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RESEARCH REVIEW

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Food-Grade Expression and Secretion Systems in *Lactococcus*

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Abstract *Lactococcus* species are noninvasive and nonpathogenic microorganisms that are widely used in industrial food fermentation and as well-known probiotics. They have been modified by traditional methods and genetic engineering to produce useful food-grade materials. The application of genetically modified lactococci in the food industry requires their genetic elements to be safe and stable from integration with endogenous food microorganisms. In addition, selection for antibiotic-resistance genes should be avoided. Several expression and secretion signals have been developed for the production and secretion of useful proteins in lactococci. Food-grade systems composed of genetic elements from lactic acid bacteria have been developed. Recent developments in this area have focused on food-grade selection markers, stabilization, and integration strategies, as well as approaches for controlled gene expression and secretion of foreign proteins. This paper reviews the expression and secretion signals available in lactococci and the development of food-grade markers, food-grade cloning vectors, and integrative food-grade systems.

Keywords: expression signal, secretion signal, food-grade selection marker, food-grade cloning vector, integrative food-grade system, *Lactococcus lactis*

Introduction

Lactic acid bacteria (LAB) are Gram-positive, nonsporulating, and nonrespiring bacteria that produce lactic acid as a major end product during carbohydrate fermentation. The historical genera of LAB are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Lactococcus*. Due to the taxonomic revision of these genera and the creation of new genera, LAB now include species of the genera *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (1).

LAB is widely used in the food industry for the production and preservation of fermented foods such as dairy products (e.g., milk, yogurt) and fermented vegetable products (e.g., *kimchi*, saukraft). The traditional use of these bacteria in the food industry confirms their lack of pathogenicity; they are generally regarded as safe (GRAS) organisms. LAB have also been used as probiotics to manage intestinal disorders such as lactose intolerance, acute gastroenteritis due to rotavirus and other enteric pathogens, inflammatory bowel disease, and food allergies (2-7). LAB or their cellular components possess antimicrobial, hypercholesterolemic, immune-enhancing, and antitumor activities (8-11).

Recombinants LAB have been developed with novel properties for applications in the health care industry and for traditional uses in food fermentation and preservation (12-26). The eventual aim of the genetic modification LAB is to produce flavor compounds or enzymes for food production, and cellular factories for the production of antimicrobials and other high-value proteins.

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Genetic engineering of *Lactococcus*

The *Lactococcus* genus comprises five species: *L. graviae*, *L. raffinolactis*, *L. plantarum*, *L. piscium*, and *L. lactis* (27, 28). *L. lactis* is the best characterized species and comprises three subspecies: *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, and *L. lactis* ssp. *hordniae*. Genetic interest has been focused exclusively on *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*, due to their value as starter cultures in industrial dairy fermentation.

Various genetic tools have been used to analyze and modify LAB, with a particular focus on the industrially important strain *Lactococcus*. The genome of *L. lactis* ssp. *lactis* was recently sequenced (29). Recent research has been focused on developing genetic tools to improve the properties and efficiency of lactococci for use in food and medicine production. Much of the effort centers on developing the molecular tools for lactococci, including genetic techniques, transformation protocols, and vectors, as well as gene integration and amplification systems (30-33). A variety of useful genetic systems have been developed for the analysis of industrially important traits (34, 35). Moreover, genetically modified *Lactococcus* species are now being developed for a variety of traditional, novel, and innovative applications related to their use as fermentation starters, probiotics, and other health-related cultures, or as industrial hosts for the production of peptides, enzymes, and metabolites (15, 36-42). Most of these improvements have exploited the abundance of cloning, integration, and expression vectors that are based on plasmids naturally occurring in *L. lactis* (31, 42). To select the appropriate transformants and to maintain selective pressure for the genetic modification, these vectors contain one or more genes coding for resistance to antibiotics such as chloramphenicol, erythromycin, tetracycline, or spectinomycin. Although such vectors are instrumental in showing proof of LAB improvements, they are not suitable for the food industry. Transferable antibiotic-resistance (Ab^r) markers should not be present in microorganisms used in

the food industry due to legal, ethical, and in some cases of scientific reasons. Alternative food-grade genetic markers and industrial strains are needed to obviate the use of Ab^r markers, and to develop so-called food-grade systems. Such approaches were proposed more than a decade ago for selection markers based on the natural properties of LAB (44).

Food-grade genetic elements

Gene expression signals Several gene expression systems are now available to further the scientific and industrial application of *Lactococcus*. These systems use lactococcal constitutive or inducible promoters (34, 38) as well as innovative protein secretion systems, which in many cases operate endogenously in *Lactococcus* (16, 45, 46).

***Constitutive expression signals*:** Transcription initiation signals have received considerable attention in various LAB due to their important roles in the efficiency and control of gene expression (31, 33, 47). However, only the *rpoD* gene in *L. lactis* has been characterized (48). *rpoD* encodes the 39-kDa major sigma factor protein (σ^{39}), which has strong similarity to the vegetative *Bacillus subtilis* σ^{43} and the carboxyl-terminal part of the *Escherichia coli* σ^{70}. The specific features of the *L. lactis* σ^{39} with regard to the lactococcal promoter region have not been determined.

Analysis of a large number of promoters revealed a consensus lactococcal promoter with specific features including: conserved -35 and -10 hexamers found in prokaryotic cells, a strict separation between these canonical hexamers of 17 nucleotides, a 'TGN' motif in the upstream of the -10 hexamer, and a region immediately upstream from the -35 sequence that is AT-rich (30, 31).

Three different strategies have been followed to isolate promoters from *L. lactis*. The first is based on screening vectors, both plasmids and transposons, carrying promoterless reporter genes such as those encoding chloramphenicol resistance, β-galactosidase, or β-glucuronidase (31-33, 49). New and useful lactococcal promoters are still being discovered as illustrated in a screening study involving the promoterless *lux* gene that yielded a set of more than 10 new promoters with vastly different efficiencies (50). For most of these, promoter strength was compared in different microorganisms using reporter proteins such as chrolamphenicol, acetyltransferase, and β-galactosidase. However, the reporter activity was substantially lower in *L. lactis* than in *E. coli*, therefore *L. lactis* is not a good candidate for comparing promoter strength in lactococci (51). The use of luciferase assays within intact cells circumvented the low activity in *L. lactis* (49). The absolute luciferase intensity of most promoters in the lactococcal host was lower than that in *E. coli*, but the values were different enough to be compared quantitatively. In addition, unlike the chloramphenicol, acetyltransferase, and β-galactosidase reporter assays, luciferase assays can be simply performed with intact cells. Therefore, the luciferase system can be successfully used in lactococcal hosts for promoter strength analysis.

