Factors affecting secretion and surface display of heterologous proteins in *Lactococcus lactis*

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ACADEMIC DISSERTATION

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Helsinki 2010
CONTENTS

SUMMARY

LIST OF ORIGINAL PUBLICATIONS

ABBREVIATIONS

1 LITERATURE REVIEW ................................................................. 1

1.1 Introduction ........................................................................... 1
  1.1.1 Taxonomy and nomenclature of the species Lactococcus lactis .... 1
  1.1.2 Fields of L. lactis application ............................................. 1

1.2 Gene expression ..................................................................... 20
  1.2.1 Preface .............................................................................. 20
  1.2.2 L. lactis promoters ............................................................ 20
  1.2.3 Heterologous promoters ................................................... 23
  1.2.4 Synthetic promoters ......................................................... 23
  1.2.5 Codon optimization ......................................................... 24

1.3 Protein secretion and targeting ........................................... 24
  1.3.1 Preface .............................................................................. 24
  1.3.2 Sec-dependent protein secretion .................................... 25

1.4 Anchoring of proteins to the bacterial surface .................. 33
  1.4.1 Preface .............................................................................. 33
  1.4.2 Membrane anchors ......................................................... 34
  1.4.3 Cell wall anchors ............................................................ 34

1.5 Examples of other strategies to enhance heterologous protein production .... 35
  1.5.1 Stabilization of messenger RNA .................................... 35
  1.5.2 Fusion protein technology .............................................. 36

1.6 Escherichia coli post-weaning diarrhoea and oedema disease in swine .... 36
  1.6.1 Preface .............................................................................. 36
  1.6.2 F18 fimbriae ................................................................. 36
  1.6.3 Vaccination against F18-positive E. coli infection ................. 37
  1.6.4 The F18 fimbriae receptor .............................................. 38
2 AIMS OF THE STUDY ...........................................................................................................39

3 MATERIALS AND METHODS ..........................................................................................40

4 RESULTS AND DISCUSSION ............................................................................................42
  4.1 Role of B. subtilis PrsA on heterologous protein secretion in L. lactis (I) .................42
     4.1.1 Construction of plasmid vectors ........................................................................42
     4.1.2 Production of PrsA ..........................................................................................42
     4.1.3 Effect of PrsA on AmyQ production and activity .........................................46
     4.1.4 Effect of PrsA on PenP production and activity ...........................................46
     4.1.5 Function of PrsA ...........................................................................................46
  4.2 Surface display of the receptor binding domain of L. brevis SlpA in L. lactis (II) ....47
     4.2.1 Adhesion assays ............................................................................................47
  4.3 Optimization of production and surface display of the FedF adhesin in L. lactis (III, IV) ..................................................................................48
     4.3.1 Secretion studies ...........................................................................................48
     4.3.2 Surface display studies .................................................................................50
     4.3.3 Adhesion studies ...........................................................................................51
  4.4 Constitutive expression of fedF for surface display of the receptor binding domain of the FedF adhesin (IV) ..........................................................51
     4.4.1 Construction of plasmid vectors and ELISA ................................................51
     4.4.2 Analysis of promoter sequences ...................................................................52

5 CONCLUSIONS AND FUTURE PROSPECTS ....................................................................53

6 ACKNOWLEDGEMENTS ........................................................................................................55

7 REFERENCES ........................................................................................................................56
Bacteria are utilized for the production of many heterologous proteins, including industrially important enzymes, pharmaceuticals and vaccines. This has resulted in an extensive research on the steps along the protein production pathway in both Gram-negative and Gram-positive bacteria, in order to understand the mechanisms involved and to develop new and better tools for optimal production. However, there is still a lack in our understanding regarding bacterial protein synthesis and the production of every target protein has to be experimentally evaluated. Even so, satisfying quantity and quality of many produced proteins have not been reached.

In some applications, including industrial production of enzymes, the aim is usually to produce high yields of the target protein. This can best be accomplished if the protein is secreted into the culture medium from where it can be purified. In other applications, e.g. bacterial delivery of an antigen to mucosal surfaces, the requirement to reach the maximum yield might not be the main goal. Instead, the production system could be optimized in order to display the target antigen on the bacterial surface, from where it could be presented to the immune system on mucosal surfaces. During the last decade, there has been an increasing interest to utilize non-pathogenic and probiotic bacteria as vehicles for the delivery of therapeutic and prophylactic molecules to mucosal surfaces. Especially, lactic acid bacteria (LAB) with putative probiotic properties are interesting candidates.

In this work, the secretion and surface display of heterologous proteins in the food-grade LAB \textit{Lactococcus lactis} was studied. In the first part, the protein secretion pathway of \textit{L. lactis} was complemented with the extracellular chaperone PrsA from \textit{Bacillus subtilis}. With the nisin-controlled gene expression (NICE) system, PrsA was produced in an active form in \textit{L. lactis} and its function was similar to its function in \textit{B. subtilis}. That is, PrsA increased the secretion yield of a PrsA-target protein, whereas it had no effect on the secretion of a PrsA-nontarget protein. Even though the secretion yield increased, some of the PrsA-target protein remained partly trapped on the trans side of the cytoplasmic membrane in an unprocessed form, indicating a problem at the late stages of secretion.

In the second part, the secretion efficiency and surface display of different proteins were optimized. The target proteins were produced as translational fusions with the lactococcal proteinase PrtP and the lactococcal autolysin AcmA cell wall binding repeats. Different fragments, spanning the H- and W-domains of PrtP were utilized as spacers, to extend the target protein through the cell wall to the bacterial surface. With this method, two target proteins were successfully surface displayed and recognized by their respective antibodies in whole-cell enzyme-linked immunosorbent assay studies. \textit{Escherichia coli} β-lactamase (Bla) was mainly used as an easily detectable reporter protein when the first constructs were made, whereas the \textit{Lactobacillus brevis} S-layer (SlpA) receptor binding domain rendered the nonadhesive \textit{L. lactis} cell the ability to adhere to fibronectin and the human intestinal epithelial cell line, Intestine 407.

In the third part of the study, the aim was to employ the surface display system to construct a live bacterial mucosal vaccine against post weaning diarrhoea and oedema disease, caused by F18-positive \textit{E. coli}. The secretion efficiency of the receptor-binding domain of FedF, the adhesin of F18 fimbriae, was evaluated by translational fusion of FedF-ProtP to either one of the signal peptides of \textit{L. lactis} Usp45 or \textit{L. brevis} SlpA. Expression in the NICE system resulted in a larger protein yield with the SlpA signal peptide, which was used in further studies. Purified secreted FedF-protein was able to bind to isolated porcine epithelial cells. For efficient surface display of the receptor binding domain of FedF several
parameters were evaluated, including length of FedF protein, length of PrtP spacer, type of cell wall anchor, and host background (wild type \textit{L. lactis} NZ9000 and an NZ9000\textit{ΔhtrA} mutant). The strongest adhesion to isolated porcine intestinal epithelial cells was attained with a construct comprising 42 amino acid residues FedF adhesin, 516 amino acid residues PrtP spacer, and the AcmA cell wall anchor, produced in the NZ9000\textit{ΔhtrA} mutant.

In the fourth study, the FedF surface display system was further developed for constitutive expression. For this, a set of artificial promoters were synthesized and used to express the gene fusion. With the strongest constitutive promoter, \textit{L. lactis} cells surface displayed FedF to the same extent as with optimized nisin induction, indicating the attainment of an optimal constitutive expression level for the construct.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numeral. The original articles are reprinted with the kind permission of the publishers.


ABBREVIATIONS

aa  amino acid
ABC  adenosine triphosphate-binding cassette
ATCC  American type culture collection
ATP  adenosine triphosphate
ATPase  adenosine triphosphatase
C-region  cleavage region
C-terminal  carboxyterminal
C-terminus  carboxyterminus
CWA  cell wall anchor
DNA  deoxyribonucleic acid
ELISA  enzyme-linked immunosorbent assay
ETEC  enterotoxigenic Escherichia coli
GC  guanine and cytosine
H-region  hydrophobic region
IgA  immunoglobulin A
IgG  immunoglobulin G
IL  interleukin
kbp  kilobase pair
LAB  lactic acid bacteria
mRNA  messenger ribonucleic acid
NICE  nisin-controlled gene expression
N-region  aminoterminal region
N-terminal  aminoterminal
N-terminus  aminoterminus
OD  optical density; oedema disease
P  promoter
PCR  polymerase chain reaction
PPIase  peptidyl prolyl cis-trans isomerase
PWD  post-weaning diarrhoea
RBD  receptor-binding domain
RNA  ribonucleic acid
Sec  secretory
S-layer  surface layer
SRP  signal recognition particle
SS  signal sequence
TAs  teichoic acids
Tat  twin-arginine translocation
TMS  transmembrane-spanning
tRNA  transport ribonucleic acid
TTFC  tetanus toxin fragment C
UTLS  untranslated leader sequence
VTEC  verotoxigenic Escherichia coli
1 LITERATURE REVIEW

1.1 Introduction

1.1.1 Taxonomy and nomenclature of the species Lactococcus lactis

*Lactococcus lactis* species are non-pathogenic, non-motile, obligatory homofermentative and facultative anaerobes with an optimum growth temperature near 30°C. Under the microscope, they appear as Gram-positive cocci in pairs or short chains.

*L. lactis* was the first organism to be isolated in pure culture (Hutkins 2006). This was done in 1873 by Joseph Lister, who isolated a bacterium from milk that he named *Bacterium lactis*. In 1909, *B. lactis* was renamed *Streptococcus lactis*, a name it carried until 1985, when an extensive reclassification of the genus Streptococcus was done to make a distinction between pathogenic streptococci and non-pathogenic lactococci (Validation List No. 20 1986). In the reclassification, *Streptococcus lactis* and *Streptococcus cremoris* were designated as two *L. lactis* subspecies: *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*, respectively. A third, but rare subspecies within the species *L. lactis* is designated *L. lactis* ssp. *hordinea*. An overview of nomenclature changes is presented in Figure 1.

In nature, *L. lactis* is found mainly on green plant and animal surfaces, but also in the gastrointestinal tract of animals and humans, where it is believed to enter with consumed plant material and dairy products. Because several *L. lactis* strains have been isolated from milk products, milk can be considered an additional “natural” habitat for this bacterium (Hutkins 2006).

1.1.2 Fields of *L. lactis* application

1.1.2.1 Dairy fermentation

The ability of *L. lactis* to ferment lactose to lactic acid, hydrolyse casein and ferment citric acid has made it one of the most important lactic acid bacteria (LAB) in the dairy industry. Especially the subspecies *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* have a long history in dairy fermentation and contribute to taste, flavour, and texture of milk products, primarily

![Figure 1](image_url). Changes in the nomenclature of *L. lactis* species from when it was first isolated in 1873 till to date. The years within brackets indicate the time point when new names were introduced, while texts within brackets indicate names of common laboratory strains.
cheeses (e.g. Brie, Camembert, Cheddar, Gruyère, Parmesan and Roquefort), buttermilk and yoghurt. Other processes using *L. lactis* fermentation include the making of wine, beer, pickled vegetables and some breads and sausages (Hutkins 2006).

The traditional use of *L. lactis* in dairy food has been given a GRAS (generally recognized as safe) status by the US Food and Drug Administration (FDA). In Europe, the safety system Qualified Presumption of Safety (QPS) is maintained by the European Food Safety Authority (EFSA), which annually updates a list of microorganisms with approved QPS status (www.efsa.europa.eu). Due to its long history of safe use, *L. lactis* has been proposed for QPS since the term was introduced in 2007 (EFSA 2007).

### 1.1.2.1 Heterologous protein production

Most of the industrially important traits of *L. lactis* strains (e.g. lactose catabolism and proteinase production) are encoded on plasmids, which might constitute a large and often unstable DNA complement to the cell. The development of the plasmid-free *L. lactis* strain MG1363 in the early 1980s opened new doors for the use of *L. lactis* in heterologous protein production (Gasson 1983). MG1363 is stable and relatively easy to modify genetically, and today *L. lactis* is “the international prototype for LAB genetics” (Wegmann *et al.* 2007) and the model LAB in heterologous protein production. Many genetic tools, including transformation protocols and new vector systems, as well as tools for protein targeting, including protein secretion and display of proteins on the cell surface, are available (Le Loir *et al.* 2005). In addition, *L. lactis* is the first LAB for which the whole genome was sequenced (Bolotin 2001). To date, the whole genome sequences of four *L. lactis* strains are available on the Entrez Genome web site.

The available tools have been used to produce a variety of proteins and peptides of plant, viral, fungal, bacterial and mammalian origin in *L. lactis*, including membrane proteins, enzymes, antimicrobials (bacteriocins, bacteriophage endolysins, defensins, fungicides), antigens and therapeutic molecules (allergens, cytokines) (Table 1). In recent years, *L. lactis* has become especially popular for the production of antigens, allergens and cytokines, which it delivers to mucosal surfaces of animals and humans.

Several reports on significant *in vivo* responses on recombinant *L. lactis* in animals are available (Wells and Mercenier 2008). To date, one recombinant *L. lactis* strain has been evaluated in human phase I and phase II clinical trials, where the therapeutic effects of *L. lactis* producing the cytokine interleukin 10 (IL-10) on inflammatory bowel disease (IBD) were studied (Braat *et al.* 2006; ActoGenix 2009). The strain used did not significantly reduce disease symptoms in patients, but this primary clinical trial demonstrated that mucosal drug delivery using *L. lactis* was clinically well tolerated and that the strain was environmentally contained. Currently, the therapeutic effects of recombinant *L. lactis*, producing human Trefoil factor, on oral mucositis are being evaluated in a phase 1 clinical trial (www.ClinicalTrials.gov, case NCT00938080).

### 1.1.2.2 Mucosal vaccine production and delivery

The first internationally published use of *L. lactis* as a carrier of an antigen in mucosal immunization was reported in Japan in 1990 by Iwaki *et al.* with the aim of developing an oral vaccine against dental caries. Oral immunization of mice with killed recombinant *L. lactis* cells loaded (cell associated, mainly cytoplasmic) with *Streptococcus mutans* surface protein antigen (PAC) resulted in significant salivary IgA and serum IgG responses to PAC.
### Table 1. Examples of heterologous proteins and peptides produced in *Lactococcus lactis*

