CHAPTER 13

Future Directions for Research on Biotherapeutic Agents

Contribution of Genetic Approaches on Lactic Acid Bacteria

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1. Introduction

Genetic tools have significantly contributed to increasing our knowledge in two fields related to biotherapeutic agents (BTA): bacterial physiology and the control of protein expression. The most striking example of an application in this new domain is the use of invasive, but nonpathogenic Salmonella with the ability to interact with lymphoid tissue in the Peyer's patches (*1*). These mutants have been shown to be an effective means of stimulating mucosal secretory IgA directed against a variety of heterologous antigens. However, these attenuated pathogens may not be acceptable in the elderly and very young, since they may translocate from the digestive tract to invade the host. Nevertheless, this demonstrates the applicability and potential of genetic engineering in expanding the applications of BTA.

Unlike Salmonella, lactic acid bacteria (LAB) used in fermented milk are not considered pathogenic, and selected LAB strains have the potential to improve human health (*2*). These strains have the capacity to be used as BTA. The main point of this chapter is to describe the future directions for research into how BTA may be improved and utilized using genetic technologies. The authors have focused on LAB in this chapter, but many of the genetic approaches could be used to develop or improve other bacterial or yeast BTA.

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Genetic approaches may allow two major breakthroughs with respect to developing improved BTA. The first might be to determine stimulated beneficial functions or inactivated deleterious functions in LAB during digestive tract transit. Until now, BTA have been isolated from natural strains, mostly yeasts or LAB, and there has been poor understanding of their mode of actions. Thus, it is difficult to search rationally for new natural strains with increased therapeutic activities. The choice of particular microorganisms as potential BTA is often based on the availability of facilities for culture of the organism or production of dietary products containing the organism. Field experiments are then performed to demonstrate benefits for human or animal health. Thus, a better understanding of the mode of action of BTA would be useful to develop new screening strategies and to isolate valuable strains. Since the action of most BTA takes place in the intestine, the study of bacterial physiology in this complex ecology must be undertaken to understand the different modes of action of BTA in the digestive tract. This approach could be very difficult without the use of genetic tools. In this chapter, we will focus on the opportunity to increase the knowledge of bacterial physiology in the digestive tract.

The second major application using genetic tools would be to introduce new functions or to improve existing functions of LAB. For example, BTA can potentially be genetically manipulated to produce molecules of therapeutic interest in the intestine. LAB may be especially suitable vectors for elaborating these molecules. We will explain in this chapter how the LAB genome can be modified to produce proteins having just such a positive role on health. These rationally designed strains may produce, transport, and release proteins of interest directly into the digestive tract.

2. Current Knowledge About LAB

2.1. Lactic Acid Bacteria (LAB)

LAB is a generic term that designates bacteria producing mainly lactic acid from sugars. They are Gram-positive, facultatively or strictly anaerobic, nonspore-forming cocci or rods. The most common generi are *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Enterococcus,* and *Pediococcus.* These bacteria are found in different ecological niches, such as dairy products (all generi), vegetables (all generi, but Streptococci), meats (mostly *Lactobacillus* and *Pediococcus*), human and animal mucosa (mostly *Streptococcus*), and digestive tract (*Streptococ-*

cus, Lactobacillus, Enterococcus). Many species are used as "starters" in fermented foods, but some species are also involved in pathology (mostly, but not exclusively *Streptococcus* and *Enterococcus*). Thus, it is interesting to note that inside each genus that contains genetically closely related strains, there are both food strains and pathogenic strains. For example, *Streptococcus thermophilus* and *Lactococcus lactis* are widely used as starters in yogurt and cheese production, respectively, whereas *Streptococcus pneumoniae* and *Lactococcus garviae* are agents for pneumonia and mastitis, respectively. Current genetic studies have been developed in two distinct areas: virulence factors from pathogenic Streptococci and Enterococci, and traits involved in food technology. There is little or no overlap between these research areas. Pathogenic strains cannot be used as BTA, but the knowledge from both areas of genetic study can be applied to developing BTA.

2.2. Safety

BTA have to be absolutely safe and well tolerated in humans to be able to be considered as practical therapeutic agents. In our opinion, this point excludes attenuated pathogens that are considered to be safe after the introduction of one or few mutations, because they might regain their pathogenicity by recovering native genes through genetic exchange with related strains in the environment. This point is particularly critical if large amounts of living bacteria are disseminated into the environment during the treatment. LAB used in food products have good safety records, and none of them have been found to be virulent. Some other LAB have rarely been associated with infection as a consequence of severe immune deficiency. Strains already widely ingested with fermented foods are safe. However, most of these strains have not been demonstrated to have useful therapeutic activities. They might be used for vectors of active molecules, however. Finally, strains isolated from human or animal mucosa, including some *Lactobacillus* species or *Streptococcus gordonii*, might also be useful as vectors, but because they have not been used in food products, they offer a lesser degree of certainty with respect to safety.

2.3. Studies on the Beneficial Effects of LAB as BTA

In a preceding review, Elmer and coworkers (2) have described the clinical trials performed with certain LAB and other BTA to prevent or treat some human diseases. Most of the publications cited were of high standard: single- or double-blind randomized experiments with placebo. On the other hand, numerous publications or reviews suggest the potential of other species of LAB based on smaller human trials

or on animal experiments. We will consider them as "promising for the future," but these should be confirmed by studies from different laboratories using well-defined strains and rigorous conditions (double-blind with placebo- and randomized-controlled studies). We will now review the more promising LAB candidates as BTA (*3–11*).

Most natural strains of LAB considered as potential BTA belong to the genus *Lactobacillus.* Indeed, some Lactobacilli have a long history as traditional therapies, and are administrated as tablets or capsules under different commercial labels. As early as 1908, Metchnikoff (*12*) suggested that some dairy products fermented with Lactobacilli were beneficial for health. Consequently, companies have selected Lactobacilli strains belonging to species that are occasionally found in the human intestine, and developed popular products potentially beneficial for human or animal health. Many of these products are called "probiotics" and are used as a preventive measure for various maladies and diseases (*13–15*). It appears that the distinguishing line between bacteria used as probiotics in food and BTA is not well defined. However, these probiotic bacteria should not be considered as therapeutic *per se,* as long as proper experimental studies have not been performed to demonstrate their efficacy.

It is well established that LAB present in fermented milk improves lactose digestion (*6,16*). However, it is not clear yet to what extent the living LAB are involved. There is still some debate to pinpoint whether this effect is owing to the cleavage of lactose by the bacterial lactase, or owing to the stimulation of the mucosal lactase by the bacteria. The effect of LAB in hypercholesteremia and in colon cancer is subject to more controversy (*3,10,17,18*). LAB have been proposed to produce enzymes reducing carcinogenic compounds or to produce substances with antitumoral effects on the mucosa, or to modify and stabilize the beneficial natural flora.

Potential for the use of LAB during intestinal disturbances or intestinal infections is frequently reported (*11*). The therapeutic effect of LAB has been proposed to be owing to an increase of intestinal immunity. The oral administration of several Lactobacilli alone or in association with *S. thermophilus* increased the level of immunoglobulins in intestinal fluid, the percentage of B-lymphocytes in the Peyer's patches, and the IgA response (*19,20*). The use of Lactinex® (Hynson, Westcott and Dunning, Inc., Baltimore) and of *Lactobacillus casei* was successful to treat some, but not all, antibiotic-related disturbances. *Lactobacillus* GG has been shown to have relevant therapeutic effects during acute gastroenteritis (*21*). It should be stressed that the reason

for the efficacy of these living bacteria is not known, but that heat-killed Lactobacilli (Lacteol fort®) was also shown to be beneficial in the treatment of gastroenteritis (*22*). Several studies have also reported the absence of an effect of LAB products on gastroenteritis (*17*). Trials on the efficacy of different LAB on traveler's diarrhea and on some gastroenteric pathogens are not convincing (*17*), but some lactic bacteria are effective against antibiotic-associated diarrhea and pseudomembranous colitis (*2,8*).