The second strategy was to exploit the large number of genes now identified, and consequently the wealth of known promoters, notably those from housekeeping genes. Several of these seem to be regulated (see regulated expression signals), while others such as the *usp45* promoter are presently uncharacterized (31). The third approach was to develop synthetic promoters (52); this involved the construction and screening of promoters from the consensus *L. lactis* promoter in which the sequences of the separating spacer regions were randomized. These promoters are suitable for metabolic engineering studies in which constitutive gene expression is modulated. Some of these promoters are not only functional in *L. lactis* but also in *E. coli*, in which some of the weak *L. lactis* promoters are still highly active. This may reflect the fact that *L. lactis* and other LAB set more stringent requirements on promoters than *E. coli*, which is a relatively promiscuous host.

***Regulated expression signals*:** Expression-vector systems based on efficient expression signals for lactococci can also be regulated by induction. Such systems allow the controllable overproduction of proteins on a large scale at specified points during industrial fermentation. Studies on regulated gene expression in *L. lactis* are described in Table 1.

The best-characterized regulated expression system is based on the lactose-inducible transcription of the *lac* operon which encodes genes in the lactose phosphotransferase system and tagatose-6-phosphate pathway (36). However, application of this system is hampered by low induction levels (less than 10-fold) and by the intermediate tagatose-6-phosphate, the concentration of which is difficult to control, especially in large-scale fermentations. Another inducible expression system is based on the *E. coli* bacteriophage T7 promoter combined with the T7 polymerase gene fused to the *lac* operon promoter. However, this system has the same drawbacks and, furthermore, it is based on a heterologous gene that has not been authorized for use in the food industry (17). Recently, the promoter of *xylT* (a gene encoding a xylose permease) which is based on the xylose operon, was efficiently induced in the *Staphylococcus aureus* release gene by xylose (53).

Regulated expression systems based on the *L. lactis* *nisA* promoter are also available (15, 18, 54-56). The promoter sequence of the *nisA* gene can efficiently regulate transcription initiation depending on the extracellular concentration of the antimicrobial peptide nisin (57). Nisin is used in some countries by the food industry as a safe and natural preservative. The *nisA* gene encodes the nisin peptide itself, and proteins encoded by *nisR* and *nisK* are involved in the regulation of *nis* gene-cluster expression. NisR and NisK belong to the class of two-component regulatory proteins: NisK is a sensor histidine kinase and NisR is the response regulator. This system is highly versatile and can deal with large-scale protein production, if required, since there is a linear relationship between the amount of nisin added to the culture medium and promoter activity. Gene expression can be modulated within a range of more than 1,000-fold (54, 57). The nisin-inducible system was applied in lactococci containing essentially the *nisR* and *nisK* genes.

Another lactococcal bacteriophage-based system was developed by combining phage-induced DNA amplification and gene expression (58). This so-called explosive gene

Table 1. Inducible expression systems for *Lactococcus lactis*

Host	Promoter	Inducible element	Expressed gene	Induction factor	References
Sugar-inducible expression systems					
L. lactis	*lacA*	Lactose	*cat-86*, *luxAB*, Lysin	<10	98-100
L. lactis	*lacR*	Lactose	*luxAB*	<10	99
L. lactis	*lacA/T7*	Lactose	TTFC, IL-2	<20	17, 101
L. lactis	*xylT*	Xylose	*nucB*	>5	53
Bacteriocin-inducible expression systems					
L. lactis	*nisA or nisF*	Nisin	*gusA*, *pepN*, *lacZ*	>1000	54, 57, 102
Other gene-inducible expression systems					
L. lactis	*φ31*	φ31 infection	*lacZ*	>1000	59
L. lactis	*dnaJ*	High temperature	*amyS*	<4	46
L. lactis	*sodA*	Aeration	*lacZ*	2	103
L. lactis	PA170	Low pH, low temperature	*lacZ*	50-100	60
L. lactis	*lactA*	pH	*lacZ*	<40	104
L. lactis	P_{zn}	Zn^{2+}, EDTA	*lacZ*	100-500	63
L. lactis	*gadC*	NaCl	*pepX*	>1000	62

expression system allows for an approximately 1,000-fold increase in protein production (59). The high inducibility coupled with lysis and release of the product of interest makes the explosive expression system highly attractive. One drawback is the phage-dependent induction, which may be difficult to realize on an industrial scale, because it is limited to specific host-phage interactions.

Several lactococcal promoters regulated by environmental conditions such as pH, temperature, and aeration have also been isolated. Most of these promoters are expressed low levels of product when induced. Among them, P170 is a strong promoter, but it is only active at low pH and when cells enter the stationary growth phase, and it has the property of being more active at low rather than at high temperatures (60, 61). A chloride-inducible promoter has also been reported, whereby the P_{gad} promoter system can be induced more than 1,000-fold by NaCl (62). Similarly, a P_{zn} promoter expression system was developed using zinc as an inducer (63).

Gene secretion signals Protein secretion by a GRAS bacterium would allow the direct production of proteins in food and thus facilitate interactions between the secreted protein (enzyme or antigen) and the environment (the food product itself or the digestive tract). *Lactococcus* species secrete relatively few proteins (46). In particular, *L. lactis* strains do not produce any extracellular proteases (64) which can hydrolyze secreted proteins, and they do not produce endotoxins (29). Therefore, *L. lactis* is a good candidate for the secretion of heterologous protein, particularly for the food industry.

