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Lactic acid bacteria: an alternative platform for live vaccine development

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Abstract

Lactic acid bacteria (LAB), a large group of food grade microorganisms, have recently been developed to express reactive antigens, which are intended to be used for vaccination. Immunological effects of these vaccines depend on the amount of the expressed antigenic protein loaded on the strains utilized for the delivery. Several factors including gene choice, cloning vector, and expression system could account for the different yield of antigens. Moreover, appropriate cellular location of the expressed gene product, either in the cytoplasm, the cell wall, or secreted into the medium can also affect the immune response. This review addresses the current knowledge of vaccine development by using lactic acid bacteria as an antigen-delivery system. The principal aim of this manuscript is to inform the reader on the processes of constructing recombinant LAB (rLAB) expressing about particular heterologous, immunologically reactive proteins, which can then be used as advantageous live vaccines.

Keywords: lactic acid bacteria, probiotic, cloning vector, heterologous expression, live vaccine

1. Rationale for using lactic acid bacteria as live vaccine

Live vaccine vectors based on attenuated pathogens such as *Salmonella typhi* and *Vibrio cholerae* have been developed to deliver the vaccine component (foreign antigen) which aimed to induce a specific immunity at mucosal level (1). However, the drawback of using attenuated bacterial pathogen as vaccines is the need to reduce their virulence leading to a concern on the risk of reversion to the original virulent organisms, including the possibility of causing disease in immune compromised individuals and young children. To overcome these obstacles, non-pathogenic bacteria especially lactic acid bacteria (LAB) offer an alternative choice for live vaccine development.

LAB, a group of Gram-positive, non-sporulating bacteria, compose of a vast array of heterogeneous

bacterial genera including Bifidobacterium. Enterococcus. Lactobacillus, Lactococcus, Pediococcus, and Streptococcus. Currently, using LAB as antigen delivery vectors to induce a specific immune response against pathogens is an attractive strategy. The majority reasons for using these bacteria as vaccine vectors including LAB are defined as "Generally Recognized As Safe (GRAS) microorganisms" because they have long term of use in food product and they have been consumed by humans without causing any recognized disease (2). Some LAB species especially lactobacilli and bifidobacterium are commensal organisms in the gastrointestinal tract (GIT) of humans, where they are thought to exert a vast array of health promoting benefits for improving the human intestinal homeostasis (3), the competitive exclusion against infection with intestinal pathogens (4), and the modulation of an immune response (5). In addition to these naturally beneficial effects of LAB, a wealth of genetic information (plasmid, transposons, prophages, and insertion element) and several genetics and proteomics tools for use in this bacterial group are now available (6), making it possible to convert LAB into live oral vaccines after intake.

2. Strategies for construction of recombinant LAB

Numerous review articles have recently been published on the construction of recombinant lactic acid bacteria (rLAB) as bacterial vectors for the delivery of proteins at the level of the human mucosa surfaces as prophylactic and therapeutic agents (7). Proteins utilized as reactive antigens derived from different microorganisms, including bacteria, viruses, and parasites have been cloned and expressed successfully in different LAB species. Some of the rLAB have further been used for vaccination purpose. Immunization experiments with these vaccines showed that rLAB was able to induce specific protective immunity, especially the secretory immunoglobulin A (sIgA), the major immunoglobulin located at mucosal area that can prevent an initial infection (8). Furthermore, it was found that the immune response induced by rLAB is principally affected by the amount of administered antigen and the localization of the expressed antigens (9). These expressed antigens can be localized both in the cytoplasm, the cell wall and at the extracellular milieu of LAB.

Several strategies for increasing yields of the heterologous protein and designing the correct location of antigen at cytoplasm, cell wall, or extracellular milieu have been investigated. The strategies for increasing yield of heterologous proteins include the use of a suitable cloning vector as well as the use of optimal promoters and expression systems, and terminators. In addition, the codon usage bias in LAB has been reported as an important factor affecting the expression efficiency of heterologous genes (10). All factors and strategies, needed for the development of rLAB, producing a given antigen as a live oral vaccine are shortly discussed in the following paragraphs.