<table>
<thead>
<tr>
<th>Protein/peptide and its origin</th>
<th>Application prospects as suggested by author</th>
<th>Properties</th>
<th>Location (SP or anchor and its origin*)</th>
<th>Promoter used (and its origin*)</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Allergens</strong></td>
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<td><em>Arachis hypogaea</em> (synthetic)</td>
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<td><em>Ara h 2</em></td>
<td>immunotherapy against peanut allergy</td>
<td></td>
<td>secreted (SP310mut2)</td>
<td>P170</td>
<td>Glenting <em>et al.</em> 2007*^a^</td>
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<td></td>
<td></td>
<td></td>
<td>cytoplasm secreted (SPUsp45)</td>
<td>PspA, PnisA</td>
<td>Chatel <em>et al.</em> 2001*^b^ Adel-Patient 2005*^c^</td>
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<tr>
<td>bovine BLG (β-lactoglobulin)</td>
<td>induction of oral tolerance against milk allergy</td>
<td></td>
<td>allergen fused to PrtB</td>
<td>secreted (SPuB of <em>L. bulgaricus</em>) anchored (SPuB/CWAuB of <em>L. bulgaricus</em>)</td>
<td>PnisA</td>
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<td></td>
<td></td>
<td></td>
<td>all allergen fused to Nuc</td>
<td>secreted (SPNuc)</td>
<td>PnisA</td>
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<td></td>
<td></td>
<td></td>
<td>all allergen fused to Nuc and/or LEISSTCDA, vaccine coadministered with IL-12 at immunization</td>
<td>secreted (SPUsp45)</td>
<td>PnisA</td>
</tr>
<tr>
<td>human (synthetic) DQ8d (DQ8-specific gliadin epitope)</td>
<td>induction of oral tolerance in celiac disease</td>
<td></td>
<td>secreted (SPUsp45)</td>
<td>P1</td>
<td>Huibregste <em>et al.</em> 2009*^b^</td>
</tr>
<tr>
<td>ovine OVA (ovalbumin)</td>
<td>induction of oral tolerance</td>
<td></td>
<td>secreted (SPUsp45)</td>
<td>P1</td>
<td>Huibregste <em>et al.</em> 2007*^b^</td>
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<td><strong>Antigens, bacterial</strong></td>
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<tr>
<td><em>Brucella abortus</em> GroEL</td>
<td>vaccine against brucellosis</td>
<td></td>
<td>antigen fused or not to LEISSTCDA</td>
<td>cytoplasm secreted (SPUsp45)</td>
<td>Miyoshi <em>et al.</em> 2006*^a^</td>
</tr>
<tr>
<td>L7/L12</td>
<td></td>
<td></td>
<td>antigen fused or not to Nuc or LEISSTCDA</td>
<td>cytoplasm secreted (SPUsp45) anchored (SPUsp45/CWA6 of <em>S. pyogenes</em>)</td>
<td>PnisA</td>
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<tr>
<td>Protein/peptide and its origin</td>
<td>Application prospects as suggested by author</td>
<td>Properties</td>
<td>Location (SP or anchor and its origin*)</td>
<td>Promoter used (and its origin*)</td>
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<td><em>Clostridium perfringens</em> β-toxoid</td>
<td>vaccine against enteritis</td>
<td>secreted (SP β-tox of C. perfringens)</td>
<td>P&lt;sub&gt;rpf&lt;/sub&gt; (Bacillus subtilis), P&lt;sub&gt;nie&lt;/sub&gt;A</td>
<td>Nijland et al. 2007&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>cytoplasm</td>
<td>P&lt;sub&gt;T7&lt;/sub&gt; (bacteriophage)</td>
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<td></td>
<td></td>
<td>secreted (SP&lt;sub&gt;Usp45&lt;/sub&gt;)</td>
<td>P&lt;sub&gt;T7&lt;/sub&gt;</td>
<td>Wells et al. 1993&lt;sup&gt;a,b&lt;/sup&gt;; Norton et al. 1996&lt;sup&gt;c&lt;/sup&gt;; Norton et al. 1997&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
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<td>anchored (SP&lt;sub&gt;Usp45&lt;/sub&gt;/CWA&lt;sub&gt;nie&lt;/sub&gt;)</td>
<td>P&lt;sub&gt;T7&lt;/sub&gt;</td>
<td>Norton et al. 1996&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>anchored (SP&lt;sub&gt;Usp45&lt;/sub&gt;/CWA&lt;sub&gt;nie&lt;/sub&gt;)</td>
<td>P&lt;sub&gt;nie&lt;/sub&gt;A</td>
<td>Robinson et al. 1997&lt;sup&gt;c&lt;/sup&gt;; 2004&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td><em>Clostridium tetani</em> TTFc (tetanus toxin fragment C)</td>
<td>vaccine against tetanus</td>
<td>antigen coproduced with IL-2 and IL-6</td>
<td>cytoplasm</td>
<td>Steidler et al. 1998&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>antigen produced in an alanine racemase mutant strain</td>
<td>cytoplasm</td>
<td>Grangette et al. 2002&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>cytoplasm</td>
<td>Grangette et al. 2004&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><em>Erysipelotrix rhusiopathiae</em> SpaA (surface protective antigen)</td>
<td>vaccine against swine erysipelas</td>
<td>anchored (SP&lt;sub&gt;SpaA&lt;/sub&gt;/CWA&lt;sub&gt;SpaA&lt;/sub&gt; of E. rhusiopathiae)</td>
<td>P&lt;sub&gt;nie&lt;/sub&gt; (E. rhusiopathiae)</td>
<td>Cheun et al. 2004&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><em>Escherichia coli</em> FaeG (fimbrial adhesin)</td>
<td>vaccine against (neonatal) diarrhoea in pigs</td>
<td>secreted (SP&lt;sub&gt;Usp45&lt;/sub&gt;)</td>
<td>P&lt;sub&gt;nie&lt;/sub&gt;A</td>
<td>Hu et al. 2009&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><em>Helicobacter pylori</em> Cag-12 (outer membrane protein)</td>
<td>vaccine against gastric disorders</td>
<td>cytoplasm</td>
<td>P&lt;sub&gt;nec&lt;/sub&gt; (NN)</td>
<td>Kim et al. 2006&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><em>UreB</em> (urease subunit B)</td>
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<td>cytoplasm</td>
<td>P&lt;sub&gt;nec&lt;/sub&gt;</td>
<td>Lee et al. 2001&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
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<td>cytoplasm</td>
<td>P&lt;sub&gt;nec&lt;/sub&gt;A</td>
<td>Zhang et al. 2009&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
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<td>secreted (SP&lt;sub&gt;310mut2&lt;/sub&gt;/CWA&lt;sub&gt;SpaA&lt;/sub&gt; of <em>Staphylococcus aureus</em>)</td>
<td>P&lt;sub&gt;170&lt;/sub&gt;</td>
<td>Gu et al. 2009</td>
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<td><em>UreBE</em> (fragment E of UreB)</td>
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<td>anchored (SP&lt;sub&gt;310mut2&lt;/sub&gt;/CWA&lt;sub&gt;SpaA&lt;/sub&gt; of <em>Staphylococcus aureus</em>)</td>
<td>P&lt;sub&gt;170&lt;/sub&gt;</td>
<td>Song &amp; Gu 2009&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Protein/peptide and its origin</td>
<td>Application prospects as suggested by author</td>
<td>Properties</td>
<td>Location (SP or anchor and its origin*)</td>
<td>Promoter used (and its origin*)</td>
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<td><em>Lactobacillus paracasei</em> GroEL</td>
<td>characterization of stress tolerance in bacteria</td>
<td>cytoplasm</td>
<td>( P_{mis} )</td>
<td>Desmond et al. 2004(^a)</td>
<td></td>
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<tr>
<td><em>Listeria monocytogenes</em></td>
<td>vaccine against meningitis and septicaemia</td>
<td>cytoplasm</td>
<td>( P_{mis} )</td>
<td>Bahey-El-Din et al. 2008(^a), Bahey-El-Din et al. 2008(^b)</td>
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<td>LLO (listeriolysin O)</td>
<td>vaccine against meningitis and septicaemia</td>
<td>cytoplasm secreted (SP(_{Usp45}))</td>
<td>( P_{44}^{PP} P_{mis4} )</td>
<td>Bahey-El-Din et al. 2008(^b), 2010(^b)</td>
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<tr>
<td></td>
<td>antigen fused to P60</td>
<td>secreted (SP(_{Usp45}))</td>
<td>( P_{mis4} )</td>
<td>Bahey-El-Din et al. 2010(^b)</td>
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<td>P60</td>
<td>antigen fused or not to LLO</td>
<td>secreted (SP(_{Usp45}))</td>
<td>( P_{mis4} )</td>
<td>Bahey-El-Din et al. 2010(^b)</td>
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<td><em>M6 CRR</em> (C-repeat region of M6 protein)</td>
<td>vaccine against pharyngitis</td>
<td>secreted (SP(<em>{Usp45})) anchored (SP(</em>{Usp45}/CWA_M6) of ( S. pyogenes ))</td>
<td>( P_{46}^{PP} P_{mis4} (Lactobacillus acidophilus) )</td>
<td>Geller et al. 2001(^a), Mannam et al. 2004(^a)</td>
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<td><em>Mycobacterium tuberculosis</em> ESAT-6 (early secreted antigenic target)</td>
<td>tuberculosis diagnostics</td>
<td>secreted (SP(_{Usp45}))</td>
<td>( P_{170} )</td>
<td>Aggerbeck and Madsen 2006(^b)</td>
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<td><em>Proteus mirabilis</em> ( MrpA ) (mannose-resistant ( Proteus )-like fimbriae subunit A)</td>
<td>vaccine against urinary tract infection</td>
<td>secreted (SP(<em>{Usp45})) anchored (SP(</em>{Usp45}/CWA_M6) of ( S. pyogenes ))</td>
<td>( P_{mis4} )</td>
<td>Seavone et al. 2007(^b)</td>
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<td><em>Salmonella enterica</em> FliC (flagellar antigen)</td>
<td>surface display of heterologous proteins with the PrtB CWA</td>
<td>anchored (SP(<em>{AmyA}) of ( Streptococcus bovis) /CWA(</em>{pB}) of ( Lactobacillus delbrueckii))</td>
<td>( P_{AmyA} (S. bovis) )</td>
<td>Kim et al. 2008(^a)</td>
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<td><em>Streptococcus agalactiae</em> Pilin island 1 and 2 proteins GBS80, GBS104</td>
<td>vaccine against infection in newborns</td>
<td>anchored (SP(<em>{GspA})/CWA(</em>{IS1}) of ( S. agalactiae))</td>
<td>( P_{GBS80/GBS104} (S. agalactiae) )</td>
<td>Buccato et al. 2006(^b)</td>
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**Table 1 cont.**

<table>
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<tr>
<td><em>Streptococcus mutans</em> PAc (surface protein antigen)</td>
<td>vaccine against dental caries</td>
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<td>PAc (S. mutans)</td>
<td>Iwaki et al. 1990b)</td>
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<td><em>Streptococcus pneumonia</em> PppA (pneumococcal protective protein A)</td>
<td>vaccine against respiratory tract infection</td>
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<td>P_{nisA}</td>
<td>Medina et al. 2008a) Villena et al. 2008b), 2009c) Vintini et al. 2009c)</td>
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<td>PspA (pneumococcal surface protein A)</td>
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<td>P_{nisA}</td>
<td>Hanniffy et al. 2007b)</td>
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<td><em>Yersinia pseudotuberculosis</em> LcrV (low calcium response V)</td>
<td>vaccine against yersiniosis</td>
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<td>P_{asp45}</td>
<td>Foligne et al. 2007b) Daniel et al. 2009c)</td>
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<td><em>Giardia lamblia</em> CWP2 (cyst wall protein 2)</td>
<td>vaccine against giardiasis</td>
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<td>cytoplasm secreted (SP_{Usp45}) anchored (SP_{Usp45}/CWA_{M6} of S. pyogenes)</td>
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<td>Lee and Faubert 2006b) Lee et al. 2009c)</td>
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<td><em>Plasmodium falciparum</em> GLURP (glutamate-rich protein)</td>
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<td>Ramasamy et al. 2006b) Moorthy and Ramasamy 2007c) Moorthy et al. 2009c)</td>
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<td>Tang and Li 2009b)</td>
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<td>Protein/peptide and its origin</td>
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<td>P&lt;sub&gt;59&lt;/sub&gt;</td>
<td>Dieye &lt;i&gt;et al.&lt;/i&gt; 2003&lt;sup&gt;b&lt;/sup&gt;)</td>
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<td>VP2 and VP3</td>
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<td>P&lt;sub&gt;S9&lt;/sub&gt;, P&lt;sub&gt;mac&lt;/sub&gt;</td>
<td>Langella &amp; Le Loir 1999&lt;sup&gt;a&lt;/sup&gt;)</td>
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<td>Sim &lt;i&gt;et al.&lt;/i&gt; 2008&lt;sup&gt;b&lt;/sup&gt;)</td>
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<td>strain coadministered with hIFN-α2b at immunization</td>
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<td>P&lt;sub&gt;nisA&lt;/sub&gt;</td>
<td>Zhang &lt;i&gt;et al.&lt;/i&gt; 2010&lt;sup&gt;b&lt;/sup&gt;)</td>
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<td>HBsAg (hepatitis B surface</td>
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<td>P&lt;sub&gt;inw&lt;/sub&gt;</td>
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<sup>a</sup> Reference for different studies.

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<td>strain coadministered with IL-12 at immunization anchored (SP&lt;sub&gt;Usp45&lt;/sub&gt;/CWA&lt;sub&gt;M6&lt;/sub&gt; of S. pyogenes)</td>
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<td>Cortes-Perez et al. 2003&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Bermudez-Humaran et al. 2007&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>antigen coproduced with Mig:IP-10 anchored (SP&lt;sub&gt;Usp45&lt;/sub&gt;/CWA&lt;sub&gt;M6&lt;/sub&gt; of S. pyogenes)</td>
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<td>Cortes-Perez et al. 2008&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>E7 mutated anchored (SP&lt;sub&gt;Usp45&lt;/sub&gt;/CWA&lt;sub&gt;M6&lt;/sub&gt; of S. pyogenes and CWA&lt;sub&gt;29da&lt;/sub&gt; of Lactobacillus plantarum)</td>
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<td>Cortes-Perez et al. 2005&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>L1</td>
<td>vaccine against diarrhoea</td>
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<td>Zhou et al. 2008&lt;sup&gt;a&lt;/sup&gt;)</td>
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<td><em>Carnobacterium divergens</em> DvnRV41 (divercin RV41)</td>
<td>food preservation</td>
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<td><em>E. coli</em> bacteriophages T4 and λ lysozyme</td>
<td>preservation of dairy products</td>
<td>cytoplasm</td>
<td>P&lt;sub&gt;T7&lt;/sub&gt; P&lt;sub&gt;32&lt;/sub&gt;</td>
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<td><em>Listeria monocytogenes</em> phage Ply118 (endolysin)</td>
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<td>Gaeng et al. 2000&lt;sup&gt;a&lt;/sup&gt;)</td>
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<td>P_{LPS} (<em>L. lactis</em> bacteriophage)</td>
<td>Pusch <em>et al.</em> 2005&lt;sup&gt;a)&lt;/sup&gt;</td>
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<td><em>Pediococcus acidilactici</em> PedA-1 (pediocin PA-1)</td>
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<td>O’Flaherty <em>et al.</em> 2005&lt;sup&gt;a)&lt;/sup&gt;</td>
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<td><em>Staphylococcus simulans</em> lysostaphin</td>
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<td>human (synthetic) IFN-α2b (interferon-alfa 2b)</td>
<td>vaccine adjuvant</td>
<td>strain coadministered with HBsA determinants at immunization</td>
<td>secreted (SP_{Usp45})</td>
<td>P_{ntr.A}</td>
<td>Zhang <em>et al.</em> 2010&lt;sup&gt;a)&lt;/sup&gt;</td>
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<td>immunotherapy, vaccine adjuvant</td>
<td>protein fused or not to LEISSTCDA</td>
<td>secreted (SP_{Usp45}; SP_{SbpA})</td>
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<td>IL-10</td>
<td>immunotherapy against IBS and Crohn’s disease</td>
<td>protein coproduced with OtsBA</td>
<td>secreted (SP_{Usp45})</td>
<td>P_{1}, P_{rhyA}</td>
<td>Steidler <em>et al.</em> 2003&lt;sup&gt;b)&lt;/sup&gt; Braat <em>et al.</em> 2006&lt;sup&gt;g)&lt;/sup&gt;</td>
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<td>secreted (SP_{Usp45})</td>
<td>P_{1}</td>
<td>Termont <em>et al.</em> 2006&lt;sup&gt;g)&lt;/sup&gt;</td>
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<td>murine IFN-γ</td>
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<td>P&lt;sub&gt;nisA&lt;/sub&gt;</td>
<td>Bermúdez-Humáran &lt;i&gt;et al.&lt;/i&gt; 2008&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>IL-2 (interleukin-2)</td>
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<td>secreted (SP&lt;sub&gt;Usp45&lt;/sub&gt;)</td>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Steidler &lt;i&gt;et al.&lt;/i&gt; 1995&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>IL-6</td>
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<td>Steidler &lt;i&gt;et al.&lt;/i&gt; 1998&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>P&lt;sub&gt;up&lt;sub&gt;5&lt;/sub&gt;&lt;/sub&gt;</td>
<td>Foligne &lt;i&gt;et al.&lt;/i&gt; 2007&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>ovine IFN-ω (interferon-omega)</td>
<td>immunotherapy against enteric viral diseases</td>
<td>cytoplasm secreted (SP&lt;sub&gt;Usp45&lt;/sub&gt;)</td>
<td>P&lt;sub&gt;nisA&lt;/sub&gt;</td>
<td>Bermúdez-Humáran &lt;i&gt;et al.&lt;/i&gt; 2003&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>porcine IFN-γ (interferon-gamma)</td>
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<td>Rupa et al. 2008⁴)</td>
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<td>IL-2</td>
<td>vaccine adjuvant</td>
<td>secreted (SP_Usp45)</td>
<td>P₄tr.A</td>
<td>Ávall-Jääskeläinen 2006⁴)</td>
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**Enzymes**

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<th>Organism</th>
<th>Description</th>
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<td><em>Bacillus subtilis</em></td>
<td>AlaDH (alanine dehydrogenase)</td>
<td>production of alanine in dairy industry</td>
<td>cytoplasm</td>
<td>P_{ldh} (Streptococcus thermophilus) P₄tr.A</td>
<td>Ye et al. 2010⁴)</td>
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<td>KatE (catalase)</td>
<td>gut therapy</td>
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<td>Rochat et al. 2005⁴)</td>
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<td>nattokinase</td>
<td>prevention of thrombosis</td>
<td>cytoplasm secreted (SP_Usp45)</td>
<td>P₃₂ P₄pt₅</td>
<td>Liang et al. 2007⁴)</td>
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<td>Npr (neutral protease)</td>
<td>cheese ripening</td>
<td>secreted (SP_NprE of <em>B. subtilis</em>)</td>
<td>P₃₂</td>
<td>Van de Guchte et al. 1990⁴)</td>
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<td>bovine plasmin</td>
<td>cheese ripening</td>
<td>cytoplasm secreted (SP_Usp45)</td>
<td>P_{prtP} P₄pt₅</td>
<td>Arnau et al. 1997⁴)</td>
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<td><em>Brevibacterium linens</em></td>
<td>MGL (methionine-γ-lyase)</td>
<td>cheese flavour</td>
<td>cytoplasm</td>
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<td>Hanniffy et al. 2009⁴)</td>
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<td><em>Cupriavidus necator</em></td>
<td>PhaC (poly hydroxyalkanoate synthase) together with PhaA (β-ketothiolase) and PhaB (acetoacetyl-CoA reductase)</td>
<td>production of poly(3-hydroxybutyrate) beads in biomedicine</td>
<td>cytoplasm</td>
<td>P₄tr.A</td>
<td>Mifune et al. 2009⁴)</td>
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<td>OtsA (trehalose-6-phosphate synthase)</td>
<td>cryoprotection of bacteria</td>
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<td>cytoplasm</td>
<td><em>P</em>&lt;sub&gt;nisA&lt;/sub&gt;</td>
<td>Termon et al. 2006&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><em>Fragaria x ananassa</em></td>
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<td>FaNES (linalool/nerolidol synthase)</td>
<td>flavour and fragrance in foodstuff</td>
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<td>SAAT (alcohol acyltransferase)</td>
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<td>GSTA1 (glutathione S-transferase A1-1)</td>
<td>substance for detoxification of carcinogens in e.g. functional foods</td>
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<td>cytoplasm</td>
<td><em>P</em>&lt;sub&gt;lacA&lt;/sub&gt;</td>
<td>Xiang et al. 2000&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>SOD (Cu/Zn superoxide dismutase)</td>
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<td>Xiang et al. 2003&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><em>Lactobacillus delbrueckii</em></td>
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<td><em>Lactobacillus gasseri</em></td>
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<td><em>P</em>&lt;sub&gt;170&lt;/sub&gt;</td>
<td>Cho et al. 2007&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>LGMA (maltogenic amylase)</td>
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<td><em>Lactobacillus acidophilus</em></td>
<td>production of oligosaccharides in prebiotics</td>
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<td>Maischberger et al. 2010&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Lactobacillus sakei&lt;sup&gt;β&lt;/sup&gt;-galactosidase</td>
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<td><em>murine</em></td>
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<td>HO-1 (heme oxygenase-1)</td>
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<td><em>Neocallimastix</em> sp. cellulase</td>
<td>biodegradation of plant material in silage</td>
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<td><em>Propionibacterium shermanii</em></td>
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<td>Leenhouts <em>et al.</em> 1998&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>HasB (uridine diphosphate glucose dehydrogenase)</td>
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<td>Chien and Lee 2007&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><em>HasABC</em> (HasC = uridine diphosphate-glucose pyrophosphorylase)</td>
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<td>Other, bacterial</td>
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<td><em>Cellvibrio japonicus</em></td>
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<td>increased cell density in industrial fermentations</td>
<td>anchored (SP&lt;sub&gt;Usp45&lt;/sub&gt;/&lt;CWA&lt;sub&gt;hyd&lt;/sub&gt; or CWA&lt;sub&gt;AcmA&lt;/sub&gt;)</td>
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<td>cytoplasm</td>
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<td>Novotny <em>et al.</em> 2005&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><em>Lactobacillus monocytogenes</em> BilE (bile resistance mechanism)</td>
<td>improved bile tolerance of bacterial strain in mucosal delivery</td>
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Table 1 cont.

