC-specific IgA-secreting cells, IL-4, IL-5, IL-10 and IFN-γ positive events among CD4⁺ lamina propria lymphocytes [serum IgA, IgM]</strong></td>
<td><strong>Robinson et al., 2004</strong></td>
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<td></td>
<td><strong>i.n (mouse)</strong></td>
<td><strong>IgG1, IgG2a, IgA, TTFC-specific IgA-secreting cells [serum IgA, IgM]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>i.p. (mouse)</strong></td>
<td><strong>IgG1, IgG2a, IL-4 production [serum IgA, IgM, IFN-γ]</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CRR, conserved C-repeat; HPV, human papillomavirus; IBDV, infectious bursal disease virus; NSP4, rotavirus nonstructural protein 4; PAc, surface protein antigen; SpaP, surface-protective antigen; TTFC, tetanus toxin fragment C; UreB, urease subunit B.

* n.d., not detected.
A number of factors have been shown to influence the immunogenicity of the antigens delivered by *Lactococcus* antigen carriers. The form in which the antigen is associated with the lactococcal cell has been shown to affect its immunogenicity. Studies by Norton et al. (1996) and Bermúdez-Humarán et al. (2004) have shown that a cell-surface-displayed form of the antigen is more immunogenic than intracellular or secreted forms of the antigen. Studies by Bermúdez-Humarán et al. (2004) and Mercenier et al. (2000) have indicated dose-dependent antigen-specific immune responses, but an increased dosage of *Lactococcus* antigen carriers has not always improved the immune responses obtained (Mannam et al., 2004). The immunogenicity of *Lactococcus* vaccine carriers is most likely not dependent upon their viability in vivo, since immunological responses of the same magnitude have been obtained in immunizations with killed and live *Lactococcus* vaccine vehicles by the mucosal route (Grangette et al., 2002; Norton et al., 1997; Robinson et al., 1997). Chatel et al. (2003) have, however, reported a failure of killed *Lactococcus* vaccine carriers, administered by the parenteral route, to elicit antigen-specific immune responses, although no comparison with corresponding live lactococcal vectors given by the same route was made in the study.

Several strategies, including the use of adjuvants and the genetic modification of the *Lactococcus* vaccine strains, have been exploited for the enhancement of immune responses elicited by *Lactococcus* vaccine vectors. Adjuvants used in *Lactococcus* vaccines include cholera toxin (Norton et al., 1997), commercial adjuvants (Enouf et al., 2001) and cytokines (Bermúdez-Humarán et al., 2003c; Steidler et al., 1998a). Cholera toxin, used as an adjuvant for *Lactococcus* vaccines delivered by the intranasal (i.n.) route, has been shown to enhance mucosal but not serum antibody responses to TTFC (Norton et al., 1997). Cytokines are being tested as adjuvants because of their involvement as immune response mediators participating in the initiation, maintenance and resolution of both innate and acquired immune responses (Taylor, 1995). Both simultaneous production of antigen and cytokine in one *Lactococcus* strain and the use of separate *Lactococcus* strains for the antigen and cytokine production have been tested in immunization studies, both strategies having shown enhancement of antigen-specific immune responses compared to immunization without the cytokine (Bermúdez-Humarán et al., 2003c; Steidler et al., 1998a). The adjuvant effect of cytokine co-expression was lost when the *Lactococcus* strains were killed before immunization, suggesting that active secretion of the cytokines in vivo by recombinant lactococci is required (Steidler et al., 1998). Grangette et al. (2004) have used alanine racemase mutant strains of lactococci with increased membrane permeability for the intracellular expression of TTFC, and the superiority of these mutant strains in eliciting TTFC-specific antibody responses compared with their wild-type counterparts was demonstrated in mucosal immunizations.

### 2.3.4. *Lactobacillus* species as vaccine vectors

The *Lactobacillus* genus, with over a hundred species recognized at present, is very heterogeneous, these species possessing differences in their metabolism, distribution in nature and phenotypic properties such as adhesion capability (Mercenier et al., 2000). The heterogeneity of the *Lactobacillus* group is reflected in the number of species currently studied as possible antigen carriers including *L. fermentum, L. plantarum, L. casei, L.
paracasei, Lactobacillus jensenii and L. johnsonii. The Lactobacillus strains studied as possible vaccine carriers are commensals of humans or other animals or of dietary origin.

When developing lactobacilli as vaccine carriers, the choice of the appropriate Lactobacillus antigen carrier strain is of utmost importance. The factors that need to be considered include the capacity of the Lactobacillus strain to adhere to and colonize the relevant epithelial surfaces of the host, adjuvant and immunostimulation properties of the strain, genetic amenability of the strain and the codon usage preferences of the strain. Lactobacilli have been shown to be capable of stimulating both non-specific host immune responses, including the enhancement of phagocytic activity of phagocytic cells, and specific host immune responses, including the enhancement of humoral immune responses (Gill et al., 2003). The intrinsic adjuvant activities of lactobacilli, leading to the stimulation of immune responses to the co-administered antigen, are most likely based on the induction of cytokines by lactobacilli (Seegers, 2002). Considerable differences among diverse Lactobacillus species and strains have been found in their ability to induce the expression of anti- and pro-inflammatory cytokines (Morita et al., 2002; Perdigón et al., 2002). The immune responses elicited by Lactobacillus vaccines can thus be focused in the required direction by selecting an appropriate Lactobacillus vaccine carrier strain. The adhesion of lactobacilli to host epithelial surfaces and subsequent colonization is most likely host-and tissue- or site-specific (Pouwels et al., 1998). If colonization is expected, it is unlikely for a single Lactobacillus strain to be used as a vaccine carrier in different hosts or to deliver antigens to different mucosal cavities within the same host. As a result of the heterogeneity of the Lactobacillus group, gene cloning and expression tools often need to be optimized for each Lactobacillus strain used, which has been reflected in the choice of strains studied for vaccine delivery (Wells et al., 1996). When efficient translation of the antigen is required the codon usage preferences, varying between different Lactobacillus species (Pouwels and Leunissen, 1994), also need to be considered.

Antigens produced by Lactobacillus vaccine carriers include those of bacterial (Zegers et al., 1999), viral (Ho et al., 2005) and eukaryotic (Maassen et al., 2003) origin. Antigens have been produced in lactobacilli intracellularly, extracellularly and surface exposed, of which the intracellular and surface displayed production seem to be the most widely studied forms, as can be concluded from strains used for immunization studies (Table 3). As in Lactococcus, the most widely studied antigen in lactobacilli is also TTFC. The capacity of many lactobacilli to stimulate the expression of cytokines probably explains why lactobacilli have not yet been studied as cytokine production hosts.
### Table 3. Immunization studies with *Lactobacillus* spp. as an antigen or antibody fragment delivery vehicle.