In bacteria, most proteins trafficked via the secretory (Sec) pathway are synthesized as precursors containing the mature protein and an N-terminal signal peptide (SP), which provides an essential signature for protein secretion (65). The SP associates with the Sec machinery and retards precursor folding together with the action of Sec-specific chaperones (66, 67). Although SP primary sequences are poorly conserved, they display a common tripartite structure that consists of a positively charged N-terminus, a hydrophobic core, and a neutral or negatively charged C-terminus containing the SP cleavage site (65). SPs from *L. lactis*, which restore the reporter protein activity, were identified by screening vectors carrying reporter genes that lack SPs, such as the α-amylase gene, β-lactamase gene, and nuclease gene (68-73). Their isolated SPs originated from an undefined gene (Table 2). Moreover, the screen revealed that protein analyses based on GenBank data do not easily identify the secreted form of a protein. Further investigation of SPs is therefore needed to distinguish whether the SPs of a defined protein are derived from the membrane protein or a secreted protein (Table 2).

Many heterologous eukaryotic (16, 19-22), prokaryotic (17, 18, 20), and viral (15, 24-26) proteins have been produced and secreted by *L. lactis* (Table 3). The best secretion efficiencies have been obtained with the SP of Usp45 (an unknown secreted protein of 45 kDa), which is efficiently recognized by the lactococcal Sec machinery. Even with the appropriate SP, secretion may be inefficient, and some heterologous proteins remain poorly secreted, or not at all, even when fused to a homologous SP (24, 71, 74). Notably, a charged N-terminus in the mature protein may significantly affect the efficiency of translocation across the cytoplasmic membrane (75). Le Loir *et al.* (45, 76) showed that the introduction of negatively charged amino acids by the insertion of a nine-residue synthetic propeptide after the SP cleavage site, improved secretion

Table 2. Putative Lactococcal secretion signal sequences with defined cleavage sites

Secretion signal	Protein	Putative secretion signal sequence	References
Derivatives from undefined genes			
ss1	Unknown	MKKILIIGLGLIGSSIALG ↑ I	72
ss30	Unknown	MKIWTKLGLLSLVGLTLTACG ↑ S	72
ss38	Unknown	MKKILITTTLALALLSLGA ↑ C	72
ss45	Unknown	MKKINLSKLIIIALIIIIAAMSAIFISA ↑ K	72
ss80	Unknown	MKKKIFIALMASVSLFTLAA ↑ C	72
AL9	Unknown	MRALMFLQRFLATIIDLIIVYLPVLLLVQGDPLEST ↑ A	69
BL1	Unknown	MNFFKNTIIFLKTIKTKPLDFVIVFVLFLASFSTIFLF ↑ ATGSTGA K ↑ A	69
BL11	Unknown	MTSKIKSLTYLLLFIVGIEIIGGLSGFF ↑ AGIIKEIYNNLILPPLAPPDYLFGIVWGDPLESSTA ↑ QA ↑ C	69
BL13	Unknown	MKRYVTGFNGLRTIGVLTVILYHLWG ↑ D	69
AL39	Unknown	MFLKHASQLLKVGVQPLVLVLALRGDPLES ↑ T	69
S405	Unknown	MNRTFIKGFLAGNALLLA ↑ GIT ↑ A	68
S407	Unknown	MEYGVLSVILVILVAFLAGL ↑ EG ↑ I	68
Derivatives from defined genes			
SPexp1	Exp1	MKNLIPKKIKQVGILVGALLMLLSVLPVNLLGVMKVDA ↑ D	69
SPexp2	Exp2	MKKIAIIFCTLLMSLSVLSSFAVSA ↑ D	69
SPexp3	Exp3	VEKVKHEKGIIAFLTVLTILLTGAVKVSA ↑ D	69
SPexp4	Exp4	MKKINLALLTLATLMGVSSTAVVFA ↑ D	69
SPexp5	Exp5	VRYSKISTKSKKNKQNKRAKRGSAKSKWWTAVKLFLIVFFSLIILGLA ↑ A	69
SPnlp1	Nlp1	MKKKIFIALMASVSLFTLAA ↑ C	69
SPnlp2	Nlp2	MKSWKKVALGGASVLALATLAA ↑ C	69
SPnlp3	Nlp3	MKKILMLFAIPAVLLLAG ↑ C	69
SPnlp4	Nlp4	LKFKKLGLVMATVFAGAALVTLSG ↑ C	69
SPprtP	PrtP	MQRKKKGLSILLAGTVALGALAVLPVGEIQAKA ↑ A	105
SPnisP	NisP	MKKILGFIVCSLGLSATVHG ↑ C	106
SPusp45	Usp45	MKKKIISAILMSTVILSAAAPLSGVYA ↑ D	46
SPnisI	NisI	MRRYJLILIVALIGITGLSG ↑ C	97

efficiency and production yields of different heterologous proteins. Other reports confirmed the role of this synthetic propeptide as an enhancer of production and secretion efficiency for heterologous proteins in *L. lactis* (18).

Food-grade markers Alternatively, food-grade selection markers have been proposed for the genetic modification of *L. lactis* strains; these can be catalogued as dominant or complementation markers (Table 4; 77-88). Dominant markers do not rely on host-expressed genes and can be used in most wild-type strains of *L. lactis*. For instance, lactococcal genes that confer immunity to nisin have been used for the selection of dominant markers (80, 82, 85). Since nisin is considered a food-grade molecule, it can be used for maintenance as long as this bacteriocin is compatible with a specific application. Resistance to heavy metals represents a second type of dominant marker (85). Although useful in the selection step, heavy metals are toxic and obviously cannot be used to maintain plasmids during food fermentation. However, maintenance of selection pressure throughout the fermentation process may not be necessary, as a vector using cadmium resistance has been shown to replicate through a theta mechanism (89), which is expected to be stable and thus not require continued selection (90). Unfortunately, with most of these dominant markers, the phenotypes selected for occur naturally in numerous lactococcal strains, thus limiting the host spectrum. In addition to resistance or immunity markers, a dominant marker based on rare sugar fermentation has also been reported (77, 83). Until now, genes involved in sugar metabolism, such as sucrose and lactose, were exploited as complementation markers. However, such phenotypes are widespread in lactococci, restricting their utilization. The phenotype of rare sugar fermentation (e.g., melibiose or raffinose) was therefore an attractive choice. Most lactococci were not fermented the melibiose and do not contain the gene encoding α-galactosidase which hydrolyze melibiose or raffinose. Therefore, α-

