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Controlled overproduction of proteins by lactic acid bacteria

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Lactic acid bacteria are widely used in industrial food fermentations, contributing to flavour, texture and preservation of the fermented products. Here we describe recent advances in the development of controlled gene expression systems, which allow the regulated overproduction of any desirable protein by lactic acid bacteria. Some systems benefit from the fact that the expression vectors, marker genes and inducing factors can be used directly in food applications since they are all derived from food-grade lactic acid bacteria. These systems have also been employed for the development of autolytic bacteria, suitable for various industrial applications.

In the past few decades, numerous genetic tools have been developed for lactic acid bacteria (LAB), Gram-positive bacteria that are used in industrial food fermentations. These include the development of transformation, transduction and conjugation procedures[1], as well as the isolation and characterization of plasmid-free strains, stable cloning vectors, efficient promoters and the functional analysis of a plethora of genes encoding relevant proteins[2–4], any number of which could improve the properties of fermented products upon overproduction. Most advances in these areas have been made with strains of *Lactococcus lactis* that are widely used as starter cultures for cheese and other dairy products[1–4].

A desirable feature of the genetic tools that are being developed to modify LAB is their suitability for safe application in food. Ideally, this means that a so-called food-grade cloning host is a microorganism with a long history of safe use in food, which is currently true for all lactic acid bacteria. These hosts should contain plasmids or other DNA elements (e.g. transposons, bacteriophage DNA) that do not harbour any undesired genes, such as those coding for antibiotic-resistance markers. When use is made of an inducing factor for gene expression, this factor should be acceptable in food products, for example small inorganic molecules like salts, organic molecules such as saccharides or fatty acids, or proteinaceous compounds originating from LAB. Alternatively, changes in growth conditions, including pH, temperature, aeration or even phage infection, would be an acceptable way to induce certain activities. For some applications, expression systems should consist of elements that all originate from a single LAB species, since this results in strains improved by self-cloning. Several systems for self-cloning have been described, including one that uses a complementing *lacF* gene as a selectable marker for growth on lactose[5] and a system based on complementation of nonsense mutants in the purine biosynthetic pathway using plasmids with an ochre suppressor as a selectable marker[6].

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Regulated gene expression systems for lactic acid bacteria

Various expression systems using stable, broad host-range vectors and constitutive promoters have been described to date for different LAB, e.g. lactococci, lactobacilli, streptococci and leuconostocs[1–4]. In addition, chromosomal integration of genes at desirable loci is commonly used in microorganisms that can be transformed with relatively high efficiencies[2]. Here we will focus exclusively on expression systems that are based on transformation of LAB with plasmids containing effective expression signals, which can be regulated by inducers, repressors or environmental factors. Clearly, there is a need for such regulated expression systems for LAB, since ideally they will allow the controllable overproduction of proteins at high levels at any desired moment during industrial fermentation. This also means that proteins that are deleterious to the cell can be produced, because only minute amounts of these proteins will be produced by cells in the uninduced state. Regulated gene expression also

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Table 1. Characteristics of inducible expression systems for LAB

LAB	Inducible element	Inducing factor	Expressed gene(s)	Ratio induced:uninduced	Refs
L. lactis	*lacA* or *lacR* promoter	Lactose	*cat-86, luxAB*	<10	7,8
L. lactis	*lacA*/T7 promoter	Lactose	Gene for TTFC	<20	9
L. lactis	*dnaJ* promoter	High temperature	*amyS*	<4	10
L. lactis	*sodA* promoter	Aeration	*lacZ*	2	11
L. lactis	*prtP* or *prtM* promoter	Absence of peptides	*gusA*	<8	12,13
L. lactis	repressor/operator ϕr1t	Mitomycin C	*lacZ*	70	14
Lb. pentosus	*xylA* promoter	Xylose	*cat-86*	60–80	15
L. lactis	PA170 promoter	Low pH, low temperature	*lacZ*	50–100	17
L. lactis	*trpE* promoter	Absence of tryptophan	*lacZ*	100	16
L. lactis	ϕ31 promoter and *ori*	ϕ31 infection	*lacZ*	>1000	18
L. lactis	*nisA* or *nisF* promoter	Nisin	*gusA, pepN, lytHA, nox estA, ptsH, pepO, ccpA*	>1000	19–21

provides the opportunity to study the effects of varying intracellular levels of protein production on metabolic pathways, provided that the level of expression is correlated with the amount of inducer added. Several studies on regulated gene expression, mainly in *L. lactis*, have been described, and some of these will be discussed here (Table 1).

