From DNA sequence to application

Venema, G; Kok, J; van Sinderen, D

Published in:
Antonie Van Leeuwenhoek: International Journal of General and Molecular Microbiology

Publication date:
1999

Antonie van Leeuwenhoek **76:** 3–23, 1999.

From DNA sequence to application: possibilities and complications

Gerard Venema[1,*], Jan Kok[1] & Douwe van Sinderen[2]
[1]*Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN, Haren, The Netherlands;* [2]*Department of Microbiology and the National Food Biotechnology Centre, University College Cork, Ireland. (*Author for correspondence)*

Key words: lactic acid bacteria, bacteriophage, inducible gene expression, regelatory DNA elements

Abstract

The development of sophisticated genetic tools during the past 15 years have facilitated a tremendous increase of fundamental and application-oriented knowledge of lactic acid bacteria (LAB) and their bacteriophages. This knowledge relates both to the assignments of open reading frames (ORF's) and the function of non-coding DNA sequences. Comparison of the complete nucleotide sequences of several LAB bacteriophages has revealed that their chromosomes have a fixed, modular structure, each module having a set of genes involved in a specific phase of the bacteriophage life cycle. LAB bacteriophage genes and DNA sequences have been used for the construction of temperature-inducible gene expression systems, gene-integration systems, and bacteriophage defence systems.

The function of several LAB open reading frames and transcriptional units have been identified and characterized in detail. Many of these could find practical applications, such as induced lysis of LAB to enhance cheese ripening and re-routing of carbon fluxes for the production of a specific amino acid enantiomer. More knowledge has also become available concerning the function and structure of non-coding DNA positioned at or in the vicinity of promoters. In several cases the mRNA produced from this DNA contains a transcriptional terminator-antiterminator pair, in which the antiterminator can be stabilized either by uncharged tRNA or by interaction with a regulatory protein, thus preventing formation of the terminator so that mRNA elongation can proceed. Evidence has accumulated showing that also in LAB carbon catabolite repression in LAB is mediated by specific DNA elements in the vicinity of promoters governing the transcription of catabolic operons.

Although some biological barriers have yet to be solved, the vast body of scientific information presently available allows the construction of tailor-made genetically modified LAB. Today, it appears that societal constraints rather than biological hurdles impede the use of genetically modified LAB.

Introduction

The central molecule of a living unit is a polymerized string of either ribonucleotides (RNA) or deoxyribonucleotides (DNA). These often huge linear or circular polymers serve two functions: (1) they encode all information needed to sustain and reproduce an individual cell and, (2) they are transmitted during cell division with high fidelity. Alterations are kept to a minimum due to the presence of powerful repair mechanisms and daughter cells are essentially identical to the parent cell, at least during asexual reproduction. When sexual or parasexual systems interfere, the genetic information can be changed more drastically. However, in the natural context, never beyond certain limits set by the degree to which the genetic exchange mechanisms operating in general recombination can tolerate.

The natural constraints on the formation of grossly changed DNA at high frequency in a short period of time were definitely raised with the advent of recombinant DNA technology which permitted to intentionally change the genetic meaning of genomes almost overnight in a way unlikely to occur in Nature. This development not only accelerated the progress of fundamental research to an impressive speed, but also opened avenues for the production of cells and organisms for the pharmaceutical and fermentation industries. In addition, automated DNA sequencing facilities and advanced computer programmes which

convert nucleotide sequences to ORF's and link together nucleotide sequences into contigs has permitted entire microbial genomes to be sequenced as well as that of the eukaryotic *Saccharomyces cerevisiae* and, very recently, that of the multicellular *Caenorhabditis elegans*. As more genomes are being sequenced, we expect to understand in a much more detailed way than before, how evolution worked in shaping the genomes in present day organisms. For the first time the genome of a LAB has been sequenced to completion. The sequence of *Lactococcus lactis* strain IL1403 is a major step toward making comparisons with the genome sequences of other LAB, such as that of *Lactobacillus acidophilus*, which is well under way in the framework of the U.S. National Dairy Foods Research Center Programme (T.R. Klaenhammer, pers. comm.). As more LAB genomes are completed, comparing their genetic organization and informational content will undoubtedly shed light on the evolutionary relationships among the diverse members of this group.

After the genomes are sequenced, determining the function of each and every gene contained in the genome of an organism is a tremendous challenge. The general strategy to determine the function of a gene is to produce an interrupted copy by a Campbell-type integration and to analyze the phenotypic effect(s). However, if the gene to be interrupted is essential, this strategy does not work and should be replaced by one in which, in addition to the interrupted copy, an intact copy remains present, which can be turned on at will. For the functional analysis of the *Bacillus subtilis* genome, the vector pMUTin2 meeting these requirements has been developed (Vagner et al. 1998). Upon integration of this vector, transcription of the non-interrupted copy of the essential gene is controlled by the Pspac promoter in an IPTG-dependent fashion. As far as we are aware, such a vector for LAB has not yet been constructed. However, since both suitable integration vectors for LAB (Leenhouts et al. 1991; Maguin et al. 1992; Biswas et al. 1993; Law et al. 1996) and tightly regulated inducible promoters are currently available, a LAB vector with properties similar to pMUTin2 could be easily constructed. Nevertheless, the shear number of all the genes of a genome makes it a formidable task to uncover their functions. Undoubtedly, this task will carry us through the next few decades, even if several groups would join forces in this enterprise for just one species of LAB. Yet, pursuing this goal is worthwhile, as it will provide a wealth of information for both fundamental and applications-oriented research.

Although the phenotype of an organism ultimately depends on its genetic potential, knowledge of the function of all its ORF's is only part of the complete understanding of the organism in its biological context. Ideally, one would like to be able to predict the behaviour of that organism from its genetic composition. This requires knowledge about non-coding DNA sequences, such as those to which regulatory molecules attach to modulate gene expression. Even then, full understanding of the organism would be incomplete without knowledge of how proteins interact to produce a particular phenotype. Rapid progress is being made on this level, owing to the computer-assisted exploitation of the large body of information available on proteins and protein domains. In those fortunate cases where the crystal structure of a protein is known, examples exist in which computer-assisted modelling of related proteins can occur and result in genetic engineering of proteins with desirable properties.

Cells are continuously adapting to their surroundings: nutrients can become limiting and, in the case of LAB, cells are exposed to increasingly lower pH values through their own metabolic activity. Moreover, chemical additions to industrial fermentations are routinely made (e.g., salt during cheese making). How these changes affect the cell's biochemistry, depends on the modulation of specific gene sets, which can be examined by Northern hybridisation at the transcriptional level or by Western blotting at the translational level. These are, of course, very time consuming approaches and in a in a majority of cases Western analysis is not feasible because suitable antibodies are lacking. An alternative to assess the protein-encoding potential of the cell is to use highly standardized 2D gel electrophoresis to construct a 2D protein index as has been done for *B. subtilis* (Bernhardt et al. 1997; Schmid et al. 1997;). However, to link particular protein spots to the corresponding genes is time-consuming and often requires microsequencing and/or mutant production. Moreover, genes which are weakly expressed will remain hidden in this type of analysis.

The recent introduction of DNA array technology to monitor RNA expression of target genes, represents a major step forward in studying genome-wide gene expression. These techniques have now advanced to a state that the transcriptional potential of complete genomes can be monitored on just one, or a very limited number of oligonucleotide chips (for review: see

Schena et al. 1998; Ramsay 1998, and elsewhere in this volume), with a resolving power of only a few mRNA molecules per cell (de Saizieu et al. 1998). In summary, a whole spectrum of sophisticated analytical tools is presently available for quickly assessing gene function, its modification in a predictable way and tracing differential gene expression under a variety of external conditions.

Due to the relatively small size of their chromosomes, the genomic analysis of LAB bacteriophages advanced much more rapidly than that of their hosts. In the following text, some aspects of LAB bacteriophage genomics will be reviewed briefly, emphasizing those bacteriophage elements with potential interest for the industry, This will be followed by a few selected examples in which the (probable) function of a lactococcal gene could be derived from sequence comparisons with bacteria distantly related to LAB. Finally, some attention will be given to non-coding DNA, as well as to those constraints which impede applications of genetically modified LAB.

LAB bacteriophage genomics

The first LAB bacteriophage chromosome sequenced to completion was that of the lytic lactococcal bacteriophage bIL67 (Schouler et al. 1994). Additional (complete) sequences of bacteriophage chromosomes, from both virulent and temperate phages infecting various LAB species, have now become available (Table 1). We are now in the position to compare their overall genetic organization and, by homology comparisons, assign probable functions to a number of their ORF's. Figure 1 displays a schematic representation of such a functional assignment for a specific group of small isometric-headed phages containing both lytic and temperate representatives which infect a variety of LAB species. Comparison of the partial functional maps has allowed a number of generalisations. Figure 2 shows that: (1) genes involved in lysogeny are transcribed divergently from those required for the lytic pathway; (2) the element governing the switch from the lysogenic to the lytic cycle of temperate bacteriophages is located between the lytic and lysogenic genes; and, (3) adjacent to the 3′-end of the genetic switch is a cluster of genes for the initiation and sustenance of bacteriophage chromosome replication. These genes are followed by gene clusters involved in packaging of the chromosomes into the phage heads, and directing bacteriophage morphogenesis and genes required for lysis of the host. Thus, these bacteriophage chromosomes are highly modular with functionally-related genes clustered together in a fixed order. Exchange of modules between interbreeding bacteriophages by recombination has been invoked to explain the variability between and evolution of bacteriophage chromosomes (Botstein 1980). However, in an extensive review on the variability of a number of *Streptococcus thermophilus* bacteriophages, this evolutionary mechanism has been questioned on the basis of the observation that exchanged segments were neither functional units (modules) nor even complete genes (Brüssow et al. 1998). A number of ORF's of bacteriophage ØSfi21 of *S. thermophilus* showed significant identities with ORF's from various LAB-infecting bacteriophages and even from a *Lb. delbrueckii* plasmid. These observations favour the idea that *S. thermophilus* bacteriophages have evolved through horizontal gene transfer between various LAB as their hosts share the same ecological environment.

It is interesting to note that the mechanism by which the chromosomes are incorporated in the bacteriophage heads may differ profoundly, even among closely-related bacteriophages. For example bacteriophage r1t uses a *cos* site and a terminase to produce chromosomes with single- strand complementary termini, whereas Tuc2009 employs the headful mechanism to produce circularly permuted chromosomes. Apparently, lytic phage varieties can be derived from temperate bacteriophages, as illustrated by inspection of the genome organization of bacteriophage LL-H, which, although having retained *attP* and part of the integrase gene, appears to lack the repressor gene and the switch governing the life cycle (Figure 1).

Apart from these general deductions, the question should be raised as to which set of genetic information could be used for (future) applications. Obvious examples are: (1) repressor-operator systems for the construction of LAB strains in which gene expression can be turned on or off at will; (2) genetic elements which could be used to provoke lysis of LAB in the context that lysis of LAB may facilitate cheese ripening; (3) elements that could be used for the stable integration of (foreign) genes in LAB; and, (4) elements which could be used to combat phage infections during fermentations. An example of each of these possibilities will be described in the following pages.