Table 3. Secretion systems for *Lactococcus lactis*

Secretion signal	Secreted protein	Origin	References
Eukaryotic protein			
usp45	Plasmin	Bovine	16
usp45	IL-2, IL-6	Murine	22
usp45	IL10	Murine	21
usp45	IL12	Murine	19
usp45	IFN-ω	Ovine	20
usp45	Bovine beta-lactoglobulin	Bovine	21
Prokayrotic protein			
usp45	Antigen L7/L12	*Brucella abortus*	18
usp45	TTFC	*Clostridium tetani*	17
slpA	Aminopeptidase N	*Lactobacillus helveticus*	23
Viral protein			
usp45	E7	Papillomavirus Type16	15
usp45	Rotavirus nonstructural protein 4	Rotavirus	24
slpA	Bacteriophage lysins	*Listeria monocytogenes* bacteriophage	25
AL9	VP8	Rotavirus	26

Table 4. Food-grade selection markers for *Lactococcus*, including the donors from which the systems originated and the hosts that allow their application

Selection system	Donor	Host	Selection marker	Food-grade vector	Reference
Sugar utilization marker					
Sucrose fermentation[1)]	*P. pentosaceus*	*L. lactis*	*scrA/scrB*	pINT125 [2)]	84
Lactose fermentation[3)]	*L. lactis*	*L. lactis*	*lacF*	pNZ2104 series	86
Lactose fermentation[3)]	*L. lactis*	*L. lactis*	*lacZ*	-[4)]	81
Melibiose fermentation[1)]	*L. raffinolactis*	*L. lactis*	*aga*	pRAF800	77
Melibiose fermentation[1)]	*Lb. plantarum*	*L. lactis*	*melA*	pFMN30	83
Auxotrophic markers					
Purine biosynthesis[3)]	*L. lactis*	*L. lactis*	tRNA (gln)	pFG1	79
Pyrimidine biosynthesis[3)]	*L. lactis*	*L. lactis*	tRNA (ser)	pFG200	87
Alanine conversion [3)]	*L. lactis*	*L. lactis*	*alr*	-[4)]	78
Resistance/immunity markers					
Nisin resistance[1)]	*L. lactis*	*L. lactis*	Nis^R	pFM011, pFK012	80, 82
Nisin immunity[1)]	*L. lactis*	*L. lactis*	*nisI*	pLEB590	88
Cadmium resistance[1)]	*L. lactis*	*L. lactis*	Cd^R	pND625	85

[1)]Dominant marker systems.
[2)]Integrative food-grade vector.
[3)]Complementation marker systems.
[4)]*E. coli*/lactococcal shuttle vector.

galactosidase gene-based selection system was not necessary to construct the gene-deficient mutant host, and the α-galactosidase-utilizing phenotype was selected dominantly (77, 83).

Complementation markers require the prior development of a bacterial mutant with a specific deficiency that is

Table 5. Food-grade integration systems for *Lactococcus lactis*

Integration gene	Replication origin	Host	Remark	References
Homologous recombination				
pepX	pWV01	*L. lactis*	Single crossover	84
pepX	pGhost+	*L. lactis*	Double crossover	94
his	*E. coli* Ori	*L. lactis*	Double crossover	95

complemented by the marker to restore the original phenotype. Two systems based on threonine- and pyrimidine-auxotroph derivative *L. lactis* strains allow the cloning and efficient expression of heterologous and homologous proteins in various industrial lactococcal strains (87, 91). These two systems are stable, and do not impair growth rates or other important properties, such as milk acidification. Because of the absence of Ab^r and foreign DNA, strains using these complementation marker systems maintain their food-grade status.

Food-grade vector systems

L. lactis is a food-grade bacterium, but this status would be compromised if expression/secretion systems using Ab^r genes as selection markers or foreign DNA are introduced. Such tools could compromise the use of engineered lactococcal strains in the food industry and in the production of purified recombinant proteins because of the instability of plasmids in the absence of selection pressure and their contamination of biomass or purified proteins. Food-grade cloning systems have therefore been developed to produce proteins directly and efficiently in food or in large-scale fermentations, without Ab^r or foreign DNA.

Food-grade cloning vector systems Food-grade cloning vector systems must meet various specifications, irrespective of whether the specifications relate to selection markers, exploitation of the chromosome, or controlled gene expression strategies. First and foremost, the vector systems should be safe, well characterized, stable, and versatile. Second, food-grade cloning vector systems should contain no Ab^r markers and be compatible with the desired food application. Third, any food-grade system must avoid the use of harmful compounds, sustain safety, and reduce the environmental load. Finally, all systems need to be applied in an efficient, timely, and cost-effective manner (92). Table 4 lists the food-grade selection markers used in vector systems for *Lactococcus*. While the limitation of dominant markers is linked to the natural occurrence of the conferred phenotype, complementation markers are inconvenient to use. Recently, a third approach was proposed that capitalizes on the advantages of both dominant and complementation systems and relies on two components (93): a cloning vector carrying a functional lactococcal replicon composed entirely of *L. lactis* DNA without a selection marker, and the 'companion', a replication-deficient plasmid carrying an erythromycin-resistance gene as a dominant selection marker. The companion construct can only replicate in *L. lactis* if it is complemented *in trans* by the cloning vector. Since only those cotransformants containing both the cloning vector and the companion can survive on plates containing erythromycin, the companion can be used transiently to select for cells that have acquired the cloning vector. Given its defective replication, the companion can be readily lost from cells by growing them on antibiotic-free medium after the selection step (93).

Food-grade integration systems Cloning systems based on plasmids and food-grade markers inherently suffer from instability. Ultimate stability is provided by integration into the chromosome. Until recently, constructed food-grade integration systems for *Lactococcus* were based on homologous chromosomal targeting (Table 5; 84, 94, 95). All systems for chromosomal integration are based on nonreplicating or conditional plasmids that are well established in lactococci (31). Since several of the nonreplicating plasmids are based on replicons of *E. coli* or microbes other than lactococci, a specific set of food-grade lactococcal integration vectors has been developed. These so-called Ori^+ vectors only contain the replication origin of the well-characterized *L. lactis* plasmid pWV01, which can replicate only when the RepA replication protein is provided *in trans* (43).