The lacA*-promoter based systems*

One of the first examples of regulated gene expression was described for the *lacA* promoter of the lactose operon of *L. lactis*[7]. Fragments containing the *lacA* promoter and increasing lengths of flanking regions were fused to the promoterless *cat-86* gene. It was shown that expression was dependent on the LacR repressor, being derepressed during growth on lactose by the formation of the inducer tagatose-6-phosphate. Sequences downstream and upstream significantly enhanced promoter activity (R. J. van Rooijen, PhD Thesis, University of Wageningen, 1993). Protein engineering studies with LacR yielded mutant repressors with improved activity. The level of *cat-86* expression in cells grown in lactose was 5–10-fold greater than in those grown in glucose (Ref. 7; R. J. van Rooijen, PhD Thesis). The *lacA* and *lacR* promoter activities were also studied by Eaton *et al.*[8], who reported a 5- and 7-fold induction of *lacA* and *lacR* promoters, respectively, by growth on lactose, using the reporter genes *luxAB*. The lactose-inducible system always shows a significant background expression on glucose, hampering the production of 'lethal' proteins. Moreover, for industrial purposes, it is not very attractive to switch the sugar during large-scale fermentations.

An improvement on the use of the *lacA* promoter was realized by Wells *et al.*[9], who described the development of a lactococcal system that uses the T7-polymerase of *Escherichia coli* bacteriophage T7 to transcribe target genes. The polymerase gene itself is expressed under control of the *lacA* promoter, and induction takes place by growing the cells in lactose, during which a significant increase in expression level of the gene of interest cloned downstream of the T7-promoter was achieved. However, significant background expression of the gene of interest does occur. The system was applied to produce substantial amounts (22% of total intracellular protein) of heterologous tetanus toxin fragment C (TTFC), with the ultimate goal to use *L. lactis* as an antigen delivery vector for oral antigen administration. Promising results were obtained when it was demonstrated that the overexpressing strain could generate antibodies in mice, which subsequently protected them from a lethal TTFC challenge[9].

The dnaJ*-promoter based system*

Another type of gene regulation was found to occur in the case of the expression of the *dnaJ* gene of *L. lactis*, which could be induced by heat shock[10]. A transcriptional fusion between the *dnaJ*-promoter fragment and a signal sequence derived from the lactococcal *usp45* gene, followed by *amyS* encoding the *Bacillus stearothermophilus* α-amylase, resulted in a 3–4-fold increase in extracellular α-amylase activity upon raising the temperature from 30 to 42°C. The mechanism by which the induction is brought about is certainly interesting, however, the applicability to industrial fermentations of a *dnaJ*-promoter based system is limited, because of the relatively low level of induction, the high background expression level in the uninduced state and the undesirable shift of temperature, which will also affect expression levels of many other genes.

The sodA*-promoter based system*

Analysis of stress response in *L. lactis* revealed that the synthesis of at least three proteins is induced at low pH values of the culture medium[11]. The gene coding for one of these proteins was cloned and sequenced, and was shown to encode a superoxide dismutase. The expression of the *sodA* gene was analysed by transcriptional fusion with a promoterless *lacZ* gene. The β-galactosidase activity in aerated cultures of *L. lactis* was twice that of nonaerated cultures[11]. Regrettably, no induction experiments at low pH values or at different time points during growth were described.

Although the mechanism of regulation of gene expression by oxygen pressure is quite relevant for industrial fermentations, the induction level that can be obtained is too low for industrial protein production. However, this work provides an attractive tool for metabolic engineering, e.g. modulating enzyme activities in *L. lactis* by aeration during growth.

The prtP*-promoter based system*

Two genes are involved in the production of the cell-envelope-associated proteinase of *L. lactis*, i.e. *prtM* and *prtP*, encoding a maturation protein and the protease, respectively. It has been shown that transcription of these divergently transcribed genes is controlled in an identical manner, and is dependent on the peptide concentration in the medium[12,13]. Transcriptional fusions of these promoters with *gusA* showed that the activity was depressed at high peptide concentrations, particularly by the dipeptides prolylleucine and leucylproline, whereas the level of expression was about eightfold higher in media that contained relatively low amounts of peptides. Although these findings are relevant for the control of industrial fermentation processes in relation to growth characteristics, the system itself might not have a high potential for regulated gene expression of other genes, because of the relatively low inducibility and the rather difficult task of controlling peptide concentrations during bacterial growth.

The repressor/operator of φr1t based system

A regulated gene expression system employing somewhat different tools was recently developed using the repressor–operator system from the completely sequenced *L. lactis* bacteriophage r1t (Ref. 14). This phage contains a DNA fragment with a gene, *rro*, that specifies a protein that represses gene expression from a promoter on the same fragment. This region was exploited for inducible gene expression in *L. lactis* by transcriptional fusion to the *lacZ* gene. By applying conditions that normally induce the switch from the lysogenic to the lytic life cycle of r1t, i.e. by the addition of the DNA-damaging agent mitomycin C, a 70-fold increase in β-galactosidase activity was obtained. Obviously, this system holds potential for overproduction of proteins by *L. lactis*, if another, more food-acceptable, induction system can be applied. This could be based on a thermosensitive repressor as has been suggested[14], although many large-scale processes do not easily permit the use of thermal switches during production.