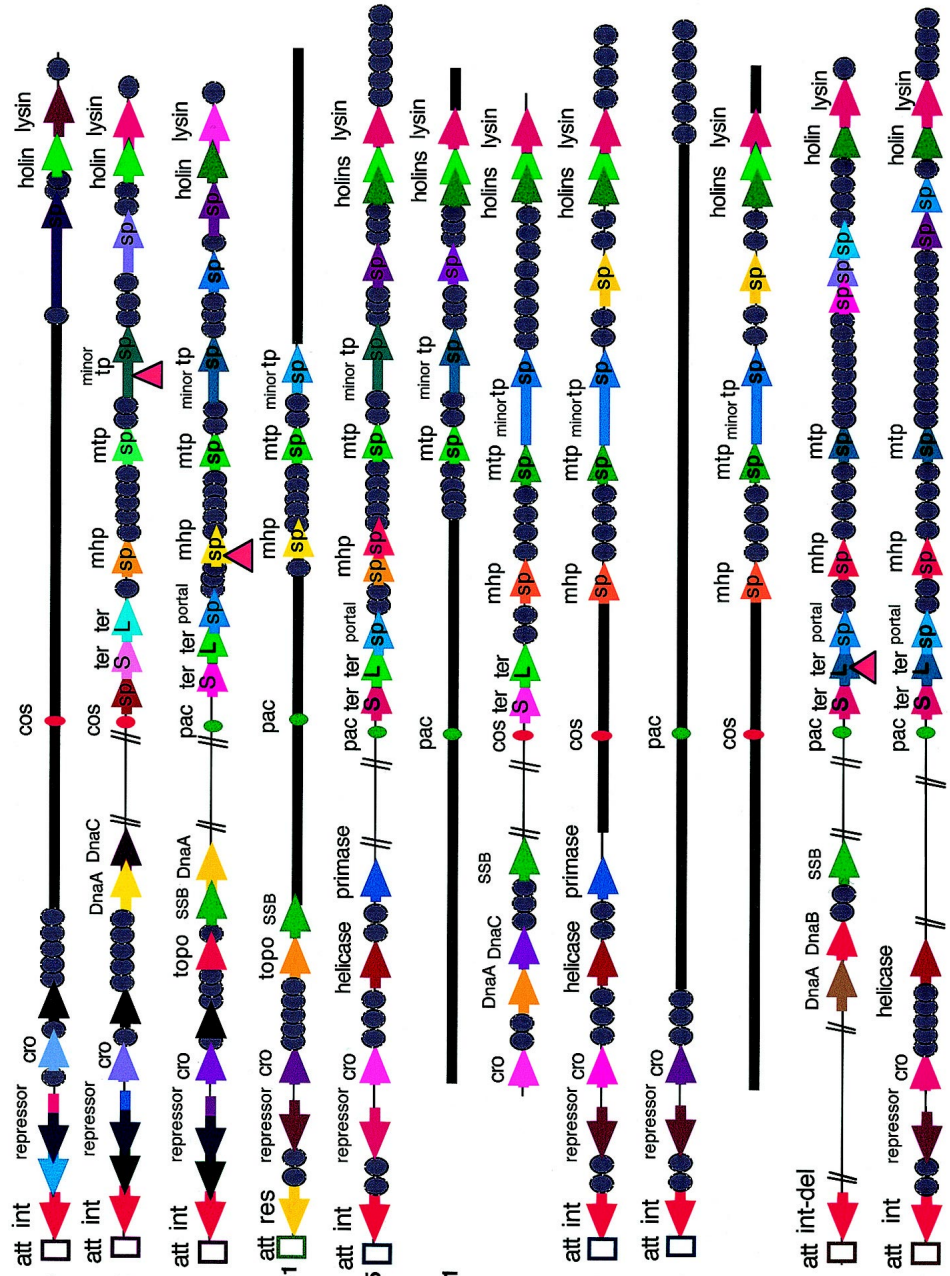

Figure 1. Schematic representation of the genomes of a number of temperate and lytic small isometric-headed bacteriophages infecting various LAB (names in the left-hand margin refer to phages; see also Table 1). Arrows and filled circles (●) depict identified open reading frames (ORF's). The direction of the arrows corresponds to the presumed direction of transcription. Triangles (▲) indicate the position at which an intron interrupts the coding coding sequence of an ORF. A thick black line (▬) indicates sections of phage genomes where no sequence information is available. Genomic sections which were not considered because of lack of known function have been placed between two sets of perpendicular lines (–//—//–). Deduced or determined positions of initiation of packaging, pac or cos, are indicated by green and red filled circles, respectively. Bacteriophage attachment sites (att) are indicated by squares (□). Other abbreviations refer to to the presumed function of the ORF and are as follows: int, integrase; cro, small DNA-binding protein probably involved in the genetic switch; DnaA, DnaB and DnaC, components of the replisome, referring to the *E. coli* replisome; sp, structural protein; terS and terL, small and large subunits of the terminase, respectively; mhp, major head protein; mtp, major tail protein; minor tp, minor tail protein; portal, portal protein; SSB, single-strand DNA binding protein; topo, topoisomerase I; int-del, deleted integrase-encoding ORF.

Table 1. List of LAB bacteriophages of which the genome has been partially or completely sequenced. This table only includes phages of which at least six kb of contiguous sequence has been determined

Phage	cos/pac[a]	Type	Complete sequence available; base pairs	ORF's present on genome	LAB host	Reference
bIL67	cos	prolate, lytic	yes; 22,195	37	*L. lactis* subsp *lactis*	Schouler et al. 1994
c2	cos	prolate, lytic	yes; 22,163	39	*L. lactis* subsp *lactis*	Lubbers et al. 1995
sk1	cos	small isometric, lytic	yes; 28,451	54	*L. lactis* subsp. *cremoris*	Chandry et al. 1997
bIL41	cos	small isometric, lytic	no	NA	*L. lactis* subsp. *lactis*	Parreira et al. 1996
BK5-T	cos	small isometric, temperate	no	NA	*L. lactis* subsp. *cremoris*	Boyce et al. 1995
r1t	cos	small isometric, temperate	yes; 33,350	50	*L. lactis* subsp *cremoris*	van Sinderen et al. 1996
Tuc2009	pac	small isometric, temperate	yes; 38,347	56	*L. lactis* subsp *cremoris*	van Sinderen et al.[1]
TP901-1	pac	small isometric, temperate	no	NA	*L. lactis* subsp *cremoris*	Christiansen et al. 1996 Johnsen et al. 1996 Madsen et al. 1998
LL-H	pac	small isometric, lytic	yes; 34,659	52	*Lb. delbrueckii* subsp. *lactis*	Mikkonen et al. 1996
øg1e	(pac)	small isometric, temperate	yes; 42,259	62	*Lactobacillus* species	Kodaira et al. 1997
øO1205	pac	small isometric, temperate	yes; 43,075	57	*S. thermophilus*	Stanley et al. 1997
ø7201	cos	small isometric, lytic	yes; 35,466	44	*S. thermophilus*	van Sinderen et al.[2]
TP-J34	pac	small isometric, temperate	no	NA	*S. thermophilus*	Neve et al. 1998
øSfi11	pac	small isometric, lytic	no	NA	*S. thermophilus*	Lucchini et al. 1998
øSfi19	cos	small isometric, lytic	no	NA	*S. thermophilus*	Lucchini et al. 1998 Desiere et al. 1998
øSfi21	cos	small isometric, temperate	no	NA	*S.thermophilus*	Desiere et al. 1998 Bruttin et al. 1997 Desiere et al. 1997

[a] cos/pac: phages using cos or pac type of DNA packaging, respectively; NA: not applicable.

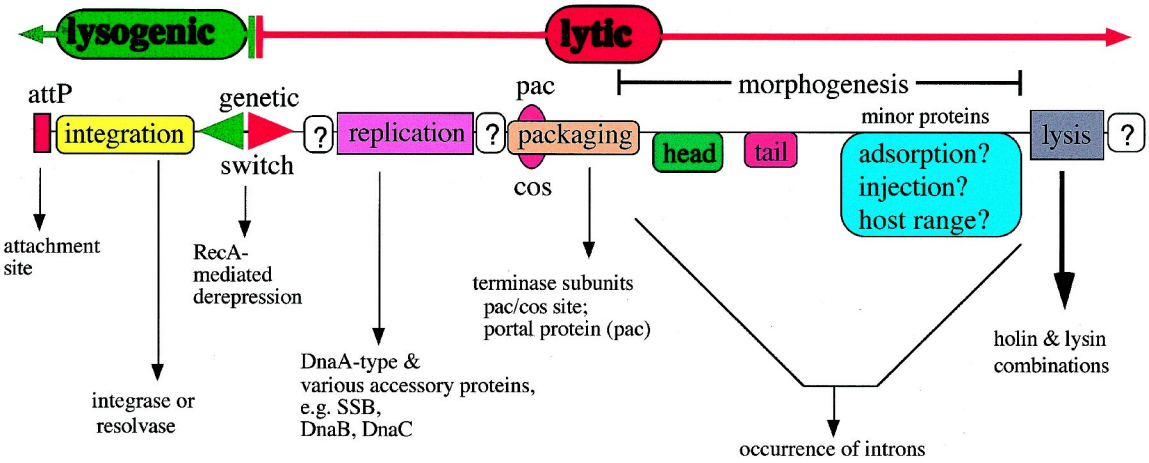

Figure 2. Modular genomic organization found in a large group of small isometric-headed bacteriophages (see Figure 1 and Table 1). Arrows positioned at the top of the figure and coloured in green and red indicate the transcription direction during the lysogenic and lytic life cycle, respectively (note that in lytic phages the DNA region transcribed from lysogenic phages is not, or only partially present). The presumed function of specific DNA modules is indicated in the middle part of the figure and regions with no known function are indicated by question marks. Vertical arrows pointing towards the bottom part of the figure point at additional information on functionality or composition of the specific DNA module.

Inducible gene expression systems derived from bacteriophage

As noted above, all genes involved in the lytic cycle of temperate phages are transcribed oppositely from those governing the lysogenic cycle. In analogy with temperate phages of other bacterial species, this suggests that important regulatory functions could be located in the intergenic region. The two divergently transcribed ORF's flanking this region are ORF 3 and ORF 4 (Figure 1, see r1t, in which ORF3 and ORF4 are indicated as repressor and cro). Examination of the nucleotide sequence of this region of the *L. lactis* phage r1t revealed that it contained two oppositely oriented promoters, P1 and P2, identical to the consensus promoter sequence used by *L. lactis* RNA polymerase. As the -35 sequence of these promoters are part of two direct repeats with dyad symmetry, the intergenic region has the characteristic features of control regions which are instrumental in the decision between the lytic and lysogenic life cycles of temperate phages. Alignments of the deduced *rro* product (Rro) with a number of known and supposed repressors showed that it shared a number of identical amino acids at specific positions with these repressors, including two adjacent amino acids, A and G, where the *E. coli* repressor LexA and the λ CI repressor cleaves during autodigestion. In addition, the protein contained a putative helix-turn-helix motif. Together these features strongly suggested that the Rro was the repressor of phage r1t. This was underscored by experimental evidence: a strain carrying a plasmid in which the transcription of *lacZ* was placed under the control of the lytic promoter P2 produced β-galactosidase when exposed to mitomycin C, whereas a frameshift mutation in *rro* led to constitutive synthesis of β-galactosidase (Nauta et al. 1996). The conclusion that Rro was indeed the repressor controlling the lytic/lysogenic switch in phage r1t provided the basis for the construction of a temperature-inducible gene expression system for lactococci.

A suitable repressor-based temperature-inducible system should meet a number of requirements: the cells should have the wild-type phenotype at low temperatures, whereas at higher temperatures the repressor should be inactive. In addition, the critical temperature interval should be small, as the cells should stay metabolically active at the elevated temperature. The successful development of such a system was facilitated by the availability of the crystal structure of the repressors of the two temperate *E. coli* bacteriophages λ and 434 (Aggarwal et al. 1988; Beamer & Pabo 1992). Characteristically, these repressors have a two domain structure with an N-terminal portion binding to the cognate operators and a C-terminal domain important for dimer formation, dimer-dimer interaction, and repressor self-cleavage (Pabo et al. 1979). The binding specificity of repressors to the cognate operators of temperate bacteriophages resides in the second helix of the helix-turn-helix motif present in the N-terminal repressor domain, whereas the amino acid sequence constituting the turn is important for the correct positioning of the second helix relative to the DNA backbone (see Ptashne 1992). The requirements to be met by a suitable temperature-labile repressor, noted above, are perfectly met by the well-known temperature-sensitive λ repressor CI857, carrying an A66Thr substitution in the N-terminal domain (Lieb 1981). Molecular modelling, using the programme WHAT IF (Vriend 1990) showed that Ala66, contained in helix 4 of the N-terminal domain, is buried in a densely packed hydrophobic pocket. Replacement of Ala66 by Thr would result in distortion of local hydrophobic interactions. Modelling studies using the 3D-structures of the N-terminal domains of the λ and 434 repressors revealed the presence of a structurally similar pocket in the N- terminal domain of Rro, despite the fact that the N-terminal domains of the three repressors shared only 5 conserved amino acids (Figure 3). By randomizing three residues predicted to destabilize the hydrophobic pocket, several temperature-sensitive repressor mutants were obtained. In cells producing one of the mutant repressors, carrying an F50S in addition to a P61A replacement, the induction factor upon raising the temperature from 24 °C to 37 °C and 40 °C was approximately 200 and 600, respectively (Nauta et al. 1997).