In the case where an integrating plasmid is equipped with two homologous regions that are physically close, two recombination events may result in gene replacement. These recombination events may occur simultaneously (double crossover) or consecutively (single crossover followed by loop-out deletion). Depending on the nature of the sequences between the homologous regions, the final result may be either a chromosomal deletion or the insertion of a gene of interest. Several gene integration strategies that allow chromosomal insertions in a food-grade manner are available, and these are based on the temperature-sensitive pGhost-series of plasmids, the Ori^+ set of integration vectors, and a *lacZ*-based system to allow blue/white screening for the second crossover event (43, 96). In some cases, gene integrations are difficult or even impossible to obtain, particularly when the gene to be replaced is essential, when there are polar effects in the chromosome due to the insertion or expression of the gene of interest, or when a preferred recombination occurs at the region of homology where the first crossover occurred, restoring the wild-type genotype. Deletions as small as four nucleotides have been obtained by gene replacement in the *L. lactis* chromosome (97).

Future prospects

Various genetic elements such as replicons, promoters, and secretion signals have been developed and used in the production and secretion of useful proteins in *L. lactis*.

Moreover, a range of food-grade systems using food-grade markers for the selection of recombinant lactococci as well as these genetic elements have also been developed for food-grade selection, stable maintenance, and over-expression of relevant genes of interest in *Lactococcus*.

Future studies should investigate the potential for food-grade recombinant lactococci systems having stability and the capacity for overexpressing technologically relevant genes in industrial starter strains on a plant scale. Also, political and public acceptance of genetically modified microorganisms including lactococci is necessary for progression in the food industrial applications of *Lactococcus*. Once this issue is resolved, some of the systems reviewed here will allow for new and rapid advances in both the efficient design and practical evaluation of novel metabolic engineering strategies aimed at overproduction in the food and medicinal industries.

Altogether, the GRAS status of lactococci is a clear advantage for their use in production and secretion of novel peptides, enzymes, and proteins for therapeutic use and vaccinations. Current genetic elements and food-grade systems will contribute to the development and improvement of new food-grade systems for lactococci and should be used to construct lactococcal strains dedicated to the efficient production of useful proteins, peptides, and metabolites for the food industry.

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References

1. Hozapfel WH, Habere P, Geisen R, Bjorkroth J, Schillinger U. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr. 73: 365S-373S (2001)
2. Bengmark S, Martindale R. Prebiotics and synbiotics in clinical medicince. Nutr. Clin. Pract. 20: 244-261 (2005)
3. Hwang K-T, Lee W, Kim G-Y, Lee S-K, Lee J, Jun W. The binding of aflatoxin B_1 modulates the adhesion properties of *Lactobacillus casei* KCTC 3260 to a HT29 colon cancer cell line. Food Sci. Biotechnol. 15: 227-231 (2006)
4. Lee N-K, Kim H-W, Chang H-I, Yun C-W, Kim S-W, Kang C-W, Paik H-D. Probiotic properties of *Lactobacillus plantarum* NK181 isolated from Jeotgal, a Korean fermented food. Food Sci. Biotechnol. 15: 227-231 (2006)
5. Lee N-K, Kim T-H, Choi S-Y, Lee SK, Paik H-D. Identification and probiotic properties of *Lactococcus lactis* NK24 isolated from Jeotgal, a Korean fermented food. Food Sci. Biotechnol. 13: 411-416 (2004).
6. Shanahan F. Probiotics in inflammatory bowel disease-therapeutic rationale and role. Adv. Drug Deliver. Rev. 56: 809-818 (2004)
7. Cunningham-Rundles S. The effect of aging on mucosal host defense. J. Nutr. Health Aging 8: 20-25 (2004)
8. Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. 3: 777-788 (2005)
9. Cross ML. Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. FEMS Immunol. Med. Mic. 34: 245-253 (2002)
10. Kim JY, Lee S, Jeong D-W, Hachimura S, Kaminogawa S, Lee HJ. Effects of intraperioneal administration of *Lactococcus lactis* ssp. *lactis* cellular fraction of immune response. Food Sci. Biotechnol. 14: 405-409 (2005)
11. Sablon E, Contreras B, Vandamme E. Antimicrobial peptide of lactic acid bacteria: mode of action, genetic and biosynthesis. Adv. Biochem. Eng. Biotechnol. 68: 21-60 (2002)
12. Giuliano M, Schiraldi C, Marotta MR, Hugenholtz J, de Rosa M. Expression of *Sulfolobus solfataricus* α-glucosidase in *Lactococcus lactis*. Appl. Microbiol. Biot. 64: 829-832 (2004)
13. Simons G, Rutten G, Hornes M, Mijhuis M, van Asseldonk M. Production of prochymosin in lactococci. Adv. Exp. Med. Biol. 306: 115-119 (1991)
14. Drouault S, Corthier G, Ehrlich SD, Renault P. Expression of the *Staphylococcus hyicus* lipase in *Lactococcus lactis*. Appl. Environ. Microbiol. 66: 588-598 (2000)
15. Bermudez-Humaran LG, Langella P, Miyoshi A, Gruss A, Guerra RT, Montes de Oca-Luna R, Le Loir Y. Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. Appl. Environ. Microbiol. 68: 917-922 (2002)
16. Arnau J, Hjerl-Hansen E, Israelsen H. Heterologous gene expression of bovine plasmin in *Lactococcus lactis*. Appl. Microbiol. Biot. 48: 331-338 (1997)
17. Wells JM, Wilson PW, Norton PM, Gasson MJ, Le Page RW. *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. Mol. Microbiol. 8: 1155-1162 (1993)
18. Ribeiro LA, Azevedo V, Le Loir Y, Oliveira SC, Dieye Y, Piard JC, Gruss A, Langella P. Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. Appl. Environ. Microbiol. 68: 910-916 (2002)
19. Bermudez-Humaran LG, Langella P, Cortes-Perez NG, Gruss A, Tamez-Guerra RS, Oliveira SC, Saucedo-Cardenas O, Montes de Oca-Luna R, Le Loir Y. Intranasal immunization with recombinant *Lactococcus lactis* secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. Infect. Immun. 71: 1887-1896 (2003)
20. Bermudez-Humaran LG, Langella P, Commissaire J, Gilbert S, Le Loir Y, L'Haridon R, Corthier G. Controlled intra-or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. FEMS Microbiol. Lett. 224: 307-313 (2003)
21. Chatel JM, Langella P, Adel-Patient K, Commissaire J, Wal JM, Corthier G. Induction of mucosal immune response after intranasal or oral inoculation of mice with *Lactococcus lactis* producing bovine beta-lactoglobulin. Clin. Diagn. Lab. Immunol. 8: 545-551 (2001)
22. Steidler L, Robinson K, Chamberlain L, Schofield KM, Remaut E, Le Page RW, Wells JM. Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. Infect. Immun. 66: 3183-3189 (1998)
23. Kahala M, Palva A. The expression signals of the *Lactobacillus brevis slpA* gene direct efficient heterologous protein production in lactic acid bacteria. Appl. Microbiol. Biot. 51: 71-78 (1999)
24. Enouf V, Langella P, Commissaire J, Cohen J, Corthier G. Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. Appl. Environ. Microbiol. 67: 1423-1428 (2001)
25. Gaeng S, Scherer S, Neve H, Loessner MJ. Gene cloning and expression and secretion of *Listeria monocytogenes* bacteriophage-lytic enzymes in *Lactococcus lactis*. Appl. Environ. Microbiol. 66: 2951-2958 (2000)
26. Gil MT, Perez-Arellano I, Buesa J, Perez-Martinez G. Secretion of the rotavirus VP8 protein in *Lactococcus lactis*. FEMS Microbiol. Lett. 203: 269-274 (2001)
27. Schleifer K-H, Kraus J, Dvorak C, Killper-Balz R, Collins MD, Fischer W. Transfer of *Streptococcus lactis* and related streptococci to the genes *Lactococcus*. Syst. Appl. Microbiol. 6: 183-195 (1985)
28. Williams AM, Fryer JL, Collins MD. *Lactococcus piscium* sp. nov. a new *Lactococcus* species from salmonid fish. FEMS Microbiol. Lett. 68: 109-114 (1990)
29. Bolotin A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissenbach J, Ehrlich SD, Sorokin A. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. 11: 731-753 (2001)
30. de Vos WM, Kleerebezem M, Kuipers OP. Expression systems for industrial Gram-positive bacteria with low guanine and cytosine

