Gene expression systems for lactic acid bacteria

Willem M de Vos

Considerable advances have been made in the genetics and molecular biology of lactic acid bacteria, including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* spp. These have resulted in the construction of constitutive gene expression cassettes, inducible gene expression systems, and specific protein targeting systems for these bacteria. These developments are important in the food industry where lactic acid bacteria can be exploited as food-grade cell factories.

Addresses
Wageningen Centre for Food Science, Wageningen, The Netherlands
Microbial Ingredients Section, NIZO Food Research, Ede, The Netherlands
Laboratory of Microbiology, Wageningen Agricultural University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands; e-mail: willem.devos@algemeen.micr.wau.nl

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Abbreviations

AT	adenine and thymidine
bp	base pairs
GC	guanine and cytosine
LAB	lactic acid bacteria
NICE	nisin-controlled expression

Introduction

Lactic acid bacteria (LAB) constitute a phylogenetically related group of anaerobic Gram-positive bacteria, including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* spp. (Figure 1), that share the capacity to ferment sugars primarily into lactic acid. Many of these LAB are associated with the traditional and industrial production of fermented foods, beverages and animal feed. Engaging examples include the manufacture of cheese, yogurt, and sausage from animal produce, or sauerkraut, soy sauce, and silage from vegetable origin. Moreover, some LAB are exploited as producers of flavoring enzymes, peptides with antimicrobial activity, or metabolites that contribute to the flavor, conservation or texture of foods. Finally, several LAB, notably *Lactobacillus* spp., are presently marketed as probiotic cultures with health-promoting capacity.

This decade has seen significant advances in the genetic study of LAB resulting in the development of a great number of genetic techniques, transformation protocols, and sophisticated vector, integration and amplification systems that have been reviewed elsewhere [1–5]. In addition, several sophisticated, sustainable and safe selection systems have been generated that can be applied in the food industry and, hence, are termed food-grade [6]. Moreover, hundreds of genes and operons of LAB have been characterized in considerable detail for their sequence, function and expression. Finally, genomics has been advanced in LAB and the genome of *Lactococcus lactis* has recently reported to be completely sequenced, while genome sequencing of several other starter and probiotic strains has been initiated [7].

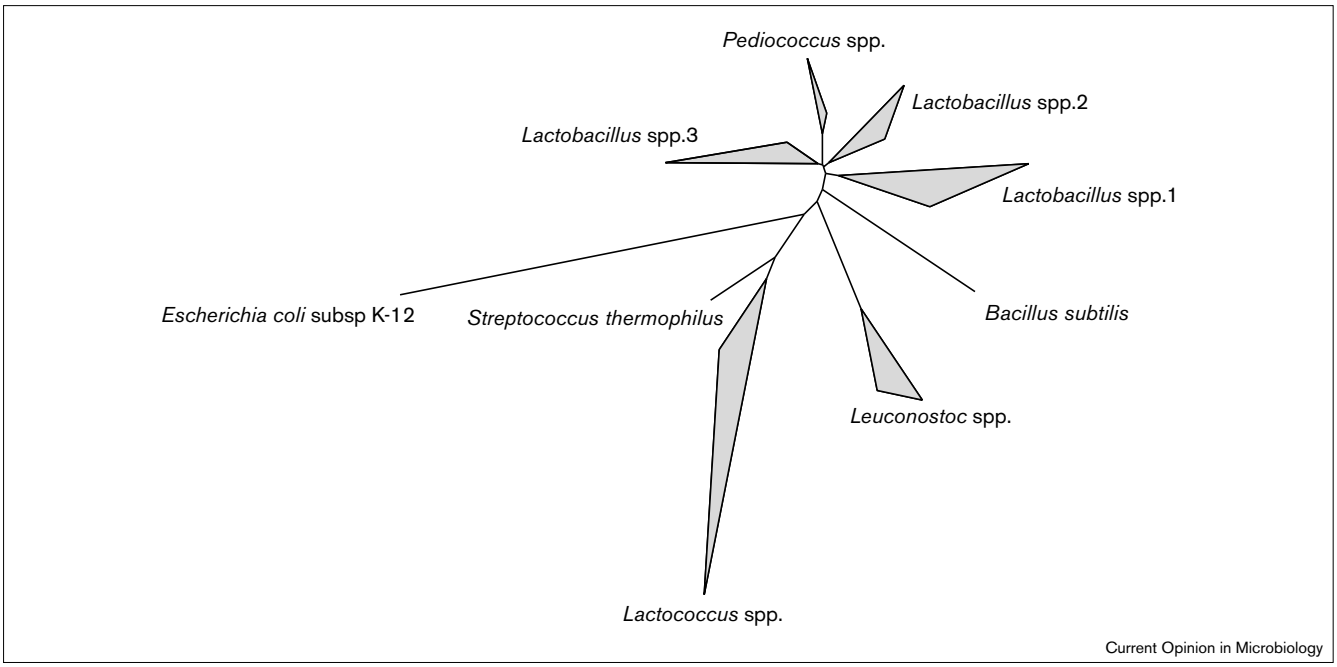
Essential for the further scientific and industrial development of LAB is the modulated expression of novel, existing or new combinations of genes, and the appropriate delivery of their gene products. For this purpose a variety of constitutive or inducible gene expression as well as protein targeting systems have been developed that in many cases are also operating in various LAB. In this review the essential features of these expression systems, their development, and their present or potential applications are discussed with specific attention to *L. lactis*, which has become the paradigm for LAB.