Using a random mutagenesis technique, a thermosensitive mutant of the repressor (Rad) of the *Lb. gasseri* bacteriophage Øadh was recently obtained, which contained three amino acid substitutions. Based on tertiary structure analysis, one of the mutations, V48A, corresponded to V54 in the hydrophobic pocket of Rro. However, induction at 42 °C appeared to be quite low. Interestingly, one of the hydrophobic pocket variants (L52D) showed a slightly cryosensitive phenotype. From this observation we may speculate that by proper engineering of the hydrophobic pocket, repressors could be obtained which effectively lose their repressing ability at low temperature, which

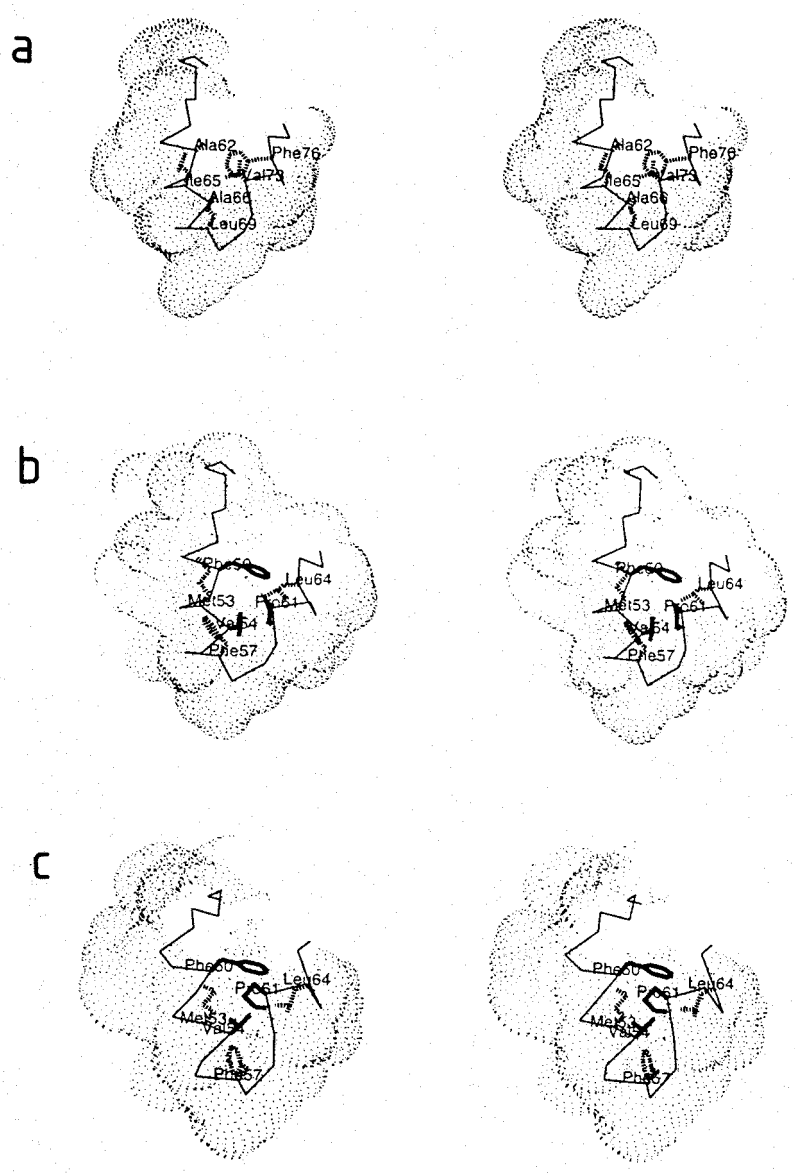

Figure 3. The hydrophobic pockets within the N-terminal domain of λ CI (a) and the Rro models that were obtained using λ CI (b) and 434 CI (c) as templates. The hydrophobic core residues supposed to be important for stability of the pockets are indicated. The side chain of the λ CI residue A66 that has been shown to be critical for stability at elevated temperatures and the side chains of Rro residues that were randimized are depicted in bold. Solvent accessible surfaces are dotted.

might be profitable for *in situ* expression of genes during actual cheese making (G. Engel, pers. comm.).

These systems, in which expression is induced by a simple physical parameter, hold great promise for the induced synthesis of both homologous and heterologous proteins in LAB, important both for fundamental research as well as possible applications.

Lytic genes

The first LAB lysis gene to be successfully cloned, sequenced and characterized was that of the lactococcal prolate-headed bacteriophage ØvML3 (Shearman et al. 1989). Several lysis genes have subsequently been identified in a number of other bacteriophages from both *Lactococcus* and *Lactobacillus* origin (for

review: see Gasson 1996). Homology comparisons strongly suggested that ORF 23 of bacteriophage r1t (denoted lysin, Figure 1) could encode the lysin. Proof was obtained by cloning the gene, *lytR*, in the IPTG-inducible plasmid pAG58 in *E. coli*. Subsequent assay of cell wall lytic activity by subjecting cell-free *E. coli* extracts to SDS-polyacrylamide gel electrophoresis showed a prominent clearing zone in the gel containing *M. leisodeikticus* cell walls, corresponding to the expected molecular size of LytR (Nauta 1997).

Phage lysins are generally synthesized without an N-terminal secretory signal peptide needed for *sec*-dependent transport across the cytoplasmic membrane. To gain access to the cell wall substrate, the phage lytic genes are accompanied by an ORF specifying a small hydrophobic protein, thought to form pores in the cytoplasmic membrane, through which the phage lysin gains access to the cell wall substrate (Young 1992; Young and Bläsi 1995). Support for the supposed function of these so-called holins is the presence of two membrane spanning domains, separated by a short sequence likely to adopt a β turn configuration. Supporting evidence that holins act by forming pores in the cytoplasmic membrane came from experiments in which the genes for the supposed holin and that for the lysin of phage r1t were separately and jointly expressed in *E. coli*. Whereas expression of just the lysin gene had little effect on cell viability (due to the absence of a signal peptide the enzyme remains cytoplasmic), expression of just the holin gene reduced the viability by almost 4 log units. When both genes were expressed, viability was reduced by two additional log units. The dramatic effect on viability of expression of the holin gene is in accordance with its presumed pore forming function in the cytoplasmic membrane, which is, apparently, not reconcilable with the cell's life (Nauta 1997). Essentially similar results were obtained in *L. lactis*; induced expression of the lysin gene of bacteriophage øUS3 did not cause significant lysis, partial lysis was observed upon expression of the holin gene, whereas simultaneous expression of both genes resulted in complete lysis of the host (de Ruyter et al. 1997). In summary, two genes appear to be required for the phage progeny to escape from their host, the phage lysin gene and the holin gene. These genes are usually, but not always, closely linked to form the lysis cassette.

Two major (potential) applications of the lysis genes or lysis cassettes have been explored: (1) provoked lysis to accelerate cheese ripening and (2) elimination of undesirable bacteria during fermentation. The former application was implemented in a cheese making experiment where the effect of nisin-induced lysis of an adjunct starter culture on lysis of cells was studied, using cytoplasmic L-lactate dehydrogenase as a marker for lysis. After 4 days of ripening the activity of the marker enzyme had increased 4-fold as compared to the controls, indicating that the system of induced lysis also functioned under actual cheese making conditions (de Ruyter et al. 1997). The latter application has been demonstrated by expression of the lysin gene of a *Listeria monocytogenes* bacteriophage in *L. lactis*. Upon induction, a range of *Listeria* strains were found vulnerable to lysis (Payne et al. 1995).

Integration of genes using bacteriophage DNA-derived sequences

The capacity of temperate bacteriophages to lysogenize their hosts by site-specific integration essentially depends on their attachment site (*attP*), the presence of an identical sequence on the host chromosome (*attB*), and a bacteriophage-encoded integrase (Int). Site-specific recombination has been used to integrate plasmids into the chromosomes of lactococci, lactobacilli and *S. thermophilus*. To make the bacteriophage-derived systems operational, first the critical genetic elements for integration had to be determined, which was established for the *Lactobacillus gasseri* bacteriophage Øadh (Raya et al. 1992), *Lb. delbrueckii* subsp. *bulgaricus* bacteriophage mv4 (Dupont et al. 1995), and the *L. lactis* subsp. *cremoris* bacteriophages Tuc2009 (van de Guchte et al. 1994), TP901-1 (Christiansen et al. 1994), r1t (van Sinderen et al. 1996) and ØLC3 (Lillehaug & Birkeland 1993) as well as for ØSfi21 of *S. thermophilus* (Bruttin et al. 1997b). By incorporating the *int* gene and *attP* into (conditionally) non-replicating plasmids, the plasmids became integrated into the target site, *attB*, of the chromosomes of transformants. Integration at the correct site was even observed when a single gene was ligated to the *int* gene-*attP* cassette to form a circular molecule (Lillehaug et al. 1997). The main advantages of using the *int-attP* cassette over systems in which integration is governed by general recombination are: (1) the integration takes place at the same site used by the bacteriophage chromosome during lysogenisation, thus at a site which disruption is likely to be harmless to the cell, (2) the genetic stability of the integrants under non-selective conditions is usually much higher than those generated by general recombination, (3) the

orientation of the integrated structure appears to be fixed and (4) the integrated structure is generally not subject to amplification. With respect to the versatility of use, there is little doubt that the *int-attP* derived system can be used for integration in LAB lysogenizable by bacteriophage from which the casette was obtained. As the integrase should recognize the cognate *attP-attB* pair, conservation of the nucleotide sequence of *attB* might allow integration in a wider range of strains or (sub)species. In fact, this has been demonstrated in *L. lactis* (van de Guchte et al. 1994) and the genus *Lactobacillus* (Dupont et al. 1995)

Combatting bacteriophage propagation with phage DNA

Bacteriophage pose a serious threat to dairy fermentations. Consequently, a considerable body of research continues to be dedicated to bacteriophage-host interrelationships with the aim to inhibit bacteriophage propagation. Various phage defence mechanisms in LAB have been uncovered (see elsewhere in this volume). The discussion below will be limited to non-native systems entirely derived from bacteriophage chromosomes.

The basis for combatting bacteriophage multiplication using a piece of bacteriophage DNA was laid down by Hill et al. (1990). A 4.5-kb DNA fragment from the virulent, small, isometric-headed *L. lactis* bacteriophage Ø50 incorporated into the shuttle vector pSA3, reduced the efficiency of plating of Ø50 by a factor of approximately 3. The plaques, however, were pinpoint in size. The bacteriophage-resistance phenotype was labelled Per (*p*hage-*e*ncoded *r*esistance). Subsequent structural and functional analyses of the *per* region revealed that Per was dependent on the presence in a 1.4-kb fragment of a series of non-coding direct and inverted repeats, hallmarks of phage replicative origins. Consistent with the idea that the *per* region was, in fact, the replication origin of Ø50, was the observation that a plasmid carrying the *per* region was subject to amplification as a concatamer upon phage infection. Also, the level of protection against phage attack correlated with the copy number of the plasmids on which *per* resided (O'Sullivan et al. 1993). Insertion of *per* from the lactococcal virulent isometric-headed bacteriophage Ø31 into a low and a high copy plasmid, reduced the EOP of Ø31 to a mere 0.3 and 7.2×10^{-7}, respectively, demonstrating the potential power of this non-native resistance mechanism. Characteristically, *per*-based systems incorporate two features: titrating out factors required for bacteriophage replication and enabling amplification of *per*-containing plasmids, especially those of the low-copy category.

