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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: **Ab^r**, 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; **SP_{Usp45}**, 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-

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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 bacteria, 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 transient bacterium in the oral and intestinal cavities (Kimoto *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 makes it an ideal host 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 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; Piard *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 inductor, 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 nisA* 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^r) 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*. 2006).

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 Cl^- (Sanders *et al*. 1998) or Zn^{2+} (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 MVDV. 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 various LAB species, including *L. lactis* (Piard *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 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 (Bermude-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 (SP_{Usp45}) 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 $SP_{Usp45,}$ 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 SP_{Usp45} also resulted in greatly improved secretion of Nuc (le Loir *et al*. 2001). The better secretion obtained by the use of SP_{Usp45} may be due to a better efficient recognition of precursor containing SP_{Usp45}

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_{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 poorly, or are not at all secreted (Chatel *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.

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 preproproteins, 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 α-amylase of *Bacillus stearothermophilus* (le Loir *et al*. 1998), BLG protein (Chatel *et al*. 2001), the UreB gene of *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 in contrast to a positive one (le Loir *et al*. 2001). However, Western blot revealed that the presence the first 15 amino acids of the Usp45 protein just after SP_{Usp45}, which resulted in the creation of a negative net charge of -3 and -1 at the E7 and E7 mutant protein(E7mm), could not favor secretion of E7 and E7mm (Cortes-Perez *et al*. 2005). But the presence the first 15 amino acids of the Usp45 protein just after SP_{Usp45}, was shown to stabilize the cell-anchored protein E7 and E7mm in *L. lactis* (Cortes-Perez *et al*. 2005). Thus secretion is not the cause of the deficiency in anchoring of E7 and E7mm without the first 15 amino acids of the Usp45 protein observed in *L. lactis*. These amino acids could represent a useful tool for the rescue of heterologous proteins 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 might optimize the charge balance around the signal cleavage site to facilitate translocation, or might affect precursor conformation to facilitate its processing by cytoplasmic secretory chaperones. Thus peptides like LEISSTCDA could be a useful tool for enhancing SE of heterologous proteins. Strikingly, the enhancement of SE was reproducibly accompanied by an overall increase of protein yields as determined in Western blot. This observation suggests that the insertion of a synthetic propeptide like LEISSTCDA could help precursor to escape the intracellular degradation thanks to a better secretion and/or could render the precursor less sensitive to intracellular degradation. Thus a better secretion could be also a way to escape proteolysis (le Loir *et al*. 2005). The combination of SP_{Usp45} and 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 (Ribeiro *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 *Leuconostoc mesenteroides* dextransucrase) (Neubauer *et al*. 2003) to 9.8 kDa (size of Afp1, the *Streptomyces tendae* 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*, through 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-E7) 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 the secretion machinery comprised fewer components in *L. lactis* than in the well-characterized secretion machinery *B. subtilis*. Unlike *B. subtilis*, *L. lactis* does not possess any SecDF equivalent, which involved in late secretion stages and is required for the high-capacity protein secretion in *B. subtilis* (Nouaille *et al*. 2006). A study by Nouaille *et al*. (2006) showed that complementation of *L. lactis* secretion machinery with SecDF from *B. subtilis* by random mutagenesis approaches had a positive effect on a secreted form of *B. abortus* L7/L12 antigen and low-level expression of *B. subtilis* secDF enhanced secretion of overproduced NucT in *L. lactis*. Similarly, a recent study by Lindholm *et al*. (2006) showed that the secretion yield of some heterologous proteins can be significantly increased in *L. lactis* when coproduced with the *B. subtilis* PrsA protein, which enhanced the yield of several homologous and heterologous exported proteins in *B. subtilis* by being involved in the posttranslocational stage of the secretion process. The

above-mentioned studies showed that secretion capacities of *L. lactis* can be increased by interspecies complementation of secretion-dedicated components. The complementation of *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 *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).

The ability of L. lactis to modify heterologous proteins: The capacity of heterologous proteins acquiring their native conformation is important for *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 *L. lactis*, even when they are in close proximity to the peptidoglycan (Alexandrescu *et al*. 1990). Future works should investigate the ability of *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-Humaran *et al*. 2003c), ovine interferon omega (Bermudez-Humaran *et al*. 2003b), and trefoil factors (Vandenbroucke *et al*. 2004) can be efficiently produced in *L. lactis*. On the other hand, other proteins requiring disulfide bond formation, such as BLG and Afp1, are poorly secreted by *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 *L. lactis* has the capacity to secrete proteins containing disulfide bond, the genome sequencing of *L. lactis* does not reveal any lactococcal homologue of *dsb* or *bdb*, which are the genes involved in disulfide bond formation in *E. coli* and *B. subtilis*, respectively. Thus, the production of proteins requiring disulfide bond formation, such as Afp1, may be still a challenge for the development of *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 *L. lactis* capacity for post-translational modifications is still to be investigated.