Constitutive expression systems

Because of its important role in the efficiency and control of gene expression, the process of transcription initiation has received considerable attention in various LAB, specifically *Lactococcus* and *Lactobacillus* spp. [1–3]. Only in *L. lactis*, however, has the *rpoD* gene encoding the major sigma (σ) factor, been characterized. *rpoD* was found to encode a 39 kD protein with high similarity to the vegetative *Bacillus subtilis* σ^{43} and the carboxy-terminal part of the *Escherichia coli* σ^{70} [8]. The specific contacts of the *L. lactis* σ^{43} with the lactococcal promoter region have not been determined. On the basis of analysis of a large number of promoters, however, a consensus lactococcal promoter can be deduced that includes the conserved –35 and –10 hexamers found in *E. coli* and *B. subtilis*; shows a strict separation between these canonical hexamers of 17 nucleotides; contains a TG dinucleotide at position –15; and includes a region immediately upstream from the –35 sequence that is AT rich [2,5]. By examining similarities between aligned promoters, including that of rRNA and tRNA operons, it has been proposed that the latter upstream sequence has a specific consensus of AGTTATTC, and a conserved sequence at +1, GTACTGG, which includes the transcription initiation site, was also identified [9,10••].

Three different strategies have been followed to isolate promoters from *L. lactis* and other LAB. The first strategy is based on screening vectors, both plasmids and transposons, carrying promoterless reporter genes such as those encoding chloramphenicol resistance (*cat-86*) or β-galactosidase (*lacZ*) or β-glucuronidase (*gusA*) [1–3]. New and useful lactococcal promoters are still being discovered as illustrated in a screening study exploiting the promoterless *lux* gene that yielded a new set of more than 10 promoters with greatly different efficiency [11]. The second strategy capitalizes on the great number of genes that now have been studied and has led to the identification of various promoters, notably those from house-keeping genes, that are

Figure 1



Unrooted phylogenetic tree based on 16S rRNA sequences showing the relative positions of *Lactococcus*, *Lactobacillus* (three main groups, designated 1, 2 and 3), *Leuconostoc*, *Pediococcus* sp. and *Streptococcus thermophilus*. The positions of *Bacillus subtilis* and *Escherichia coli* are shown for comparisons.

strong and constitutive [1–3]. In addition, several controlled promoters have been discovered in this way and some of the most promising ones are discussed below. A third and exciting novel approach has been reported recently, and comprises the construction and screening of synthetic promoters obtained from the consensus *L. lactis* promoter (Figure 2) in which the sequences of the separating spacer regions were randomized [10••]. A set of 38 promoters that differed in strength by 3–4 logs of activity, including several very strong promoters, was analyzed and found to be constitutive. Both of these properties render this set of promoters specifically suitable for metabolic engineering studies in which gene expression should be modulated in a constitutive way. Some of these promoters were not only functional in *L. lactis* but also in *E. coli*, in which some of the weak *L. lactis* promoters were 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. A high selectivity of LAB has also been described for the *Lactobacillus* promoters [1]. This phenomenon may be due to the fact that the GC content of LAB is lower than that of *E. coli* and, hence, there may be a bias in LAB against the high fraction of AT-rich sequences found in the consensus promoter (see above). It will be of interest to see how this strategy using synthetic promoters could be applied to other LAB in order to obtain species-specific promoters and increase the understanding of initiation of gene expression.