By combining the latter feature with a regulated middle promoter of bacteriophage Ø31, a system was developed resulting in explosive plasmid amplification and gene expression triggered by infection with a lytic bacteriophage (O'Sullivan et al. 1996). Analysis of the phage-inducible promoter revealed the presence of two sets of phage-inducible tandem transcription start sites in addition to a complete ORF (ORF2) on a 888 nt fragment. When fused to the promoterless *lacZ* gene from *S. thermophilus* in a low copy number plasmid, more than 2000 units of β-galactosidase were produced in *L. lactis* upon infection with Ø31. ORF2 appeared to be a transcriptional activator and a large part of the 5′ end of the 888 bp promoter fragment could be deleted without loss of inducibility. A shortened promoter fragment, carrying only one set of transcriptional start sites, prevented basal expression of β-galactosidase and, after a few further changes, was used in a vector which directed the synthesis of 11,000 units of β-galactosidase upon infection with Ø31. As no synthesis of β-galactosidase was observed in uninfected cells, the system would potentially allow the synthesis of toxic gene products in *L. lactis* (Walker & Klaenhammer 1998). To further exploit this system in phage defence, a 239 bp promoter fragment of the middle promoter of Ø31, designated Ø31*p*, was transcriptionally fused to the restriction cassette *Lla*IR$^+$, consisting of the genes *llaI.1*, *llaI.2* and *llaI.3*. These genes are all essential for restriction activity and when expressed, are lethal for *L. lactis* (O'Sullivan et al., 1995). The efficiency of plating of Ø31 on *L. lactis* expressing the suicide cassette on a high copy-number plasmid was lowered to 10^{-4}, whereas the burst size decreased fourfold (Djordjevic et al. 1997). Bacteriophages that escaped this suicide system appeared to have lost their capacity to effectively fire the suicide cassette. This data prompted the use of a fourfold stronger version of Ø31*p* which reduced the EOP by another factor of 2.2, but somewhat retarded the growth of the host. Combining the suicide cassette with the restriction enhancer *llaC* on the same replicon, which elevates the restriction activity of the suicide cassette, resulted in a further 5-fold reduction of the EOP. In hosts carrying this system and plasmids specifying the abortive phage defences *per31* and *abiA*, the EOP was shown to be less than 10^{-10} (Djordjevic & Klaenhammer 1997).

An essentially different method to combat phage infections concerns the production of antisense RNA directed against bacteriophage mRNA. The potential of this approach was demonstrated in a series of experiments in which anti-sense RNA of a cryptic ORF encoding a protein of 449 amino acids was produced from a constitutive lactococcal promoter. This ORF appeared to be conserved among a number of lactococcal bacteriophages. The complementary RNA produced from the ORF of one of these reduced the EOP by 99.6%, but was also effective against two other bacteriophages in which the ORF was conserved (Kim & Batt 1991). However, subsequent study (Chung et al. 1992) showed that the target RNA largely determined the efficiency of the system. Antisense RNA directed against the activator of the middle genes of Ø31, referred to above, was ineffective against Ø31 propagation (Walker & Klaenhammer 1998). Notwithstanding the failure in some systems to inhibit bacteriophage propagation by means of the antisense methodology, this approach would seem potentially useful, provided the antisense could be targeted to sufficiently conserved pivotal (regulatory) bacteriophage genes which are not abundantly transcribed.

Potentially useful applications of ORF's from LAB

Recently the complete chromosome of *L. lactis* IL1403 has been sequenced (for details see elsewhere in this volume). Undoubtedly, the genetic information derived from the sequence will be helpful in advancing our knowledge of this organism, other lactococcal strains and, likely, LAB species in general. In the text that follows, we will discuss a number of lactococcal ORF's, identified by means of standard genetic analyses, which may be useful for practical applications.

Salt and pH-inducible ORF's

During cheese making procedures NaCl is often added to the curd. This, in addition to the fact that LAB produce lactic acid during growth, resulting in a decreasing pH of the medium, has prompted considerable interest in LAB gene expression in response to varying salt concentration and pH.

In their search for NaCl-inducible ORF's, Sanders et al (1998a) employed a chromosome integration strategy in which a conditionally non-replicating integration vector, pORI13, was used to insert a promoterless *E. coli lacZ* gene randomly in the genome of *L. lactis*. The integrants were assayed for a blue colour on plates containing X-gal and salt, while remaining white in the absence of salt. One integrant showed this phenotype, which depended on the presence of Cl^- rather than of Na^+ ions. Genetic analysis of the integrant showed that pORI13 had integrated in an ORF whose putative product shared homology with GadC of *Shigella flexneri* and *E. coli*. Both Gad's are homologous to a number of amino acid antiporters. Similar to GadC of *S. flexneri*, the lactococcal GadC contains a cluster of 12 highly hydrophobic domains in addition to a conserved domain present in glutamate transporting proteins. GadC probably represents an integral membrane protein involved in glutamate transport. Further analysis of the sequences around the integrated pORI13 revealed the presence of two ORF's flanking *gadC*, labelled *gadR* and *gadB*. GadB is homologous to glutamate decarboxylase from *Synechocystis* sp. (48% identity) and two glutamate decarboxylases from *E. coli* (43 and 44% identity). Northern blot analysis revealed that the *L. lactis gadC* and *gadB* genes are in one operon. Immediately upstream of *gadC*, *gadR* could encode a protein with similarity to positive regulator proteins from *Streptococcus gordonii* (Rgg, 25% identity) and that from *Bacillus thuringiensis* (PlcR, 19% identity). GadR was shown to be the activator of the *gadCB* operon as a *gadR* insertional mutant failed to transcribe the operon. Transcription of *gadCB* was not induced upon lowering of the pH of the medium. However, in the presence of salt and glutamate, the activity of the operon was considerably higher at pH 4.5 than at pH 5.5, suggesting that it might be involved in protection against acid stress. This idea proved to be correct. When wild-type cells were challenged with lactic acid, such that the pH dropped to 3.5, the percent survival was only $5\times10^{-4.}$ Under the same conditions in the presence of NaCl and glutamate, survival increased by a factor of 1000. Increased survival was not observed in a *gadR* mutant which does not produce *gadCB* mRNA (Sanders et al. 1998b). The most plausible explanation for the function of the *gad* system is depicted in Figure 4, in which it is assumed that glutamate is taken up by the amino acid antiporter GadC. After conversion to γ-aminobutyrate by glutamate decarboxylase, it is subsequently externalized by GadC in antiport with a molecule of glutamate. Since the conversion of glutamate to γ-amino butyrate consumes protons, this helps to stabilize the internal pH under acidic external conditions.

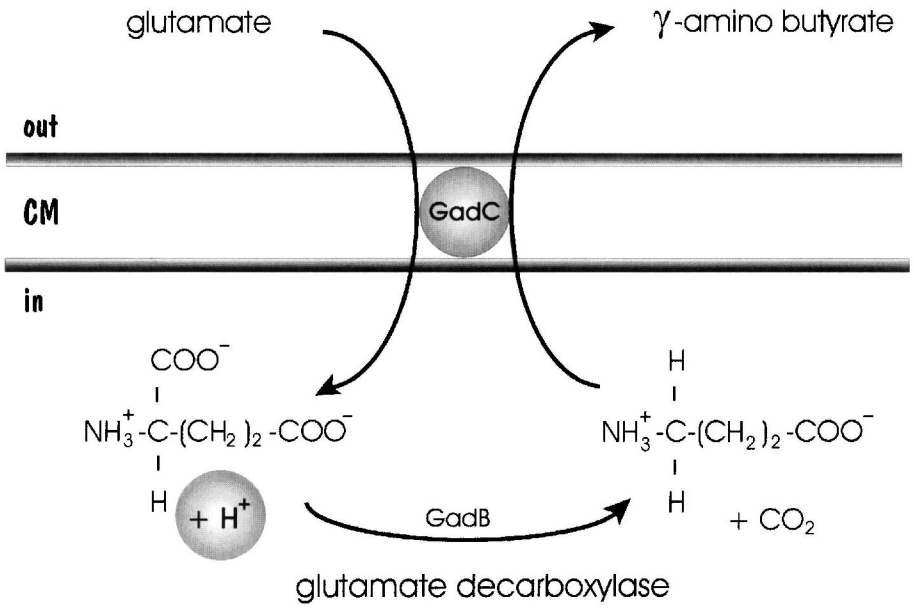

Figure 4. Schematic representation of the stabilization of the internal pH through the activity of the *L. lactis gadBC* operon. The antiporter GadC in the cytoplasmic membrane (CM) internalizes glutamate, which, after conversion to γ-aminobutyrate by GadB, is externalized by GadC in antiport with the optake of glutamate. The conversion of glutamate to γ-aminobutyrate consumes a proton, thus helping in stabilization of the internal pH.

The results of the research summarized above may have at least two practical applications. First, salt and glutamate might be added to protect commercial starter cultures for dairying purposes from adverse effects of low pH. Second, the *gad* cassette, consisting of *gadR* and the *gadCB* promoter could be used for NaCl-induced production of heterologous and homologous proteins. In this case, the induced expression is effected by a simple chemical often used in cheese making. In fact, this cassette is quite effective in inducing lysis *L. lactis* when transcriptionally fused to the lysis cassette of bacteriophage r1t and the *acmA* gene of *L. lactis*, which specifies the autolysin (Sanders et al., 1997).

Based on homology comparisons the function of genes of the *gad* system could be defined reasonably easy. However, when no homology exists with ORF's of other organisms, or because no function could as yet be assigned to homologous ORF's, the situation becomes less clear. An example of the latter of these two possibilities concerns an ORF which is expressed under conditions of low pH at the start of the stationary growth phase of *L. lactis* (Madsen et al. 1999). The putative ORFX product shows a high degree of sequence identity (57.3%) with the hypothetical *B. subtilis* Yybh protein, to which, unfortunately, no function has as yet been ascribed. The P170 promoter of OrfX, like that of *gadCB*, lacks the -35 canonical promoter sequence TTGACA, present in the majority of the promoters so far identified in *L. lactis* (van de Guchte et al. 1992; de Vos & Simons 1994). Interestingly, the inducibility of the promoter of OrfX could be conferred on a constitutive lactococcal promoter (CP25) by substituting the -35 sequence together with 22 bp upstream of this sequence for the corresponding 27 bp of the inducible promoter. Inducible expression of OrfX was increased 150–200 fold upon deletion of 72 bp of the 270 bp untranslated leader of the OrfX mRNA. The modulating activity of this fragment could be conferred on two other constitutive lactococcal promoters. Potentially, the 27 bp element could be used for those situations in which it would be desirable to turn a constitutively expressed gene into an inducible one, whereas the untranslated 72 bp OrfX leader fragment might be exploited for the inhibition of late genes that are not targeted for expression.

LAB protein modules

Analysis of derived amino acid sequences has revealed that proteins are often composed of functional domains and/or motifs such as involved in catalysis,

substrate recognition and co-factor binding. A specific example of this modularity with potentially interesting possibilities for application concerns the composition of the major autolysin AcmA of *L. lactis*. The amino acid sequence of the N-terminal moiety of this enzyme (amino acids 65–220) shows approximately 56% identity with muramidase-2 of *Enterococcus hirae* and the autolysin of *Streptococcus faecalis*, whereas the identity in the C-terminal part (amino acid 222–437) was only approximately 24%. Inspection of the amino acid sequence of this part of the lactococcal enzyme showed that it contained three interspersed repeated sequences of 45 amino acids, each containing 16 identical amino acids and 18 conservative replacements (Buist et al., 1995). These repeated motifs are also present in the C-terminal moieties of the corresponding enzymes of *S. faecalis* and *E. hirae*, although in different numbers – 4 in the former and 5 in the latter of these species. Fusing the C-terminal *L. lactis* AcmA domain to TEM β-lactamase, provided with a *Lactococcus*-specific signal peptide, resulted in association of the hybrid protein with the cell wall. Since β-lactamase without the C-terminal repeats was secreted into the medium, the C- terminal part of AcmA apparently functions as a cell wall anchor (Buist 1997), which agrees with data obtained in other lytic systems (López et al. 1995; Sheehan et al. 1997). As discussed in more detail elsewhere in this volume, such anchoring motifs may find use for applications.

