Progress in the Development of *Lactococcus lactis* as a Mucosal Vaccine Delivery Vehicle

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**ABSTRACT**

*Lactococcus lactis*, a food-grade, non-pathogenic, non-invasive, non-colonizing and “generally regarded as safe” lactic acid bacteria, is widely used in food, medicine, and husbandry industry, and it is a potential and promising candidate as a mucosal vaccine delivery vehicle (MVDV). This review describes the latest research progress of *L. lactis* as an MVDV and its potential improvements. Firstly, the review introduces the advantages of using *L. lactis* as an MVDV, emphasizing the efficient controlled protein expression and protein-targeting systems developed for production of a desired antigen. Secondly, an extensive overview is given of the progress made in improving production yield and stability of the heterologous proteins expressed in *L. lactis*. Thirdly, an overview is provided of the efficiency of *L. lactis* as MVDV for mucosal immunization. Finally, the problems limiting the use of *L. lactis* as MVDV are introduced and probable methods to solve them are brought forward.

**Keywords:** immune response, MVDV, protein expression systems, protein-targeting systems, secretion

**Abbreviations:** Ab₃, antibiotic resistance; BLG, bovine β-lactoglobulin; GM, genetically modified; HPV-16, human papillomavirus type 16; IL, interleukin; MVDV, mucosal vaccines delivery vehicles; NICE, nisin-controlled gene expression; NSP4, non-structural protein 4; Nuc, nuclease; SE, secretion efficiency; SP, signal peptide; SPUsp45, SP of usp45; Th, T-helper; TTFc, tetanus toxin fragment C; UreB, urease subunit B

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**INTRODUCTION**

The development of efficient mucosal vaccines delivered by mucosal routes (e.g., oral, nasal, rectal and vaginal routes) is one of the hotspots in modern vaccinology. Mucosal vaccines offer several benefits over parenteral routes of vaccination from both immunological and practical points of view. Firstly, they can mimic the route of entry of many pathogens and activate the mucosal immune response at the site of primary infection which can determine a better influx of immunocompetent cells at the mucosal level and secrete a large amount of IgA onto the mucosal surfaces. Secondly, they can be administered orally or nasally eliminating the chance of injection with infected needles and need for a professional healthcare infra structure, therefore they are much more suitable for mass vaccinations. Mucosal vac-
cines represent a promising approach in vaccinology and may partly replace injectable vaccines provided that potent and relevant responses are elicited.

However, before reaching the mucosal immune system, mucosal vaccines have to overcome several formidable barriers in the form of significant dilution and dispersion; low pH and enzymatic degradation; competition with a myriad of various live replicating bacteria, viruses, inert food and dust particles. Thus, efficient mucosal vaccine delivery systems are very important, and a multitude of such delivery vehicles have been developed, including various inert systems as well as live bacterial or viral vector system, to deliver antigens to mucosal surfaces (Holmgren et al. 2003a; Detmer and Glenting 2006). Among them, live bacterial vector system is an attractive vaccine strategy (Medina and Guzman 2001). It implies the use of live bacteria including attenuated pathogenic and food related bacteria as vehicles for the production and delivery of vaccine component, such as antigens from infectious diseases, allergy promoting proteins and therapeutic proteins. These vaccines are referred as live bacterial vaccines. Using attenuated pathogenic bacteria, such as Listeria monocytogenes, Mycobacterium bovis, Salmonella typhimurium, Shigella flexneri, Vibrio cholerae and Yersinia enterocolitica, as MVDV can induce a specific immune response against the heterologous antigen and simultaneous protection against the pathogen (Medina and Guzman 2001; Nouaille et al. 2003). However, these attenuated strains maintain certain invasive and virulence properties and could recover their pathogenic potential and tend to disseminate in the body, therefore they are not totally safe for use in humans, especially in children, older people, immunosuppressed and immunodeficient individuals (Nouaille et al. 2003; Bermudez-Humaran et al. 2004). Thus, non-pathogenic food grade bacteria are being developed as alternatives (Grangette et al. 2001; Nouaille et al. 2003; Detmer and Glenting 2006). Much research has led to the development of Lactococcus lactis as MVDV for mucosal immunization (Nouaille et al. 2003; Detmer and Glenting 2006). In the last decade, the efficacy of L. lactis as MVDV to deliver antigens to the mucosal immune system has been extensively studied. This review will give an overview of the use of L. lactis as MVDV.

**ADVANTAGES OF L. LACTIS AS MVDV**

*L. lactis*, a food-grade, non-pathogenic, non-invasive, non-colonizing and “generally regarded as safe” lactic acid bacterium, is widely used in food, medicine, and husbandry industry, and it is a better potential and promising candidate as vaccine delivery vehicles (Nouaille et al. 2003; Steidler and Rottiers 2006). There are following advantages of using *L. lactis* as a vaccine delivery vehicle: (1) Compared to attenuated pathogenic bacteria, *L. lactis* has low antigenicity (Norton et al. 1996; Cheun et al. 2004), and does not produce toxic substances, which eliminates inflammatory reactions like those observed in the use of attenuated pathogenic bacteria as vaccine delivery vehicles. (2) *L. lactis* is a non-invasive bacterium. It cannot on ingestion, invade the tissues and never causes infection; even when given overt opportunity, as would be the case following *L. lactis* consumption during an ongoing intestinal disease, it displays no health risk (Steidler and Rottiers 2006). Therefore the use of *L. lactis* as a vaccine delivery vehicle in children, older people, immunosuppressed and immunodeficient individuals is particularly attractive. (3) *L. lactis* is a non-colonizing and transmissible enteric organism on the oral and intestinal epithelium (Yamamoto et al. 2003). Therefore the risk of eliciting a tolerance response to the antigen delivered is diminished compared with persistent bacteria.