Thus, some LAB have potential for the prevention and treatment of selected diseases. However, there is a real lack of knowledge on their mechanisms of action. Rigorous trials to define their potential should be established for well-defined pathologies. It will then be possible to perform experiments to determine their mechanism of action. As an alternative, LAB without known beneficial effects could be modified to produce substances of interest for health. In both cases, a good knowledge of the bacterial and the host physiology is required to develop efficient treatments.

2.4. Genetics of Food LAB

Most genetic studies concerning LAB are related to pathogenic Streptococci and on strains used in dairy technology. Among food strains, *L. lactis* has emerged as the model for the genetics of LAB. Approximately 6–10% of its genome is already available in databases, reflecting the great interest of the industry and the dynamism of research in this field. Data are available on various aspects of its sugar metabolism, nitrogen assimilation, biosynthesis of amino acids and bases, response to stress, and to various environmental changes (*23*). Thus, many aspects of the metabolism of *L. lactis* are now well established, especially with regard to factors involved in growth in milk (*24*). Moreover, a number of tools have been developed to allow controlled gene expression and food-grade genetic modification (*25–27*). Many examples of cell engineering are now described demonstrating the metabolic potential of this bacteria (*28*). In the few last years, the genetics of other LAB, mostly dairy strains, have received increased attention, and data are now available for *S. thermophilus* (*29*) and some other Lactobacilli (*30*). However, only little is known (or available in the literature) about the genetics of potential BTA, such as *Lactobacillus casei, Lactobacillus acidophilus,* and almost nothing is known about the Lactobacilli that have been investigated clinically as BTA, i.e., *Lactobacillus* GG, *Lactobacillus reuteri,* and *Lactobacillus gasseri.*

3. Animal Models and Human Trials to Study Lab in the Digestive Tract

BTA are usually administrated by the oral route. Therefore, appropriate animal models must be developed for initial testing of efficacy. To address this point, several models involve the use of gnotobiotic or conventional animals. Human studies have been performed with the most promising strains found in these animal models.

3.1. Conventional and Gnotobiotic Animals

An animal model was developed to test the establishment of a BTA as dominant flora in the intestinal tracts of animals devoid of bacterial flora (gnotobiotic model). This model requires germ-free isolators and facilities (*31,32*). Germ-free animals can be obtained from different animal species, such as rodents, quail, hare, sheep, piglet, and calf. Germ-free animals are kept in sterile conditions and receive pure strains of LAB by the oral route. Once gnotobiotic animals have a pure strain introduced, they are termed gnotoxenic. During the experiments, animals are protected from any other microbial contamination (Fig. 1). In this way, the bacteria established in the intestine remain stable in the absence of any antagonistic effects of the normal flora. In gnotoxenic models, reproduction of a BTA may be slow, but sufficient to overcome bacterial death and intestinal tract motility, leading to high-level colonization of the BTA. The physiological state of bacteria in gnotoxenic models usually operates differently than when in the presence of normally established complex flora. This model was previously used to demonstrate the efficacy of *Saccharomyces boulardii* (a BTA) to protect rodents from *Clostridium difficile* pathology (*33,34*). More recently, the physiology of *L. lactis* established in the intestine was studied with this model.

Another model was developed with conventional animals to mimic LAB transit in the digestive tract. In conventional animals, the normal microflora prevents extensive proliferation of the BTA. For example, at least 90% of LAB are eliminated from the intestine 2 days after oral administration. As a consequence, daily dosing of LAB is required for therapeutic effects. The animal model employed should have transit characteristics as close as possible to those of humans, although this goal may be difficult to obtain. The alimentation process of conventional rodents does not resemble the human's, since these animals nibble all night long. However, rodents can be adapted to receive one feeding per day. In this situation, they eat the diet in 1 or 2 h, and

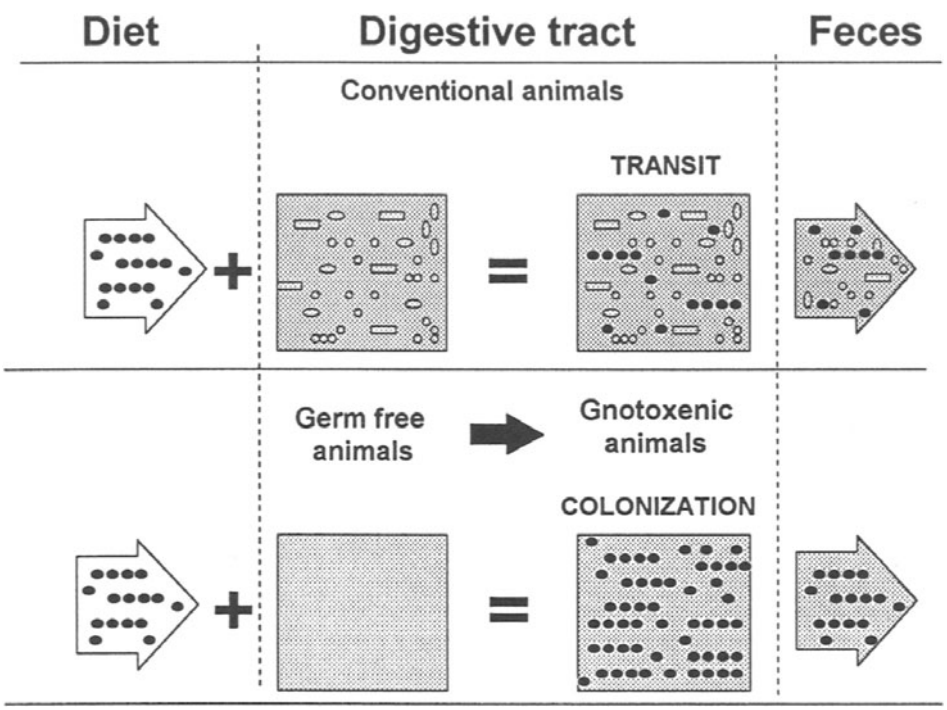

Fig. 1. The two animal models used to study bacteria in the digestive tract. **(Top)** Pure bacterial culture given in the diet encounters the normal microflora naturally present in the lower digestive tract. Bacteria *(L. lactis)* do not multiply and are gradually eliminated with food transit. **(Bottom)** Germ-free animals are inoculated with a pure bacterial culture. The absence of normal microflora permits colonization. The introduced bacteria (LAB) persists in the digestive tract.

fill up their stomach rapidly, followed by the release of stomach contents into the small intestine. This schedule of alimentation is similar to human digestion schedules. The microorganisms in liquid suspension (the tested BTA) are mixed with a powdered diet, so that the microbial transit of the BTA occurs during digestion. Using such a model, we observed that *L. lactis* viability in the small intestine was only slightly reduced (10–40% survival) compared to forced-feeding of living bacteria without food (<0.1% survival). In the lower parts of the digestive tract, viability was not affected by the presence or absence of food (Corthier, unpublished results).

This model could approximate the human situation more closely by the use of gnotoxenic rodents colonized with a human flora (*32,35*). The model uses germ-free rodents that have received a sample of dominant human flora. The animals are then protected from any other

microbial contamination by the same type of facilities used for germ-free animals. After 1–2 wk, their global microbial population of the digestive tract was similar to the human flora that was originally administrated. After the human flora is established in the animal, the BTA may be administered and tested for bacterial interactions.

