29 Development of Mucosal Vaccines Based on Lactic Acid Bacteria

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29.1 Introduction

Today, sufficient data are available to support the use of lactic acid bacteria (LAB), notably lactococci and lactobacilli, as delivery vehicles for the development of new mucosal vaccines. These non-pathogenic Gram-positive bacteria have been safely consumed by humans for centuries in fermented foods. They thus constitute an attractive alternative to the attenuated pathogens (most popular live vectors actually studied) which could recover their pathogenic potential and are thus not totally safe for use in humans. This chapter reviews the current research and advances in the use of LAB as live delivery vectors of proteins of interest for the development of new safe mucosal vaccines. The use of LAB as DNA vaccine vehicles to deliver DNA directly to antigen-presenting cells of the immune system is also discussed.

29.2 Potential Applications of Mucosal Immunisation

Mucosal surfaces are the primary interaction sites between an organism and its environment and they thus represent the major portal of entry for pathogens. In the last 10 years, there have been several reports of successful immunisation with a variety of mucosal vector vaccines (Holmgren and Czerkinsky, 2005). The choice of this route of immunisation is governed by the efficiency of vaccines at different Mucosa-Associated Lymphoid Tissue (MALT): lymphoid structures associated with the nasopharynx, tonsils, salivary glands, and upper respiratory tract, termed Nasal-Associated Lymphoid Tissues (NALT), the Bronchoepithelium and Lower respiratory Tract (BALT), Gastrointestinal tract (GIT), and
male and female genital tracts (Cesta, 2006; Corr et al., 2008). Each MALT is covered by epithelium containing specialized cells known as follicle-associated epithelium or microfold (M) cells and plays an important role in the maintenance of the mucosal surface barrier and initiation of mucosal immune reactions (Corr et al., 2008). M cells transport soluble and particulate matter across the mucosal epithelium and perform sampling of luminal antigens; they thus constitute potential inductive sites to stimulate immune responses. Furthermore, mucosal immunisation also induces efficient systemic immune responses and presents less collateral side effects than systemic vaccines. Finally, mucosal immunisation is more easily performed without the need of needles and syringes and thus trained personnel (important feature for mass vaccination programs).

29.3 Brief Description of the Various Delivery Systems for Mucosal Administration

Recent advances in biotechnology and in the understanding of the immune system have now rendered possible the design of new mucosal delivery systems. Such vehicles include inert systems in which purified antigens or naked DNA are associated in microspheres, liposomes, nanoparticles, immunostimulating complexes as well as live bacterial or viral vector systems (Christensen et al., 2007; Daudel et al., 2007; Hu et al., 2001; Illum and Davis, 2001; Jennings and Bachmann, 2008; Mielcarek et al., 2001; Singh et al., 2008). Live bacteria and viruses are more immunogenic than inert vectors and thus represent better candidates to induce both mucosal and systemic immune responses against infectious agents.

Vaccinia virus and its derivatives are the most frequently used virus vaccines (Moss, 1991; Ulaeto and Hruby, 1994); however in the last years, these vectors have been progressively replaced by other poxviruses, such as canary and fowl pox viruses, and by adenoviruses (Beukema et al., 2006; Karkhanis and Ross, 2007; Patterson and Robert-Guroff, 2008). The live bacterial vectors are either based on attenuated pathogens or on non-pathogenic bacteria (Daudel et al., 2007; Wells and Mercenier, 2008). Compared to viruses genomes which are limited in their capacity to encapsulate several foreign DNA, the genomes of live bacterial vectors can harbor many such heterologous genes. Recombinant bacteria can thus produce many different heterologous antigens which may allow the development of multivalent vaccines.
29.4 **Lactic Acid Bacteria as Carrier Systems**

The immunogenicity of soluble proteins orally and intranasally administered is low and it can be significantly enhanced by either coupling the protein to a bacterial carrier or by genetic engineering of bacteria resulting in the production of the desired antigen. Attenuated pathogen bacteria such as derivatives of *Mycobacterium*, *Salmonella* and *Bordetella* spp. are particularly well adapted to interact with mucosal surfaces that most of them use to initiate the infection process. Unfortunately, these organisms could recover their pathogenic potential and are not totally safe for use in humans, especially in children and immunosuppressed patients (Alexandersen, 1996). Gram-positive food-grade or commensal bacteria (belonging to commensal flora of the human MALT) constitute an attractive alternative to attenuated pathogenic bacteria (Wells and Mercenier, 2008). In particular, the food-grade lactic acid bacteria (LAB) such as *Lactococcus lactis* and certain species of lactobacilli possess a number of properties which make them attractive candidates for the development of mucosal vaccines (Bermúdez-Humarán et al., 2004a). Indeed, LAB have been used for centuries in the fermentation and preservation of food and they are considered as safe organisms with a GRAS (Generally Recognized As Safe) status. Moreover, several antigens and/or cytokines have been successfully expressed in LAB, and mucosal
administration of these genetic engineered LAB has been shown to elicit both systemic and mucosal immunity (Table 29.2).

The production of a desired antigen by LAB can, in theory, occur in three different cellular locations: (1) intracellular, this location allows the protein to escape the drastic environmental conditions (such as gastric juices in the stomach after oral administration of the recombinant strain) but it requires cellular lysis for protein delivery; (2) extracellular, this location allows the release of the protein in external medium and thus a direct interaction with environment (food product or the digestive tract); and (3) cell surface-attached, a cellular location that combines the advantages of the first ones, i.e., interaction between the cell wall-anchored protein and the environment, and protection from proteolysis degradation. In this context, several studies have compared the production of different antigens in LAB using these three localisations and evaluated the subsequent immunological effects (reviewed in Bermúdez-Humarán et al., 2004a; Wells and Mercenier, 2008). These studies have shown that most of the highest immune responses are obtained with antigens exposed to the surface of LAB. Therefore, most of recent studies have selected surface exposure of the antigen of interest, rather than intra- or extracellular production.

29.5 **Lactococcus lactis as Live Vaccine Delivery Vector**

*Lactococcus lactis* is the most widely used LAB in the production of fermented milk products and is considered as the model LAB because many genetic tools have been developed and its complete genome is sequenced (Bolotin et al., 2001). *L. lactis* is considered as a good candidate for heterologous proteins production because it secretes relatively few proteins (van Asseldonk et al., 1993). In addition, the most commonly used laboratory strain (*L. lactis* MG1363) is plasmid-free and does not produce extracellular proteases (Gasson, 1983). However, the major advantage of the use of *L. lactis* as live vector for mucosal delivery of therapeutic proteins resides in its extraordinary safety profile since this bacterium is catalogued as a non-invasive and non-pathogenic organism with a GRAS status. Finally, the capacity of *L. lactis* to produce antigens has been clearly demonstrated in the last 2 decades (Table 29.2). These features make *L. lactis* a potential candidate for the development of new safe mucosal vaccines.
### Table 29.2