Moreover, *L. lactis* strains show advantages of production of heterologous proteins: (1) only a few proteins are naturally secreted in *L. lactis* and only one, Usp45 (an unknown secreted protein of 45 kDa) is secreted in quantities detectable by Coomassie blue staining (Poquet et al. 1998), which eliminates the chance of disturbing functions of heterologous protein. (2) compared with the well-known protein producer *Bacillus subtilis*, *L. lactis* strains have the low extracellular proteinase activity and possess only two extracellular proteinases PrtP and HtrA (Kunji et al. 1996; Poquet et al. 2000), and even laboratory *L. lactis* strains do not produce any extracellular proteases, which avoids extracellular degradation of proteins and stabilize secreted proteins; (3) *L. lactis* is a Gram-positive bacterium and therefore has only one cellular membrane, which is ideal for exporting protein from cytoplasm, with subsequent to membrane- or cell-wall- anchoring, or the extracellular medium; (4) Last but not least, great progress has been made in the development of the molecular characterization of *L. lactis*, and a number of highly efficient and elaborate genetic engineering tools, including transformation protocols, gene integration, gene knockout, conjugation, different vectors, protein expression and targeting systems have already been developed for *L. lactis* in the last 30 years. So *L. lactis* can nowadays be genetically engineered quite efficiently and elaborately to express and stably produce heterologous proteins. Especially a series of efficient controlled protein expression and protein-targeting systems have already been developed for stable production of the desired antigen (de Ruyter et al. 1996; Pfard et al. 1997; Steidler et al. 1998b; de Vos 1999a; Dieye et al. 2001; le Loir et al. 2001; Ribeiro et al. 2002).

**Controlled protein expression systems for L. lactis**

Although a large number of constitutive expression systems are available for *L. lactis*, (de Vos 1999a) continuous high level production of a protein, based on lactococcal constitutive expression systems, could lead to intracellular accumulation, aggregation, or degradation of this protein in the cytoplasm, which could, in some cases, be deleterious to the cell. To circumvent these problems, inducible expression systems have been developed, and through these expression systems, gene expression can be controlled by an inducer, a repressor or by environmental factors (de Ruyter et al. 1996; Sanders et al. 1998; Madsen et al. 1999; Llull and Poquet 2004). They constitute powerful tools to control heterologous protein production in terms of quantities, conditions and timed expression.

The best-characterized and most successful and commonly used controllable expression system is the nisin-controlled gene expression (NICE) system, based on a combination of the *L. lactis* nisF promoter and the nisRK regulatory genes, which can be induced by nisin (Zhou et al. 2006). The NICE system for controllable gene expression has proven to be highly versatile and has many desirable advantages: (1) The inducer nisin is a food-grade inducer; (2) The expression appears to be very tightly controlled, leading to undetectable protein levels in the uninduced state, and the level of expression is controllable in a dynamic range of 1000-fold which is directly dependent on the concentration of nisin added to the culture medium (de Ruyter et al. 1996); (3) Very high protein expression levels, which can go up to 60% of the total intracellular protein level, can be reached; and (4) The NICE system containing the food-grade selection marker instead of an antibiotic resistance (Ab’) gene has been developed. The NICE system has already been used for over-expressing a wide variety of heterologous proteins, including antigens, allergens and cytokines, peptides, enzymes, biopreservatives (Zhou et al. 2004).

Moreover, several lactococcal promoters regulated by environmental factors have also been isolated. A few examples are the promoters can be up or down regulated by the extracellular concentration of ions, such as Ca2+ (Sanders et al. 1998) or Zn2+ (Llull and Poquet 2004); and the promoters are regulated by pH or temperature (Madsen et al. 1999). For example, promoter P170, which is a strong promoter, only active at low pH and when cells enter the stationary growth phase (Madsen et al. 1999). Antigen hybrid GLURP–MSP3 protein has been successfully expressed in...
an inducible expression system based on promoter P170, which support the value of this system for vaccine development (Theisen et al. 2004).

**Cellular targeting systems for heterologous proteins in *L. lactis***

Targeting heterologous proteins to the cell wall or the extracellular medium (herein referred as protein export) is regarded as the preferable mode because it facilitates interaction between antigen and immune system. The ability of *L. lactis* to target a given protein to different cellular locations (the cytoplasm, the membrane, the cell wall, or the extracellular medium) using the same backbone vector, the same induction level and promoter strength, allows a rigorous comparison of the preferred antigen localization for mucosal immune response in humans or animals.

Several systems for *L. lactis* have been developed to target a given protein to specific cellular locations (Dieye et al. 2001). One kind of system is protein secretion system based on secretion-dependent machinery. The secretion-dependent machinery is a ubiquitous secretion system comprised of a set of proteins that mediate translocation of a precursor protein, consisting of the mature protein and an N-terminal signal peptide (SP), across the cytoplasmic membrane (von Heijne 1990). The precursors are firstly recognized by the host secretion machinery and translocated across the cytoplasmic membrane. Upon translocation across the membrane, the SP, an essential signature for protein secretion, is cleaved off by signal peptidase, and then the mature protein is released in the culture supernatant. Sometimes, secreted proteins require subsequent folding and maturation steps to acquire their active conformation (le Loir et al. 2005).