3.2. Human Studies

The viability of different LAB was investigated in several human studies (*5,17,36*). The recovery in stool was found to be between 1 and 10% except for *Bifidobacterium* sp., which reached about 30% recovery (*37*). A limitation of these studies is that stool levels may not reflect BTA levels in the intestinal lumen. The practical problem of sample collection of the contents of the human intestinal tract was solved by using a plastic apparatus introduced through the nose. This apparatus allowed collection of fluid from the lumen of any part of intestine (*17,38*). This procedure was successfully used to study LAB survival following ingestion of fermented milk (*39*). The ability of a strain of Bifidobacterium sp. to survive passage through the upper gastrointestinal tract when ingested in fermented milk (10^{10} bifidobacteria in 400 g) was investigated in six fasting healthy adults by using in vivo ileal sampling. The average number of bifidobacteria recovered from the terminal ileum during the 8 h after fermented-milk ingestion was 10^9 and constituted 23.5% of the number ingested. These results indicate that in healthy adults, *Bifidobacterium* sp. survive transit through the stomach and small intestine when ingested in fermented milk (*39*).

4. LAB as a Producer of New Molecules

Genetic tools are now available to produce heterologous proteins in different microorganisms (yeasts, *Bacillus subtilis, Streptococcus,* tuberculosis bacillus, Salmonella, and viruses used as vaccines or baculovirus from insects). Most of the time, the proteins produced are extracted and used in a purified form. However, *Escherichia coli* and *Salmonella* have been used to produce heterologous molecules inside the digestive tract in order to elicit an immune response (*1,40*). More recently a similar approach was extended to LAB. Genetically, modified LAB ingested by an oral or a nasal route were found to elicit a local immune response to tetanus toxins (*36,41,42*).

4.1. Molecules of Interest for Production by BTA

BTA may be used to produce potent molecules in vivo. In the case of proteins, they must be produced in sufficient quantities to be effective. Levels must be high enough to overcome their degradation by digestive proteases, but not so high that they produce unwanted secondary effects. Below we discuss three areas of where genetically engineered BTA may have great potential—oral vaccines, enzymes, and biological mediators.

4.1.1. Oral Vaccines

In the digestive tract (especially in the small intestine), the immune system is well developed and defined as Gut-Associated Lymphoid Tissue (GALT). Peyer's patches are the largest lymphoid aggregates of the GALT. They can be triggered with antigens from the lumen and initiate a local immune response of specific local IgA production in the digestive secretions. The transit of lymphocytes via lymph nodes, spleen, and blood transports IgA to different exocrine secretions, such as saliva, tears, and milk (*43–46*).

The vaccines that could be developed using BTA as a delivery vector in the digestive tract must stimulate a local immune response to prevent disease. Ongoing predominant areas of research in this area include the following:

1. Local immunity against enteric viruses. Rotavirus and coronavirus are etiologies for severe infant diarrhea. Few effective vaccines (killed or attenuated virus) are currently available. Delivery of viral antigens by BTA might be of interest. An attempt of that type was performed with *E. coli* expressing pig coronavirus epitopes in the pilli (*47*). The authors included some small epitopes in the coding sequence of pilli. They obtained immunogenic constructions, but the oral doses required to elicit an immune response were found to be restrictively high. This promising approach needs to be developed further and should be tested using different BTA.
2. Local immunity against bacterial toxins. It is worthwhile investigating local immunity against bacterial toxins if no effective alternative vaccination exists. For example, efficient vaccines already exist against tetanus toxins, but none exist against *Clostridium difficile* or *Clostridium perfringens toxins,* which cause sporadic but dangerous diseases in human and domestic animals. Synthesis in the digestive tract by BTA of selected toxin epitopes (i.e., immunogenic toxin fragments devoid of toxic activity) may lead to a protective immune response.

3. Immune response to block food allergy. The process involving hypersensitivity reactions to food proteins is initialized in the digestive tract. Feeding with large quantities of the incriminating protein has been suggested to modify the immune response (*46,48*). BTA constructed to provide adapted quantities of an allergen to desensitize the GALT might be helpful to attenuate food allergy reactions.

4.1.2. Enzymes

Some BTA may counteract host deficiencies, such as human intestinal lactase. When people with lactase deficiency ingest milk, the nondigested lactose is fermented by the microflora leading to gas production (flatulence) and sometimes diarrhea. LAB are known to produce high amounts of lactase. Consumption of fermented milk containing LAB has been shown to reduce lactose malabsorption in the digestive tract. This feature is one of the most established beneficial characteristics of fermented milk (*see* Section 2.3.), although it is not known if this is owing to the release of the bacterial enzyme or to another mechanism (*6,49*).

There are other enzyme deficiencies, such as pancreatic insufficiency, which adversely affect digestion. Among them, the most important is the lack of intestinal lipase after pancreas ablation, which leads to steatorrhea. Lipase-deficient patients are treated by supplementation of enzyme supplements, including lipase. In the future, lipase could be provided with the diet using a genetically modified BTA. The expressed lipase gene could be of bacterial origin if it can be shown to exert similar activities and is stable in the digestive tract compared to the native human lipase (*50*). The efficacy of the bacterial lipase was shown in dogs which received purified bacterial lipase in with the diet successful treatment of experimental steatorrhea (*51*). No BTA-producing high levels of lipase have been constructed yet, but research is currently ongoing in this field. In the future, BTA producing other enzymes may be investigated, for example, enzymes which detoxify dietary components or modify carcinogenic bile salts in human colon, which would reduce cancer risks.

4.1.3. Biological Mediators

A number of biological mediators (such as hormones or interleukins) are produced in the digestive tract. Interleukins, e.g., interferon, are associated with the prevention of several diseases by inducing cell resistance to viral attacks. It may be hypothesized that an increased level of a specific biological mediator may improve the resistance of the host. BTA could provide the desired mediator in the digestive tract. At pres-

ent, interleukins and growth factors are the best candidates to initiate fundamental research in this field, especially antiviral interferons. For example, the α- or β-interferons are able to block viral multiplication. If an appropriate quantity of interferon could be produced by LAB in the digestive tract, it could elicit a resistant state in the enterocyte and a reduced sensitivity to viral enteric infections. The pharmacodyndamics of interferons or other cell mediators in the digestive tract must be studied carefully, because the host response to these mediators is known to be dose-dependent. Interferons also are resistant to low pH, but sensitive to degradation by digestive proteases.

4.2. The State-of-the-Art on Heterologous Protein Expression in LAB

The area of the expression of homologous or heterologous proteins in LAB was stimulated by the identification of the genetic mechanisms in these bacteria (*52,53*). The first studies were performed with model molecules, such as reporter genes, or molecules of industrial interest. Then molecules having a medical interest, such as the *Clostridium tetani* toxin fragment (*36,54*), viral proteins (*55,56*), or interleukins (*57*), were investigated.