Antigens and cytokines (adjuvants) successfully expressed in lactic acid bacteria *(Cont’d p. 1104)*

| Antigens | Source                  | Vector     | Indication/Potential use                  | Reference                  |
|----------|-------------------------|------------|------------------------------------------|----------------------------|
| **Bacterial**                              |            |                                        |                            |
| PA       | Bacillus anthracis      | Lb. casei  | Anthrax vaccine                          | Zegers et al. (1999)       |
|          |                         | L. lactis  | Anthrax vaccine                          | Unpublished data           |
| LpA      | Borrelia burgdorferi    | Lb. plantarum | Lyme disease vaccine                    | del Rio et al. (2008)     |
| L7/L12   | Brucella abortus        | L. lactis  | Brucellosis vaccine                      | Ribero et al. (2002)      |
| GroEL    | Brucella abortus        | L. lactis  | Brucellosis vaccine                      | Miyoshi et al. (2006)     |
| TTFC     | Clostridium tetani      | L. lactis  | Tetanus vaccine                          | Wells et al. (1993)       |
|          |                         | Lb. casei  | Tetanus vaccine                          | Maassen et al. (1999)     |
|          |                         | Lb. plantarum | Tetanus vaccine                         | Grangette et al. (2001)  |
| ß-toxin  | Clostridium perfringens | L. lactis  | C. perfringens type B and C vaccine      | Nijland et al. (2007)     |
| K99      | Enterotoxigenic         | Lb. acidophilus | Enteric colibacillosis treatment        | Chu et al. (2005)        |
|          | Escherichia coli (ETEC)|           |                                           |                            |
| SpaA     | Erysipelothrix rhusiopathiae | L. lactis | Swine erysipelas vaccine                | Cheun et al. (2004)      |
| UreB     | Helicobacter pylori     | L. lactis  | Helicobacter vaccine                    | Lee et al. (2001)         |
|          |                         | Lb. plantarum | Helicobacter vaccine                  | Corthèsy et al. (2005)   |
| Cag12    | Helicobacter pylori     | L. lactis  | Helicobacter vaccine                    | Kim et al. (2006)         |
| FliC     | Salmonella enterica     | Lb. casei  | SE vaccine                               | Kajikawa et al. (2007)   |
|          | serovar Enteritidis (SE)|           |                                           |                            |
| PAc      | Streptococcus mutans    | L. lactis  | Dental caries vaccine                   | Iwaki et al. (1990)      |
| M6       | Streptococcus pyogenes  | L. lactis  | Dental caries vaccine                   | Mannam et al. (2004)     |
| PsaA     | Streptococcus pneumoniae| L. lactis  | Pneumococcal vaccine                    | Hanniffy et al. (2007)   |
| Antigens                        | Source                  | Vector       | Indication/Potential use                      | Reference                  |
|--------------------------------|-------------------------|--------------|----------------------------------------------|----------------------------|
| L. plantarum                   |                         |              | Pneumococcal vaccine                         |                            |
| L. helveticus                  |                         |              | Pneumococcal vaccine                         |                            |
| Pili                            | Streptococcus agalactiae GBS | L. lactis   | Streptococcal vaccine                        |                            |
|                               |                         |              |                                              |                            |
| Viral                           |                         |              |                                              |                            |
| NSP4                            | Bovine coronavirus      | L. lactis    | Coronavirus vaccine                          | Enouf et al. (2001)        |
| SARS                            | Coronavirus             | Lb. casei    | SARS-CoV vaccine                             | Lee et al. (2006)          |
| Spike glycoprotein S            | Coronavirus             | Lb. casei    | Gastroenteritis coronavirus vaccine          | Ho et al. (2005)           |
| EDIII                           | Dengue virus serotype 2 | L. lactis    | Dengue vaccine                               | Sim et al. (2008)          |
| V3                              | Human immunodeficiency  | L. lactis    | HIV vaccine                                  | Xin et al. (2003)          |
| Human papillomavirus type-16 (HPV-16) | L. lactis     |              | Cervical cancer therapeutic vaccine          | (Bermúdez-Humarán et al., 2002) |
|                                 |                         | Lb. casei    | Cervical cancer therapeutic vaccine          | Poo et al. (2006)          |
|                                 |                         | L. plantarum | Cervical cancer therapeutic vaccine          | Cortes-Perez et al. (2007) |
| L1                              | HPV-16                  | L. lactis    | Cervical cancer prophylactic vaccine         | Cho et al. (2007) and Cortes-Perez et al. (Unpublished data) |
|                                 |                         | Lb. casei    | Cervical cancer prophylactic vaccine         | Aires et al. (2006)        |
| VP2 and VP3                     | Infectious bursal       | L. lactis    | Coronavirus vaccine                          | Dieye et al. (2003)        |
|                                | disease virus (IBDV)    |              |                                              |                            |
| Cap                             | Porcine circovirus type 2 (PCV2) | L. lactis | PCV2 vaccine                                 | Wang et al. (2008)         |
| VP2                             | Porcine parvovirus      | Lb. casei    | Parvovirus vaccine                           | Xu and Li (2007)           |
| VP7                             | Rotavirus               | L. lactis    | Rotavirus vaccine                            | Perez et al. (2005)        |
| Antigens       | Source                         | Vector  | Indication/Potential use         | Reference                        |
|---------------|--------------------------------|---------|----------------------------------|----------------------------------|
| Others        |                                |         |                                  |                                  |
| MSP-1         | Plasmodium yoelii              | L. lactis| Malaria vaccine                   | Zhang et al. (2005)              |
| MSA2          | Plasmodium falciparum          | L. lactis| Malaria vaccine                   | Ramasamy et al. (2006)           |
| Sm28          | Schistosoma mansoni            | L. lactis| Schistosomiasis vaccine           | Wells et al. (1995)              |
| beta-lactoglobulin | Bovine blactoglobulin       | L. lactis| Allergy modulations              | Chatel et al. (2001)             |
|               |                                |         |                                  | Hazebrouck et al. (2006)          |
| Der p 5 allergen | Dermatophagoides pteronyssinus | Lb. acidophilus| Allergy treatment              | Charng et al. (2006)             |
| CWP2          | Giardia lamblia                | L. lactis| Giardiasis vaccine               | Lee and Faubert. (2006)          |
| Cytokines     |                                |         |                                  |                                  |
| IL-2          | Mus musculus                   | L. lactis| TTFC vaccine adjuvant             | Steidler et al. (1995)           |
| IL-6          | Mus musculus                   | L. lactis| TTFC vaccine adjuvant             | Steidler et al. (1998)           |
| IL-10         | Mus musculus                   | L. lactis| Colitis treatment                | Steidler et al. (2000)           |
|               | Homo sapiens                   | L. lactis| Crohn's disease treatment        | Steidler et al. (2003)           |
| IL-12         | Mus musculus                   | L. lactis| E7 vaccine adjuvant              | Bermúdez-Humarán et al. (2003a)  |
|               |                                | Lb. plantarum| E7 vaccine adjuvant             | Bermúdez-Humarán et al. Unpublished data |
| IFN-ω         | Mus musculus                   | L. lactis| Antiviral treatment              | Bermúdez-Humarán et al. (2003b)  |
| IFN-γ         | Mus musculus                   | L. lactis| Antiviral/antitumoral treatment  | Bermúdez-Humarán et al. (2008)   |
| Sus scrofa    | L. lactis                      |         | Antiviral/antitumoral treatment  | Rupa et al. (2008)               |
Immune Response to Antigens Delivered by Lactococcus lactis

Today, a number of studies support the use of recombinant *L. lactis* to induce mucosal and systemic immune response against a desired antigen (Bermúdez-Humarán et al., 2004a). The first attempt to analyze the potential of *L. lactis* as mucosal vaccine was performed with killed recombinant lactococci producing a cell wall-attached form of a *Streptococcus mutans* protective antigen (PAc). Mice immunized orally with this recombinant strain developed PAc-specific serum IgG and mucosal IgA antibodies (Iwaki et al., 1990). These results showed for the first time that *L. lactis* can be used as a delivery vector to present an antigen to the immune system. However, Wells et al. (1993) reported for the first time the use of live recombinant *L. lactis*, producing the tetanus fragment C (TTFC), to protect mice via subcutaneous injection against a lethal challenge with tetanus toxin. Later, the same group evaluated the effect of oral or intranasal administration with live recombinant lactococci producing TTFC in mice (Norton et al., 1997; Robinson et al., 1997). Oral immunization in mice resulted in a lower serum IgG and mucosal IgA antibodies response than nasal immunization, whereas the protective efficacy (i.e., challenge with tetanus toxin) was the same.