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<td>DNA-vaccine development</td>
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<td>characterization of adhesion</td>
<td>anchored (SP&lt;sub&gt;ClfA&lt;/sub&gt;/CWA&lt;sub&gt;ClfA&lt;/sub&gt; of <em>S. aureus</em>)</td>
<td>P&lt;sub&gt;23&lt;/sub&gt;</td>
<td>Que et al. 2000&lt;sup&gt;a&lt;/sup&gt;, Sinha et al. 2000&lt;sup&gt;c&lt;/sup&gt;, Que et al. 2001&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>ClfA</strong> (clumping factor A)</td>
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<td>protein coproduced or not with FnBPA</td>
<td>anchored (SP&lt;sub&gt;ClfA&lt;/sub&gt;/CWA&lt;sub&gt;ClfA&lt;/sub&gt; of <em>S. aureus</em>)</td>
<td>P&lt;sub&gt;23&lt;/sub&gt;</td>
<td>Que et al. 2005&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>FnBPA</strong> (fibronectin binding protein A)</td>
<td>characterization for adhesion and invasion</td>
<td>protein coproduced with GFP</td>
<td>anchored (SP&lt;sub&gt;FnBPA&lt;/sub&gt;/CWA&lt;sub&gt;FnBPA&lt;/sub&gt; of <em>S. aureus</em>)</td>
<td>P&lt;sub&gt;23&lt;/sub&gt;</td>
<td>Que et al. 2001&lt;sup&gt;b&lt;/sup&gt;, Sinha et al. 2000&lt;sup&gt;c&lt;/sup&gt;, Buck et al. 2010&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>C and D domains coproduced or not with ClfA</td>
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<td>P&lt;sub&gt;23&lt;/sub&gt;</td>
<td>Innocentin et al. 2009&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>protein coproduced with GFP</td>
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<td>P&lt;sub&gt;23&lt;/sub&gt;</td>
<td>Que et al. 2005&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>P&lt;sub&gt;23&lt;/sub&gt;</td>
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<td><strong>HtrA&lt;sub&gt;1&lt;/sub&gt; and HtrA&lt;sub&gt;2&lt;/sub&gt;</strong> (high temperature requirement)</td>
<td>characterization of bacterial stress responses</td>
<td>anchored (membrane anchor of HtrA)</td>
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<td>P&lt;sub&gt;abcA&lt;/sub&gt;</td>
<td>Rigoulay et al. 2004&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>ZZ (IgG binding domain)</td>
<td>biomedicine</td>
<td>displayed on the surface of poly(3-hydroxybutyrate) beads produced in <em>L. lactis</em></td>
<td>$P_{nisA}$</td>
<td>Mifune <em>et al.</em> 2009&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><em>Staphylococcus epidermidis</em></td>
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<td>anchored (SP&lt;sub&gt;Fbe&lt;/sub&gt;/CWA&lt;sub&gt;Fbe&lt;/sub&gt; of <em>S. epidermidis</em>)</td>
<td>$P_{LPS2}$ (<em>L. lactis</em> bacteriophage)</td>
<td>Hartford <em>et al.</em> 2001&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Fbe (SdrG)</td>
<td>characterization of adhesion</td>
<td>anchored (SP&lt;sub&gt;SspA&lt;/sub&gt; of <em>S. gordonii</em> / CWA&lt;sub&gt;Aap&lt;/sub&gt; of <em>S. epidermidis</em>)</td>
<td>$P_{1}$</td>
<td>Macintosh <em>et al.</em> 2009&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><em>Aap</em> (accumulation-associated protein)</td>
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<td>secreted (SP&lt;sub&gt;CspA&lt;/sub&gt; of <em>S. agalactiae</em>)</td>
<td>$P_{nisA}$</td>
<td>Shelver and Bryan 2008&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><em>Streptococcus agalactiae</em></td>
<td>protein characterization</td>
<td>anchored (SP&lt;sub&gt;Usp45&lt;/sub&gt;/CWA&lt;sub&gt;Aap&lt;/sub&gt; of <em>S. gordonii</em>)</td>
<td>$P_{1}$</td>
<td>Holmes <em>et al.</em> 1998&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><em>CspA</em> (cell-envelope protease)</td>
<td>characterization of adhesion</td>
<td>anchored (SP&lt;sub&gt;M6&lt;/sub&gt;/CWA&lt;sub&gt;M6&lt;/sub&gt; of <em>S. pyogenes</em>)</td>
<td>$P_{23}$, $P_{59}$</td>
<td>Piard <em>et al.</em> 1997&lt;sup&gt;d&lt;/sup&gt;</td>
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<td><em>Streptococcus pyogenes</em></td>
<td>characterization of cell wall anchor</td>
<td>anchored (SP&lt;sub&gt;Usp45&lt;/sub&gt;/CWA&lt;sub&gt;SA&lt;/sub&gt; of <em>S. aureus</em>)</td>
<td>$P_{T7}$</td>
<td>Steidler <em>et al.</em> 1998&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>M6</td>
<td></td>
<td>cytoplasm secreted (SP&lt;sub&gt;Yab&lt;/sub&gt; of <em>B. subtilis</em>)</td>
<td>$P_{slpA}$ (<em>L. acidophilus</em>)</td>
<td>Yeh <em>et al.</em> 2008&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><em>Streptococcus sp.</em> (NN)</td>
<td>immobilization of bacteria in industrial fermentations</td>
<td>protein coproduced with E7</td>
<td>secreted (SP&lt;sub&gt;Usp45&lt;/sub&gt;)</td>
<td>$P_{nisA}$</td>
<td>Bermúdez-Humarán <em>et al.</em> 2007&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><em>LZ-8</em> (Ling Zhi-8)</td>
<td>obesity therapy, vaccine adjuvant</td>
<td>.Anchor reduces inflammation and cancer therapy</td>
<td>secreted (SP&lt;sub&gt;Usp45&lt;/sub&gt;)</td>
<td>$P_{nisA}$</td>
<td>Yuvaraj <em>et al.</em> 2008&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>human leptin</td>
<td>oral therapy against mucositis</td>
<td>secreted (SP&lt;sub&gt;Usp45&lt;/sub&gt;)</td>
<td>$P_{nlA}$</td>
<td>Caluwaerts <em>et al.</em> 2010&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>scFv SIgA</td>
<td>exotherapy against colon cancer</td>
<td>anchored (SP&lt;sub&gt;MclF&lt;/sub&gt;/MA&lt;sub&gt;MclP&lt;/sub&gt;)</td>
<td>$P_{nisA}$</td>
<td>Yuvaraj <em>et al.</em> 2008&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>TFF (trefoil factors)</td>
<td></td>
<td>secreted (SP&lt;sub&gt;Usp45&lt;/sub&gt;)</td>
<td>$P_{nlA}$</td>
<td>Caluwaerts <em>et al.</em> 2010&lt;sup&gt;d&lt;/sup&gt;</td>
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Table 1 cont.

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<th>Protein/peptide and its origin</th>
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<th>Properties</th>
<th>Location (SP or anchor and its origin*)</th>
<th>Promoter used (and its origin*)</th>
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<tr>
<td>murine (synthetic) TFF1, 2 and 3</td>
<td>exotheraphy against and prevention of acute colitis and epithelial damage</td>
<td>secreted (SP (_{Usp45}))</td>
<td>P(_1)</td>
<td>Vandenbroucke 2004(^b)</td>
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<tr>
<td>NN, synthetic AFP (type I antifreeze protein analogue)</td>
<td>cryopreservation of food and pharmaceutical materials</td>
<td>secreted (SP (<em>{Usp45}) or SP (</em>{SpA}) of (L.\ acidophilus); SP (_{SpAcB}) of (B.\ subtilis))</td>
<td>(P(_{slpA}) (L. acidophilus))</td>
<td>Yeh \textit{et al.} 2008(^a)</td>
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<td></td>
<td>cryopreservation of meat and dough</td>
<td>secreted (SP (_{SpA}) of (L.\ acidophilus))</td>
<td>(P(_{slpA}) (L. acidophilus))</td>
<td>Yeh \textit{et al.} 2009(^a)</td>
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<td>porcine EFG (epidermal growth factor)</td>
<td>dietary supplement for intestinal development</td>
<td>secreted (SP (_{310mut2}))</td>
<td>(P(_{170}) )</td>
<td>Cheung \textit{et al.} 2009(^b) Kang \textit{et al.} 2010(^c)</td>
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<td>Other, plant</td>
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<td>Pentadiplandra brazzeana brazzein (synthetic)</td>
<td>food sweetener</td>
<td>cytoplasm secreted (SP (_{Usp45}))</td>
<td>(P(_{mutA}) )</td>
<td>Berlec \textit{et al.} 2006(^a); 2008(^c)</td>
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<td></td>
<td></td>
<td>secreted (SP (_{Usp45}))</td>
<td>(P(_{mutA}) )</td>
<td>Berlec and Strukelj 2009(^a)</td>
<td></td>
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</table>

a) strain produced in this study (with or without in vitro experimentation on strain functionality)
b) strain produced and tested in vivo in this study
c) earlier published strain tested in vitro or in vivo
d) clinical trial
NN, not mentioned
*the origin is mentioned if it is non-lactococcal
The use of the first live *L. lactis* vaccine was reported in 1993 by Wells et al. (1993a), who constructed a recombinant *L. lactis* strain that produced the highly immunogenic tetanus toxin fragment C (TTFC) of *Clostridium tetani* intracellularly at a level of up to 22% of total soluble cell protein after controlled expression of the protein encoding gene. In this early study, mice were vaccinated subcutaneously with the induced recombinant *L. lactis* strain to evaluate its immunogenicity and protection against lethal challenge, both of which gave positive results. Later, TTFC has been widely used as a model antigen to study different parameters involved in attaining an optimal *in vivo* response to the vaccine. These include evaluation of the preferred cellular location of the antigen in the bacterial vehicle (cytoplasm, surface displayed, secreted), vaccine dose, route of administration (intranasal, oral, intragastric, genital) and co-expression of antigen and cytokine (Wells and Mercenier 2008). For *L. lactis* producing TTFC, the best dose-response ratio was achieved when the antigen was anchored to the bacterial surface and inoculated by the nasal route (Norton et al. 1996; Robinson et al. 1997).

Another antigen that has been produced in *L. lactis* and intensively tested in preclinical immunization studies is the human papillomavirus type 16 (HPV-16) E7 protein, with the aim of developing a vaccine against cervical cancer. *L. lactis* strains producing E7 at different cellular locations have been used in immunization studies on mice, and, similar to the results for TTFC, the best immune responses have been attained with the surface-anchored form (Bermúdez-Humarán et al. 2004, 2009). The results of challenge studies and therapeutic immunizations with E7 producing *L. lactis* in mice have been promising in protecting against cancer tumours and regression of palpable tumours, respectively (Bermúdez-Humarán 2009). However, no results from human clinical trials with this vaccine have yet been published. In addition, more than 50 other putative vaccine antigens and therapeutic molecules produced in *L. lactis* have been published in peer-reviewed research papers (Table 1).

### 1.1.2.2 Advantages and disadvantages of *L. lactis* in protein production

Compared with other bacterial cell factories, like *Escherichia coli* and *Bacillus subtilis*, *L. lactis* provides both advantages and disadvantages as a host for heterologous protein production. Advantages include: 1) a relatively simple metabolism that is well known, 2) the presence of only one chromosome-encoded extracellular housekeeping protease (HtrA) (compared with 12 in *B. subtilis*) that can easily be deleted without compromising the vitality of the cell (Miyoshi et al. 2002; Rigoulay et al. 2004), 3) a relatively simple and inexpensive purification of heterologous secreted proteins (Mierau et al. 2005b) because secreted proteins are exported directly into the medium and *L. lactis* secretes only one native major protein, Usp45 (van Asseldonk et al. 1990).

However, compared with *E. coli* and *B. subtilis*, the protein yields produced with *L. lactis* are modest. One reason for this is the fermentative metabolism in *L. lactis*. Because of sugar fermentation, accumulation of lactic acid decreases pH of the culture medium, with a concomitant increase in undissociated lactate, which is toxic to the cell. As a result, cell division is hampered and cell densities remain under one gram dry cell mass per litre. This corresponds to an optical density of less than one at 600 nm, when the dry cell weight factor [0.3 g/L at OD600 = 1] is used (Mierau et al. 2005c). The cell density can be increased by increasing the buffering capacity of the culture medium. Dry cell masses of up to 5 g/L and 10 g/L have been reached in optimized culture conditions when NaOH or NH₄OH has been added to the culture medium (Mierau et al. 2005c) or by using a more concentrated
medium (3 x M17 medium) (Oddone et al. 2007), respectively. However, the largest increase in biomass yield has been reached by removing growth inhibitory substances from the fermentation medium. With this method, a cell density up to 54 g/L has been reached with L. lactis (Madsen et al. 2006).

By optimizing culture conditions, including temperature, pH, medium composition, aeration, addition of heme and the concentration and time of inducer addition, the production yields of heterologous proteins have been significantly increased in large-scale applications (Mierau et al. 2005c; Oddone et al. 2007; Berlec et al. 2008). A production yield of 300 mg/L secreted protein was reached with L. lactis secreting Staphylococcus simulans biovar lysostaphin (Mierau et al. 2005b). Despite these improvements, the production yields with L. lactis have not reached the same levels as for aerobic bacteria, which can grow to cell densities above 100 g/L dry cell mass in conventional culture tanks and produce several grams of protein per litre culture (Riesenberg and Guthek 1999; Knoll et al. 2007). However, when therapeutic and prophylactic proteins are produced to be delivered to mucosal surfaces, a high production yield might not always be the main goal. Instead, a balance between the different stages along the protein production pathway becomes important to generate a system that induces an efficient and safe response in the host.

Contrary to conventional parental vaccines that stimulate only the systemic immune response, bacterial delivery of antigens to the mucosal surfaces can induce both systemic and mucosal immune responses (Villena et al. 2008b; Wells and Mercenier 2008). Vaccination with attenuated pathogens always includes a risk for infection especially in immunocompromised individuals. LAB (including L. lactis) present several advantages over attenuated pathogens including: 1) a long history of safe use in food and feed, 2) non-recombinant strains induce only low-level immune responses against themselves and 3) some species themselves induce improved resistance against pathogens.

Contrary to some other LAB used as mucosal vaccine vehicles, L. lactis does not colonize the gut, but passes through the human gut in less than a week (Klijn et al. 1995). However, comparative immunization studies with dead and live L. lactis producing the model antigen TTFC suggest that prolonged in situ antigen synthesis is not essential to induce a significant immune response (Robinson et al. 1997). If needed, the persistence and survival of L. lactis in the gastrointestinal tract can be increased by increasing the tolerance of the cell to gastric acid and bile (Termont et al. 2006; Watson et al. 2008).

### 1.1.2.2.3 Safety concerns

Safety concerns with recombinant L. lactis as mucosal delivery vehicles mainly include transfer of undesired genetic material (e.g. antibiotic resistance genes) to other bacteria and release of genetically modified DNA/organisms into the environment (Detmer and Glenting 2006). These problems can be minimized by using plasmid-free strains (inserting the antigen-encoding gene into the genomic DNA of the host strain), using strains without antibiotic selection markers, and by impeding survival of the recombinant bacterium outside the host (Steidler et al. 2003; Kim et al. 2010). Both active and passive containment systems have been used in bacteria (Steidler et al. 2003). For example, the L. lactis strain used in human clinical trials was engineered by inserting the hIL-10 gene in place of the thymidylate synthase (thyA) gene (Ross et al. 1990b), which is essential for DNA replication and survival outside the host gut (Ross et al 1990a; Steidler et al. 2003).
1.1.2.4 Bottlenecks
The production of heterologous proteins in bacteria meets several bottlenecks along the
pathway from gene transcription to secretion (Bolhuis et al. 1999; Li et al. 2004). Therefore,
in order to improve protein production, it is important to have a basic understanding of all
key parameters in the process: gene expression and peptide/protein targeting, secretion,
folding, anchoring and stability. Here, the bacterial protein production process will be
discussed with emphasis on research of *L. lactis* as a producer and carrier of antigens and
therapeutic molecules.

1.2 Gene expression

1.2.1 Preface
Transcription of a gene is initiated when the sigma factor part of the holoenzyme recognizes
and binds to specific promoter regions on the gene sequence. As a result, messenger RNA
(mRNA) is synthesized. Contrary to *E. coli* and *B. subtilis*, only one major sigma factor
has been identified from the genome sequences of *L. lactis* laboratory strains (Bolotin et al.
2001; Wegmann et al. 2007). This sigma factor, designated sigma$_{39}$ (Araya et al. 1993), is
a homolog to the housekeeping sigma$_{70}$ and sigma$_{43}$ of *E. coli* and *B. subtilis*,
respectively (Doi and Wang 1986).

In the conventional model for promoter recognition, sigma$_{70}$ and its homologues
bind to two conserved DNA regions, TATAAT and TTGACA, located at positions -10
and -35 upstream of the transcription initiation start point of the gene, respectively. This
Pribnow box is also well conserved in *L. lactis* promoters (Jensen and Hammer 1998). The
optimal number of bases between the 3’end of the -10 hexamer and the 5’end of the -35
hexamer in sigma$_{70}$ is 17 +/-1, whereas drastic alterations of the spacer length decrease its
activity (Dombroski et al. 1996). Promoter strength refers to the frequency of initiation of
transcription. A strong promoter includes the consensus hexamers and usually an optimal
number (17) of nucleotides between them, while a weak promoter has substitutions in the
consensus regions and/or differences in spacer length.

In some bacteria, transcription initiation is also possible without the -35 region. In *E.
coli* and *L. lactis*, the -35 region is compensated by a TGN motif that is located directly
upstream of the -10 hexamer (Kumar et al. 1993; Jensen and Hammer 1998). It has been
suggested that this simpler extended -10 promoter has evolved to the more complex bipartite
sigma$_{70}$-type promoter (Shultzaberger et al. 2007). Both promoters appear to be equally able
to stabilize the holoenzyme and initiate transcription (Shultzaberger et al. 2007).