4.2.1. Genetic Approaches to Produce Heterologous Protein in LAB

The biosynthesis of proteins in bacteria follows the general rules of all living organisms. A gene carried on a DNA fragment produces a messenger RNA, which is then processed by the cell machinery and translated into a protein. The first step is under the control of the promoter, which is a nucleotide sequence placed upstream of the gene coding sequence for a specific protein. The strength of the promoter may vary according to its sequence and specific regulation depending on environmental conditions. Some promoters may be constantly open, a property that might be of interest unless the continuous production of the desired protein is toxic. Actually the ideal promoter for driving gene expression should be closed in most media, but specifically open in the proper place of the digestive tract. Such an ideal promoter has not been characterized or constructed yet, but work is in progress in this field. Promoters induced by a precise metabolite or protein have been isolated (*26*). Their use in the BTA field might be effective if the inducer is nontoxic, stable in the diet, and slowly metabolized in the digestive tract. Among the described systems, the P_{nisA} induced by nisin A has promising properties to drive gene expression in LAB (*58,59*). Nisin A is a peptide with bacteriocin properties produced by LAB. It is used as a food additive to prevent

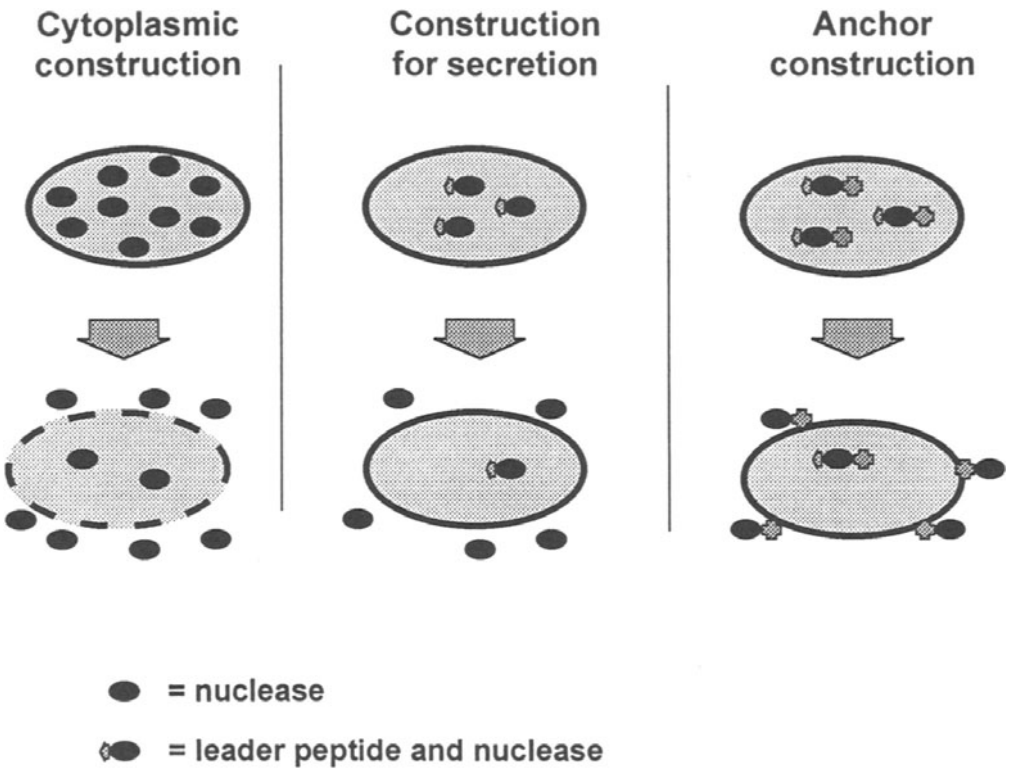

Fig. 2. Localization of proteins produced by microorganisms. Depending on the vector used to produce a protein, it can be accumulated in cytoplasm **(left),** secreted in the medium **(middle),** or bound to the cell outside of the cell **(right).** Protein accumulated in the cytoplasm can be found in the medium if a significant part of the population lyses.

bacterial growth. P_{nisA} promoter drives the expression of the enzyme machinery to produce nisin A. This strong promoter is closed in the absence of nisin A and fully open on its addition. However, it has not been tested yet in the digestive tract, and it is feared that nisin A could be digested by enzymatic activity in the digestive tract. Other promoter-inducer systems that might be developed in the future, are those involved in malolactic (*60, 60a*), xylose (*61*) or lactose (*62,63*) fermentations.

Another consideration in the development process is related to the location of the molecule in the BTA. Targeted molecules could be produced in the cytoplasm, secreted in external medium or anchored onto the bacterial outer membrane (Fig. 2). In LAB, various vectors have been constructed to achieve the targeting of proteins at different locations (*64–68*). It seems obvious that exclusion of cytoplasmic pro-

duction would result in minimum protein release in the digestive tract. However, some LAB do not survive in the digestive tract, a process that could be followed by the cell lysis and release of the desired protein. In such conditions, the bacteria could be considered as a bag containing the active protein. The challenge then is to obtain cell lysis at the right place. This possibility has to be checked. The anchorage of protein to the cell (*67*) could be of value especially in the field of local immunity. In this case, the cell-bound antigen would presumably be more immunogenic than the unbound antigen.

The production of a heterologous protein that remains in the bacterial cytoplasm is easier to achieve than a protein that must be either secreted or secreted and anchored onto the bacterial cell surface (*69*). Secretion of a protein requires the presence of a leader peptide to transport the protein to the membrane. This requirement might limit the rapidity of the development process. In *L. lactis,* Steidler et al. (*70*) have tested the secretion of different interleukins and cytokines under the control of the same promoter, expression signal, and plasmid vector. The amount in the external medium varied from 0 to 100 ng/mL. This suggests that other factors interfere with the secretion of these proteins and that further studies are required to optimize the export of protein outside the cell. The anchor option is more complicated, since it requires a second peptide in addition to the leader peptide already mentioned. The function of this second peptide is to bind the molecule to the cell wall after secretion. Some specialized vectors were constructed for this purpose and were found to be efficient in lactic bacteria (*67,68*).

The use of genetically modified bacteria as BTA raises the question of the eventual gene dissemination in the environment. Indeed BTA are designed to be delivered to the digestive tract in a viable state. Therefore, the risk of gene transfer to other microflora, the host, or the environment must be as limited as possible. Recently, new genetic systems were constructed for LAB, allowing precise insertion of DNA into the chromosome (*27*). It is a gene replacement system. Therefore no plasmid or transposon or foreign gene sequences remain except the altered gene. This procedure is, to our knowledge, the safest technique available. Other bacterial containment systems are also under study. For example, the introduction of conditional lethal mutations in the chromosome of the bacteria (*71–73*), which would destroy the BTA after the therapeutic action was achieved, has been investigated.

4.2.2. Expression of Heterologous Protein in Digestive Tract by LAB

There are numerous examples of expression of heterologous proteins in LAB (*see* Table 1). Some proteins were mostly used as models such as *Streptococcus pyogenes* M6 protein (*64,68,74,75*) or *Staphylococcus aureus* nuclease (*76*). Others have application in disease prevention, such as tetanus (*36,69*) or may involve viral fragments (*56,74*). The latter field is quite new (*36,70*), and many of the findings mentioned in this chapter are from personal communications or unpublished data. Some of these findings are listed in Table 2 and should be considered as emerging research.

The next more difficult question is the production of these heterologous proteins in the digestive tract. To our knowledge, no studies have dealt with the direct quantification of the heterologous protein in the digestive tract. It can be shown indirectly that production has occurred since immune responses are demonstrated after the oral inoculation of protein producing LAB. Beyond the technical problems of producing a specific protein in LAB, a particular issue relating to the complexity of the digestive tract is the need for released heterologous proteins to be sufficiently stable in the digestive tract to ensure the desired effect. In the small intestine, large quantities of proteases are secreted by the pancreas. In the large bowel, most of the proteases from the host are inactivated, but additional proteases are produced by the normal microflora. All these enzymes may substantially reduce the quantities of available heterologous protein.

