Use of Lactobacilli and their Pheromone-Based Regulatory Mechanism in Gene Expression and Drug Delivery

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Abstract: Lactobacilli are common microorganisms in diverse vegetables and meat products and several of these are also indigenous inhabitants in the gastro-intestinal (GI) tract of humans and animals where they are believed to have health promoting effects on the host. One of the highly appreciated probiotic effects is their ability to inhibit the growth of pathogens by producing antimicrobial peptides, so-called bacteriocins. Production of some bacteriocins has been shown to be strictly regulated through a quorum-sensing based mechanism mediated by a secreted peptide-pheromone (also called induction peptide; IP), a membrane-located sensor (histidine protein kinase; HPK) and a cytoplasmic response regulator (RR). The interaction between an IP and its sensor, which is highly specific, leads to activation of the cognate RR which in turn binds to regulated promoters and activates gene expression. The HPKs and RRs are built up by conserved modules, and the signalling between them within a network is efficient and directional, and can easily be activated by exogenously added synthetic IPs. Consequently, components from such regulatory networks have successfully been exploited in construction of a number of inducible gene expression systems. In this review, we discuss some well-characterised quorum sensing networks involved in bacteriocin production in lactobacilli, with special focus on the use of the regulatory components in gene expression and on lactobacilli as potential delivery vehicle for therapeutic and vaccine purposes.

Keywords: Lactic acid bacteria, lactobacilli, pSIP vectors, bacteriocins, gene expression, protein delivery.

INTRODUCTION

Lactobacilli have frequently been referred to as "probiotics" because of their health promoting effects to the human gastro-intestinal (GI) tract. Some of the much appreciated effects of lactobacilli have been attributed to their ability to modulate the immune response of the host and to control pathogens by affecting the diversity of the microbial flora in the GI-tract [1-3]. Several lactobacilli are commensals in the GI tract of humans and animals and are also often used in diverse protocols for fermentation and/or preservation of food and feed. These bacteria are therefore generally recognized as safe (GRAS) to human consumption. Because of their GRAS status and their indigenous presence in the human GI-tract, lactobacilli are considered highly attractive for therapeutic applications, in particular as vehicle for *in situ* delivery of antigens or other bioactive compounds in the GI-tract [4].

Lactobacillus plantarum is one of the most studied and best understood *Lactobacillus* species. This species has been isolated from a diverse range of habitats such as fermented food, decaying plant materials, faeces, and the vaginal and intestinal tracts. Potential probiotic effects of *L. plantarum* and the high survival rate during passage through the GI-tract make this species a promising candidate for delivery of interesting molecules to the human cavities [5, 6]. *L. sakei* is mainly found in meat products, but occurs also in a wide range of other habitats, such as fermented fish, plant material and rice wine. This species is widely used as starter cultures for dry fermented sausages in Europe and is quite resistant to low temperatures, high salt concentrations, smoke, ethanol, low water activity and radiation [7]. The genome sequences of both *L. plantarum* [8] and *L. sakei* [9] are known. Other *Lactobacillus* species with known genomes are *L. acidophilus, L. brevis, L. casei, L. delbrueckii, L. gasseri, L. reuteri* and *L. salivarius.* Most of these species are found in the GI-tract of humans. In fact, lactobacilli are among the dominant bacteria in some part of the human GI-tract such as the small intestine [10]. Consequently, lactobacilli are of special interest both as probiotics and as possible delivery vehicles.

As for other lactic acid bacteria, lactobacilli produce a number of acids, e.g., acetic acid and lactic acid, that have broad and unspecific antimicrobial effects. In addition, several of these bacteria produce ribosomally synthesized antimicrobial peptides, often referred to as bacteriocins, that have a more specific killing mechanism and that normally act on species closely related to the producers [11-14]. The most common classes of bacteriocins are the so-called lantibiotics (class I bacteriocins), characterized by post-translational modifications including formation of lanthionine rings [15], and the so-called class II bacteriocins that do not contain the post-translational modifications [13]. Some of the best known class II bacteriocins are the so-called pediocin-like bacteriocins (subclass IIa), known for their strong activity towards *Listeria* [16, 17].

It is generally believed that bacteriocins kill target cells by permeabilizing the cytoplasmic membrane via pore-formation, leading to leakage of cellular components and

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solutes and eventually cell death. Two main mechanisms in cell targeting have been uncovered. Lantibiotics employ the cell wall precursor lipid II as a docking molecule, to approach sensitive cells. Once bound to lipid II, these bacteriocins disrupt the cell wall integrity by blocking the synthesis of peptidoglycan layer or by forming lethal pores in the cytoplasmic membrane, in a concentration-dependent manner [18, 19]. The second mechanism, known to be employed by several class II bacteriocins, involves the mannose-phosphotransferase system (man-PTS) as a receptor [20-23]. The man-PTS is the key uptake machinery for mannose and glucose in bacteria and it is ubiquitous in the bacterial world but absent in eukaryotes. Such a mannose-uptake complex normally comprises three different proteins, the cytoplasm-located IIAB, and the membrane-located IIC and IID. By genetic and protein analyses, both IIC and IID (but not IIAB) were found to be required for man-PTS to function as a receptor for class II one-peptide bacteriocins [23].

Production of many bacteriocins involves a quorum-sensing mode of regulation, mediated by a secreted peptide pheromone (induction peptide; IP), a histidine protein kinase (HPK) and a response regulator (RR) [24-28]. Genes of these regulatory determinants are normally organized within the same operon and have a low basal expression at low cell density. This regulatory operon can be activated by some cues, e.g., the presence of some specific bacteria, in the environment [29, 30] or by a critical threshold concentration of the secreted IP when cell density reaches a certain level (hence the term "quorum sensing") [24, 25, 27]. The IP signal activates the gene activator RR via a series of phosphorylation reactions mediated by the sensor HPK. Subsequently, the phosphorylated RR binds to regulated promoters and activates a defined set of genes including its own operon, which eventually triggers a burst in bacteriocin production [31, 32]. A schematic view of the regulatory network is shown in Fig. (**1**). The signaling pathway taking place between the IP, HPK and RR is well understood [28, 33, 34]. Furthermore, components of such regulatory systems have been exploited in construction of a number of gene expression systems successfully applied in diverse settings (see below). In this review, we provide an overview of some of the well-characterized regulatory systems involved in quorum-sensing based bacteriocin production in lactobacilli and of the use of these regulatory systems for protein expression in lactobacilli. The potential application of lactobacilli as drug delivery vehicles will also be discussed.