1.2.2 *L. lactis* promoters
Several lactococcal promoter sequences have been isolated, characterized and made
available to establish *L. lactis* strains for the production of heterologous proteins. Before
whole *L. lactis* genome sequences were available, *L. lactis* promoters were mainly identified
by random screening of the genome (van der Vossen et al. 1985, 1987; Koivula et al. 1991;
Platteuw et al. 1994; Waterfield et al. 1995; Jeong et al. 2006). In this method, randomly cut
*L. lactis* chromosomal DNA fragments were inserted upstream of a promoterless reporter
gene in a promoter probe vector. Relative promoter strength was determined by measuring
the activity of the gene product. Both strong (P$_1$, P$_{21}$, P$_{23}$, P$_{59}$) and weak (P$_{32}$ and P$_{44}$) *L. lactis*
promoters have been isolated and characterized with this method (van der Vossen et al. 1987; Waterfield et al. 1995) and some of them have been used to produce heterologous proteins in \textit{L. lactis} (Table 1).

Other \textit{L. lactis} promoters have been identified from specific characterized \textit{L. lactis} genes. These include the promoters of \textit{usp45} (van Asseldonk et al. 1990) and \textit{nisA} (Kuipers et al. 1993), encoding Usp45 (unknown secretory protein) and nisin, respectively. Today, with the availability of whole genome sequences, new promoter sequences can easily be identified by screening documented genome sequences with bioanalytical methods. Promoters can also be selected from microarray data (Kim et al. 2009).

1.2.2.1 Constitutive gene expression
Constitutive expression of a gene means that the gene is not regulated in any way and that the expression is constant. Constitutive expression of the gene of interest is preferred in some applications, including \textit{in situ} production of a protein at mucosal surfaces in animals and humans and large-scale production of proteins, where the addition of an inducer increases production costs.

The constitutive lactococcal promoter \( P_1 \) (Waterfield et al. 1995) has been used to produce the model antigen TTFC and the cytokine IL-10 in immunization studies (Table 1). Other constitutive promoters used to produce heterologous proteins in \textit{L. lactis} include \( P_{32} \) and \( P_{55} \) (van der Vossen et al. 1987) (Table 1).

1.2.2.2 Controlled gene expression
Regulation of gene expression has evolved as a mechanism for bacteria to adapt to the environment in which they live. Examples of environmental factors that induce gene expression are changes in temperature or pH, phage attack, and the presence of specific sugars and chemicals in the growth medium.

In heterologous protein production, the inducibility of gene expression allows controlled expression of a gene, and expression can be switched on at a chosen time point of proliferation. This becomes especially important if the protein production process or the protein product causes stress or death to the cell. In addition, in cases where the produced protein is exported out of the cell, the balance between transcription, translocation and secretion can be more easily optimized with an inducible expression system.

1.2.2.2.1 The NICE system
The most commonly used inducible expression system in \textit{L. lactis} today is the nisin-controlled gene expression (NICE) system (Kuipers et al. 1995; de Ruyter et al. 1996b). The anti-microbial peptide nisin is a food-grade bacteriocin, and due to its broad host spectrum, it is widely used as a preservative in the food industry (for a recent review, see de Arauz et al. 2009). Nisin synthesis is encoded by a cluster of 11 genes, \textit{nisABTCIPRKFEG} (or \textit{nisZBTCIPRKFEG}) present in some \textit{L. lactis} strains (Siegers and Entian 1995; Ra et al. 1996). The first gene in the cluster, \textit{nisA}, encodes the peptide nisin, the \( B, C, T \), and \( P \) genes are involved in modification, translocation and processing of nisin, the \( I, F, E \) and \( G \) genes are involved in the bacterial host’s immunity against nisin while the \( R \) and \( K \) genes are involved in regulation and expression of the nisin gene cluster (for references see Mierau and Kleerebezem 2005). The gene cluster includes three promoters, \( P_{nisA} \), \( P_{nisF} \) and \( P_{nisK} \), \( P_{nisA} \) and \( P_{nisF} \) that drives the expression of \textit{nisABTCIP} and \textit{nisFEG}, respectively, are subject to
auto-regulation by nisin while $P_{nisR}$ drives constitutive transcription of the two regulator genes (de Ruyter et al. 1996a).

The NICE system utilizes the promoter of nisA and requires the presence of the genes nisR and nisK, which can be integrated into the genome or added to the strain on a plasmid (Figure 2). The transcriptional fusion of the gene of interest to the nisA promoter sequence allows transcription initiation by addition of sub-inhibitory levels of nisin to the culture medium. Today, the system includes several different host strains and plasmids constructed for various applications, including food-grade systems (Mierau and Kleerebezem 2005; Mierau et al. 2005a, 2005b; Zhou et al. 2006). A recent observation demonstrated that incorporation of nisI, the gene encoding NisI required for nisin immunity, into the NICE system improved production of a reporter protein by increased tolerance to the concentration of nisin for induction (Oddone et al. 2009b).

The NICE system has been used to study metabolic and enzyme functions, to produce prokaryotic and eukaryotic integral membrane proteins and toxic proteins and to over-produce, secrete and anchor both homologous and heterologous (plant, protozoan, viral, bacterial and mammalian) proteins in L. lactis (Table 1) and other bacterial hosts (Mierau and Kleerebezem 2005; Zhou et al. 2006). The system is easy to use at a laboratory scale and has potential in large-scale applications as well. A protein yield of up to 100 mg/L was achieved in the 3000-L scale and the yield was further increased to 300 mg/L in optimized culture conditions (Mierau et al. 2005a, 2005b).

![Figure 2](image-url). The nisin-controlled gene expression (NICE) system. Expression of the promoter (P) is induced in the presence of nisin in the culture medium, through the two-component signal transduction system, involving the proteins NisR and NisK. NisK, a histidine-protein kinase located in the cytoplasmic membrane, binds nisin, becomes autophosphorylated and transfers a phosphate group to NisR. The NisR response regulator located in the cytoplasm becomes activated and induces transcription of the gene of interest (gene X) from the nisA promoter (P). The resulting mRNA is translated into protein (not shown). Depending on the presence of a signal peptide, the protein product can be targeted to remain in the cytoplasm (dotted ovals) or secreted through the cytoplasmic membrane (striped ovals). The Figure is a reprint from Mierau and Kleerebezem 2005 with permission granted by Springer.
1.2.2.2 Other regulated *L. lactis* promoters

The promoter P170 of an uncharacterized gene *orfX* was originally isolated from *L. lactis* MG1363 (Israelsen et al. 1995). Transcription from P170 is induced by lactate, when pH in the culture medium decreases below 6, because of lactic acid accumulation at the transition from growth phase to stationary phase (Israelsen et al. 1995; Madsen et al. 1999). The qualities of self-inducibility and separation of protein production from the growth phase have made the P170 expression system an attractive alternative in up-scaled applications (e.g., industrial fermentations). Yields of up to 100 mg/L have been achieved with secreted *S. aureus* nuclease (Nuc) in up-scaled experiments (Ravn et al. 2000). The P170 promoter has been used to produce bacterial and viral antigens and allergens for delivery to mucosal surfaces (Table 1). Today, the *L. lactis* P170 expression system includes expression vectors and production hosts. It utilizes the lactococcal signal peptide 310mut2 for secretion of proteins (Ravn et al. 2000, 2003).

Other described controlled promoters are regulated by the extracellular concentration of ions, such as Cl\(^-\) (Sanders et al. 1998) and Zn\(^{2+}\) (Llull and Poquet 2004). In addition, a self-inducible expression system based on phosphate starvation was recently described (Sirén et al. 2008). However, these promoters have, thus far, found only limited use in heterologous protein production.

Early immunization studies with TTFC-producing *L. lactis* strains used the lactose-inducible promoter of the *L. lactis* lac operon in combination with the *E. coli* bacteriophage T7 promoter and T7 polymerase gene (Wells et al. 1993a; Wells et al. 1993b). However, this system was not food-grade and was soon replaced by the constitutive *L. lactis* P1 promoter (Robinson et al. 1997).

1.2.3 Heterologous promoters

In addition to native *L. lactis* promoters, several non-lactococcal promoters have been used to produce heterologous protein in *L. lactis*. These include promoters of genes encoding proteins of *Lactobacillus acidophilus* (P6, P15, P16, PslpA), *Lactobacillus bulgaricus* (PprtB), *Lactobacillus brevis* (PslpF), *Lactobacillus fermentum* (Psep), *Staphylococcus aureus* (Pnuc, Pstaf), *B. subtilis* (Ppsf) and *B. subtilis* bacteriophage (pSP02). For references and details on studies where these promoters have been used to produce heterologous proteins, see Table 1.

1.2.4 Synthetic promoters

An alternative and a rather convenient approach to find a suitable promoter for the production of a protein of interest is to make a library of synthetic promoters. Different methods to create synthetic promoter libraries in bacteria have been described (Santos and Stephanopoulos 2008). They all are based on the fact that promoter strength can be altered by randomizations of bases in the promoter sequence.

The first synthetic promoter library in *L. lactis* was created by Jensen and Hammer (Jensen and Hammer 1998). In this study, the promoter sequence was designed as an oligonucleotide, including consensus sequences of known *L. lactis* promoters surrounded by undefined spacer regions, and a region with homology to the target gene downstream of the promoter. Synthesis of the oligonucleotide resulted in a mixture of different promoter sequences that were introduced to express the gene of interest through a single cloning. When introduced into the production host, the gene of interest was expressed at different levels by the different promoters.
Synthetic promoter libraries have mainly been used for fine-tuning gene expression in metabolic optimization and metabolic control analysis. However, the method has great potential for finding an optimal promoter for constitutive expression of genes at heterologous protein production in *L. lactis* and other bacterial protein production hosts (Miksch et al. 2005; Hammer et al. 2006). Optimization of gene expression is especially important when produced proteins are exported and secreted at high levels to minimize accumulation and degradation of the produced protein due to misfolding and stress.

### 1.2.5 Codon optimization

The same amino acid may be preferentially encoded by different codons in different organisms. For example, bacteria with a high genomic GC content prefer codons with guanines (Gs) and cytosines (Cs), while bacteria with a low genomic GC content (including *L. lactis*) prefer codons with adenines (As) and thymines (Ts) (Fuglsang 2003). The codon bias is especially pronounced in highly expressed genes and expression efficiency is directly dependent on the abundance of tRNA, corresponding to codons in the gene. Therefore, if the codons of the heterologous gene differ considerably from the codon preferences of the bacterial host, translation is likely slow and prone to errors as a result of ribosomal stalling at positions requiring incorporation of amino acids corresponding to minor codon tRNAs. Consequently, the expression of a heterologous gene can be improved by changing its nucleotides so that they correspond to the natural codon usage of the host (Fuglsang 2003). For example, this was demonstrated in a recent experiment when the expression of 94 full-length human wild type and sequence-optimized genes in *E. coli* were compared (Maertens et al. 2010). The results indicated that codon optimization increased the level of mRNA and protein yields for 70% of the genes tested.

The availability of reliable and inexpensive computer programs for codon optimization and DNA synthesis services has made codon optimization an easily accessible method for increasing the expression of heterologous genes in bacteria. Among LAB, especially heterologous protein production in *L. lactis* has been suggested to benefit from this approach (Fuglsang 2003; Steidler et al. 2003; Vandenbroucke et al. 2004; Berlec et al. 2006; Glenting et al. 2007; Yeh et al. 2008; Zhang et al. 2010).

As an alternative method, the concentration of the rare tRNA needed for protein synthesis can be increased. For example, this strategy was successfully used in the production of strawberry flavour components in *L. lactis* by adding the tRNA<sup>Arg</sup> (AGG) gene to the cell in an independent plasmid under the control of the nisin inducible *nisF* promoter (Hernández et al. 2007). However, generally this method has not been as efficient as codon optimization (Maertens et al. 2010).

### 1.3 Protein secretion and targeting

#### 1.3.1 Preface

The ribosomally synthesized protein can in Gram-positive bacteria be targeted to remain in the cytoplasm or be secreted. Secreted proteins can either be anchored to the cell surface or released to the extracellular medium.

Typically, bacteria secrete 5-10% of the proteins encoded on their genome. From the genome sequence of *L. lactis* MG1363, 184 genes (7.5% of the total genome) have been predicted to encode secreted proteins (Wegmann et al. 2007). Most native secreted proteins
are essential for cell viability, including macromolecular hydrolases, which provide cells with simple nutrients, and other enzymes involved in cell-wall synthesis and cell division. Furthermore, many important virulence factors of pathogenic bacteria are secreted proteins.

Several pathways for protein export from the cytoplasm have been described in Gram-positive bacteria (Tjalsma et al. 2004). At least three of them are present in L. lactis: pathways using ATP-binding cassette (ABC) transporters, the twin-arginine translocation Tat pathway and the common (general, major) secretory (Sec) pathway (Bolotin et al. 2001, Wegmann et al. 2007). In Gram-positive bacteria, including L. lactis, majority of known proteins are secreted via the Sec pathway and detailed knowledge is available about the Sec pathway and its components in B. subtilis, the model for Gram-positive protein secretion studies (Tjalsma et al. 2004; Ling et al. 2007). Details regarding the L. lactis secretion machinery have mainly been extracted from comparing the genome and protein databases of L. lactis with those of B. subtilis and E. coli. Here, only the Sec pathway for protein secretion will be discussed.

1.3.2  Sec-dependent protein secretion
Secretion via the Sec pathway is divided into three stages (Tjalsma et al. 2000). The early stage involves the synthesis and elongation of the precursor protein (preprotein), transport of the preprotein to the cytoplasm membrane and anchoring of the preprotein to the membrane. The second part of the secretion pathway includes the translocation of the preprotein through the cytoplasm membrane by a preprotein translocase complex. In the late stage of protein secretion, the secreted protein attains its mature form through cleavage of the signal peptide and proper folding, followed by release from the cytoplasm membrane.

1.3.2.1  Sec-dependent signal peptides
Proteins secreted via the Sec pathway are synthesized as preproteins with a hydrophobic N-terminal extension, the signal peptide. The structure and function of the Gram-positive signal peptide has been covered in several reviews (e.g. Simonen and Palva 1993; Tjalsma et al. 2000; Tjalsma et al. 2004; Harwood and Cranenburgh 2008). In Gram-positive bacteria, Sec-type signal peptides are on average 28 amino acid residues long, but can vary between 19 and 44 amino acid residues. They have a specific structural organization, including a positively charged N-terminus (N-region), a hydrophobic core (H-region) and a short hydrophilic cleavage (C) region. Typically, the N-region contains two or three positively charged amino acids (arginine or lysine), which have been suggested to interact with the translocation machinery and the negatively charged phospholipids in the lipid bilayer of the membrane. The H-region is formed by a stretch of hydrophobic residues that can form an α-helical structure, which in the majority of the signal peptides is broken by a glycine residue to allow the formation of a hairpin-like structure that can insert into the membrane. Lastly, the hydrophilic C-region contains the consensus sequence A-X-A, a target site for type I signal peptidase at positions -3 to -1 relative to the cleavage site. Generally, the structure is well conserved, while the amino acid sequences differ between signal peptides.

The search for optimal signal peptides to increase secretion efficiencies of heterologous proteins has often been unsuccessful (Ravn et al. 2003; Brockmeier et al. 2006). The effect of a signal peptide on secretion efficiency varies depending on the protein to which it is fused, but there is still a lack of understanding of the interactions between the signal peptide and the mature protein. Therefore, the choice of a signal peptide for the secretion of a
specific heterologous protein should be made on a case-to-case basis (Brockmeier et al. 2006).

Several signal sequences have been isolated by screening L. lactis genomes for genes encoding exported proteins or by characterizing signal sequences of specific protein-encoding genes. At least two different approaches have been used by using the Staphylococcus aureus Nuc as a reporter protein. In the first approach, a shuttle vector was designed to identify genes encoding exported proteins as translational fusions to Nuc devoid of its own signal peptide (Poquet et al. 1998). In the second approach, a TnNuc transposon was transpositioned into the L. lactis chromosome to allow the generation of gene fusions in-frame with nuc (Ravn et al. 2000). As for promoters, the availability of whole-genome sequences and highly developed databases and computer programs simplifies the identification of signal peptides (Tjalsma et al. 2000; Brockmeier et al. 2006).

The by far most common signal peptide used in heterologous protein secretion in L. lactis is the signal peptide of the lactococcal Usp45 protein (SP Usp45) (van Asseldonk et al. 1993). It has been reported to be functional for the secretion of a variety of proteins (Table 1). In addition, several signal peptides of secreted proteins from non-lactococcal bacteria have been used to secrete foreign proteins in L. lactis. Some of the foreign proteins produced (e.g. surface proteins) are secreted with the help of their own signal peptide. Details about these and other L. lactis signal peptides that have been used are listed in Table 1.

Lipoproteins that have a lipoprotein signal peptide are also secreted through the Sec pathway. The lipoprotein signal peptide has a similar structural organization as the Sec-type signal peptide, but it is shorter and the C-region contains a consensus lipobox sequence L-(A/S)-(A/G)-C. After translocation, the signal sequence is cleaved off by a type II signal peptidase in front of the cysteine in the lipobox (Tjalsma et al. 2004; Venema et al. 2003).

1.3.2.2 Intracellular targeting
The newly synthesized protein has to remain in an unfolded state in the cytoplasm to be targeted to the translocation site. In E. coli, the majority of Sec-dependent nascent preproteins are stabilized and transported by the molecular chaperone SecB, while some preproteins with a very hydrophobic signal peptide and most inner membrane proteins utilize the signal recognition particle (SRP) -mediated pathway (de Keyzer et al. 2003). The lack of a SecB homologue in Gram-positive bacteria, including B. subtilis and L. lactis, suggests that the SRP-mediated pathway is the only recognized mechanism for intracellular recognition, chaperoning and targeting of Sec-dependent preproteins (Bolotin et al. 2001; Wegmann et al. 2007; Zanen et al. 2006; Harwood and Cranenburgh 2008).