The xylA*-promoter based system*

The *xyl* genes of *Lactobacillus pentosus*, involved in xylose catabolism, are induced by xylose and repressed by glucose, ribose and arabinose[15]. Both the repressor XylR and the mechanism of catabolite repression are responsible for the reduced expression on glucose. Fusions have been made between the *xylA* promoter fragment and the promoterless *cat-86* gene and these were transformed into *Lactobacillus pentosus*. Growth on xylose resulted in a 60–80-fold higher CAT activity than growth on glucose[15]. This system provides a valuable tool for controlled gene expression in *L. pentosus*. However, switching to xylose as a carbon and energy source during industrial fermentations is less attractive for economic reasons.

The tryptophan controlled system

Transcriptional studies on the regulation of the tryptophan (Trp) biosynthetic genes *trpEGDCFBA* of *L. lactis* showed that the *trp* genes are organized in a single operon and that the synthesis of this transcript is regulated by a factor of about 100 by Trp, through the mechanism of transcription termination[16]. Other expression studies using transcriptional fusions between the *trp* promoter and the *lacZ* gene showed that β-galactosidase activity was negatively affected by Trp (Ref. 16). This interesting system could be valuable for laboratory applications to express genes, but probably has limited value for biotechnological applications in view of the difficult and expensive task of modulating the Trp concentrations in commercial media used for fermentation.

The PA170-promoter based system

Transposon insertions that result in fusions of different chromosomal loci of *L. lactis* to a promoterless *lacZ* gene have been reported to yield hundreds of β-galactosidase-producing integrants[17]. Several of the integrants were analysed for regulated gene expression and one of these, PA170, was shown to produce considerably more β-galactosidase under different conditions. The β-galactosidase activity was higher when cells were grown at pH 5.2 than at pH 7.0, higher at 15°C than at 30°C and higher in stationary phase than in logarithmic phase. A 9.7 kb DNA fragment from PA170 was cloned on a multicopy vector and introduced into *L. lactis*. This strain showed a similar mode of regulation; differences in induction levels ranged from 8–50-fold, depending on the conditions used[17]. This property might be valuable for the construction of genetically improved strains for industrial applications, since the regulation takes place through naturally occurring processes; for example, during cheese ripening. Further characterization and engineering of this promoter region might improve the induction factor and the final level of expression. In addition, a combined effect of low temperature and low pH could be expected to yield tighter control of gene expression.

The φ31-middle-promoter and ori *based system*

An original strategy for regulated gene expression in *L. lactis* has been based on two combined features of a lytic bacteriophage[18]. Plasmids were constructed that contained the *lacZ* reporter gene under control of a φ31-promoter region. After introduction into *L. lactis*, the resulting strain was infected by φ31, resulting in considerable overproduction of β-galactosidase. A further improvement of this system was achieved by introduction of the phage origin of replication on

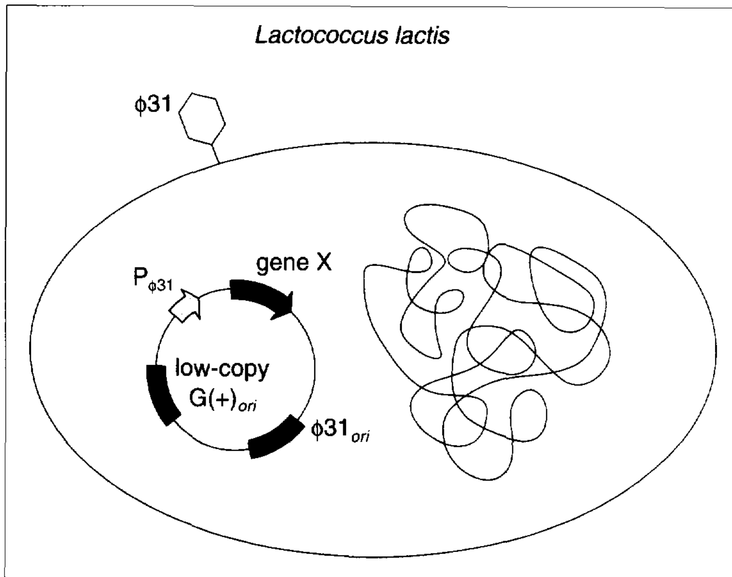

Figure 1
Sequence of events following ϕ31 infection of lactococcal cells harbouring the plasmid for explosive gene expression (Ref. 18). 0 min: Induction by ϕ31 infection; 20 min: effective plasmid amplification reached; 40 min: high gene transcription/translation reached; 60 min: lysis and release of overproduced proteins.

