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Review

Heterologous protein production and delivery systems for *Lactococcus lactis*

Sébastien Nouaille[1], Luciana A. Ribeiro[1,2], Anderson Miyoshi[2], Daniela Pontes[2], Yves Le Loir[3], Sergio Costa Oliveira[2], Philippe Langella[1] and Vasco Azevedo[2]

[1]Unité de Recherches Laitières et de Génétique Appliquée, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy en Josas Cedex, France
[2]Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[3]Laboratoire de Microbiologie UMR1055, Ecole Nationale Supérieure Agronomique de Rennes, Institut National de la Recherche Agronomique, 65, rue de Saint Brieuc, CS84215, 35042 Rennes cedex, France
Corresponding author: V. Azevedo
E-mail: vasco@mono.icb.ufmg.br

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ABSTRACT. Lactic acid bacteria (LAB), widely used in the food industry, are present in the intestine of most animals, including humans. The potential use of these bacteria as live vehicles for the production and delivery of heterologous proteins of vaccinal, medical or technological interest has therefore been extensively investigated. *Lactococcus lactis*, a LAB species, is a potential candidate for the production of biologically useful proteins. Several delivery systems have been developed to target heterologous proteins to a specific cell location (i.e., cytoplasm, cell wall or extracellular medium). A promising application of *L. lactis* is its use as an antigen delivery vehicle, for the development of live mucosal vaccines. The expression of heterologous proteins and antigens as well as the various delivery systems developed in *L. lactis*, and its use as an oral vaccine carrier are discussed.

Key words: *Lactococcus lactis*, Secretion, Mucosal vaccine, Antigens, Cytokines

INTRODUCTION

Lactic acid bacteria (LAB), daily ingested by humans, are widely used in the food industry for the production and the preservation of fermented products. Lactic acid bacteria include a large number of Gram-positive cocci or bacilli belonging to a phylogenetically heterogeneous group. Their traditional use in the food industry confirms their lack of pathogenicity; they are considered to be generally regarded as safe (GRAS) organisms.

Since the 1980's, many efforts have been made to better understand the molecular basis of LAB's technological properties and to obtain better control of industrial processes involving LAB. This knowledge has led researchers to investigate their potential use for new applications, such as the production of heterologous proteins in bio-reactors, in fermented food products or directly in the digestive tract of humans and other animals. Some LAB, used as probiotic strains, naturally exert a positive action in lactose-intolerant consumers by providing lactase in the gut (de Vrese et al., 2001; Seegers, 2002). Besides such natural benefits, another and innovative application of LAB is the delivery of digestive enzymes to supplement pancreatic deficiency in humans. Some recombinant strains producing lipase have already been used with success in animals (Drouault et al., 2000, 2002). A new application for LAB, and probably the most promising, is their use as live delivery vectors for antigenic or therapeutic protein delivery to mucosal surfaces. Such engineered LAB are able to elicit both mucosal and systemic immune responses. Several research projects have used *Lactobacillus* sp. and *Lactococcus* sp. as vectors. Efficient expression systems have already been developed for controlled and targeted production of the desired antigen for exposure to the gastrointestinal mucosal immune system (Wells et al., 1993b; Norton et al., 1995; Le Loir et al., 2001).

In this review, we describe new advances concerning the use of LAB as live delivery vectors, focusing on *Lactococcus lactis*, the LAB model. Various molecular tools have been developed to efficiently express antigens and therapeutic molecules at different cellular localizations. We report systems developed to use *L. lactis* as a live vaccine and the effects of such an antigen presentation mode on the mucosal immune system.

PRODUCTION SYSTEMS OF HETEROLOGOUS PROTEINS IN *LACTOCOCCUS LACTIS*

Lactococcus lactis is the model LAB: many genetic tools have been developed and its complete genome was recently sequenced (Bolotin et al., 2001). Protein secretion by this GRAS bacterium would allow production directly in a food product and thus an interaction between the secreted protein (enzyme or antigen) and the environment (the food product itself or the digestive tract). *Lactococcus lactis* can be considered a good candidate for heterologous protein secretion for at least two reasons: i) to date, relatively few proteins are known to be secreted by *L. lactis* and only one, Usp45 (an unknown secreted protein of 45 kDa) is secreted in quantities detectable by Coomassie blue staining and ii) secreted proteins are prone most of the time to extracellular degradation, even in multi-deficient protease strains (Wu et al., 1991; Wu et al., 1998; Lee et al., 2000), and laboratory *L. lactis* strains do not produce any extracellular proteases.