Fusion expression to stabilize production of heterologous proteins in *L. lactis*

Instability of heterologous proteins in *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 *L. lactis*. Stabilization by fusion to Nuc was observed for several secreted proteins such as NSP4 (Enouf *et al*. 2001), E7 (Bermudez-Humaran *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 epitopeprotein (Langella and Le Loir 1999). *Lactobacillus bulgaricus* proteinase PrtB is also successfully used as fusion partner to stabilize production of BLG in *L. lactis* (Bernasconi *et al*. 2002). Protein fusion has also been successfully used to optimize the production of the two subunits of heterodimeric complexes as demonstrated with murine IL-12 in *L. lactis* (Bermudez-Humaran *et al*. 2003c). Similarly, *Plasmodium falciparum* Glutamate-rich protein (GLURP) genetically coupled to Merozoite surface protein 3 (MSP3) was also successfully produced in *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 are increased. Thus protein fusion can be envisioned when *L. lactis* is used as MVDV, and fusion could be a valuable strategy for future vaccine development.

EFFICIENCY OF *L. LACTIS* AS MVDV FOR MUCOSAL IMMUNIZATION

Efficiency of *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 *L. lactis* (**Table 1**). Most of antigens produced in *L. lactis* are proteins. However, a study by Gilbert *et al*. (2000) showed that capsular polysaccharide antigen had also been successfully produced in *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 *L. lactis* as a antigen delivery vehicle is highly immunogenic tetanus toxin fragment C (TTFC) from *Clostridium 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 *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 *L. lactis* strains expressing the Env protein from HIV (Xin *et al*. 2003), the conserved C-repeat region of M protein from *S. pyogenes* (Mannam *et al*. 2004), the MSP-1_{19} from *Plasmodium yoelii* (Zhang *et al*. 2005), the SpaA antigen from *Erysipelothrix rhusiopathiae* (Cheun *et al*. 2004) and the L7/L12 antigen from *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 *L. lactis* strains expressing relevant protective antigen. In conclusion, these studies confirmed that the efficiency of *L. lactis* for the presentation of antigen to the mucosal immune system, to elicit a specific immune response, and mucosal immunization with these recombinant *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.

However, when mice were immunized orally recombinant *L. lactis* strains expressing the UreB gene of *H. pylori*, no protective effect was observed, which implied that *L. lactis* strains are likely to be insufficient to produce an effective immune response to protect against *H. pylori* challenge, when used to deliver a weak immunogen like UreB (Lee *et al*. 2001). Oliveira *et al*. (2006) also observed a similar phenomenon in which low levels of IgA and IgG was induced and a decrease in *Streptococcus pneumoniae* recovery was not observed in mice immunized nasally recombinant *L. lactis* MG1363 expressing the PsaA antigen of *S. pneumoniae*.

Table 1 Microbial antigens, allergens and cytokines expressed in *L. lactis*.

Protein	Origin	Location[1]	Model[2]	References
Bacterial antigens				
L7/12	*Brucella abortus*	C/S/A	M	Ribeiro *et al*. 2002; Pontes *et al*. 2003
GroEL	*Brucella abortus*	C/S	—	Miyoshi *et al*. 2006
TTFC	*Clostridium tetani*	C/S/A	M	Norton *et al*. 1995, 1996, 1997; Robinson *et al*. 1997; Grangette *et al*. 2002, 2004
M Protein	*Streptococcus pyogenes*	A	M	Mannam *et al*. 2004
UreB	*Helicobacter pilori*	S	M	Lee *et al*. 2001
SpaA	*Erysipelothrix rhusiopathiae*	S	M	Cheun *et al*. 2004
CPS	*Streptococcus pneumoniae*	S	M	Gilbert *et al*. 2000
PsaA	*Streptococcus pneumoniae*	A	M	Oliveira *et al*. 2006
Parasitical antigens				
GLURP-MSP3	*Plasmodium falciparum*	S	M	Theisen *et al*. 2004;
MSA2	*Plasmodium falciparum*	A	R	Ramasamy *et al*. 2006
$MSP\text{-}1_{19}$	*Plasmodium yoelii*	A	M	Zhang *et al*. 2005
Viral antigens				
NSP4	bovine rotavirus	C/S	R	Enouf *et al*. 2001
E7	HPV type-16	C/S/A	M	Bermudez-Humaran *et al*. 2002, 2003a, 2004; Cortes-Perez *et al*. 2003, 2005
VP7	Rotavirus	C/S/A	M	Perez *et al*. 2005
Nucleocapsid	SARS–Coronavirus	C/S	M	Pei *et al*. 2005
Env	HIV	A	M	Xin *et al*. 2003
Allergens				
Blg	Bovine	C/S/A	M	Chatel *et al*. 2001; Bernasconi *et al*. 2002; Chatel *et al*. 2003; Adel-Patient *et al*. 2005; Nouaille *et al*. 2005
Cytokines				
IL-2	Mouse	S	M	Steidler *et al*. 1995
IL-6	Mouse	S	M	Steidler *et al*. 1998a
IL-10	Mouse/Human	S	M	Schotte *et al*. 2000; Steidler *et al*. 2000, 2003
IL-12	Mouse	S	M	Bermudez-Humaran *et al*. 2003c; Wu *et al*. 2006
interferon-ω	Ovine	C/S	—	Bermudez-Humaran *et al*. 2003b
TFF	Mouse	S	M	Vandenbroucke *et al*. 2004
Co-express of antigen and cytokine				
TTFC +IL-2	Mouse	C+S	M	Steidler *et al*. 1998a
TTFC +IL-6	Mouse	C+S	M	Steidler *et al*. 1998a

