Inducible gene expression and environmentally regulated genes in lactic acid bacteria

Jan Kok
Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, the Netherlands

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Summary

Relatively recently, a number of genes and operons have been identified in lactic acid bacteria that are inducible and respond to environmental factors. Some of these genes/operons had been isolated and analysed because of their importance in the fermentation industry and, consequently, their transcription was studied and found to be regulatable. Examples are the lactose operon, the operon for nisin production, and genes in the proteolytic pathway of Lactococcus lactis, as well as xylose metabolism in Lactobacillus pentosus. Some other operons were specifically targeted with the aim to compare their mode of regulation with known regulatory mechanisms in other well-studied bacteria. These studies, dealing with the biosynthesis of histidine, tryptophan, and of the branched chain amino acids in L. lactis, have given new insights in gene regulation and in the occurrence of auxotrophy in these bacteria. Also, nucleotide sequence analyses of a number of lactococcal bacteriophages was recently initiated to, among other things, specifically learn more about regulation of the phage life cycle. Yet another approach in the analysis of regulated genes is the ‘random’ selection of genetic elements that respond to environmental stimuli and the first of such sequences from lactic acid bacteria have been identified and characterized. The potential of these regulatory elements in fundamental research and practical (industrial) applications will be discussed.

Introduction

Bacteria continuously monitor the availability of essential nutrients (e.g. sources of carbon, nitrogen, phosphorus and sulphur, trace metals and certain ions) by measuring extracellular concentrations and (fluxes in) intracellular pools. They have evolved complex regulatory networks enabling quick responses to changes in their environment. Generally, the response involves modulation of gene expression, allowing the bacteria to adapt to a wide variety of stimuli.

Gene expression in lactic acid bacteria (LAB) has received much attention during the last decade. These studies have addressed transcription initiation and termination as well as translation initiation and codon usage. Details of this work can be found in a number of excellent reviews covering this field, and the literature references therein (de Vos & Simons, 1994; van de Guchte, 1992; Chopin, 1993; Mercenier et al., 1994). For a number of LAB species, notably L. lactis, this has led to the definition of the canonical vegetative promoter. The lactococcal promoter consensus conforms to that of the Escherichia coli and Bacillus subtilis vegetative promoters: TTGACA–17/18–TATAAT. A number of the L. lactis promoters have been used to construct gene expression vectors for LAB with which many homologous and heterologous proteins in LAB have been (over)expressed (de Vos & Simons, 1994).

Only relatively recently studies on the control of gene expression in LAB were initiated. This is a reflection of the fact that, with the expanding genetic ‘tool box’ for LAB, increasingly more genes and operons from LAB are being cloned and sequenced. As a next step, the in depth analysis of many of these genes and their products is now undertaken. Methods are available for the targeted (knock-out) mutation of genes on the chromosome or the introduction of transcriptional or translational fusions of a gene of interest with
reporter genes such as *E. coli lacZ* in one copy in the chromosome (Leenhouts & Venema, 1993). Both techniques are very useful for answering specific questions relating to regulation of gene expression. In a number of cases gene regulation has been directly studied at the mRNA level. In this way, several important industrial traits have been examined and the results of these studies will be detailed here.

Approaches which specifically address regulation of gene expression in LAB include the cloning from these organisms of genes that are known to be controlled in other organisms (e.g. amino acid biosynthesis operons). Transposons and integration plasmids carrying a promoterless *lacZ* reporter gene have been used to randomly target environmentally regulated or stress-induced chromosomal loci. Also, the effect of a variety of stress conditions on protein production in LAB is presently being investigated by one- and two-dimensional gelelectrophoresis (Kunji et al., 1993; Ayffray et al., 1992; Hartke et al., 1994, 1995). Several genes involved in one of the stress conditions, heat shock, have been cloned and sequenced (van Assel-donk et al.; 1993, Eaton et al., 1993; Kim & Batt, 1993).

The field of control of gene regulation in LAB is rapidly expanding as will be clear from the present evaluation. Only those cases which have been examined into appreciable detail at the DNA and mRNA levels will be discussed here, as well as some of the strategies employed to isolate regulated genes.

**Random selection of regulated promoters**

Several transposons with promoterless reporter genes have been developed for the analysis of expression of single-copy (chromosomal) genes in bacteria. For instance, derivatives of the *Enterococcus faecalis* transposon Tn917 carrying the *E. coli lacZ* gene devoid of its own promoter have been used in the analysis of control of gene expression in *B. subtilis* (Youngman, 1987). Variants of Tn917 were shown to also randomly transpose in *L. lactis* (Israelsen & Hansen, 1993). A collection of *L. lactis* strains with random fusions of *lacZ* to chromosomal loci was screened for β-galactosidase (*LacZ*) activity under standard growth conditions (Israelsen et al., 1995). A number of clones showed altered LacZ activities when growth conditions, such as temperature, medium pH and/or arginine content, were changed. One of the integrants appeared to produce β-Gal at pH 5.2 but not at pH 7.0, produced more of the enzyme at 15 °C than at 30 °C, while the highest activities were observed in the stationary phase. Chromosomal DNA (9.7 kb) upstream of the integrated *lacZ* gene was rescued from the integrant and introduced in a promoter-probe vector. On this plasmid the fragment displayed the same pattern of gene regulation as in the single-copy situation on the chromosome of the integrant. At present, no data are available as to the identity and the molecular details of regulation of this interesting promoter.

From the lactococcal plasmid vector pWV01 a series of non-replicative derivatives have been constructed that can be used for chromosomal integration in, among others, LAB (Leenhouts & Venema, 1993). One derivative, pORI13, carries a promoterless *lacZ* gene preceded by lactococcal translation signals and a multiple cloning site. Stop codons were introduced upstream of *lacZ* to allow for transcriptional fusions only (J.W. Sanders, pers. comm.). A library of random partial restriction fragments of *L. lactis* chromosomal DNA in this vector was made in *E. coli* and subsequently used to make a bank of integrants of *L. lactis*. Screening for LacZ activity in the presence or absence of 0.3 M NaCl gave colonies with all four possible phenotypes. One colony displaying LacZ activity only on plates with NaCl was taken for further analysis. The original insert in pORI13 in this clone was rescued and shown to comprise 10 kb of chromosomal DNA. The salt-inducible promoter was identified by deletion mapping, nucleotide sequence analysis and primer extension experiments. *lacZ* had been fused to the first 360 bp of an open reading frame X (ORFX) of which the deduced product shows homology with membrane proteins. The minimal region needed for salt-induced LacZ activity contained a gene for a regulator, *rggL*, followed by a 21-bp inverted repeat structure at position –35 of the actual promoter. No canonical –35 hexanucleotide could be identified, but a good –10 sequence was present, 7 bp downstream of which transcription started in the presence of salt. No primer extension product was observed when cells were grown in the absence of NaCl and only a very weak signal was obtained in Northern blots. In an *rggL* insertion mutant very little transcript was detected in Northern blots of mRNA from cells grown both in the presence or absence of NaCl, indicating that RggL acts as an activator in the NaCl-dependent expression of ORFX. The transcript was approximately 3 kb in size (J.W. Sanders, pers. comm.).
Screening for two-component regulatory systems