<table>
<thead>
<tr>
<th>Host</th>
<th>Antigen or antibody fragment/origin</th>
<th>Cellular location in lactobacilli</th>
<th>Route of immunization (animal)</th>
<th>Immune responses obtained [studied but not obtained]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. plantarum</em></td>
<td>TTFC/Clostridium tetani</td>
<td>Intracellular</td>
<td>s.c. (mouse)</td>
<td>IgG</td>
<td>Pavan et al., 2000</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>TTFC/C. tetani</td>
<td>Intracellular</td>
<td>i.g., i.n. (mouse)</td>
<td>IgG (oral, i.n.), IgA (i.n.), antigen-specific T-cell activation (i.n.) and antigen-specific antibody secreting cells (i.n.)</td>
<td>Shaw et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surface displayed</td>
<td></td>
<td>IgG (i.n.), IgA (i.n.)</td>
<td></td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>TTFC/C. tetani</td>
<td>Intracellular</td>
<td>i.n. (mouse)</td>
<td>IgG1, IgG2a, IgG2b, IgA, TTFC protection, antigen specific T-cell proliferative response [IgG3]</td>
<td>Grangette et al., 2001</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>T cell epitope of Der p 1/house dust mite</td>
<td>Intracellular</td>
<td>i.n. (mouse)</td>
<td>Induction of Der p 1–specific T-cells producing IFN-(\gamma) [IL-5, IL-10]</td>
<td>Kruisselbrink et al., 2001</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>TTFC/C. tetani</td>
<td>Intracellular</td>
<td>i.g. (mouse)</td>
<td>IgG1, IgG2a, IgG2b, protective humoral response [IgA]</td>
<td>Grangette et al., 2002</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>TTFC/C. tetani</td>
<td>Intracellular</td>
<td>s.c., i.n. or i.g. (mouse)</td>
<td>IgG (s.c., i.n., i.g.), IgA (i.n., i.g.)</td>
<td>Reveneau et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extracellular</td>
<td></td>
<td>IgG (s.c., i.n., i.g.), IgA (i.n., i.g.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surface displayed</td>
<td></td>
<td>IgG (s.c., i.n., i.g.), IgA (i.n., i.g.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c. priming followed by oral immunization (mouse)</td>
<td></td>
<td>Booster effect in serum</td>
<td>Pouwels et al., 1996</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>Hackett epitope/ influenza virus</td>
<td>Intracellular</td>
<td>s.c., i.n. or i.g. (mouse)</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>TTFC/C. tetani</td>
<td>Intracellular</td>
<td>i.v. (mouse)</td>
<td>IgG1, IgG2a, IgG2b, protective humoral response, local IgA [IgG3, serum IgA]</td>
<td>Grangette et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.g. (mouse)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>TTFC/C. tetani</td>
<td>Surface displayed</td>
<td>s.c. (mouse)</td>
<td>IgG</td>
<td>Maassen et al., 1999</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>PA/Bacillus anthracis</td>
<td>Intracellular</td>
<td>Oral, i.n, i.p. (mouse)</td>
<td>IgG (i.p.) [PA specific antibody response by oral or i.n. immunization]</td>
<td>Zegers et al., 1999</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>TTFC/C. tetani</td>
<td>Intracellular</td>
<td>i.g., i.n. (mouse)</td>
<td>IgG (i.n.)</td>
<td>Shaw et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surface displayed</td>
<td></td>
<td>n.d.* [IgG]</td>
<td></td>
</tr>
<tr>
<td>L. casei</td>
<td>Guy’s 13 scFv/mouse monoclonal IgG1</td>
<td>Surface expression</td>
<td>Oral (rat)</td>
<td>Reduction in <em>Streptococcus mutans</em> bacterial counts and dental caries</td>
<td>Krüger et al., 2002</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------</td>
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<td>---------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>L. casei</td>
<td>gpMBP/guinea pig</td>
<td>Extracellular</td>
<td>i.n., i.g. (rat)</td>
<td>Induction of intranasal tolerance [gpMBP specific antibody response by i.g. or i.n. immunization, oral tolerance induction]</td>
<td>Maassen et al., 2003</td>
</tr>
<tr>
<td>MBP&lt;sub&gt;72-85&lt;/sub&gt;/guinea pig</td>
<td>Extracellular</td>
<td>i.n., i.g. (rat)</td>
<td>Induction of intranasal and oral tolerance [gpMBP specific antibody response by i.g. or i.n. immunization]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intracellular</td>
<td>i.g. (rat)</td>
<td>Induction of oral tolerance [gpMBP specific antibody response by i.g. or i.n. immunization]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. casei</td>
<td>hCGβ/human</td>
<td>Extracellular</td>
<td>i.v. (mouse)</td>
<td>IgA, antigen-specific lymphocytes, IFN-γ and IL-4 production</td>
<td>Yao et al., 2004</td>
</tr>
<tr>
<td>L. casei</td>
<td>Fragment of spike glycoprotein S/TGEV</td>
<td>Extracellular</td>
<td>i.g. (mouse)</td>
<td>IgA, IgG, induction of antibodies with neutralizing effects on TGEV infection</td>
<td>Ho et al., 2005</td>
</tr>
<tr>
<td>L. johnsonii</td>
<td>TTmim-PrtB/C. tetani, <em>Lactobacillus delbrueckii</em> subsp. bulgaricus</td>
<td>Surface displayed</td>
<td>i.g. (mouse)</td>
<td>PrtB specific IgG and IgA</td>
<td>Scheppler et al., 2002</td>
</tr>
<tr>
<td>L. johnsonii</td>
<td>Anti-idiotypic scFv/human</td>
<td>Surface displayed</td>
<td>s.c., i.n. (mouse)</td>
<td>Anti-α-IdscFv2 IgG (s.c., i.n.), anti-IgE IgG (s.c., i.n.)</td>
<td>Scheppler et al., 2005</td>
</tr>
<tr>
<td></td>
<td>IgE mimotope/human</td>
<td></td>
<td></td>
<td>Anti-α-IdscFv2 IgG (i.n.), anti-IgE IgG (s.c., i.n.) [Anti-α-IdscFv2 IgG (s.c.)]</td>
<td></td>
</tr>
</tbody>
</table>

* der P, an allergen of house dust mite; hCGβ, β subunit of human chorionic gonadotropin; gpMBP, guinea pig myelin basic protein; PA, protective antigen; PrtB, proteinase B; scFv, single chain fragment variable; TTFC, tetanus toxin fragment C; TTmim, tetanus toxin mimotope; TGEV, transmissible gastroenteritis coronavirus.
* n.d., not detected
2.3.5. Immune responses to *Lactobacillus* vaccines

Immunization studies conducted with using *Lactobacillus* spp. as an antigen or antibody fragment delivery vehicle are listed in Table 3. The most commonly used route of immunization has been the intragastric (i.g.) or oral delivery of *Lactobacillus* vaccines, and other routes of mucosal delivery, including the i.n. or intravaginal (i.v.) routes, and parenteral delivery, have also additionally been tested. When the mucosal route of antigen delivery has been used, antigen-specific mucosal as well as systemic antibody responses have been obtained, although sometimes either of the responses has been lacking (Table 3). The IgG isotypic profiles obtained after mucosal delivery of the *Lactobacillus* vaccines suggest the activation of both Th1 and Th2 T helper cell subsets (Grangette et al., 2001; 2002; 2004). Humoral mucosal responses at distant mucosal sites with *Lactobacillus* vaccine carriers have not been studied, except by Shaw et al. (2000), who by nasal priming followed by an oral boost detected no IgA responses in bronchoalveolar lavage fluids of mice immunized by *L. plantarum* vaccine carriers. With the parenteral route of antigen delivery an antigen-specific IgG response has always been obtained (Table 3).

In addition to humoral responses, other types of responses to *Lactobacillus* vaccine vectors have also been detected. The cellular immune responses occurring in the recipient after the delivery of *Lactobacillus* vaccine vectors have been studied in several reports. Immunization with *Lactobacillus* vaccines has been shown to lead to antigen-specific T-cell proliferative responses (Grangette et al., 2001; Kruisselbrink et al. 2001; Shaw et al., 2000) or lymphocyte proliferative responses (Yao et al., 2004). Other cellular responses observed include the detection of antigen-specific antibody secreting cells (Shaw et al., 2000) and the production of cytokines by the induced T cells (Kruisselbrink et al., 2001). The efficacy of *Lactobacillus* as a mucosal delivery vehicle in therapeutics has been demonstrated by Krüger et al. (2002) and Maassen et al. (2003). Lactobacilli surface expressing a single-chain Fv antibody fragment, which recognizes the adhesion molecule of *Streptococcus mutans*, was shown to reduce the number of *S. mutans* cells and caries scores in a rat model of dental caries development (Krüger et al., 2002). A reduction in experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis, was found when lactobacilli expressing guinea pig myelin antigens were used in mucosal immunization (Maassen et al., 2003). A method applicable for the tracing of *Lactobacillus* antigen carriers after administration and for analyzing host immune cell-*Lactobacillus* interactions was described by Geoffroy et al. (2000), who by using green fluorescent protein carrying lactobacilli showed their active phagocytosis by macrophages in vitro and also after administration ex vivo.

Factors shown to influence to the immunogenicity of the antigens delivered by *Lactobacillus* include the cellular location of the antigen, production level of the antigen and the viability of the carrier strain. When effects of the cellular location of the antigen in *Lactobacillus* vaccines have been studied, contradictory results have been reported. Reveneau et al. (2002) obtained the highest humoral responses to TTFC delivered mucosally with an intracellular form of TTFC, but the cell-surface associated and secreted forms of TTFC were found to be more immunogenic than the intracellular form of TTFC. In a study by Shaw et al. (2000), the intracellularly produced TTFC was, however, concluded to be more effective in producing antigen-specific humoral responses than the
surface-expressed form of TTFC delivered mucosally. When the parenteral route of delivery was used, the cellular location of TTFC had no effect on the immune responses obtained, since they were of similar levels with all of the constructs (Reveneau et al., 2000). Several studies (Grangette et al., 2001; 2002; Pavan et al., 2000; Reveneau et al., 2002) have shown that the absolute level of the antigen produced by Lactobacillus vaccine strains is a key factor in determining the level of immune responses obtained, and that the addition of the antigen dose leads to an enhancement of the immune response. The effect of the viability of Lactobacillus vaccine strains to the immune responses obtained has been studied with strains expressing TTFC intracellularly (Grangette et al., 2001; 2002). The results from these studies have shown that the antibody responses elicited by live and inactivated vectors are of similar levels, although the level of protective responses against TTFC was found to be higher with live carriers.

Lactococcus and Lactobacillus antigen carriers have been compared in their ability to elicit immune responses to the co-expressed antigen in studies by Grangette et al. (2002; 2004). Similar levels of intracellular antigen production in lactococci and lactobacilli were found to lead to significantly lower immune responses with Lactococcus vaccine strains compared to L. plantarum vaccine strains, determined by humoral responses and by the protective capacity of anti-TTFC serum antibodies in a neutralization assay (Grangette et al., 2001; 2004). The isotypic profiles of anti-TTFC antibodies were similar in both Lactococcus and Lactobacillus antigen carriers (Grangette et al., 2001; 2004). When alanine racemase mutant strains of Lactobacillus and Lactococcus antigen carrier strains were used, the immunogenicity of the Lactococcus vaccines delivered by the i.g. route was enhanced to a similar level with the Lactobacillus vaccines, but by the i.v. route of delivery the immunogenicity of Lactococcus mutant vaccine strain remained at a lower level than obtained with the equivalent Lactobacillus strain (Grangette et al., 2004).

2.3.6 Streptococcus gordonii as a vaccine vector

S. gordonii is a nonpathogenic bacterium belonging to the normal microbiota of the human oral cavity (Lee, 2003) and has been widely studied as a putative vaccine vector. The colonization potential of S. gordonii vaccine vector strains should be excellent, since in humans S. gordonii is one of the first organisms to colonize the oral cavity (Lee, 2003). Streptococcus gordonii has been shown to stably colonize the oral (Lee et al., 2002) and vaginal cavities (Medaglini et al., 1997) of mice and the vaginal mucosa of rats (Beninati et al., 2000), while persisting only transiently in the vaginal cavity of monkeys (Di Fabio et al., 1998). The murine intestinal mucosa has also been shown to be colonized by S. gordonii (Ricci et al., 2000) for a few weeks, whereas the colonization of the murine oral and vaginal mucosa can continue for up to twelve (Sharma et al., 2001) or six (Medaglini et al., 1997) weeks, respectively. No differences in colonization capacities have been found between recombinant and wild-type S. gordonii in the vaginal or oral mucosa of mice (Medaglini et al., 1995; 1997; Oggioni et al., 1995). The colonization properties of S. gordonii allow a long-lasting exposure of the host to the antigens delivered by the S. gordonii vaccine carriers, since the antigen is constantly expressed in vivo (Medaglini et al., 1998). Due to its persistent nature, a single oral inoculation with a S.
S. gordonii vaccine vector could in principle provide life-long immunity to a desired antigen (Lee, 2003).