4.2.3. Immune Response to Heterologous Proteins Produced by LAB

Some LAB have been investigated to develop oral vaccines. Therefore, the immune response was determined after local inoculation (oral or nasal) of the tested LAB (Table 3) (*36,77–79*). The intranasal route of immunization is unexpected for LAB, which are absent in the respiratory tract. However, the authors demonstrated that this route was effective in stimulating an immune response (Table 3).

Streptococcus gordonii elicits a systemic immune response against different antigens when given orally or intranasally (*74,80*). These bacteria are a natural resident of the oral cavity and persist in oral/pharyngeal mucosa for 10–11 wk. These bacteria were engineered to express an allergen from hornet venom by fusion with the anchor region of the M6 protein of *S. gordonii* at its surface (*80*). A significant increase of specific IgA was detected in saliva and lung washes, suggesting that the antigen production was sufficient to stimulate the immune system. Mice were immunized via an oral route with

Table 1
Heterologous Proteins Expressed in Lactic Bacteria (Published Investigations)

Lactic bacteria expression host	Antigen and source	Location of antigen, amount if known	Reference
Lactococcus lactis	Egg lysozyme	Intracellular	*100*
L. lactis	Fragment of tetanus toxin *Clostridium tetani*	Intracellular (20% soluble protein)	*36*
Lactobacillus plantarum	VP7 protein of rotavirus	Intracellular (0.1% total protein)	*56*
L. casei	VP1 protein of FMD virus	Intracellular (0.2% total protein)	*56*
L. lactis	Murine interleukin-2	Secreted	*57*
L. lactis	*S. aureus* nuclease	Secreted	*76*
L. lactis and various *Lactobacilli*	β-lactamase	Secreted (80 mg/L)	*66*
L. lactis	*Bacillus stearothermophilus* α-amylase	Secreted	*101*
Lactobacillus curvatus	*Staphylococcus hyicus* lipase	Secreted	*102*
S. gordonii	M6 protein of *Streptococcus pyogenes*	Displayed on cell surface	*64*
S. gordonii	Allergen hornet venom	Displayed on cell surface	*80*
S. gordonii	Fragment of HIV-1 envelope protein	Displayed on cell surface	*55*
L. lactis	Fragment of tetanus toxin *Clostridium tetani*	Displayed on cell surface (0.2% total protein)	*36*
L. lactis	M6 protein of *Streptococcus pyogenes*	Intracellular, or displayed on cell surface or secreted	*68*

Table 2
Heterologous Proteins Expressed in Lactic Bacteria (Mentioned in Reviews or Unpublished)

Lactic bacteria expression host	Antigen and source	Location of antigen, amount if known	Reference
L. lactis	Fragment of tetanus toxin and fragment of HIV-1 envelope protein	Intracellular or displayed on cell surface	Litt from *36*
L. plantarum	Fragment of HIV-1 envelope protein	Displayed on cell surface or secreted	Mercenier A. from *36*
L. lactis	Murine interleukin-2	Secreted (100 mg/L)	*70*
L. lactis	Murine interleukin-2	Secreted (100 mg/L)	*70*
L. lactis	Murine interleukin-4	Secreted (3 mg/L)	*70*
L. lactis	Murine interleukin-6	Secreted (100 mg/L)	*70*
L. lactis	Human interleukin-2	Secreted (50 mg/L)	*70*
L. lactis	Murine interferon-β	Secreted (0 mg/L)	*70*
L. lactis	*Staphylococcus hyicus* lipase	Intracellular and secreted	*60a*
L. lactis	Ovin interferon ω	Intracellular	*60a*
L. lactis	Bovine β-lacto globulin	Intracellular	*60a*

Table 3
Immune Response to Heterologous Proteins Expressed by Lactic Bacteria

	Expression		Route of immunization			Immune response			
Strain	**Antigen**	**Localization**	**Systemic**	**Intranasal**	**Oral route**	**General**	**Local**	**Protection**	**Reference**
L. lactis	Tetanus toxin fragment	Intracellular, displayed on cell surface, or secreted	+			+	ND[a]	+	*54*
L. lactis	Tetanus toxin fragment	Intracellular, displayed on cell surface, or secreted	+			+	ND[a]	+	*69*
L. lactis	Tetanus toxin fragment	Intracellular		+		+	+	+	*41*
L. lactis	Tetanus toxin fragment	Intracellular, displayed on cell surface, or secreted		+	+	+	+	+	*36*
L. lactis	Tetanus toxin fragment			+	+	+	+	+	*42*
S. gordonii	Allergen from hornet venom	Displayed on cell surface		+	+	+	+		*80*
S. gordonii	Human papillomavirus protein	Displayed on cell surface		+	+	+			*74*
Lactobacilli	Influenza virus antigen	Intracellular	+				+		*56*

[a]ND = not done.

S. gordonii expressing the E7 protein of human papillomavirus type 16 by fusion with the M6 protein (*74*). The immune response depended on the effective colonization by live bacteria, since killed bacteria did not induce such a response.

Since *L. lactis* does not become established in the digestive tract, passive transit of this specific bacteria is observed. Regardless of this challenge, experiments have been done using *L. lactis* strains expressing a tetanus toxin fragment, which is a potent immunogen. If a protective response occurs, this can be demonstrated by challenging test animals with the natural toxin. Intranasal immunization of mice with *L. lactis,* which intracellularly expressed the toxin fragment, induced a significant systemic response (*36,41,79*). Similar results were obtained with mice immunized orally (*42*). The elicited antibody titers were lower than those following intranasal immunization, but the protective efficacy was on the same order of magnitude. Antitoxin-specific fecal IgA responses could be detected following both oral or intra-nasal immunization.

The development of immune responses using oral or intranasal routes is very encouraging, since it indicates that the heterologous proteins produced by LAB are able to reach and stimulate the immune system. In the future, we can look forward to the results of oral immunization with a wider variety of antigens.

4.3. Conclusions About the Use of LAB as Producer of New Molecules in the Digestive Tract

We have shown in this chapter that there may be numerous genetic tools to express heterologous proteins in LAB. They allow the development of new LAB-producing antigens, enzymes, or mediators, which may have positive effects on health. Different studies performed in animal models show that these new genetically modified LAB are good candidates for oral vaccines against different pathogens. This promising field will continue to develop in the near future and is an original direction for future research with BTA.

5. LAB Metabolism with Special Attention to Digestive Tract Conditions

The production of heterologous proteins and the efficiency of a BTA are likely to be dependent on the metabolism of the bacteria during transit or after colonization of the digestive tract. Thus, the knowledge of how bacteria behave in the digestive tract is necessary to develop their full potential as BTA. The digestive tract content, in

term of culture medium, varies from the mouth to the rectum and is not homogenous within each compartment. Consequently, the conditions encountered by the bacteria are very diverse; in addition LAB are diverse, and the results obtained with one special strain cannot be extrapolated to all bacteria.

5.1. Survival, Lysis, and Level of Population of Ingested LAB During Transit

The viability of LAB in the digestive tract has been studied extensively for many different species (Table 4). Different techniques can be applied to recover the contents and make viable counts on proper selective media. The presence of resident LAB may interfere with a simple count, because proper selective media do not exist to discriminate all LAB from each other. However, the human flora does not contain high levels of LAB compared to the ingested bacteria present in fermented food or tablets. Nevertheless, significant errors might occur if the intrinsic resistance to digestion of the ingested bacteria is low. Nucleotide probes might be used to discriminate ingested bacteria from other different species of the same genus, but they do not allow the differentiation of other bacteria. The difficulties in identifying ingested LAB from resident strains may be overcome by marking the strains. The introduction of antibiotic resistance genes on a plasmid using transformation could be a simple method to achieve this, although some endogenous bacteria may already carry antibiotic resistance genes, and high plasmid instability has been reported in ingested bacteria. Finally, other types of markers could be used, but they necessitate technical experience in the genetic engineering of the bacteria studied (*see* Section 5.2.).