Systems used by bacterial cells to adapt to changes in their environment often involve two families of signal transduction proteins, namely sensory kinases and response regulators. The first component monitors an environmental parameter while the latter generally directs changes in gene expression (for review, see Stock et al., 1995). A complementation strategy was employed to randomly clone sensory kinases from *L. lactis* (M. O’Connell Motherway, G.F. Fitzgerald and D. van Sinderen, pers. comm.). An *E. coli* phoR creC double mutant lacks the two sensory kinases needed for expression of the phosphate regulon. Consequently, the strain is alkaline phosphatase negative (PhoA−), and grows to white colonies on LB plates containing the chromogenic PhoA substrate 5-bromo 4-chloro 3-indolyl phosphate. Chromosomal DNA fragments of *L. lactis* cloned in an *E. coli* replicon which were able to complement the PhoA− deficiency were identified in several blue *E. coli*(phoR creC) colonies. In this way, the (partial) genes of five different kinases were isolated and sequenced. In two of the clones the corresponding response regulators were present in the upstream DNA sequences. The next important step will be to determine, on the basis of protein homologies and by making mutants and analysing their phenotypes, in which signalling pathway the genes are involved (M. O’Connell Motherway, G.F. Fitzgerald & D. van Sinderen, pers. comm.).

T7 RNA polymerase overexpression system

Pending the identification of homologous highly inducible gene expression systems for LAB, the T7 RNA polymerase overexpression system has been developed for use in *L. lactis* (Studier et al., 1990; Wells et al., 1993). Use is made of the lactose-inducible lac promoter and lacR repressor gene (see below) for the controled expression of the *E. coli* bacteriophage T7 RNA polymerase in *L. lactis*. This is accomplished by shifting cells from a glucose- to a lactose-containing medium. Target genes cloned behind the phage T7 promoter of gene 10 are overexpressed to up to 22% of total soluble protein (Wells et al., 1993). In a further advancement of the system two inducible expression-secretion vectors have been constructed, one of which was used to secrete the model protein tetanus toxin fragment C (Wells et al., 1993). Presently, the system employs three plasmids to provide all the necessary functions.

Lactose metabolism

Lactose metabolism in LAB is either initiated by a lactose permease system or a phosphoenolpyruvate dependent lactose phosphotransferase system (lactose PEP-PTS) (McKay et al., 1970). Lactococci used in dairy industry transport lactose exclusively via the lactose PEP-PTS. In this system, the lactose-specific components are the membrane-associated enzyme EIIlac (LacE) and the soluble protein EIIIlac (LacF). Lactose is phosphorylated during uptake and the resulting lactose–6-phosphate is converted by phospho-β-galactosidase (LacG) to glucose, which is further taken down by the Embden-Meyerhof-Parnass route, and galactose–6-phosphate (McKay et al., 1970). Galactose–6-phosphate is metabolized via the tagatose–6-phosphate pathway by the consecutive action of galactose–6-phosphate isomerase (LacAB), tagatose–6-phosphate kinase (LacC), and tagatose 1,6-diphosphate aldolase (LacD) (Bissett & Anderson, 1974).

Although it was already known for quite some time that key enzymes in the breakdown of lactose by *L. lactis* could be induced by growth on lactose or galactose (Bisset & Anderson, 1974; Leblanc et al., 1979; McKay; Maeda & Gasson, 1989), it is only of recent date that the underlying mechanism is understood in considerable detail. The unraveling of the control of lactose utilization had to await the identification and isolation of the genes involved. In *L. lactis* ssp. *cremoris* strain NCD0712, the structural genes for the lactose-specific PTS enzymes (LacEF) as well as those for phospho-β-galactosidase and the enzymes of the tagatose–6-phosphate pathway (LacABCDFEGX) are organized in a 7.8-kb lac operon: lacABCDFEGX (de Vos & Gasson, 1989; de Vos et al., 1990; van Rooijen et al., 1991; see Figure 1). An oppositely oriented gene immediately upstream of lacA, lacR, specifies a repressor protein (van Rooijen & de Vos, 1990). Transcriptional analyses revealed that two lactose-inducible transcripts of 6 and 8.5 kb are formed, comprising the lacABCDFE and lacABCDFEGX genes, respectively (de Vos et al., 1990; van Rooijen & de Vos, 1990, van Rooijen et al., 1991). Both transcripts are initiated at a single lactose-inducible promoter upstream of lacA (van Rooijen et al., 1992). An inverted repeat between lacE and lacG could act as an intracistronic terminator for the 6-kb
transcript with partial readthrough explaining the presence of the 8.5-kb mRNA (de Vos & Gasson, 1989; de Vos et al., 1990). lacR is transcribed as a monocistronic mRNA of 1.2 kb in the presence of glucose, while synthesis of this mRNA is repressed 5-fold during growth on lactose. The intercistronic non-coding region between lac and lacR contains the promoters for both the lac operon and lacR. lac operon mRNA initiates at a G residue 94 bp upstream of the AUG start codon of lacA, both in the presence of glucose or lactose, although 5 to 10 times more of the transcripts was present in lactose-grown cells. The relevance of low levels of constitutive transcription remains to be elucidated (van Rooijen et al., 1992). The transcription start site (TSS) of lacR was located at an A residue 305 bases upstream of the AUG start codon both in the presence of lactose or glucose. Both promoters conform to the lactococcal promoter consensus. The activity of the lac promoter was enhanced 5- to 38-fold by immediate down- and upstream sequences. A possible stem-loop structure in the 5' non-coding region of the lac transcript was postulated to be involved in mRNA stability. The stimulating region upstream of lacTSS may either be involved in DNA bending or could be the target for a transcription activating protein (van Rooijen et al., 1992).

Transcriptional control of the lac operon is provided by LacR (van Rooijen & de Vos, 1990; van Rooijen et al., 1992). The protein specifically represses the lactose catabolic genes of L. lactis: when multiple copies of lacR were present in cells grown on lactose reduced growth rates were observed, whereas no effect was seen during growth on glucose. When the lac promoter was fused to the reporter gene cat–86 for chloramphenicol acetyl transferase (CAT), reduced CAT activities were measured when lacR was present both in cis and in trans. In a strain carrying a chromosomal copy of lacR and multiple copies of the lac promoter on a plasmid, derepression of the chromosomal lac operon was observed as a result of titration of LacR by the lac promoter. Conversely, super-repression and reduced LacG activities were seen during growth on glucose and lactose, respectively, in a strain carrying a single copy of the lac promoter on the chromosome and multiple copies of lacR. DNAse I footprinting and DNA homology searches identified two operators in the lac promoter region (van Rooijen & de Vos, 1990; see Figure 1). LacR protected the sequences from –31 to +6 (lac01), and –313 to –279 (lac02) relative to the lac operon TSS. A TGT TT motif present in both operators was postulated to constitute the LacR recognition sequence. In lac01, but not in lac02, the motif is part of an inverted repeat that shows homology with the deo01 operator (van Rooijen & de Vos, 1990, 1992). From gel mobility shift assays it was concluded that lac01 has a higher affinity for LacR than lac02. Both operators are needed to repress transcription initiation of lac during growth on glucose, indicating that LacR binds to both lac01 and lac02. The latter might be involved in the formation of a repression loop, such as has been proposed for the control of several E. coli operons (van Rooijen & de Vos, 1990; Matthews, 1992). The gel mobility shift assay was also used to identify the inducer of lac operon expression: of the phosphorylated intermediates formed during growth on lactose and galactose (both derepress lac operon expression [LeBlanc et al., 1979; Park & McKay, 1982]) only tagatose-6-phosphate inhibited formation of the LacR-operator complex.