Several studies were conducted to analyze the expression of many viral, bacterial or eukaryotic heterologous proteins in *L. lactis* (Bermúdez-Humarán et al., 2004a and Table 29.2). The immunogenicity of the resulting recombinant strains has been evaluated in some cases in mouse models with very promising results. Among them, one of the best documented projects is based on the use of recombinant *L. lactis* producing Human Papillomavirus type-16 (HPV-16) E7 antigen.

### Table 29.2

| Antigens | Source      | Vector | Indication/Potential use                  | Reference                          |
|----------|-------------|--------|------------------------------------------|------------------------------------|
| IFN-β    | *Homo sapiens* | *L. lactis* | Antiviral/anti-inflammatory treatment     | Zhuang et al. (2008)               |
| MIG/IP-10 | *Mus musculus* | *L. lactis* | Novel vaccine adjuvant                   | Cortes-Perez et al. (2008)         |
| Leptin   | *Homo sapiens* | *L. lactis* | Novel vaccine adjuvant                   | Bermúdez-Humarán et al. (2007)     |
This viral protein is considered as a major candidate antigen for vaccines against HPV-related cervical cancer, the second cause of cancer death in women. The intracellular production of E7 antigen model led to its rapid degradation in the cytoplasm of *L. lactis* even when produced in a protease-free strain (Bermúdez-Humarán et al., 2002). In contrast, secreted and cell wall-anchored forms are rescued from proteolysis and produced a higher level of E7 in *L. lactis* (Bermúdez-Humarán et al., 2002, 2004b). Antigen-specific humoral (production of E7 antibodies) and cellular (secretion of IL-2 and IFN-γ cytokines) responses were observed after intranasal administrations to mice of recombinant lactococci expressing E7 antigen at different levels and cellular locations. They were significantly higher in mice immunized with *L. lactis* expressing E7 as a cell wall-anchored form (Bermúdez-Humarán et al., 2004b). These first reports of E7 production in a food-grade LAB represent one more step towards the development of a therapy against HPV-related cervical cancer. Indeed, the protective effects of mucosally co-administered live *L. lactis* strains expressing cell wall-anchored E7 and a secreted form of interleukin-12 to treat HPV-16-induced tumors in a murine model were then evaluated (Bermúdez-Humarán et al., 2005). When challenged with lethal levels of tumor cell line TC-1 expressing E7, 50% of pre-treated mice showed full prevention of TC-1-induced tumors. Therapeutic immunization with these recombinant strains, i.e., 7 days after TC-1 injection, induced regression of palpable tumors in 35% of treated mice. These preclinical results suggest the feasibility of mucosal vaccination and/or immunotherapy against HPV-related cervical cancer using genetically engineered lactococci.

Although most immunological studies have been performed with *L. lactis* producing TTFC and E7 antigen, the reports supporting recombinant lactococci as mucosal vaccine continue to grow, and today, approximately 50 peer-reviewed publications validated this potential (Table 29.2).

### 29.7 Lactobacilli as Live Vaccine Delivery Vector

In contrast to lactococci, some lactobacilli species can persist longer in the GIT and sometimes colonize certain regions of the mucosa and induce a local immune response. A second benefit of the use of lactobacilli is that some strains are considered probiotics (i.e., show health-promoting activities for humans and animals) (Seegers, 2002). Indeed, this genus is widespread and contains over 60 species differing in biochemical, ecological and immunological properties. This biodiversity...
rendered the use of *Lactobacillus* spp. as vaccine vehicles more complex compared to *L. lactis*, for which only one single strain (MG1363) was used. However, the capacity of the genus *Lactobacillus* to produce antigens has also been demonstrated.

### 29.8 Immune Response to Antigens Delivered by *Lactobacillus* spp

The use of genetically modified lactobacilli (i.e., *L. fermentum*, *L. acidophilus*, *L. casei* and *L. plantarum*) to produce heterologous proteins and to develop a new generation of mucosal vaccines was first proposed in the 90s decade (Pouwels et al., 1996; Rush et al., 1995). By the end of the 1990s and early 2000s, several laboratories used recombinant strains of *Lb. casei* and *Lb. plantarum* as vehicles for medical proteins delivery at mucosal surfaces; both stimulated strong local immune responses (reviewed in Seegers, 2002; Wells and Mercenier, 2008). Approximately 30 peer-reviewed publications have been published confirming the advantages of the genus *Lactobacillus* as a live mucosal vaccine.

As for *L. lactis*, several studies were also conducted to analyze the expression of a variety of viral, bacterial or eukaryotic origins in *Lb. plantarum* and *Lb. casei* (Table 29.2). The immunogenicity of recombinant *Lb. plantarum* producing E7 antigen has been evaluated in mouse models with promising results (Cortes-Perez et al., 2007).

### 29.9 Recombinant Lactic Acid Bacteria as DNA Delivery Vehicles

In contrast to bacteria-mediated delivery of protein antigens, bacteria-mediated delivery of DNA vaccines leads to the expression of post-translationally modified antigens by the host cells and therefore to the presentation of conformationally restricted epitopes (Fouts et al., 2003). As for protein delivery, the use of food-grade LAB as DNA delivery vehicles is a promising alternative to attenuated pathogens as DNA vaccines carriers.

*L. lactis* strains have been used to deliver an expression cassette encoding for bovine β-lactoglobulin (BLG) cDNA, one of the major cow’s milk allergen, under the transcriptional control of the viral promoter CMV into the epithelial cell line Caco-2. The expression cassette was inserted in one *L. lactis* replicating plasmid.
Production and secretion of BLG was observed in Caco-2 cells after incubation with *L. lactis* carrying the expression plasmid, demonstrating that non invasive *L. lactis* is able to deliver fully functional plasmid into epithelial cells. Interestingly, no production of BLG was observed when Caco-2 cells were co-incubated with purified plasmid alone or mixed with *L. lactis*, suggesting that the plasmid should be inside the bacterium to achieve transfer and subsequent BLG production into epithelial cells (Guimaraes et al., 2006).

After oral administration of *L. lactis* carrying the eukaryotic expression cassette encoding for BLG, BLG cDNA and protein were detected in the small intestine 72 h after the last administration. No BLG was detected 6 days after the last oral administration. The mice developed a BLG specific Th1 primary immune response characterized by a weak and transitory IgG2a response in serum. In sensitized pre-treated mice, IgE and IL-5 concentrations decreased 70 and 40% respectively compared to sensitized naive mice. Moreover, only splenocytes from pre-treated mice secreted IFN-γ after BLG specific re-activation (Chatel et al., 2008). Mice were effectively protected against further sensitization by a specific Th1 response.

Immune response to *L. acidophilus* carrying a DNA vaccine against VP1 antigen of food-and-mouth-diseases virus (FMDV) was investigated after administration by systemic and mucosal routes. The route of administration had a significant impact on the magnitude of the systemic immune response. Indeed, strong immune response to the vaccine antigen was detected only for injected routes of administration although mucosal administration could prime a specific immune response. The intramuscular administration generated the highest level of FMDV VP1 antibodies followed by the intraperitoneal, intranasal and oral routes (Li et al., 2007).