The acidic content of the stomach is not usually the most significant factor affecting LAB viability, especially when LAB are ingested with food. Pancreatic secretion decreases the viability of the two yogurt bacteria, *S. thermophilus* and *L. bulgaricus*. However, some Lactobacilli, such as *L. acidophilus, L. casei* and *Lactobacillus sake*, may resist degradation in the small bowel and reach significant levels in the colon. It should be emphasized that even if ingested LAB may transiently represent a dominant part of the flora (up to 10^7 CFU/mL) in the parts of the digestive tract that normally contain low levels of bacteria, such as the jejunum, these bacteria will represent only a small fraction of the population of the colon. In the colon, LAB population may be around 10^8 bacteria/mL compared to 10^{11} for the dominant endogenous flora (5).

Table 4
Survival of *L. lactis* Along the Digestive Tract and Expression of Luciferase Genes by *L. lactis* Along the Digestive Tract (Corthier et al., Unpublished Results)

			Contents of the different parts of digestive tract				
		Inoculum	**Stomach**	**Duodenum**	**Jejunum**	**Ileum**	**Colon**
Forced-fed mice	Transit ratio[a]	1/1	1/10	1/300	1/50	1/1	1/1
	Luciferase/*L. lactis* $\times 10^8$ (log)	4.10^6	5.10^6	7.10^6	3.10^6	2.10^6	1.10^6
Transit of *L. lactis* with diet	Transit ratio[a]	1/1	1/1	1/5	1/1	1/1	1/1
	Luciferase/*L. lactis* $\times 10^8$ (log)	3.10^6	3.10^6	4.10^6	4.10^6	4.10^6	3.10^6

[a]Transit ratio was estimated as follows: thermoresistant spores of *B. subtilis* were inoculated with *L. lactis*. The two bacteria were enumerated 2 h after administration and the ratio of viable *L. lactis* per *B. subtilis* spore was calculated. The enumeration of *B. subtilis* spores and *L. lactis* used to calculate the ratio were given for the group of forced-fed mice.

Luciferase expression was carried out in *L. lactis* containing the *lux* genes under the control of a strong constitutive promoter on a high-copy-number plasmid.

The death of the bacteria is usually accompanied by the lysis of the cell, thus liberating the cellular content into the lumen of the gut. Assessment of these data in essential to understanding the pharmacokinetics of native or heterologous proteins if the bacteria is used as a delivery vector. However, very few experiments have been done to verify the assumption that cell death is associated with cellular lysis, despite the fact that this is not always the case in laboratory media. Indeed, there is no established methodology to carry out this measure in the digestive tract.

5.2. Physiological States of LAB

5.2.1. Latency, Growth, and Stationary Phase

The life of a nonspore-forming bacteria is divided into four phases after it is inoculated in a fresh laboratory medium. The first period, the latency period, is required before the bacteria reproduces. Then the bacterial cells proliferate in the expontential period, using components of the medium to grow and support cell division. Once an essential component of the medium is depleted, the cells stop growing and enter into the stationary phase until they are inoculated into fresh medium. The last phase or death period occurs when essential medium components are exhausted and not replaced. This ideal scheme of bacterial life is nevertheless much more complex, even in laboratory medium. Indeed, the development of the cells will differ as a function of the composition of the medium and other environmental factors. As the growth of bacteria occurs, nutrients are taken up from the medium, and nonuseful metabolites are excreted. For example, LAB consume glucose to produce energy and secrete lactate as an end product. As growth progresses, bacteria face changing environmental conditions, and spend increasing amounts of energy to utilize rare nutrients and excrete end products present in increasing amount in the medium. In most cases, high amounts of end products exert a toxic effect for the cells. To face the changing conditions, the bacteria activate the expression of new pathways, including high-affinity transport systems, biosynthesis pathways, and new degradative pathways to exploit the different resources of the medium. The physiological state of the bacteria changes with the evolution of the culture medium. Consequently, as a function of the age of the culture, cells will have different enzymatic profiles, membrane and cell-wall compositions, and different potentials to produce molecules or to resist the stress of environmental changes.

The knowledge of the variation in the metabolism is of particular importance for BTA at the time of administration and during transit. Indeed the cell state before ingestion will influence the survival in the digestive tract. Moreover, the capacity of the cell to produce high amounts of biotherapeutic molecules will depend on the metabolic activity of the cell, its energetics, and the expression system introduced in the cell.

The variations of this metabolism have been extensively studied for bacteria, such as *E. coli* and *Bacillus subtilis,* that serve as models for Gram-negative and Gram-positive bacteria, respectively. Studies on LAB, although less advanced, show that the data obtained with *E. coli* and *B. subtilis* could not always be extrapolated to LAB. Moreover, most of the metabolic studies were done in controlled laboratory or industrial media that are completely different from the digestive tract content as medium. It thus appears necessary to study specifically the metabolism of LAB in the in vivo environment where it will be used as BTA.

5.2.2. New Tools to Assess the Physiology of LAB in Complex Media

Traditional methods to assess the physiology of bacteria include assays for enzymatic activities, production or consumption of metabolites, cell content, and composition. None of these methods can be applied to study of the bacteria in the digestive tract content for several reasons. The heterogeneity, low quantity, and the poor accessibility of cells in the digestive tract content make cell recovery extremely difficult. Moreover, the presence of indigenous bacteria in the digestive tract contents does not allow the measurement of metabolic activities of a single population of bacteria, because it is difficult to separate the ingested bacteria from the bacteria already established in the digestive tract. The distinction between metabolites produced by the ingested bacteria from those of the indigenous flora is not possible either. Finally, metabolites specifically produced by the ingested bacteria can be further transformed by the natural flora or absorbed by the mucosa. It follows that new methodologies should be used.

The physiological state of bacteria in the digestive tract can be investigated by the use of target rRNA probes in epifluorescence microscopy. A study with *E. coli* suggested that the actively reproducing cells were located in the mucus, but were static when in the lumen (*81*). This confirms that there are different ecological niches in the digestive tract, a fact that complicates the study of bacterial physiology. Methods based on other types of fluorescent probes by flow

cytometry or microscopy allow the measurement of a limited number of parameters related to physiology, such as growth rate or cell viability. However, the use of fluorescent oligonucleotide probes is not sensitive enough to measure the strength of the promoter by direct mRNA hybridization (*82*). Moreover, although rRNA probes are available for LAB, fluorescent microscopy has not been used yet to determine the state of this bacteria as well as its localization in the mucus or in the lumen. Indeed, concentrations of the ingested LAB might be not sufficient compared to the autochthonous flora to allow this type of study.

To trace bacteria in their environment, light-emitting genes, such as luciferase genes (*lux*AB), can also be used. For example, the dissemination of invasive Salmonella, in which the *lux* operon (complete operon including substrate biosynthesis) was introduced, was successfully followed in living mice using a very sensitive video camera (*83*).