A sequence 5 bp downstream of the lac01 operator shows strong homology to CRE, a catabolite-responsive element postulated to be involved in catabolite repression in Bacillus species (van Roojen, 1993; see below for a more detailed explanation). Whether this sequence is the binding site for a lactococcal equivalent of the catabolite control protein CcpA and whether lac expression is under catabolite control is currently under investigation. A gene for a CcpA-
like protein has recently been cloned using antibodies against *B. megaterium* CcpA (E.J. Luesink & O.P. Kuipers, pers. comm.).

LacR is a 29-kDa protein with a high overall homology to repressors of the DeoR family (van Rooijen & de Vos, 1990). The homology was most pronounced with the helix-turn-helix (HTH) region in DeoR, the repressor of the *E. coli* deoxyribose operon. This region in DeoR is thought to bind to the operator of the *deo* operon (Lehming et al., 1988). The HTH motif was mutagenized to identify amino acid residues in LacR involved in operator binding. The methionine and arginine residues at positions 34 and 38, respectively, were shown to be directly involved in DNA binding. These studies also confirmed what had been shown before in vitro, namely that LacR is most probably active as a dimer or multimer in vivo (van Rooijen & de Vos, 1993; van Rooijen, 1993). Another region of high homology is present between amino acid residues 212 and 222 of LacR. This region also showed homology with enzymes of pro- and eukaryotic organisms involved in sugar or nucleoside metabolism. The postulate that the latter region in LacR might, thus, be involved in the response to tagatose–6-phosphate was substantiated by site-directed mutagenesis experiments (van Rooijen & de Vos, 1990; van Rooijen et al., 1993). Replacement of the lysine residues at positions 72, 80, and 213, as well as the aspartic acid at position 210, by alanine led to repressed LacG activities during growth on lactose. The mutated LacR proteins did bind normally to the lac operators but they did not dissociate from the operators in the presence of tagatose–6-phosphate. Whether this behaviour of LacR has to be attributed to a decreased affinity for the inducer or to the inability to undergo a possibly essential conformational change upon inducer binding still has to be sorted out.

All of the above results were unified in a three-stage model for regulation of *lac* operon expression in *L. lactis* (van Rooijen, 1993):

First, binding of LacR repressor to operator *lacO1* during growth on glucose results in autoactivation of *lacR* expression. Although not proven directly, it is very likely that LacR and *lacO1* are both involved in the activation of transcription of *lacR*. Repression of *lac* operon expression probably does not occur at this stage in the process. Second, at increasing concentrations of LacR during growth on glucose, *lacO2* will be bound by repressor. Binding of LacR to both operators results in repression of transcription of the *lac* operon, which might involve the formation of a DNA loop between *lacO1* and *lacO2*. Activation of *lacR* expression by *lacO1* and repression of *lacR* expression by binding of LacR to *lacO2* leads to a certain steady state concentration of the repressor. The third step in the process is taken upon binding of the inducer tagatose–6-phosphate to LacR. When this happens, the LacR- *lacO1lacO2* complex dissociates resulting in the initiation of transcription of the *lac* operon.

**Xylose metabolism**

Pentoses are usually fermented by heterofermentative LAB. These sugars are internalized by specific permeases and converted to the central intermediate D-xylulose–5-phosphate which is subsequently fermented to lactate and acetate (Kandler, 1983). *Lactobacillus pentosus* is one of the very few *Lactobacillus* species able to use the pentose D-xylose as an energy source. Once intracellular, D-xylose is substrate for D-xylose isomerase and D-xylose kinase. It has been shown already a long time ago that D-xylose isomerase of *Lb. pentosus* is induced during growth in xylose-containing media (Mitsuhashi & Lampen, 1953). Lokman & coworkers (1991, 1994) have recently cloned and analyzed the genetic elements involved in xylose metabolism in *Lb. pentosus* MD353. Five genes in the order *xylPQRAB* were identified (see Figure 2). The putative function of the *xylP* and *xylQ* genes will be discussed below. *xylR*, *xylA* and *xylB* encode a repressor protein, D-xylose isomerase, and D-xylose kinase, respectively.

Northern blot analyses revealed that two xylose-inducible transcripts are produced from *xylA* (Lokman...
et al., 1994; see Figure 2). The major transcript of 1.5 kb only encompasses \( \text{xylA} \), while a minor mRNA of 3 kb is produced from \( \text{xylA} \) and \( \text{xylB} \). Neither transcript is found when cells are grown on glucose. Ribose and arabinose also repress transcription of \( \text{xylAB} \), which is indicative of the operation of a general catabolite repression mechanism. \( \text{xylR} \) is transcribed as a 1.2-kb mRNA both in the presence of glucose and xylose. In addition, a transcript of more than 7 kb encompassing \( \text{xylR} \), \( \text{xylQ} \) and \( \text{xylP} \) is synthesized during growth with xylose, but not with glucose. At least ten-fold more \( \text{xylR} \) mRNA, nearly all part of this polycistronic messenger, is present under inducing conditions. The long mRNA is initiated at a xylose-inducible promoter 140 bp upstream of \( \text{xylP} \) (S. Chaillou & P. Pouwels, pers. comm.). Xylose-specific transcripts of 5 and 2.4 kb also reacted with \( \text{xylP} \)- and \( \text{xylQ} \)-specific probes.

The promoters and TSSs of \( \text{xylR} \) and \( \text{xylAB} \) have been determined using mRNA isolated from xylose-grown cells (Lokman et al., 1994). The \( \text{xylAB} \) TSS is located 42 bp upstream of the AUG start codon of \( \text{xylA} \), while that for \( \text{xylR} \) is 83 bp away from the GUG start codon. Fusions of both promoters with the \( \text{cat} \)-86 reporter gene were made and CAT activities were measured during growth of cells with xylose or glucose. The \( \text{xylAB} \) promoter was induced 60- to 80-fold in the presence of xylose. It was repressed 15 to 25 times when glucose was added to the xylose-containing medium. \( \text{cat} \)-86 gene expression was independent of the sugar source (glucose and/or xylose) when it was controlled by the \( \text{xylR} \) promoter. Expression from the \( \text{xylR} \) promoter was 10-fold less efficient than from the \( \text{xylAB} \) promoter. No promoter activity was detected with a DNA fragment encompassing the \( \text{xylA} \)-\( \text{xylB} \) intergenic region, which is in agreement with the fact that no mRNA was found to be initiated in this region.

