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

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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, nanoparticules, 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) (*Table 29.1*). 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.

Table 29.1

Bacterial vectors used as live vaccines

Bacterial vector	Reference
Attenuated pathogens	
Mycobacterium bovis BCG	Stover et al. (1992)
Listeria monocytogenes	Jensen et al. (1997)
Salmonella spp.	Curtiss et al. (1994)
Vibrio cholera	Killeen et al. (1999)
Shigella spp.	Brahmbhatt et al. (1992)
Bordetella spp.	Stevenson and Roberts (2003)
Non-pathogenic bacteria	
Streptococcus gordonii	Lee (2003)
Lactococcus lactis	Bermúdez-Humarán et al. (2004a)
Lactobacillus spp.	Seegers (2002)
Staphylococcus spp.	Ståhl et al. (1997)

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). *Lo. 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)
β-toxin	*Clostridium perfringens*	*L. lactis*	*C. perfringens* type B and C vaccine	Nijland et al. (2007)
K99	Enterotoxigenic *Escherichia coli* (ETEC)	*Lb. acidophilus*	Enteric colibacillosis treatment	Chu et al. (2005)
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	Corthésy et al. (2005)
Cag12	*Helicobacter pylori*	*L. lactis*	Helicobacter vaccine	Kim et al. (2006)
FliC	*Salmonella enterica* serovar Enteritidis (SE)	*Lb. casei*	SE vaccine	Kajikawa et al. (2007)
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)

Table 29.2 (*Cont'd* p. 1105)

Antigens	Source	Vector	Indication/ Potential use	Reference
		Lb. plantarum	Pneumococcal vaccine	Oliveira et al. (2006)
		Lb. helveticus	Pneumococcal vaccine	Oliveira et al. (2006)
Pili	*Streptococcus agalactiae* GBS	*L. lactis*	Streptococcal vaccine	Buccato et al. (2006)
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 immunodefficiency virus (HIV-1)	*L. lactis*	HIV vaccine	Xin et al. (2003)
E7	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)
		Lb. 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 disease virus (IBDV)	*L. lactis*	Coronavirus vaccine	Dieye et al. (2003)
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)

Table 29.2 (*Cont'd* p. 1106)

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-lacto-globulin	Bovine blacto-globulin	*L. lactis*	Allergy modulations	Chatel et al. (2001)
		Lb. casei	Allergy modulations	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	Bermudez-Humaran 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)

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)

29.6 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.

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 (Guimarães 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 immure 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 electro-competents 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 $DO_{600=}$ 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 DO_{600}. 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 1×10^9 colony-forming units (CFU). Three mice (6–8 weeks) are immunized intranasally with 1×10^9 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_{600} 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). 2×10^5 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 lyzed 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 (Huibregtse 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.

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