Another kind of system is protein cell wall anchoring system based on secretion-dependent machinery and sortase-dependent machinery, which can mediate translocation of a precursor protein across the cytoplasmic membrane with subsequent anchoring the protein to the cell wall. The combination of secretion and anchoring systems provide the needed versatility for protein targeting in *L. lactis* using as MV DV. The sortase machinery has been characterized for *Staphylococcus aureus* (Marraffini et al. 2004). Cell surface-anchored proteins are first synthesized as a preprotein containing an N-terminal SP and a C-terminal ~30-amino-acid cell wall anchor domain which consists of a conserved LPXTG motif, a transmembrane fragment, and a charged C terminus. Proteins are covalently anchored by their C terminus to the peptidoglycan by a transpeptidation mechanism based on sortase (Marraffini et al. 2004). Homologs to sortase in *S. aureus* and the same C-terminal structure of many cell surface-located proteins are present in many Gram-positive bacteria, including LAB (Ton-That et al. 2004; Marraffini et al. 2006). Anchoring of heterologous proteins using the cell wall anchor of protein A from *S. aureus*, protein M6 from *Streptococcus pyogenes*, protein AcmA or PrtP from *L. lactis* was demonstrated to be efficient in LAB species (not in *Lactococcus lactis* Pianta et al. 1997; Steidler et al. 1998b; Leenhouts et al. 1999; Dieye et al. 2001, 2003; Ribeiro et al. 2002; Cortes-Perez et al. 2003; Bermudez-Humaran et al. 2004; Lindholm et al. 2004; Ramasamy et al. 2006). For example, the fusion of *Brucella abortus* ribosomal protein L7/L12 or human papillomavirus type 16 (HPV-16) E7 protein with the cell wall anchor region of the *S. pyogenes* M6 protein led to efficient cell wall anchored form of L7/L12 or E7 protein in *L. lactis* (Ribeiro et al. 2002; Cortes-Perez et al. 2003; Bermudez-Humaran et al. 2004). However, in some cases the anchoring step proved to not be totally efficient in *L. lactis*, as considerable amounts of protein remained membrane associated. Studies suggest that the defect may be due to limiting sortase in the cell (Dieye et al. 2001, 2003). Components of the sortase machinery could be overexpressed in order to achieve a better anchorage of cell surface proteins.

**IMPROVEMENTS FOR HETEROLOGOUS PROTEINS PRODUCTION IN *L. lactis***

Secretory expression for better production yields

Heterologous proteins produced in *L. lactis* are prone to intracellular degradation, and intracellular proteolysis in *L. lactis* remains poorly understood. *L. lactis* possesses a wide range of enzymes (peptidases, housekeeping proteases) dedicated to intracellular proteolysis. Until recently, only two cytoplasmic proteases, FtsH (Nilsson et al. 1994) and ClpP (Frees and Ingmer 1999), have been identified in *L. lactis*. ClpP is reportedly the major house keeping protease (Frees and Ingmer 1999). However, expression of L7/L12 and E7 protein in ClpP deficient strains indicated that ClpP was not involved in intracellular proteolysis of L7/L12 and E7 protein. The existence of a third, as yet unidentified protease, was postulated by studies of a ClpP mutant suppressor (Frees et al. 2001).

The nuclease (Nuc) from *S. aureus* was the first heterologous protein expressed in *L. lactis* where higher protein yields were obtained with the secreted form rather than cytoplasmic form. Similar results were obtained for the production of other heterologous proteins, such as bovine β-lactoglobulin (BLG) protein (Chatel et al. 2001), bovine rotavirus non-structural protein 4 (NSP4) (Enouf et al. 2001), the urease subunit B (UreB) gene of *Helicobacter pylori* (Lee et al. 2001), *B. abortus* ribosomal protein L7/L12 (Ribeiro et al. 2002), HPV-16 E7 protein (Bermudez-Humaran et al. 2002) and ovine interferon omega (Bermudez-Humaran et al. 2003b). The results suggested that: (1) better production yields could be expected when secretion is used versus cytoplasmic production; (2) secretion could be a way to escape intracellular proteolysis and thus secretion could help to stabilize and avoid disturbing functions of heterologous proteins (le Loir et al. 2005).

**Factors involved in protein secretion and stability**

Protein secretion was very inefficient in some cases, possibly due to inefficient precursor translocation or inefficient precursor processing. The improvement of secretion and stability of heterologous proteins produced in *L. lactis* has been researched and is now intensified by the elucidation of the genome information of many Gram-positive bacteria. The factors affecting secretion and stability of heterologous proteins produced in *L. lactis* mainly include the features of the precursor itself and host factors (le Loir et al. 2001, 2005).

**The features of the precursor**

*Nature of the signal peptide*: The SP associates with the secretion machinery and also retards precursor folding, together with the action of secretion-specific chaperones (Tjalsma et al. 2000). Secretion of a protein can vary with the SP chosen to direct its secretion (Ravn et al. 2000). To enhance protein secretion, the nature of the SP has been optimized. Although the SP primary sequences are poorly conserved, they display a common tripartite structure including a positively charged N-terminus, a hydrophobic core and a neutral or negatively charged C-terminus containing the SP cleavage site (von Heijne 1990). To date, the SP of the major lactococcal-secreted protein Usp45 (SPUsp45) is the most widely used SP to direct protein secretion (le Loir et al. 2001). A panel of new homologous protein secretion signal peptides in *L. lactis* was searched and developed by screening and mutagenesis works (Poquet et al. 1998; Ravn et al. 2000, 2003). However, compared with SPUsp45, the newly described SPs were less efficient to direct secretion of Nuc (Ravn et al. 2000, 2003). In addition, Replacement of the native SP of Nuc by SPUsp45 also resulted in greatly improved secretion of Nuc (le Loir et al. 2001). The better secretion obtained by the use of SPUsp45 may be due to a better efficient recognition of precursor containing SPUsp45.
by the lactococcal secretion machinery. However, a recent study showed that a *Lactobacillus brevis* SP (originated from a Slayer protein) drove the secretion of the *Escherichia coli* FedF adhesin more efficiently than SP 15_Usp45_ (Lindholm et al. 2004). Better secretion might thus result, at least in part, from good adequacy between the mature protein and the SP used to direct secretion. Even with the appropriate SP, secretion may be inefficient, and some heterologous proteins remain intracellularly, or are not produced at all. Indeed, secretion of the DsrD protein of *Lactobacillus sake* (Neubauer et al. 2003) to 9.8 kDa (size of Afp1, the *Streptomyces tendai* antifungal protein) (Freitas et al. 2005) through secretion-dependent machinery. This suggests that protein size is not a serious bottleneck for heterologous protein secretion in *L. lactis*. Le Loir et al. (2005) brought forward that protein conformation is a major problem for heterologous protein secretion in *L. lactis* as well in *E. coli* and *B. subtilis*, though analyzing the production of heterologous proteins: NSP4 of the bovine rotavirus (Enouf et al. 2001), BLG protein (Chatel et al. 2001; Nouaille et al. 2005) and the *B. abortus* GroEL chaperone protein (Miyoshi et al. 2006).