### 29.10 Recombinant Invasive Lactic Acid Bacteria as DNA Delivery Vehicles

As demonstrated with recombinant *E. coli*, invasion of the host cell is a limiting step to achieve an efficient DNA vaccine delivery (Grillot-Courvalin et al., 1998). To increase LAB DNA vaccine delivery efficiency, *L. lactis* has been modified in order to become invasive by expression of the *InlA* gene of *Listeria monocytogenes* *InlA* gene coding for the Internalin A surface protein, which mediates the invasion of non phagocytic cells by *Listeria monocytogenes* (Gaillard et al., 1991; Mengaud et al., 1996). InlA binds to an extracellular domain of E-cadherin,
a transmembrane cell-to-cell adhesion molecule (Mengaud et al., 1996). InlA is necessary for invasion of epithelial cells and is sufficient to reconstitute invasion when expressed in non-pathogenic and non-invasive species of *Listeria innocua* (Mengaud et al., 1996). Moreover, when InlA is expressed in *L. lactis*, it can promote the internalization of lactococci into the human epithelial line Caco-2 *in vitro* and into enterocytes *in vivo* after oral administration to guinea pigs. In addition, *L. lactis* InlA+ is able to deliver a functional plasmid coding for GFP and about 1% of Caco-2 cells express GFP after co-culture with this strain (Guimarães et al., 2005).

**29.11 Methodologies and Techniques for Genetic Manipulations of Lactic Acid Bacteria**

**29.11.1 Genetic Engineering of LAB to Produce Heterologous Proteins**

The expression system used (based on constitutive or inducible promoters) is an important feature that must be considered for *in vivo* delivery by live bacterial vectors. High production level of heterologous proteins in *L. lactis* can be achieved using constitutive promoters. However, continuous high-level production of certain proteins, such as cytokines (our observations) could lead to intracellular accumulation or degradation which could be deleterious to the cell. Thereby, to prevent possible negative effects caused by high production, inducible promoters have been developed. In these systems, gene expression can be controlled by an inductor, a repressor or by environmental factors, such as pH, temperature or ion concentrations (Morello et al., 2008).

Today, one of the best characterized expression systems is the NICE (*Nisin-Controlled Expression*) system (Mierau and Kleerebezem, 2005). NICE is a system that allows controlled gene expression by addition of nisin, an antimicrobial peptide used as a natural preservative in the food industry. With this versatile system, the level of gene expression can be controlled by the amount of nisin used for the induction and can be up-regulated more than 1,000-fold (Mierau and Kleerebezem, 2005). As *L. lactis* is a non-colonizing bacterium, therefore, a system that allows preload of the organism with the antigen before *in vivo* application is highly desirable. Induction with NICE system can be considered as a good strategy to obtain high levels of heterologous antigen production *in vitro*. In addition, even after an *in vitro* nisin-pulse, recombinant *L. lactis*
continue to produce heterologous proteins and to evoke an antigen-specific response when administered in mice (Bermúdez-Humarán et al., 2003b, 2004b). These observations support the use of NICE system for the expression of heterologous proteins in *L. lactis*.

Several delivery systems have been developed to target heterologous proteins at different levels and cellular locations within *L. lactis* (Bermúdez-Humarán et al., 2004a; Wells and Mercenier, 2008; Wells et al., 1995). In this context, a family of new vectors which allow heterologous antigens expression in *L. lactis* either intracellularly, extracellularly or cell wall-attached were designed (Bermúdez-Humarán et al., 2003b; Cortes-Perez et al., 2003). These small vectors, based on the broad-host-range pGK plasmid (Kok et al., 1984), are composed of cassettes that allow easy exchange of different expression signals and/or genes. Moreover, as these vectors have the capacity to replicate in Gram-positive bacteria (including *Bacillus subtilis*, *L. lactis* and *Lactobacillus* spp.) and *Escherichia coli*, the procedure of DNA cloning (sometimes laborious in Gram-positive organisms) can be performed in *E. coli*. Once the recombinant vector is established in this bacterium, it can be transferred to the desired Gram-positive bacterium. Most importantly, these vectors have been used to produce successfully different heterologous antigens in *L. lactis* (Table 29.2). A well illustrated example of the efficiency of our system to produce heterologous antigens is the expression of E7 antigen from human papillomavirus type-16 (HPV-16). Initially, DNA plasmid constructions were performed in *E. coli* for E7 expression at different levels and cellular locations using the family of vectors described above (i.e., pCYT:E7, pSEC:E7 and pCWA:E7). After confirmation of the sequence of these recombinant vectors, they were transferred successfully to *L. lactis* and E7 production was evaluated by western blot analysis.

### 29.11.2 Transformation of LAB

The following protocol to prepare electrocompetent cells of *L. lactis* (1 × 10^7 CFU/µg DNA, approximately) is recommended: the strain is grown overnight in 5 ml of M17 medium (Difco) supplemented with 0.5% of glucose (GM17), 0.5 M sucrose and 2% glycine (GM17SG) at 30°C without agitation. Then 1/200 from this culture is inoculated in 200 ml of the same medium and incubated at 30°C until optical density (DO_{600}) at 0.5–0.8 is reached. The culture is then incubated immediately in ice for 15 min and stirred every 5 min before the cells are pelleted by centrifugation at 7,000 rpm for 10 min at 4°C. Cellular pellet is washed twice in
100 ml of washed buffer (0.5 M sucrose, 10% glycerol). A third washing is performed with 20 ml of the same buffer. The final pellet is resuspended in 1 ml of PEG-Gly (polyethylene-glycol 3000, 10% glycerol). Aliquots of 100 µl are made in 1.5 ml microcentrifuge tubes, frozen immediately in liquid nitrogen and stored at −80°C until further use. For the transformation, 100 µl of electrocompetent cells are mixed with 1 µl of DNA (10 µl in the case of ligation), transferred to chilled electroporation cuves (2 mm), and exposed to a single electric pulse (Gene-Pulser, BIORAD Laboratories), 25 µF, 200 Ω, 2.4 kV. Immediately after electric discharge, 900 µl of medium GM17S (GM17, 0.5 M sucrose) are added and incubated for 1 h to plasmid expression. Finally, different dilutions are plated in GM17 (1% agar) plus the antibiotic marker. Recombinant colonies are selected after 48 h of incubation at 30°C.

For Lactobacillus spp., we have adapted the protocol used for L. lactis; briefly, an overnight culture of the strain of Lactobacillus (we have tested successfully three different Lactobacillus species) are grown in MRS medium (DIFCO) supplemented with 1% of glycine (MRSG) at 37°C without agitation. Then 1/20 of the culture is inoculated in 200 ml of the same medium and culture continued at 37°C until DO₆₀₀= 0.6–0.7. The culture is then incubated immediately in ice for 15 min and stirred every 5 min before pelleting the cells by centrifugation at 7,000 rpm for 8 min at 4°C. The pellet is washed once with 1 volume of 1 mM of cold magnesium chloride (MgCl₂) and once with 1 volume of cold polyethylene glycol 3000, glycerol 10% (PEG-Gly). The final pellet is suspended in 1/100 of the initial volume with PEG-Gly. Aliquots of 50 µl are immediately frozen in liquid nitrogen and stored at −80°C until use. The optimal conditions of the electrotransformation are: a single electric pulse (Gene-Pulser, BIORAD Laboratories), 25 µF, 400 Ω, 1.5 kV with a chilled electroporation cuve (1 mm). Immediately after the electric shock, 500 µl of MRSSM medium (MRS, plus 500 mM sucrose, 100 mM MgCl₂) are added and incubated for 3 h for plasmid expression. Finally, different dilutions are plated in MRS (agar 1%) plus the antibiotic. Recombinant clones are selected after 48–72 h of incubation at 37°C.

29.11.3 Nisin Induction, Protein Samples Preparation and Immunoblotting for LAB

Expression of a desired antigen in L. lactis using NICE system is recommended as follows: heterologous protein expression can be performed using either 1
or 10 ng/ml of nisin (SIGMA) for a 1- or 3-h period as previously described (Bermúdez-Humarán et al., 2002, 2003c). Protein samples are then prepared from 2 ml of induced cultures. Cell pellet and supernatant are treated separately. To inhibit proteolysis in supernatant samples, 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol are added. Proteins are precipitated by addition of 100 µl of 100% trichloroacetic acid, incubated 10 min on ice, and centrifuged 10 min at 13,000 rpm at 4°C. For the cell fraction, TES- Lys buffer (25% sucrose, 1 mM EDTA, 50 mM Tris-HCl [pH 8.0], lysozyme [10 mg/ml]) is complemented with 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, western blotting, and immunorevelation with antibodies can be then performed.