A wide variety of antigens with a bacterial (Sharma et al., 1996), viral (Di Fabio et al., 1998) or eukaryotic (Ricci et al., 2003) origin have been produced by S. gordonii. Research has focused on studying the possible use of S. gordonii vaccine carriers for the prevention of sexually-transmitted diseases and several antigens of sexually-transmitted pathogens have therefore been expressed by recombinant S. gordonii (Table 4).

The majority of antigens expressed by S. gordonii have been surface displayed, the other expression site under study being the extracellular location (Table 4). The most widely studied system for the heterologous surface expression of antigens in S. gordonii is based on a translational gene fusion encoding the signal sequence and anchor of the S. pyogenes M6-protein with the heterologous protein (Pozzi et al., 1992). Transformation of the recombinant vectors to recipient strains of S. gordonii leads to the chromosomal integration of the gene fusion downstream of a strong resident promoter (Oggioni et al., 1996, Pozzi et al., 1992). Extracellular production of the heterologous antigens with the aforementioned system can be achieved by inserting a translational stop codon sequence before the anchor sequence of M6 (Sharma et al., 1997). A factor that aids in the construction of S. gordonii recombinant strains is the natural capability of S. gordonii for genetic transformation (Pozzi et al., 1990).

Some potential problems related to the safety of S. gordonii as vaccine carriers exist, which need to be evaluated if these vectors are intended to be used in humans. The M6 protein, used as the fusion partner in most of the expression constructs, is considered to be a virulence determinant of S. pyogenes (Mercenier et al., 2000). S. gordonii has also been associated with some diseases, such as infective endocarditis in humans (Douglas et al., 1993) and dental caries in rats (Tanzer et al., 2001). However, the results of a recent clinical study with human volunteers exposed to a S. gordonii strain not bearing any vaccine antigens further encourage the development of a S. gordonii mucosal vaccine vector (Kotloff et al., 2005). In this study the S. gordonii strain was given by combined nasal and oral inoculation and was found to be well tolerated, highly colonizing and removable either spontaneously or by antibiotic treatment.

### 2.3.7. Immune responses to Streptococcus vaccines

S. gordonii was the first recombinant commensal bacterium used as a live vaccine vector (Potzi et al., 1992). Immunization studies performed with S. gordonii antigen and/or cytokine carriers are listed in Table 4. Most often the mucosal route of delivery has been used, although the parenteral route of delivery has also been widely studied in the context of S. gordonii vaccines. In a few studies two different mucosal routes have been simultaneously used (Bolken et al., 2002; Lee et al., 2002; Oggioni et al., 1995). A local IgA response has almost always been obtained after the mucosal immunization with S. gordonii antigen carriers, and likewise after the parenteral route of delivery a systemic humoral response has almost always been obtained (Table 4). In most of the studies conducted with S. gordonii vaccines, the mucosal route of delivery has also elicited antigen-specific systemic humoral responses (Table 4). When the parenteral route of delivery has been used, the predominant or only IgG isotype observed has been the IgG1, suggesting
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a Th2 type of response to the antigen delivered by this route (Medaglini et al., 2001; Oggioni et al., 1999; Ricci et al., 2000). The isotypic profiles obtained after the mucosal delivery of *S. gordonii* antigen carriers have, however, suggested the recruitment of Th1 responses (Oggioni et al., 1999) or the activation of both Th1 and Th2 T helper cell subsets (Medaglini et al., 2001; Ricci et al., 2000). The route of antigen delivery thus seems to have an important role in determining the nature of the immune responses elicited by *S. gordonii* antigen carriers.

Dendritic cells are the most likely mediators of immune responses to *S. gordonii* antigen carriers (Corinti et al., 2000). Human and mouse dendritic cells have been shown to internalize both wild-type *S. gordonii* cells and *S. gordonii* cells expressing a heterologous antigen by phagocytosis, leading to the maturation and activation of dendritic cells (Corinti et al., 1999; Rescigno et al., 1998). The subsequent antigen presentation to CD4+ T lymphocytes has been demonstrated by in vitro analyses (Corinti et al., 1999; 2000). Recombinant antigens expressed on the *S. gordonii* cell surface were shown to be presented to T lymphocytes with the major histocompatibility complex class I molecules 10^6 times more efficiently than the same antigen in a soluble form (Rescigno et al., 1998). The activation of T-cells after encountering *S. gordonii* antigen carriers in vivo has been demonstrated by Di Fabio et al. (1998), who obtained a T cell proliferative response after the i.v. immunization of monkeys.

A novel approach contrasting with the active immunization with *S. gordonii* antigen carriers aims at the selective delivery of therapeutic agents, such as antibody fragments, for the prevention and therapy of mucosal diseases. Experimental vaginitis, caused by *Candida albicans*, has been successfully treated with *S. gordonii* secreting or displaying a microbicidal single-chain antibody, resulting in the enhancement of the infection clearance by both of the recombinants (Beninati et al., 2000). Another example of the successful delivery of a therapeutic agent by *S. gordonii*, leading to the reduction of disease severity in a mouse model of human ulcerative colitis, has been reported by Ricci et al. (2003).

Factors shown to influence to the immunogenic properties of *S. gordonii* therapeutic protein/antigen carriers include the cellular location of the heterologous protein and the viability of the *S. gordonii* carrier strains. Beninati et al. (2000) have shown that a secreted form of a therapeutic antibody fragment led to a more pronounced therapeutic effect than the surface-displayed form of the same molecule. Due to the fact that all *S. gordonii* antigen carriers used in immunizations have had a surface-displayed form of the antigen (Table 4), the effects of different cellular locations of the antigen on the immune responses elicited have not been compared. The colonization of *S. gordonii* antigen carriers seems to be a prerequisite for the induction of mucosal immune responses. When a mucosal route of delivery has been used, antigen-specific mucosal IgA responses have not been obtained by using killed *S. gordonii* antigen carriers (Medaglini et al., 1995; Oggioni et al., 1995) and the induction of systemic IgG responses have only been described in one study (Medaglini et al., 1997). When given parenterally, killed *S. gordonii* vaccine carriers have been reported to induce a systemic humoral response (Lee et al., 1999; 2004).
<table>
<thead>
<tr>
<th>Antigen, cytokine or antibody fragment/origin</th>
<th>Cellular location in streptococci</th>
<th>Route of immunization (animal)</th>
<th>Immune responses obtained [studied but not obtained]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7/HPV16</td>
<td>Surface displayed</td>
<td>s.c. (mouse)</td>
<td>Production of E7-reactive antibodies</td>
<td>Pozzi et al., 1992</td>
</tr>
<tr>
<td>E7/HPV16</td>
<td>Surface displayed</td>
<td>Partly i.n., partly oral (mouse)</td>
<td>E7-specific IgG [M6-specific IgG]</td>
<td>Oggioni et al., 1995</td>
</tr>
<tr>
<td>M6/Streptococcus pyogenes</td>
<td>Surface displayed</td>
<td></td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>Ag5.2 allergen/hornet venom</td>
<td>Surface displayed</td>
<td>i.n., s.c., oral (mouse)</td>
<td>IgA (oral, i.n.), IgG (oral, i.n., s.c.)</td>
<td>Medaglini et al., 1995</td>
</tr>
<tr>
<td>FimA N- or C-terminal epitope /Porphyromonas gingivalis</td>
<td>Surface displayed</td>
<td>s.c. (rabbit)</td>
<td>Production of <em>P. gingivalis</em> fimbrillin specific antibodies</td>
<td>Sharma et al., 1996</td>
</tr>
<tr>
<td>E7/HPV16</td>
<td>Surface displayed</td>
<td>i.v. (mouse)</td>
<td>IgG, IgA</td>
<td>Medaglini et al., 1997</td>
</tr>
<tr>
<td>V3 domain of gp120/HIV-1</td>
<td>Surface displayed</td>
<td>i.v. (monkey)</td>
<td>IgG, IgA, T-cell proliferative response</td>
<td>Di Fabio et al., 1998</td>
</tr>
<tr>
<td>E7/HPV16</td>
<td>Surface displayed</td>
<td>i.v. (monkey)</td>
<td>IgG, IgA, T-cell proliferative response</td>
<td></td>
</tr>
<tr>
<td>SpaP-S1 of PT/Streptococcus mutans, Bordetella pertussis</td>
<td>Surface displayed</td>
<td>s.c. (rabbit), i.p. (mouse)</td>
<td>Production of PT specific antibodies (s.c.), protection against PT (i.p.)</td>
<td>Lee et al., 1999</td>
</tr>
<tr>
<td>V3 domain of gp120/HIV-1</td>
<td>Surface displayed</td>
<td>s.c., i.v. (mouse)</td>
<td>IgG1 (s.c., i.v.), IgG2a (i.v.) [IgG3 (s.c), IgG2b (s.c., i.v.), IgA (i.v.)]</td>
<td>Oggioni et al., 1999</td>
</tr>
<tr>
<td>ScFv-H6/mouse mAb KT4</td>
<td>Extracellular or surface displayed</td>
<td>i.v. (rat)</td>
<td>Therapeutic activity against experimental vaginal candidiasis</td>
<td>Beninati et al., 2000</td>
</tr>
<tr>
<td>E7/HPV16</td>
<td>Surface displayed</td>
<td>s.c., i.g. (mouse)</td>
<td>IgG (s.c.) [E7-specific antibodies by i.g. immunization]</td>
<td>Ricci et al., 2000</td>
</tr>
<tr>
<td>B monomer of LTB/Escherichia coli</td>
<td>Surface displayed</td>
<td>s.c., i.g. (mouse)</td>
<td>IgG1 (s.c., i.g.), IgG2a (s.c., i.g.) IgG2b(s.c., i.g.), IgG3 (s.c., i.g.), IgA (i.g.)</td>
<td></td>
</tr>
<tr>
<td>Antigen</td>
<td>Route of display</td>
<td>Antibodies</td>
<td>Notes</td>
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<tr>
<td>-------------------------------</td>
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</tr>
<tr>
<td>TTFC/Clostridium tetani</td>
<td>Surface displayed</td>
<td>s.c., i.n. (mouse)</td>
<td>IgG1 (s.c., i.n.), IgG2a (s.c., i.n.), IgG2b (s.c.) IgA (i.n.), TTFC protection (s.c., i.n.)</td>
<td>Medaglini et al., 2001</td>
</tr>
<tr>
<td>FimA N- or C-terminal epitope/P. gingivalis</td>
<td>Surface displayed</td>
<td>Oral (rat)</td>
<td>IgG, IgA, protection from <em>P. gingivalis</em>-induced alveolar bone loss</td>
<td>Sharma et al., 2001</td>
</tr>
<tr>
<td>CRR region of M6/S. pyogenes</td>
<td>Surface displayed</td>
<td>i.n. and oral simultaneously (mouse)</td>
<td>IgG, IgA</td>
<td>Bolken et al., 2002</td>
</tr>
<tr>
<td>CRR region of M6/S. pyogenes with IL-2 or IFN-γ/murine</td>
<td>Extracellular (IL-2, IFN-γ), surface displayed (CRR)</td>
<td>s.c. (mouse)</td>
<td>Immunomodulating effect, IL-2 and IL-4 production [IgG, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ ]</td>
<td>Byrd et al., 2002</td>
</tr>
<tr>
<td>SpaP-S1 of PT/S. mutans, Bordetella pertussis</td>
<td>Surface displayed</td>
<td>i.n. and i.g. simultaneously (mouse)</td>
<td>IgA (anti-PT, anti-SpaP) [IgG (anti-PT, anti-SpaP)]</td>
<td>Lee et al., 2002</td>
</tr>
<tr>
<td>LTB/E. coli and V3 domain of gp120/HIV-1 separately or together</td>
<td>Surface displayed</td>
<td>s.c. (mouse)</td>
<td>V3- and LTB-specific IgG</td>
<td>Maggi et al., 2002</td>
</tr>
<tr>
<td>IL-1 ra/human</td>
<td>Extracellular</td>
<td>i.g. (mouse)</td>
<td>Therapeutic effect in ulcerative colitis</td>
<td>Ricci et al., 2003</td>
</tr>
<tr>
<td>Diphtheria toxin fragment A/Corynebacterium diphtheriae</td>
<td>Surface displayed</td>
<td>i.p., i.m., i.n., oral (mouse)</td>
<td>IgA (i.n., oral⁵), IgG (i.p., i.m., i.n.) [no anti-diphtheria toxin antibodies when only oral immunization was used]</td>
<td>Lee et al., 2004</td>
</tr>
</tbody>
</table>