In B. subtilis, the SRP complex consists of the small cytoplasmic RNA molecule and the proteins Ffh (fifty-four homologue) and HBsu (Harwood and Cranenburgh 2008). In the cytoplasm, the signal peptide of the nascent precursor protein is recognized by and bound to SRP, which maintains the nascent protein in an unfolded state and transports it to the translocase with the help of the docking protein FtsY (for references, see Tjalsma et al. 2004). Homologues to the highly conserved genes encoding FtsY and Ffh have also been recognized from the genome sequence of L. lactis, suggesting that the same targeting system functions in L. lactis (Bolotin et al. 2001).

Recent sequence analysis of streptococcal and staphylococcal signal peptides suggested that some cell wall-anchored proteins are directed to and secreted at specific locations of the cell (Carlsson et al. 2006; DeDent et al. 2008). Surface proteins that carry an YSIRK/
GS in the signal peptide are directed to the cross wall, the peptidoglycan layer that forms during cell division to separate new daughter cells. These proteins are distributed as a ring surrounding the cell. Surface proteins without the YSIRK/GS motif are directed to the cell pole and display at discrete assembly sites (DeDent et al. 2008).

1.3.2.3 Intracellular quality control
Even under normal conditions, abnormal and misfolded proteins are formed inside the cell. Environmental factors including heat, pH and oxidative stress as well as overproduction of foreign proteins further increase the formation of aberrant proteins. Cells have evolved mainly two strategies to avoid the accumulation of these proteins in the cytoplasm: molecular chaperones and proteases.

Proteins that are destined to stay in the cytosol or exported by e.g. the Tat pathway are folded into their active conformation inside the cell. Comparison of E. coli and B. subtilis genomes reveals that the intracellular chaperone systems GroEL/GroES and DnaK/DnaJ/GrpE are present in both of them. However, deletion and knock out studies have suggested that the systems function differently in different bacteria (Molière and Turgay 2009). The systems have been best studied in E. coli (Baneyx and Mujacic 2004; Sabate et al. 2010). In E. coli, the ribosome-associated trigger factor (TF) is the first chaperone to interact with and hold newly synthesized peptide chains in an unfolded state when they emerge from the ribosome. The action of TF is partially overlapped with the action of the DnaK/DnaJ/GrpE chaperone complex. In addition, the DnaK/DnaJ/GrpE complex assists folding, prevents misfolding and has the ability to repair protein damage. Successfully folded proteins are released into the cytosol while others are transferred to the large cylindrical chaperonin GroEL/GroES complex. The central cavity of the GroEL/GroES complex provides a physically defined compartment inside which the protein or a protein domain can fold without being affected by other components in the cytosol (Sabate et al. 2010).

Aberrant and/or aggregated proteins are degraded by proteases to avoid accumulation and cell death. In low GC Gram-positive bacteria, the Clp protease complex is the major housekeeping proteolytic system, responsible for intracellular quality control in both non-stress and stress conditions (Frees et al. 2007). The proteolytic complex consists of a central proteolytic core consisting of 14 ClpP subunits arranged in two heptameric rings associated with one or two hexameric rings of Clp ATPases (Frees et al. 2007).

The Clp ATPases, designated ClpB, ClpC, ClpE, ClpL and ClpX, recognize aberrantly folded and aggregated proteins, dissolve the aggregates formed, unfold the proteins and transfer them into the ClpP chamber, where they are degraded (Kirstein et al. 2009). The expression of clpP is known to increase in different stress conditions that induce production of aberrant proteins (Frees et al. 2007). However, in B. subtilis it was shown that 20-30 % of newly synthesized native proteins aggregated in the cytoplasm of a clpP mutant even under non-stress conditions, underlining the importance for continuous quality control by the ClpP complex (Kock et al. 2004).

The intracellular chaperone complexes (DnaK/DnaJ and GroEL/GroES but not TF) and components in the Clp protease complex have also been identified in L. lactis, and the effects of clpP inactivation on housekeeping proteolysis have been studied. Production of misfolded proteins by heat-stress and puromycin treatment resulted in increased sensitivity to heat and puromycin and the accumulation of puromycyl polypeptides in the cytoplasm of the clpP mutant (Nilsson et al. 1994; Frees and Ingmer 1999). However, in heterologous protein production studies, neither mutational inactivation of clpP nor silencing of clpP by
antisense RNA inhibition had any effects on protein production (Cortes-Perez et al. 2006; Oddone et al. 2009a).

1.3.2.4 Translocation

1.3.2.4.1 The translocation complex
The next step in the Sec-dependent secretion process is the translocation of the preprotein through the translocase. The Sec-dependent translocase complex (Figure 3) is an integral membrane pore through which the nascent protein is exported. In *E. coli* and *B. subtilis*, three proteins, SecY, SecE and SecG, form the core of the translocation channel (for a review, see de Keyzer et al. 2003). This heterotrimeric complex interacts with a fourth protein, SecA, which acts as a motor for protein translocation by ATP binding and hydrolysis. Besides the Sec components, the translocase includes a number of accessory proteins. Comparison of the protein database of *L. lactis* IL1403 with those of *E. coli* and *B. subtilis* secretomes revealed that the essential Sec components of the translocation machinery (SecA and SecYEG) are present in *L. lactis* (Bolotin et al. 2001). However, *E. coli* and *B. subtilis* also have a second heterotrimeric protein complex including SecD and SecF, which does not have any homologues in *L. lactis*. In *E. coli*, SecDF appears to be important for SecA cycling, maintaining the forward momentum of the preprotein and its release on the trans side of the membrane (Nouwen and Driessen 2005). In *B. subtilis*, inactivation of *secDF* under conditions of hyperproduction of AmyQ resulted in slower precursor processing and accumulation of the precursor protein inside the cell, suggesting that SecDF plays a role in late secretion steps and is required for efficient secretion in *B. subtilis* (Bolhuis et al. 1998).

Complementation of the Sec-dependent secretion machinery of *L. lactis* with SecDF of *B. subtilis* has been studied (Nouaille et al. 2006). Two forms of the *S. aureus* Nuc protein, NucB and NucT, which are known to be efficiently and poorly secreted in *L. lactis*, respectively (Le Loir et al. 2001), were used as reporters. The presence of SecDF had no effect on the production of NucB. However, the total amount of NucT increased significantly with a 10-fold increase in secreted protein and an even larger increase in the accumulation of precursor in the cell fraction, suggesting that SecDF did not increase secretion efficiency. The presence of SecDF also increased the accumulation of *Brucella abortus* L7/L12 antigen precursor in *L. lactis*, but had no effect on secretion of this protein (Nouaille et al. 2006). The authors concluded that SecDF is involved in stabilization of the precursor in the cytoplasm in *L. lactis*.

Immunolabelling of components in the Sec translocation machinery together with light and electron microscopy techniques have suggested that *E. coli* and *B. subtilis* have several translocons that are organized in a spiral-like fashion along the cell membrane (Shiomi et al. 2006; Campo et al. 2004). Similar studies on the coccoid bacteria *Streptococcus pyogenes* and *Enterococcus faecalis* (both closely related to *L. lactis*) have suggested that Sec-dependent proteins are secreted via a single microdomain in the cytoplasmic membrane called the ExPortal, which is located at the septum (Rosch and Caparon 2004; 2005; Kline et al. 2009). However, in the case of *S. pyogenes*, the results do not correspond to findings in a later study on signal peptide directed localized secretion, which suggests that Sec-dependent proteins are secreted either at the cell pole or at the cross wall (Carlsson et al. 2006).
Figure 3. Components involved in Sec-dependent protein export in *B. subtilis*. Secretory proteins are ribosomally synthesized as precursor proteins with an N-terminal signal peptide (SP). Cytoplasmic chaperones, such as SRP/FtsY, keep the precursors in a translocation-competent state and facilitate their targeting to the translocase in the membrane, consisting of SecA, SecY, SecE, SecG, and SecDF (lacking in *L. lactis*). During or shortly after translocation, the preprotein is cleaved by one of the type I signal peptidases (here SipS-W; SipL in *L. lactis*) or lipid modified by the diacylglycerol-transferase (Lgt) and cleaved by the lipoprotein-specific signal peptidase (Lsp). SppA and TepA may be involved in the degradation of cleaved signal peptides, whereas the folding of several secreted proteins depends on the activities of PrsA, BdbBC, and/or SpoIIIJ/YqjG. Extracellular proteases (HtrA, HtrB, and WprA) are involved in the quality control of secretory proteins. It should be noted that for reasons of simplicity, HtrAB are depicted in the cell wall, although HtrA is detected in both the membrane and the medium. On passage through the cell wall, the mature protein is released into the environment. The Figure is a reprint from Tjalsma et al 2004 with permission granted by the American Society for Microbiology. The Figure text is modified from the original Figure.
1.3.2.4.2 The translocation process

The translocation process has been described in several reviews (e.g. Tjalsma et al. 2000; 2004). In the first steps of translocation, SecA binds to the SecYEG core in the cytoplasmic membrane. The arriving precursor-SRP complex binds to SecA and the preprotein is transferred to SecA, followed by translocation of a short stretch of the preprotein into the SecYEG pore. Next, SecA hydrolyses ATP and the preprotein is released from the SRP complex. Repeated cycling of SecA through ATP-binding and hydrolysis drives the translocation of the rest of the preprotein. At the membrane, the positively charged N-region of the signal peptide interacts with negatively charged phospholipids in the membrane, leading to a looping insertion of the H-region. After the H-region unloops, the first part of the preprotein is pulled through the membrane. During or shortly after translocation, the signal peptide is cleaved off by type I signal peptidases at the consensus cleavage site A-X-A and the mature protein is released from the translocase.

Contrary to five type I signal peptidases produced in *B. subtilis* (Tjalsma et al. 2004), *L. lactis* has only one enzyme, SipL, for the processing of secretory preproteins (Bolotin et al. 2001). This might compromise maturation of proteins and secretion efficiency in *L. lactis*. However, overproduction of SipL did not improve the secretion of NucT in *L. lactis* (Morello et al. 2008).

1.3.2.5 Post-translocational folding in the microenvironment of the cell envelope

After translocation across the cytoplasmic membrane, the signal peptidase-processed protein enters the last and perhaps the most challenging step in the secretion process – folding into its authentic structural configuration. Outside the membrane, the unfolded (or loosely folded) proteins are encountered in the microenvironment of the Gram-positive cell wall. Normally, native proteins fold rapidly into their proteolysis-safe conformation, assisted by extracellular folding factors and chaperons. However, the microenvironment of the cell envelope might be unfamiliar to heterologous proteins and result in compromised folding, followed by degradation by housekeeping proteases (Harwood and Cranenburgh 2008).

1.3.2.5.1 Main components of the Gram-positive cell wall

The structure of the Gram-positive cell wall constitutes a thick layer of peptidoglycan, teichoic acids and wall-associated proteins, all of which contribute to the properties of the cell wall. Together the components of the cell envelope function as a complex anionic matrix which controls metal cation homeostasis and assists in trafficking of metal cations, nutrients, proteins and antibiotics (Neuhaus and Baddiley 2003). In addition, the cell wall matrix functions as a platform for cell wall-anchored proteins and adhesins.

Peptidoglycan, the major structural polymer of the cell wall is made up of glycan strands of repeating disaccharide residues that are cross-linked via peptide side chains. A coiled-coil model for cell wall peptidoglycan architecture in *B. subtilis* was recently described (Hayhurst et al. 2008). The peptidoglycan mesh, also called the murein succulus, functions as an exoskeleton responsible for cell shape and prevents the cell from bursting due to osmotic pressure. Constant peptidoglycan synthesis at the cross wall is crucial for cell growth and is a target for many antibiotics.

Teichoic acids (TAs) are the other main constituent of the cell wall. The structure of TAs can differ, varying between species and even between strains of the same species. The backbone is a polymer of glycerol phosphate units either covalently linked to the
peptidoglycan (wall teichoic acid, WTA) or anchored to the membrane via a glycolipid (lipoteichoic acid, LTA).

TAs extend through the peptidoglycan to the cell surface, and their hydrophilic nature has a strong impact on the physiochemical properties of the cell surface. Due to the presence of phosphate groups in TAs, they have a negative charge and ion exchanger-like properties. The negative net charge of the cell wall attracts cations, especially Ca²⁺, Mg²⁺ and Fe³⁺, which are important for the folding of some secreted proteins (Thwaite et al. 2002; Harwood and Cranenburgh 2008).

1.3.2.5.2 D-alanylation of teichoic acids
The negative net charge of the cell wall is mainly controlled by D-alanylation of TAs. Protonated D-alanyl ester residues link covalently to the negatively charged polyglycerophosphate chains, resulting in a decrease in negative charge and a concomitant decrease in the capacity to bind cations in the cell wall (Neuhaus and Baddiley 2003).

In Gram-positive bacteria, inactivation of genes in the dlt operon, encoding proteins required for D-alanylation of TAs, resulted in a variety of phenotypes that differ in between species (Neuhaus and Baddiley 2003). Observed phenotypes include increased sensitivity to bacteriocins and antibiotics, lower growth rate, defective cell separation and loss of acid tolerance. In L. lactis, inactivation of dltD resulted in decreased growth rate, increased UV sensitivity and increased bacterial chain length (Duwat et al. 1997). In addition, an increased sensitivity to nisin and lysozyme was observed in the dltD mutant, whereas increased D-alanylation resulted in increased resistance towards these cationic antimicrobials (Giaouris et al. 2008).

The effect of dlt inactivation on heterologous protein production has been intensely studied in B. subtilis. In this species, the lack of D-alanylation resulted in an increase in the concentration of cations in the cell wall environment, which in turn increased the secretion yield of some proteins and decreased the secretion yield of others (probably those dependent on divalent cations for proper folding) (Hyyryläinen 2000; Thwaite et al. 2002; Vitikainen et al. 2005). In L. lactis, the secretion of only one protein has been studied in the dlt mutant background. The inactivation of dltA had no effect on the secretion efficiency or yield of NucT (Nouaille et al. 2004).

1.3.2.5.3 Extracellular folding factors
Native secreted proteins have evolved with the host and adapted to the qualities of the host and its habitat. Therefore, they normally fold rapidly into their mature/active conformation assisted by folding factors/chaperones. At least three types of folding factors have been identified in Gram-positive bacteria: intramolecular chaperones, peptidyl prolyl cis-trans isomerasers, and the earlier-mentioned metal cations.

Intramolecular chaperones are usually 60 to 200 amino acid residues long propeptides encoded in the primary sequence of the protein and located at either the N-terminus or C-terminus of the peptide sequence. Propeptides do not contribute to protein function. Instead, they are thought to accelerate folding of the protein by stabilizing an intermediate folding state during the folding process. Proteins with propeptides are often proteases that attain their full activity only after the propeptide is cleaved and degraded after the protein has attained its authentic conformation (Chen and Inouye 2008; Harwood and Cranenburgh
Several bacterial proteins with propeptides have been identified (Eder and Fersht 1995; Shinde and Inouye 2000). Examples in LAB and related bacteria are cell-envelope proteinases including PrtP of *L. lactis* (for references, see Siezen 1999). In Gram-positive bacteria, propeptides shorter than 60 residues are found in some secreted enzymes including the α-amylose AmyE of *B. subtilis* (Takase et al. 1988) and nuclease Nuc of *S. aureus* (Davis et al. 1977).

Both non-lactococcal and synthetic propeptides have been used to improve the yields of secreted heterologous proteins in *L. lactis*. The *S. aureus* Nuc protein, which has been widely used as a reporter protein for secretion studies in *L. lactis*, includes a 21-residue N-terminal propeptide (Davis et al. 1977; Le Loir et al. 1998). Cleavage of the propeptide by the extracellular housekeeping protease HtrA resulted in an enzymatically active nuclease. Deletion of the propeptide encoding gene fragment decreases secretion efficiency of Nuc in *L. lactis* (Le Loir et al. 1998). However, the secretion efficiency can be restored by replacing the Nuc propeptide with a synthetic sequence that is acidic or neutral (Le Loir et al. 1998, 2001). The synthetic propeptide LEISSTCDA has been used in combination with both native and non-native lactococcal signal peptides (e.g. SP_	ext{Usp45}, SP_	ext{Nuc}) to improve secretion efficiency of several heterologous proteins in *L. lactis* (Le Loir et al. 1998, 2001, 2005; Ribeiro et al. 2002). Unlike native propeptides, LEISSTCDA is not cleaved off, but remains fused to the mature protein. The function of the synthetic propeptide in heterologous protein production is unclear, but could be addressed to improved charge balance around the signal peptide cleavage site and/or conformation of the precursor (Le Loir et al. 2001).

**Peptidyl prolyl cis-trans isomerases** (PPIase) are proteins that increase the rate of folding of proteins through cis/trans isomerisation of peptidyl-proline bonds. The lipoprotein PrsA of *B. subtilis* is a PPIase, and the function of *B. subtilis* PrsA on heterologous protein production has been extensively studied. In this host, PrsA is vital for cell growth, which makes it impossible to study the effects of *prsA* deletion (Kontinen and Sarvas 1993; Vitikainen et al. 2001). However, the reduced production of active PrsA, by approximately 10-fold compared with the wild type strain, was made possible by a single mutation in the C-terminus of PrsA (Kontinen and Sarvas 1988). In this PrsA mutant, the secretion of PrsA-dependent proteins was reduced by up to 98%, compared with the wild type, when expressed at a high level (Kontinen and Sarvas 1988; Jacobs et al. 1993). This decrease in yield depended on an increase in the post-translocational degradation of PrsA-dependent proteins because of misfolding (Jacobs et al. 1993). A comparative study on protoplasts, made from wild type and PrsA depleted cells, indicated that post-translocational folding, stability and secretion of the normally PrsA-dependent protein AmyQ were independent of PrsA when the cell wall was lacking (Wahlström et al. 2003). Summarized, the results from research on *B. subtilis* suggest that PrsA functions as a cell-associated extracellular folding chaperone/foldase that protects its substrate proteins from degradation by preventing unfavourable interactions of the protein with components in the microenvironment of the cytoplasmic membrane-cell wall interface (Wahlström et al. 2003; Colomer-Pallas et al. 2004).