For lactobacilli, induction is essentially performed as follows: an overnight culture is diluted 1/20 and after 1 h of growth, nisin is added at 25 ng/ml and the culture continued for 5 h. Protein extractions and immunoblotting assays with antibodies are performed as previously described.

### 29.11.4 Immunofluorescence Microscopy (IFM)

To corroborate protein surface expression in lactococci and lactobacilli, recombinant strains are grown and induced as described above and analysed by immunofluorescent microscopy (IFM). For this, at the end of the induction phase, 2 ml of culture are harvested and suspended in 1 ml of sterile PBS-BSA (bovine serum albumin 3%) containing the corresponding antibody (1/500) and incubated overnight at room temperature. After three washes with PBS-T (PBS-Tween 0.05%), the cell-antibody complex is incubated for 5 h at room temperature (avoiding light exposure) with a solution (1/50 dilution in PBS-BSA) of goat-derived anti-mouse immunoglobulin G (IgG, H + L), conjugated to Alexa Fluor 546 dye (Molecular Probes, Europe BV). Cells are washed three times in PBS-T and the pellet is suspended in 100X µl of PBS from final DO₆₀₀. Afterwards, different dilutions are performed to determine the optimal quantities to obtain a clear field in the microscopy, usually 2 µl from a dilution 1:10. To visualize the entire cell population, bacteria are stained with 40,6-diamidino-2-phenylindole (DAPI, 2.5 mg/ml; SIGMA) laid on a glass slide, air dried and heat fixed. Pictures of cells are taken with an IFM equipped with a three band filter set for emission light (Nikon, Tokyo, Japan) and Sensia 400 film (Fuji, Tokyo, Japan). Filters appropriate for red excitation light are used to visualize cells, which
are stained with an Alexa Fluor 546 fluorophore. In addition, bacterial images are taken without a filter for excitation light; this allows cells stained with DAPI and Alexa Fluor 546 to be compared simultaneously. Hybridized cells are counted on images captured with the image analysis system Visiolab 1000 (Biocom, Les Ulis, France).

29.11.5 Preparation of Live Bacterial Inoculum and Immunization Protocol

Bacterial cultures are induced as described above and cell pellets are harvested and washed three times with sterile PBS. The pellets are suspended in 10 μl of PBS to obtain a final concentration of 1 × 10⁹ colony-forming units (CFU). Three mice (6–8 weeks) are immunized intranasally with 1 × 10⁹ CFU of induced recombinant LAB strains (5 μl are administered with a micropipette into each nostril) on days 0, 14 and 28. Mice are partially anesthetized by intra-peritoneal injection of a combination of xylazine and ketamine (0.40 ml for 10 kg of weight). Plate counts are performed to check the amount of CFU administered. The control mice received identical quantities of wild-type LAB strain.

29.11.6 Invasiveness Assays of Bacteria into Human Epithelial Cells

Bacterial entry into human epithelial cells was assayed using the human colon carcinoma cell line Caco-2 (ATCC number HTB37), as described by Dramsi et al. (1995). Eukaryotic cells were cultured in RPMI supplemented with 2 mM L-glutamine (BioWhittaker, Cambrex Bio Science, Verviers, Belgium) and 20% fetal calf serum (FCS). The gentamicin survival assay was used to estimate bacteria survival: *L. lactis* strains were grown to an OD<sub>600</sub> of 0.9–1.0, washed in PBS, and diluted such that the multiplicity of infection (MOI) was about 1,000 bacteria per cell. The bacterial suspension was added to mammalian cells grown in P-24 plates (Corning Glass Works). 2 × 10⁵ cells were seeded in each well the day before the experiment. After 1 h of contact (internalization), gentamicin (20 mg/l) was added to the culture medium. After 2 h of incubation, the cells were washed, then lysed in 0.2% Triton-X100, and serial dilutions of the lysate were plated for bacterial counting. Gentamicin invasiveness assays were done in triplicate.
29.12 Conclusion

Therapeutic applications of LAB have progressed rapidly in the last years, and following the demonstration that IL-10-producing LAB (i.e., *L. lactis*) could treat colitis in mouse models (Steidler et al., 2000) a successful phase I clinical trial was recently conducted in patients with Crohn’s disease (Braat et al., 2006). However, before the approval of this clinical study the development of a containment system for the genetically modified *L. lactis* was necessary. To address safety concerns with the use of IL-10-secreting *L. lactis* in humans, the chromosomal thymidylate synthase (*thyA*) gene was replaced by the gene encoding for IL-10 to generate a thymine auxotroph phenotype. Viability of the *thyA* hIL-10\(^+\) strain was reduced by several orders of magnitude in the absence of thymidine or thymine and containment was validated *in vivo* in pigs (Steidler et al., 2003). Strikingly, the phase I clinical trial conducted with the *thyA* hIL10\(^+\) strain in patients with Crohn’s disease showed that the containment strategy was effective (Braat et al., 2006). These studies open new doors for the use of recombinant LAB as delivery vehicles in the future.

Other exciting applications on the horizon concern the delivery of DNA vaccines using LAB (Chatel et al., 2008), allergen-specific immunotherapy of allergic diseases (Huibrengtse et al., 2007) and anti-infectives molecules such as scFv antibodies and microbiocides (Chang et al., 2003; Chancey et al., 2006; Krüger et al., 2002; Liu et al., 2006). With the possibility to express factors such as ScFv antibodies, host targeting molecules and immunomodulators in LAB, we can hope to see more applications and progress towards studies in humans.

References

Aires KA, Cianciarullo AM, Carneiro SM, Villa LL, Boccardo E, Pérez-Martinez G, Perez-Arellano I, Oliveira ML, Ho PL (2006) Production of human papillomavirus type 16 L1 virus-like particles by recombinant *Lactobacillus casei* cells. Appl Environ Microbiol 72:745–752

Alexandersen S (1996) Advantages and disadvantages of using live vaccines risks and control measures. Acta Vet Scand Suppl 90:89–100

Bermúdez-Humará L, Langella P, Miyoshi A, Gruss A, Guerra RT, Montes de Oca-Luna R, Le Loir Y (2002) Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. Appl Environ Microbiol 68:917–922

Bermúdez-Humará L, Langella P, Cortes-Perez NG, Gruss A, Támez-Guerra RS, Oliveira SC, Saucedo-Cardenas O, Montes de Oca-Luna R, Le Loir Y (2003a) Intranasal immunization with recombinant
Lactococcus lactis secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. Infect Immun 71:1887–1896

Bermúdez-Humaran LG, Langella P, Commis-saire J, Gilbert S, Le Loir Y, L’Haridon R, Corthier G (2003b) Controlled intrac- or extracellular production of staphylococcal nuclease and ovine omega interferon in Lactococcus lactis. FEMS Microbiol Lett 224:307–313

Bermúdez-Humaran LG, Cortes-Perez NG, Le Loir Y, Gruss A, Rodriguez-Padilla C, Saucedo-Cardenas O, Langella P, Montes de Oca-Luna R (2003c) Fusion to a carrier protein and a synthetic propeptide enhances E7 HPV-16 production and secretion in Lactococcus lactis. Biotechnol Prog 19:1101–1104