**Host factors**

Besides the features of the precursor itself, host factors are also involved in protein secretion and stability. Current research works are focusing on the analysis of host factors that involved in protein secretion and stability in *L. lactis*.

**Construction of proteinase-deficient *L. lactis* strains:**

To date, there are only two extracellular proteinases known in *L. lactis*: the cell-wall-anchored proteinase PrtP (200 kD) (Kunji et al. 1996) and the surface housekeeping proteinase HtrA (Poquet et al. 2000). PrtP is plasmid-encoded and does not be produced in a plasmid-free host strain (Gasson 1983). HtrA-mediated proteolysis is now well-characterized in *L. lactis* (Poquet et al. 2000) and can be overcome by use of a HtrA deficient *L. lactis* strain constructed by a single crossover recombinant event (Miyoshi et al. 2002; Lindholm et al. 2004). Expression analyses revealed that the amounts of secreted or anchored fusion proteins produced by the HtrA-deficient strain differed substantially from those produced by wild-type *L. lactis* NZ9000 (Miyoshi et al. 2002; Lindholm et al. 2004). A *L. lactis* strain deficient in both intracellular protease ClpP and extracellular protease HtrA, was constructed and called clpP-htrA. The secretion rate of two heterologous proteins (Nuc and Nuc-7E) was higher in clpP-htrA than in the wild-type strain. In addition, the clpP-htrA double mutant showed both higher stress tolerance (e.g. high temperature and ethanol resistance) and higher viability than single clpP or htrA mutant strains (Cortes-Perez et al. 2006). These proteinase-deficient *L. lactis* strains should be useful hosts for high-level and stable production of heterologous proteins.

**Complementation of secretion machinery with secretion-dedicated components:** Complete genome sequence analysis revealed that many host factors that are rapidly degraded when exposed at cell-surface as E7 (Cortes-Perez et al. 2005).

Insertion of a properly designed synthetic propeptide like LEISSTCDA could be a valuable tool for enhancing SE of heterologous proteins and has been successfully used for enhanced SE of *B. abortus* ribosomal protein L7/L12 (Ribiero et al. 2002), HPV-16 E7 protein (Bermudez-Humaran et al. 2003a).

**Protein conformation rather than protein size:** *L. lactis* is able to secrete proteins from molecular mass of 165 kDa (size of DsrD, the cell wall-anchored secretion protein) (Neubauer et al. 2003) to 9.8 kDa (size of Afp1, the *Streptomyces tendai* antifungal protein) (Freitas et al. 2005) through secretion-dependent machinery. This suggests that protein size is not a serious bottleneck for heterologous protein secretion in *L. lactis*. Le Loir et al. (2005) brought forward that protein conformation is a major problem for heterologous protein secretion in *L. lactis* as well in *E. coli* and *B. subtilis*, though analyzing the production of heterologous proteins: NSP4 of the bovine rotavirus (Enouf et al. 2001), BLG protein (Chatel et al. 2001; Nouaille et al. 2005) and the *B. abortus* GroEL chaperone protein (Miyoshi et al. 2006).

**Nature of protein N terminus:** Notably, the N terminus of the mature moiety may greatly affect the translocation efficiency across the cytoplasmic membrane and secretion of heterologous proteins in *L. lactis* can be enhanced by altering the N-terminal sequence of the mature protein (le Loir et al. 1998, 2001). Numerous secreted proteins including Nuc are synthesized as preproteins, in which the SP is followed by an N-terminal propeptide that is cleaved after translocation, giving rise to the mature protein (Shinde and Inouye 2000). Deletion of the native Nuc propeptide dramatically reduces Nuc secretion efficiency (SE) in *L. lactis*, regardless of which SP is used. However, replacement of the native Nuc propeptide by a 9-residue synthetic propeptide, LEISSTCDA, can restore or even enhance Nuc SE (le Loir et al. 1998). Introduction of this synthetic propeptide just after the SP cleavage site was also shown to enhance the SE of other heterologous proteins in *L. lactis*: the *H. pylori* (Lee et al. 2001), the ribosomal protein L7/L12 of *B. abortus* (Ribeiro et al. 2002) and the Nuc-E7 hybrid protein (Bermudez-Humaran et al. 2003a). Moreover, the synthetic propeptide insertion did not interfere with antigenic properties or biology activity of these heterologous proteins. A study demonstrated other acidic and neutral propeptides were equally effective in enhancing Nuc SE as well as LEISSTCDA, whereas basic propeptide strongly reduced Nuc SE (le Loir et al. 2001). These experiment results showed that a negative or neutral net global charge of the first amino acids of the N-terminal part favors efficient secretion (le Loir et al. 2001; Enouf et al. 2001). So the use of a SP may be necessary, but not sufficient, to guarantee efficient protein secretion and information in the mature region of a secreted protein is also important for protein secretion.
above-mentioned studies showed that secretion capacities of \textit{L. lactis} can be increased by interspecies complementation of secretion-dedicated components. The complementation of \textit{L. lactis} secretion machinery developed the above-mentioned studies can be extended to other components involved in late secretion steps, such as heterologous signal peptidases, to improve the precursor maturation step, absent in lactococci and present in other Gram-positive bacteria. Random mutagenesis approaches also can be used for the identification and characterization of genes of unknown functions specifically involved in production yields of the secreted proteins in \textit{L. lactis}. Similar approaches revealed that features of the cell wall, such as lipoteichoic acid D-alanylation, also play an important role in the protein secretion process (Nouaille et al. 2004).