REGULATED BACTERIOCIN PRODUCTION IN LACTOBACILLI

The Plantaricin System in *L. plantarum*

L. plantarum C11 produces two bacteriocins of the subclass IIb, i.e., non-modified bacteriocins whose activity is dependent on the action of two different peptides [35]. The regulatory mechanism behind its bacteriocin production involves the action of four different proteins, the IP plantaricin A (PlnA), the HPK PlnB and two homologous RRs PlnC and PlnD, through a quorum sensing (QS)-based pathway as outlined in Fig. (**1**) (for a review, see [13, 28]). PlnA is a small peptide (25 aa) with several physico-chemical properties resembling a bacteriocin. It is cationic and amphiphilic, and applies the same transport system (an ABC-transporter) as for bacteriocins [24, 32, 36]. In fact, PlnA has been shown to exhibit some antimicrobial activity [37]. Whether PlnA is a true bacteriocin is still disputable because the antimicrobial activity is chirally independent as both the L- and D-enantiomers of synthetic PlnA can kill sensitive cells [37] and because it does not involve a cognate immunity protein, a feature which is characteristic for most bacteriocins [13, 36]. Unlike its antimicrobial activity, the inducibility can only be achieved with the natural D-form of PlnA [37]. From a practical point of view, it is important to note that PlnA and all other peptide pheromones discussed in this section can be easily obtained by peptide synthesis.

The HPK PlnB belongs to the so-called HPK_{10} subfamily whose members are involved as sensors in various peptide-pheromone based QS pathways [38, 39]. Members of this protein family are characterized by a large N-terminal integral membrane domain [13, 36, 40]. Using a reporter gene fusion approach, Johnsborg *et al*. [39, 41] demonstrated that the PlnB's membrane-located domain contains seven TMSs (Fig. (**1B**)) and that, not surprisingly, this domain is responsible for the specific recognition of the cognate IP. PlnB can be activated by exogenously added PlnA at nanomolar concentrations [24]. The activation is then conveyed from PlnB, through a series of phosphorylation reactions, to the RRs PlnC and PlnD which in turn bind to and modulate gene expression from regulated promoters [39, 42, 43]. Five different operons, including the regulatory operon *plnABCD* are activated during this process, resulting in the bacteriocin producing phenotype [32].

The *pln* RRs bind DNA as dimers, in a cooperative manner, onto two direct repeats located in the -40 and -80 region of the regulated promoters [44]. Although non-phosphorylated forms of PlnC and PlnD can also bind DNA, phosphorylation of PlnC and PlnD significantly improves their DNA affinity [42, 43]. The nature of the regulatory repeats has been elucidated in great detail [32, 43, 44]. The individual repeats consist of 9 nucleotides (nt) and of the consensus sequence TACGTTAAT (Fig. (**1D**)). The pair-wise repeats are separated by a 12 nt-stretch which approximately corresponds to one helical turn of the DNA double-helix; this spacing allows the two repeats to face towards the same side of the DNA facilitating a cooperative dimeric binding of RR. DNA binding assay with mutated promoters have shown that the invariant bases in positions 2, 3, 6 and 8 of the individual repeats are important for (strong) RR binding and that also the 12 nt-spacing is critical for an efficient cooperative binding [44].

Other Plantaricin Producers

To date, three other *L. plantarum* strains, WCFS1, NC8 and J23, have been reported to contain a *pln* locus in their genomes [8, 45, 46]. Most of the encoded proteins from these loci share more than 95 % sequence identity with their counterparts in C11, and the genetic organization as well as the features of the regulated promoters are almost identical or identical to that in C11, suggesting that all these strains are recently derived from a common ancestor. An interesting feature worth to mention here is that the bacteriocin production in NC8 and J23 has been reported to be induced by co-

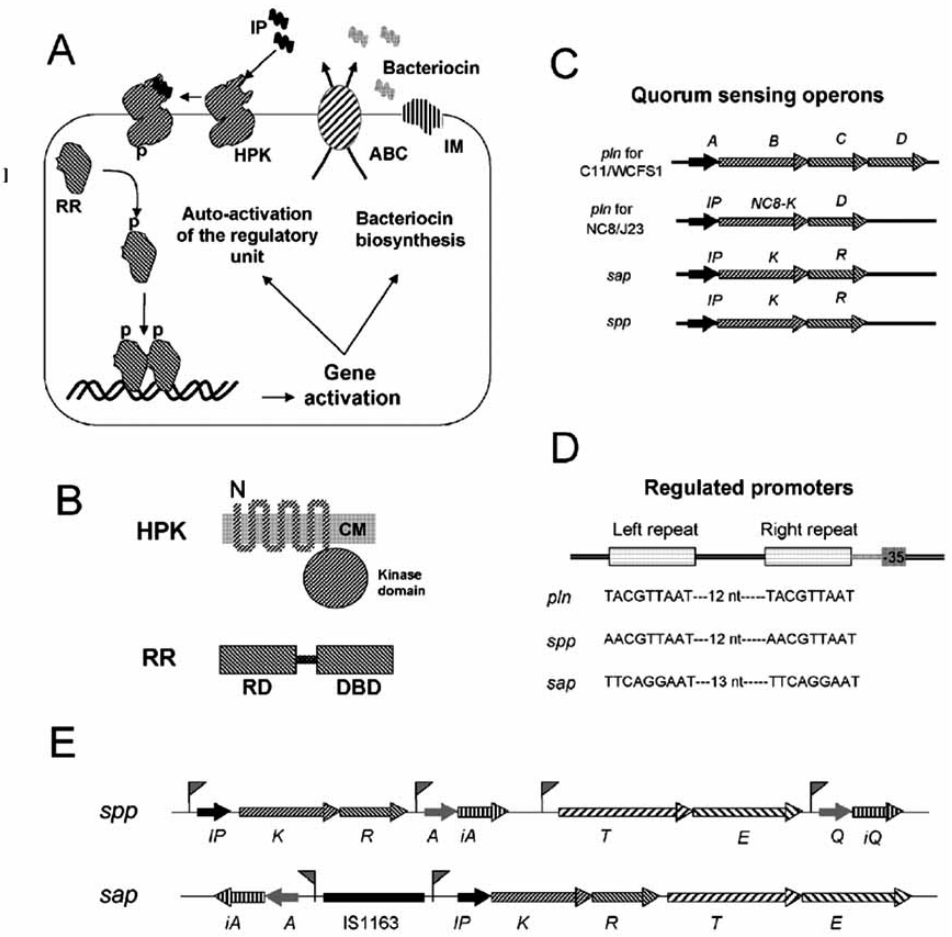

Fig. (1). A) A schematic view of an IP-based quorum sensing pathway involved in bacteriocin production. ABC, transporter protein exporting both the inducing peptide IP , and the bacteriocin (Nb. Note that transport in fact involves one more protein mostly referred to as "accessory protein"); IM, immunity protein for the bacteriocin; HPK, histidine protein kinase consisting of a domain located in the cell membrane (CM) and a cytoplasmic kinase domain that becomes phosphorylated (P) upon binding of IP; RR, response regulator, consisting of a receiver domain (RD) to be phosphorylated by the kinase, and a DNA-binding domain (DBD). See text for further description of the different steps. **B)** Topologies of an HPK and an RR. **C)** Genetic organization of the regulatory operons involved in the production of plantaricins (*pln*), sakacin A (*sap*) and sakacin P (*spp*). **D)** Regulatory elements in the regulated promoters. **E)** The *spp* and *sap* regulons. *IP*, *K* and *R* code for an IP, an HPK and an RR; *A* and *Q* for bacteriocin precursors; *iA* and *iQ* for immunity; *T* and *E* for an ABC-transporter and an accessory protein; IS1163, an IS-element; flags for regulated promoters.