2.1 Cloning vectors

A suitable cloning vector is an essential tool for carrying the gene to be expressed into the bacterial host of interest. When a particular expression vector is needed for each bacterial species, the construction of the expression cassette in the cloning vector is a prerequisite step. DNA purification and manipulation (extraction and transformation), as well as vector amplification, are to perform easier in *Escherichia coli* than in Gram-positive bacteria including all LAB species. Thus, a vector multiplying in both *E. coli* and LAB, which is called *E. coli*/LAB shuttle vector, is generally preferred (6). In general, *E. coli*/LAB shuttle vectors must contain at least three DNA elements in the vector backbone. These include a replicon (a DNA region essential for plasmid replication in each bacterial host) of both *E. coli* (e.g. pUC-, pBR-based replicon) and LAB (pWV01, pSH71-based replicon), a selective marker, and unique restriction endonuclease recognition sites for cloning. Moreover, vector properties such as structural and segregation stability (plasmid transfer to daughter cells without plasmid loss) in the expression host are also essential to certify that the vector could be maintained during bacterial division and without DNA rearrangements.

The basic replicons of LAB have been categorized into two types, either rolling cycle (RC) or theta-type (For detail of plasmid biology, see reference 6). RC-replicating plasmids have usually a wider host range, and some of them replicate not only in LAB but also in Gram-negative bacteria, including E. coli. Plasmids of this type frequently have higher copy number than that of theta-replicating mode. Contrastively, RC plasmids have frequently lower stability of both structural and segregational than theta plasmids. The size of RC plasmids is usually smaller than that of theta-replicating plasmids. Since the size mediates electro-transformation efficiency, RC plasmids are usually easier to transform and give the higher number of transformants with the same amount of DNA. However, in general, the selection of RC- or theta-type plasmids for vector construction greatly depends on the application. For example, working with a large DNA inserts requires a theta replicon-based vector. In contrast, if the overexpression of a heterologous gene is required, RC-based vectors with a high copy number enable to increase gene dosage and, thus, are attractive choices (6). To date, both RC and theta replicons derived from Lactobacillus sp., Lactococcus sp., and Pediococcus sp. have been used for the construction of E. coli/ LAB cloning vectors, leading to the development of rLAB expressing reactive antigen as live vaccine (6). Recently, a series of Bifidobacterium/E. coli shuttle vectors based on pBC1 derived from *Bifidobacterium catenulatum* have been constructed. The pBC1 has been used for the overexpression of an a-1-arabinofuranosidae gene derived from *B. longum* B667 in both *E. coli* and *Bifidobacterium* strains (11).

In addition to a basic replicon, the selective marker used for transformant selection and plasmid maintenance is needed. The commonest marker genes used in LAB are those encoding resistance to antibiotics, such as erythromycin, tetracycline and chloramphenicol. However, for the development of oral vaccines, the utilization of antibiotic resistance genes as selective markers should be avoided, because resistances could be transferred horizontally into other gut microorganisms, including pathogens. Thus, cloning vectors containing alternative selective markers are required. Recently, a number of food-grade vectors, containing only DNA from food-related microorganisms and lacking antibiotic resistances have been developed. Among the food-grade marker genes allowing selection in LAB, those encoding a bile salt hydrolase, bacteriocin immunity, phage resistance, and cadmium and copper resistances can be mentioned (6). Food-grade vectors based on selective markers have been successfully constructed for LAB species such as Lactobacillus plantarum (12)

2.2 Promoter and expression systems

To ensure that the gene of interest is transcripted into messenger RNA (mRNA), a functional promoter recognized by the host is needed. In addition to a promoter, the presence of an efficient Shine Dalgarno sequence or ribosome binding site (RBS) should locate downstream of promoter to mediate translation of mRNA into an amino acid sequence. Two major types of bacterial promoters can be distinguished, constitutive and inducible promoters. A constitutive promoter does not need an inducer molecule to lead expression because the expression occurred all along the bacterial growth phases; usually with the highest expression through the exponential phase. Constitutive external inducers. However, this production could lead promoters derive from a number of genes, including the surface layer protein gene (slp) of Lactobacillus acidophilus; the L-(+)-lactate dehydrogenase (ldhL) gene of L. acidophilus and the Lactobacillus casei; erythromycin ribosomal methylase (ermB) (13). The Ldh promoter has been successfully used to control the expression of bacterial and viral antigens, such as the tetanus toxin fragment C (TTFC) derived from Clostidium tetani in L. plantarum (9). The Ldh promoter has also been shown to be able to control the expression in E. coli, because of the facts that; 1) The Ldh promoter has two hexameric regions, -35 (CTGTCA) and -10 (GATAAT), which are similar to those of E. coli, and thus they can surely be recognized by the transcriptional factors of *E. coli*; moreover, the RBS (AGAAGGATGAT) of the Ldh promoter is similar too to that of E. coli(14). In vivo studies in mice have found that the ldhL promoter can mediate the production of luciferase in L. casei during the transit of the cells through the GIT of mice, leading to the possibility to use the promoter of the *ldhL* gene in order to control the production of antigens in LAB (15). Other constitutive promoters, including the strong constitutive promoter P59 of L. lactis, and the natural promoters of Lactobacillus helveticus aminopeptidase genes (such as pepC, pepN, pepX, pepO, pepE, and pepO2), have completely been shown to enable the control in expression of heterologous genes in L. casei and L. lactis (13). In Bifidobacterium species, a strong constitutive promoter P191 of Bifidobacterium bifidum BGN4 has been used for heterologous expression in its origin strain and other bifidobacteria species, such as Bifidobacterium dentium, B. longum, Bifidobacterium adolescentis, and Bifidobacterium animalis (11).