\textbf{The ability of \textit{L. lactis} to modify heterologous proteins:} The capacity of heterologous proteins acquiring their native conformation is important for \textit{L. lactis} used as MVDV, since spatial structure of conformational epitopes is crucial for immune response. Nuc with a globular structure can fold properly in \textit{L. lactis}, even when they are in close proximity to the pepotidoglycan (Alexandrescu et al. 1990). Future works should investigate the ability of \textit{L. lactis} to modify heterologous proteins, such as disulfide bond formation. Proteins that require disulfide bond to acquire their native conformation, such as murine interleukin-2 (IL-2) (Steidler et al. 1995), IL-6 (Steidler et al. 1998a), IL-10 (Schotte et al. 2000), IL-12 (Bermudez-Humara et al. 2003c), ovine interferon omega (Bermudez-Humara et al. 2003b), and trefoil factors (Vandenbroucke et al. 2004) can be efficiently produced in \textit{L. lactis}. On the other hand, other proteins requiring disulfide bond formation, such as BLG and Afp1, are poorly secreted by \textit{L. lactis}, and the proportion of secreted BLG with a proper conformation has been very low (Chatel et al. 2001; Nouaille et al. 2005; Freitas et al. 2005). Although \textit{L. lactis} has the capacity to secrete proteins containing disulfide bond, the genome sequencing of \textit{L. lactis} does not reveal any lactococcal homologue of \textit{dsb} or \textit{bba}, which are the genes involved in disulfide bond formation in \textit{E. coli} and \textit{B. subtilis}, respectively. Thus, the production of proteins requiring disulfide bond formation, such as BLG and Afp1, may be still a challenge for the development of \textit{L. lactis} strains engineered for high-level production of proteins of interest. Similarly, other elements involved in post-translational modifications are still to be identified and the \textit{L. lactis} capacity for post-translational modifications is still to be investigated.

\textbf{Fusion expression to stabilize production of heterologous proteins in \textit{L. lactis}:} Instability of heterologous proteins in \textit{L. lactis} can be overcome in part by fusion. It is difficult to postulate any rule concerning the stabilization effect. Nuc is reportedly a stable protein and is the fusion partner most commonly tested so far for stabilization in \textit{L. lactis}. Stabilization by fusion to Nuc was observed for several heterologous proteins such as NSP4 (Enouf et al. 2001), E7 (Bermudez-Humara et al. 2002, 2003a), L7/L12 (Ribeiro et al. 2002), BLG (Chatel et al. 2001, 2003; Adel-Patient et al. 2005; Nouaille et al. 2005), bovine coronavirus epoiteproteine (Langella and Le Loir 1999). \textit{Lactobacillus bulgaricus} proteinase PrtB is also successfully used as fusion partner to stabilize production of BLG in \textit{L. lactis} (Bermasconi et al. 2002). Protein fusion has been successfully used to improve the production of the two subunits of heterodimeric complexes as demonstrated with murine IL-12 in \textit{L. lactis} (Bermudez-Humara et al. 2003c). Similarly, \textit{Plasmodium falicarparum} Glutamate-rich protein (GLURP) genetically coupled to Merozoite surface protein 3 (MSP3) was also successfully produced in \textit{L. lactis} as a secreted recombinant GLURP–MSP3 fusion protein (Theisen et al. 2004). The above-mentioned studies also demonstrated that both moieties of these fusion proteins expressed are still recognized by the corresponding antiserum and are immunogenic, even immunogenicity of some fusion proteins is increased. Thus protein fusion can be envisioned when \textit{L. lactis} is used as MVDV, and fusion could be a valuable strategy for future vaccine development.

\textbf{EFFICIENCY OF \textit{L. lactis} AS MVDV FOR MUCOSAL IMMUNIZATION}

\textbf{Efficiency of \textit{L. lactis} as antigen delivery vehicles for mucosal immunization}

To date, diverse vaccine components, such as bacterial antigens, viral antigens, parasitical antigens and allergens have been expressed in \textit{L. lactis} (Table 1). Most of antigens produced in \textit{L. lactis} are proteins. However, a study by Gilbert et al. (2000) showed that capsular polysaccharide antigen had also been successfully produced in \textit{L. lactis}. The potential of these recombinant strains expressing antigens as vaccines against the associated diseases has been evaluated. The results showed that mucosal immunization with these recombinant strains preloaded with vaccine components can activate the mucosal immune system to elicit protective secretory IgA antibodies and cellular immunity.

The most frequently used model antigen to test the efficiency of \textit{L. lactis} as an antigen delivery vehicle is highly immunotogenic tetanus toxin fragment C (TTFC) from \textit{Closstridium tetanus} (Norton et al. 1995, 1996, 1997; Robinson et al. 1997; Grangette et al. 2002, 2004). Mice immunized orally as well as intranasally with recombinant \textit{L. lactis} strains expressing TTFC develop significantly higher levels of protective systemic antibody IgG and protective serum antibody IgA against TTFC. These mice become more resistant to a lethal challenge with the tetanus toxin than did nonimmunized mice (Norton et al. 1996, 1997; Robinson et al. 1997; Grangette et al. 2002, 2004). Similarly, mucosal immunization with recombinant \textit{L. lactis} strains expressing the Env protein from HIV (Xin et al. 2003), the conserved C-repeat region of M protein from \textit{S. pyogenes} (Mannam et al. 2004), the MSP-1,9 from \textit{Plasmodium yoelii} (Zhang et al. 2005), the SpaA antigen from \textit{Erysipelothrix rhusiaphathiae} (Cheun et al. 2004) and the L7/L12 antigen from \textit{B. abortus} (Pontes et al. 2003), can also activate the mucosal immune system to elicit protective secretory IgA antibodies and/or cellular immunity, which significantly reduces the relevant pathogens load following challenge with pathogens. These immunized animals become more resistant to infection of relevant pathogens than did nonimmunized animals, i.e., protection against pathogens can be obtained through mucosal immunization with recombinant \textit{L. lactis} strains expressing relevant protective antigen. In conclusion, these studies confirmed that the efficiency of \textit{L. lactis} for the presentation of antigen to the mucosal immune system, to elicit a specific immune response, and mucosal immunization with these recombinant \textit{L. lactis} strains expressing protective antigen can reduce infection of relevant pathogens. They seem particularly useful for the development of vaccines against pathogens invading the body through the mucosal surface.
Table 1 Microbial antigens, allergens and cytokines expressed in \textit{L. lactis}