cultivation with some specific bacteria [30, 46], a phenomenon which might reflect the *in vivo* sensing of competitors and a mechanism for the induced bacterial warfare. Not surprisingly, the inducing bacteria identified comprise different lactic acid bacteria as well as species of Gram-positive *Listeria* and *Staphylococcus*, bacteria that often coexist with lactobacilli in nature [30, 47]. The co-cultivation-induced bacteriocin production was found to be mediated through the action of the *pln* regulatory unit by an unknown mechanism. The exact nature of inducing factor(s) from inducing bacteria also remains to be identified. Some preliminary studies suggest that these factors are membrane-associated, heat-labile and probably of proteinaceous nature [30, 47].

The Sakacin Systems in *L. sakei*

Genetic determinants for bacteriocin production of sakacins A and P have been identified in a number of *L. sakei* strains and the best characterized producers of these two bacteriocins are strains Lb706 (sakacin A, *sap* locus) and LTH673 (sakacin P, *spp* locus), respectively [31, 48-53]. Both bacteriocins belong to the pediocin-like family and their production is regulated by quorum sensing, based on mechanisms that are machanistically very similar to those described for *L. plantarum* C11 (Fig. (**1**)).

The IPs for the production of sakacin P (spp-IP) and sakacin A (sap-IP) are cationic and small in size (19 and 23 aa residues, respectively), and the individual IPs act specifically on their cognate HPKs to activate bacteriocin production [28, 31, 48]. The small size and cationic property of IPs are characteristics that they share with other IPs and bacteriocins and are probably important for the initial approach to the anionic environment of the phospholipid layer of the membrane where they subsequently interact with the HPK sensor domain (Fig. (**1**)). In terms of sequence similarity between the different HPKs and RRs, the *spp* regulon seems more closely related to the *pln* regulon than to the *sap* regulon (Fig. (**2**)).

Furthermore, the *spp* regulon also shows much higher resemblance to the *pln* system in the way the regulated promoters are built up: the consensus sequence of the regulatory repeats in the *spp* system shares only 4 out of 9 nucleotides with the *sap*'s consensus sequence but 8 of 9 nucleotides with the *pln*'s consensus sequence, and the spacing between the pair-wise repeats is 12 nucleotides in both *spp* and *pln* promoters but 13 nucleotides in *sap* promoters (Fig. (**1D**)). Furthermore, the *spp* regulated promoters have also been shown to be recognized and efficiently up-regulated by the *pln* regulatory system in *L. plantarum* C11 [31, 54, 55].

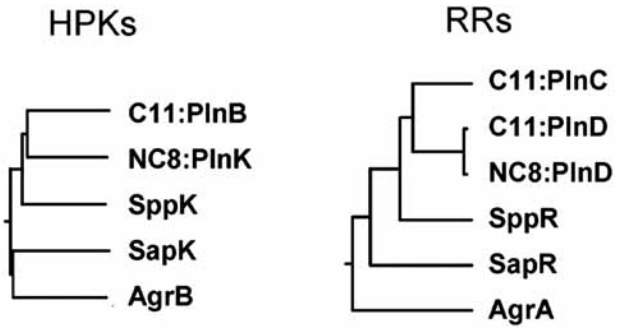

Fig. (2). Phylogenetic trees of the HPKs and RRs involved in IP-based quorum sensing pathways for bacteriocin production. The HPK AgrB and RR AgrA involved in toxin production in *Staphylococcus aureus* are included as a reference.

The production of sakacin A and P has been shown to be regulated by environmental changes, via the action of the quorum-sensing regulatory units in a manner resembling that described for *L. plantarum* NC8 and J23 above. Sakacin A is constitutively produced by Lb706 in the temperature range 25-30ºC, but production is significantly reduced or aborted at 33 ºC or higher. However, production of sakacin A can be reestablished at the elevated temperatures by adding sufficient amounts of the *sap*-IP into the growth medium [56]. The bacteriocin-producing phenotype of sakacin P-producing *L. sakei* Lb673 and also of plantaricin-producing *L. plantarum* C11 is lost under some conditions (such as extreme dilution on standard culture medium) but the bacteriocin-producing phenotype can be restored by adding the corresponding IPs [24, 25]. Furthermore, while non-producing cells of Lb673 (obtained by extreme dilution) kept the Bac^- phenotype under aerobic growth on certain types of media, production was resumed when the plates were kept under anaerobic conditions or when other types of media were used [31]. The impact of environmental conditions on the functionality of the QS-based regulatory system is important to keep in mind when exploiting these systems for gene expression (see below). On the other hand, all data so far show that the partly unknown effects of environmental factors may be "overruled" by addition of sufficient (but still low, i.e., in the 2 - 20 ng/ml concentration range) amounts of the appropriate synthetic IP.

The loss of QS-based bacteriocin production, as observed with sakacins A, P and plantaricins under laboratory growth conditions is not unique for these producers as it also occurs for producers outside lactobacilli, e.g., the enterocin P production in *Enterococus faecium* [57] and the production of carnobacteriocins and piscicolin 126 by some species of *Carnobacterium* [58, 59].