Controlled expression systems

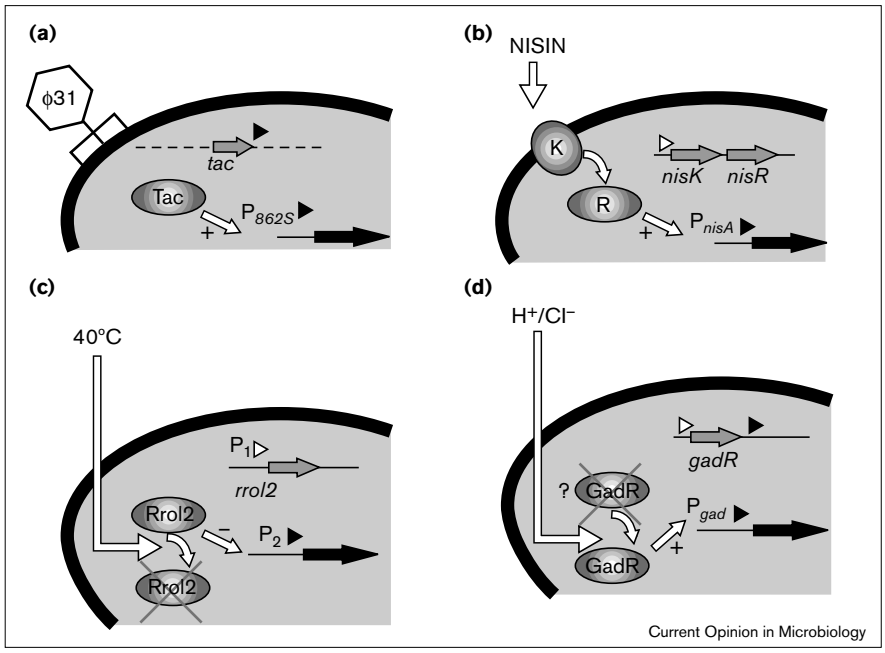
The structural and functional analysis of specific genes and operons has allowed for a detailed insight in the regulation of transcription initiation in LAB [12–14]. Some of these promoters, however, are less useful than others in the development of controlled expression systems. Therefore, regulated promoters with low transcriptional efficiency, promoters that are only controlled to a limited extent, or promoters that have limited application potential are not

Table 1

Sugar-inducible expression systems for LAB.

LAB	Sugar	Promoter	Regulator	Expressed genes	Induction factor	References
Lactococcus lactis	Lactose	*lacA*	LacR	*cat-86, luxAB,* Lysin gene	~ 10	[64–66]
Lactococcus lactis	Lactose	*lacR*	LacR	*luxAB*	~ 10	[65]
Lactococcus lactis	Lactose	*lacA/T7*	LacR	TTFC gene, IL2 gene	~ 20	[27,67]
Lactobacillus pentosus	Xylose	*xylA*	xylR	*cat-86*	~ 60	[28,68]
Streptococcus thermophilus	Lactose	*lacS*	GalR	*cat-86*	~ 10	[25]

Figure 2



General outline of the molecular architecture of the inducible gene expression systems developed for *L. lactis* and other LAB. **(a)** The explosive expression system. The expression of the *tac* gene is induced by bacteriophage ϕ31 infection resulting in the synthesis of the Tac transcriptional activator. The middle promoter of ϕ31, p_{8625}, is also induced by bacteriophage infection as well as the Tac transcriptional activator. Expression from P_{8625} results in protein over-production and lysis of the expression host. **(b)** The NICE system. The P_{nisA} promoter is regulated by the two-component regulatory system, consisting of the sensor kinase NisK and the response regulator NisR, which responds to extracellular nisin. Addition of nisin to the extracellular media results in induction of gene expression from P_{nisA}. **(c)** A temperature-inducible system. The thermolabile Rro12 repressor of P_2 is inactive at 40°C, resulting in induction of gene expression controlled by P_2. **(d)** A pH-inducible system. The induction of the P_{gad} by chloride or acid is mediated by GadR by an unknown mechanism. Arrowheads indicate location and orientation of promoters (white, not controlled; black, controlled). Black arrows indicate genes of interest that can be expressed by the inducible systems.

further discussed here. Specific cases that are not further discussed here, but may be useful in some applications, are globally controlled promoters identified in LAB; for example, heat-shock promoters controlled by the negative regulator HcrA and CIRCE operators [15,16], cold-shock promoters [17,18], other stress-controlled promoters affected by CtsR or other factors [19•,20], promoters repressed or activated by catabolite repression mediated by Hpr/CcpA acting on *cre* elements [21,22], and promoters under control of FNR- or possibly OxyR-like proteins [23•,24].

Sugar-inducible expression systems

Sugar utilization has been extensively studied in LAB because of its important role in the industrial fermentations. Most genes involved in sugar transport and catabolism are organized into operons that are strongly expressed and controlled at the level of transcription initiation. Most systems are subject to CcpA-dependent catabolite repression, but many are also specifically control by a dedicated regulator, which in most cases has been identified. Several of the latter control circuits have been used to develop sugar-dependent gene expression systems that have been tested with a number of reporter and other relevant genes. These sugar-inducible expression systems vary in efficiency, gene location, and inducing sugar (Table 1). Although most promoters are subject to repression, it appears that the *Streptococcus thermophilus lac* promoter is under control of a transcriptional activator (Vaughan EE, Catzeddu P, van den Boogert P, de Vos WM, unpublished data). This system is of specific interest since, in contrast to the other systems, it is not located on high copy number plasmids but provides induction of single copy genes inserted by gene replacement in the chromosomal *lac* region [25].