<table>
<thead>
<tr>
<th>Protein</th>
<th>Origin</th>
<th>Location$^1$</th>
<th>Model$^2$</th>
<th>References</th>
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<td>Bacterial antigens</td>
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<td>\textit{Helicobacter pilori}</td>
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<td>Oxine</td>
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<td>TFF</td>
<td>Mouse</td>
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<td>Co-express of antigen and cytokine</td>
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<td>TTFC + IL-2</td>
<td>Mouse</td>
<td>C+S</td>
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<td>TTFC + IL-6</td>
<td>Mouse</td>
<td>C+S</td>
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$^1$Location of protein in \textit{L. lactis}: C (cytoplasmic), S (secreted), A (anchored).
$^2$Animal model in immune trials: M (mouse), R (rabbit).

Factors affecting immune responses elicited by recombinant \textit{L. lactis} strains expressing antigens

**Immunization routes**

Immune response elicited by recombinant \textit{L. lactis} strains expressing antigens can be affected by immunization routes. Oral or nasal administration is preferable to injections from the point of view of ease of administration, safety and compliance. Furthermore, oral administration would be a practical approach for the immunization of wildlife and a large number of animals. Good results have been obtained in mice models using oral immunization in some cases (Robinson et al. 1997; Xin et al. 2003; Pontes et al. 2003; Cheun et al. 2004; Zhang et al. 2005). Studies showed that oral immunization with recombinant \textit{L. lactis} expressing the SARS coronavirus nucleocapsid protein, MSA2 antigen of \textit{P. falciparum} merozoites, led to higher levels of serum antibodies than did the corresponding nasal immunization; and intestinal antibodies to MSA2 were produced only after oral immunization (Pei et al. 2005; Ramasamy et al. 2006). The results suggest that immunization routes can influence the magnitude and type of immune response. Similarly, there are also good results obtained in mice models using nasal immunisation procedure (Norton et al. 1997; Mannam et al. 2004; Cheun et al. 2004). Therefore oral or nasal administration is useful against pathogens.

**The dose of antigen**

Immune response may be correlated to the dose of antigen delivered by recombinant \textit{L. lactis} strains. Compared with the constitutive system based on the control of a lactococcal constitutive promoter \(P_{59}\), a higher-level of E7 was obtained with the NICE system. An antigen-specific cellular response (i.e. secretion of IL-2 and interferon-gamma cytokines) was evoked and was substantially higher in mice receiving \textit{L. lactis} producing E7 with the nisin inducible system than the constitutive system based on promoter \(P_{50}\) (Bermudez-Humaran et al. 2004). This suggests a direct correlation between the amount of produced E7 and the intensity of the desired immune response. Adel-Patient et al. (2005) also observed a similar phenomenon that a direct correlation between the amount of produced BLG and the intensity of the desired immune response. Both of them are in agreement with previous study demonstrating that the immunogenicity of TTFC produced via lactobacilli depends on their production levels (Grangette et al. 2001). Thus high-level production of heterologous proteins in \textit{L. lactis} plays an important role in the use of \textit{L. lactis} as MVDV.

**The location of the antigen**

Immune response may be also correlated to the location of the antigen. In some cases, antigen export may be of interest since it allows a direct contact between the antigen and the immune system. A study by Perez et al. (2005) showed recombinant \textit{L. lactis} strains secreting VP7 proved to be more immunogenic than strains containing the antigen in the cytoplasm or anchored to the cell wall. The higher immunogenicity of antigens anchored to the cell wall of \textit{L. lactis} cells as opposed to intracellular expression also has been demonstrated. TTFC and E7 in cell-surface presentation required lower antigen doses to be immunogenic than intracellular, secreted form of TTFC and E7 (Norton et al. 1996; Reveneau et al. 2002). This was attributed either to a better accessibility to the immune system when the antigen was exposed at the cell surface, or to some adjuvant proper-
ties of *L. lactis* itself would that enhance the immunological response of hosts (Vitini et al. 2000; Adel-Patient et al. 2005). Another advantage of the anchored antigen is less exposed to degrading or denaturing agents such as proteases or acid-rich environments such as the stomach of man and animals than secreted form of antigen. The highest IgG serum antibody titers were obtained with the strain producing large amounts of TTFC in the cytoplasm (Reveneau et al. 1998). The highest immune response was elicited by administration of *L. lactis* producing an inducible cell-wall-anchored form of E7 protein (Bermudez-Humaran et al. 2004). Thus the greater immune response could thus be due to a combination of cell surface display and a dose-dependent response. Some studies focused on the cell wall presentation of the antigen and showed that the highly immune response was elicited by administration of *L. lactis* producing an inducible cell-wall-anchored form of antigen (Xia et al. 2003; Bermudez-Humaran et al. 2004; Cheun et al. 2004; Mannam et al. 2004; Pei et al. 2005; Ramasamy et al. 2006).