⁴CRR, conserved C-repeat; FimA, fimbrillin encoding protein; HPV, human papillomavirus; IL-1 ra, interleukin-1 receptor antagonist; LTB, B monomer of *E. coli* heat-labile toxin; PT, pertussis toxin; scFv, single chain fragment variable; TTFC, tetanus toxin fragment C.  
⁵priming parenterally with a commercial vaccine against diphtheria, tetanus and acellular pertussis.
To modulate the magnitude and direction of immune responses elicited by *S. gordonii* antigen carriers, the use of different adjuvants including Freund’s incomplete (Lee et al., 1999) and Freund’s complete adjuvant (Lee et al., 2004; Pozzi et al., 1992), B monomer of *E. coli* heat-labile toxin (LTB) (Maggi et al., 2002), cholera toxin subunit B (Lee et al., 2004), an immunostimulating CpG oligonucleotide (Lee et al., 2004), and cytokines (Byrd et al., 2002) have thus far been tested. The co-expression of LTB simultaneously at the cell surface with the V3 domain of human immunodeficiency virus (HIV)-1 gp 120 resulted in a four-fold higher V3-specific IgG titer compared to the titers induced by *S. gordonii* expressing only the V3 domain alone or titers induced by a mixture of strains expressing LTB and V3 separately (Maggi et al., 2002). The extracellular expression of two different cytokines together with a surface-displayed form of antigen in *S. gordonii* has been reported by Byrd et al. (2002), who demonstrated several immunomodulatory effects, including the up or down regulation of several genes associated with the immune response, due to the cytokine and antigen co-expression.
3. AIMS OF THE STUDY

Encouraging results from immunization studies with LAB vaccine vectors further support the establishment of new and even more efficient antigen display systems. This study was aimed at the development of LAB vaccine vectors and adjuvant cytokine production systems. As a potential antigen display vehicle, the S-layer protein SlpA of \textit{L. brevis} ATCC 8287 was chosen for this study. In addition, the S-layer of \textit{L. brevis} ATCC 14869 was chosen for basic characterization to aid structure-function analysis of \textit{L. brevis} S-layers and to clarify the binding characteristics of these structures. For further characterization of the S-layer protein binding properties, the receptor binding domain of the \textit{L. brevis} SlpA was also studied using a non-adhesive heterologous host.

The specific aims of the present work were the following:

1. To characterize the genes encoding the S-layer protein of \textit{L. brevis} ATCC 14869 and study their expression in different growth conditions.
2. To investigate the use of S-layer protein SlpA of \textit{L. brevis} ATCC 8287 as a carrier of foreign epitopes.
3. To examine the expression of the SlpA receptor-binding region of \textit{L. brevis} ATCC 8287 in nonadhesive lactococci.
4. To create an expression system for porcine IL-2 in \textit{Lactococcus lactis} and study the specific biological activity of the expressed IL-2.
4. MATERIALS AND METHODS

4.1. Bacterial strains, plasmids, cell lines, and growth conditions

The microbial strains, plasmids, cell lines, antibiotics and growth conditions used in this study are described in publications I-IV.

4.2. DNA methods and transformation

Routine molecular biology techniques were applied (Sambrook and Russell, 2001). DNA restriction enzymes and modification enzymes were used as recommended by the manufacturers (Promega, Madison, Wis.; New England Biolabs Inc., Beverly, Mass.). Chromosomal DNA was isolated from *L. brevis* essentially as described by Vidgrén et al. (1992). Plasmid isolation from *E. coli* was performed with Wizard Minipreps (Promega) and from *L. lactis* and *L. brevis* with the QIAfilter Plasmid Midi Kit (Qiagen GmbH, Hilden, Germany) and using increased lysozyme concentration (8 mg/ml). *L. brevis* was transformed essentially as described by Bhowmik and Steele (1993). Transformation of *L. lactis* and *E. coli* was performed by the methods of Holo and Nes (1989) and Sambrook and Russell (2001), respectively. The polymerase chain reaction (PCR) was carried out using the manufacturer’s recommended reaction conditions (Finnzymes). Real-time quantitative PCR was performed with an iCycler (Bio-Rad Laboratories) in reaction conditions presented in Study I. The Vectorette system (Sigma Genosys Ltd.) was used for amplifying and sequencing the *L. brevis* ATCC 14869 *slp* gene regions. DNA sequencing and sequence analyses performed are described in detail in Study I. Southern blotting was carried out by the method of Southern (1975) and hybridizations were performed as described in Studies I and II.

4.3. RNA methods and RT-PCR

Total RNA (ribonucleic acid) was isolated with the Rneasy minikit (Qiagen) from *L. brevis* cells lysed by a cell homogenizator with glass beads as described in Study I. From stimulated PBMC total RNA was extracted as described in Study IV. RNA gel electrophoresis and Northern blotting was performed with the method of Hames and Higgins (1985). Oligonucleotide probes were labelled with digoxigenin-dUTP by using the DIG-High Prime kit (Boehringer Mannheim). Detection of the probe-specific hybrids was carried out by using the DIG luminescence kit (Boehringer Mannheim). Primer extension reactions for the 5’end determination of transcripts and the reverse transcriptase reactions were performed as described in Study I.

RT-PCR of RNA samples was done either by using the iScript cDNA synthesis kit (Bio-Rad Laboratories) or the RobusT RT-PCR-kit (Finnzymes) as recommended by the manufacturers.
4.4. Protein and enzyme assays

Sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis and immunoblot analyses were carried out as described in Studies I-IV. Intracellular aminopeptidase activities were determined as described in Study I. Surface-exposed epitopes or polypeptides were detected by whole-cell enzyme-linked immunosorbent assay (ELISA) as outlined in Studies II-III, respectively. The N-terminal sequencing of intact proteins and tryptic peptides was performed with the method of Kalkkinen and Tilgmann (1988). The molecular mass analyses were carried out as described in Studies I-II. His-tagged fusion proteins were purified by using a HisTrap HP affinity chromatography column as recommended by the manufacturer (Amersham Biosciences). The expression and purification of His-tagged fusion proteins is described in more detail in Study IV.

4.5. Immunofluorescence

The surface accessibility of epitopes or polypeptides on the bacterial cell surface was investigated with immunofluorescent assays as described in Studies II-III.