The 43-kDa XylR repressor of \( \text{Lb. pentosus} \) is similar to \( \text{B. subtilis} \) XylR, a repressor involved in regulation of D-xylose gene expression (Lokman et al., 1991). In the N-terminus of \( \text{Lb. pentosus} \) XylR, from amino acid residues 30 to 50, an HTH motif was identified. Deletion of \( \text{xylR} \) results in constitutive expression of \( \text{xylAB} \), indicating that XylR is a repressor (C. Lokman & P. Pouwels, pers. comm.). A region immediately upstream of the \( \text{xylA} \) Shine-Dalgarno sequence shows (limited) homology with the \( \text{B. subtilis} \) \( \text{yl} \) operator, and may bind XylR. The \( \text{xylR} \) mutant showed an increased lag-phase in xylose-containing medium, suggesting that under these conditions XylR is an activator.

A second region in the \( \text{xylRA} \) intergenic region, located 68 bp upstream of the \( \text{xylA} \) start codon and overlapping the -35 sequence of the \( \text{xylAB} \) promoter (element II, Lokman et al., 1994), shows homology with CRE, the consensus sequence involved in catabolite (glucose) repression (Weickert & Chambliss, 1990; for reviews, see Fisher & Sohnschein, 1991; Hueck & Hillen, 1995). When the \( \text{xylRA} \) intergenic region was introduced in \( \text{Lb. pentosus} \) on a plasmid, 5 times more \( \text{xylA} \) transcript was found in the presence of xylose as compared to the wild-type strain. This is suggestive of the titration of a \textit{trans}-acting negative factor. It also suggests that in the wild-type strain, even under inducing conditions, a repression factor is bound to specific sequences in the \( \text{xylRA} \) intergenic region. Catabolite repression in various species of Gram-positive bacteria is affected by the \textit{trans}-acting factors CcpA (the catabolite control protein) and Hpr (the heat-stable protein involved in PEP-PTS phosphate transfer). CcpA is a member of the GalR/LacI family of transcriptional regulators and, probably, specifically interacts with CRE (Hueck & Hillen, 1995). Using a PCR strategy, the gene specifying \( \text{Lb. pentosus} \) CcpA was isolated and sequenced. In an \( \text{Lb. pentosus} \) ccpA disruption mutant, glucose repression was almost completely eliminated (C. Lokman & P. Pouwels, pers. comm.), supporting the idea of a globally active mechanism of catabolite repression in Gram-positive bacteria (Hueck & Hillen, 1995).

As to the function of XylP and XylQ, the following has recently been observed (S. Chaillou & P. Pouwels, pers. comm.). Mutants with disrupted \( \text{xylQ} \) or \( \text{xylPQ} \) genes grow, despite a longer lag-phase, better than a wild-type strain in the presence of xylose. Whereas \( \text{xylAB} \) and \( \text{xylPQ} \) mRNA levels are low during lag-phase in the wild-type strain, and strongly increased once exponential growth commences, both disruption mutants showed higher expression levels during the lag-phase and sub-maximal transcriptional activity during exponential growth. Also, D-xylose uptake was shown to be increased in the \( \text{xylPQ} \) mutant. XylQ is thought to be required for high-level expression of the \( \text{xyl} \) genes. Although the protein shows homology with membrane transport proteins, XylP is not considered to be the actual xylose permease but, together with XylQ, is thought to be involved in regulation of xylose transport. In support of this hypothesis is the fact that XylQ shows similarities to domains conserved in two-component regulator proteins and, more specifically, with class III transcriptional activators (S. Chaillou & P. Pouwels, pers. comm.).
Nisin production

Nisin is a ribosomally synthesized antimicrobial peptide which undergoes extensive posttranslational modification leading, ultimately, to the mature, extracellular, membrane-active bacteriocin used so widely in the food industry. A cluster of eleven genes in L. lactis, nisABTCIPRKFEG, is involved in all aspects of biosynthesis of this lantibiotic (see Figure 3). nisA is the structural gene, nisBC are postulated to be involved in the modification reactions, nisT and nisP translocate and cleave precursor nisin, respectively, while nisl and nisFEG have been implicated, by different mechanisms, in immunity towards nisin. Regulation of nisin biosynthesis is provided by the proteins encoded by nisR and nisK. These proteins belong to the class of two-component regulatory systems in which, in this case, NisR is the response regulator and NisK the signal sensor or sensor histidine kinase (for excellent reviews, see Schnell et al., 1988; Jung 1991; Sahl et al., 1995; de Vos et al., 1995).

Three promoters, upstream of nisA, nisR and nisF, have been mapped by primer extension (Kuipers et al., 1993; Kuipers et al., 1995; de Ruyter et al., 1996; P. Saris, pers. comm.). From the nisA gene a 260-bp transcript is produced which is initiated at a G residue 42 bp upstream of the AUG start codon (Kuipers et al., 1993). At a proper distance from this TSS a canonical L. lactis promoter sequence was identified. Interestingly, nisA transcription was completely abolished when a 4-bp internal deletion was made in the chromosomally located nisA gene. When a plasmid carrying the intact nisA gene was introduced in the ΔnisA strain, a transcript of ΔnisA was observed. Apparently, nisin or one of its precursors is required for transcription of its own gene. In a follow-up study (Kuipers et al., 1995), this phenomenon was further analyzed. Northern blotting using mRNA from the ΔnisA strain revealed that, indeed, ΔnisA transcription was restored upon addition of nisin to the culture medium. The amount of transcript was proportional to the amount of nisin added. Nisin Z, a natural His27Asn variant of nisin A (Mulders et al., 1991), and several of its mutants were also able to induce transcription. Transcriptional activation varied several 100-fold depending on the actual mutation, with the Dhb2Dha and Met17Trp mutants of nisin Z being more potent inducers than nisin Z itself. Related peptides like the lantibiotics subtilin, lactacin 481, and Pep5, as well as the unmodified synthetic precursor of nisin A did not induce transcription. By fusing a nisA promoter fragment to the promoterless E. coli reporter gene gusA for β-glucuronidase, induction capacities could be quantitated and it was established that less than 5 molecules of the best inducer (nisin Z mutant Dhb2Dha) are sufficient to activate ΔnisA transcription. Induction capacity and antimicrobial potency are two different, independent characteristics of the nisin molecule. Synthetic nisin A fragments were used to show that induction capacity resided in the first 11 residues, comprising the first two ring structures, of nisin A. Moreover, a hampered biosynthesis (modification) of nisin interferes with nisA transcription: an in-frame deletion of nisB, one of the putative modification genes, completely abolished nisA transcription, which could be restored by the extracellular addition of nisin (Kuipers et al., 1996).