The best characterized lactococcal promoter is that of the *L. lactis lac* operon. This strong *lacA* promoter, which is virtually not subject to catabolite repression, is controlled by the autoregulated LacR repressor. Induction by lactose is effected by the intermediate tagatose-6-phosphate that inactivates the LacR repressor. The critical residues of the LacR repressor involved in this process have been identified [26]. By cloning the *E. coli* T7 RNA polymerase gene under control of the *lacA* promoter, a sophisticated system has been developed for *L. lactis* that exploits the efficiency of the dedicated T7 promoter, which indirectly is stimulated by growth of the cells on lactose [27]. This system has been reported to have the highest efficiency of all the sugar-inducible expression systems, but its induction is surpassed by that of the *Lactobacillus pentosus xylA* promoter, which is strongly sensitive to catabolite repression [27,28].

Other gene expression systems developed for LAB

Although the sugar-controlled systems are useful, mainly because of their high efficiency, there is a need for efficient expression systems that are better and easier to control. Four completely different systems that allow gene expression to be induced between 100–1000-fold have recently been developed on the basis of molecular analysis of genetic regulatory circuits (Figure 2).

ϕ31-induced explosive expression system

An elegant system has been developed on the basis of the middle promoter of the lactococcal bacteriophage ϕ31, which is induced by infection by ϕ31 (Figure 2a). This molecular switch is turned on by bacteriophage infection and, hence, results in lysis of the production strain. An additional boost to the expression was provided by using an expression plasmid containing the replication origin of ϕ31 (*ori31*) that is also induced upon bacteriophage infection and results in runaway replication. Because of the lysis that is ultimately realized, this system was termed explosive expression [29].

Recent analysis of this regulatory circuit, which is also functional in *E. coli*, provided evidence for the involvement of a ϕ31-encoded *trans*-acting transcriptional activator (Tac), which is encoded by the *tac* gene preceding the inducible ϕ31-middle promoter (Figure 2a). Analysis of the 322 bp minimal promoter fragment revealed that a mutation in an inverted repeat upregulates the promoter while maintaining an induction factor of >1000-fold after two hours of infection [30••]. Because of its high inducibility coupled to lysis and release of the product of interest, the explosive expression system is highly attractive. A 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.

Nisin-controlled expression system

A highly versatile, strongly controlled, and food-grade process for controlled over-production of proteins is based on the nisin-controlled expression (NICE) system [31] (Figure 2b). The NICE system has been derived from the molecular characterization of the production of nisin, a post-translationally modified antimicrobial peptide, produced by several strains of *L. lactis* that are widely used in the food industry [32]. Nisin production is under control of the *nisA* promoter that is subject to autoregulation by a two-component regulatory system, consisting of the sensor kinase NisK and the response regulator NisR, which responds to extracellular nisin [33]. This regulatory system belongs to the well-known peptide pheromone-dependent quorum sensing systems that are widely distributed in Gram-positive bacteria [34].

It was shown that *nisA* promoter, which could be trimmed down to less than 50 bp, could be employed in a series of transcriptional and translational fusion vectors. These appeared to be extremely useful for the expression of a variety of genes resulting in hyper-production of enzymes such as the debittering aminopeptidase N to more than 50% of the total protein [35–37]. The induction could be realized in a dynamic range to 10,000-fold by the addition of subinhibitory amounts of the signaling molecule nisin, mutants thereof, or simply by adding a supernatant of a nisin-producing strain. In addition, density-dependent expression of genes of interest was realized by using a nisin-producing *L. lactis* strain as production host. Finally, the NICE system could be successfully implemented in a variety of LAB and other industrially relevant bacteria, including *Bacillus*, *Enterococcus*, *Lactococcus*, *Lactobacillus* and *Streptococcus* spp. [38,39••].

Thermoinduction systems

Determination of the complete nucleotide sequence of the temperate *L. lactis* bacteriophage ϕr1t allowed the identification of a control region involved in the lysogenic switch, strongly resembling that of bacteriophage lambda [40]. It consists of two divergent genes promoters, P_1 and P_2, controlling expression of the *rro* gene, encoding a repressor, and the *tec* gene (topological equivalent to *cro*), respectively (Figure 2c). Experimental evidence was provided for the repression of P_2 expression by Rro, which could be alleviated by the addition of mitomycin C [41].

An improvement of this system was realized by designing a thermolabile variant of the Rro repressor, Rro12, that upon shifting from a permissive growth temperature of 24°C to 42°C resulted in an approximately 500-fold induction of gene expression controlled by P_2 [42]. Although thermoinduction is not easy to apply on an industrial scale and also leads to the induction of various heat-shock genes, this system may have similar potential as the highly similar *E. coli* P_L promoter.

pH-dependent systems

Because acidification is one of the properties of LAB, several screening approaches have uncovered pH-dependent promoters that usually also responded to other stress factors [43,44]. A specific case is the *gad* promoter driving expression of the *gadCB* operon predicted to encode a glutamate-γ-aminobutyrate antiporter and glutamate decarboxylase, respectively, that operate in glutamate-dependent acid stress resistance (Figure 2d). The induction of the *gad* promoter by chloride, glutamate or acid is mediated in an unknown way by the GadR protein that is assumed to be a transcriptional activator [45••]. On the basis of this environmentally regulated control circuit an inducible expression system was developed that was induced more than 1000-fold by growth in the presence of 0.5 M NaCl [46].