Non-coding sequences

Obviously, the identification, characterization and functional analysis of genes is crucial for successful genetic modification. Equally important is detailed knowledge as to how genes targeted for use in genetic modification are regulated. In many cases, non-coding DNA or mRNA is the target for regulatory molecules. The function of such nucleotide sequences will be briefly reviewed because they could be used in many conceivable ways for modulation of gene expression.

Analysis of non-coding sequences is inherently more difficult than that of coding sequences because one needs to consider the significance of the ordering of individual bases rather than of base triplets.

Presently, functional genome analysis is almost completely limited to efforts to determine the function of ORF's, usually by disrupting the ORF's by the integration of specially designed plasmids into the respective genes and subsequent analyses of the phenotype under a variety of different conditions. If the vectors designed for gene interruption are also provided with a reporter gene, differential gene expression may be monitored under different conditions or during growth. Thus ORF's can be identified that are either positively regulated by transcription activators or negatively regulated by repressors binding near to the start of transcription. Fortunately, regulatory proteins often bind as dimers or tetramers to their recognition sites. At the DNA level the target sequences are reflected by the presence of inverted or direct repeats. However, the absence of such repeats does not guarantee that the promoter is *not* regulated, as exampli fied by the pH-inducible promoter P170, referred to above, in which no secondary structural elements could be detected. Both the pH-inducible P170 as well as the salt inducible P*gad* lack the -35 promoter sequence. Whether or not the absence of -35 sequences are characteristic features of regulated host promoters remains to be established.

The presence of long stretches of untranslated, or partially translated mRNA 5′ to the encoded gene may also be indicative of regulation, especially when these leaders contain (potential) secondary structure. Examples in *L. lactis* illustrating this notion concern a number of operons involved in amino acid biosynthesis. In the 439 nt leader of *trp* operon mRNA several stem-loops could be formed. Two of these, the terminator and antiterminator, mutually exclude each others formation, because they use the same nucleotide stretch in the formation of their stems. Under Trp limitation, the 3′-end of uncharged $tRNA^{trp}$, the acceptor (GCCA), is believed to base pair with the complementary sequence (anti-acceptor), embedded in the so-called T-box, residing in the stem of the antiterminator. This interaction, together with basepairing of the anticodon of $tRNA^{trp}$ with the specifier codon UGG in a stem-loop structure (numbered I) close to the 5'-end of the leader, is believed to stabilize the antiterminator. This prevents the formation of the terminator, and allows transcription to continue over the entire length of the structural genes of *trp* operon (van de Guchte et al., 1998). Apparently, the *his* operon of *L. lactis* is subject to a similar mode of attenuation regulation, as the specifier codon (CAC) is positioned at the same location in stem-loop I as the UGG codon for tryptophan biosynthesis and a T-box is present containing three nucleotides complementary to the 3′-end of $tRNA^{his}$. By comparing the nucleotide sequences of the leaders of *his* mRNA of various distantly related lactococci, it was observed that nucleotide variations

generally did not affect the proposed secondary structure elements, thus lending support to the folding model of the leader (Delorme et al. 1999).

The leader of the *leu* operon appears to be partially translated into a 16 amino acid-containing peptide with 3 consecutive leucines. The leader also contains a rho-independent transcription terminator which, under conditions of leucine starvation, will not be formed due to stalling of the ribosomes. This form of regulation clearly represents attenuation regulation of the operon on the basis of the specific codons present in the translated part of the leader (P. Renault, pers. comm.)

Although little is known about the regulation of the other amino acid biosynthetic pathways in LAB, characterization of the 5′ portions of the mRNA involved in these pathways will probably prove valuable in defining whether or not they are subject to similar modes of regulation.

In the examples referred to above, the formation of the terminator is prevented by antiterminator stabilization either by interaction of uncharged tRNA, or by ribosome stalling at the leader. The formation of terminator structures residing in certain mRNA leaders can also be prevented by interaction with Bgl-type regulator proteins, which recognize a conserved nucleotide sequence, labelled RAT (*r*ibonucleic *a*nti*t*erminator; consensus: GGATTGTTACTGCA AAGCAGGCAAAACC, Schnetz et al., 1996) that is part of the 5′-portion of the terminator. This mechanism is well documented in *E. coli* as well as a number of Gram-positive bacteria (for references see Bardowski et al. 1994), and also appears to operate in LAB. The regulatory protein BglR of *L. lactis* classifies as a member of the Bgl family of antiterminator proteins, where its expression is positively regulated by β-glucosides (Bardowski et al. 1994). Antitermination by interaction of Bgl-type proteins with mRNA leaders also appears to operate in lactobacilli. For example, in *Lb. casei*, *lacT* is the first gene of the Lac-PTS operon and encodes a Bgl-type antiterminator protein which destabilizes a transcription terminator on the mRNA preceding *lacT* in a way similar to that in *L. lactis* (Alpert & Siebers 1997). These antiterminator proteins belong to a large class of regulators, which contain a duplicated, conserved domain (PRD), and are controlled by PTS-dependent phosphorylation (for review see Stülke et al. 1998). A model for PTS-mediated control of PRD-containing regulators postulates that in the absence of both glucose and the PTS-specific substrate, phosphorylation of both PRD's inactivates the regulator. In the absence of glucose, but in the presence of the substrate, phosphorylation of one of the PDR's would render the regulator active, while in the presence of both glucose and the substrate, neither of the PDR's would be phoshorylated, resulting in inactivation (Stülke et al. 1998).

A different signature of regulated genes in Gram-positive bacteria is the *c*is-acting *c*atabolite *r*esponsive *e*lement (*cre*), a sequence potentially able to form a hairpin (consensus sequence TGT/ANANCGNTNA/TCA, Weickert & Chambliss 1990; for review, see Hueck & Hillen 1995). As implied in its name, this element plays an important role in carbon catabolite repression (CCR), the phenomenon where expression of many catabolic genes is repressed when a readily metabolizable sugar is available. In contrast to the regulatory secondary structure elements described above, which seem to be invariably positioned in mRNA leaders, *cre*'s are either present upstream or overlap with the promoters, or are found in the 5′-coding moieties of the genes or operons they control. The salient model for the mechanics of CCR in Gram-positive bacteria is that the PTS component, HPr, becomes phosphorylated at Ser-46 by an ATP-dependent kinase in the presence of a readily metabolizable carbon source. Phosphorylated HPr interacts with the key regulatory protein of CCR, CcpA, such that the latter binds to the target *cre*, thus affecting expression of sugar catabolic operons (Hueck & Hillen 1995). Using a variety of experimental approaches including DNase I footprinting and gel retardation assays, convincing support for this model has been obtained (Fujita et al. 1995; Ramseier et al. 1995). Migration of a 26-mer containing *cre* was effectively retarded in the presence of wild-type CcpA and Hpr-Ser46-P, but not in the presence of unphosphorylated HPr. Also, a number of specific CcpA mutants completely failed to bind to the probe in the presence of phoshorylated HPr (Kraus et al. 1998). In further support of the model, when HPr-Ser-46P with a radioactively labeled phosphoryl group was used together with CcpA, the radioactivity was co-retarded with the *cre*-containing probe (W. Hillen, pers. commun.).

The data on the mechanisms underlying CCR in Gram-positive bacteria were obtained with a number of bacilli. Recently *cre* sequences have also been examined in LAB and the data strongly suggest that they are also the targets for CcpA-type proteins. Polyclonal antibodies raised against CcpA from *B. megaterium* cross-reacted with specific proteins of many

Gram-positives, including *L. lactis*, *Lb. casei* and *Lb. plantarum* (Küster et al. 1996). The gene encoding CcpA of *L. lactis* was cloned and sequenced and the protein was shown to repress transcription of the *gal* operon, which contains a *cre* site in the untranslated leader. CcpA activated the *las* operon, encoding the glycolytic enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase (Luesink et al. 1998). This operon also contains a *cre* located upstream of the -35 promoter sequence. A *cre* sequence is also present in the leader of *sacB* specifying EII^{Suc} of the sucrose gene cluster, but whether this site is required for CCR remains to be established (Luesink et al. 1999a). The gene encoding the protein modulating the *cre* binding activity of CcpA, HPr, was recently also isolated from *L. lactis*. Functional analysis of the protein using site-directed mutagenesis of the serine residue at position 46 demonstrated the role of HPr(Ser-P) as the co-repressor in CCR of the *gal* operon and as co-activator in catabolite activation of the *las* operon in *L. lactis* (Luesink et al. 1999b).

The main players in catabolite repression, *cre*, HPr and CcpA, are also present in lactobacilli. In *Lb. pentosus ccpA* as well as a *cre* sequence overlapping with the -35 sequence of the *xylA* promoter were identified (Lokman et al. 1994; Lokman et al. 1997). Two *cre*'s were found in *Lb. amylovorus*, one overlapping with, the other close to the 3′-end of the -10 sequence of the α-amylase gene (Lokman et al. 1997). In addition, evidence was obtained that both EII^{Man} and EII^{Fru} are important components of CcpA-mediated CCR in *Lb. pentosus* (Chaillou 1999). The *ccpA* gene has been isolated from *Lb. casei* and a mutational analysis indicated that disruption of *ccpA*, or deletion of *cre*, resulted in relief of catabolite repression of the *lacTEGF* operon (Monedero et al. 1997). The overlap of *cre* with various promoters suggests that their interaction with CcpA inhibits the initiation of transcription by RNA polymerase. It might be speculated that binding of CcpA to *cre*'s located upstream of promoters facilitates RNA polymerase binding and thus transcription initiation, whereas association of CppA with *cre*'s located within coding sequences might inhibit mRNA elongation through ribosome stalling.

Another example of a protein that can act as a repressor or as an activator is the global regulator protein, FNR (*f*umarate and *n*itrate as *r*educible substrates), which in *E. coli* is activated to a functional repressor or functional activator under conditions of anoxia (for review see Spiro & Guest 1990; Spiro 1994). FNR is homologous to the cyclic AMP receptor protein CRP, which mediates catabolite repression in Gram-negative bacteria. FNR differs from CRP in being an iron containing monomeric molecule, rather than a metal-free dimer, and in having an N-terminal extension containing four cysteine residues (Melville & Gunsalus 1990; Sharrocks et al. 1990; Green et al. 1991). Recently, the gene encoding an FNR-like protein (FLP) has been isolated from *Lb. casei*. The protein, when expressed in *E. coli*, failed to activate or to interfere with transcription from FNR-dependent promoters. This could be related to differences in the binding sites for FNR and FLP (TTGAT-N4-ATCAA and CCTGA-N4-TCAGG, respectively), in which the symmetrical TGA half-site motifs (underlined) in the former are separated by 6, and in the latter by 4 base pairs. Gel-retardation experiments showed that the reduced (dithiol-containing) form, rather than the disulphide containing form of FLP retarded target DNA, and it was proposed that the interconversion of the two forms, in which cupric ions may have a role, provides a redox-mediated transcriptional switch in *Lb. casei* (Gostic et al. 1998). *L. lactis* contains at least two *flp* genes, *flpA* and *flpB* (J. Kok, unpublished; Gostic et al. 1999). The derived amino acid sequences of FlpA and FpB show a high degree of similarity with that of *Lb. casei*. The *flpB* gene is located in an operon preceded by an FNR-binding site which also contains the transcriptional start site. As the TGA motifs in this site are separated by 6 bp, it resembles a 'true' FNR binding site rather than a FLP binding site. FlpB contains 3 His doublets in the first 14 N-terminal amino acids, providing a 'natural' His tag that allowed easy purification of the protein by means of Ni^{2+} column chromatography. Gel retardation experiments showed that the reduced form of the lacococcal FlpB bound to its FNR box at low pH. Transition metals enhanced FlpB binding and a chelator of transition metals (phenanthrolin) completely abolished binding (J. Kok and A. de Jong, unpublished). *FlpA* is located in an operon analogous to that containing *flpB*. The promoter regions of these two operons are associated with potential FNR sites at positions +4.5 for the *flpA* operon and -42.5 for the *flpB* operon, possibly suggesting that the respective operons are subject to positive and negative regulation, respectively. A *flp* double mutant led to a lower zinc content and increased hydrogen peroxide sensitivity, suggesting that FlpA and FlpB respond to metal-ion or oxidative stress and include systems concerned with the transport and storage of zinc (Gostic et al. 1999).