Inactivation of the chromosomal copy of nisK led to the inability of any of the nisin variants added externally to induce transcription of ΔnisA (Kuipers et al., 1995). Also, introduction in the nisK deletion strain of a plasmid with an intact nisin structural gene did not lead to nisin production, whereas such a plasmid did restore bacteriocin production in the ΔnisA strain (which carries an intact copy of nisK). These results indicate that NisK is an essential component in the signal transduction pathway and, most probably, directly interacts with the nisin molecule. Deletion studies have shown that nisR is also essential for the production of nisin (van der Meer et al., 1993). One of the strains used in this study carried the nisABTCIR genes on a multicopy plasmid and was shown to secrete fully modified precursor nisin. This result indicates, among other things, that overexpression of nisR alone is enough for activation of transcription of nisA and the downstream genes, the latter through partial read-through of an inverted repeat in the nisAB intergenic region (see Fig-
Figure 3). When a DNA fragment carrying the DNA region from the nisA promoter down to the 5'-part of nisB (including ΔnisA) was fused to gusA, β-glucuronidase activity was only detected after induction with nisin. The level of activity, however, was 50-fold lower than that observed when gusA was fused directly to the nisA promoter. Deletion of the nisA promoter completely abolished GusA activity, both in the presence and absence of nisin, showing that nisB and probably also nisTCIP expression depend on read-through from the nisA promoter and are under nisin control. When the nisA promoter fragment was present in multicopies next to a single chromosomal copy of the nis operon, 50-fold less nisin was produced and immunity was severely reduced. Probably, NisR is titrated by a putative NisR binding site in the nisA promoter region (Kuipers et al., 1995). The nisRK genes have their own promoter. The TSS was localized to an A residue 26 nucleotides upstream of the start codon of nisR. nisRK are sufficient for signal transduction: integration of only these two genes into the chromosome of L. lactis results in a nisin-inducible strain.

Recently, it has been shown that a promoter upstream of nisF shows homology to the nisA promoter sequence. This suggests that both promoters are under the same mechanism of control. Indeed, externally added nisin increases nisin immunity (O. Kuipers et al., 1995; de Ruyter et al., 1996; P. Saris, pers. comm.).

Proteolysis

Proteolysis by lactic acid bacteria has received much attention through the years. After the identification and characterization of some 20 genes involved in this trait, the next challenge in this field is the elucidation of the interrelationship, if any, between all these genes. In a number of cases, genes for putative regulator proteins have been identified in the vicinity of genes involved in casein utilization. Immediately downstream of the gene for the general aminopeptidase PepC of L. lactis MG1363 two genes are present, the products of which show similarity to LysR- and MerR-type regulators (I. Mierau, pers. comm.). Upstream of the opp operon encoding the oligopeptide transport system of the same strain a possible regulator gene was identified encoding a protein of the FNR/CRP family of regulators (A. de Jong, S. Tynkkynen, & J. Kok, unpublished). In the immediate vicinity of the Lb. delbrueckii sspp. lactis peptidase genes pepI and pepQ two genes for putative regulators were identified (J.R. Klein and B. Henrich, pers. comm.). In all of these cases, actual involvement of the genes in regulation has still to be proven.

Some strains of L. lactis produce more proteinase (PrtP) in a milk-based medium than in rich broth, while strain-specific differences in proteinase production have also been observed (Hugenholtz et al., 1984; Exterkate, 1985; Bruinenberg et al., 1992). In these studies, regulation was examined at the enzyme level with the pitfall that active enzyme production could be influenced by unrelated factors in proteinase secretion, processing and/or stability. Indeed, growth rate-dependent autoproteolysis of PrtP has recently been observed, with a decrease in specific growth rate leading to a sharp increase in PrtP breakdown (Meijer et al., 1996). Marugg et al. (1995) have made transcriptional fusions of the prtP and prtM promoters with the gusA gene in order to study transcriptional control of proteinase gene expression. The prtP and prtM genes are divergently orientated and their promoters are partially overlapping in a face-to-face fashion (see Figure 4). Both promoters were shown to be regulated by the peptide content of the medium. GusA activities under control of either promoter decreased approximately 10-fold with increasing peptide concentrations. Minimal expression was seen in a rich broth medium. These results were matched by the outcome of quantitative primer extension analyses: highest prtP- and prtM-specific mRNA levels were observed in media containing low amounts of peptides, with an approximately 8-fold decrease upon growth in a medium with a high peptide concentration (J. Marugg, pers. comm.). These data show that medium-dependent expression of the prtP and prtM promoters is controlled at the level of transcription initiation.

Peptide-dependent regulation of prt was further examined by adding specific (di- and tri)peptides to the growth medium of a strain carrying the prtP-gusA fusion (Marugg et al., 1995). Of 11 peptides tested only leucylproline and prolylleucine negatively affected GusA activity. Repression was a very rapid and transient process: addition of prolylleucine to a steady state chemostat culture led to an immediate halt in GusA production which resumed after a number of hours. Like a wild-type strain, a strain of L. lactis in which the di-/tripeptide transporter DtpT was mutated produced high levels of GusA from the prtP-gusA fusion under low-peptide conditions. However, GusA levels were not as low as in the wild-type under high-peptide conditions. This effect was even more extreme in a dtpT alaT double mutant in which GusA levels
Figure 4. Organization of the \( \text{prtP} \) and \( \text{prtM} \) genes of \( L. \) lactis. Right and left turn arrows represent the \( \text{prtP} \) and \( \text{prtM} \) promoters, \( P_P \) and \( P_M \), respectively. The \(-10\) and \(-35\) sequences of both promoters, as well as their TSSs (asterisks), are indicated in the nucleotide sequence at the top. The major TSSs are shown by the large asterisks. A region of dyad symmetry overlapping the transcription initiation sites of both promoters is also indicated.

were approximately four- to five- fold derepressed in a rich broth medium. A mutant in Opp, the oligopeptide transport system, behaved like the wild-type under all growth conditions tested. Apparently, uptake of small (di/tri)peptides, and possibly amino acids, plays an important role in the control of \( \text{prtP} \) promoter activity.

Deletion and mutation analyses of the \( \text{prt} \) promoter region revealed that a sequence of 90 bp containing both \( \text{prt} \) promoters is sufficient for their full expression and regulation (J. Marugg, pers. comm.). A region of dyad symmetry positioned around the transcription initiation sites of the two promoters is present in all \( \text{prtPM} \) determinants sequenced so far and was shown to be involved in \( \text{prt} \) regulation. Removal of half of the dyad repeat resulted in a nearly constitutive expression from the \( \text{prtP} \) promoter. Insertion of small linkers disrupting the palindrome led to increased mRNA levels and derepressed GusA activities at high peptide concentrations, while expression of both promoters at low peptide concentrations was hardly affected.

The following model was proposed to accommodate all the above results (Marugg et al., 1995; Meijer et al., 1996). A repressor protein is postulated to bind to the \( \text{cis} \) sequence of dyad symmetry in the \( \text{prt} \) promoter region (operator). Interaction of effector molecule(s) with this putative repressor would increase its affinity for the operator, leading to repression of transcription (J. Marugg, pers. comm.). Specific dipeptides (such as prolylleucine) or their derivatives may constitute the effector molecules. Because of the high intracellular peptidase levels in \( L. \) lactis, the possibility that the cell senses a temporary increase in intracellular peptide concentration is favoured.