Bermúdez-Humaran LG, Corthier G, Langella P (2004a) Recent advances in the use of Lactococcus lactis as live recombinant vector for the development of new safe mucosal vaccines. Recent Res Devel Microbiol 8:147–160

Bermúdez-Humaran LG, Cortes-Perez NG, Le Loir Y, Alcocer-González JM, Tamez-Guerra RS, de Oca-Luna RM, Langella P (2004b) An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci. J Med Microbiol 53:427–433

Bermúdez-Humaran LG, Cortes-Perez NG, Lefèvre F, Guimarães V, Rabot S, Alcocer-Gonzalez JM, Gratadoux JJ, Rodriguez-Padilla C, Tamez-Guerra RS, Corthier G, Gruss A, Langella P (2005) A novel mucosal vaccine based on live Lactococci expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papillomavirus type 16-induced tumors. J Immunol 175:7297–7302

Bermúdez-Humaran LG, Nouaille S, Zilberfarb V, Corthier G, Gruss A, Langella P, Issad T (2007) Effects of intranasal administration of a leptin-secreting Lactococcus lactis recombinant on food intake, body weight, and immune response of mice. Appl Environ Microbiol 73:5300–5307

Bermúdez-Humaran LG, Cortes-Perez NG, L’Haridon R, Langella P (2008) Production of biological active murine IFN-gamma by recombinant Lactococcus lactis. FEMS Microbiol Lett 280:144–149

Beukema EL, Brown MP, Hayball JD (2006) The potential role of fowlpox virus in rational vaccine design. Expert Rev Vaccines 5:565–577

Bozolar A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissbach J, Ehrlich SD, Sorokin A (2001) The complete genome sequence of the lactic acid bacterium Lactococcus lactis ssp. lactis IL1403. Genome Res 11:731–753

Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJ, Neirynck S, Peppelenbosch MP, Steidler L (2006) A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn’s disease. Clin Gastroenterol Hepatol 4:754–759

Brahmbhatt HN, Lindberg AA, Timmis KN (1992) Shigella lipopolysaccharide: structure, genetics, and vaccine development. Curr Top Microbiol Immunol 180:45–64

Buccato S, Maione D, Rinaudo CD, Volpini G, Taddei AR, Rosini R, Telford JL, Grandi G, Margarit I (2006) Use of Lactococcus lactis expressing pili from group B Streptococcus as a broad-coverage vaccine against streptococcal disease. J Infect Dis 194 (3):331–340

Cesta MF (2006) Normal structure, function, and histology of mucosa-associated lymphoid tissue. Toxicol Pathol 34:599–608

Chancey CJ, Khanna KV, Seegers JF, Zhang GW, Hildreth J, Langan A, Markham RB (2006) Lactobacilli-expressed single-chain variable fragment (scFv) specific for intercellular adhesion molecule 1 (ICAM-1) blocks cell-associated HIV-1
transmission across a cervical epithelial monolayer. J Immunol 176:5627–5636

Chang TL, Chang CH, Simpson DA, Xu Q, Martin PK, Lagenaur LA, Schoolnik GK, Ho DD, Hillier SL, Holodniy M, Lewicki JA, Lee PP (2003) Inhibition of HIV infectivity by a natural human isolate of Lactobacillus jensenii engineered to express functional two-domain CD4. Proc Natl Acad Sci USA 100:11672–11677

Chang YC, Lin CC, Hsu CH (2006) Inhibition of allergen-induced airway inflammation and hyperreactivity by recombinant lactic-acid bacteria. Vaccine 24:5931–5936

Chatel JM, Langella P, Adel-Patient K, Commissaire J, Wal JM, Corthier G (2001) Induction of mucosal immune response after intranasal or oral inoculation of mice with Lactococcus lactis producing bovine beta-lactoglobulin. Clin Diagn Lab Immunol 8:545–551

Chatel JM, Pothelune L, Ah-Leung S, Corthier G, Wal JM, Langella P (2008) In vivo transfer of plasmid from food-grade transiting lactococci to murine epithelial cells. Gene Ther 15:1184–1190

Cheun HI, Kawamoto K, Hiramatsu M, Tamaoki H, Shirahata T, Igimi S, Makino SI (2004) Protective immunity of SpaA-antigen producing Lactococcus lactis against Erysipelothrix rhusiotpathiae infection. J Appl Microbiol 96:1347–1353

Cho HJ, Shin HJ, Han IK, Jung WW, Kim YB, Sul D, Oh YK (2007) Induction of mucosal and systemic immune responses following oral immunization of mice with Lactococcus lactis expressing human papillomavirus type 16 L1. Vaccine 25:8049–8057

Christensen D, Korsholm KS, Rosenkrands I, Lindenstrøm T, Andersen P, Agger EM (2007) Cationic liposomes as vaccine adjuvants. Expert Rev Vaccines 6:785–796

Chu H, Kang S, Ha S, Cho K, Park SM, Han KH, Kang SK, Lee H, Han SH, Yun CH, Choi Y (2005) Lactobacillus acidophilus expressing recombinant K99 adhesive fimbriae has an inhibitory effect on adhesion of enterotoxigenic Escherichia coli. Microbiol Immunol 49:941–948

Corr SC, Gahan CC, Hill C (2008) M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. FEMS Immunol Med Microbiol 52:2–12

Cortes-Perez NG, Bermúdez-Humárn LG, Le Loir Y, Rodriguez-Padilla C, Gruss A, Saucedo-Cárdenas O, Langella P, Montes-de-Oca-Luna R (2003) Mice immunization with live lactococci displaying a surface anchored HPV-16 E7 oncoprotein. FEMS Microbiol Lett 229:37–42

Cortes-Perez NG, da Costa Medina LF, Lefèvre F, Langella P, Bermúdez-Humárn LG (2008) Production of biologically active CXC chemokines by Lactococcus lactis: evaluation of its potential as a novel mucosal vaccine adjuvant. Vaccine 26:5778–5783

Cortes-Perez NG, Lefèvre F, Corthier G, Adel-Patient K, Langella P, Bermúdez-Humárn LG (2007) Influence of the route of immunization and the nature of the bacterial vector on immunogenicity of mucosal vaccines based on lactic acid bacteria. Vaccine 25:6581–6588

Cortheşy B, Boris S, Isler P, Grangette C, Mercenier A (2005) Oral immunization of mice with lactic acid bacteria producing Helicobacter pylori urease B subunit partially protects against challenge with Helicobacter felis. J Infect Dis 192:1441–1449

Curtiss R III, Kelly SM, Tinge SA, Tacket CO, Levine MM, Srinivasan J, Koopman M (1994) Recombinant Salmonella vectors in vaccine development. Dev Biol Stand 82:23–33

Daudel D, Weidinger G, Spreng S (2007) Use of attenuated bacteria as delivery vectors for DNA vaccines. Expert Rev Vaccines 6:97–110