In *L. lactis* IL1403, at least ten genes encoding proteins involved in protein modification have been identified (Bolotin et al. 2001). These include two uncharacterized PPIase protein-encoding genes, designated *ppiA* and *ppiB*, and the *pmpA* (for protein maturation protein A) gene encoding a PrsA-like protein. Like PrsA, PmpA is a lipoprotein,
but it lacks the typical PPlase motif (Drouault et al. 2002). Still, it has been suggested that PmpA has foldase activity because a slight (< twofold) overproduction of PmpA decreased degradation and improved secretion of a model protein known to fold incorrectly in the corresponding L. lactis wild type strain (Drouault et al. 2002a). Contrary to the importance of PrsA for B. subtilis survival, the inactivation of pmpA demonstrated that PmpA is not required for cell vitality in L. lactis.

1.3.2.5.4 Extracellular quality control
Proteins that fail to fold into their mature active configuration are susceptible to degradation by housekeeping proteases in order to avoid aggregation and accumulation of proteins at the translocation sites and/or cell wall growth sites that are vital to the cell. However, the housekeeping property of degradation can be a problem in heterologous protein production (Bolhuis et al. 1999; Li et al. 2004).

Contrary to B. subtilis, which secretes several extracellular proteases (Simonen and Palva 1993; Stephenson and Harwood 1998), only one housekeeping protease, HtrA, has been identified in L. lactis (Poquet et al. 2000; Bolotin et al. 2001). While HtrA belongs to a family of proteases present in many bacterial species, the function of HtrA seems to differ between species. The L. lactis HtrA is a membrane-anchored protein with large serine protease domains exposed to the cell surface (Poquet et al. 2000), and its production increases when the cell is stressed by heat and puromycin treatment (Foucaud-Scheunemann and Poquet 2003). Studies on an htrA mutant strain producing a truncated HtrA, which lack its catalytic site, suggested that L. lactis HtrA is involved in proteolysis of secreted aberrant proteins, maturation of native proteins and processing of propeptides (Poquet et al. 2000). While the htrA mutant was fully viable under normal laboratory growth conditions (30°C), the lack of HtrA resulted in increased chain lengths and greater sedimentation in liquid growth medium, probably due to incomplete processing/maturation of the autolysin AcmA (Foucaud-Scheunemann and Poquet 2003). Still, htrA mutant L. lactis strains have proved to be promising hosts for the production of several secreted heterologous proteins (Morello et al. 2008). Suppression of the acid tolerance response (through an increase in phosphate level and buffering capacity in the culture medium in wild-type recombinant cultures) was recently introduced as a method to circumvent the negative effects of htrA deletion (Sriraman and Jayaraman 2008).

1.4 Anchoring of proteins to the bacterial surface

1.4.1 Preface
Many native secreted proteins in bacteria are anchored to the outer surface of the cell. These proteins are fundamental to biological processes, including surface adherence, colonization, immunoreactions, signal transduction and cell-cell interactions (Samuelson et al. 2002).

From the genome sequence of L. lactis MG1363, it has been predicted that 184 genes encode secreted proteins (Wegmann et al. 2007). Of these, 39 genes encode a protein with a typical lipoprotein membrane-anchoring domain, while 38 genes encode proteins with domains that mediate either covalent (n=9) or noncovalent (n=29) binding to the cell wall. The rest of the proteins either are secreted to the extracellular medium or bind to the cell surface through a transmembrane-spanning (TMS) domain.
Research on different anchoring methods to display heterologous proteins on the surface of bacteria has increased, with the growing interest in using bacteria for different applications, including biocatalysis, biosensor technology, bioremediation, selection technologies and vaccine development (Samuelson et al. 2002). In Gram-positive bacteria, the absence of an outer membrane allows proteins to be anchored either to the cytoplasmic membrane, the cell wall matrix or as part of the surface layer (S-layer) (Leenhouts et al. 1999). Different anchoring modes used in heterologous protein production in LAB are presented in Figure 4.

Figure 4. Modes of anchoring of chimeric proteins to the cell surface of LAB. The light grey areas represent a heterologous (poly) peptide. The darker grey regions are the different types of anchoring domains. 1: transmembrane anchor, 2: lipoprotein anchor, 3: LPXTG-type cell-wall anchor, 4: AcmA-repeats cell-wall binding, 5: surface-layer-protein attachment. The black lines extending from anchors 2 and 3 represent the covalent bond between the anchors and the lipid bilayer and the peptide crossbridge that connect the peptidoglycan layers, respectively. Components of the cell surface such as (lipo)teichoic acids and oligosaccharides are not shown. The Figure is a reprint from Leenhouts et al. 1999 with permission granted by Springer.

1.4.2 Membrane anchors
Membrane binding anchors link the protein to the cell membrane with either a TMS domain or a lipoprotein-anchoring domain. As the name indicates, the transmembrane anchor spans the cell membrane, while the lipoprotein anchor binds to the lipid bilayer (for references, see Tjalsma et al. 2004). Only a few membrane protein anchors have been used in heterologous protein production in *L. lactis*, but no surface accessibility has been described (Leenhouts et al. 1999).

1.4.3 Cell wall anchors

1.4.3.1 Covalent cell wall binding
Typically, proteins that bind covalently to the cell wall include an N-terminal signal peptide for secretion and a C-terminal cell wall-anchoring (CWA) motif. The CWA of *Staphylococcus aureus* Protein A (SPA) has often been used as a model system to study covalent surface binding of Gram-positive bacteria. The consensus CWA consists of three domains: a conserved LPXTG sequence followed by a hydrophobic part and a short positively charged tail (Fischetti et al. 1990). All parts of the CWA are important
for efficient sorting of the protein to the cell wall. Deletion of the hydrophobic part or the charged tail causes secretion of the protein to the extracellular medium, whereas deletion of the LPXTG domain retains the protein in the membrane (Schneewind et al. 1992, 1993).

At the translocation complex, newly translocated peptides are scanned by sortases, specific cell wall-anchored enzymes that recognize the LPXTG motif and catalyse the covalent binding between LPXTG and peptidoglycan in the cell wall (for references, see Ton-That et al. 2004). Genes encoding two sortase homologues, SrtA and SrtB, have been identified in the genome sequence of L. lactis (Bolotin 2001; Wehmann et al. 2007).

Translational fusion of the CWA motif to the C-terminus of a heterologous protein containing an N-terminal signal peptide allows secretion and anchoring of the hybrid protein to the cell wall (Steidler et al. 1998b). Covalent cell wall binding is one of the most commonly used binding types when heterologous proteins are anchored to the surface of L. lactis. Especially, the CWAs of the Streptococcus pyogenes M6 protein (M6) (Piard et al. 1997) and S. aureus Protein A (SPA) (Steidler et al. 1998) have been applied (Table 1).

1.4.3.2 Non-covalent cell wall binding
Non-covalent cell wall binding with the lactococcal AcmA-repeats anchor has also been used to anchor heterologous protein on the surface of L. lactis (Table 1). AcmA, the major autolysin of L. lactis is responsible for cell separation and lysis of cells in the stationary phase (Buist et al. 1995). It consists of an active domain, followed by a C-terminal region containing three highly homologous repeats of 45 amino acids each, also called lysine motif (LysM) domains because they were originally identified in bacterial lysins (Buist et al. 1995). The LysM domains bind to peptidoglycan with high specificity and affinity, but the molecular mechanisms of interaction of LysM and the peptidoglycan have not been characterized (Andre et al. 2008). Proteins with an AcmA anchor are detected in the culture medium as well as on the bacterial surface, suggesting an interaction of the LysM attachment region with the cell wall that is weaker than that of the LPXTG type anchor. Indeed, it has been demonstrated that the binding of LysM to peptidoglycan on L. lactis cells is partly hindered by cell wall components (Steen et al. 2003; Andre et al. 2008). The LysM motifs of proteins from other LAB have also been used to display heterologous proteins on the surface of L. lactis (Turner et al. 2004; Hu et al. 2010).

The AcmA cell wall anchor has also been used to bind recombinant proteins to non-living L. lactis cells (Bosma et al. 2006). These non-living L. lactis particles called Gram-positive Enhancer Matrix (GEM) were generated by trichloroacetic acid treatment and have been used to deliver externally added antigens to mucosal surfaces (Ramasamy et al. 2006; Audouy et al. 2007; Ramirez et al. 2010).

1.5 Examples of other strategies to enhance heterologous protein production

1.5.1 Stabilization of messenger RNA
The stability of mRNA has an impact on the protein yield. In recombinant systems, the degradation of mRNA by cytoplasmic ribonucleases (RNases) can be minimized by using ribosome-binding sites (RBS) that ensure efficient binding of the host ribosome to mRNA. By doing this, both mRNA stabilisation and efficient translation initiation, with concomitant increase in protein production can be achieved (van de Guchte et al 1991; Jana and Deb 2005; Sørensen and Mortensen 2005).
The fusion of the promoter to a 5′-untranslated leader sequence (UTLS), including a 5′-stem-loop structure and an RBS, can increase heterologous protein production by stabilizing mRNA after transcription. The presence of an UTLS has been reported to stabilize mRNA in *B. subtilis*, *E. coli* and LAB (Boot et al. 1999; Daguer et al. 2005; Komarova et al. 2005; Narita et al. 2006). In *L. lactis*, the UTLS of the *Lactobacillus acidophilus* *slpA* gene was fused to the promoters of *Lactobacillus casei* *ldh* and *L. lactis* *clpC* to produce a reporter protein, but only the fusion promoter P<sub>clpC</sub>-UTLS resulted in an increase in protein production (Narita et al. 2006). Messenger RNA can be further stabilized by placing a transcription terminator sequence that forms a stem loop downstream of the protein-encoding gene (Jana and Deb 2005; Sørensen and Mortensen 2005). The transcription terminator also contributes to plasmid stability by preventing transcription through the origin of replication.

### 1.5.2 Fusion protein technology

A commonly used method to improve the production of heterologous proteins in bacteria is to fuse the protein to a known stable partner protein to protect the protein from intracellular proteolysis and/or enhance solubility (Sørensen and Mortensen 2005). The Nuc protein of *S. aureus* is commonly used to stabilize heterologous proteins in *L. lactis* due to its stability (Bermúdez-Humarán et al. 2002; Chatel et al. 2003; Dieye et al. 2003; Adel-Patient et al. 2005; Nouaille et al. 2005). It is a small, non-toxic, intracellularly inactive protein with a simple monomeric structure lacking disulphide bonds (Poquet et al. 1998). In addition, the codon usage in *nuc* has been considered appropriate for high-level expression in *L. lactis* (Ravn et al. 2000).

### 1.6 *Escherichia coli* post-weaning diarrhoea and oedema disease in swine

#### 1.6.1 Preface

Post-weaning diarrhoea (PWD) and oedema disease (OD) are responsible for economic losses on pig farms due to mortality, morbidity, decreased growth rate and increased cost of treatment of production animals in Finland and worldwide (Fairbrother et al. 2005). These diseases are caused primarily by fimbriated *E. coli* strains that either produce enterotoxins (ETEC), Shiga toxins (Stx2e) (VTEC) or both (VTEC/ETEC). While enterotoxins cause diarrhoea, shigatoxins cause OD. Today, these serious diseases are mainly treated with antibiotics, which include risks of bacterial drug-resistance and residues in food products made from the production animals. Several attempts have been made to produce safe and functional vaccines against porcine PWD and OD causing *E. coli* (Fairbrother et al. 2005), but to date no effective commercial vaccines are available.

*E. coli* strains that cause porcine PWD and OD share as a common virulence factor F18-type fimbriae that adhere to specific receptors on intestinal epithelial cells and specifically or non-specifically to the mucus coating the epithelium.

#### 1.6.2 F18 fimbriae

F18 fimbriae are 1-2 μm long flexible protein filaments with a diameter of 3-7 nm that occur as two different antigenic variants, F18ab and F18ac (Rippinger et al. 1995). Generally, F18ab fimbriae are more frequently found on VTEC and VTEC/ETEC strains, while the F18ac variant is found more frequently on ETEC strains (Nagy et al. 1997; Fekete et
The biosynthesis is F18 fimbriae is encoded by the fedABCEF (fimbriae associated with edema disease) gene cluster located on a plasmid, with fedA encoding the major structural protein, fedB the outer membrane usher, fedC the periplasmic chaperone, fedE the minor linker protein and fedF the adhesin (Imberechts et al. 1992; Imberechts 1996; Smeds et al. 2001). The minor subunit protein FedF, located on the tip of the fimbriae, mediates the adhesion of the fimbriae to specific receptors (see below 1.6.4) on epithelial cells in the porcine intestine (Imberechts et al. 1996; Smeds et al. 2001). Deletion studies have indicated that the adhesive part of FedF is located between amino acid residues 60 and 109 of the mature protein (Smeds et al. 2003). Furthermore, FedF is highly conserved among F18-positive E. coli strains in Finland and worldwide (Smeds et al. 2003; Tiels et al. 2005), which makes FedF a good target for vaccine development, as immunity induced with one form will cross-react with many others, broadening the range of efficacy of the vaccine.

1.6.3 Vaccination against F18-positive E. coli infection

Both passive and active oral immunization studies against F18-fimbriated E. coli have been performed. Passive immunization with egg yolk antibodies, raised against F18 fimbriae, reduced excretion of challenge bacteria and protected experimentally challenged pigs from diarrhoea and death (Imberechts et al. 1997b; Yokoyama et al. 1997). However, the production of egg yolk antibodies is relatively expensive, and thus, is not a practical choice for pig farms.

An ideal way to prevent infection would be to block attachment of the pathogen to mucosal surfaces by inducing adhesin-specific antibody production at the mucosal site and/or directly exclude binding to the receptor site (Wizemann et al. 1999; van Ginkel et al. 2000). Active oral immunization of pigs with isolated F18 fimbriae resulted in the production of antibodies mainly towards the major structural protein FedA, and did not protect pigs from challenge with the pathogenic strain (Verdonck et al. 2007). Studies on type 1 fimbriae of uropathogenic E. coli have indicated that presenting the immune system exclusively the adhesin subunit FimH resulted in specific antibody production against FimH that effectively blocked attachment of the pathogen to its receptor (Langermann et al. 1997, 2000). In vitro studies have indicated that a MBP-FedF fusion protein and FedF-specific antibodies can block binding of F18-positive E. coli to isolated porcine intestinal epithelial cells (Smeds et al. 2001, 2003). Furthermore, by fusing FedF to F4 fimbriae a weak FedF specific immune response was reported (Tiels et al. 2008). However, no complete protection against F18-positive E. coli challenge could be observed (Tiels et al. 2008).

E. coli strains with F4 fimbriae cause neonatal diarrhoea in pigs. Contrary to F18 fimbriae, isolated F4 fimbriae administered by the oral route protect pigs from challenge infection with F4 positive ETEC (van den Broech et al. 1999). Several possible reasons for the differences between the immunogenicity of F4 and F18 fimbriae has been indicated. They include: 1) the adhesin of F18 fimbriae is a minor subunit protein located only at the tip of the fimbrial filament (Imberechts et al. 1992; Smeds et al. 2001), whereas the adhesin of F4 fimbriae is the major structural protein present in 100 to 1000 subunits, 2) binding of fimbriae to its receptor on enterocytes results in endocytosis and transcytosis of F4 fimbriae but not F18 fimbriae, 3) F4 fimbriae induce maturation of dendritic cells (for references, see Cox et al. 2010).
1.6.4 The F18 fimbriae receptor

F18-positive *E. coli* utilize blood group determinants as receptors to mediate adhesion and initiate colonization at the host epithelium (Coddens *et al.* 2009). On porcine enterocytes, the FedF adhesin selectively interact with glycosphingolipids having blood group H or A type 1 determinants in pigs with blood group O or A, respectively (Coddens *et al.* 2009).

It has been well established that F18-positive *E. coli* does not cause disease in pigs less than three weeks of age (Nagy *et al.* 1992; Coddens *et al.* 2007). The reason for this age-dependent infection has been unclear, and traditionally neonatal pigs were thought to lack a functional F18 receptor. Nevertheless, a recent study suggested that F18-binding glycosphingolipids are present in both neonatal and adult pigs, but the ability of the glycosphingolipids to interact with F18 fimbriae depend on the acidity of the glycosphingolipids. In newborn pigs, the proportion of non-binding acid glycosphingolipids is higher than in adult pigs, which could cause resistance to infection (Coddens *et al.* 2009).

Some pigs are resistant to infection by F18-positive *E. coli*. Sequencing of the FUT1 gene, which encodes the enzyme α(1,2)-fucosyltransferase involved in receptor expression of resistant and susceptible pigs, revealed a polymorphism at nucleotide 307 of the gene (Meijerink *et al.* 2000). Resistant pigs carries the nucleotide A on both alleles at this location (FUT1A/A genotype), while pigs susceptible to infection have the genotype FUT1G/A or FUT1G/G. Pigs with the FUT1A/A genotype show a decreased FUT1 activity that results in an almost complete absence of histo-blood group antigens (HBGAs) on their intestinal epithelial cells (Meijerink *et al.* 2000; Coddens *et al.* 2007). These findings open new doors for preventing infections by F18-positive *E. coli* through selection of resistant pigs for breeding (Coddens *et al.* 2008). However, the influence of FUT1 and HBGA deficiency on the normal development on pigs must first be established (Coddens *et al.* 2008). Meanwhile, there is an urgent need to develop other strategies, including probiotics and vaccines, to prevent F18-positive *E. coli* infections and minimize the use of antibiotics.
2 AIMS OF THE STUDY

The goal of this study was to study factors affecting heterologous protein production and secretion in order to increase heterologous protein yields in *L. lactis* and to develop a surface display system that can be used to display adhesins and antigens on the surface of *L. lactis*. The long-term goal of the project is to develop a live bacterial vaccine vector against porcine post weaning diarrhoea and oedema disease caused by F18-positive *E. coli*.

The studies have focused on the following subprojects:

I. The role of the chaperone-like PrsA protein of *B. subtilis* on heterologous protein secretion in *L. lactis*.

II. The expression and surface display of the receptor-binding domain of *L. brevis* SlpA protein in *L. lactis* – making a non-adhesive *L. lactis* strain adhesive.

III. Optimization of parameters affecting secretion and binding efficiency of the receptor-binding domain of the FedF adhesin of *E. coli* F18 fimbriae in *L. lactis*.