4.6. Bacterial adhesion assays

Bacterial adhesion to Intestine 407 monolayer and human plasma fibronectin was determined as described in Study III. The statistical methods used in evaluating the results are described in Study III.

4.7. Electron microscopy

Transmission electron microscopy analyses, carried out as described in Studies I-II, were used in analyzing the surface structure of bacterial cells.

4.8. Isolation of peripheral blood mononuclear cells and stimulation

Isolation of peripheral blood mononuclear cells (PBMC) from the whole blood of a large white pig, collected into heparinized tubes, was performed as described in Study IV. For stimulation of the PBMC, phytohemagglutinin was used as described in Study IV.

4.9. Proliferation assays with the cell line CTLL-2

The biological activity of porcine rIL-2 proteins was examined by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay as recommended by the manufacturer (Promega) and is described in more detail in Study IV.
5. RESULTS AND DISCUSSION

5.1. Characterization of the genes encoding the S-layer protein of *L. brevis* ATCC 14869 (I)

*L. brevis* strains ATCC 8287 and ATCC 14869 show differences in their ability to bind porcine epithelial cells (M. Jakava-Viljanen and A. Palva, unpublished results). To resolve the basis of these differences, the S-layer protein gene content and the slp gene expression in ATCC 14869 were characterized in detail in this study.

5.1.2. The S-layer protein profile

During the course of the study, *L. brevis* ATCC 14869 cells were observed to form two colony types, smooth (S) and rough (R), on MRS plates when growing under aerobic conditions for longer periods, whereas under anaerobic conditions only S-type colonies were formed. This phenomenon has not earlier been described for this *L. brevis* neotype strain. Identical 16S ribosomal DNA (rDNA) sequences with *L. brevis* identity from the cells of both colony types excluded the possibility of strain contamination. Two putative S-layer protein bands of 50 and 43 kDa were detected by whole-cell SDS-PAGE analyses from R-colony type cells propagated on MRS plates and cells grown under aeration in MRS broth. The 50 kDa band was predominant in cells of the S-colony type and in cells grown anaerobically in MRS broth. Electron microscopy (EM) analysis revealed the presence of only a single S-layer with an oblique structure on top of the peptidoglycan layer on the cells of both colony types. Thus, the EM data do not explain the difference in the colony morphology but suggest that the S-layer structures are not directly involved.

5.1.3. Cloning and sequence analysis of the slp genes

Degenerate sequencing primers were designed according the N-terminal amino acid sequences from the purified 50- and 43-kDa S-layer proteins and their tryptic peptides and the PCR products obtained were used as probes in Southern hybridizations. Based on the blotting data obtained, *HindIII*-cut chromosomal DNA was ligated with the *HindIII* Vectorette units and the amplifying and sequencing of the slp gene regions were performed with the Vectorette system.

The amino acid sequence predicted from the *orf* encoding the 50-kDa S-layer protein included the N-terminal and internal amino acid sequences determined from this protein. This *orf* was designated *slpB* and was found to encode a polypeptide of 483 aa with a signal sequence of 30 aa. The molecular weight of the mature SlpB (48 kDa) was found to be in good agreement with the molecular mass of the 50-kDa protein estimated by SDS-PAGE. Sequence analysis of the *slpB* revealed the presence of two putative promoter regions, a possible RBS and a putative rho-independent type transcription terminator sequence.

Downstream of the *slpB* sequence an *orf* designated *slpC* with a capacity to encode a mature protein of 46 kDa was found. Sequence analysis revealed the presence of two
putative -10 regions for a promoter and a possible RBS, but the gene was not followed by transcription terminator-like sequences.

Because the predicted DNA sequence and coding capacity of the \textit{slpC} did not correspond to the N-terminal sequence and size of the 43-kDa protein S-layer protein from R-colony type cells, new primers were designed for the isolation of the third gene, \textit{slpD}, from the Vectorette library. The \textit{orf} of \textit{slpD} gene was found to encode a polypeptide of 413 aa with a signal sequence of 30 aa. The molecular weight of the mature SlpD (42 kDa) was found to be in good agreement with the molecular mass of the 43-kDa protein estimated by SDS-PAGE. Two putative -10 regions for a promoter and a possible RBS were identified from the upstream region of \textit{slpD} and downstream of the stop codon a putative rho-independent type transcription terminator sequence could be recognized. Southern blotting and PCR analysis revealed that the \textit{slpD} gene is not closely linked to the \textit{slpB}-\textit{slpC} gene region.

The genetic organization of the three GRL62 \textit{slp} genes is distinct from that described so far for other lactobacilli. In \textit{L. acidophilus} ATCC 4356, the two S-layer protein genes, \textit{slpA} and \textit{slpB}, are in opposite orientation to each other and interspaced with a 3 kb DNA-region (Boot et al., 1996c). The putative S-layer protein genes \textit{apf1} and \textit{apf2} of \textit{L. gasseri} and \textit{L. johnsonii} are also separated by a short intergenic DNA-region, but these genes are in parallel orientation (Ventura et al., 2002). The spacing and orientation of the \textit{L. crispatus} S-layer protein genes, \textit{cbsA} and \textit{cbsB}, have not yet been deduced (Sillanpää et al., 2000). From \textit{L. brevis} ATCC 8287, we have been able to find only one S-layer protein gene, \textit{slpA} (Vidgrén et al., 1992; Palva et al., unpublished results). The current data on the genetic organization of the \textit{Lactobacillus} S-layer protein genes thus suggest that they are generally located in a rather close proximity to each other.

The S-layer proteins of \textit{L. brevis} ATCC 14869 were characterized in this study in order to find putative conservative regions with the SlpA protein of \textit{L. brevis} ATCC 8287. These conservative regions, implicative of regions with similar structural functions, could have been utilized, for example, in the further optimization of the ATCC 8287 S-layer protein antigen display system. Homology comparisons of the \textit{L. brevis} Slp proteins revealed that the intraspecies identity was mainly restricted to the N-terminal regions of these proteins, whereas the C-terminal regions were rather divergent. By pairwise protein sequence alignments computed to the \textit{L. brevis} Slp proteins a few middle and C-terminal regions with considerable sequence similarity have, however, been found between the SlpB and SlpD proteins (Åvall-Jääskeläinen and Palva, 2005). The amino acid sequence identities of the \textit{L. brevis} ATCC 14869 Slp proteins to the SlpA protein of \textit{L. brevis} ATCC 8287 were lower than expected and negligible compared to other S-layer proteins of the \textit{Lactobacillus} species studied. Secondary structure predictions computed for the \textit{L. brevis} Slp proteins have revealed these proteins to be quite dissimilar when compared to each other and also to other S-layer proteins of lactobacilli (Åvall-Jääskeläinen and Palva, 2005). Except for the α-helices located at the signal sequences of \textit{L. brevis} Slp proteins, the locations of the other observed secondary structures (extended strands and random coils) in the \textit{L. brevis} Slp proteins have not been found to be directly comparable to each other (Åvall-Jääskeläinen and Palva., 2005). Due to the dissimilarity between the S-layer proteins of the two studied \textit{L. brevis} strains and also due to the dissimilarity between the ATCC 14869 Slp and other lactobacillar S-layer proteins, the location of the putative
receptor-binding domain and the cell wall binding domain of the ATCC 14869 Slp protein/s cannot be directly determined. On the other hand, sequence alignments between the S-layer proteins of \textit{L. crispatus} and \textit{L. acidophilus} and \textit{L. helveticus} have shown that the C-terminal sequences between the S-layer proteins of these lactobacilli are highly similar (Åvall-Jääskeläinen and Palva., 2005) and contain a putative carbohydrate-binding consensus sequence, suggested to be involved in cell wall binding (Smit et al., 2001). For the S-layer proteins of \textit{L. brevis} ATCC 14869 the adhesin function and location of the adhesion domain needs to be experimentally confirmed.

5.1.4. Expression studies on the \textit{slp} genes

The expression of the \textit{slp} genes of \textit{L. brevis} ATCC 14869 was studied as a function of growth. Northern blot analysis confirmed the monocistronic nature of the \textit{slpB} and \textit{slpD} transcripts. With primer extension analysis, a transcription start site of the \textit{slpD} gene could also be localized. Under aerated growth conditions, both \textit{slpB} and \textit{slpD} were found to be expressed, whereas under anaerobic growth conditions only expression of \textit{slpB} was detected. Under the growth conditions tested, \textit{slpC} was found to be a silent gene. \textit{L. acidophilus} and \textit{L. crispatus} have also been found to have silent S-layer protein genes (Boot and Pouwels, 1996; Sillanpää et al., 2000).

For studying a possible chromosomal rearrangement involved in the activation of the \textit{slpD} gene expression, real-time quantitative PCR was performed with primers spanning the \textit{slpD} promoter region and with chromosomal DNA from ATCC 14869 cells grown under aerated or anaerobic conditions. The results indicated that chromosomal rearrangements are not likely to be involved in the regulation of the \textit{L. brevis slpD} gene expression. Regulation of S-layer gene expression by DNA arrangements have been reported to occur in one \textit{Lactobacillus} species, \textit{L. acidophilus} (Boot et al., 1996), but the conditions that would induce this change have not been established.

Expression studies with a PepI reporter protein under the control of the \textit{slpD} promoter showed that under aerated conditions the specific PepI activities were growth stage dependent. Under anaerobic conditions no similar response was observed and the specific PepI activities remained low.