Dieye Y, Hoekman AJ, Clier F, Juillard V, Boot HJ, Piard JC (2003) Ability of Lactococcus lactis to export viral capsid
antigens: a crucial step for development of live vaccines. Appl Environ Microbiol 69: 7281–7288
Dramsi S, Biswas I, Maguin E, Braun L, Mastroeni P, Cossart P (1995) Entry of Listeria monocytogenes into hepatocytes requires expression of inIB, a surface protein of the internalin multigene family. Mol Microbiol 16:251–261
Enouf V, Langella P, Commissaire J, Cohen J, Corthier G (2001) Bovine rotavirus non-structural protein 4 produced by Lactococcus lactis is antigenic and immunogenic. Appl Environ Microbiol 67:1423–1428
Fouts TR, DeVico AL, Onyabe DY, Shata MT, Bagley KC, Lewis GK, Hone DM (2003) Progress toward the development of a bacterial vaccine vector that induces high-titer long lived broadly neutralizing antibodies against HIV-1. FEMS Immunol Med Microbiol 37:129–134
Gaillard JL, Berche P, Frehel C, Gouin E, Cossart P (1991) Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell 65:1127–1141
Gasson MJ (1983) Plasmid complements of Streptococcus lactis NCDO 712 and other lactic streptococci after protoplast-induced curing. J Bacteriol 154:1–9
Grangette C, Müller-Alouf H, Goudercourt D, Geoffroy MC, Turneer M, Mercenier A (2001) Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant Lactobacillus plantarum. Infect Immun 69:1547–1553
Grillot-Courvalin C, Goussard S, Huetz F, Ojcius DM, Courvalin P (1998) Functional gene transfer from intracellular bacteria to mammalian cells. Nat Biotechnol 16:862–866
Guimarães VD, Gabriel JE, Lefevre F, Cabanes D, Gruss A, Cossart P, Azvedo V, Langella P (2005) Internalin-expressing Lactococcus lactis is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. Microbes Infect 7:836–844
Guimarães VD, Innocentin S, Lefevre F, Azvedo V, Wal JM, Langella P, Chatel JM (2006) Use of native lactococci as vehicles for delivery of DNA into mammalian epithelial cells. Appl Environ Microbiol 72:7091–7097
Hannify SB, Carter AT, Hitchin E, Wells JM (2007) Mucosal delivery of a pneumococcal vaccine using Lactococcus lactis affords protection against respiratory infection. J Infect Dis 195:185–193
Hazebruck S, Oozeer R, Adel-Patient K, Langella P, Rabot S, Wal JM, Corthier G (2006) Constitutive delivery of bovine beta-lactoglobulin to the digestive tracts of gnotobiotic mice by engineered Lactobacillus casei. Appl Environ Microbiol 72:7460–7467
Ho PS, Kwang J, Lee YK (2005) Intragastric administration of Lactobacillus casei expressing transmissible gastroenteritis coronavirus spike glycoprotein induced specific antibody production. Vaccine 23:1335–1342
Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11: S45–S53
Hu KF, Lövgren-Bengtsson K, Morein B (2001) Immunostimulating complexes (ISCOMs) for nasal vaccination. Adv Drug Deliv Rev 51:149–159
Huibregtse IL, Snoeck V, de Creus A, Braat H, De Jong EC, Van Deventer SJ, Rottiers P (2007) Induction of ovalbumin-specific tolerance by oral administration of Lactococcus lactis secreting ovalbumin. Gastroenterology 133:517–528
Illum L, Davis SS (2001) Nasal vaccination: a non-invasive vaccine delivery method that holds great promise for the future. Adv Drug Deliv Rev 51:1–3
Iwaki M, Okahashi N, Takahashi I, Kanamoto T, Sugita-Konishi Y, Aibara K, Koga T (1990) Oral immunization with recombinant Streptococcus
mutans surface protein antigen gene. Infect Immun 58:2929–2934
Jennings GT, Bachmann MF (2008) The coming of age of virus-like particle vaccines. Biol Chem 389:521–536
Jensen ER, Shen H, Wettstein FO, Ahmed R, Miller JF (1997) Recombinant Listeria monocytogenes as a live vaccine vehicle and a probe for studying cell-mediated immunity. Immunol Rev 158:147–157
Kajikawa A, Satoh E, Leer RJ, Yamamoto S, Igimi S (2007) Intragastric immunization with recombinant Lactobacillus casei expressing flagellar antigen confers antibody-independent protective immunity against Salmonella enterica serovar Enteritidis. Vaccine 25:3599–3605
Karkhanis LU, Ross TM (2007) Mucosal vaccine vectors: replication-competent versus replication-deficient poxviruses. Curr Pharm Des 13:2015–2023
Killeen K, Spriggs D, Mekalanos J (1999) Bacterial mucosal vaccines: Vibrio cholerae as a live attenuated vaccine/vector paradigm. Curr Top Microbiol Immunol 236:237–254
Kim SJ, Jun DY, Yang CH, Kim YH (2006) Expression of Helicobacter pylori cag12 gene in Lactococcus lactis MG1363 and its oral administration to induce systemic anti-Cag12 immune response in mice. Appl Microbiol Biotechnol 72:462–470
Kok J, van der Vossen JM, Venema G (1984) Construction of plasmid cloning vectors for lactic streptococci which also replicate in Bacillus subtilis and Escherichia coli. Appl Environ Microbiol 48:726–731
Krüger C, Hu Y, Pan Q, Marcotte H, Hultberg A, Delwar D, van Dalen PJ, Pouwels PH, Leer RJ, Kelly CG, van Donkweerd C, Ma JK, Hammarström L (2002) In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. Nat Biotechnol 20:702–706
Lee JS, Poo H, Han DP, Hong SP, Kim K, Cho MW, Kim E, Sung MH, Kim CJ (2006) Mucosal immunization with surface-displayed severe acute respiratory syndrome coronavirus spike protein on Lactobacillus casei induces neutralizing antibodies in mice. J Virol 80:4079–4087
Lee MH, Roussel Y, Wilks M, Tabaqchali S (2001) Expression of Helicobacter pylori urease subunit B gene in Lactococcus lactis MG1363 and its use as a vaccine delivery system against H. pylori infection in mice. Vaccine 19:3927–3935
Lee P, Faubert GM (2006) Expression of the Giardia lamblia cyst wall protein 2 in Lactococcus lactis. Microbiology 152: 1981–1990
Lee SF (2003) Oral colonization and immune responses to Streptococcus gordonii: Potential use as a vector to induce antibodies against respiratory pathogens. Curr Opin Infect Dis 16:231–235
Li YG, Tian FL, Gao FS, Tang XS, Xia C (2007) Immune responses generated by Lactobacillus as a carrier in DNA immunization against foot-and-mouth disease virus. Vaccine 25:902–911
Liu X, Lagenaar LA, Simpson DA, Essennacher KP, Frazier-Parker CL, Liu Y, Tsai D, Rao SS, Hamer DH, Parks TP, Lee PP, Xu Q (2006) Engineered vaginal lactobacillus strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N. Antimicrob Agents Chemother 50:3250–3259
Maassen CB, Laman JD, den Bak-Glshouwer MJ, Tielen FJ, van Holten-Neelen JC, Hoogeijling L, Antonissen C, Leer RJ, Pouwels PH, Boersma WJ, Shaw DM (1999) Instruments for oral disease-intervention strategies: recombinant Lactobacillus casei expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. Vaccine 17:2117–2128
Mannam P, Jones KF, Geller BL (2004) Mucosal vaccine made from live, recombinant Lactococcus lactis protects mice against pharyngeal infection with Streptococcus pyogenes. Infect Immun 72:3444–3450
Mengaud J, Ohayon H, Gounon P, Mege R-M, Cossart P (1996) E-cadherin is the
receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. Cell 84:923–932

Mielcarek N, Alonso S, Locht C (2001) Nasal vaccination using live bacterial vectors. Adv Drug Deliv Rev 51:55–69

Mierau I, Klerebezem M (2005) 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. Appl Microbiol Biotechnol 68:705–717

Miyoshi A, Bermúdez-Humarán LG, Ribeiro LA, Le Loir Y, Oliveira SC, Langella P, Azevedo V (2006) Heterologous expression of Brucella abortus GroEL heat-shock protein in Lactococcus lactis. Microb Cell Fact 23:5:14

Morello E, Bermúdez-Humarán LG, Llull D, Solé V, Miraglio N, Langella P, Poquet I (2008) Lactococcus lactis, an efficient cell factory for recombinant protein production and secretion. J Mol Microbiol Biotechnol 14:48–58

Moss B (1991) Vaccinia virus: a tool for research and vaccine development. Science 252:1662–1667