High-level production of heterologous proteins in *L. lactis* has been obtained using lactococcal constitutive or inducible promoters (Kuipers et al., 1997; de Vos, 1999). Thirty-eight promoters that differ in their capacity to induce strong protein expression have been

analyzed and classified as constitutive (de Vos, 1999). However, continuous high level production of a protein could lead to intracellular accumulation, aggregation, or degradation of this protein in the cytoplasm (Makrides, 1996). These phenomena can be deleterious to the cell. To prevent possible negative effects caused by high production, inducible expression promoters have been developed. In this way, gene expression can be controlled by an inductor, a repressor or by environmental factors, such as pH, temperature or ion concentrations.

The best-characterized controllable expression system is based on the lactose-inducible transcription of the *lac* operon encoding the lactose phosphotransferase system and tagatose-6-phosphate pathway (de Vos and Vaughan, 1994). However, application of this system is hampered by a low level of induction (less than 10-fold) and is mediated by the intermediate, tagatose-6-phosphate, the concentration of which cannot be controlled easily, especially in large-scale fermentations. Another inducible expression system based on the *Escherichia coli* bacteriophage T7 promoter combined with the T7 polymerase gene fused to the *lac* operon promoter has the same drawbacks and furthermore, it is based on the use of a heterologous gene unauthorized in the food industry (Wells et al., 1993b). Another lactococcal bacteriophage-based system was developed by combining phage-induced DNA amplification and gene expression (O'Sullivan et al., 1996). This so-called explosive gene expression system allows for an approximately 30-fold increase in protein production but it could eventually result in uncontrolled complete lysis, which is not always a desirable feature.

To circumvent those problems, a regulated expression system was developed based on the use of the *L. lactis nisA* promoter (de Vos and Gasson, 1989; Wells et al., 1993a; Eichenbaum et al., 1998). The *nisA* gene contains a promoter sequence that can efficiently control transcription initiation, depending on the extracellular concentration of the antimicrobial peptide nisin (de Ruyter et al., 1996). Nisin is used in some countries by the food industry as a safe and natural preservative. The *nisA* gene encodes the nisin peptide itself and proteins encoded by *nisR* and *nisK* are involved in the regulation of *nis* gene cluster expression. NisR and NisK belong to the class of two-components regulatory systems: NisK is a sensor histidine kinase and NisR is the response regulator. This system is very versatile and can deal with high protein production if required, since there is a linear dependency between the amount of nisin added to the culture medium and the promoter activity. Gene expression can be achieved within a dramatic range of more than 1,000-fold (de Ruyter et al., 1996).

Several lactococcal promoters regulated by environmental conditions have also been isolated. Among them, P170 is a strong promoter, only active at low pH and when cells enter the stationary growth phase. It has the property of being more active at low than at high temperatures (Israelsen et al., 1995; Madsen et al., 1999). It can therefore be used for expression of toxic proteins by controlling pH, with the expression at low temperature of proteins prone to aggregation. Promoters can be up or down regulated by the extracellular concentration of ions, such as Cl^- (Sanders et al., 1998) or Zn^{2+} (Poquet, I., personal communication).

Although *L. lactis* is a food-grade bacterium, this status can be compromised by all the expression systems mentioned above, based on high copy number plasmids, antibiotic resistance (Ab^r) genes as selection markers and the use of foreign DNA. Those tools could compromise the use of engineered lactococcal strains in the food industry and for the production of purified recombinant proteins because of the loss of expression plasmids in the absence of selection pressure and contamination of the biomass or purified proteins by Ab^r. Food-grade cloning systems have been developed to efficiently produce proteins directly in food or in large scale fermentations, without Ab^r and foreign DNA. Two systems based on threonine- and pyrimi-

dine-auxotroph derivative *L. lactis* strains allow cloning and efficient expression of heterologous and homologous protein expression in various industrial strains (Sorensen et al., 2000; Glenting et al., 2002). These two systems are stable, and do not impair growth rates and important properties, such as milk acidification. Because of the absence of Ab^r and foreign DNA, strains using those systems maintain their food-grade status.