Meijer et al. (1996) studied the activities of PrtP and two intracellular peptidases, the X-prolyl-dipeptidyl aminopeptidase PepXP and the general aminopeptidase PepN, under various growth conditions and in two different strains of \( L. \) lactis. In both strains, specific PrtP activities were highest at the end of the exponential phase of cells growing in milk. In a milk-based medium, specific PrtP activities decreased when the peptide content was increased. In contrast, a difference in the activity levels of PepXP and PepN was observed between the two strains. While PepXP and PepN activity levels showed a medium dependency similar to that of PrtP in strain MG1363, PepN seemed to be hardly affected and PepXP only slightly so in strain SK1128. PepN and PepXP activity levels in MG1363 were repressed by the addition of 0.5 mM of the dipeptide prolylleucine. In glucose- limited continuous culture experiments, addition of peptides resulted in an immediate decline in the level of PepN activity in MG1363 and, to a much lesser extent, of that of PepXP. Prolylleucine had a similar repressive effect on PepN, whereas PepXP seemed to be insensitive to the addition of the dipeptide.

**Amino acid biosynthesis**

Amino acid biosynthesis has been studied in many organisms and these studies have revealed a wealth of information regarding gene organization and gene regulation. Recently, the operons for three amino acid biosynthetic pathways from \( L. \) lactis have been cloned, sequenced, and analyzed (Bardowski et al., 1992; Delorme et al., 1992, 1993; Godon et al., 1992, 1993). Tryptophan biosynthesis has been studied in a dairy strain, while the histidine and branched chain amino acid pathways were elucidated in a prototrophic non-dairy strain of \( L. \) lactis. These studies have given insight in why dairy lactococcal strains are more fastidious than \( L. \) lactis strains isolated from plant material (Godon et al., 1993; Delorme et al., 1993), and have
The tryptophan biosynthesis operon of \textit{L. lactis}. Transcription terminators T1 and T2 are indicated as well as the mRNAs produced in media with or without Trp. An ORF of unknown function (1) precedes the operon while it is followed by \textit{bglR}, encoding a regulator involved in $\beta$-glucoside utilization (see text). Adapted from Chopin (1993).

Figure 5. The tryptophanes biosynthesis operon of \textit{L. lactis}. Transcription terminators T1 and T2 are indicated as well as the mRNAs produced in media with or without Trp. An ORF of unknown function (1) precedes the operon while it is followed by \textit{bglR}, encoding a regulator involved in $\beta$-glucoside utilization (see text). Adapted from Chopin (1993).

also provided data on the regulation of these operons, as will be discussed now.

The tryptophan biosynthesis operon of \textit{L. lactis} IL403 contains seven structural genes in the order \textit{trpEGDCFBA} (Bardowski et al., 1992; see Figure 5). All genes are preceded by proper ribosome binding sites, while a consensus vegetative promoter sequence is located upstream of \textit{trpE}. The \textit{trp} mRNA contains a 439-bp non-coding leader region. Three transcripts of 8000, 290, and 160 bases are initiated from the \textit{trp} promoter. The 8-kb transcript encompasses the entire \textit{trp} operon. The transcripts are produced by termination at a specific terminator (T2 for the long mRNA, T1 for the two short products, see Figure 5) and by site-specific endoribonucleolytic activity for the 160-b transcript. Several factors affect production of these transcripts: (1) the 8-kb transcript is only produced in the absence of tryptophan; (2) energy limitation of the cells prevents the production of the 8-kb and the 290-b mRNAs, even in the absence of Trp; (3) transfer of cells to fresh medium strongly stimulates transcription initiation at the \textit{trp} promoter (A. Chopin & R.R. Raya, pers. comm.).

The lactococcal \textit{trp} leader sequence displays sequence and predicted secondary structure conservation with leader regions of aminoacyl-tRNA synthetase genes and some amino acid biosynthesis operons in a number of Gram-positive bacteria. Upon starvation of bacterial cells for a certain amino acid, the cognate tRNA synthetase is generally induced. This response is specific: induction is only seen after limitation of the corresponding amino acid and not after general amino acid starvation (Nass & Neidhardt, 1967). A model has recently been proposed for the regulation of tRNA synthetase genes in \textit{B. subtilis} (Grundy & Henkin, 1993, 1994). In this model, an antiterminator structure in the tRNA synthetase leader sequence involving a conserved 14-bp 'T-box' sequence is thought to be stabilized by direct interaction with the corresponding non-charged tRNA, resulting in transcription of the synthetase gene. Interaction is through the so-called 'specifier codon', a sequence present in a conserved part of the leader which determines the specificity of the response to amino acid limitation, and through a sequence that is complementary to sequences in the acceptor arm of tRNA. Several aspects of this model can be applied to lactococcal \textit{trp} operon control but it does not fully explain all intricacies of \textit{trp} operon expression (M. van de Guchte & A. Chopin, pers. comm.). A transcription terminator and possible antiterminator can be identified in the \textit{trp} leader. Limited homology to TRAP (formerly MtrB protein) binding sequences of \textit{B. subtilis} is also present, but it is not located in the antiterminator structure. The 8-kb \textit{trp} mRNA is induced 50-fold under Trp limitation. This was shown to be due to transcription attenuation. Spontaneous cis-mutations resulting in constitutive \textit{trp} operon expression were almost exclusively located in the terminator structure upstream of the structural genes (H. Frenkiel, pers. comm.). tRNA^{trp} appears to play a key role in the sensing of Trp levels in the cell and, under Trp limitation, the uncharged form of tRNA^{trp} is thought to bind to the non-translated leader transcript. This binding would stabilize the antiterminator structure, allowing transcription to proceed past T1 and over the entire length of the \textit{trp} operon. Two elements are important in the mRNA-tRNA interaction, namely the specifier codon and a sequence in the antiterminator that shows complementarity with the acceptor arm of tRNA^{trp}. The specifier codon largely determines the efficiency and specificity of the response. Whereas the codon-anticodon interaction is not strictly indispensable, that involving the tRNA acceptor arm seems to be essential. In addition to these RNA interactions, results have been obtained that suggest the involvement of additional factor(s) (M. van de Guchte, pers. comm.).

The genes for the biosynthesis of the branched chain amino acids leucine, isoleucine, and valine (BCAA) of \textit{L. lactis} NCDO2118 form a large cluster of two units (\textit{leu} and \textit{ilv} separated by 121 bp) with the gene order \textit{leuABCD-ilvDBNCA} (Godon et al., 1992; see Figure 6). The entire cluster is needed for the biosynthesis of leucine while the \textit{ilv} genes are required for the synthesis of isoleucine and valine. An \textit{ilvE} homologue is absent from the operon indicating...
that the last step in BCAA synthesis (a transamination) is performed by a nonspecific transaminase or is specified elsewhere on the chromosome. Between leuD and ilvD an ORF is present the product of which shows homology to ATP-binding-cassette (ABC) proteins. The role, if any, of this putative protein in BCAA biosynthesis is unknown. The last gene of the cluster is followed by aldB, encoding \( \alpha \)-acetolactate decarboxylase and an ORF of unknown function (ORF12).