As mentioned above, a constitutive promoter has the advantage that it can drive a continuous production of a given protein without the need for

into the intracellular accumulation and degradation afterward of the expressed protein if it is deleterious for the cells. Inducible promoters are an alternative of choice for heterologous protein production, due to the fact that it is only active when the inducer is present. Several inducible promoters have been used to develop expression systems for LAB. Among these, it could mention about the promoter of the nisin structural gene (nisA) (16), and the promoter of genes involved sugar utilization (17). The Nisin-Controlled gene Expression System (NICE) is a well-known inducible expression system that was firstly developed for L. lactis and then transferred to species of other genera of LAB including Lactococcus sp., Lactobacillus sp., and Leuconostoc sp. (16). The NICE system has already been successfully used to produce antigens from various medical important microorganisms such as VP8 of rotavirus (18) and E7 protein of human papilomavirus type 16 (HPV-16) (19), This indicates the powerful nature of the NICE for heterologous gene expression in LAB. Recently, a stress-inducible controlled expression system (SICE), based on the inducible promoter of the L. lactis groESL operon has been developed (20). This promoter is encouraged by several stress condition found in gastrointestinal tract such as heat stress, acid stress, and bile-salt stress, leading to specific expression without the need of a supplementary inducer. Consequently, it can be used in real situation in GIT. The SICE system has been successfully used to express the murine IL-10 cytokine and the HPV-16 E7 antigen (20).

2.3 Codon usage in LAB

Another considerable factor affecting the heterologous gene expression in LAB is the codon bias. In general the translation efficiency of protein depends on the ability of the bacterial tRNA to recognize the codon(s) of the newly introduced genes. It has been demonstrated that the expression level of genes containing higher than that of genes containing a different set of codons (21). Additionally, Johnston and co-workers (22) demonstrated that by changing codon sequence of the gene coding for the MAP-specific antigen of Mycobacterium avium ssp. paratuberculosis (MptD), the production yield of MptD peptide in Lactobacillus salivarius was enhanced over 37 times, as compared to that obtained with its native codon sequence. Other genes, including those encoding the L1 from HPV (23) and the Japanese cedar pollen allergen (Cry-j) (24) have been expressed successfully in LAB by this codon optimization approach. This finding indicates that the coding sequence of the introduced genes should be modified according to the codon usage in the bacterial host of choice. Comparative analysis and gene annotation of LAB genomes will allow the prediction of tRNA genes in all LAB species, which may help in subsequent codon optimization for successful expression of heterologous proteins in this bacterial group. Data on codon usage by bacterial species of many different genera, including LAB have recently been published, and they can be accessed through the Internet (http://www.kazusa. or.jp/codon/). In addition, at least a codon optimization web-based free program is now available through the following web address, http://sg.idtdna.com/CodonOpt. Thus, codon optimization is an important step before cloning and expression of antigen-encoding genes in LAB for the development of oral vaccines.

2.4 Design of a protein targeting system in

LAB

Besides the amount of antigen produced by the rLAB, the production of the antigen in different cellular locations, either in the cytoplasm, the cell wall, or the extracellular milieu, can also affect the immune response. Immunization of animal models with antigens which are produced in all these fashions by rLAB has been found that the highest immune response

codons matching those of a given bacterial host is much higher than that of genes containing a different set of codons (21). Additionally, Johnston and co-workers (22) demonstrated that by changing codon sequence of the gene coding for the MAP-specific antigen of *Mycobacterium avium* ssp. *paratuberculosis* (MptD), the production yield of MptD peptide in *Lactobacillus salivarius* was enhanced over 37 times, as compared to that obtained with its native codon sequence. Other genes, including those encoding the L1 from HPV (23) in the intestine (25).