A large number of expression systems are now available in *L. lactis* with different strength and regulation systems; some of them exerting strong activity and tight regulation. They constitute powerful tools to control heterologous protein production in terms of quantities, timed expression and conditions.

CELLULAR TARGETING OF HETEROLOGOUS PROTEINS IN *LACTOCOCCUS LACTIS*

To evaluate the incidence by cell location of heterologous proteins, several systems have been designed to specifically target these proteins to different locations (i.e., the cytoplasm, the cell wall or the extracellular medium) in *L. lactis*.

In bacteria, most proteins that are secreted via the secretion (Sec) pathway are synthesized as precursors containing the mature protein and an N-terminal signal peptide (SP) that is an essential signature for protein secretion (von Heijne, 1990). The SP associates with the Sec machinery and also retards precursor folding, together with the action of Sec-specific chaperones (for a review of Sec mechanisms, see Pugsley, 1993; Tjalsma et al., 2000). Although the SP primary sequences are poorly conserved, they display a common tripartite structure including a positively charged N-terminus, a hydrophobic core and a neutral or negatively charged C-terminus containing the SP cleavage site (von Heijne, 1990).

Many heterologous eukaryotic (Arnau et al., 1997; Steidler et al., 1998; Chatel et al., 2001), prokaryotic (Drouault et al., 2000; Gaeng et al., 2000; Lee et al., 2001; Ribeiro et al., 2002) and viral (Langella and Le Loir, 1999; Enouf et al., 2001; Bermudez-Humaran et al., 2002) proteins have been produced and secreted by *L. lactis*. The best secretion efficiencies were obtained by the use of SP of Usp45 (the main secreted protein in *L. lactis*) efficiently recognized by the lactococcal Sec machinery. Even with the appropriate SP, secretion may be inefficient, and some heterologous proteins remain poorly, or are not at all secreted, even when fused to a homologous SP (Puohiniemi et al., 1992; Ravn et al., 2000; Enouf et al., 2001). Notably, charges at the N-terminus part of the mature moiety may greatly affect the translocation efficiency across the cytoplasmic membrane (Andersson and von Heijne, 1991). Le Loir et al. (1998, 2001) showed that the introduction of negative charges, by the insertion of a nine-residue synthetic propeptide after the SP cleavage site, improved secretion efficiency and production yields of different heterologous proteins. Other reports confirmed the role of this synthetic propeptide as an enhancer of production and secretion efficiency of other heterologous proteins in *L. lactis* (Ribeiro et al., 2002).

When required, heterologous proteins can be targeted to the cell wall by an anchoring signal sequence (cell wall anchor, CWA), which consists of a conserved LPXTG motif, a transmembrane fragment and a charged C-terminus. When CWA is fused to the carboxyl-terminal end of the protein, it remains covalently attached to the cell wall by transpeptidation between the LPXTG motif and peptidoglycan. Anchoring of heterologous proteins using the CWA of proteins A and M6 from *Staphylococcus aureus* and *Streptococcus pyogenes*, respectively, was demonstrated to be efficient in various LAB species (Medaglini et al., 1995; Piard et al., 1997; Stahl and Uhlen, 1997; Dieye et al., 2001; Ribeiro et al., 2002).

FROM HETEROLOGOUS PROTEIN EXPRESSION TO LIVE MUCOSAL VACCINES

The concept of a specialized immune system associated to the mucosa is relatively new. Mucosal epithelia constitute barriers between the internal and the external environment and consequently are the first line of defence of the organism against most of the pathogens that use this way of entry (Salminen et al., 1998). The entry of pathogens by natural infection routes (oral, respiratory, genital) allows the exposition of antigens to the mucosa, inducing the secretion of IgAs, the main class of antibodies that can be efficiently secreted by epithelium, neutralizing the toxicity of pathogens (McGhee and Mestecky, 1990; Salminen et al., 1998). Antibodies are produced by migration of lymphocytes to the mucosal lymphocytic tissue (Perdigon et al., 2001).