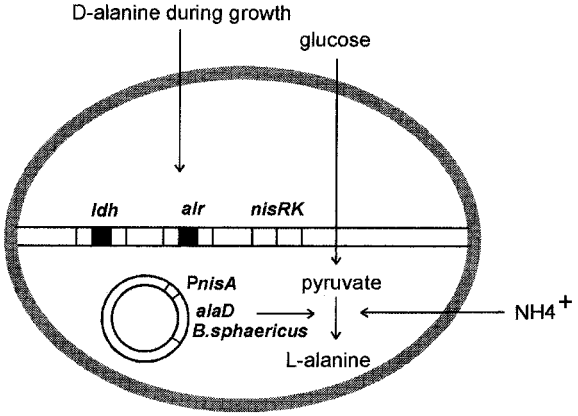

Figure 5. Schematic representation of the genetic structure of *L. lactis* for the production of enantiopure L-alanine. By disrupting the gene encoding lactate dehydrogenase (*ldh*), resting cells at elevated pH in the presence of excess ammonium convert pyruvate almost exclusively to L-alanine, catalysed by *alaD*-encoded alanine dehydrogenase from *B. sphaericus*. The conversion of L- to D-alanine was prevented by disruption of *alr*, specifying the synthesis of alanine racemase. High AlaD production was obtained by nisin activation of P*nisA* (for further details: see text). □■□: interrupted gene

ORF's, regulatory elements and metabolic engineering

The rapidly expanding knowledge of the function of ORF's from LAB and the way they are controlled, offers many opportunities for rational and efficient metabolic engineering. An elegant example, in which packages of initially unrelated knowledge were successfully integrated, concerns the conversion of *L. lactis* from homolactic to homoalanine fermentation. An interesting perspective of alanine-producing dairy strains would be their use as *in situ* producers of the natural sweetener alanine in cheese, buttermilk and yoghurt. Pyruvate, which plays a pivotal role in the sugar metabolism of LAB (for review see de Vos 1996) is the substrate for a number of enzymes which convert this intermediate to various compounds. Conversion of pyruvate to L-lactate by L-lactate dehydrogenase (L-LDH) with NADH as the cofactor was prevented by interrupting of the *ldh* gene through a Campbell-type recombination of an integration plasmid. To re-route the carbon flux towards alanine, the gene encoding alanine dehydrogenase (L-AlaDH) from *Bacillus sphaericus* was expressed in this mutant using the highly efficient nisin- controlled expression system (de Ruyter et al. 1996; Kuipers et al. 1997). The choice for this enzyme was prompted by the fact that it uses pyruvate as substrate, NADH as cofactor, and the km of the *B. sphaericus* enzyme and NADH are very close to that of *L. lactis* L-LDH. Alanine production was measured in a suspension of cells (first pre-loaded with L-AlaDH through nisin induction) in a phosphate buffer containing glucose with an excess of ammonium at alkaline pH, as the pH optimum for amination is between 8.5 and 9. Under these conditions glucose was completely and exclusively converted to alanine. However, the alanine produced still contained 15% of the D-isomer, which is required for peptidoglycan biosynthesis and is produced from the L-form by alanine racemase. To prevent the racemization, the alanine racemase gene in the *L. lactis* strain was disrupted by Campbell-type recombination. To ensure growth of the production cultures, D-alanine was supplied externally, which, after growth, was washed away before the cells were resuspended in the supplemented buffer for production purposes. This procedure resulted in the exclusive production of the L-enantiomer (over 99.5 purity) and represents, for the first time, an example of re-routing pathways towards a metabolite which is not a natural end product of the host (Hols et al. 1999). This example, schematically represented in Figure 5, also demonstrates that the knowledge acquired on ORF's and regulatory elements has created new possibilities for metabolic engineering which only a few years ago would have been considered quite unrealistic.

Complications

Present-day organisms are equipped with genomes that are optimally adapted to their environments. The question arises, why would we then like to improve them. The answer is, of course, that man uses organisms for his own benefit, and consequently, tries to modify them by breeding programmes, selection and, with the advent of the recombinant DNA technology, by genetic modification. With sufficient knowledge about genes and their regulation, which will undoubtedly be accelerated now that the complete sequence of genome of *L. lactis* has been determined, and that of *Lb. acidophilus* will be soon available, almost all modifications to suit the fermentation and pharmaceutical industry are within reach. However two constraints exist, one arising from biological considerations, the other from societal perception.

Biological constraints

Many biological constrains exist, or are conceivable, which preclude the production of GMO's with properties designed for industrial use. Some obvious obstacles will be mentioned briefly. First, procedures should exist for the introduction of (sufficient) DNA into the host to be modified. If not available, well-transformable intermediary hosts can sometimes be used as conjugal donors for the DNA to be transferred. The search for proper transfer systems can be tedious, as examplified by finding the conditions and the proper vector for making *Lb. delbrueckii* subsp. *bulgaricus* transformable (Sasaki et al. 1993). Such searches, in which all possible variables are examined, do not always meet with success as is, for instance, the case for *Oenococcus oeni*, which remains as yet untransformable (M. Zuñiga, pers. comm.).

Second, the expression of heterologous genes in LAB may not be efficient when the codon usages of the host and the donor of the heterologous DNA are seriously mismatched. Conceivably, attempted overexpression of the heterologous material might even titrate away amino acids required for the synthesis of the host's own proteins, thus interfering in a negative way with cellular metabolism. In principle, such complications can be circumvented by redesigning the gene to be expressed, such that the codon content is more comparable to that used by the host, but even for medium-sized genes this is a laborious and costly affair.

Third, the gene product to be synthesized could form insoluble aggregates and, when required to be exported from the cell, fails to do so, or when transported, is degraded in the medium. Whether such problems relate to LAB, has not yet been thoroughly investigated. However, *B. subtilis*, which efficiently secretes various homologous exo-enzymes, often fails to produce exported proteins from heterologous sources (Simonen & Palva 1993).

Fourth, the presence of paralogous genes may impede functional analysis, as the mutant phenotype may be only produced when all paralogues have been inactivated.

In principle, given time and understanding, several of these and other barriers may be surmounted. Fantastic progress has been made during the past 15 years, where our almost complete ignorance of the genetics and molecular biology of *L. lactis* has been converted into a vast body of scientific information. This progress makes one optimistic about overcoming these barriers for the production of genetically modified LAB for improving dairy processing, food products, and making these organisms useful for human and animal health care purposes.

Societal constraints

In the present space of time a substantial proportion of the conscious consumers prefer 'natural' foods. Manipulation of food induces an attitude of caution by the consumer towards these foods. This concern immediately touches on the use of genetically modified LAB for food production. What scientists and industries could do to convince consumers and regulatory public bodies that this concern is unnecessary and unfounded was excellently reviewed by Verrips & van den Berg (1996). In our opinion, to address the consumer's concern and guide modified LAB's through regulatory procedures, modified LAB should retain their original properties as closely as possible, in that: (1) the improvement or new property should preferably be caused by mutation(s) also occurring in Nature; (2) the new property should preferably be caused by rearrangements of the existing genetic information (self-cloning); (3) when the incorporation of heterologous genes is required to produce the desired LAB, these genes should preferentially be obtained from other food-grade or at least harmless organisms; (4) the presence of antibiotic markers should be avoided; and (5) heterologous genes should be stably integrated into the chromosome, rather than carried on plasmids to reduce the possibility of dissimination of the property to other bacteria present in food, the digestive tract, or the environment. Fortunately, a collection of well-defined integration vectors and genetic procedures exists which do not leave behind a trace of vector DNA and meet the points raised in (2) and (3) (de Vos et al. 1989; Leenhouts 1995; Leenhouts et al. 1996; Leenhouts et al. 1998). In addition, when induced gene expression is required, several completely harmless means are at our disposal, such as induction by nisin, temperature, salt and pH shifts.

The following example illustrates how recombinant DNA technology was used by a starter culture company attempting to obtain an improved strain of *L. lactis* in which the improvement was due to a spontaneous mutation. The aim was to isolate a spontaneous mutant of the *L. lactis* biovar. *diacetylactis ald* gene, specifying acetolactate decarboxylase, which converts acetolactate (ALA) into acetoin. A mutation in *ald* would lead to an increased pool of ALA, the imme-

diate precursor of diacetyl, and result in increased diacetyl production. As ALA is also a precursor for the synthesis of the branched amino acids isoleucine, leucine and valine, and the presence of leucine allosterically activates ALD, the pool of ALA is depleted and, consequently, will not be available for the synthesis of diacetyl and the branched amino acids. Thus the cells will cease to grow. However, mutants that produce no ALD accumulate ALA and, therefore, continue to synthesize the branched amino acids even in the presence of leucine. Such mutants will continue to grow in the absence of valine (Swindell et al. 1996; Goupil-Feuillerat et al. 1997). Unfortunately, this selection procedure can not be applied to many dairy lactococci, because they have irreversibly lost their capacity to synthesize branched amino acids. To overcome this problem, several strains of the *diacetylactis* biovar were first transformed with a plasmid carrying the genes for isoleucine and valine production. By subsequent selection for transformants capable of growth in the presence of leucine but in the absence of valine, *aldB* mutants were obtained. Finally, curing of the strain of the plasmid used to make the selection possible, yielded a strain with a plasmid complement that was indistinghuisable from the wild-type and produced approximately 27 times more diacetyl (Curic et al. 1999). Although the modified phenotype of the strain is caused by just one spontaneous mutation, nevertheless its application in the manufacture of dairy products must, according to current EU regulations, still be approved, because it descended from a progenitor bearing a recombinant plasmid.

In our opinion, the need for an approval process is unnecessary. The product rather than the way in which the modification was produced should be taken as the sole criterion for approval, as is currently practiced by US regulatory agencies.

Concluding remarks

Since the development of the first plasmid cloning vector from a small cryptic lactococcal plasmid, which was capable of replication in both Gram-positive and Gram-negative bacteria (Kok et al. 1984) and the development of efficient electro-transformation protocols (Chassy & Flickinger 1987; Luchansky et al. 1988; Holo & Nes 1989), the field of LAB genetics and molecular biology has developed explosively. In Europe, this development was further catalysed by the generous and constant support from the EU, which, through its conditions for proposal granting, united individual research groups into tightly cooperating consortia. With the advent of automated DNA sequencing facilities and the development of advanced computer software for the analysis of ORF's, operons, regulatory elements and even complete genomes, the means has become available to quickly obtain a view of the functionalities of these genetic entities. Extrapolating the past developments to the future, one should anticipate that knowledge of LAB and their interactions with the environment will increase at even a faster rate than before. The recent developments in DNA chip technology represents an extremely valuable asset to analyse ways in which bacteria adapt their metabolism when transcending to stationary growth or after exposure to stress conditions.