IV. Optimization of constitutive gene expression for surface displaying the receptor-binding domain of the FedF adhesin of *E. coli* F18 fimbriae *L. lactis*. 
# 3 MATERIALS AND METHODS

Strains, plasmids and cell line used in this work are listed in Table 2. Methods used in this work are listed in Table 3.

**Table 2.** Strains, plasmids and cell line used in this work.

<table>
<thead>
<tr>
<th>Strain, plasmid, cell line</th>
<th>Characteristics</th>
<th>Source or reference</th>
<th>Article</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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</tr>
<tr>
<td><em>L. lactis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCDO712</td>
<td><em>lac</em> + and <em>prt</em>+</td>
<td>NCDO*</td>
<td>II,III</td>
</tr>
<tr>
<td>NZ9000</td>
<td>MG1363 derivative with <em>nisR</em> and <em>nisK</em> integrated into the chromosome</td>
<td>de Ruyter <em>et al.</em> 1996</td>
<td>I,II,III,IV</td>
</tr>
<tr>
<td><strong>L. brevis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 8287</td>
<td>Strain with surface layer protein SlpA, isolated from green fermented Sevillano variety olives</td>
<td>Orla-Jensen <em>et al.</em> 1934, ATTC*</td>
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<tr>
<td><strong>E. coli</strong></td>
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<td>DH5α</td>
<td>Transformation host</td>
<td>Hanahan 1983</td>
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<tr>
<td>107/86</td>
<td>Clinical isolate with F18 fimbriae</td>
<td>Imberechts <em>et al.</em> 1992</td>
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<td>One Shot® TOP10F'</td>
<td>Chemically competent cells</td>
<td>Invitrogen</td>
<td>III</td>
</tr>
<tr>
<td><strong>C. crescentus</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>One Shot® B5 BAC</td>
<td>Electrocompetent cells</td>
<td>Invitrogen</td>
<td>III</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC19</td>
<td><em>Amp</em>'; <em>E. coli</em> cloning vector</td>
<td>Yanisch-Perron 1985</td>
<td>I</td>
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<tr>
<td>pNG101his</td>
<td><em>Cm</em>'; 4.6 kb; pNG101 derivative carrying His-tag and autolysine anchor transcriptionally fused to the <em>nisA</em> promoter</td>
<td>Leenhouts <em>et al.</em> 1999</td>
<td>III</td>
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<tr>
<td>pNZ8032</td>
<td><em>Cm</em>'; pSH71; 4.8 kb</td>
<td>de Ruyter <em>et al.</em> 1996</td>
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<tr>
<td>pNZ8037</td>
<td><em>Cm</em>'; pSH71; 4.1 kb</td>
<td>de Ruyter <em>et al.</em> 1996</td>
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</tr>
<tr>
<td>pIH120</td>
<td><em>Amp</em>'; pUC18 derivative carrying the entire F18 fimbria-encoding region</td>
<td>Imberechts <em>et al.</em> 1996</td>
<td>III</td>
</tr>
<tr>
<td>pLP712</td>
<td><em>lac</em>’ and <em>prt</em>‘</td>
<td>Gasson <em>et al.</em> 1983</td>
<td>III</td>
</tr>
<tr>
<td>pIL277</td>
<td><em>Ery</em>'; pAMβ1; 4.3 kb</td>
<td>Simon and Chopin 1988</td>
<td>I</td>
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<tr>
<td>pCX-TOPO</td>
<td><em>Cm</em>'; 5.4 kb; vector for high-level expression of Invitrogen RsaA fusion proteins</td>
<td>Invitrogen</td>
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<td>pG’HOST4</td>
<td><em>Em</em>'; thermosensitive derivative of pWV01 replicon</td>
<td>Appligene-Oncor</td>
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<td>pKTH10</td>
<td>Kan*'; pUB110; 4.5 kb</td>
<td>Palva 1982</td>
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<tr>
<td>pKTH2121</td>
<td><em>Cm</em>’, <em>Ery</em>’; pWV01; 6 kb</td>
<td>Savijoki <em>et al.</em> 1997</td>
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<td><strong>Cell line</strong></td>
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<tr>
<td>Intestine 407</td>
<td>Human intestinal epithelial cell line ATCC CCL-6</td>
<td>ATCC*</td>
<td>II</td>
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</tbody>
</table>

*a National Collection of Dairy Organisms

*b American Culture Collection

*r = resistance
Table 3. Methods used in this study.

<table>
<thead>
<tr>
<th>Method</th>
<th>Article</th>
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<tr>
<td>Adhesion of recombinant <em>L. lactis</em> strains to human intestine 407 cell line*</td>
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<td>Adhesion of recombinant <em>L. lactis</em> strains to human plasma fibronectin</td>
<td>II</td>
</tr>
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<td>Adhesion of recombinant <em>L. lactis</em> strains to porcine enterocytes</td>
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<td>Adhesion of secreted FedF proteins to porcine enterocytes</td>
<td>III</td>
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<tr>
<td>AmyQ activity determination</td>
<td>I</td>
</tr>
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<td>Cloning of recombinant plasmid constructs</td>
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<td>Construction of <em>L. lactis</em> NZ9000ΔhtrA*</td>
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<td>Construction of plasmid vectors for secretion studies</td>
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<tr>
<td>Construction of plasmid vectors for surface display studies</td>
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</tr>
<tr>
<td>Construction of plasmid vectors for constitutive surface display of FedF</td>
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<tr>
<td>DNA sequencing</td>
<td>I, II, III, IV</td>
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<tr>
<td>Immunoblotting</td>
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<td>Immunofluorescence assay for detection of peptide on the cell surface of <em>L. lactis</em></td>
<td>III</td>
</tr>
<tr>
<td>Immunoprecipitation</td>
<td>I</td>
</tr>
<tr>
<td>Isolation of genes used in cloning by PCR</td>
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</tr>
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<td>Nisin induction (NICE)</td>
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<td>Plasmid isolation</td>
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</tr>
<tr>
<td>Preparation of protoplasts</td>
<td>I</td>
</tr>
<tr>
<td>Production of FedF-RsaA antisera in rabbits*</td>
<td>III</td>
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<tr>
<td>Production of FedF-RsaA protein for antibody production*</td>
<td>III</td>
</tr>
<tr>
<td>Pulse-chase experiments</td>
<td>I</td>
</tr>
<tr>
<td>Purification of His-tagged proteins</td>
<td>III</td>
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<tr>
<td>Quantitation of cell lysis</td>
<td>I</td>
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<tr>
<td>SDS-PAGE</td>
<td>I, II</td>
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<td>Transformation of <em>C. crescentus</em></td>
<td>III</td>
</tr>
<tr>
<td>Transformation of <em>E. coli</em></td>
<td>I</td>
</tr>
<tr>
<td>Transformation of <em>L. lactis</em></td>
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<tr>
<td>Whole-cell ELISA for the detection of peptide on the cell surface of <em>L. lactis</em></td>
<td>II, III, IV</td>
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* Method not conducted by author
4 RESULTS AND DISCUSSION

All plasmids and strains produced in this work are listed in Table 4.

4.1 Role of *B. subtilis* PrsA on heterologous protein secretion in *L. lactis* (I)

The *B. subtilis* protein PrsA is known to increase the yield of some secreted proteins in *B. subtilis* through a chaperone-like function on the trans side of the cytoplasmic membrane. PrsA has been suggested to protect newly translocated proteins from degradation by extracellular proteases by preventing interactions of the naive protein with components on the cytoplasmic membrane and in the cell wall (Wahlström et al. 2003; Colomer-Pallas et al. 2004). To determine whether PrsA could increase the yield of heterologous exported proteins in *L. lactis*, we studied the effect of PrsA on the secretion of two different heterologous proteins: the *Bacillus amyloliquefaciens* α-amylase (AmyQ) as a target protein for PrsA and *Bacillus licheniformis* penicillinase (PenP) as a non-target protein for PrsA (Kontinen and Sarvas 1988; Vitikainen et al. 2005).

4.1.1 Construction of plasmid vectors

To co-express prsA with either amyQ or penP, three plasmids were constructed. The *B. subtilis* prsA gene was inserted into the low-copy plasmid pIL277 (Simon and Chopin 1988), while amyQ and penP were inserted into a high copy plasmid of pNZ8032 origin (de Reuter et al. 1996b). All genes were inserted downstream of the nisin-inducible nisA promoter to allow for controlled expression (de Ruyter et al. 1996a). To ensure secretion of the encoded proteins, prsA included its native signal sequence, while the signal sequence of *Lactobacillus brevis* slpA (Vidgren et al. 1992) was inserted upstream and in frame with amyQ and penP. *L. lactis* NZ9000 was transformed with two plasmids for co-expression of either prsA and amyQ or prsA and penP. Two corresponding control double-plasmid NZ9000 strains lacking prsA were also prepared. For low-, to high-level expression of the genes, three different nisin levels were used: 0.1, 1 and 10 ng per ml culture medium.

4.1.2 Production of PrsA

The production of PrsA was studied from the disrupted cells of nisin-induced strains with immunoblotting. At the lowest induction level, no PrsA could be detected. However, at the middle-, and high-induction levels, the yield of PrsA increased as a function of induction time to approximately 3×10^5 molecules per cell after 1.5 and 3 h of induction with 1 ng and 10 ng nisin per ml, respectively (Fig. 1C, I). The amount of PrsA was comparable with or even exceeded, the amount of PrsA detected in *B. subtilis* with a multicopy expression system (Vitikainen et al. 2001). The molecular size of PrsA on immunoblots indicated that PrsA was processed in *L. lactis*, and the amount of PrsA was presumably enough to have an effect on AmyQ production.
<table>
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**Results and discussion**
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a  GRS = *Lactococcus lactis* strain  

b  ERF = *Escherichia coli* strain  

c  NZ9000 derivatives  

d  NZ9000ΔhirA derivatives  

e  PEL = *Caulobacter crescentus* strain
4.1.3 Effect of PrsA on AmyQ production and activity

As expected from the results of PrsA immunoblots, no effect of the presence of prsA on the production and activity of AmyQ could be detected at the lowest induction level.

At the middle-induction level, differences between the strains expressing prsA+amyQ or amyQ alone started to appear. The highest total yield of AmyQ was reached after 5 h of induction at the early stationary phase. At this time point, the presence of PrsA resulted in an up to 20-fold increase in total AmyQ, as detected with immunoblotting (Fig. 2, I). The largest accumulation (30-fold) was seen in the cell fraction. Further studies on protoplasts and pulse-chase experiments revealed that most of the cell-associated AmyQ was in the precursor form located on the trans side of the cytoplasmic membrane. These findings indicated that AmyQ was successfully translocated through the cytoplasmic membrane, but that there was some problem with cleavage of the signal peptide, and hence, the release of mature protein to the medium was delayed. Still, the amount of AmyQ in the extracellular medium was up to nine-fold higher in the strain co-expressing amyQ and prsA than in the strain lacking prsA. A further increase in the expression level from 1 ng nisin/ml to 10 ng nisin/ml resulted in a 2- to 4-fold increase in AmyQ in the cell and supernatant fractions of both strains. Thus, the difference in the amount of AmyQ between strains remained essentially the same.

AmyQ activity studies showed that AmyQ was correctly processed and folded in L. lactis. However, the activity of AmyQ in the strain expressing amyQ alone was rather low. The presence of prsA increased the activity of AmyQ in the supernatant fraction as a function of induction time. The largest difference in AmyQ activity (six-fold) between the amyQ+prsA and amyQ expressing strains was reached after 5 h of induction. However, contrary to the results of immunoblotting, no increase occurred in AmyQ activity when the amount of nisin was increased from 1 ng to 10 ng per ml culture medium. This indicated that at higher expression levels a fraction of the processed and secreted AmyQ molecules in the medium were incorrectly folded, and thus, not fully active despite the presence of PrsA.

4.1.4 Effect of PrsA on PenP production and activity

The gene penP was expressed under the same induction conditions as amyQ. Both immunoblotting and activity assays demonstrated that PrsA had no effect on the production or activity of PenP in L. lactis. These results are in perfect agreement with PenP studies on B. subtilis showing, that PenP is not a target protein for PrsA (Kontinen and Sarvas 1988; Vitikainen et al. 2005).

4.1.5 Function of PrsA

Our results suggested that PrsA plays a similar role in the heterologous host L. lactis as it does in B. subtilis, executing a chaperone-like function on exported target proteins. A gene encoding a PrsA-like protein has also been identified from the genome sequence of L. lactis IL1403 (Bolotin et al. 2001), indicating that PrsA-like chaperones are also present in other Gram-positive bacteria. The protein PmpA, encoded by this gene, shows 27% identity to PrsA (Drouault et al. 2002), and overproduction of PmpA was found to increase the yield of secreted Staphylococcus hyicus lipase, probably by preventing degradation of misfolded protein (Drouault et al. 2002).

It is clear that the use of PrsA is limited to its target proteins, but because the substrate profiles of extracellular chaperones are probably different, it could be of great value to
characterize and further develop different extracellular chaperones for the bacterial production of industrially and pharmaceutically important proteins.

### 4.2 Surface display of the receptor binding domain of *L. brevis* SlpA in *L. lactis* (II)

Several immunization studies, using LAB vehicles for the delivery of an antigen to mucosal surfaces, have suggested that cell surface-located antigens give the best immune response (for references see Wells and Mercenier 2008). In this study, the surface display of the receptor-binding domain (RBD) of *L. brevis* ATCC 8287 surface layer (S-layer) protein SlpA was investigated in *L. lactis*.

To increase the surface accessibility of the SlpA RBD on the bacterial surface, two spacers, including the H-domain and parts of the W-domain of the *L. lactis* subsp. cremoris Wg2 PrtP protein (Kok *et al.* 1988), were used as spacers. These domains of PrtP were chosen because the alpha-helical H-domain has been suggested to function as a spacer directing other PrtP domains further from the cell wall (Siezen 1999).

The function of the two PrtP spacers, 215 and 516 amino acids residues (aa) long, were first tested by using the β-lactamase (Bla) reporter for surface display. For this, two plasmids were constructed by cloning the genes encoding Bla and the spacers into the surface display vector pNG101his (Leenhouts, unpublished) downstream of the nisin-inducible promoter P_{nisA}. The resulting plasmids, encoding a fusion protein consisting of the AcmA signal peptide, the mature Bla, the 215 or 516 aa spacer and the AcmA cell wall anchor, were expressed in *L. lactis* NZ9000. The surface accessibility of Bla in the resulting strains was tested with the enzyme-linked immunosorbent assay (ELISA) on whole cells with anti-Bla antibodies. With the 516 aa spacer, cross-linking between Bla and antibodies was observed, whereas no surface-displayed Bla could be detected with the shorter 215 aa spacer.

Due to its superiority in Bla studies, the 516 aa spacer was employed to surface-display the SlpA fragment. DNA encoding the AcmA signal peptide and mature Bla was replaced with DNA encoding the first 247 N-terminal amino acids of preprotein SlpA (SlpA_{1-247}) in the pNG101his plasmid vector, resulting in plasmid pKTH5056. *L. lactis* NZ9000 was transformed with pKTH5056 and the expression of the gene construct was induced with nisin. The surface location and accessibility of SlpA_{1-247} in induced cells was verified by immunological assays with anti-SlpA antibodies. All three experiments, including immunoblotting of isolated cell wall extracts, immunofluorescence microscopy and whole-cell ELISA, confirmed that the SlpA protein was located on the cell surface.

#### 4.2.1 Adhesion assays

Flagellar display experiments with *E. coli* have shown that SlpA of *L. brevis* ATCC 8287 mediates adhesion to human intestinal cell lines and fibronectin *in vitro* via a binding region located within the N-terminal part of the protein (Hynönen *et al.* 2002). The adherence ability of the SlpA-PrtP-AcmA-producing strain was demonstrated by *in vitro* adhesion assays using the human intestinal cell line, Intestine 407 and human fibronectin. *L. lactis* cells surface-displaying SlpA_{1-247} bound to Intestinal 407 cells approximately 7-fold better than the plasmid-free strain NZ9000, but only 3- to 4-fold better than the strain carrying the control plasmid pKTH5046 lacking *slpA*. To make sure that the binding was truly mediated
by SlpA, adhesion inhibition studies were performed by treating nisin-induced recombinant \textit{L. lactis} cells with either anti-SlpA antibodies or PBS before the adhesion assay. Pre-treatment with anti-SlpA antibodies inhibited the binding of induced NZ9000 cells carrying pKTH5056 to the level of the negative control lacking \textit{slpA} (NZ9000 carrying pKTH5046), while pre-treatment with PBS had no effect on binding.

To characterize the binding specificity of SlpA$_{1-247}$ on the \textit{L. lactis} cell surface further, fibronectin adhesion assays were performed. The presence of SlpA$_{1-247}$ on the cell surface resulted in a 7-fold increase in binding to fibronectin immobilized on glass slides, when compared with the control strain NZ9000 without plasmid or with pKTH5046.

The discrepancy in binding of the control strains to Intestinal 407 cells might be explained by aggregation of induced cells, a phenomenon observed to a lesser degree in fibronectin adhesion assays, where cells were subjected to gentle agitation during propagation.

Even though the surface display of SlpA$_{1-247}$ gave non-adherent \textit{L. lactis} cells a significant ability to adhere to human Intestine 407 cells, the binding efficiency was substantially lower than that of wild type \textit{L. brevis}, coated by approximately 500 000 SlpA subunits (Palva \textit{et al.} unpublished; Hynönen \textit{et al.} 2002). One obvious explanation for this is that the amount of SlpA displayed on the surface of recombinant \textit{L. lactis} was only a small percentage of the abundance of SlpA subunits present in the native S-layer lattice in \textit{L. brevis}. Other putative explanations might be insufficient folding and/or activity of the RBD of SlpA when displayed as a fusion protein on the surface of \textit{L. lactis}.

4.3 Optimization of production and surface display of the FedF adhesin in \textit{L. lactis} (III, IV)

The best defence against intestinal infection would be to block the initial attachment of the pathogen to its receptor in the intestine (van Ginkel \textit{et al.} 2000). This could be accomplished by using bacteria with surface displayed adhesins. Furthermore, if the adhesin is immunogenic a bacterial mucosal vaccine could induce both systemic and mucosal immunity.