By definition, the term *quorum sensing* refers to cells monitoring their own density, in the present case by help of a secreted IP. When the cell density, reflected by the IP concentration, reaches a certain threshold ("quorum"), a change in gene expression is induced. The findings that environmental cues, such as the presence of specific bacteria, a change in temperature or oxygen tension, can affect bacteriocin production via the action of the regulatory unit, strongly suggest that such QS pathways are probably rather interactive with the environment and that the regulatory unit (IP, HPK and RR) itself is used as a means to monitor the cues and to coordinate an adaptive response. From an ecological point of view, this interactive regulation makes sense because bacterial habitats in nature are constantly subjected to changes (e.g., changes in pH due to acid production, changes in toxic pressure due to antimicrobial metabolites produced by the bacterium itself or its competitors, limited nutrition sources, etcetera.). Consequently, bacteria that can sense these signals and then launch a coordinated attack on other bacteria or fine-tune their growth pattern to the prevailing growth conditions will have a better chance to survive. It is challenging in future work to identify the nature of inducing cues and the underlying mechanism by which these cues interact with the QS pathway. The signaling cues could act either in an unspecific manner, e.g., a change in the pH or water activity across the membrane that might affect the affinity between the IP and the sensor HPK, or in a more specific way as shown for bacteriocin production in NC8 and J23 which is induced by an (unknown) proteinaceous factor from some specific bacteria.

USE OF BACTERIOCIN-BASED REGULATORY SYSTEMS FROM LACTOBACILLI IN GENE EXPRESSION

The discovery of regulated strong promoters driving massive production of bacteriocins in lactobacilli and the discovery of an analogous "nisin-"system driving lantibiotic production in lactococci [27, 60] in the mid-nineties were landmarks in bacteriocin research. Furthermore, and most importantly, these findings opened up new avenues for development of gene expression systems in lactic acid bacteria that would allow expression to be both highly efficient and strongly regulated. The availability of gene expression systems in LAB is very important for the exploitation of these bacteria, not only because of their potential roles as cell factories but also because gene expression systems are important tools in metabolic engineering and advanced studies in bacterial physiology.

The lactococcal nisin system (called "NICE" for NIsin Controlled Expression) was developed very rapidly after the initial discovery of the regulatory mechanism and has a long history of successful use in lactococci and other lactic acid bacteria [60]. The nisin system is particularly well-suited for use in lactococci, which are attractive as food-grade delivery vehicles (see below) but which do not colonize the human GI-tract. Successful use of the nisin system in lactobacilli has also been described [61-66]. Obviously, when it comes to the admissibility of genetically modified microorganisms

outside the laboratory, the use of non-self genes, such as in the case of using the lactococcal nisin system in lactobacilli, is preferably avoided. The options and applications of the lactococcal nisin system have been extensively described elsewhere, e.g. [60].

The first application of the promoters and regulatory mechanisms depicted in Fig. (**1**) was described by Axelsson *et al*. [67] who developed a versatile two-plasmid system for heterologous expression of bacteriocins in non-bacteriocin producing lactobacilli, based on genes and promoters from the *sap* regulon in *L. sakei* Lb706 [48]. One plasmid (pSAK20) harbours an operon consisting of the *IP-K-R* genes followed by two genes needed for transport and processing of the bacteriocin- and IP-precursors. The other plasmid contains the strong sakacin A promoter translationally fused to a bacteriocin structural gene followed by its immunity gene. This expression system contains all the genes that are naturally involved in auto-induction of bacteriocin production (in this case sakacin A), hence, no external IP needs to be added to activate expression of the gene of interest.

This two-plasmid expression system allows bacteriocin production levels that are higher than those routinely obtained with wild-type producer strains. The system can easily be adapted to any bacteriocin of interest as long as the typical bacteriocin "core operon" (i.e. bacteriocin gene followed by the immunity gene) is known. Indeed, the two-plasmid system has been used to produce a large variety of class II bacteriocins and has been instrumental for site-directed mutagenesis studies of bacteriocins, e.g. [51, 67-73]. It remains to be seen whether the two-plasmid system for bacteriocin overexpression also may be used to express other, non-bacteriocin peptides.

Interestingly, Corr *et al*. [1] very recently showed that *in situ* production of an anti-listerial Abp118 bacteriocin is a major cause of the well-documented probiotic effect of *Lactobacillus salivarius* UCC118. This shows that, when it comes to engineering lactobacilli for health, manipulation of the bacteriocin production spectrum is one of the options.

pSIP Vectors

In another approach Sørvig *et al.* [74, 75] developed series of versatile expression vectors based on the *sap* (pSIP300 series) and the *spp* (pSIP400 series) regulon (Fig. (**3**)). For comparison, analogous vectors based on genes from the lactococcal NICE system were also constructed (pSIP500 series; [74]). The vectors were constructed in a modular fashion, permitting easy exchange of different parts, such as the gene of interest, the promoter, the replicon, and the selection marker (Fig. (**3**)). An additional variable tested was the promoter driving the expression of the *KR* operon. In one series, expression of *KR* was driven by read-through from the upstream resistance marker gene (*ermB*) (Fig. (**3**)). In another series, such as in pSIP403 depicted in Fig. (**3**), expression was driven both by *ermB* read-through and by the original inducible promoter (P_{sppIP} in Fig. (**3**)). In all variants, the structural gene for the IP, which naturally precedes the K-R genes, was inactivated by deletion. With respect to the choice of promoter, it must be noted that IP-regulated bacteriocin regulons contain several IP-controlled promoters (Fig. (**1E**)), which in principle all can be used to drive expression of the gene of interest. Usually, the promoters driving expression and bacteriocin structural genes are the strongest and most tightly regulated [31, 55]. Therefore, only such promoters were used in the pSIP series, i.e., P_{sapA} in the pSIP300 series and P_{sppA} or P_{sppQ} in the pSIP400 series (Nb. P_{sppQ} has been called P_{orfX} in previous publications; see [72]).

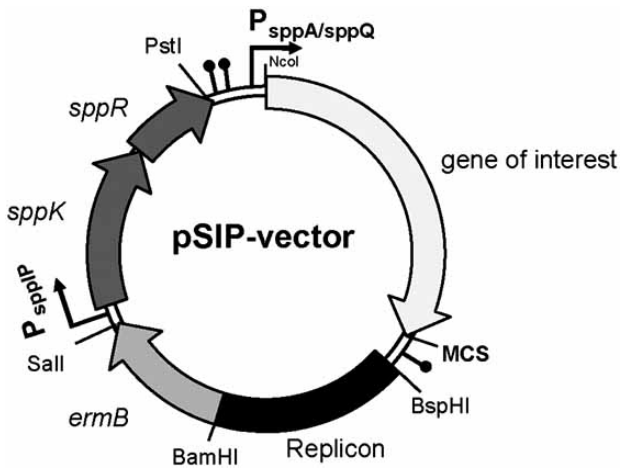

Fig. (3). Schematic overview of the modular pSIP-vector expression system. The picture shows the main outline of the pSIP400 series, based on the *spp* regulon. The *sppK* and *sppR* genes encode the proteins in the two-component regulatory system. The gene of interest is under control of one of the strictly regulated bacteriocin promoters, P_{sppA} (sakacin P) or P_{sppQ} (sakacin Q) and is translationally coupled to the promoter via an *Nco*I restriction that incorporates the ATG start codon. Unique restriction sites for easy replacement of different modules are indicated. The replicon region consist of two determinants; pUC(pGEM)-ori for *E. coli* and 256rep for *L. sakei* and *L. plantarum*. Lollipops indicate transcription terminators. Note that the vectors vary with respect to the promoter driving the transcription of the *KR* operon (see text).