Protein targeting systems

Following the production of proteins or peptides by LAB using the described expression systems, they should be properly folded, targeted, and, if appropriate, recovered. Protein secretion via the sec-dependent pathway has been subject of recent studies resulting in optimization of previously established secretion cassettes, such as those based on the Usp45 signal sequence, with specific prokaryotic but also various eukaryotic proteins in *L. lactis* [47,48,49•] and *Lactobacillus* spp. [50,51,52•,53]. Also new secretion signals in various LAB have been screened and exploited for protein targeting systems [53–55]. In addition, the use of dedicated, non-sec-dependent secretion via ABC exporters is receiving increasing attention and has led to the export of novel

peptides, such as colicin V, mesentericin and pediocin, by *L. lactis* [56–58].

In addition, a new emerging area is to specifically deliver proteins to the cell envelope, in order to facilitate protein recovery, immobilize cells, or present bioactive molecules, such as antigens. Attempts to anchor proteins using *L. lactis* signals, such as the PrtP carboxy-terminal domain, have been described [50]. Recently, the carboxy-terminal cell wall anchor of *Staphylococcus aureus* protein A was found to be effective in specifically anchoring streptavidin to the cell wall of *L. lactis* [59••]. Another approach has been based on the newly identified carboxy-terminal anchor sequence of the *L. lactis* autolysin AcmA that could be used to anchor proteins to lactococcal cell walls even after they had been produced in another bacterium [60].

Finally, specific lysis systems have been designed based on the inducible expression signals (Figure 2) that were coupled to lysin and/or holin genes from bacteriophages or the lactococcal autolysin gene *acm*A [61–63]. In all cases successful lysis was obtained in *L. lactis* resulting in the release of intracellular enzymes, including those that were simultaneously over-produced. Although resembling the explosive expression triggered by phage infection [29], these systems have superior control and industrial potential, notably the food-grade NICE system that was applied in model cheese production [61].

Conclusions

A variety of highly sophisticated expression systems have now been developed for LAB. These will be instrumental in providing an improved understanding of gene function and expression in LAB that is essential in the post-genomic era. In addition, these tools may be implemented in other Gram-positive and even Gram-negative bacteria. Finally, these systems may be used to rationally improve the existing food applications of LAB resulting in superior traditional fermentations or novel functional foods. Also LAB could be developed for the production of new compounds (varying from enzymes or peptides to metabolites and neutraceuticals), or to promote specifically the *in situ* production of desired compounds, either in food products or in the gastrointestinal tract, following their consumption.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Pouwels PH, Leer RJ: **Genetics of Lactobacilli. Plasmids and gene expression.** *Antonie van Leeuwenh* 1993, **64**:85-107.

2. De Vos WM, Simons G: **Gene cloning and expression systems in Lactococci.** In *Genetics and Biotechnology of Lactic Acid Bacteria.* Edited by Gasson MJ, de Vos WM. Oxford: Chapman and Hall; 1994: 52-105.

3. Mercenier A, Pouwels PH, Chassy BM: **Genetic engineering of lactobacilli, leuconostocs and *Streptococcus thermophilus.*** In *Genetics and Biotechnology of Lactic Acid Bacteria.* Edited by Gasson MJ, de Vos WM. Oxford: Chapman and Hall; 1994: 252-293.

4. Renault P: **Genetic Engineering Strategies.** In *Lactic Acid Bacteria: Current Advances in Metabolism, Genetics and Applications.* Edited by Bozoglu TF, Ray B. NATO Symposium Series; 1996:1-37.

5. De Vos WM, Kleerebezem M, Kuipers OP: **Expression systems for industrial Gram-positive bacteria with low guanine and cytosine content.** *Curr Opin Biotechnol* 1997, **8**:547-553.

6. De Vos WM: **Safe and sustainable systems for food-grade fermentations by genetically modified lactic acid bacteria.** *Int Dairy* J 1999, in press.

7. Courvalin P, de Vos WM, Rubens CE: **Genetics of Streptococci, Enterococci and Lactococci.** *ASM News* 1998, **64**:501-504.

8. Araya T, Ishinashi N, Shimamura S, Tanaka T, Takahashi H: **Genetic and molecular analysis of the *rpoD* gene from *Lactococcus lactis.*** *Biosc Biotech Biochem* 1993, **57**:88-92.