In this work, our aim was to develop an efficient cell surface display system in \textit{L. lactis} for FedF, the adhesin of F18 fimbriae, to develop a bacterial mucosal vaccine against porcine PWD and OD caused by \textit{E. coli} with F18 fimbriae. First, we evaluated the secretion efficiency of FedF in \textit{L. lactis}. Our attempt to produce a secreted form of the full-length FedF protein as a fusion to a carrier in \textit{L. lactis} NZ9000 resulted in inefficient secretion and degradation of the fusion protein. Later studies on the production of full-length FedF by Tiels \textit{et al.} (2007, 2008) have demonstrated that FedF forms insoluble aggregates in the cytoplasm and requires fusion to a stable carrier protein in order to remain in a soluble and active form. In our further studies, several parameters influencing secretion and surface-display of FedF were evaluated. In secretion optimization studies, these included: length of FedF, signal peptides (SPs), and \textit{L. lactis} host backgrounds, whereas in surface display optimization the length of FedF, length and origin of spacer and anchor were studied in different \textit{L. lactis} host backgrounds.

4.3.1 Secretion studies

To minimize proteolysis of the heterologous protein, the \textit{htrA} gene, encoding the major housekeeping protease HtrA of \textit{L. lactis}, was inactivated by chromosomal deletions in the
promoter and 5’end in the L. lactis strain NZ9000. The NZ9000ΔhtrA strain was named GRS1090.

To enhance correct folding and secretion of FedF, the fedF gene was truncated to consist of only the part that encodes the domain essential for binding to the receptor (RBD) on porcine intestinal epithelial cells (Smeds et al. 2003). Two different truncated forms of fedF, encoding 42 or 62 aa residues, were fused to the prtP fragment encoding the helix (H)-domain of L. lactis subsp. cremoris Wg2 PrtP protein (Siezen 1999). The H-domain was chosen as a fusion partner to stabilize FedF bearing in mind future surface display studies. The PrtP H-domain has been suggested to function as a spacer directing other PrtP domains farther from the cell wall (Siezen et al. 1999).

To increase the flexibility of the FedF fragment (mainly for surface display studies, see below) in the fusion protein, nucleotides encoding the dipeptide Gly-Pro were inserted on both sides of the fedF gene. In order to direct the synthesized protein to the secretion machinery, the signal sequences of L. brevis slpA or L. lactis usp45 were placed translationally in front of fedF. To enhance the processing and secretion capacity further, the synthetic sequence encoding the propeptide LEISSTCDA (Le Loir et al. 1998, 2001) was inserted between the signal sequence and fedF. A sequence encoding six histidine residues was included at the 3’end of each sequence to enable purification and detection of the protein. A negative control strain carrying a plasmid with a fedF fragment encoding a non-adhesive 42 aa residue peptide was also constructed. The fedF-prtP gene constructs (Fig. 1, III) were expressed from the plasmid vector pNZ8037 in strain NZ9000, or its htrA mutant, to enable controlled expression from the nisA promoter.

The ability of induced L. lactis strains to secrete FedF-PrtP fusion proteins was determined by immunoblotting of supernatants using anti-His6 antibodies for detection. An affinity-purified FedF-PrtP protein was used for quantification of secreted protein yields. In all strains, the highest secretion yield was obtained with the signal peptide (SP) of SlpA (Fig. 2, III). Compared with strains with the Usp45 SP, the level of secretion was 4- to 6-fold higher, depending on the length of FedF or whether the fusion protein was produced in NZ9000 or GRS1090. In NZ9000, the highest protein yield (2.3 μg/mL) was reached with FedF42aa-PrtP, while production of the fusion protein with FedF62aa was 1.7-fold less. On the other hand, with the htrA mutant strain GRS1090, both fusion proteins were produced to approximately the same level, 2.6 and 2.3 μg/mL for FedF62aa-PrtP and FedF42aa-PrtP, respectively. These results indicated that FedF62aa-PrtP was more susceptible to degradation by HtrA, probably due to its more complicated structure, owing to its longer length.

4.3.1.1 Adhesion of FedF-PrtP proteins to isolated porcine intestinal epithelial cells

Secreted FedF-PrtP proteins with His-tags were isolated from induced cultures with Nickel-affinity chromatography, and equal amounts of protein were used in binding studies with isolated porcine intestinal epithelial cells. Bound FedF-PrtP proteins were visualized with indirect immunofluorescence using anti FedF-RsaA antibodies produced in rabbit and fluorescein isothiocyanate-labelled anti-rabbit antibodies. Fluorescence microscopy of labelled proteins revealed that both FedF42aa-PrtP and FedF62aa-PrtP proteins, including the FedF receptor binding domain equally strongly adhered to epithelial cells, while the fusion protein with the non-adhesive FedF (FedFna42aa) did not (Fig. 3, III).
4.3.2 Surface display studies

4.3.2.1 Construction of plasmid vectors
For surface display, the secreted FedF-PrtP proteins were anchored to the cell wall of *L. lactis* with the cell wall-anchoring domain of either *L. lactis* PrtP or AcmA. The PrtP anchor binds covalently to the cell wall with an LPXTG domain (Siezen 1999), while the AcmA repeats binds to the cell wall in a non-covalent manner that has not been fully characterized (Buist *et al.* 1995). Three PrtP spacers, composed of 210, 270 and 516 aa residues were used to optimize surface accessibility of FedF RBD. The shortest spacer spanned the whole alpha-helical Helix (H)-domain of PrtP, while the 270 and 516 aa spacers spanned the H-domain and parts of the wall (W)-domain of PrtP. The longest spacer was the same 516 aa spacer that was used earlier in SlpA-surface-display studies (III).

To study all parameters, three sets of plasmids were constructed with two lengths of FedF, three lengths of spacers and two cell wall anchors (Fig. 1, III). In the first set, the gene fragments encoding the 210 aa H-domain of PrtP and the AcmA anchor were inserted downstream of *fedF* in the secretion plasmids. These plasmids encoded the fusion proteins SP_{SlpA}LEISSSTCDA-FedF_{42aa}-PrtP_{210aa}-AcmA or SP_{SlpA}LEISSSTCDA-FedF_{62aa}-PrtP_{210aa}-AcmA. In the second set, the two receptor-binding FedF fragments were produced in fusion with the H, W and anchor domains of PrtP, and in the third set, the *fedF* gene construct was inserted into plasmid pKTH5056 upstream of the 516 aa PrtP spacer and AcmA anchor encoding genes. For all three sets, a plasmid with the corresponding gene construct, but lacking *fedF*, was also constructed. All nine plasmids were expressed in both NZ9000 and GRS1090, resulting in 18 different strains.

4.3.2.2 Surface accessibility of FedF
The surface accessibility of FedF RBD was assayed in all strains by whole-cell ELISA with anti FedF-RsaA antibodies. Over-production of secreted heterologous proteins often results in accumulation, aggregation and degradation of the produced protein. To avoid this and to find a balance between expression-translocation-secretion, the expression level of the *fedF* gene construct was optimized by using different nisin levels for induction. The maximal nisin induction level that did not affect growth of an individual strain was thereafter used in ELISA studies. The ELISA results are presented in Fig 4, III.

Of the three spacer lengths tested, the shortest 210 aa PrtP spacer, in combination with the AcmA anchor, allowed the best surface accessibility of both FedF fragments in *L. lactis* GRS1090. However, there was an approximately 15 % difference in surface accessibility between FedF_{42aa} and FedF_{62aa}, favouring FedF_{62aa}. With the longest 516 aa spacer and AcmA anchor, the level of surface-displayed FedF RBD could not be distinguished from that of the negative control in the wild-type NZ9000 background. However, when expressed in GRS1090, both FedF constructs were surface-displayed at a high level, reaching approximately 60% of the level with the 62 aa FedF and 210 aa spacer.

In all NZ9000 strains where the FedF fusion protein was anchored with the AcmA anchor, the surface accessibility of FedF was significantly reduced compared with GRS1090, indicating degradation of proteins by HtrA.

With the last group of FedF constructs, including the native PrtP anchor and 270 aa spacer, the poorest overall performance was observed. In this group, surface accessibility remained at a low level in the GRS1090 background, but was, surprisingly, somewhat higher in the NZ9000 background. It has been reported that covalent binding of PrtP to the
lactococcal cell wall is not always efficiently formed with high-level expression of exported heterologous proteins (Norton et al. 1995, Leenhouts et al. 1999). We did not further characterize whether the low surface accessibility obtained was due to release of FedF fusion proteins into the culture medium or to lower expression-translocation efficiency.

### 4.3.3 Adhesion studies

Based on the ELISA results, the best-performing GRS1090 clones with both FedF-fragment sizes were chosen for adhesion studies. Surprisingly, the binding assays of the surface-displayed FedF fusions with the porcine epithelial cells revealed that the two clones with the shorter PrtP spacer (210 aa) were unable to adhere, although they were most efficiently recognized by FedF antibodies in whole-cell ELISA (Fig. 6, III). These results suggest that with relatively short peptides, this PrtP spacer was insufficient or the folding of the fusion protein was compromised to allow receptor binding competent exposure of the FedF adhesin fragments. Instead, the longer PrtP spacer (516 aa) allowed the surface presentation of FedF proteins that were effectively recognized by the F18 receptors on porcine intestinal cells (Fig. 5, III). The clone producing the fusion protein with the shorter (42 aa) FedF was clearly more adhesive than the clone with the identical construct carrying the 62 aa FedF fragment, although, neither the immunofluorescence assays performed with purified FedF<sub>62aa</sub>-PrtP and FedF<sub>42aa</sub>-PrtP fusion proteins nor the surface display assays showed any significant difference. These results indicated that the shorter FedF fragment, in combination with the 516 aa spacer attained a more correct configuration for binding to the F18 receptor on the epithelial cells.

### 4.4 Constitutive expression of fedF for surface display of the receptor binding domain of the FedF adhesin (IV)

To make a vaccine with in situ production of FedF RBD at the intestinal mucosal surface, the next step was to replace the inducible nisA promoter with a constitutive promoter for the expression of the slpA-leiss-fedF<sub>42aa</sub>-prtP<sub>516aa</sub>-acmA gene construct.

In our first attempt to find an optimal constitutive expression system for our application, we used the strong promoter of L. brevis slpA (Vidgren et al. 1992). However, the plasmid carrying this promoter for the expression of the fedF construct was unstable, and no production of FedF could be confirmed.

To find a suitable promoter for the constitutive production of FedF RBD on the surface of L. lactis, we constructed a library of synthetic promoters based on known lactococcal promoter sequences according to the method of Jensen and Hammer (1998).

#### 4.4.1 Construction of plasmid vectors and ELISA

In order to construct plasmids with different promoters, first, an oligonucleotide with defined L. lactis consensus promoter regions and randomized spacer regions was designed (Fig 1, IV). Synthesis of the 78-bases-long oligonucleotide resulted in a mixture of different promoter sequences that were used to express the slpA-leiss-fedF<sub>42aa</sub>-prtP<sub>516aa</sub>-acmA gene construct from pKTH5056 in L. lactis GRS1090. Plating of the transformants resulted in 250 chloramphenicol-resistant clones that were further screened with whole-cell ELISA using anti FedF-RsaA antibodies and PCR (with the forward primer in the plasmid and reverse primer in the slpA signal sequence) to identify clones with different promoters. Finally, DNA sequencing of isolated plasmids revealed that several plasmids carried
identical promoter sequences and the number of unique promoters was reduced to nine (Fig. 2, IV). With these nine promoters, different amount of FedF was displayed on the cell surface (as judged by whole-cell ELISA). The best-performing promoter clone (GRS1155) reached the same level of FedF surface accessibility as GRS1134 that surface displayed the FedF RBD after optimal induction with nisin (Fig 3, III).

4.4.2 Analysis of promoter sequences

Sequencing of isolated promoter plasmids surprisingly revealed that the consensus regions were modified in all promoters (Fig 2, IV). Only one of the promoters (in clone GRS1158) corresponded to the synthetic oligonucleotide, except for a deletion in the -10 region that changed the consensus sequence TATAAT to TATAAA. Analysis of E. coli sigma_70 promoters has demonstrated that the last T in the -10 region is highly conserved (Shultzaberger 2007). It is therefore likely that the substitution of T to A at this position contributed to weakening of the promoter, making it one of the weakest in our set. The rest of the active promoters showed major deletions that completely or partly removed oligonucleotide-defined bases upstream of the extended -10 region TGNTATAAT. This extended -10 region is sufficient to initiate gene transcription in L. lactis (Jensen and Hammer 1998; Madsen at al 1999), but whole-cell ELISA results clearly suggested up to 3-fold consistent differences in the amount of surface-displayed FedF indicative of differences in promoter activity between the clones. In order to recognize alternative bipartite promoter candidates, all promoter sequences were therefore analysed by using Prokaryote Promoter Prediction (PPP) software (Molecular Genetics, the Netherlands). This program identified the extended -10 promoter and a bipartite promoter in all nine fedF-expressing clones (Fig. 2, IV). When the new promoters were compared with each other, minor variations in bases in the spacers between the -35 and -10 regions and/or downstream of the -10 region was detected, which might explain differences in the amount of FedF produced on the cell surface (Jensen and Hammer 1998).

The reasons for the major DNA deletions are unclear. Over-production of secreted proteins may cause stress to the cell, resulting in deletions and rearrangement of genes in the plasmid or degradation of produced protein (Bolhuis et al. 1999). Nevertheless, we observed no fluctuation in FedF production between repeated experiments, suggesting that the nine promoter plasmids were stable. Neither could we detect any degradation products of produced protein in immunoblots. A potential explanation of the deletions could be that a restriction endonuclease site used in cloning was formed in the undefined spacer between consensus hexamers during oligonucleotide synthesis. However, these weakened promoters being selected for surface display of FedF, clearly suggested that the consensus promoter was too strong to obtain a balance between expression of the fedF gene construct and translocation and secretion of the encoded protein. Despite the obvious requirement for a weak promoter, a production level comparable with the optimized production with the nisin-inducible expression system was reached and a strain was produced that can be used for in situ production of FedF in immunization studies.
5 CONCLUSIONS AND FUTURE PROSPECTS

*L. lactis* has defined its place as an important host for the production of heterologous proteins. The possibility to utilize *L. lactis* as a producer and vehicle of antigens and therapeutics destined to mucosal surfaces has stirred great interest among researchers, and the first human clinical trials have been performed with a recombinant *L. lactis* strain producing human interleukin-10 (IL-10) (Braat *et al.* 2006). Trials with several other strains are under way.

When developing a mucosal vaccine, it is crucial that the immune system is presented with enough antigen to induce an immune response that blocks the attachment of the pathogen to mucosal surfaces and/or neutralizes toxins produced by the pathogen. Even though several reports are available describing methods to increase heterologous protein production in *L. lactis*, many immunization studies imply that the lactococcal protein yields are too low to induce a protective immune response in the host. Therefore, further studies are needed to improve the capacity of *L. lactis* to produce heterologous proteins.

Studies on *B. subtilis* have suggested that problems in secretion of heterologous proteins are often associated with events following translocation (Bolhuis *et al.* 1999). In the first part of this thesis, the effect of the *B. subtilis* extracellular chaperone-like protein PrsA on heterologous protein production was evaluated. The results demonstrated that active PrsA can be produced in relatively high concentrations in *L. lactis* and that the presence of this heterologous chaperone on the trans side of the cytoplasmic membrane increased the amount of translocated AmyQ molecules. Although majority of translocated protein was trapped in the cell wall in its precursor form the yield of mature AmyQ in the culture medium increased when PrsA was present.

It is well established in *B. subtilis* that PrsA is needed only to secure the correct folding of its (over)produced target proteins, which limits its use in heterologous protein production. However, characterization of the PrsA-like protein PmpA in *L. lactis* suggests that extracellular folding factors are also present in LAB (Drouault *et al.* 2002). The substrate proteins of PmpA have not been determined, but one might speculate that different chaperones have different target proteins. Therefore, the characterization of different extracellular chaperones and their substrates could be of great benefit in bacterial production of industrially and pharmaceutically important proteins. A step further could be to construct strains that produce several different chaperones e.g. as chimeras, capable of assisting folding of several substrate proteins.

A good mucosal vaccine should induce an immune response that protects the host from infection by blocking attachment of bacteria to the mucosal surface of the host by the production of adhesin specific antibodies and/or by competitive exclusion at receptor sites (Wizemann *et al.* 1999; van Ginkel *et al.* 2000). Several parameters have been shown to influence the immunogenicity of LAB vaccines. These include antigen immunogenicity, amount and localization of the antigen, as well as intrinsic immunogenicity and persistence of the bacterial vehicle in the host (Wells and Mercenier 2008).

Here, we developed a surface-display system in order to present heterologous proteins on the surface of *L. lactis*. Several parameters, including secretion efficiency, length of cell wall spacers and cell wall anchoring modes were evaluated. In the process three different polypeptides were successfully displayed on the surface of *L. lactis*, the reporter β-lactamase and the two receptor binding domains (RBDs) of *L. brevis* surface layer protein SlpA and
E. coli F18 fimbrial adhesin FedF. The RBDs of SlpA and FedF mediated adhesion of L. lactis to human epithelial cells and fibronectin as well as porcine epithelial cells in vitro, respectively.

The long-term aim of the project is to develop a vaccine towards porcine post-weaning diarrhoea and oedema disease caused by enteropathogenic F18-positive E. coli. Mucosal immunization studies against F18-positive E. coli infections have hitherto been performed either with isolated F18 fimbriae or with soluble adhesin (FedF), alone or fused to a carrier protein (Verdonck et al. 2007; Tiels et al. 2008). In those studies, the immune response against FedF has been very weak.

Delivery of the RBD of FedF to the intestinal mucosa on the surface of a bacterial vehicle could induce a stronger immune response by protecting the adhesin from degradation in the gut. In addition, the RBD on the bacterial surface could bind specifically to its receptor site on epithelial cells in the intestine and exclude adhesion of pathogenic F18-positive E. coli. To date, we have tested one of our L. lactis strains surface displaying the RBD of FedF in a preliminary immunization study on mice. However, only a very weak (statistically insignificant) FedF-specific secretory IgA response could be detected in intestinal lavage from a few of the immunized individuals. Therefore, further immunization studies are needed to determine the immunogenicity of the produced strains. These could include modulation of the vaccination protocol and co-administration of the vaccine strain with a vaccine adjuvant.
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