Transcription analyses and luxAB fusion studies have revealed the presence of three functional promoters and two transcription terminators, one between P1 and leuA and one downstream of ORF12 (Godon et al., 1993; Renault et al., 1995; P. Renault, pers. comm.; see Figure 6). Promoters P1 (upstream of the entire cluster) and P2 (upstream of ilvD) initiate transcription only in the absence of isoleucine, resulting in 14.5- and 7.7-kb transcripts, respectively. Both transcripts terminate downstream of ORF12. Isoleucine represses both promoters approximately 10- to 20-fold and constitutive mutants are presently analysed to identify the putative repressor protein (P. Renault, pers. comm.). Leucine negatively influences transcription from P1. A transcript initiated at P1 could fold in either of two ways. One of these leads to the formation of a rho-independent transcription terminator upstream of leuA whereas the other does not. This configuration strongly suggest that the cluster is controlled by transcriptional attenuation (Kolter & Yanofski, 1982). Indeed, a small ORF, leuL, present in the leader of the leu-ilv transcript specifies a leader peptide of 16 amino acids with three consecutive leucines followed by an isoleucine (Godon et al., 1992). The model for regulation of biosynthesis of BCAA in *L. lactis* is the classical one (Yanofski, 1987): limited BCAA results in limited availability of the cognate charged tRNA. The consequent reduction in translation of the corresponding codons in the coding region for the LeuL leader peptide will cause ribosome stalling on the mRNA. Due to the position of the LeuL coding region, ribosome stalling will prevent the formation of the transcription terminator and the leu-ilv operon will be transcribed. In the presence of excess BCAA rapid translation of leuL mRNA will allow the terminator structure to be formed and to stop transcription (N. Goupil & P. Renault, pers. comm.).

The position of aldB immediately downstream of the leu-ilv cluster and the fact that it is cotranscribed with these genes (Godon et al., 1993) suggests a role of \( \alpha \)-acetolactate decarboxylase (AldB) in BCAA biosynthesis. Indeed, the enzyme converts the leucine- and valine precursor acetolactate to acetoin and, thus, influences the flux of acetolactate. AldB enzyme activity is subject to allosteric activation by leucine (Phalip et al., 1994). This observation could explain why *L. lactis* cannot grow in a leucine-rich medium without valine: acetolactate degradation by AldB leads to valine starvation. aldB is transcribed from P3 in the presence of BCAA but transcription is shut off when all three BCAA are lacking. The isoleucine-repressible transcripts initiated at P1 and P2 also cover aldB. aldB translation is negatively influenced by a strong stem-and-loop structure encompassing the ribosome binding site of aldB, sequences immediately upstream of this structure, and translation of the upstream ilvA gene. A 500-fold increase in aldB mRNA translation (as measured with a translational fusion to luxAB) was observed when the upstream region was deleted.
The histidine operon of *L. lactis* NCDO2118 has the gene order *hisCGDBHAFIE*. Interestingly, four additional ORFs are present in what seems to be one transcriptional unit of 10 kb (C. Delorme et al., 1992, 1993; see Figure 7). The function of ORFs 7 and 9 is unknown. The ORF8 product is possibly involved in dephosphorylation of histidinol phosphate, a function that seems to be missing from the lactococcal *hisB* product. The role of ORF6 will be detailed now.

The first gene in the operon, *hisC*, is preceded by a canonical lactococcal promoter. In the absence of His, two transcripts of 10 kb and 250 b are initiated from this promoter (Delorme et al., 1993; Renault et al., 1995). The long mRNA covers the entire *his* operon, while the short one stops at the rho-independent terminator T1 immediately upstream of *hisC* (see Figure 7). His virtually completely abolished synthesis of the 10-kb transcript. Like the trp operon leader sequence, the *his* leader does not specify a leader peptide but contains a consensus T-box immediately upstream of T1. A histidine codon at the proper position and all structural features proposed to be involved in T-box-mediated attenuation are present (Renault et al., 1995). The role of the putative ORF6 product in regulation of *his* operon expression has recently been examined (Renault et al., 1995). This work was initiated following the observation that the ORF6 protein shows homology with *E. coli* histidinyl-tRNA synthetase, which catalyzes aminoacylation of tRNA\textsubscript{his} (Delorme et al., 1992). The ORF6 product is about 150 amino acids shorter, lacking one of the conserved motifs required for the activity of class II type tRNA synthetases and will probably not charge tRNA. ORF6 can be disrupted without affecting cell viability. Cells in which the ORF6 protein is overexpressed grow normally in rich broth and in a chemically defined medium with His. They grow slowly in the absence of His and the activity of the *hisD* product, histidinol dehydrogenase, is reduced 4- to 5-fold under these conditions. Overexpression of the ORF6 product decreases the amount of the 10-kb transcript to a level that is below detection even in the absence of His. All these results suggest that the ORF6 product inhibits *his* operon expression by increasing termination at T1. Alternatively, it may inhibit the tRNA\textsubscript{his}-dependent antitermination at this terminator (Renault et al., 1995). As described above for trp regulation, T-box regulation involves the stabilization of an antiterminator structure by uncharged tRNA (increased due to the low level of the cognate synthetase or amino acids). The simplest model entertained at the moment for *L. lactis* *his* operon regulation assumes binding of the ORF6 protein to uncharged tRNA\textsubscript{his} (increased due to a low His level). This interaction would prevent association of the tRNA with the T-box structure, allowing terminatìr to be fired and to prevent *his* expression. The level of attenuation control is approximately 10-fold. An additional level of control (approximately 20-fold) is exerted by a repressor of the *his* promoter (C. Delorme & P. Renault, pers. comm.).

**β-Glucoside utilization**

Next to ‘classical’ attenuation of the *leu-ilv* operon by ribosome stalling during translation of a leader peptide, and antitermination promoted by uncharged tRNAs (*trp* and *his* operons), a third mechanism controlling transcript elongation exists in *L. lactis*. A gene immediately downstream of the *trp* operon, *bgIR* (see Figure 5) encodes a functional regulator of the BglG family (Bardowski et al., 1994). Proteins in this family positively control utilization of different sugars. Transcription antitermination of *E. coli* BglG synthesis is
exerted by binding of the protein to a conserved RNA sequence partially overlapping the transcription terminator (Houman et al., 1990). This configuration, a transcription terminator and a 5' overlapping sequence with high similarity to the RNA binding site of these systems was present upstream of bglR (Bardowski et al., 1994). bglR is positively autoregulated: in a strain carrying a bglR::lacZ fusion on the chromosome, constitutive overexpression of bglR from a plasmid resulted in 2- to 3-fold increased LacZ levels in the presence of β-glucoside sugars. L. lactis BglR can functionally replace E. coli BglG. Expression of a bglR::lacZ fusion was increased by β-glucosides, while bglR disruption mutants are impaired in growth on some β-glucosides. All of these data indicate that BglR is an RNA-binding antiterminator protein (Bardowski et al., 1994).