[1]Location of protein in *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 *L. lactis* strains expressing antigens

Immunization routes

Immune response elicited by recombinant *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 *L. lactis* expressing the SARS coronavirus nucleocapsid protein, MSA2 antigen of *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 *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 *L. lactis* producing E7 with the nisin inducible system than the constitutive system based on promoter P_{59} (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 *L. lactis* plays an important role in the use of *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 *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 *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 that would 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*. 2002), whereas 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 cell-wall-anchored form of antigen (Xin *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 Alanine 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 Alanine racemase deficient mutant resulted in a major improvement in the mucosal delivery of antigens and the Alanine racemase deficient mutant thus could be used as a useful host to enhance the potential 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 they 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 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

IL-12, a heterodimeric glycoprotein composed of two disulfide-linked chains (p35 and p40), is an important cytokine that plays a key role in the regulation of Th1/Th2 balance. It induces Th1 and suppresses Th2 responses. IL-12 was expressed as two separate polypeptides (p35 and p40) or as a single recombinant polypeptide by linking the p35 to the p40 subunit in *L. lactis* (Bermudez-Humaran *et al*. 2003b). Intranasal administration of *L. lactis* strains producing IL-12 resulted in interferon-γ production in mice. The activity was greater with the single recombinant polypeptide.

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 (al-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*. 2003c). 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 and cell wall-anchored HPV-16 E7 antigen to treat HPV-16-induced tumors in a murine model. HPV-16 E7 antigen has been implicated in the progression of cervical cancer and is considered a potential candidate antigen for anticancer vaccine development. The antigen-specific cellular response measured by secretion of Th1 cytokines (IL-2 and interferon-γ) elicited by a recombinant *L. lactis* strain expressing a cell wall-anchored E7 antigen alone 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

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. Oral 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 preventive 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 a 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*. 2004; Steidler and Rottiers 2006).

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^r 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 encoding unit carried by *L. lactis* for human 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 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^r genes as selection markers in vaccines is not encouraged as these genes may transfer to in the end humans and thus hamper the use of therapeutic antibiotics. Various alternatives to Ab^r marker genes and food-grade cloning systems without Ab^r genes have been developed to efficiently produce proteins directly in food or in large scale fermentations (de Vos 1999b; Sorensen *et al*. 2000; Bron *et al*. 2002; Glenting *et al*. 2002; Takala and Saris 2002; Mills *et al*. 2006). Because of the absence of Ab^r genes, *L. lactis* strains using those food-grade systems maintain their food-grade status. Some food-grade cloning systems having a new attractive selection strategy which based on pyrimidine-, alanine- and threonine-auxotroph derivative *L. lactis* strains, allow cloning and efficient expression of heterologous protein in auxotroph derivative *L. lactis* strains (Sorensen *et al*. 2000; Bron *et al*. 2002; Glenting *et al*. 2002; Mills *et al*. 2006).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^r genes as selection markers, Steidler *et al*. (2003) constructed GM *L. lactis*, Thy12, by replacing the chromosomal thymidylate synthase gene *thyA* 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 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 Thy12 can avoid the deliberate release of GM organisms into the environment. Furthermore, acquirement of an intact *thyA* gene would recombine the transgene out of the genome, resulting in reversion to its wild type state. This approach may eliminate some of the drawbacks associated with the use of GM bacterial delivery systems, and has received approval from the Dutch authorities for the conduct of the first clinical trial ever that utilizes a live GM bacterium as a therapeutic.

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