The functionality of the pSIP vectors has been analyzed using several homologous and heterologous genes of interest (reporter genes), primarily *gusA* (β-glucoronidase) from Gram-negative *Escherichia coli* and *pepN* (aminopeptidase N) from Gram-positive *Lactococcus lactis*. Expression studies have been conducted using *L. plantarum* and *L. sakei* as host strains. Several of the pSIP vectors gave very good results in the sense that: (1) expression levels were high when induced, (2) basal gene expression (i.e., in the absence of IP) was low, and (3) the amount of IP needed for maximum induction was as low as 10-25 ng/ml and clear dose-response effects were observed at lower IP concentrations [74, 75]. The functionality of the different vectors (in terms of expression levels and strictness of regulation) depended in a non-predictable manner on the combination of host strain, gene of interest, promoter and vector copy number. This indicates that in some applications it may be useful to test various vectors for expressing a gene of interest. The nisin-based analogues (pSIP500 series) consistently functioned less well than the other pSIP vectors, both in terms of yield and strictness of regulation [74].

The potential of the pSIP vectors to express large amounts of protein in lactobacilli is illustrated in Fig. (**4**).

Panel A shows expression of *pepN* in *L. sakei* using a pSIP400 variant (Fig. (**3**)) in which the gene of interest is *pepN* and expression is under control of the P_{sppQ} promoter. In this set-up PepN levels amounted to as much as 46 % of total cellular protein [75]; see also [76]. The pepN levels obtained with the optimised vectors in *L. sakei* were similar to levels obtained with optimised variants of the lactococcal NICE system in *Lactococcus lactis* [77]. This PepN level is almost twice as high as the maximum levels obtained with strong constitutive promoters in *L. plantarum* (see below). Likewise, expression levels obtained with the *gusA* reporter gene were among the highest ever reported in LAB and in the same range as the levels that were obtained with the lactococcal NICE system in *L. lactis* [77].

Fig. (**4B**) shows results from a recent study by Halbmayr *et al.* [78] who tried to produce heterodimeric β-galactosidases from four *Lactobacillus* species in a food-grade manner by expressing them in *L. plantarum* and *L. sakei* using vectors from the pSIP400 series. This study showed that high levels of protein expression could be obtained (Fig. (**4B**)) but also confirmed that expression levels are difficult to predict. Expression levels varied in an unpredictable manner, depending on the gene of interest and the host strain used. Apparently, the effectiveness of the expression systems depends on subtle properties, affecting for example parameters such as mRNA stability or protein folding efficiency, that are not straightforward to predict.

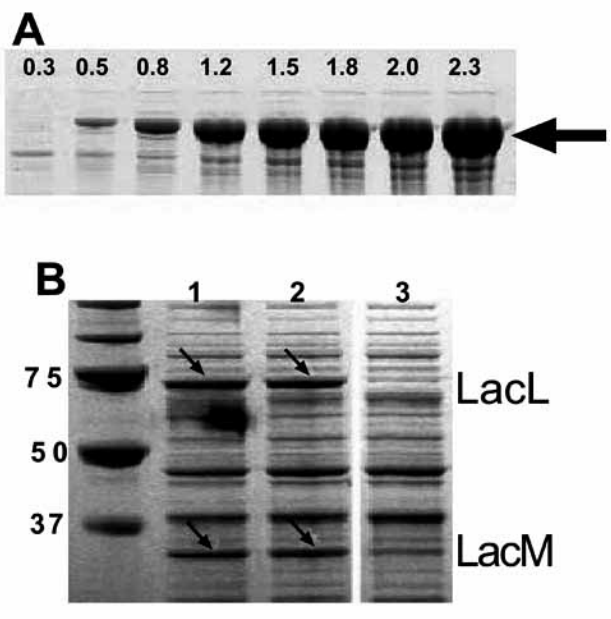

Fig. (4). SDS-PAGE analysis of intracellular production of heterologous proteins using pSIP-vectors in *Lactobacillus*. **A)** Production of *L. lactis* aminopeptidase N in induced *L. sakei* Lb790/ pSIP412 harvested at various OD_{600}. **B)** Production of both subunits of β-galactosidase (LacL & LacM) from *L. reuteri* in *L. plantarum* WCFS1 using pSIP403 (lane 1) and pSIP409 (lane 2) derivatives. Lane 3 shows non-induced cells.

Other Systems

In the past decade a range of constitutive promoters has been exploited for heterologous protein production in lactobacilli [79-86]. One of most used and strongest constitutive promoters is the promoter driving the expression of Surface-Layer-Protein, P_{slpA}. Kahala and Palva [80] have shown that the *slpA* promoter from *L. brevis* can drive expression of PepN to levels reaching up to 28% of the total protein content in cells. Also in this case, it was noticed that production levels varied considerably between different hosts and the reporter genes. Interestingly, Rud *et al.* [82] have recently constructed a library of synthetic promoters for *L. plantarum*, where the strongest promoter yielded PepN levels amounting to 10-15% of the total cellular protein. Using this library, the strength of constitutive expression can be regulated by selecting a promoter of choice.

The regulable NICE system is one of the most used for gene expression in LAB, including lactobacilli [60]. This is due to the following reasons: (1) many of the successful stories of expressing therapeutic proteins in LAB (see below) concern *L. lactis*, for which the NICE system is perfectly suited; (2) the NICE system may yield expression levels that are higher than those obtained with the best constitutive promoters; and (3) the NICE system has been developed extensively, including adaptations to use in other LAB than *L. lactis*. Finally, from a general point of view, it may be advantageous to use regulable promoters for production of heterologous, potentially toxic proteins. When using inducible systems, it is possible to generate a considerable amount of cell mass before expression of the gene of interest is induced. In this case, considerable amounts of protein can be obtained, even if this protein hampers growth of the host cell. Next to inducible nisin promoters, inducible promoters involved in sugar metabolism are often exploited [87-90].