The results of Northern analyses, expression of a PepI reporter protein under the control of the \textit{slpD} promoter and quantitative real-time PCR analysis of \textit{slpD} expression under aerated and anaerobic conditions obtained in the study indicated that the variation in S-layer protein content in GRL62 occurs at the transcriptional level rather than with DNA rearrangements. When SlpD is produced, the amount of SlpB is lowered and the level of \textit{slpB} transcripts is also somewhat decreased. However, there appears to be no marked repression of \textit{slpB} transcription, although it might be partly masked by the long half-life of \textit{slpB} transcripts. Thus, this suggests that the variation in the S-layer protein content takes place by a unique mechanism involving activation of \textit{slpD} transcription in the exponential growth phase by a soluble factor as a result of an environmental change in \textit{L. brevis} ATCC 14869. How this response is triggered, and what all the participating components are remains to be elucidated. It is also unclear whether the variation in the colony forms and \textit{slp} gene expression are connected by the same regulon or whether they are independent events. Furthermore, it cannot yet be predicted whether the change in

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redox potential or amount of dissolved oxygen has a direct effect on the induction of slpD expression or whether some other changes in environmental factors under these conditions affect the control of slpD transcription. Most likely, a stress-related cascade of events may be involved.

The further construction of an L. brevis ATCC 14869 S-layer protein antigen display system has not yet been employed, since the phase variation responsible for the differential expression of Slp proteins under diverse growth conditions has not yet been characterized in detail. The phase variation observed in the Slp protein expression of ATCC 14869 in addition to the observed limitation of ATCC 14869 to tolerate higher growth temperatures do not encourage the use of this strain as an antigen delivery vector. Further characterization of the binding specificity and the binding domain may provide additional possibilities to modify this function in L. brevis S-layer applications.

5.2. Surface display of model epitopes on the Lactobacillus brevis S-layer (II)

The large number of S-layer protein subunits present in an S-layer and the possibility to detach the S-layer from the cell to a cell-free self-assembling S-layer protomer would make the use of antigen display system based on a Lactobacillus S-layer an interesting alternative to the other surface-display systems developed for lactobacilli. The S-layer protein of L. brevis ATCC 8287 was chosen for this study due to its well-characterized status. L. brevis strain ATCC 8287 is a possible candidate for a live mucosal antigen delivery vector, since it has been shown to possess several properties required for a probiotic bacterium (Rönkä et al., 2003) and it adheres to several types of human intestinal epithelial cell (Hynönen et al., 2002), which may be beneficial in the elicitation of immune responses to the antigen displayed by the L. brevis.

5.2.1. Construction of epitope-expressing L. brevis strains producing chimeric S-layers

The four most hydrophilic parts determined from the hydrophilicity profile of the SlpA protein of L. brevis ATCC 8287 were chosen to be used as epitope insertion sites and were named as I to IV. The number of insertion sites to be tested was restricted due to the fact that the L. brevis slpA gene contains a high amount of inverted and direct repeats (Vidgrén et al., 1992), and it has been found to be very difficult to clone due to its instability and toxicity in heterologous hosts. As model epitopes, an 11-aa immunodominant region of the VP1 capsid protein of enteroviruses (Hovi and Roivainen, 1993) and a 10-aa c-Myc epitope (Evan et al., 1985) were used. Expression cassettes carrying under the control of the nisA promoter the slpA gene and the VP1 epitope DNA in slpA insertion sites I-IV and c-myc in insertion site II were constructed in L. lactis and subsequently transferred into L. brevis, harboring pNZ9530 for nisin induction.

The surface accessibility of the model epitopes expressed by the nisin induced double-plasmid strains was studied with whole-cell ELISA and immunofluorescence microscopy using anti-VP1 or anti-c-Myc antibodies. The results from the whole-cell ELISA assays indicated that with insertion site II the best surface accessibility of the model epitopes could be obtained and that also with insertion site I VP1 could be surface displayd. By
immunofluorescence assays the surface display of the model epitopes could not be detected in any of the double-plasmid strains, indicating a low expression level of epitopes in this system. This was probably due to a much lower expression level of the epitope-SlpA constructs under the *nisA* promoter compared to the high level expression of the chromosomal *slpA* gene, which was still present in these double-plasmid strains. With this nisin-inducible double-plasmid system a chimeric and active S-layer structure could thus be obtained containing, however, only a few copies of the epitope-SlpA subunit.

### 5.2.2. Gene replacement of the native *L. brevis* *slpA* gene with the *slpA*-c-*myc* fusion construct and characterization of the integrant strain GRL1046

To increase the expression level of the epitope-SlpA protein obtained with the double plasmid *L. brevis* strains, several thermosensitive-integration vectors were tested to obtain gene replacement by two subsequent single crossover events (data not shown). With these vectors, a primary integration of the model epitopes to *slpA* could be demonstrated but the transformants obtained turned out to be highly unstable (data not shown). Development of a gene replacement system was also hampered by the lack of certainty over whether the epitopes in the chosen insertion sites would be toxic or retard cell growth when produced as part of a uniform S-layer. Finally, a gene replacement system consisting of a pORI280 derivative pKTH5115 harboring c-*myc* in insertion site II and a thermosensitive plasmid pVE6007 was chosen to be tested. This system was selected to minimize the concomitant *slpA* transcription and plasmid replication functions in the *L. brevis* chromosome, which are likely to result in DNA rearrangements. By supplying the RepA protein in another plasmid, a tighter replication control can be achieved compared to the direct thermosensitive-integration vectors, where the restrictive temperature in *L. brevis* does not completely shut down the replication functions. This integration strategy has been utilized previously in *Lactococcus* for the generation of chromosomal mutations (Law et al., 1995), and a similar two plasmids system was recently modified for *Lactobacillus gasseri* and *Lactobacillus acidophilus* to widen the applicable gene inactivation temperatures (Russell and Klaenhammer, 2001).

A transformant harboring pKTH5115 and pVE6007 was grown at a restrictive temperature (39°C) under antibiotic selection to find clones with the pKTH5115 integrated into the bacterial chromosome through homologous recombination between the wild-type and c-*myc*-slpA. PCR and DNA-sequencing analysis of an erythromycin-resistant and chloramphenicol-sensitive strain, indicating the loss of pVE6007, verified a direct double-crossover integration of c-*myc* into the *L. brevis* chromosome. However, by PCR-analyses this strain, designated *L. brevis* GRL1045, was shown to also harbor the unintegrated plasmid pKTH5115. A possible explanation for this was that the resident plasmid of unknown function, harbored by *L. brevis* GRL1 (Rönkä et al., 2003), could provide some residual replication function to pKTH5115. The final integrant strain GRL1046, harboring no unintegrated pKTH5115, was obtained by culturing the GRL1045 for approximately 50 generations without antibiotics. The strain GRL1046 was further characterized in detail with DNA sequencing and PCR analyses. The results from these assays verified that the integrant strain GRL1046 harbored no unintegrated pKTH5115 and that the DNA region encoding the c-Myc epitope was located in insertion site II. A
Southern hybridization with a \( slpA \) specific probe was also performed indicating that no chromosomal rearrangements on the \( slpA \) gene region had occurred in strain GRL1046.

By whole-cell SDS-PAGE analyses from GRL1046 cells a single putative recombinant S-layer protein band of 46 kDa with a slightly lower electrophoretic mobility compared with that of native SlpA was detected. The exact molecular masses of of the LiCl-isolated SlpA proteins of GRL1046 and GRL1 were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and were found to be in accordance with their calculated molecular masses. Whole-cell ELISA and immunofluorescence microscopy with anti-Myc antibodies confirmed that the c-Myc epitope was surface exposed in GRL1046. Transmission electron microscopy analyses with LiCl-isolated S-layer proteins and cells from GRL1046 and GRL1 indicated that the S-layer lattice symmetry or the self-assembly of isolated S-layer protein subunits was not affected by the presence of c-Myc epitopes, since both \( L. \ brevis \) strains formed an oblique structure. The negative staining technique only allowed visualization of disrupted cells and isolated self-assembled S-layers because of the high staining background of intact cells. To visualize the cell surface of intact \( L. \ brevis \) cells, the freeze-etching technique and certain stainings in the thin-sectioning technique could be applied. With these techniques, resolution problems could, however, arise due to the nature of this S-layer and because it is superimposed with the irregular structures (e.g. the polymers) of the cell wall.

Various recombinant \( Lactobacillus \) strains producing surface-exposed antigens have been constructed and used in immunization experiments (Maassen et al., 1999; Reveneau et al., 2002; Scheppler et al., 2002). On the surface of \( L. \ casei \), approximately \( 1.4 - 3.9 \times 10^3 \) TTFC molecules have been produced with vectors driving the TTFC expression under control of an \( \alpha \)-amylase or L-(+)-lactate dehydrogenase promoter, respectively (Maassen et al., 1999). It has been theoretically calculated that the encompassing S-layer on an average size cell consists of approximately \( 5 \times 10^5 \) monomers (Sleytr and Messner, 1988). Thus, with the surface display system developed in this study, it is possible to present at least such a high number of antigen epitope molecules on the cell surface of each \( L. \ brevis \) cell. Surface displaying of vaccines as part of an S-layer would thus be a very efficient method for presenting antigens to the mucosa-associated lymphoreticular tissue. This study has thus shown that at least small epitopes can be successfully surface-displayed as part of the S-layer protein of \( L. \ brevis \), and that an intact S-layer without any changes in the crystalline structure can be formed in spite of the presence of a heterologous epitope in every S-layer subunit.