content. Curr. Opin. Biotech. 8: 547-553 (1997)
31. de Vos WM, Simons G. Gene cloning and expression systems in Lactococci. pp. 52-105. In: Genetics and Biotechnology of Lactic Acid Bacteria. Gasson MJ, de Vos WM (eds). Chapman & Hall, London, UK (1994)
32. Mercenier A, Pouwels PH, Chassy BM. Genetics engineering of lactobacilli, leuconostocs and *Streptococcus thermophilus*. pp. 252-293. In: Genetics and Biotechnology of Lactic Acid Bacteria. Gasson MJ, de Vos WM (eds). Chapman & Hall, London, UK (1994)
33. Pouwels PH, Leer RJ. Genetics of lactobacilli. Plasmids and gene expression. Antonie van Leeuwenhoek 64: 85-107 (1993)
34. de Vos WM. Gene expression systems for lactic acid bacteria. Curr. Opin. Microbiol. 2: 289-295 (1999)
35. Gasson MJ, de Vos WM. Genetics and biotechnology of lactic acid bacteria. Chapman & Hall, London, UK. pp. 1-245 (1994)
36. de Vos WM, Vaughan EE. Genetics of lactose utilization in lactic acid bacteria. FEMS Microbiol. Rev. 15: 217-237 (1994)
37. Hugenholtz J, Smid EJ. Nutraceutical production with food-grade microorganisms. Curr. Opin. Biotech. 13: 497-507 (2002)
38. Kuipres OP, de Ruyter PG, Kleerebezem M, de Vos WM. Controlled overproduction of proteins by lactic acid bacteria. Trends Biotechnol. 15: 135-140 (1997)
39. Ouwehand AC, Salminen S, Isolauri E. Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek 82: 279-289 (2002)
40. Robinson K, Chamberlain LM, Schofield KM, Wells JM, Le Page RW. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nat. Biotechnol. 15: 653-657 (1997)
41. Roy DG, Klaenhammer TR, Hassan HM. Cloning and expression of the manganese superoxide dismutase gene of *Escherichia coli* in *Lacotoccus lactis* and *Lactobacillus gasseri*. Mol. Gen. Genet. 239: 33-40 (1993)
42. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 289: 1352-1355 (2000)
43. Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Miereau I, Dabrowska M, Venema G, Kok J. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol. Gen. Genet. 253: 217-224 (1996)
44. de Vos WM. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. 46: 281-295 (1987)
45. Le Loir Y, Gruss A, Ehrlich SD, Langella P. A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. J. Bacteriol. 180: 1895-1903 (1998)
46. van Asseldonk M, de Vos WM, Simon G. Functional analysis of the *Lactococcus lactis* usp45 secretion signal in the secretion of a homologous proteinase and a heterologous α-amylase. Mol. Gen. Genet. 240: 428-434 (1993)
47. McCracken A, Timms P. Efficiency of transcription from promoter sequence variants in lactobacillus is both strain and context dependent. J. Bacteriol. 181: 6569-6572 (1999)
48. Araya T, Ishinashi N, Shimamura S, Tanaka T, Takahashi H. Genetic and molecular analysis of the *rpoD* gene from *Lactococcus lactis*. Biosci. Biotech. Bioch. 57: 88-92 (1993)
49. Jeong D-W, Choi YC, Lee JM, Kim JH, Lee JH, Kim KH, Lee HJ. Isolation and characterization of promoters from *Lactococcus lactis* ssp. *cremoris* LM0230. Food Microbiol. 23: 82-89 (2006)
50. Waterfield NR, LePage PWF, Wilson PW, Wells JM. The isolation of lactococcal promoters and their use in investigating bacterial luciferase synthesis in *Lactococcus lactis*. Gene 165: 9-15 (1995)
51. Koivula T, Sibakov M, Palva I. Isolation and characterization of *Lactococcus lactis* subsp. *lactis* promoters. Appl. Environ. Microbiol. 57: 333-340 (1991)
52. Jensen PR, Hammer K. The sequences of spacers between the consensus sequences modulates the strength of prokaryotic promoters. Appl. Envrion. Microbiol. 63: 82-87 (1998)
53. Miyoshi A, Jamet E, Commissaire J, Renault P, Langella P, Azevedo V. A xylose-inducible expression system for *Lactococcus lactis*. FEMS Microbiol. Lett. 239: 205-212 (2004)
54. Chandrapati S, O'sullivan DJ. Characterization of the promoter regions involved in galactose- and nisin-mediated induction of the *nisA* gene in *Lactococcus lactis* ATCC 11454. Mol. Microbiol. 2: 467-477 (2002)