Strategies designed to fight against pathogens at mucosal surfaces are not only desirable but, in some instances, can be the only way to prevent infection. For example, it would be easier to block infections by intracellular parasites and viruses at the mucosa than to develop schemes to eliminate the organism after infection. Actually, one of the most important contributions to the development of new mucosal vaccines is the use of adjuvants, substances able to increase the immune response. They are also able to activate different immunologic system ways, to increase the time of immune response and to modulate the specificity and the immune response type (cellular or humoral). Presently, the most efficient known adjuvants are *Vibrio cholerae* toxin and *Escherichia coli* thermostable enterotoxin (Dickinson and Clements, 1995; Koprowski et al., 2000; Pizza et al., 2001). Cytokines have also been administrated as adjuvant vaccines to induce mucosal and systemic immune responses, and to increase the magnitude of such responses (Boyaka et al., 1999; Kovarik et al., 2000; Hultgren et al., 2001; Bermudez-Humaran et al., 2002).

To develop efficient live mucosal vaccines, two components are essential: the bacterial vector strain and a well-adapted antigen presentation system. Ideal mucosal vaccines should promote an effective contact between the antigen and the immune system, stimulate humoral and cellular immune responses, produce long-term protection after a single dose and be stable and nontoxic (Jennings et al., 1998). Different strategies could be used as vaccines to present antigens in the mucosa. Among them, the use of pathogenic bacteria as vaccine vectors, is probably the best studied (Ellis, 2001; Medina and Guzman, 2001). The use of an attenuated pathogen is particularly interesting, being a powerful tool to present antigens, since they can induce a specific immune response against the heterologous antigen and simultaneous protection against the pathogen. Various attenuated mutants have been produced and tested, such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella flexneri*, *Mycobacterium bovis*, *Yersinia enterocolitica* and *Vibrio cholerae* (Shata et al., 2000; Dietrich et al., 2001; Medina and Guzman, 2001). Although these attenuated strains have been extensively engineered to reduce their pathogenicity, they maintain certain invasive and virulence properties (Fischetti et al., 1996), and therefore their use in children and immunodeficient individuals should be limited (Foss and Murtaugh, 2000).

To circumvent some of the safety and environmental issues inherent to the wide-scale dissemination of engineered pathogens, nonpathogenic Gram-positive vectors have been developed (Pozzi et al., 1992; Wells et al., 1993a; Bermudez-Humaran et al., 2002; Ribeiro et al., 2002). In addition to their GRAS status, some LAB are able to stimulate the immune system

of the host as adjuvants due to their probiotic proprieties and their immunomodulation capacity (Neuman et al., 1998; Perdigon et al., 2001; Seegers, 2002; Shu and Gill, 2002). For example, the probiotic *Lb. delbrueckii* (UFV-H2b20, previously described as *Lb. acidophillus*) resists the stress condition in the gastrointestinal tract and stimulates the mononuclear phagocytic system of the host (Neuman et al., 1998).

The combination of these properties makes LAB very advantageous live vaccines and many studies are under way to express antigens in LAB and to evaluate the effect of this antigen presentation system on the immune system. In some systems, the antigen is expressed in the cytoplasm of the host cell or is secreted (Wells et al., 1993b). Others systems have been developed to display the foreign antigen on the cell surface (Nguyen et al., 1993; Gunneriusson et al., 1996; Dieye et al., 2001; Ribeiro et al., 2002). This latter construct is particularly interesting because antigen export allows a direct contact between the antigen and the immune system (Norton et al., 1994).

Some LAB, such as *Streptococcus gordonii* (Medaglini et al., 2001), *Lactobacillus plantarum* (Grangette et al., 2001; Reveneau et al., 2002), *Lactobacillus johnsonii* (Scheppler et al., 2002) and *Lactobacillus rhamnosus* (Shu and Gill, 2002) have been shown to induce a specific immune response in mice. No toxic activity or harmful effects were observed in mice after oral or subcutaneous administrations of these bacteria.