We have proposed a methodology, based on the use of the luciferase genes as a biosensor, to study the physiology of colonized bacteria or in transit with the diet. The bacterial luciferase was shown to be a potential reporter gene in *L. lactis* to measure promoter strengths (*63,84,85*). The emission of light with intact cells requires nonaldehyde as substrate and reduced flavin mononucleotide (FMN) as a cofactor (*63*). The cofactor is supplied by the bacteria, whereas the substrate is added at the time of measurement. The availability of the reduced cofactor depends on the physiological state of the cell, since it is present in detectable amounts only in fully viable and metabolically active cells. Thus, dying bacteria, as well as those that are metabolically inactive or unenergized, will not produce light, even if the gene product of *lux*AB, the luciferase, is present in the cells. This reporter gene has an advantage over classical systems based on enzymatic assay or nucleic hybridization in that no interference with any activity of the resident flora that do not have light-emitting activity is observed. This eliminates the problem of nonspecific signals obtained with fluorescent metabolic probes, which may react with a large number of microorganisms (*86*).

Luciferase can also be used to measure the activity state of the cell, using an indirect measure of the reduced cofactor, which is depleted when the cell's activity is low. We have shown also that it was possible to measure specifically the activity of a promoter in active cells. From the information about an active or inactive promoter, it is possible to deduce the metabolic activities of the bacteria. Indeed the organisms

adapt their metabolism to the environment by synthesizing proteins, the activities of which are required for bacterial survival and eventual proliferation. The level of transcription of regulated promoters reflects the need of the cell for a particular function according to its physiological state and environmental stimulation. The measurement of the promoter activity in the digestive tract would contribute to our knowledge of the physiological state of added BTA.

5.2.3. Physiology of LAB in the Digestive Tract

Little is known about the physiology of LAB in the digestive tract. It is known that some strains may naturally colonize the digestive tract, but their concentration is very low compared to the dominant flora, which is always present at least at three orders of magnitude (1000) over LAB. Nevertheless, these bacteria have developed some metabolic activities in order to survive and remain in the intestine. Most LAB, as well as other BTA, are present only during transit, and thus, their population level is dependent on the amount ingested. It is expected that BTA metabolism could be different from the metabolic activities of the resident flora.

The physiology of a strain of *L. lactis* has been investigated with the help of the luciferase genes (Fig. 3) by the methodology described earlier. An early encouraging finding for the use of LAB as BTA was that in fecal samples, viable *L. lactis* are fully active as suggested by the level of luciferase activities per bacteria, which was similar in the feces and in the inoculum. Moreover, the concentration of LAB was relatively high 1 d after ingestion, but dropped to nondetectable levels after the second day (*60a*).

The specific effect of each compartment of the digestive tract on the bacteria could be investigated by administering bacterial suspension containing the Lux gene to mice. During transit, the cells face different conditions in each segment of the digestive tract as the organism is exposed to gastric juice, bile salts, and mucosal secretions, which might affect its metabolic activities and viability. At the end of the digestive tract, there is intense competition with the complex resident flora. This may be owing to bile salt secretions and/or defensins produced by enterocyte (*87–89*).

In forced fed mice (gavage), *L. lactis* and the thermoresistant spores rapidly traversed the digestive tract so that some *L. lactis* and spores reached the lower tract within 15 minutes. Thereafter, a stasis occurred and while most of the *L. lactis* was found in the lower tract, some

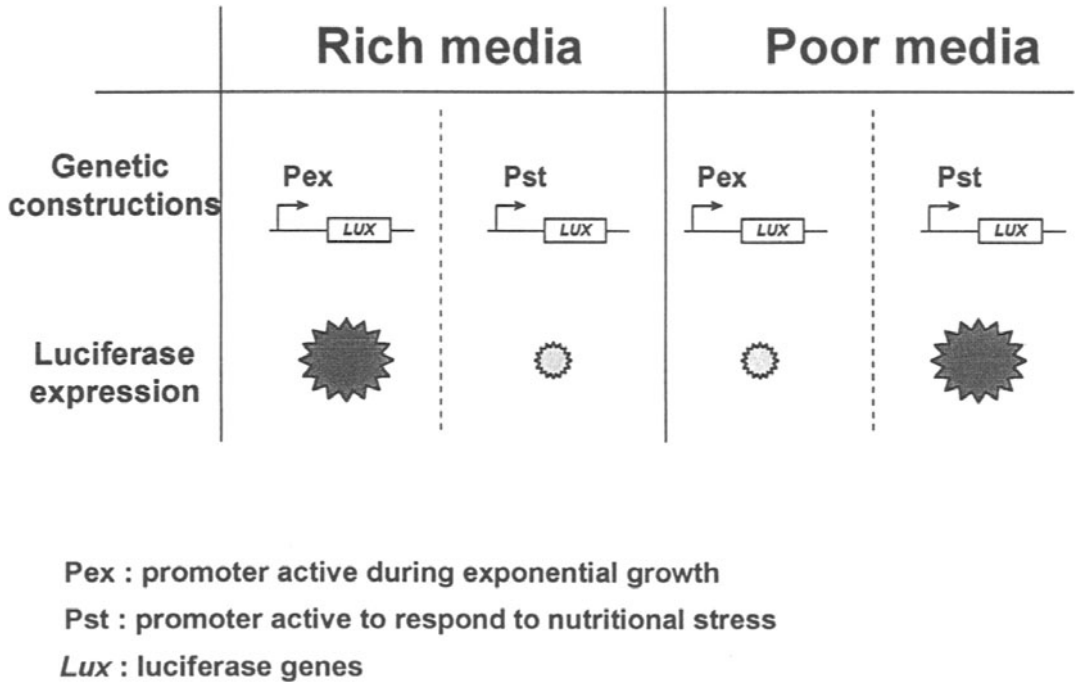

Fig. 3. Use of luciferase as reporter gene in *L. lactis*. The emission of light depends on the activity of the promoter controlling its expression. The activity of the promoter may depend on the environmental conditions, such as the medium composition (shown in the figure), but also on other factors (temperature, pH, oxygen, and digestive tract conditions).

remained throughout. The data in Table 4 were obtained by measuring the numbers of viable *L. lactis* and spores from the indicated intestinal section at 2 h after gavage. The results indicate that the *L. lactis* that had reached the ileum and colon remained viable (1/1 ratio), whereas *L. lactis* that remained in the upper digestive tract (stomach, duodenum, jejunum) lost viability (ratio < 1). The duodenum was an especially important site of destruction. When *L. lactis* was given together with the food, there was much reduced loss of viability in the upper digestive tract. This observation will have to be taken in account if bacteria are used as a vector to produce substances during transit. Interestingly, the luciferase probe demonstrates that surviving *L. lactis* are able to produce normal luciferase activity in each compartment during transit (Table 4). This property suggests that these bacteria are still active during transit and would be able to produce desirable proteins in the digestive tract.

Table 5
Expression of Luciferase of *L. lactis* Under the Control of Different Promoters in Culture Broth and in Gnotoxenic Mice (Colonization) (*60a*)

		Luciferas/*L. lactis* × 10^8, log		
		P_{his}[c]	P_{hisD}[d]	P_{aldB}[e]
CDM[a]	Amino acid starvation[b]	ND	ND	3.9
	Histidine starvation	3.5	4.3	3.9
	No amino acid starvation	2.5	4.4	4.9
Digestive tract		1.8	4.9	3.5

[a]CDM: chemically defined medium, ND: not done.
[b]Methionine, serine, arginine, isoleucine, leucine, valine (deleted amino acids).
[c]P_{his} drives the expression of the histidine biosynthesis enzymes.
[d]P_{hisD} is the same promoter deleted from the sequence involved in histidine response
[e]P_{aldB} drives the expression of acetolactate decarboxylase, an enzyme produced during exponential growth of the cells

In the studies of *L. lactis* in conventional mice, the physiology has not been probed with different promoters, the activity of which could vary in different compartments of the digestive tract. Thus we have no real idea of the different activities of the cells during transit. This was investigated using gnotobiotic mice (*60a*). These mice were made to contain a stable level of *L. lactis* population in fecal samples (*90*).