9. Nilsson D, Johansen E: **A conserved sequence in tRNA an rRNA promoters of *Lactococcus lactis.*** *Biochim Biophys Acta* 1994, **1219**:139-144.

10. •• Jensen PR, Hammer K: **The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters.** *Appl Environ Microbiol* 1998, **63**:82-87.
This paper describes the development of a large set of synthetic promoters for *L. lactis* that differ more than 1000-fold in efficiency. This approach, which is based on conserving consensus sequences and randomizing spacers, may be useful in many lactic acid bacteria.

11. Waterfield NR, LePage RWF, Wilson PW, Wells JM: **The isolation of lactococcal promoters and their use in investigating bacterial luciferase synthesis in *Lactococcus lactis.*** *Gene* 1995, **165**:9-15.

12. Kok J: **Inducible gene expression and environmentally regulated genes in lactic acid bacteria.** *Ant van Leeuwenh* 1996, **70**:129-145.

13. Kuipers, OP, de Ruyter PGGA, Kleerebezem M, de Vos WM: **Controlled overproduction of proteins by lactic acid bacteria.** *Trends Biotechnol* 1997, **15**:135-140.

14. Djordjevic GM, Klaenhammer TR: **Inducible gene expression systems in *Lactococcus lactis.*** *Mol Biotechnol* 1998, **9**:127-139.

15. van Asseldonk M, de Vos WM, Simons G: **Cloning, nucleotide sequence and regulatory analysis of the *Lactococcus lactis dnaJ* gene.** *J Bacteriol* 1993, **175**:1637-1644.

16. Nauberhaus F: **Negative regulation of bacterial heat shock genes.** *Mol Microbiol* 1999, **31**:1-8.

17. Mayo B, Derzelle S, Fernandez M, Leonard C, Ferain T, Hols P, Suarzez JE, Delcour J: **Cloning and characterization of *cspL* and *cspP*, two cold-inducible genes from *Lactobacillus plantarum.*** *J Bacteriol* 1997, **179**:3039-3042.

18. Wouters JA, Sanders JW, Kok J, de Vos WM, Kuipers OP, Abee T: **Clustered organization and transcriptional analysis of a family of five *csp* genes of *Lactococcus lactis* MG1363.** *Microbiology* 1998, **144**:2885-2893.

19. • Derre I, Rapoport TG, Msadek T: **CtsR, a novel regulator of stress and heat shock response, controls *clp* and molecular chaperone expression in Gram-positive bacteria.** *Mol Microbiol* 1999, **31**:117-131.
This paper provides evidence for a novel regulator and *cis*-acting target sequence that is widely spread in Gram-positive bacteria and lactic acid bacteria.

20. Smeds A, Varmanen P, Palva A: **Molecular characterization of a stress-inducible gene from *Lactobacillus helveticus.*** *J Bacteriol* 1998, **180**:6148-6153.

21. Deutscher J, Kuster E, Bergstedt U, Charrier V, Hillen W: **Protein-kinase dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria.** *Mol Microbiol* 1995, **15**:1049-1053.

22. Luesink EJ, van Herpen REMA, Grossiord BP, Kuipers OP, de Vos WM: **Transcriptional activation of the glycolytic *las* operon**

and catabolite repression of the gal operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. *Mol Microbiol* 1998, **30**:789-798.

23. • Gostick DO, Green J, Irvine AS, Gasson MJ, Guest JR: **A novel regulatory switch mediated by the FNR-like protein of *Lactobacillus casei*.** *Microbiology* 1998, **144**:705-717.
Functional analysis of a novel global regulator in *Lactobacillus casei* demonstrates that it resembles the well-studied *E. coli* FNR and that it is likely to control gene expression in other lactic acid bacteria.

24. Hertel C, Schmidt G, Fischer M, Oeller K, Hammes WP: **Oxygen-dependent regulation of the expression of the catalase gene *katA* of *Lactobacillus sake* LTH677.** *Appl Environ Microbiol* 1998, **64**:1359-1365.

25. Mollet B, Knol J, Poolman B, Marciset O, Delley M: **Directed genomic integration, gene replacement, and integrative gene expression in *Streptococcus thermophilus*.** *J Bacteriol* 1993, **175**:4315-4324.

26. van Rooijen RJ, Dechering KJ, Wilmink CNJ, de Vos WM: **Lysines 72, 80, 213, and aspartic acid 210 of the *Lactococcus lactis* LacR repressor are involved in response to the inducer tagatose-6-phosphate leading to induction of the *lac* operon expression.** *Prot Engin* 1993, **6**:201-206.

27. Wells JM, Wilson PW, Norton PM, Gasson MJ, Le Page RWP: ***Lactococcus lactis*: high level expression of tetanus toxin fragment C and protection against lethal challenge.** *Mol Microbiol* 1995, **8**:1155-1162.