The cytoplasmic production can protect the antigen from proteolytic degradation and environmental stress encountered in the upper digestive tract. During intestinal transit, *L. lactis* will then be lysed, and the accumulated antigen will thus be released. Alanine racemase deficient mutant has been constructed by genetic modification of the cell wall, which renders *L. lactis* more permeable. When oral route used, Alanine racemase deficient mutants expressing TTFC were far more immunogenic than their wild type counterparts. One explanation could be that the Alamine racemase deficient mutant increase the in vivo release of cytoplasmic TTFC antigen and oral immunization is very dependant on a sufficiently large dose of the antigen (Grangette et al. 2004). The design and use of Alamine racemase deficient mutant resulted in a major improvement in the mucosal delivery of antigens and the Alamine racemase deficient mutant thus could be used as a useful host to enhance the potencial of *L. lactis* as MVDV.

**Use of *L. lactis* as vehicles for production and delivery of cytokines**

When co-administered with vaccines, adjuvant systems can promote and direct the mucosal immune response toward the desired effect. Because a number of subunit antigens are poorly immunogenic, the use of adjuvants is of particular interest for new formulations of mucosal vaccines against infectious diseases. Presently, the best-studied and most potent mucosal adjuvants in experimental systems are *V. cholerae* toxin and E. coli heat-labile enterotoxin (Dickinson and Clements 1995; Holmgren et al. 2003b), and then indeed induce potent T-helper1 (Th1) and T-helper2 (Th2) cell responses. However, these adjuvants cause severe diarrhea and are not suitable for use as mucosal adjuvants in humans. Recently, much effort has been made to develop novel mucosal adjuvants, such as cytokines, with prospects for human use. Cytokines can influence the balance between humoral and cell-mediated types of immune responses and lead to a change in immune status. But how to deliver cytokines to the immune system of humans and animals was its Achilles heel (Steidler and Rottiers 2006). The use of *L. lactis* to deliver cytokines to the mucosal surfaces may have clear adheel (Steidler and Rottiers 2006). The use of *L. lactis* to deliver cytokines to the mucosal surfaces may have clear advantages over a systemic therapy approach because it reduces toxic side effects and provides a low-cost, simple method of administration, and it may even maximize the immune response. The design of *L. lactis* for the expression of cytokines as well as antigens and the use of such recombinant strains for the redirection of the immune system have been main research focuses. Mucosal immunization with recombinant strains expressing cytokines can activate different immunologic system ways, increase the magnitude of mucosal and systemic immune responses and modulate the specificity and the immune response type (Steidler et al. 1995, 1998a, 2000, 2003; Bermudez-Humaran et al. 2003c, 2005; Vandenbroucke et al. 2004; Wu et al. 2006; Cortes-Perez et al. 2007). These studies showed that a striking fact that a pulse of cytokines have been successfully produced and delivered by recombinant lactococci and the immune responses can be potentiated and modulated by coadministration of cytokines using *L. lactis* as delivery vectors.

**Mucosal immunization with recombinant *L. lactis* expressing IL-2 or IL-6**

In general, both IL-2 and IL-6 act as potent stimulators in the onset and maintenance of immune reactions. Mice immunized intranasally with the recombinant *L. lactis* strains coexpressing TTFC and IL-2 or IL-6 produced a 10- to 15-fold higher anti-TTFC immune response than did mice immunized intranasally with the strains expressing only TTFC (Steidler et al. 1998a). This demonstrated that *L. lactis* can deliver both IL-2 and IL-6 at the respiratory mucosa in quantities, which substantially enhanced immune responses to a coexpressed antigen. This is the first example that mucosal immunization with the recombinant *L. lactis* strains expressing a cytokine to enhance immune responses to a coexpressed antigen and it points the way to immunization with the recombinant *L. lactis* strains expressing cytokines to enhance the immune response.

**Mucosal immunization with recombinant *L. lactis* expressing IL-12**

It is well known that during the pathogenesis of advanced cervical cancer the density of Th2 cells is elevated, while the level of Th1 cells is dramatically diminished (Saleh et al. 1998; Ghim et al. 2001); and the Th1/Th2 balance deregulation towards a Th2 immune response plays a central role in allergy; asthma is a chronic lung disease characterized by allergen-induced airway inflammation and orchestrated by Th2 cells. So some researchers believed that successful immunotherapeutic treatments of cervical cancer, allergy and asthma patients required vaccines that could switch the immune response from the default Th2 class to the Th1 class (Bermudez-Humaran et al. 2003b). Therefore, on the basis of this belief, *L. lactis* strains secreting IL-12 were used to enhance Th1 immune responses in a murine tumor model (Bermudez-Humaran et al. 2005), a murine model of allergy (Cortes-Perez et al. 2007) and a murine asthma model (Wu et al. 2006).

Bermudez-Humaran et al. (2005) used mucosally coadministered *L. lactis* strains expressing a secreted form of IL-12 in a cell wall-anchored form of antigen (Xia et al. 2003). *L. lactis* expressing a cell wall-anchored E7 antigen was dramatically increased by coadministration with an *L. lactis* strain secreting IL-12 protein (Bermudez-Humaran et al. 2003c, 2005). When challenged with lethal levels of tumor cell line TC-1 expressing E7, these immunized mice showed full prevention of TC-1-induced tumors, even after a second challenge, suggesting that this prophylactic immunization can provide long-lasting immunity (Bermudez-Humaran et al. 2005). This showed the adjuvant effect of a recombinant *L. lactis* strain producing IL-12 protein which can enhance the mucosal immune responses against a coadministered

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antigen, and shows that immune modulation, shifting the default Th2 response towards a Th1 response during the pathogenesis of advanced cervical cancer, is now a feasible option. The results presented also suggest that it may be possible to tailor the type of immune response elicited to antigens delivered by L. lactis, through coadministration with L. lactis strains expressing appropriate cytokines and in such way lead to an appropriate vaccination strategy against a particular pathogen. On administration of some recombinant L. lactis strains expressing BLG, a major cow’s milk allergen, was demonstrated to induce a specific Th1 response down-regulating a further Th2 one and partially prevents mice from sensitization induced by intra-peritoneal injection of BLG (Adel-Patient et al. 2005). This preventative effect was improved, and the induction of a protective Th1 response which inhibited the elicitation of the allergic reaction to BLG, was obtained in mice by co-administration of some recombinant L. lactis strain producing BLG and a second recombinant L. lactis strain producing biologically active IL-12 (Cortes-Perez et al. 2007). Intranasal administration with a recombinant L. lactis strain secreting IL-12 resulted in a shift of immune responses from Th2 to Th1, inhibited lung inflammation and reduced anaphylactic symptoms in ovalbumin -induced asthma model mice (Wu et al. 2006).