5.3. Surface expression of the SlpA receptor-binding region of \( L. \ brevis \) ATCC 8287 in nonadhesive lactococi (III)

The aim of this study was to determine whether a nonadhesive LAB can be provided with a specific adhesion capability with the receptor-binding region of SlpA, mediating adhesion to various epithelial cells of human origin. The transfer of adhesin molecules to nonadherent LAB may be beneficial for several applications and it allows the study of whether the delivery of a bioactive molecule of interest is affected by the change concomitant with the adherence potential of the LAB vector.
We chose 247 amino acids long N-terminal region, including the signal peptide of 30 residues, from the *L. brevis* SlpA protein for surface expression and adhesion studies in *L. lactis*. This region was previously shown to include the receptor-binding region of SlpA, which adheres to fibronectin and several human epithelial cell types, by using a flagella display system in *E. coli* (Hynönen et al., 2002). With flagella display experiments the binding domain of SlpA could be limited to up to 81-amino-acid residues representing residues 96 through 176 in the unprocessed SlpA protein (Hynönen et al., 2002). *L. lactis* was used as the host in this study for reasons including the following: this organism does not adhere to Intestine 407 cells (Palva et al., unpublished results), a wide range of genetic tools to study it are available, it has been successfully used for surface display of heterologous proteins (Steidler et al., 1998; Bermúdez-Humarán et al., 2004; Mannam et al., 2004,) and it is currently being developed as a mucosal delivery vehicle (Cheun et al., 2004; Robinson et al., 2004).

To properly extend the SlpA receptor-binding region out of the cell surface, it was decided to use parts of the *L. lactis* subsp. *cremoris* Wg2 *prtP* gene (Kok et al., 1988) as spacers. The optimal size of the PrtP spacer was studied by constructing plasmids pKTH5050 and 5051, which encode a fusion protein consisting of the AcmA signal peptide, the mature β-lactamase (Bla), a PrtP spacer of 215 aa (pKTH 5050) or 515 aa (pKTH5051) and the AcmA cell wall anchor. Whole-cell ELISA with anti-Bla antibodies indicated that the surface accessibility of Bla was better with the PrtP spacer of 515 aa than with the 215 aa spacer, so the 515 aa spacer was selected for use for the surface display of the SlpA adhesion domain. The 515-amino-acid spacer, amplified from the C-terminus encoding part of the *prtP* gene, spans the putative H-domain, and part of the W-domain determined from the PrtP of *L. lactis* strain SK11 (Siezen, 1999). The function of the putative H-domain has not been determined, but it has been suggested to act as a spacer directing other PrtP domains further from the cell wall (Siezen, 1999). In putative H-domains, alpha-helixes are the dominant secondary structure. Spacers consisting of alpha-helixes of different length and origin have recently been used in enhancing the expression of M6 protein on the cell surface of *S. gordonii* (Bolken et al., 2002).

Based on the surface display vector pNG101his, an expression cassette was constructed that carries under the control of the *nisA* promoter the signal sequence of the *L. brevis slpA*, the *slpA* adhesion-mediating region (aa 31 to 247), an *L. lactis* proteinase (PrtP) spacer of 515 aa and the *L. lactis* AcmA cell wall-anchoring sequences (Buist, 1997). This construct was designated as pKTH5056 (Fig. 1 of III) and transferred into *L. lactis* NZ9000.

Western blot analysis of lactococcal cell wall extracts with anti-SlpA antibodies from nisin-induced *L. lactis* NZ9000 cells harboring pKTH5056 confirmed that the SlpA adhesion domain of the fusion protein was expressed and located within the cell wall layer. Whole-cell ELISA and immunofluorescence microscopy with anti-SlpA antibodies were used in studying the surface accessibility of the SlpA adhesion-mediating region. Both of these analyses confirmed that the pKTH5056-encoded SlpA receptor-binding region was successfully displayed on the recombinant *L. lactis* cell surface.
5.3.1. Adhesion of recombinant lactococci to Intestine 407 cells and human plasma fibronectin in vitro

An in vitro adhesion assay using the human intestinal epithelial cell line Intestine 407, derived from a small intestine of a 2-month-old human embryo (Henle and Deinhardt, 1957), indicated that nisin-induced recombinant \textit{L. lactis} NZ000 harboring pKTH5056 possessed an approximately sevenfold [statistically significant (P < 0.01)] higher ability to adhere to Intestine 407 cells than wild type NZ9000 cells. A statistically significant (P < 0.01) increase in adherence to Intestine 407 cells between \textit{L. lactis} NZ9000 cells harboring pKHT5056 and pKTH5046, which lacks the SlpA adhesion domain, was also observed. A serum inhibition assay further confirmed that adhesion of recombinant lactococci to Intestine 407 cells was indeed mediated by the SlpA receptor-binding region of the \textit{slpA} gene. The S-layer protein of \textit{L. brevis} ATCC 8287 has also been reported to mediate binding to human fibronectin (Hynönen et al 2002), a structural glycoprotein component of the extracellular matrix (Hay, 1981). The adhesion to immobilized fibronectin of the recombinant \textit{L. lactis} cells with pKTH5056 or pKTH5046 and the wild type NZ9000 cells was tested. The results indicated an approximately sevenfold, statistically significantly (P < 0.01) better ability of the nisin-induced recombinant \textit{L. lactis} with pKTH5056 to adhere to fibronectin compared to that of \textit{L. lactis} with pKTH5046 or all of the uninduced counterparts.

Even though a significant adhesion capacity to human epithelial Intestine 407 cells could be provided to \textit{L. lactis} host with the aid of the SlpA adhesion-mediating region, the epithelial cell binding efficiency of \textit{L. lactis}, harboring pKTH5056, was substantially lower than that of the wild type \textit{L. brevis} cells synthesizing a surface layer estimated to be composed of approximately 0.5 million SlpA subunits (Palva et al., unpublished results; Hynönen et al., 2002). One obvious explanation for this is that the quantity of pKTH5056 encoded SlpA molecules in \textit{L. lactis} is approximately only a few percent of that present in the native S-layer in \textit{L. brevis}. We cannot, however, rule out that the receptor-binding domain of SlpA also has a higher affinity in the S-layer conformation. The assumption that entire S-layers possess more efficient binding capacity than their protein subunits is supported also by the results from the expression studies of the collagen-binding CbsA protein of \textit{L. crispatus} in \textit{Lactobacillus casei}. CbsA was anchored with the cell wall sorting signal of PrtP to the cell wall and successfully expressed in \textit{L. casei}, but no S-layer was formed and the amount of collagen bound was lower than that observed with wild type \textit{L. crispatus} (Martínez et al., 2000).

In this study, an experimental approach to apply the receptor binding domain of the \textit{L. brevis} ATCC 8287 SlpA protein was demonstrated. LAB with an adhesion capability could be utilized, for example, in the development of mucosal antigen delivery vehicles or in targeting molecules/cells to a receptor of interest. The use of receptor-binding domains of \textit{Lactobacillus} origin may offer a safe alternative for the targeted delivery of various effectors on mucosal membranes.
5.4. Secretion of porcine interleukin-2 by *Lactococcus lactis* (IV)

Cytokines are currently being considered as vaccine adjuvants and several cytokines have already been expressed in *L. lactis*. This study forms the basis for the construction of an *L. brevis* vaccine vector strain co-expressing a cytokine adjuvant in addition to the antigen.

In this study, our aim was to develop an inducible extracellular expression system for porcine IL-2 protein in *L. lactis*. The IL-2 was chosen to be expressed in a secreted form to obtain the rIL-2 proteins in a soluble form that would ease the purification of the rIL-2 proteins with the TagHis6, and also ease the biological activity measurement. Moreover, for the possible adjuvant use of porcine IL-2 expressed by *L. lactis*, a secreted form is believed to be preferable to the other two possible cellular locations (intracellular or surface displayed) of a heterologously expressed protein in *L. lactis*. All of the thus far reported studies in which cytokines expressed by *L. lactis* have been used as adjuvants or therapeutic agents have relied on extracellular production of the cytokine (Steidler et al., 1998; 2000; 2003; Bermúdez-Humarán et al., 2003a), and the production of a cytokine in a surface-displayed form in *Lactococcus* has not yet been described.

Two plasmid vectors for the extracellular expression of porcine IL-2 were constructed. Total RNA of phytohemagglutinin stimulated PBMC was used as a template for the amplification of IL-2 cDNA. The sequence encoding the mature IL-2 was cloned into a pNZ8037 derivative vector giving rise to pKTH5186. In order to change the signal peptide from that of the *L. brevis* slpA gene (SS_{slpA}) (Vidgén et al., 1992) in pKTH5186 to the signal peptide of *L. lactis* major secreted protein Usp45 (SS_{usp}), the sequence encoding mature IL-2 was further amplified from pKTH5186 and cloned into pKTH5146, giving rise to plasmids pKTH5187 and pKTH5189 (Fig. 1 of IV). The expression cassette in pKTH5187 consists of the SS_{usp}, a synthetic sequence encoding the propeptide LEISSTCDA (Le Loir et al., 1998), the sequence encoding mature porcine IL-2 and a His-tag at the 3’ end, encoding rIL-protein designated as Pro_{leiss}-IL-2-Tag. The expression cassette in pKTH5189 consists of the SS_{usp}, a synthetic sequence encoding the propeptide LEISSTSDA, the sequence encoding mature porcine IL-2, sequence encoding the putative helix (H)-domain of PrtP of *L. lactis* (Siezen, 1999) and a His-tag at the 3’ end, resulting in the expression Pro_{leiss}-IL-2-PrtP-Tag protein. The putative H-domain, included in the spacer protein used in Study III, was cloned in pKTH5189 in order to study whether additional sequences in the rIL-2 protein have any effect on folding and thus on the biological activity of the rIL-2 fusion protein, anticipating the construction of a surface-displayed alternative of the IL-2. The *L. lactis* nisin promoter (P_{nis}) derived from pKTH5146 was used for the inducible expression of both rIL-2 proteins.