28. Lokman BC, Heerikshuizen M, van den Broek A, Borsboom Y, Chaillou S, Postma PW, Pouwels PH: **Regulation of the *Lactobacillus pentosus xylAB* operon.** *J Bacteriol* 1997, **179**:5391-5397.

29. 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.** *Bio/Technology* 1996, **14**:82-87.

30. •• Walker SA, Klaenhammer TR: **Molecular characterization of phage inducible middle promoter and its transcriptional activator from the lactococcal bacteriophage ϕ31.** *J Bacteriol* 1998, **180**:921-931.
This paper provides the molecular framework for the explosive expression system that couples over-production and lysis of the expression host *L. lactis*. Induction is not triggered by the addition of a chemical but by bacteriophage infection.

31. Kuipers, OP, de Ruyter, PGGA, Kleerebezem M, de Vos WM: **Quorum sensing controlled gene expression in lactic acid bacteria.** *J Biotechnol* 1998, **64**:15-21.

32. De Vos WM, Kuipers OP, van der Meer JR, Siezen RJ: **Maturation pathway of nisin and other lantibiotics: post-translationally modified antimicrobial peptides exported by Gram-positive bacteria**. *Mol Microbiol* 1995, **17**:427-437.

33. Kuipers OP, Beerthuyzen MM, de Ruyter PGGA, Luesink EJ, de Vos WM: **Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction.** *J Biol Chem* 1995, **270**:27299-27304.

34. Kleerebezem M, Quadri LEN, Kuipers OP, de Vos WM: **Quorum sensing by peptide pheromones and two-component signal transduction systems in Gram-positive bacteria.** *Mol Microbiol* 1997, **24**:895-905.

35. De Ruyter PGGA, Kuipers OP, de Vos WM: **Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin.** *Appl Environ Microbiol* 1996, **62**:3662-3667.

36. De Ruyter PGGA, Kuipers OP, Beerthuyzen MM, van Alen-Boerrigter I, de Vos WM: **Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*.** *J Bacteriol* 1996, **178**:3434-3439.

37. De Ruyter PGGA: **Development, molecular characterisation and exploitation of the nisin controlled expression system in *Lactococcus lactis* [PhD Thesis]**. Wageningen Agricultural University, The Netherlands; 1998.

38. Kleerebezem M, Beerthuyzen MM, Vaughan EE, de Vos WM, Kuipers OP: **Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc* and *Lactobacillus* spp.** *Appl Environ Microbiol* 1997, **63**:4581-4584.

39. •• 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* 1998, **64**:2763-2769.
The authors illustrate the potential of controlled expression systems, such as the nisin-controlled expression (NICE) system, to be implemented in bacteria other than lactic acid bacteria.

40. van Sinderen D, Karsens H, Kok J, Terpstra P, Ruiters MHJ, Venema G, Nauta A: **Sequence analysis and molecular characterization of the temperate lactococcal bacteriophage r1t.** *Mol Microbiol* 1996, **19**:1343-1355.

41. Nauta A, van Sinderen D, Karsens H, Smit E, Venema G, Kok J: **Inducible gene expression mediated by a repressor-operator system isolated from *Lactococcus lactis* bacteriophage r1t.** *Mol Microbiol* 1996, **19**:1331-1341.

42. Nauta A, van den Burg B, Karsens H, Venema G, Kok J: **Design of thermolabile bacteriophage repressor mutants by comparative molecular modeling.** *Nat Biotechnol* 1997, **15**:980-983.

43. Israelsen H, Madsen SM, Vrang A, Hansen EB, Johansen E: **Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn*917-lacZ* integrants with the new promoter probe vector, pAK80.** *Appl Environ Microbiol* 1995, **61**:2540-2547.

44. Sanders JW, Venema G, Kok J, Leenhouts K: **Identification of a sodium chloride-regulated promoter in *Lactococcus lactis* by single-copy chromosomal fusion with a reporter gene.** *Mol Gen Genet* 1998, **257**:681-685.

45. •• Sanders JW, Leenhouts K, Burghoorn J, Brands JR, Venema G, Kok J: **A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation.** *Mol Microbiol* 1998, **27**:299-310.
The first detailed description of a promoter that is controlled by chloride, glutamate and pH. In addition, evidence is provided for the functionality of a transcriptional activator that modulates the activity of this promoter.

46. Sanders JW, Venema G, Kok J: **A chloride-inducible gene expression cassette and its use in induced lysis of *Lactococcus lactis*.** *Appl Environ Microbiol* 1997, **63**:4877-4882.

47. van Asseldonk M, de Vos WM, Simons G: **Functional analysis of the *Lactococcus lactis usp45* secretion signal in the secretion of a homologous proteinase and a heterologous a-amylase.** *Mol Gen Genet* 1993, **240**:428-434.

48. Arnau J, Hjerl-Hansen E, Israelsen H: **Heterologous gene expression of bovine plasmin in *Lactococcus lactis*.** *Appl Microbiol Biotechnol* 1997, **48**:331-338.