Nijland R, Lindner C, van Hartskamp M, Hamoen LW, Kuipers OP (2007) Heterologous production and secretion of Clostridium perfringens beta-toxoid in closely related Gram-positive hosts. J Biotechnol 127:361–372

Norton PM, Wells JM, Brown HW, Macpherson AM, Le Page RW (1997) Protection against tetanus toxin in mice nasally immunized with recombinant Lactococcus lactis expressing tetanus toxin fragment C. Vaccine 15:616–619

Oliveira ML, Arêas AP, Campos IB, Monedero V, Perez-Martinez G, Miyaji EN, Leite LC, Aires KA, Lee Ho P (2006) Induction of systemic and mucosal immune response and decrease in Streptococcus pneumoniae colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A. Microbes Infect 8:1016–1024

Patterson LJ, Robert-Guroff M (2008) Replicating adenovirus vector prime/protein boost strategies for HIV vaccine development. Expert Opin Biol Ther 8:1347–1363

Perez CA, Eichwald C, Burrone O, Mendoza D (2005) Rotavirus vp7 antigen produced by Lactococcus lactis induces neutralizing antibodies in mice. J Appl Microbiol 99(5):1158–1164

Poo H, Pyo HM, Lee TY, Yoon SW, Lee JS, Kim CJ, Sung MH, Lee SH (2006) Oral administration of human papillomavirus type 16 E7 displayed on Lactobacillus casei induces E7-specific antitumor effects in C57/BL6 mice. Int J Cancer 119: 1702–1709

Pouwels PH, Leer RJ, Boersma WJ (1996) The potential of Lactobacillus as a carrier for oral immunization: development and preliminary characterization of vector systems for targeted delivery of antigens. J Biotechnol 44(1–3):183–192

Ramasamy R, Yasawardena S, Zomer A, Venema G, Kok J, Leenhouts K (2006) Immunogenicity of a malaria parasite antigen displayed by Lactococcus lactis in oral immunisations. Vaccine 24:3900–3908

Ribeiro LA, Azevedo V, Le Loir Y, Oliveira SC, Dieye Y, Piard JC, Gruss A, Langella P (2002) Production and targeting of the Brucella abortus antigen L7/L12 in Lactococcus lactis: a first step towards food-grade live vaccines against brucellosis. Appl Environ Microbiol 68:910–916

del Rio B, Dattwyler RJ, Aroso M, Neves V, Meirelles L, Seegers JF, Gomes-Solecki M (2008) Oral immunization with recombinant Lactobacillus plantarum induces a protective immune response in mice with Lyme disease. Clin Vaccine Immunol 15:1429–1435

Robinson K, Chamberlain LM, Schofield KM, Wells JM, Le Page RW (1997) Oral vaccination of mice against tetanus with recombinant Lactococcus lactis. Nat Biotechnol 15:653–657

Rupa P, Monedero V, Wilkie BN (2008) Expression of bioactive porcine interferon-gamma by recombinant Lactococcus lactis. Vet Microbiol 129:197–202
Rush CM, Hafner LM, Timms P (1995) Lactobacilli: vehicles for antigen delivery to the female urogenital tract. Adv Exp Med Biol 371B:1547–1552

Seegers JF (2002) Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol 20:508–515

Sim AC, Lin W, Tan GK, Sim MS, Chow VT, Alonso S (2008) Induction of neutralizing antibodies against dengue virus type 2 upon mucosal administration of a recombinant Lactococcus lactis strain expressing envelope domain III antigen. Vaccine 26:1145–1154

Singh M, Chakrapani A, O’Hagan D, Wendorf J, Chesko J, Kazzaz J, Ugozzoli M, Vajdy M, O’Hagan D, Singh M (2008) A comparison of anionic nanoparticles and microparticles as vaccine delivery systems. Hum Vaccin 4:44–49

Stahl S, Samuelson P, Hansson M, Andréoni C, Goetsch L, Libon C, Liljeqvist S, Gunneriusson E, Binz H, Nguyen TN, Uhlen M (1997) Development of non-pathogenic staphylococci as vaccine delivery vehicles. In: Wells J, Pozzi G (eds) Recombinant gram-positive bacteria as vaccine vehicles for mucosal immunization. R. G. Landes Biomedical Publishers, Springer, New York, pp 61–81

Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E (2000) Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science 289:1352–1355

Steidler L, Neirynck S, Huyghebaert N, Snoeck V, Vermeire A, Goddeers B, Cox E, Remon JP, Remaut E (2003) Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. Nat Biotechnol 21:785–789

Steidler L, Robinson K, Chamberlain L, Schofield KM, Remaut E, Le Page RW, Wells JM (1998) Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of Lactococcus lactis coexpressing antigen and cytokine. Infect Immun 66:3183–3189

Steidler L, Wells JM, Raeymaekers A, Vandekerckhove J, Fiers W, Remaut E (1995) Secretion of biologically active murine interleukin-2 by Lactococcus lactis subsp. lactis. Appl Environ Microbiol 61:1627–1629

Stevenson A, Roberts M (2003) Use of Bordetella bronchiseptica and Bordetella pertussis as live vaccines and vectors for heterologous antigens. FEMS Immunol Med Microbiol 37:121–128

Stever Ck, de la Cruz VF, Bansal GP, Hanson MS, Fuerst TR, Jacobs WR Jr, Bloom BR (1992) Use of recombinant BCG as a vaccine delivery vehicle. Adv Exp Med Biol 327:175–182

Ulaeto D, Hruby DE (1994) Uses of vaccinia virus in vaccine delivery. Curr Opin Biotechnol 5:501–504

van Asseldonk M, de Vos WM, Simons G (1993) Functional analysis of the Lactococcus lactis usp45 secretion signal in the secretion of a homologous proteinase and a heterologous alpha-amylase. Mol Gen Genet 240:428–434

Wang K, Huang L, Kong J, Zhang X (2008) Expression of the capsid protein of porcine circovirus type 2 in Lactococcus lactis for oral vaccination. J Virol Methods 150:1–6

Wells JM, Mercenier A (2008) Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol 6:349–362

Wells JM, Norton PM, Le Page RW (1995) Progress in the development of mucosal vaccines based on Lactococcus lactis. Int Dairy J 5:1071–1079

Wells JM, Wilson PW, Norton PM, Gasson MJ, Le Page RW (1993) Lactococcus lactis high-level expression of tetanus toxin fragment C and protection against lethal challenge. Mol Microbiol 8:1155–1162

Xin KQ, Hoshino Y, Toda Y, Igimi S, Kojima Y, Jounai N, Ohba K, Kushiyo A, Kiwaki M, Hamajima K, Klinman D, Okuda K (2003) Immunogenicity and protective efficacy of orally administered recombinant...
**Lactococcus lactis** expressing surface-bound HIV Env. Blood 102:223–228
Xu Y, Li Y (2007) Induction of immune responses in mice after intragastric administration of *Lactobacillus casei* producing porcine parvovirus VP2 protein. Appl Environ Microbiol 73:7041–7047
Zegers ND, Kluter E, van Der Stap H, van Dura E, van Dalen P, Shaw M, Baillie L (1999) Expression of the protective antigen of Bacillus anthracis by *Lactobacillus casei*: towards the development of an oral vaccine against anthrax. J Appl Microbiol 87:309–314
Zhang ZH, Jiang PH, Li NJ, Shi M, Huang W (2005) Oral vaccination of mice against rodent malaria with recombinant *Lactococcus lactis* expressing MSP-1(19). World J Gastroenterol 11:6975–6980
Zhuang Z, Wu ZG, Chen M, Wang PG (2008) Secretion of human interferon-beta 1b by recombinant *Lactococcus lactis*. Biotechnol Lett 30:1819–1823