**Bacteriophages**

Bacteriophages attacking lactic acid bacteria represent a potential pool of regulatory mechanisms. Recently, the molecular analysis of a number of these phages has been undertaken and nucleotide sequencing has revealed several genes specifying putative regulatory proteins. One of these, bpi, was identified in the genome of the temperate lactococcal bacteriophage BK5-T. The product of this gene has been shown to inhibit the activity of a number of BK5-T promoters (Lakshmidevi et al., 1990). The mechanism by which Bpi works is unknown. Another putative repressor encoded by BK5-T is the product of ORF297 (Boyce et al., 1995). A putative repressor has also been described for phage Tuc2009 (van de Guchte et al., 1994). The only phage-encoded protein that has been shown to function as a repressor of gene expression is Pro (repressor of rlt) of the L. lactis temperate bacteriophage rlt (Nauta et al., 1996).

The regulatory region of bacteriophage rlt consists of two divergently oriented genes, rro and tec (van Sinderen et al., 1996; Nauta et al., 1996; see Figure 8). The rro gene is driven by promoter P1, while P2 drives tec and downstream genes. The TSSs of both consensus lactococcal promoters have been determined. The intergenic region between rro and tec contains two almost perfectly matching 21-bp direct repeats with internal dyad symmetry. These repeats, O2 and O3, overlap with the -35 sequences of P2 and P1, respectively, and may function as binding sites (operators) for Rro. A third putative operator (O1) is located in the coding region of tec. Alignment of the operator sequences led to the identification of an 11-bp consensus half-site with 7 invariable nucleotides. Rro was overproduced in E. coli and shown to specifically bind to a 21-bp synthetic double-stranded O1 site in gel mobility shift assays (Nauta et al., 1996). It was unable to bind to a mutated copy of O1 in which one of the invariable residues had been changed. Rro binding to synthetic 21-bp O2 and O3 fragments is less efficient (A. Nauta, pers. comm.).

Rro displays similarity with the CI and C2 repressors of the E. coli bacteriophages 434 and P22, especially with the regions involved in RecA-mediated autocleavage (Sauer et al., 1982; Little, 1993). Significant homology was also observed with regulator proteins involved in SOS induction. An HTH motif was identified in the extreme N-terminus of Rro by computer analysis. The C-terminal part of Rro is almost identical to the C-termini of the putative repressors CI and the product of ORF297 of the L. lactis bacteriophages Tuc2009 and BK5-T (van de Guchte et al., 1994; Boyce et al., 1995). Homology is restricted to the C-terminal halves of the proteins and does not include the HTH region, which is thought to be involved in target DNA recognition. The putative tec gene product contains 80 amino acid residues, may contain a HTH region, and is speculated to be the analogue of the E. coli Cro protein, hence its name topological equivalent of Cro (Nauta et al., 1996). The possible Cro-like proteins specified by ORF63 and ORF5 of BK5-T and Tuc2009 are completely different from Tec (Boyce et al., 1995; van de Guchte et al., 1994; Nauta et al., 1996).

By making a transcriptional fusion of E. coli lacZ with the ORF immediately downstream of tec it was shown that conditions that normally cause bacteriophage rlt to enter the lytic cycle (e.g. DNA damage...
caused by mitomycin C) induce expression of lacZ. Within three hours after mitomycin C addition to the culture carrying the plasmid in question, LacZ activity had increased 70-fold. When rro was inactivated by a frameshift mutation, lacZ was expressed constitutively at a high level, suggesting that Rro represses promoter P2 (Nauta et al., 1996).

In conclusion, it seems that the strategy of developmental control by phage rlt is similar to that used by the lambdoid phages of E. coli. Rro most probably binds to the operators and shuts off expression of the lytic genes that are all located downstream of and in the same orientation as tec. In fact, this involves 47 of the 50 ORFs recognized in the rlt genome sequence. The genes likely to be required for the establishment and maintenance of lysogeny, namely rro and the integrase gene, and an ORF of unknown function, are located together on the opposite DNA strand (van Sinderen et al., 1996). Upon induction, tec is probably the first gene to be transcribed. Although DNA binding studies have not yet been performed with Tec, it is anticipated that the protein binds to the operators. Tec would thus prevent transcription of the lysogenic genes and direct the phage into the lytic cycle (Nauta et al., 1996).

Conclusions

Regulation of gene expression in lactic acid bacteria has received much attention over the last few years. As described here, this has resulted in a wealth of new information regarding several important traits of LAB. Both from a fundamental point of view as well as from a more applied angle, studies regarding gene expression and the control mechanisms involved are highly important. From the fundamental viewpoint, in addition to all that has been described already for the two prototype bacteria E. coli and B. subtilis, we can still learn and discover new principles in gene regulation. Studies on the genetics of amino acid biosynthesis, for instance, have uncovered that gene regulation mechanisms in L. lactis can markedly differ from those present in the other bacteria. The E. coli, B. subtilis and L. lactis trp operons appear to be regulated by three different mechanisms of termination and antitermination, namely translation of an RNA leader sequence, binding of a regulator protein to a non-translated leader, and binding of an uncharged tRNA to a non-translated leader, respectively. Also, the autoregulation of nisin is the first report of a peptide that induces transcription of its own structural gene via signal transduction. As for the industrial aspects of this work, a better understanding of the regulatory constraints of metabolic pathways would ultimately allow to willingly steer the routes and, thus, the actual fermentation processes in which the organism under study is employed. One example of such intervention is the redirection of α-acetolactate towards diacetyl in aldB mutants. These mutants could be positively selected for due to the knowledge of regulation of BCAA biosynthesis. A better knowledge of bacteriophage gene regulation holds the exiting possibility of (near) future targeting of specific stages in the phage life cycle with the aim to conferring bacteriophage resistance to the bacterial host.

Whereas until recently genes in LAB could only be expressed via constitutive promoters, a number of the above described systems have been put to use for the expression of genes in a controllable way. The nis promoter can be used in a strain carrying the nis-RK genes to overproduce proteins by the addition to the medium of small amounts of nisin or spent medium of a nisin-producer. As the promoter is tight, it was possible to even produce very toxic proteins that could not be expressed under a constitutive promoter (O. Kuipers, pers. comm.). The rroltec system of phage rlt is currently further developed by the isolation of temperature-sensitive derivatives of the repressor. Their potential in temperature-induced gene expression in LAB is presently being examined (A. Nauta, pers. comm.). The salt-inducible promoter isolated from the chromosome of L. lactis has been used to induce expression of lytic genes in this organism by salt, with the consequent lysis of the cells and release of intracellular proteins and enzymes (J.W. Sanders, pers. comm). The vector systems are presently being used both for the elucidation of fundamental questions and for more applied purposes. They represent only the first generation of regulatable gene expression systems and refinements of these structures as well as new systems, controllable by other (food-compatible) effectors, are certainly foreseen in the near future.

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