RECOMBINANT *LACTOCOCCUS LACTIS* STRAINS AS LIVE VACCINES

Lactococcus lactis does not belong to the human microflora and, different from *S. gordonii* and some lactobacilli, does not colonize oral and intestinal cavities. Mucosal vaccination with colonizing microorganisms could lead to tolerance towards the antigen. When required, *L. lactis* is a good alternative candidate.

The majority of studies using *L. lactis* as a live vector uses tetanus toxin fragment C (TTFC), a highly immunogenic model antigen. Norton et al. (1995) observed a significant increase in the level of IgA after oral immunization in mice with recombinant strains producing TTFC. Other studies showed that mice immunized with recombinant strains of *L. lactis* producing intracellular TTFC develop significantly higher levels of IgG and TTFC-specific fecal IgA. These mice become more resistant to a lethal challenge with the tetanus toxin than did nonimmunized mice (Wells et al., 1993a; Robinson et al., 1997; Grangette et al., 2001).

Expression of cytokines by *L. lactis* can stimulate the immune response. Clinical trials have revealed that IL-10 can reduce diarrheic disorders associated with inflammatory bowel disease. In mice, gastric administration of *L. lactis* secreting IL-10 reduced by about one half the diarrheic symptoms and prevented their appearance (Steidler et al., 2000). As immune response stimulators, the cytokines IL-2 and IL-6 are co-expressed with TTFC in *L. lactis*. After intranasal administration, mice that received *L. lactis* co-expressing cytokine and TTFC produced a 10- to 15-fold higher anti-TTFC immune response than did mice that received *L. lactis* strains expressing only TTFC (Steidler et al., 1995, 1998). Other studies confirmed the efficiency of *L. lactis* for the presentation of antigens to the mucosal immune system, to elicit a specific response (Chatel et al., 2001; Enouf et al., 2001). To date, several antigens (bacterial and viral) and cytokines have been efficiently produced in *L. lactis* (Table 1).

Table 1. Some antigens and cytokines produced in *Lactococcus lactis*. Final localization of the heterologous protein in *L. lactis* is indicated: S: secreted, C: cytoplasmic, A: anchored.

Proteins	Gene	Origin	Localization	References
Bacterial antigens				
L7/L12	*L7/L12*	*Brucella abortus*	S/C/A	(Ribeiro et al., 2002)
TTFC	*ttfc*	*Clostridium tetani*	S	(Wells et al., 1993b)
Viral Antigens				
E7	*E7*	HPV type16	S	(Bermudez-Humaran et al., 2002)
NSP4	*NSP4*	*Bovine rotavirus*	C	(Enouf et al., 2001)
BCV epitope	*BCV*	*Bovine coronavirus*	S	(Langella and Le Loir, 1999)
Cytokines				
IL-2	*IL-2*	mice	S	(Steidler et al., 1995)
IL-6	*IL-6*	mice	S	(Steidler et al., 1998)
IL-10	*IL-10*	mice	S	(Schotte et al., 2000)
IL-12	*IL-12*	mice	S	(Bermudez et al., 2003)

CONCLUSION

In conclusion, we consider that a complete tool box is now available for heterologous protein production and targeting in *L. lactis*. These tools could lead to the construction of new food-grade live vaccines based on lactococci. Such uses for vaccination purposes are promising for future therapeutic employment of lactococci. The most difficult tasks of this type of vaccination are to produce each antigen or cytokine in the precise cell location (cytoplasmic, anchored or secreted) and to regulate expression levels to induce the highest efficiency in the immune response in mice. For the moment, *L. lactis* is still the model LAB and it is the easiest LAB to manipulate. Lactobacilli are being more and more studied because of their adjuvanticity properties but, in spite of these efforts, they are still harder to work with than lactococci. Two strategies should be followed in the future: i) the first one consists in replacing the laboratory production systems based on antibiotic resistance genes by food grade systems much better accepted by potential consumers, and ii) the second will be to use cocktails of recombinant lactococci producing antigens and cytokines, and lactobacilli as probiotic adjuvants.

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