Although a tremendous arsenal of techniques, biological tools and equipment is presently available to increase our global and detailed knowledge of LAB, an overwhelmingly large body of work remains to be done. For instance, if hybridization-based expression studies using DNA micro-arrays would show that certain groups of genes are switched on under particular conditions, one would like to know the function of such genes and how they are controlled. If no clues are available as to their possible functions, only a mutation or expression approach will yield that information. To facilitate this, it would be extremely advantageous to structure a programme for gene function analysis, similar to that presently employed for yeast and *B. subtilis*. Now that the complete genome of *L. lactis* has been sequenced and the sequencing of the *Lb. acidophilus* genome is well under way, excellent conditions exist for pursuing this goal.

With our present knowledge, the construction of tailor-made LAB strains for use in the dairy industry is already possible. Ingenious inducible gene expression tools and integration systems, that do not leave behind a trace of vector DNA upon insertion into the LAB chromosomes, have been developed. Yet, these are applied much less than desired, the obvious barrier being the hesitation to use genetically modified LAB, which, in turn derives from public fear for new technologies. The main obstacle for application is, in many cases, not the present state of our scientific knowledge and technical capabilities, but rather public perception. Much needs to be done in terms of frank and open information to modify this perception. Yet, it would seem that this process is already under way. Permission was granted recently to allow a tasting panel to judge the quality of experimental

cheeses produced with a LAB which contained and expressed a non-LAB gene. When this encouraging trend continues, we anticipate that the fundamental knowledge on LAB acquired at universities, research institutions and industries will be channelled much more quickly to provide for improved strains, not only for the manufacturing of safer, more wholesome and tastier products, but also for applications in the human and veterinary health care sector.

Acknowledgements

We gratefully acknowledge the permission of several colleagues to quote from preprints or unpublished results and helpful suggestions otherwise, notably M.J. Gasson, M. van de Guchte, W. Hillen, H. Israelsen, E. Johansen, T.R. Klaenhammer, O.P. Kuipers, P.H. Pouwels and P. Renault.

References

Aggarwal AK, Rodgers DW, Drottar M, Ptashne M & Harrison SC (1988) Recognition of a DNA operator by the repressor of phage 434: a view at high resolution. Science 242: 899–907

Alpert CA & Siebers U (1997) The *lac* operon of *Lactobacillus casei* contains *lacT*, a gene coding for a protein of the BglG family of transcriptional antiterminators. J. Bacteriol. 179: 1555–1562

Bardowski J, Ehrlich SD & Chopin A (1994) BglR protein, which belongs to the BglG family of transcriptional antiterminators, is involved in β-glucoside utilization in *Lactococcus lactis*. J. Bacteriol. 176: 5681–5685

Beamer LJ & Pabo CO (1992) Refined 1.8 Å crystal structure of the lambda repressor-operator complex. J. Mol. Biol. 227: 177–196

Bernhardt J, Volker U, Volker A, Antelmann H, Schmidt R, Mach H & Hecker M (1997) Specific and general stress proteins in *Bacillus subtilis* – A two dimensional protein electrophoretic study. Microbiology 143: 999–1017

Biswas I, Gruss A, Ehrlich SD & Maguin E (1993) High efficiency gene inactivation and replacement system for gram-positive bacteria. J. Bacteriol. 175: 3628–3635

Botstein D (1980) A theory of modular evolution for bacteriophages. Ann. N.Y. Acad. Sci. 354: 484–491

Boyce JD, Davidson BE & Hilier AJ (1995) Sequence analysis of the *Lactococcus lactis* temperate bacteriophage BK5-T and demonstration that the phage DNA has cohesive ends. Appl. Environ. Microbiol. 61: 4089–4098

Brüssow H, Bruttin A, Desiere F, Lucchini S & Foley S (1998) Molecular ecology and evolution of *Streptococcus thermophilus* bacteriophages – a review. Virus Genes 16: 95–109

Bruttin A, Desiere F, Lucchini S, Foley S & Brüssow H (1997a) Characterization of the lysogeny DNA module from the temperate *Streptococcus thermophilus* bacteriophage øSfi21. Virology 233: 136–148

Bruttin A, Foley S & Brussow H (1997b) The site-specific integration system of the temperate *Streptococcus thermophilus* bacteriophage ØSfi21. Virology 237: 148–158

Buist G (1997) AcmA of *Lactococcus lactis*, a cell-binding major autolysin. Doctoral thesis, University of Groningen

Buist G, Kok J, Leenhouts KJ, Drabowska M, Venema G & Haandrikman AJ (1995) Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation. J. Bacteriol. 177: 1554–1563

Challou S (1999) Regulation of xylose and α-xyloside transport and metabolism in lactobacilli. Doctoral thesis, University of Amsterdam

Chandry PS, Moore SC, Boyce JD, Davidson BE & Hillier AJ (1997) Analysis of the DNA sequence, gene expression, origin of replication and modular structure of the *Lactococcus lactis* lytic bacteriophage sk1. Mol. Microbiol. 26: 49–64

Chassy BM & Flickinger JL (1987) Transformation of *Lactobacillus casei* by electroporation. FEMS Microbiol. Lett. 44: 173–177

Christiansen B, Johnsen MG, Stenby E, Fogensen FK & Hammer K (1994) Characterization of the lactococcal phage TP901-1 and its site-specific integration. J. Bacteriol, 176: 1069–1076

Christiansen B, Brønsted L, Vogensen FK & Hammer K (1996) A resolvase-like protein is required for the site-specific integration of the temperate lactococcal bacteriophage TP901-1. J. Bacteriol. 178: 5164–5173

Chung K, Chung SK & Batt CA (1992) Antisense RNA directed against the major capsid protein of *Lactococcus lactis* subsp. *cremoris* 4-1 confers partial resistance to the host. Appl. Microbiol. Biotechnol. 37: 79–83

Curic M, Stuer-Lauridsen B, Renault P & Nilsson D (1999) A general method for selection of α-acetolactate decarboxylase deficient mutants of *Lactococcus lactis* to improve diacetyl formation. Appl. Environ. Microbiol. 65: 1202–1206

Delorme C, Ehrlich SD & Renault P (1999) Regulation of the expression of the *Lactococcus lactis* histidine operon. J. Bacteriol. 181: 2026–2037

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

de Ruyter PGGA, Kuipers OP, Meyer WC & de Vos WM (1997) Food- grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening. Nature Biotechn. 15: 976–979

de Sazieu A, Certa U, Warrington J, Gray C, Keck W & Mous J (1998) Bacterial transcript imaging by hybridization of total RNA to oligonucleotide arrays. Nature Biotech. 16: 45–48

Desiere F, Lucchini S, Bruttin A, Zwahlen M-C & Brüssow H (1997) A highly conserved DNA replication module from *Streptococcus thermophilus* phages is similar in sequence and topology to a module from *Lactococcus lactis* phages. Virology 234: 372–382

Desiere F, Lucchini S & Brüssow H (1998) Evolution of *Streptococcus thermophilus* bacteriophage genomes by modular exchanges followed by point mutations and small deletions and insertions. Virology 241: 345–356

de Vos WM, Vos P, Simons G & David S (1989) Gene organization and expression in mesophilic lactic acid bacteria. J. Dairy Sci. 72: 3398–3405

de Vos WM & Simons G (1994) Gene cloning and expression systems in lactococci. In: Gasson MJ & de Vos WM, (Eds) (pp. 52–105). Genetics and biotechnology in lactic acid bacteria. Blackie Academic and Professional, Glasgow

de Vos WM (1996) Metabolic engineering of sugar metabolism in lactic acid bacteria. Antonie van Leeuwenhoek 70: 232–242

Djordjevic GM, O'Sullivan DJ, Walker A, Concling MA & Klaenhammer TR (1997) A triggered suicide system designed as a defence against bacteriophages. J. Bacteriol. 179: 6741–6748

Djordjevic GM & Klaenhammer TR (1997) Bacteriophage-triggered defence systems: phage adaptations and design improvements. Appl. Environ. Microbiol. 63: 4370–4376

Dupont L, Boizet-Bonhoure B, Coddeville M, Auvray F & Ritzenthaler P (1995) Characterization of genetic elements required for site-specific integration of *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteriophage mv4 and construction of an integration-proficient vector for*Lactobacilus plantarum*. J. Bacteriol. 177: 586–595

Fujita Y, Miwa Y, Galinier A & Deutcher J (1995) Specific recognition of the *Bacillus subtilis gnt cis*-acting catabolite- responsive element by a protein complex formed between CcpA and seryl-phosphorylated HPr. Mol. Microbiol. 17: 953–960

Gasson MJ (1996) Lytic systems in lactic acid bacteria and their bacteriophages. Antonie van Leeuwenhoek 70: 147–159

Gostick DO, Green J, Irvine AS, Gasson MJ & Guest JR (1998) A novel regulatory switch mediates the FNR-like protein of *Lactobacillus casei*. Microbiology 144: 715–717

Gostick DO, Griffin HG, Shearman CA, Scott C, Green J, Gasson MJ & Guest JR (1999) Two operons that encode FNR-like proteins in *Lactococcus lactis*. Mol. Microbiol. 31: 1523–1535

Goupil-Feuillerat N, Cocaign-Bousquet M, Godon JJ, Ehrlich SD & Renault P (1997) Dual role of α-acetolactate decarboxylase in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. 179: 6285–6293

Green J, Trageser M, Six S, Unden G & Guest JR (1991) Characterization of the FNR protein of *Escherichia coli*, an iron binding transcriptional regulator. Proc. R. Soc. Lond. Ser B 244: 137–144

Hill C, Miller LA & Klaenhammer TR (1990) Cloning, expression and sequence determination of a bacteriophage fragment encoding resistance in *Lactococcus lactis*. J. Bacteriol. 172: 6419–6426

Holo H & Nes IF (1989) High frequency transformation, by electroporation of *Lactococcus lactis* subsp. *lactis* with glycine in osmoticaly stabilized media. Appl. Environ. Microbiol. 55: 3119–3123

Hols P, Kleerebezem M, Schanck AN, Ferain T, Hugenholtz J, Delcour J & de Vos WM (1999) Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. Nature Biotech. 7: 588–592

Hueck CJ & Hillen W (1995) Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the Gram-positive bacteria? Mol. Microbiol. 15: 395–401

Johnsen MG, Appel KF, Madsen PL, Vogensen FK, Hammer K & Arnau J (1996) A genomic region of lactococcal temperate phage TP901-1 encoding major virion proteins. Virology 218: 306–315

Kim SG & Batt CA (1991) Antisense mRNA-directed bacteriophage resistance in *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 57: 1109–1113

Kodaira K-I, Oki M, Kakikawa M, Watanabe N, Hirakawa M, Yamada K & Taketo A (1997) Genome structure of the *Lactobacillus* temparate phage øg1e: the whole genome sequence and the putative promoter/repressor system. Gene 187: 45–53

Kok J, van der Vossen JMBM & Venema G (1984) Construction of plasmid cloning vectors which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. 48: 726–731

Kraus A, Küster E, Wagner A, Hoffmann K & Hillen W (1998) Identification of a co-repressor binding site in catabolite repressor protein CcpA. Mol. Microbiol. 30: 955–963

Kuipers OP, de Ruyter PGGA, Kleerebezem M & de Vos WM (1997) Controlled overexpression of proteins by lactic acid bacteria. Trends Biotechn. 15: 135–140

Küster E, Luesink EJ, de Vos WM & Hillen W (1996) Immunological crossreactivity to the catabolite control protein CcpA from *Bacilus megaterium* is found in many Gram-positive bacteria. FEMS Microbiol. Lett. 139: 109–115

Law J, Buist G, Haandrikman A, Kok J, Venema G & Leenhouts K (1995) A system to generate chromosomal mutations in *Lactococcus lactis* which allow fast analysis of targetted genes. J. Bacteriol. 177: 7011–7018

Leenhouts K (1995) Integration strategies and vectors. In: Ferretti JJ, Gilmore MS, Kleinhammer TR & Brown F, (Eds) Genetics of Streptococci, Enterococci and Lactococci. (pp 523–530). Dev. Biol. Stand. Basel, Karger

Leenhouts KJ, Kok J & Venema G (1991) Lactococcal plasmid pWVO1 as an integration vector for lactococci. Appl. Environ. Microbiol. 57: 2562–2567

Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M, Venema G & Kok J (1996) A general system for generating unlabelled gene replacements for lactococci. Mol. Gen. Genet. 353: 217–224

Leenhouts K, Bolhuis A, Venema G & Kok J (1998) Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 49: 417–423

Lieb M (1981) A fine structure map of spontaneous and induced mutations in the lambda repressor gene, including insertions of IS elements. Mol. Gen. Genet. 184: 364–371

Lillehaug D & Birkeland NK (1993) Characterization of genetic elements required for site-specific integration of the temperate lactococcal bacteriophage ØLC3 and construction of integration-negative ØLC3 mutants. J. Bacteriol. 175: 1745–1755

Lillehaug D, Nes IF & Birkeland N-K (1997) A highly efficient and stable system for site-specific integration of genes and plasmids into ØLC3 attachment site (*attB*) of the *Lactococcus lactis* chromosome. Gene 172: 6419–6426

Lokman BC, Leer RJ, van Sorge R & Pouwels PH (1994) Promoter analysis and transcriptional regulation of *Lactobacillus pentosus* genes involved in xylose catabolism. Mol. Gen. Genet. 245: 117–125

Lokman BC, Heerikhuisen M, Borsboom Y & Pouwels PH (1997) Characterization of the L*actobacillus pentosus ccpA* gene and its involvement in catabolite repression. J. Bacteriol. 179: 5391–5397

López R, García E, García P & García JL (1995) Architecture and domain interchange of the pneumococcal cell wall lytic enzymes. In: Ferretti JJ, Gilmore MS, Klaenhammer TR & Brown F, (Eds) Genetics of Streptococci, Enterococci and Lactococci. (pp. 273–281). Dev. Biol. Stand., Basel, Karger.