49. • Leloir Y, Gruss A, Ehrlich SD, Langella P: **A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*.** *J Bacteriol* 1998, **180**:1895-1903.
This paper provides evidence for the possibility that secretion efficiency of heterologous proteins may be increased by inserting a specific peptide.

50. Wells JM, Robinson K, Chamberlain LM, Schofield KM, Le Page R: **Lactic acid bacteria as vaccine delivery vehicles.** *Ant v Leeuwenh* 1996, **70**:317-330.

51. Hols P, Slos P, Dutot P, Reymund J, Chabot P, Delplace B, Delcour J, Mercenier A: **Efficient secretion of the model antigen M6-gp41E in *Lactobacillus plantarum* NCIMB 8826.** *Microbiology* 1997, **143**:2733-2741.

52. • Slos P, Dutot P, Reymund J, Kleinpeter P, Prozzi D, Kieny MP, Delcour J, Mercenier A, Hols P: **Production of cholera toxin B subunit in *Lactobacillus*.** *FEMS Microbiol Lett* 1998, **169**:29-36.
Lactobacillus spp. can be used as a potential host for the delivery of protein fragments that may evoke an immune response.

53. Savijoki K, Kahala M, Palva A: **High level heterologous protein production in *Lactococcus* and *Lactobacillus* using a new secretion systems based on the *Lactobacillus brevis* S-layer signals.** *Gene* 1997, **186**:255-262.

54. Satoh E, Ito Y, Sasaki Y, Sasaki T: **Application of the extracellular α-amylase gene from *Streptococcus bovis* 148 to construction of a secretion vector for yoghurt starter strains.** *Appl Environ Microbiol* 1997, **63**:4593-4596.

55. Poquest I, Ehrlich SD, Gruss A: **An export-specific reporter designed for Gram-positive bacteria: application to *Lactococcus lactis*.** *J Bacteriol* 1998, **180**:1904-1912.

56. van Belkum MJ, Worobo RW, Stiles ME: **Double-glycine-type leader peptides direct secretion of bacteriocins by ABC transporters: colicin V secretion in *Lactococcus lactis*.** *Mol Microbiol* 1997, **23**:1293-1301.

57. Horn N, Dodd H, Gasson MJ: **Production of pediocin PA-1 by *Lactococcus lactis* using the lactococcin A secretory apparatus**. *Appl Environ Microbiol* 1998, **64**:14-20.

58. Biet F, Berjeaud JM, Worobo RW, Cenatiempo Y, Fremaux C: **Heterologous expression of the bacteriocin mesentericin Y105 using the dedicated transport system and the general secretion pathway.** *Microbiology* 1998, **144**:2845-2854.

59. •• Steidler L, Viaene J, Fiers W, Remaut E: **Functional display of a heterologous protein on the surface of *Lactococcis lactis* by means of the cell wall anchor of *Staphylcoccus aureus* protein A.** *Appl Environ Microbiol* 1998, **64**:342-345.
Novel cell-wall anchoring by using the carboxy-terminal domain of protein A. This technique has potential to be used for stable surface expression of proteins in lactic acid bacteria.

60. Buist G: **The N-acetylmuramidase of *Lactococcus lactis* binds to the cell wall by means of repeated motifs located in the C-terminal domain [PhD Thesis].** Groningen University, The Netherlands; 1997.

61. De Ruyter PGGA, Kuipers OP, Meijer WC, de Vos WM: **Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese-ripening.** *Nat Biotechnol* 1997, **15**:976-979.

62. Buist G, Karsens H, Nauta A, van Sinderen D, Venema G, Kok J: **Autolysis of *Lactococcus lactis* caused by induced overproduction of its major autolysin, AcmA.** *Appl Environ Microbiol* 1997, **63**:2722-2728.

63. Sanders JW, Venema G, Kok J: **A chloride-inducible gene expression cassette and its use in induced lysis of *Lactococcus lactis.*** *Appl Environ Microbiol* 1997, **63**:4877-4882.

64. 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* 1992, **174**:2273-2280.

65. Eaton TJ, Shearman CA, Gasson MJ: **The use of bacterial luciferase genes as reporter genes in *Lactococcus lactis*: regulation of the *Lactococcus lactis* subsp. *lactis* lactose genes.** *J Gen Microbiol* 1993, **139**:1495-1501.

66. Payne J, MacCormick CA, Griffin HG, Gasson MJ: **Exploitation of a chromosomally integrated lactose operon for controlled gene expression in *Lactococcus lactis.*** *FEMS Microbiol Lett* 1996, **136**:19-24.

67. 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* 1995, **61**:1627-1629.

68. Lokman BC, Leer RJ, van Sorge R, Pouwels P: **Promoter analysis and transcriptional regulation of the *Lactobacillus pentosus* xylose regulon.** *Mol Gen Genet* 1994, **245**:117-125.