**Mucosal immunization with recombinant L. lactis expressing IL-10 or trefoil factor**

Inflammatory bowel disease is the result of breach of immune tolerance towards intestinal microbiota. In a variety of mouse models, chronic colon inflammation can be successfully treated with L. lactis strains secreting IL-10, and L. lactis strains secreting trefoil factor have also been shown to be very effective in the treatment of acute colitis (Steidler et al. 2000; Vandenbroucke et al. 2002; Glenting et al. 2002; Takala and Saris 2002; Mills et al. 2006). The existence of genes in L. lactis coding for such potential allergens and other injurious peptides can be checked beforehand searching for homologies to known allergens, as the full sequence of the bacteria and plasmid could be known. However, the route of administration of the live bacterial vaccines may also be important when evaluating hazards. Ingestion of foreign DNA occurs every day with our food, so ingestion of plasmid-encoded antigens is as such not new through oral administration (Detmer and Glenting 2006).

The use of Ab′ genes as selection markers in vaccines is not encouraged as these genes may transfer to the end humans and thus hamper the use of therapeutic antibiotics. Various alternatives to Ab′ marker genes and homologous sequences and eliminating these can limit the integrative possibility. Finally, peptides can be absorbed through the mucosa and some may induce an allergic reaction.

**LIMITATIONS OF USING L. LACTIS AS MVDV AND PROBABLE MEASURES**

Vaccination using recombinant bacteria results in the deliberate release of live recombinant organisms into nature. Furthermore, future live bacterial vaccines will most likely be either targeted mutagenised or equipped with foreign antigens and therefore considered recombinant. As such, they fall into the debate on releasing genetically modified (GM) organisms into nature and considerable safety issues against live bacterial vaccines and legitimate concerns are raised. The feasibility of this new vaccine strategy will therefore in particular depend on considerations of safety issues. Considering safety issues alongside the scientific consideration early in vaccine development will facilitate its public acceptance and its entrance to the market (Detmer and Glenting 2006).

In live bacterial vaccines, the antigen-encoding gene is either plasmid located or integrated in to the chromosome. Although L. lactis is a food-grade bacterium, this status can be compromised by all the protein expression systems mentioned above, based on high copy number plasmids, the use of foreign DNA and Ab′ genes as selection markers. Using L. lactis as MVDV may also result in the release of these bacteria in nature, as L. lactis is more suited to survive in the nature. Their recombinant nature calls for a biology containment strategy and precautions to eliminate their spread into nature. The use of auxotrophic mutants unable to replicate in the environment may be the answer. Before ever being used in vaccine, recombinant L. lactis strains will evidently need to be redesigned to reconcile medical effectiveness and biological safety.

Plasmids for heterologous gene expression are usually preferred due to its multicopy nature and higher gene dosage. However, placing the antigen encoding genes on to the bacterial chromosome may limit the spread of the foreign genes. For plasmid-encoded antigens the fate of the plasmid in the vaccine must be evaluated. Firstly, the use of a prokaryote plasmid replication unit of narrow host range can limit the probability of horizontal plasmid transfer to other bacteria present in the vaccinated individual and prevent undesired persistence of the plasmid. Furthermore, the plasmids should be evaluated for sequences facilitating integration into the human genome. The recombinant plasmid harbored by L. lactis may integrate in the genome of the recipient and potentially cause hazards. Analyzing the antigen expression pattern by L. lactis, allows elimination of homologous sequences and eliminating these can limit the integrative possibility. Finally, peptides can be absorbed through the mucosa and some may induce an allergic reaction. The use of these auxotrophic mutants unable to replicate in the environment can eliminate the corresponding safety issues of deliberate release of live recombinant L. lactis into nature. In addition, these food-grade cloning systems are stable, and do not impair growth rates and important properties of L. lactis. Thus they should be used as soon as possible in the developmental process of a vaccine.

To avoid the deliberate release of GM organisms into the environment, the use of plasmid-encoded antigens and Ab′ genes as selection markers, Steidler et al. (2003) constructed GM L. lactis, Thy12, by replacing the chromosomal thymidylate synthase gene thyd with the expression cassette for human IL-10. Thymidylate synthase is a mandatory enzyme in the synthesis of the DNA constituents thymidine and thymine. Thymidine starvation of Thy12 leads to induced cell death due to increased DNA damage and subsequent induction of SOS repair genes and fragmentation of the DNA. This phenomenon was first reported almost 50 years ago and is known as thymine-less death. Thy12 is strictly dependent on the presence of thymidine or thymine as thymine for its growth and survival, which is present in low amounts in nature and in the human body. The resulting GM L. lactis, Thy12, no longer carries its GM traits on a plasmid, nor does it require antibiotic selection for their stable inheritance. So the use of this status can be compromised by all the protein expression systems mentioned above, based on high copy number plasmids, the use of foreign DNA and Ab′ genes as selection markers. Using L. lactis as MVDV may also result in the release of these bacteria in nature, as L. lactis is more suited to survive in the nature. Their recombinant nature calls for a biology containment strategy and precautions to eliminate their spread into nature. The use of auxotrophic mutants unable to replicate in the environment may be the answer. Before ever being used in vaccine, recombinant L. lactis strains will evidently need to be redesigned to reconcile medical effectiveness and biological safety.

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