Lubbers MW, Waterfield NR, Beresford TPJ, Le Page RWF & Jarvis AW (1995) Sequencing and analysis of the prolate-headed lactococcal bacteriophage c2 genome and identification of the structural genes. Appl. Environ. Microbiol. 61: 4348–4356

Lucchini S, Desiere F & Brüssow H (1998) The structural gene module in *Streptococcus thermophilus* bacteriophage øSfi11 shows a hierarchy of relatedness to Siphoviridae from a wide range of bacterial hosts. Virology 246: 63–73

Luchansky JB, Muriana PM & Kaenhammer TR (1988) Application of electroporation for transfer of plasmid DNA into *Lactobacillus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Bacillus*, *Staphylococcus*, *Enterococcus* and *Propionibacterium*. Mol. Microbiol. 2: 637–646

Luesink EJ, van Herpen REMA, Grossiord BP, Kuipers OP and de Vos WM (1998) 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. 30: 789–798

Luesink EJ, Marugg JD, Kuipers OP & de Vos WM (1999a) Characterization of the divergent *sacBK* and *sacAR* operons involved

in sucrose utilization by *Lactococcus lactis*. J. Bacteriol. 181: 1924–1926

Luesink EJ, Beumer CMA, Kuipers OP & de Vos WM (1999b) Molecular characterization of the *Lactococcus* lactis *ptsH1* operon and analysis of the regulatory role of HPr. J. Bacteriol. 181: 764–771

Madsen PL & Hammer K (1998) Temporal transcription of the lactococcal temperate phage TP901-1 and DNA sequence of the early promoter region. Microbiology 144: 2203–2215

Madsen SM, Arnau J, Vrang A, Givskov M & Israelsen H (1999) Molecular analysis and characterization of a pH and stationary phase inducible promoter P170 from *Lactococcus lactis*. Mol. Microbiol. 32: 75–87

Maguin E, Duwat P, Hege T, Ehrlich D & Gruss A (1992) New thermosensitive plasmid for Gram-positive bacteria. J. Bacteriol. 174: 5633–5638

Melvile SB & Gunsalus RB (1990) Mutations in *fnr* that alter anaerobic regulation of electron- associated genes in *Escherichia coli*. J. Biol. Chem. 265: 18733–18736

Mikkonen M (1996) Doctoral thesis, University of Oulu, Finland

Monodero V, Gosalbes MJ & Pérez-Martínez G (1997) Catabolite repression in *Lactobacillus casei* is mediated by CcpA. J. Bacteriol. 179: 6657–6664

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

Nauta A, van den Burg B, Karsens H, Venema G & Kok J (1997) Design of thermolabile bacteriophage repressor mutants by comparative molecular modelling. Nature Biotech. 15: 980–983

Nauta A (1997) Molecular characterization and exploitation of the temperate *Lactococcus lactis* bacteriophage r1t. Doctoral thesis, University of Groningen

Neve H, Zenz KI, Desiere F, Koch A, Heller KJ & Brüssow H (1998) Comparison of the lysogeny modules from the temperate *Streptococcus thermophilus* bacteriophages TP-J34 and Sfi21: implications for the modular theory of phage evolution. Virology 241: 61–72

O'Sullivan DJ, Hill C & Klaenhammer TR (1993) Effect of increasing the copy number of bacteriophage origins of replication, in *trans*, on incoming-phage proliferation. Appl. Environ. Microbiol. 5449–2456

O'Sullivan DJ, Zagula K & Klaenhammer TR (1995) *In vivo* restriction by *Lla*I is encoded by three genes, arranged in an operon with *llIM*, on the conjugative *Lactococcus* plasmid pTR2030. J. Bacteriol. 177: 134–143

O'Sullivan DJ, Walker SA, West SG & Klaenhammer TR (1996) Development of an expression strategy using a lytic phage to trigger explosive plasmid amplification and gene expression. Biotechnology 10: 82–87

Pabo CO, Sauer RT, Sturtevant JM & Ptashne M (1979) The λ repressor contains two domains. Proc. Natl. Acad. Sci. USA 76: 1608–1612

Parreira R, Valyasev R, Lerayer ALS, Ehrlich SD & Chopin M-C (1996) Gene organisation and transcription of a late-expressed region of a *Lactococcus lactis* phage. J. Bacteriol. 178: 6158–6165

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

Ptashne M (1992) A genetic switch. Phage λ and higher organisms. Blackwell Scientific Publications and Cell Press, Cambridge, Mass.

Ramseier TM, Reizer J, Küster E, Hillen W. & Saier MH (1995) In vitro binding of the CcpA protein of *Bacillus megaterium* to *cis*-acting catabolite responsive elements (CRE's) of Gram- positve bacteria. FEMS Microbiol. Lett. 129: 207–214

Ramsay G (1998) DNA chips: state-of-the-art. Nature Biotech. 16: 40–48

Raya RR, Fremaux C, De Antoni GL & Klaenhammer TR (1992) Site- specific integration of the temperate bacteriophage Øadh into the *Lactobacillus gasseri* chromosome and molecular characterization of the phage (*attP*) and bacterial (*attB*) attachment sites. J. Bacteriol. 174: 5584–5592

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

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

Sanders JW, Leenhouts K, Burghoorn J, Brands JR, Venema G & Kok J (1998b) A chloride-inducible acid resistance mechanism in*Lactococcus lactis* and its regulation. Mol. Microbiol. 27: 299–310

Sasaki T, Ito Y & Sasaki Y (1993) Proc. 4th Symp. on lactic acid bacteria; genetics, metabolism and applications; de Vos WM, JMJ Huis in 'tVeld, & Poolman B., (Eds). Special issue FEMS Microbiol. Rev., poster abstract P8

Schena M, Heller RA, Theriault TP, Konrad K, Lachenmeier E & Davis RW (1998) Microarrays: biotechnology's discovery platform for functional genomics. Trends in Biotechn. 16: 301–306

Schmid R, Bernhardt J, Antelmann H, Volker A, Volker U & Hecker M (1997) Identification of vegetative proteins for a two-dimensional protein index of *Bacillus subtilis*. Microbiology 143: 991–998

Schnetz K, Stülke J, Gertz S, Krüger S, Krieg M, Hecker M & Rak B (1996) LicT, a *Bacillus subtilis* transcriptional antiterminator protein of the BglG family. J. Bacteriol. 178: 1971–1979

Schouler C, Ehrlich SD & Chopin M-C (1994) Sequence and organization of the lactoccal prolate-headed bIL67 phage genome. Microbiology 140: 3061–3069

Sharrocks AD, Green J & Guest JR (1990) *In vivo* and *in vitro* mutants of FNR, the anaerobic transcriptional regulator of *E. coli*. FEBS Lett. 270: 119–132

Shearman CA, Underwood HM, Jury K & Gasson M (1989) Cloning and DNA sequence analysis of a *Lactococcus* bacteriophage lysis gene. Mol. Gen. Genet. 218: 214–221

Sheehan MM, García JL, López R & García P (1997) The lytic enzyme of the pneumococcal phage DP-1: a chimeric lysin of intergeneric origin. Mol. Microbiol. 25: 717–725

Simonen S & Palva I (1993) Protein secretion in *Bacillus* species. Microbiol. Rev. 57: 109–137

Spiro S & Guest JR (1990) FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. FEMS Microbiol. Rev. 75: 399–428

Spiro S (1994) The FNR family of transcriptional regulators. Antonie van Leeuwenhoek 66: 23–36

Stanley E, Fitzgerald GF, Le Marrec C, Fayard B & van Sinderen D (1997) Sequence analysis and characterization of øO1205, a temperate bacteriophage infecting *Streptococcus thermophilus* CNRZ1205. Microbiology 143: 3417–3429

Stülke J, Arnaud M, Rapoport G & Martin-Verstraete I (1998) PRD-a protein domain involved in PTS-dependent induction and carbon catabolite repression of catabolic operons in bacteria. Mol. Microbiol. 28: 865–874

Swindell SR, Benson KH, Griffin HG, Renault P, Ehrlich SD & Gasson MJ (1996) Genetic manipulation of the pathway for diacetyl metabolism in *Lactococcus lactis*. Appl. Environ. Microbiol. 62: 2641–2643

Vagner V, Dervyn E & Ehrlich SD (1998) A vector for systematic gene inactivation. Microbiology 144: 3097–3104

van de Guchte M, Kok J & Venema G (1992) Gene expression in*Lactococcus lactis*. FEMS Microbiol. Rev. 88: 73–92

van de Guchte M, Daly C, Fitzgerald GF & Arendt EK (1994) Identification of *int* and *attP* on the genome of lactococcal bacteriophage Tuc2009 and their use for site-specific plasmid integration in the chromosome of Tuc2009-resistant *Lactococcus lactis* mg1363. Appl. Environ. Microbiol. 60: 2324–2329

van de Guchte M, Ehrlich SD & Chopin A (1998) $tRNA^{TRP}$ as a key element of antitermination in the *Lactococcus lactis trp* operon. Mol. Microbiol. 29: 61–74

van Sinderen D, Karsens H, Kok J, Terpstra P, Ruiters MHJ, Venema G & Nauta A (1996) Sequence analysis and molecular organization of the temperate lactococcal bacteriophage r1t. Mol. Microbiol. 19: 1343–1355

van Sinderen D, Seegers J, McGrath S, Arend E, Leach S, van de Guchte M, Daly C & Fitzgerald[1] GF, (unpublished)

van Sinderen D, Walsh L, Stanley E & Fitzgerald[2] GF, (unpublished)

Verrips CT & van den Berg DJC (1996) Barriers to application of genetically modified lactic acid bacteria. Antonie van Leeuwenhoek 70: 299–316

Vriend G (1990) WHAT IF, a molecular modelling and drug design program. J. Molec. Graph. 8: 52–56

Walker SA & Klaenhammer TR (1998) Molecular characterization of a phage-inducible middle promoter and its transcriptional activation from the lactococcal bacteriophage Ø31. J. Bacteriol. 180: 921–931

Weickert MJ & Chambliss GH (1990) Site directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA 87: 6238–6242

Young R (1992) Bacteriophage lysis: mechanisms and regulation. Microbiol. Rev. 56: 430–481

Young R & Bläsi (1995) Holins: form and function in bacteriophage lysis. FEMS Microbiol. Rev. 17: 191–205