> As shown in Figure 1, different expression systems to locate heterologous proteins at either the cytoplasm, the extracellular milieu, or anchored at the cell wall have been constructed in different LAB species. To this purpose, if the protein is going to be produced at the cytoplasm, the expression cassette must contain a functional promoter, a recognizable ribosome binding site, a start codon (notably, ATG or GTG) of the open reading frame spanning the introduced gene, and a terminator. Extracellular and cell surface localizations of an antigen will additionally require a signal peptide (SP) for its translocation across the cell membrane. Recently, several native SPs derived from different LAB have been characterized. The majority extracellular protein (Usp45) of L. lactis has been used as SP for protein secretion in many strains of LAB (26). Among the lactobacilli, it is worth noting the SPs of an alpha-amylase and that of PrtP from L. casei (27), as well as the SP of the S-layer protein (SlpA) from Lactobacillus brevis (28). Moreover, heterologous SPs have been used to drive secretion in LAB, such as that of the fibrillar surface protein M6 from Streptococcus pyogenes (29). All of these SPs utilized the sec-dependent (Sec) machinery for protein translocation across the cell membrane. Typically, the native SP of the Usp45 protein and its derivatives has been widely used for heterologous protein production in Lactococcus and Lactobacillus (30). However, in L. casei, it was found that the Usp45 SP failed to secrete the β-lactoglobulin.

Instead, it increased the production yield of the protein, which accumulated in the cytoplasm. In this case, this SP might be a good fusion element for enhanced protein yield rather than for protein secretion. However, the secretion efficiency mediated by the Usp45 SP in L. casei can be improved by the insertion of a short peptide of nine amino acids (LEISSTCDA) at N-terminus of gene (30). By using the same strategy, the secretion of interleukin (IL-10) in L. casei was improved when the signal peptide derived from PrtP gene was enlarged with the pentapeptide DTNSD (27). Compared to those in other species, the SPs from *Bifidobacterium* species have yet to be characterized. The SP of and exo-xylanase (XynF) from B. longum has been used to secrete the enterovirus 71 viral capsid protein (EV71-VP1) in B. longum (31). Furthermore, seven SPs have been identified in the genome Bifidobacterium breve UCC2003 have been utilized for the construction of secretion vector. Therefore, the search for efficient SPs

in bifidobacteria species is of interest for the exporting of other proteins, such as viral proteins intended as vaccines, tumor suppressor proteins, and other biological molecules.

In addition to the promoter and SP, cell wall anchorage domain (CWA) is needed for direction of antigen to bacterial cell surface. CWA mediates the attachment of expressed protein to peptidoglycan of bacterial vehicle via covalent manner. Thus, CWA is cloned at the downstream region of the expressed gene in the cloning vector. Many CWA derived from gram positive bacteria such as M6 protein from *S. pyogenes* (32), and mucus-binding protein (Mub) from *L. acidophilus* (33) have been used successfully as a fusion partner for the localization of antigen in cell wall, leading to the possibility of using these proteins as a fusion partner for cell surface expression of other antigen in LAB.



Figure 1. Illustration of possible cellular locations of a heterologous-expressed protein. The protein can be expressed intracellularly and located at the cytoplasm (A), secreted into the medium (B), and located at the cell wall via an anchoring sequence (C). P, promoter; RBS, ribosome binding site; SP, signal peptide; CWA, cell wall anchoring domain; TT, transcription terminator; Sec, secretion machinery. Arrows indicate the starting point and direction of transcription process.

3. LAB vaccine is of safe and effective treatments

The efficiency of rLAB-based vaccine has been evaluated in animal models. Some studies demonstrated that oral administration of rLAB-based vaccine is able to induce the specific protective immune response against an infection from the corresponding pathogen (7). In addition to the experiments in animal models, a human clinical trial with rLAB expressing a therapeutic molecule has recently been established for their efficiency and safety. Braat and coworkers (34) have demonstrated that oral administration with recombinant L. lactis expressing interleukin-10 in patients with Chron's disease is able to decrease the disease activity and only minor adverse effects were observed. Other human diseases including inflammatory bowel disease (IBD) have also been successfully treated with rLAB (35). For these promising results, it is possible to conclude that rLAB expressing antigenic or therapeutic proteins are suitably considered as a safe approach, leading to the support of using these bacteria as live vaccines.

4. Conclusions and future prospects

In conclusion, the genetic toolbox available for cloning and expression in LAB has demonstrated to facilitate the heterologous expression of a vast array of antigenic proteins. The use of LAB species as a host in recombinant DNA technologies offers a bunch of potential and beneficial applications for the development and administration of vaccines, including safe and effective treatments. The construction of innovative cloning, expression and presentation vectors, including food-grade systems, will open new avenues for the future application of LAB species and strains as

functional agents for vaccination. The empirical evidence suggesting a key role of LAB in this field should be substantiated through randomized, double-blind placebo-controlled clinical trials in order to establish the efficacy immune protection of each antigen/strain couple and on the different presentations.

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