Expression of the rIL-2 proteins was induced by the addition of nisin. Secreted rIL-2 proteins carrying a histidine tag were purified from the concentrated supernatants of induced *L. lactis* NZ9000 cells harboring pKTH5187 or pKTH5189 with a His Trap column. With SDS-PAGE analysis bands corresponding to the expected molecular masses of Pro_{leiss}-IL-2-Tag protein and Pro_{leiss}-IL-2-PrtP-Tag protein could be detected in purified protein preparates.
The secretion yields of the rIL-2 proteins were determined according to the protein concentrations of the purified rIL-2 samples and were found to be quite similar for both of the rIL-2 producer strains. *L. lactis* with pKTH5187 secreted approximately 0.58 µg/ml Pro<sub>leiss</sub>-IL-2-Tag and *L. lactis* with pKTH5189 secreted approximately 0.52 µg/ml Pro<sub>leiss</sub>-IL-2-PrtP-Tag. The putative H-domain of PrtP and the single amino acid substitution from C to S in the propeptide LEISSTCDA, which were the only differences between the Pro<sub>leiss</sub>-IL-2-Tag protein and the Pro<sub>leiss</sub>-IL-2-PrtP-Tag protein, thus did not seem to have any major effects to the secretion efficiency of the Pro<sub>leiss</sub>-IL-2-PrtP-Tag protein. The propeptide LEISSTCDA has a net global negative charge of 2, which was not affected by the amino acid substitution from C to S in the Pro<sub>leiss</sub>-IL-2-PrtP-Tag protein. In this study the effect of the Pro<sub>leiss</sub> on the protein secretion efficiency was not studied, since an IL-2 expression cassette lacking the synthetic propeptide sequence was not constructed.

A Western blot analysis was performed to verify the specificity of the protein bands detected with SDS-PAGE. The purified rIL-2 proteins Pro<sub>leiss</sub>-IL-2-Tag and Pro<sub>leiss</sub>-IL-2-PrtP-Tag were blotted after separation by SDS-PAGE and probed with anti-porcine IL-2 antibodies. Bands of the expected molecular mass were detected for both of the rIL-2 proteins in the Western blot analysis, thus confirming that the rIL-2 proteins reacted specifically with the antibodies raised against porcine IL-2. No background was observed either in the SDS-PAGE or Western assays, indicating a successful purification with the His trap column. The absence of breakdown products also indicates stability of the rIL-2 proteins, although this was not experimentally studied.

5.4.1. The biological activity of rIL-2 proteins produced by *L. lactis*

The biological activity of both of the rIL-2 proteins was studied by a cell proliferation assay using CTLL-2 cell line, which is dependent upon IL-2 for proliferation. A method based on the bioreduction of a MTS tetrazolium compound into a coloured formazan product was used for the measurement of cell proliferation. According to the average results from two bioactivity assays, the specific biological activity of the Pro<sub>leiss</sub>-IL-2-Tag was determined to be approximately 2.2 × 10<sup>7</sup> U/µmol and the specific biological activity of the Pro<sub>leiss</sub>-IL-2-PrtP-Tag protein was determined to be approximately 1.8 × 10<sup>7</sup> U/µmol. Hence, no substantial differences between the specific biological activities of Pro<sub>leiss</sub>-IL-2-Tag and Pro<sub>leiss</sub>-IL-2-PrtP-Tag proteins were observed in this study. The additional H-domain present in Pro<sub>leiss</sub>-IL-2-PrtP-Tag protein thus did not seem to affect the folding or conformation of the rIL-2 protein, demonstrating that additional sequences can be included in porcine rIL-2 proteins without affecting the biological activity. These results also encourage the development of a surface-displayed form of the porcine IL-2 in a chosen host strain.

This study thus showed that cytokines of porcine origin can also be produced in a biologically active form in *L. lactis* and further developed and tested as adjuvants to be used in immunizations with vaccines aimed at porcines.
6. CONCLUSIONS AND FUTURE ASPECTS

This thesis describes the characterization of two new S-proteins and three new slp genes from the *L. brevis* neotype strain ATCC 14869, the utilization of the S-layer protein or its receptor-binding domain of *L. brevis* ATCC 8287 in applications related to more efficient mucosal antigen delivery as well as the development of an expression system for a porcine cytokine in *L. lactis*. This study was part of a larger project aimed at developing live oral LAB vaccines. Advantages in employing LAB as vaccine vectors include the claimed health-promoting properties (Vaughan et al., 1999) and the intrinsic adjuvant activities (Seegers, 2002) possessed by several strains of LAB, the GRAS status of LAB (Adams and Marteau, 1995) as well as the lack of lipopolysaccharides in the cell wall of LAB, eliminating the risk of an endotoxic shock (Mercenier et al., 2000). *L. brevis* ATCC 8287 has potential to be used as a live antigen delivery vector due to its probiotic properties (Rönkä et al., 2003) and capacity to adhere to human intestinal epithelial cells (Hynönen et al., 2002). As the first target antigen to be expressed in *L. brevis*, the receptor binding domain of the FedF adhesin of *E. coli* strains carrying F18 fimbriae will be employed in our laboratory. F18 fimbrial *E. coli* strains cause porcine post-weaning diarrhea and oedema disease that often are fatal to weaned pigs and are responsible for considerable economic losses in the pig breeding industry (Verdonck et al., 2002). So far, commercial vaccines against infections caused by the F18 fimbriae carrying *E. coli* strains are lacking.

By surface displaying epitopes or antigens as part of an S-layer, up to $5 \times 10^5$ epitope monomers can be obtained to surround a single *Lactobacillus* cell, making such lactobacilli very attractive candidates for mucosal vaccine delivery vectors. Due to the well-characterized status of the S-layer protein of *L. brevis* ATCC 8287, the SlpA protein was chosen to be further developed as an antigen carrier. Neither the S-layer lattice symmetry nor the self-assembly of isolated S-layer were affected by the presence of the c-Myc epitope in every S-layer subunit. As only the use of relatively short epitopes as part of the *L. brevis* SlpA was tested, further characterization of the structural and functional domains of SlpA is needed to determine whether larger antigens or epitopes can also be successfully surface-displayed in the SlpA without effecting lattice self-assembly or symmetry. This characterization will include the determination of amino acids crucial for the self-assembly of the S-layer subunits as well as elucidation of the amino acids responsible for the binding of the S-layer to the underlying cell wall. Identification of the location of amino acid residues in the S-layer lattice will be studied in our laboratory by using site-directed mutagenesis. Amino acids located preferentially on the outer surface of the S-layer and in a location where the insertion does not affect the binding and assembly pattern of the S-layer will subsequently be utilized as immunogen insertion sites.

Binding of the LAB vaccine vector to the mucosal membranes of the host may be an advantage in general when the construction of a mucosally delivered vaccine is considered. The S-layer protein of *L. brevis* ATCC 8287 performs as an adhesin mediating attachment to various human epithelial cell types and fibronectin. Most likely the S-layer proteins characterized from the *L. brevis* ATCC 14869 are also adhesins responsible for the adhesion of this strain to porcine intestinal epithelial cells although this needs to be experimentally confirmed. The two *L. brevis* strains characterized regarding their S-layer proteins show
dissimilar binding affinities to epithelial cells of different origin (M. Jakava-Viljanen, A. Palva, unpublished results; Hynönen et al., 2002). Thus it is relevant to obtain knowledge of the binding domains of the \textit{L. brevis} S-layer subunits. Future tasks hence include the structural comparison of the S-layer binding regions in the Slp proteins of \textit{L. brevis}, which still awaits further data from the structure analyses planned for the S-layer proteins of \textit{L. brevis}. The functioning of the SlpA receptor-binding region was demonstrated in a heterologous environment, demonstrating that receptor-binding regions of S-layer proteins can be used as separate domains or entities apart from the S-layer context.

With an antigen providing the binding function to the epithelium, like the \textit{E. coli} F18 receptor-binding domain, the role of binding provided by the S-layer carrier molecule may be of less importance. With an antigen having no binding capacity, the role of the S-layer binding property to the epithelium may also become important, for example, for the prevention of colonization of the invading pathogen by competitive exclusion in addition to eliciting antibody responses against the pathogen. Further modification of the S-layer binding properties, such as the generation of hybrid S-layer molecules with altered binding domains, may also be required to match the demands of the chosen host animal or tissue.

There is currently a need to identify new generation adjuvants also applicable to mucosal immunization. By using cytokines as adjuvants the desired immunological effects, specific for each cytokine, can be obtained and thus the selection of the appropriate cytokine for each antigen plays a critical role. For the maximal induction of the mucosal immune system an efficient adjuvant capable of enhancing and/or inducing the specific mucosal immune responses to the co-administered vaccine antigen is often required. Porcine IL-2 was produced in a biologically active form in \textit{L. lactis} and can first be used as an adjuvant in future immunizations aimed against infections caused by F18 fimbriae carrying \textit{E. coli}. Our future aims include the production of porcine IL-2 in a biologically active form in \textit{L. brevis}, either as a secreted form or a surface-displayed molecule. Considering the putative use of IL-2 as a vaccine adjuvant, it also needs to be transferred from the current nisin-inducible promoter under the regulation of a constitutive promoter allowing continuous production of the cytokine.

Immunization studies with \textit{Lactobacillus} S-layer antigen constructs are still lacking, but the results of the few immunization studies utilizing recombinant S-layer proteins of other bacteria (Umelo-Njaka et al., 2001; Riedmann et al., 2003) further encourage the development of \textit{Lactobacillus} S-layer based antigen carriers. Immunization studies with piglets may be conducted with \textit{Lactobacillus} vaccine vectors surface-displaying the receptor-binding domain of \textit{E. coli} F18 fimbriae as part of the S-layer and co-expressing porcine cytokines as adjuvants.
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