55. Eichenbaum Z, Federle MJ, Marra D, de Vos WM, Kuipers OP, Kleerebezem M, Scott JR. Use of the lactococcal *nisA* promoter to regulate gene expression in Gram-positive bacteria: comparison of induction level and promoter strength. Appl. Environ. Microbiol. 64: 2763-2769 (1998)
56. Mierau I, Kleerebezem M. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl. Microbiol. Biot. 68: 705-717 (2005)
57. de Ruyter PGGA, Kuipers OP, de Vos WM. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. 62: 3662-3667 (1996)
58. O'Sullivan DJ, Walker SA, West SG, Klaenhammer TR. Development of an expression strategy using a lytic phage to trigger explosive plasmid amplification and gene expression. Biotechnology 14: 82-87 (1996)
59. Walker SA, Klaenhammer TR. Molecular characterization of phage inducible middle promoter and its transcriptional activator from the lactococcal bacteriophage Φ31. J. Bacteriol. 180: 921-931 (1998)
60. Israelsen H, Madsen SM, Vrang A, Hansen EB, Johansen E. Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn917-lacZ integrants with the new promoter probe vector, pAK80. Appl. Environ. Microbiol. 61: 2540-2547 (1995)
61. Madsen SM, Arnau J, Vrang A, Givskov M, Israelsen H. Molecular characterization of the pH-inducible and growth phase-dependent promoter P170 of *Lactococcus lactis*. Mol. Microbiol. 32: 75-87 (1999)
62. Sanders JW, Venema G, Kok J. A chloride-inducible gene expression cassette and its use in induced lysis of *Lactococcus lactis*. Appl. Environ. Microbiol. 63: 4877-4882 (1997)
63. Llull D, Poquet I. New expression system tightly controlled by zinc abailability in *Lactococcus lactis*. Appl. Environ. Microbiol. 70: 5398-5406 (2004)
64. Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic Streptococci after protoplast-induced curing. J. Bacteriol. 154: 1-9 (1983)
65. von Heijne G. The signal peptide. J. Membrane Biol. 115: 195-201 (1990)
66. Pugsley AP. The complete general secretory pathway in Gam-negative bacteria. Microbiol. Rev. 57: 50-108 (1993)
67. Tjalsma H, Bolhuis A, Jongbloed JD, Bron S, van Dijl JM. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol. Mol. Biol. R. 64: 515-547 (2000)
68. Jeong D-W, Choi YC, Lee JM, Kim JH, Lee JH, Kim KH, Lee HJ. Screening and characterization of secretion signals from *Lactococcus lactis* ssp. *cremoris* LM0230. J. Microbiol. Biotech. 14: 1052-1056 (2004)
69. Perez-Martinez G, Kok J, Venema G, van Dijl J.M, Smith H, Bron S. Protein export elements from *Lactococcus lactis*. Mol. Gen. Genet. 234: 401-411 (1992)
70. Poquet I, Ehrlich SD, Gruss A. An export-specific reporter designed for Gram-positive bacteria: application to *Lactococcus lactis*. J. Bacteriol. 180: 1904-1912 (1998)
71. Ravn R, Arnau J, Madsen SM, Vrang A, Israelsen H. The development of TnNuc and its use for the isolation of novel secretion signals in *Lactococcus lactis*. Gene 242: 347-356 (2000)
72. Sibakov M, Koivula T, von Wright A, Palva I. Secretion of TEM β-lactamase with signal sequences isolated from the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 57: 341-348 (1991)
73. Smith H, Bron S, van Ee J, Venema G. Construction and use of signal sequence selection vectors in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 169: 3321-3328 (1987)
74. Puohiniemi R, Simonen M, Muttilainen S, Himanen JP, Sarvas M. Secretion of the *Escherichia coli* outer membrane proteins OmpA and OmpF in *Bacillus subtilis* is blocked at an early intracellular step. Mol. Microbiol. 6: 981-990 (1992)
75. Andersson H, von Heijne G. A 30-residue-long "export initiation domain" adjacent to the signal sequence is critical for protein translocation across the inner membrane of *Escherichia coli*. P. Natl. Acad. Sci. USA 88: 9751-9754 (1991)