Protein Targeting

Intuitively, it would seem that delivery of therapeutic proteins is most effective if these proteins are secreted out of the producer cell. In some cases, anchoring to the bacterial cell wall may be advantageous. Secretion of proteins in lactobacilli has been achieved using both heterologous and homologous signal peptides [86, 91-96]. Fundamental studies on secretion in *L. lactis*, e.g. focussing on the properties of signal peptides, have provided some insight into what governs efficient secretion in LAB [95, 97-100], but generally such insight is lacking for lactobacilli. It should be noted that despite tremendous efforts in understanding secretion in other Gram-positive bacteria, in particular *Bacillus subtilis* [101, 102], it is still generally difficult to predict how efficient protein secretion can be genetically engineered. One of the factors that can be most easily manipulated is the signal peptide. However, the efficiency of signal peptides is unpredictable and highly dependent on the secreted protein, as was shown by a genome-wide study of signal peptides from the Gram-positive *Bacillus subtilis* [103]. In a recent study, Mathiesen *et al.* [92] further developed the pSIP400 series by replacing the gene of interest (Fig. (**3**)) with a secretion cassette (Fig. (**5**)). This cassette is constructed in such a way that vectors containing different combinations between signal peptide and gene of interest can be engineered by simple restriction cloning. The ability to rapidly test several signal peptides is important because it is not possible to predict which signal peptide functions best for a certain protein. As part of a genome wide-study of the functionality of signal

peptides in *L. plantarum* WCFS1, a preliminary screen of seven signal peptides was conducted with two reporter proteins. The results confirmed the observation from the *B. subtilis* genome wide study [103] that the efficiency of a signal peptide depends strongly on the protein which is being secreted. Furthermore, this study yielded some signal peptides (Lp_0373 and Lp_0600; see Fig. (**6**)) that performed well with both reporters and that were as efficient or even better than the most commonly used heterologous signal peptides [92]; (Fig. (**6**)).

There are two principally different ways of anchoring a secreted protein to the bacterial cell wall: covalently, via the sortase pathway, or non-covalently, via a protein domain that interacts strongly with cell wall components or the cell membrane. Both systems have been used in LAB, primarily *L. lactis* and lactobacilli.

In sortase-mediated anchoring, the secreted protein contains a C-terminal anchor containing the so-called LPXTG motif followed by a hydrophobic domain and a positively-charged tail. A membrane-anchored sortase cleaves the peptide-bond between threonine and the glycine in the LPXTG motif and links the now C-terminal threonine of the surface protein to a pentaglycine in the cell wall [104]. There exist several examples of successful sortase-based anchoring in lactobacilli, including model proteins such as the *Streptococcus pyogenes* M6 protein [105], the *Staphylococcus aureus* nuclease [95] and potentially therapeutic proteins such as tetanus toxin fragment C [81], peptide fragment of the human cystic fibrosis transmembrane conductance regulator (CFTR) protein [106] and E7 antigen from human papillomavirus type-16 [93]. C-terminal anchors show quite some variation in length (i.e., the length of the linker between the mature protein of interest and the LPXTG motif). This implies that anchored proteins can have different positions in the cell wall matrix, which again may affect their functionality (e.g. [87] discussed below).

One non-covalent cell display system for LAB is based on fusing the protein of interest to the PgsA protein from *B. subtilis*. Naturally, the PgsA protein is part of the poly-γ-glutamate synthetase complex. PgsA contains one N-terminal transmembrane helix anchoring it to the cell membrane, whereas most of the protein located outside the cell wall. Several proteins have been successfully displayed in lactobacilli by fusing them to the C-terminus of the PgsA protein. These successful studies include examples where antigens were expressed and where the resulting recombinant strains elicited promising immune responses [84, 107-109].

Another surface-display strategy is based on the BspA protein from *L. fermentum* which is thought to be anchored to acidic groups on the cell surface by electrostatic interac-

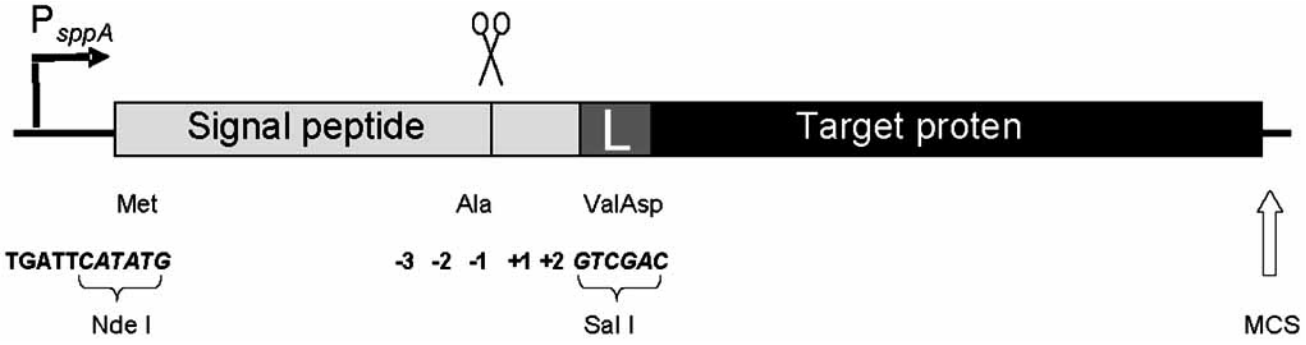

Fig. (5). Schematic overview of the modular secretion cassette which replaces the gene of interest in Fig. (**3**). Note that in secretion vectors the unique *SalI* site in the original pSIP-vector (Fig. (**3**)) has been deleted, whereas the *NcoI* site for translational fusion has been replaced by an *NdeI* site. The signal peptides followed by the first two amino acids of their cognate mature gene product were cloned into the *NdeI*/*SalI*-sites downstream of the sakacin P promoter (P_{sppA}). The *SalI* site was generated by inserting a linker (L) encoding the amino acids valine and aspartic acid. The scissors indicate the signal peptidase cleavage site. MCS, a multiple cloning site.