Several promoters were used to study different environmental stimuli. The promoter specific for the acetolactate decarboxlase gene, P_{ald}, had reduced activity during the stringent response (a cellular response to amino acid starvation) (*91*). During this response, the growth of the bacteria was blocked. The activity of most promoters involved in the transcription of catabolic genes is reduced, and those for the biosynthetic genes were activated. The similar activity of this promoter (P_{ald}) in the digestive tract and a medium depleted of some amino acids (Table 5) suggests that a stringent response is induced in the digestive tract, although the limiting factor remains to be characterized (*60a*). Amino acid starvation is probably not responsible for the stringent response in the digestive tract, since it contains a large amount of free amino acids. Indeed the stringent response might also be induced by other factors, such as carbohydrate deprivation (*92,93*) or stress (*94*).

Since established *L. lactis* cells colonizing the digestive tract are regulated by this stringent response, it will be necessary to choose a promoter active during this response in order to express new molecules. Promoters involved in the transcription of amino acid biosynthesis are usually repressed by the amino acid itself or intermediary compounds, but not by the global stringent response. For example, the region involved in this specific control is known as the promoter for histidine biosynthesis genes in *L. lactis* (P_{his}). It is closed when histidine is added to chemically defined medium (about 1 log; Table 5) and closed to a higher degree in the mouse digestive tract, which contains histidine (about 1.7 log; Table 5). A derivative of this promoter (P_{hisD}) drives a very strong expression of the luciferase gene in gnotoxenic mice (Table 5), suggesting that *L. lactis* cells are metabolically active and contain nonlimiting amounts of reduced FMN. This allows *L. lactis* to maintain its population concentrations in gnotoxenic mice even when dilution owing to transit occurs.

The metabolic activities of these bacteria in the digestive tract are not restricted to a simple steady state, and *L. lactis* is also able to respond to new stimuli, such as substrate induction. This was shown by the use of a promoter (P_{mle}) whose control is dependent on the action of a cell activator and on malate in the medium (*60*). The activity of this promoter can be activated 40-fold by the addition of malate to the diet in gnotoxenic mice (*60a*).

Until now, knowledge of the metabolism of *L. lactis* in the digestive tract has been limited by the number of promoters tested. However, the use of properly regulated promoters as biosensors for the metabolic state of the bacteria is a promising approach that will allow a more extensive understanding of the physiology of the cells in this complex environment. Finally, it will help to choose appropriate promoters to deliver proteins for therapeutic use at the required level.

Similar studies with other LAB, such as *Lactobacillus, Enterococcus,* or *Bifidobacterium,* which have better survival in the digestive tract (*37,95*), will be of great interest in the future. These bacteria may be better delivery vectors for new molecules, because they remain in the digestive tract longer. Moreover, these studies may help to identify the functions that are necessary for a bacteria to become established in the digestive tract. This will allow the development of methods to control the survival and the activity of bacteria during transit or on colonization, and will have a real impact on the use of bacteria as a delivery vector for targeted molecules.

6. Relevant Characteristics for Future Investigations

6.1. Colonization

The colonization of bacteria in the digestive tract is still not well understood; it depends not only on bacterial factors, but also on the host itself, as well as on the nature of the indigenous flora. An important factor for the use of bacteria as BTA is the establishment of the therapeutic agent in the digestive tract. We are not so sure that this assumption is valid. A BTA introduces a variety of new substances into the digestive tract and may modify the environment; these features could be dangerous if such modifications became permanent. For example, in the field of local immune response, the colonization of the BTA producing a specific antigen will initially lead to an expected local immune response followed by undesirable tolerance immune response. The same deleterious effects could be imagined for a permanent enzyme or mediator production. Therefore, the long-term presence of a BTA might be desirable or undesirable, depending on the nature of the therapy. We believe that continuous ingestion of the living BTA during a limited period of time may be more appropriate.

Most oral LAB cannot permanently colonize the digestive tract. Strains isolated from the human digestive tract may be good candidates for recolonization of the gut. However, it seems that once isolated in a laboratory medium, and even more after large-scale production, these bacteria are usually altered so that they can no longer colonize the digestive tract. There is also a tendency in Europe to increase the levels of LAB already present in the digestive tract, especially Bifidobacterium. However, no one knows the long-term effect of a permanent high population of Bifidobacterium on human health.

6.2. Adhesion on Mucosal Surface

The idea is widespread that a microorganism that will adhere to the mucosa would be a better candidate for BTA. To our knowledge, bacteria that adhere in large quantities to the upper digestive tract are pathogenic. Adhesion may be useful to mimic the normal pathological process, such as with an attenuated Salmonella, to be used as a vaccine. This approach is not ideal for BTA. Some Lactobacilli have been described that adhere to human digestive mucosa (*96*), but in limited numbers. Adhesion has often been measured in vitro with Caco-2 cells. For example, factors involved in the adherence *L. acidophilus*,

L. gasseri, and *Lactobacillus delbrueckii* subsp. *bulgaricus* were investigated in this cell line (*97*). However, it is not known whether adhesion really occurs in the upper or lower digestive tract or whether adhesion is essential for beneficial effects with Lactobacillus. Further research is needed before these important questions can be answered.

6.3. Survival During Transit

BTA administrated orally will have to face severe stresses, such as:

1. Exposure to acid in the stomach.
2. Exposure to pancreatic secretions and defensins from duodenum enterocyte.
3. Antagonist effects from indigenous bacteria in the colon and competition for nutrients.

Taken all together, these conditions may be crippling for the use of BTA in the digestive tract, but the survival (estimated in fecal samples) has been found to be from 1 to 30%, depending on the species. The survival is species-dependent and even strain-dependent. Moreover, the physiological state of the bacteria before ingestion has a critical effect on its resistance to stress. Finally, the dose of BTA ingested and the mode of administration are also critical factors. Diet or additives might have a protective effect after ingestion. In the future, survival of BTA during transit could be an interesting variable to consider in order to improve the therapeutic potential of a BTA.

6.4. Metabolic Activity

BTA are living microorganisms and will thus exert metabolic activities during their transit unless they are in a dormant state. The cell consumes and produces a number of metabolites to assure its maintenance and proliferation. The effect of this metabolism may be beneficial; for example, the cleavage of lactose in the case of lactose deficiency. However, in some cases, it could be potentially deleterious, and it has been pointed out that massive deconjugation of bile salts in the small bowel can lead to fat malabsoption and diarrhea. Our present understanding reveals that LAB do not seem to produce significantly high amounts of metabolites to have a negative effect on the host. Moreover, it seems that in many cases, killing the bacteria before ingestion abolished their therapeutic activity. In the future, the metabolic activities of BTA in the digestive tract must be investigated further to enhance BTA efficacy. This approach has been studied with another BTA, a yeast, *S. boulardii.* It had been shown (*98,99*) that the yeast can modify *C. difficile* toxin receptors and produce a protease that may inactivate *C. difficile* toxins.

7. Summary

LAB and other microbes might have a potential as BTA either as natural strains or as recombinant strains for molecule delivery. Indeed, most studies on BTA use in human health are very recent; lack of information on the metabolism of bacteria in the digestive tract precludes their comprehensive use. In this chapter, we have presented the methodology that could be applied to assess the bacterial physiology in the digestive tract, a very complex ecosystem. Knowledge of the metabolism of LAB in the digestive tract is just beginning to emerge.

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