76. Le Loir Y, Nouaille S, Commissaire J, Bretigny L, Gruss A, Langella P. Signal peptide and propeptide optimization for heterologous protein secretion in *Lactococcus lactis*. Appl. Environ. Microbiol. 67: 4119-4127 (2001)
77. Boucher I, Parrot M, Gaudreau H, Champagne CP, Vadeboncoeur C, Moineau S. Novel food-grade plasmid vector based on melibiose fermentation for the genetic engineering of *Lactococcus lactis*. Appl. Envrion. Microbiol. 68: 6152-6161 (2002)
78. Bron PA, Benchimol MG, Lambert J, Palumbo E, Deghorain M, Delcour J, de Vos WM, Kleerebezem M, Hols P. Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. Appl. Environ. Microbiol. 68: 5663-5670 (2002)
79. Dickely F, Nilsson D, Hansen EB, Johansen E. Isolation of *Lactococcus lactis* nonsense suppressors and construction of a food-grade cloning vector. Mol. Microbiol. 15: 839-847 (1995)
80. Froseth BR, Mckay LL. Development and application of pFM011 as a possible food-grade cloning vector. J. Dairy Sci. 74: 1445-1453 (1991)
81. Han TU, Jeong D-W, Cho SH, Lee J-H, Chung DK, Lee HJ. Construction of a lactococcal shuttle/expression vector containing a β-galactosidase gene as a screening marker. Korean J. Microbiol. Biotech. 33: 241-247 (2005)
82. Hughes BF, McKay LL. Deriving phage-insensitive lactococci using a food-grade vector encoding phage and nisin resistance. J. Dairy Sci. 75: 914-923 (1991)
83. Jeong D-W, Lee J-H, Kim KH, Lee HJ. A food-grade expression/secretion vector for *Lactococcus lactis* that uses an α-galactosidase gene as a selection marker. Food Microbiol. 23: 468-475 (2006).
84. Leenhouts K, Bolhuis A, Venema G, Kok J. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. Appl. Microbiol. Biot. 49: 417-423 (1998)
85. Liu C-Q, Leelawatcharamas V, Harvey ML, Dunn NW. Cloning vectors for lactococci based on plasmid encoding resistance to cadmium. Curr. Microbiol. 33: 35-39 (1996)
86. Platteeuw C, van Alen-Boreeigter I, van Schalkwijk S, de Vos WM. Food-grade cloning expression system for *Lactococcus lactis*. Appl. Environ. Microbiol. 62: 1008-1013 (1996)
87. Sorensen KI, Larsen R, Kibenich A, Junge MP, Johansen E. A food-grade cloning system for industrial strains of *Lactococcus lactis*. Appl. Environ. Microbiol. 66: 1253-1258 (2000)
88. Takala TM, Saris PEJ. A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene *nisI*. Appl. Microbiol. Biot. 59: 467-471 (2002)
89. Liu C-Q, Khunajakr N, Chia LG, Deng Y-M, Charoenchai P, Dunn NW. Genetic analysis of regions involved in replication and cadmium resistance of the plasmid pND302 from *Lactococcus lactis*. Plasmid 38: 79-90 (1997)
90. Kiewiet R, Kok J, Seegers JFML, Venema G, Bron S. The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*. Appl. Environ. Microbiol. 59: 358-364 (1993)
91. Glenting J, Madsen SM, Vrang A, Fomsgaard A, Israelsen H. A plasmid selection system in *Lactococcus lactis* and its use for gene expression in *L. lactis* and human kidney fibroblasts. Appl. Environ. Microbiol. 68: 5051-5056 (2002)
92. de Vos WM. Safe and sustainable systems for food-grade fermenta-tions by genetically modified lactic acid bacteria. Int. Dairy J. 9: 3-10 (1999)
93. Emond E, Lavallee R, Drolet G, Moineau S, LaPointe G. Molecular characterization of a theta replication plasmid and its use for development of a two-component food-grade cloning system for *Lactococcus lactis*. Appl. Environ. Microbiol. 67: 1700-1709 (2002)
94. Henrich B, Klein JR, Weber B, Delorme C, Renault P, Wegmann U. Food-grade delivery system for controlled gene expression in *Lactococcus lactis*. Appl. Environ. Microbiol. 68: 5429-5436 (2002)
95. Simoes-Barbosa A, Abreu H, Silva Neto A, Gruss A, Langella P. A food-grade delivery system for *Lactococcus lactis* and evaluation of inducible gene expression. Appl. Microbiol. Biot. 65: 61-67 (2004)
96. Leenhouts K, Kok J, Venema G. Replacement recombination in *Lactococcus lactis*. J. Bacteriol. 173: 4794-4798 (1991)
97. Kuipers OP, Beerthuyzen MM, Siezen RJ, de Vos WM. Characterization of the nisin gene cluster nis*ABTCIPR* of *Lactococcus lactis*. Eur. J. Biochem. 216: 281-291 (1993)
98. van Rooijen RJ, Gasson MJ, de Vos WM. Characterization of the promoter of the *Lactococcus lactis* lactose operon: contribution of flanking sequences and LacR repressor to its activity. J. Bacteriol. 174: 2273-2280 (1992)
99. Eaton TJ, Shearman CA, Gasson MJ. The use of bacterial luciferase genes as reporter genes in *Lactococcus lactis*: regulation of the *Lactococcus lactis* lactose gene. J. Gen. Microbiol. 139: 1495-1501 (1993)
100. Payne J, MacCormick CA, Griffin HG, Gasson MJ. Exploitation of a chromosomally integrated lactose operon for controlled gene. FEMS Microbiol. Lett. 136: 19-24 (1996)
101. Steidler L, Wells JM, Raemakers A, Vanderkerckhove J, Fiers W, Remaut E. Secretion of biologically active murine interleukin-2 by *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 61: 1627-1629 (1995)
102. de Ruyter PGGA, Kupers OP, Beerthuyzen MM, van Alen-Boerrigter IJ, de Vos WM. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J. Bacteriol. 178: 3434-3439 (1996)
103. Sanders JW, Leenhouts KJ, Haandrikman AJ, Venema G, Kok J. Stress response in *Lactococcus lactis*: Cloning, expression analysis, and mutation of the lactococcal superoxide dismutase gene. J. Bacteriol. 177: 5254-5260 (1995)
104. Hindre T, Le Pennec J-P, Haras D, Dufour A. Regulation of lantibiotic lacticin 481 production at the transcriptional level by acid pH. FEMS Microbiol. Lett. 231: 291-298 (2004)
105. Vos P, Simons G, Siezen RJ, de Vos WM. Primary structure and organization of the gene for a prokaryotic, cell-envelope located serine proteinase. J. Biol. Chem. 264: 13579-13585 (1989)
106. van der Meer JR, Rollema HS, Siezen RJ, Kuipers OP, de Vos WM. Influence of amino acid substitutions in the nisin leader peptide on biosynthesis and secretion of nisin by *Lactococcus lactis*. J. Biol. Chem. 269: 3555-3562 (1994)