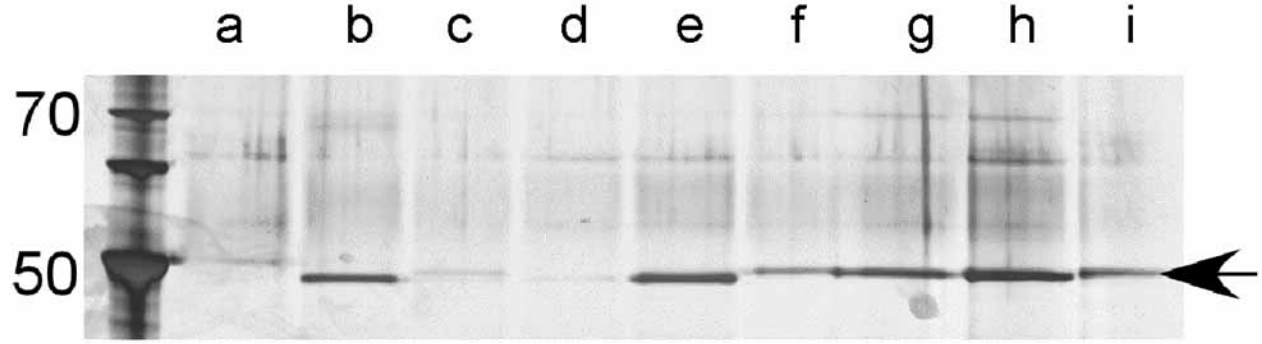

Fig. (6). Silver-stained gel after SDS-PAGE of cell-free supernatants of *L. plantarum* secreting α-amylase from *L. amylovorus*, directed by different heterologous and homologous signal peptides. Lane **(a)**, expression with no signal peptide; lanes **(b)** to (i) expression with signal peptide from: its own protein **(b)**, the *L. lactis* Usp45 protein **(c)**, the *Streptococcus pyogens* M6 protein **(d)**, exported proteins (Lp_2588, Lp_2578, Lp_2958, Lp_0373 and Lp_0600) from *L. plantarum* WCFS1 **(e-i)**. The arrow indicates the α-amylase.

tions [110]. Several studies have demonstrated successful display of proteins in lactobacilli by fusing these to the BspA protein [111-113]. Another non-covalently anchoring system exploits so-called LysM domains that are known to promote cell wall association of several natural proteins, e.g. in about 10 proteins in *L. plantarum* WCFS1 [114]. LysM domains bind non-covalently to peptidoglycan layer of Gram-positive bacteria [115]. Literature contains several examples of successful use of these domains to display proteins in LAB by fusing the Lys M domains N- or C-terminally to the target protein, including lactococci and lactobacilli [94, 115-118].

An interesting variant of the LysM option is the use of the C-terminal anchoring domain from the major autolysin from *L. lactis*, AcmA [116]. The C-terminal non-catalytic domain (cA domain) of AcmA binds to peptidoglycan and consists of three 45-amino-acid-long regions that resemble (50 to 63% amino acid identity) LysM domains. When three repeats of the cA domain were fused to an α-amylase 82% of activity was detected on the cells.

LACTOBACILLI AS HOST FOR GENE EXPRESSION AND DELIVERY TOOLS IN MAMMALS

The potential of LAB as cell factories and delivery vehicles was initially explored primarily using *L. lactis*, due to the fact that this was the first LAB for which a well-developed genetic toolbox became available. More recently, tools for lactobacilli have become available, opening up for use of these bacteria as delivery vector. In addition to the many beneficial properties mentioned above, lactobacilli have other advantages as delivery agents compared to *Lactococcus*: they have relatively high resistance against bile acid and low pH and they are more persistent in or may even colonize the GI-tract. When it comes to the type of applications of LAB as delivery vehicles, the delivery of antigens has received lot of attention. Immunization via the mucosal surfaces of the GI-tract may be an effective immunization strategy and the idea of immunizing humans by simply feeding them with antigen-delivering LAB is highly attractive.

Due to this history of events, some landmark studies in the field come from work on lactococci which have been successfully used to produce tetanus antigens [119], cancer antigen [120, 121], and cytokines [122-124] One of the most interesting results comes from Steidler *et al.* [124] who generated a *L. lactis* strain secreting murine interleukin-10, a cytokine known to be useful for treatment of inflammatory bowel disease. In their initial work, they used a colitis mouse model and showed that the onset of (provoked) colitis could be drastically reduced if the mice were intragastric administrated with interleukin secreting *L. lactis*. This work has been followed up by generating *L. lactis* strains that are deficient in thymidylate synthase and that produce human interleukin-10. These strains are biologically contained, in the sense that they can only survive and produce interleukin when inside the human body [125, 126]. Clinical trials in patients with Crohn's disease that have shown promising results have been reported [126]. Another landmark study concerns successful immunizations of mice with *L. lactis* expressing the non-toxic C fragment of tetanus vaccine (TTFC) [119]. Another highlight concerns the protection of mice against human papillomavirus type 16-induced tumors with recombinant *L. lactis* strains producing the E7 virus antigen and interleukin-12 (see below) [127].

In recent years, the number of papers reporting the expression of medicinal proteins in lactobacilli has increased. Such papers may simply report successful expression of e.g. a potentially interesting antigen, or they may report expression as well as follow-up studies showing that the expressed protein and/or the recombinant *Lactobacillus* strain has the expected biological properties, such as elicitation of a measurable immune response after oral or nasal administration. Studies reporting measurable (and expected) biological effects concern proteins such as TTFC [81, 128], an immunodominant T-cell epitope of house dust mite [85], single chain antibodies against *S. mutans* [87], a vaccine against human cervical carcinoma [84, 90, 93], IgE mimotopes provoking allergy-inhibiting anti-IgE responses [129], the proteinaceous HIV inhibitor cyanovirin-N [91], a *B. pertussis* antigen [89] and peptides for oral delivery [86]. Below, two examples are described in detail. Interestingly, available data indicate that recombinant *L. plantarum* strains tend to give better immune responses than *L. lactis* strains presenting the same antigen [130].

Several of the most promising studies in the field were focused on the human papillomavirus type-16 (HPV-16) E7 protein. The E7 protein is a viral oncoprotein which is involved in cervical cancer and may therefore function as a target for immunotherapy. Early work, done with *L. lactis*, has shown very promising results. Bermudez-Humaran *et al.* [127] immunized mice by intranasal administration of two recombinant *L. lactis* strains expressing cell-wall anchored E7 and secreted interleukin-12 (IL-12), respectively. IL-12 is a potent cytokine which modulates the immune system and has adjuvant properties. These authors showed that administration of the recombinant strains reduced tumor development in mice. Fifty percent of the co-vaccinated mice remained tumor free 100 days after challenging and in mice with tumors, the size of the tumors was significantly reduced. Furthermore, these authors demonstrated that administration of these lactococci to mice with established tumors results in total tumor regression in 35 % of the immunized mice. After the initial work on *L. lactis*, several studies have reported successful secretion and anchoring of the E7 antigen to the surface of lactobacilli [84, 93, 130]. When Cortes-Perez *et al.* [130] compared intragastric and intranasal routes of administration with *L. lactis* or *L. plantarum* as delivery vectors they found that the intranasal route was most effective and that the immunogenicity of *L. plantarum* was higher compared to *L. lactis*. The authors suggested that the differences in immune response can be explained by the fact that *L. plantarum* can persist on the mucosal surface for longer time. Alternatively, the better performance of *L. plantarum* could be due to the intrinsic beneficial immunomodulating properties of lactobacilli ("adjuvant" effect; e.g. [131]). In another approach, the E7 antigen was surface anchored to *L. casei* using the *Bacillus subtilis* PgsA transmembrane anchor [84]. Administration of the *L. casei* strain to mice evoked systemic and local immunity and reduced tumor size and increased survival rate for mice challenged with tumor cells.

In an elegant study by Krüger *et al.* [87] passive immunization against caries was carried out using *L. zeae* strains

producing cell wall anchored single chain antibody fragments which can target the *Streptococcus mutans* antigen I/II (SAI/II). This antigen is involved in the adhesion process of *S. mutans* in the oral cavity. These authors used the α-amylase promoter and signal peptide from *L. amylovorus* to drive expression and translocation, and the C-terminal anchoring sequences were derived from the proteinase P of *L. zeae* (two variants, 117 amino acids and the 244 amino acids). *L. zeae* containing the long-anchor expression vector rapidly co-agglutinated with *S. mutans*, while no co-agglutination occurred with *S. mutans* with SAI/II knock-outs. To test therapeutic effects, desalivated rats were orally inoculated with the transgenic *L. zeae* over a period of two weeks, while living on a sucrose-rich diet. Rats treated with *L. zeae* expressing the antibody with the long membrane anchor had significantly lower numbers of *S. mutans* in their oral cavity than control strains. These rats also showed a considerable lower occurrence of caries. This shows that lactobacilli harbouring the antibody on the surface can function as a "docking station" which binds *S. mutans* and thereby clears the environment for these bacteria. This study was the first study that used *Lactobacillus* as a producer of antibody fragments with prophylactic effects. Subsequently, several other promising studies on the use of *Lactobacillus* for antibody production have appeared (e.g. [132-134]). As pointed out by Krüger *et al.* [87], their *Lactobacillus*-based display strategy circumvents problems caused by the fact that free antibodies tend to have short half-lives on mucosal surfaces.

CONCLUDING REMARKS/FUTURE PERSPECTIVES

Lactic acid bacteria have been used for ages in the production of safe fermented food and they are an important part of the human diet and intestinal flora. The advent of genetic engineering has opened the perspective of engineering these bacteria such as to make them produce useful compounds *in situ*, at mucosal surfaces. Such LAB may be engineered to affect the body compartment that they reside in, or they may affect other parts of the body by provoking immune responses. They may enter the body via normal food, via specialized food products (e.g. “probiotic drinks”), or by more advanced forms of administration such as nasal spray delivery. The perspective of employing these bacteria as drug or drug carriers is highly attractive and has been explored intensely in the past ten years. Recent literature contains some very promising results, e.g. [87, 91, 119, 124, 126, 127, 129]. Furthermore, genetic tools are being continuously improved, e.g. by exploiting the regulable, highly effective expression systems describe above.

In the past decade, the nisin-based NICE system has become one of the dominating “systems-of-choice” for gene expression in LAB, in particular lactococci [60]. Other systems derived from the bacteriocin operons described above provide a valuable addition to the NICE toolbox because they perform very well, they are homologous (i.e. derived from lactobacilli), and because they do not rely on a bacteriocin (nisin) for induction, but on a non-antibiotic peptide whose only function seems to be to act as inducer.

Despite these promising results, the road towards large scale therapeutic use of lactobacilli is still quite long. Some key issues for future research include:

1. Construction of fully food-grade “self-cloning” systems that are considered 100 % safe and that are acceptable from a legislative point of view. This means for example that plasmid-based systems should be replaced by systems based on genes integrated into the host strain chromosome and that antibiotic selection markers should be avoided. Food-grade markers are available for LAB (e.g. [135, 136]), as are tools for chromosomal integration such as the promising Cre-lox-based system [137]. Integration within the chromosome will often reduce the gene dose and thereby the expression level. For example, a recent study [91] showed that a single copy integration in the *L. jensenii* genome lead to a 50 to 70 % reduction in the production level of the target protein, HIV inhibitor cyanovirin-N (CV-N), compared to a plasmid-based expression system. This example illustrates that the ”not-yet-food-grade” examples in the current literature may not look equally promising when they are adapted to the limits set by legislation and safety issues. It should be noted though, that several types of medical applications do not necessarily require maximization of protein production levels.

2. A higher success rate in protein production. Behind the successful stories appearing in the scientific literature, there are many less successful attempts to express therapeutic proteins in LAB. Eukaryotic proteins are notoriously difficult to express in bacteria, including LAB. Whereas problems caused by suboptimal codon usage nowadays can easily be overcome by using synthetic genes, many other complications can not be resolved that easily, in part because simply too little is known about the factors that steer (or prevent) protein production. Potentially important factors include: mRNA stability and secondary structure, optimized coordination of the rates of protein synthesis, folding and translocation, and the contribution of chaperones (see [138], for a review of limiting factors in *B. subtilis*). All these factors may to some extent be manipulated, but there is no clear recipe for success.

3. Better control of protein localization and a better understanding of localization effects on therapeutic effectiveness. As described above, tools for secretion and anchoring are known and have been successfully used in several contexts. However, these tools are not standard in the sense that it is difficult to predict which secretion and anchoring signals will work well for a certain protein of interest. The secretion efficiency of foreign proteins depends on many factors, like efficient processing of the signal peptide, passage through the cell wall, (unwanted) degradation of the target protein during processing and translocation (intra- or extracellularly), and the spatial and temporal coordination between production, folding and translocation. When it comes to anchoring, there is the additional question of anchor length which will affect the position of the protein in the bacterial cell wall and with that, perhaps, the therapeutic functionality of this protein (e.g. an immunization reaction). Most importantly, current literature does not provide a consistent picture with respect to what is the best localization and administration route for a therapeutic protein to be effective [81, 130, 139]. Notably, some studies show that intracellular production may be favorable for therapeutic functionality (e.g. [81, 139]).

Finally, despite major progress and some convincing data in recent years [140], there still is limited knowledge about

the magnitude of and the mechanisms behind the probiotic effects of lactobacilli, including their potential immunomodulating effects [4]. The molecular mechanisms of the interaction between lactobacilli and human intestinal cells are not known in detail nor is the impact of this interaction on behavior of the bacterial and the human cells. It has been claimed that some lactobacilli may act as adjuvants [131] which obviously is of great importance for their exploitation as vaccine delivery vehicles. Improved understanding of these mechanisms, which is likely to come from current ongoing functional genomics projects, may permit "rational design" of lactobacilli displaying an optimum combination of immunomodulatory effects, other probiotic effects, and production of a controlled amount of an intracellular, extracellular, or anchored functional protein. This will improve our options to exploit lactobacilli as live therapeutic agents.

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