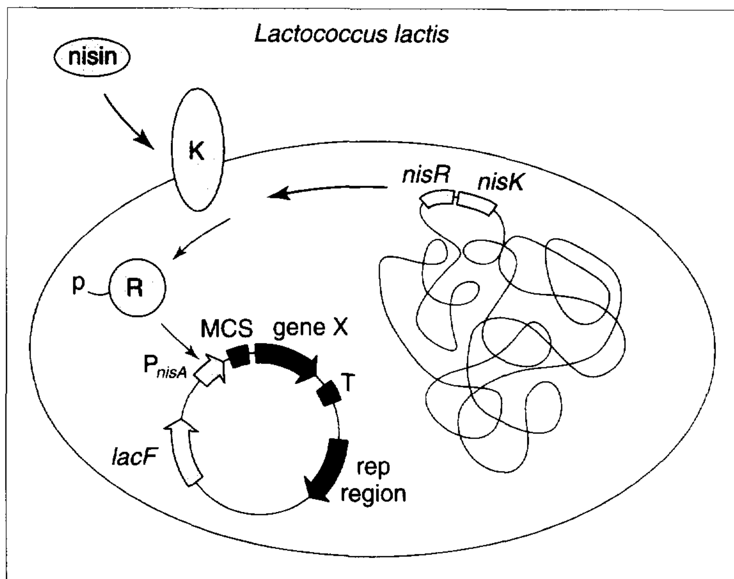

Figure 2
Model for nisin signal transduction involving the sensor histidine-kinase NisK and the response regulator NisR (Refs 19 and 22–25). T: Transcriptional teminator.

a low-copy number expression plasmid, to allow phage-inducible plasmid amplification. The expression vector is maintained at low-copy number in the host, allowing the cloning of genes encoding toxic proteins. After a deliberate phage infection, gene expression was 'explosively' triggered within 60 min, resulting in at least a 1000-fold increase in β-galactosidase activity[18] (Fig. 1). This illustrates the power of such a system to produce high amounts of protein in a relatively short time. The proteins can be maintained within the host if the cells are harvested prior to lysis, or allowed to be released into the medium if lysis is permitted. This expression system can, in principle, be constructed in a food-grade manner. A drawback for broader application might be that modulation of gene expression seems to be hard to achieve with this system.

The **nisA/nisF***-promoter based system*

A new system that offers significant potential for regulated gene expression in lactic acid bacteria is based on signal transduction by the two-component regulatory system, consisting of the response-regulator protein NisR and the sensor histidine-kinase NisK, found in the nisin gene cluster of *L. lactis*. Nisin is a 3.5 kDa highly modified antimicrobial peptide, which is widely applied as a food preservative. Extracellular nisin acts as the signal for transcriptional activation of its own structural gene and of several other biosynthetic and immunity genes[19–21]. A model of the signal transduction pathway of the nisin genes, based on the general characteristics of two-component regulatory systems[19,22–25], is presented in Fig. 2. When plasmids with genes under control of the *nisA* promoter fragment are introduced in strains that cannot produce nisin but do contain the NisR and NisK proteins, which are necessary for signal transduction, these genes will be expressed at a very low level. This expression level is in most cases undetectable, even when using sensitive assays based on β-glucuronidase production. When nisin is added to the growth medium during logarithmic growth, transcription of the genes that are controlled by the *nisA* promoter is activated. The level of expression is proportional to the amount of nisin added[19]. Recently, it was found that the *nisF* promoter, driving expression of the *nisFEG* genes, which were shown to be involved in immunity to nisin[26,27], can be activated by nisin in a way similar to the *nisA* promoter[20]. To date, this system has been applied to express several homologous and heterologous genes, e.g. *gusA* of *E. coli*, *pepN* (Ref. 28) of *L. lactis*, and *lytH* and *lytA* (Ref. 29) of ϕUS3 of *L. lactis*. A series of expression strains and vectors has been developed that allows high nisin-sensitivity of the signal transduction pathway and high production of the proteins of interest, e.g. by construction of vectors especially suited for making translational fusions at the start codon of *nisA*, thereby employing the strong translation-initiation signals of this gene[19,21]. Examples of the efficiency of the expression system are shown for *gusA* and *pepN* expression in Fig. 3. Levels of intracellular production of different proteins typically range between 10 and 60% of total soluble protein.

The nisin-inducible system has several advantages for application:

- Nisin is an attractive molecule for use as an inducer, as it already has a history of safe use in food products and therefore can be considered food-grade.
- The system is easy to use and low-cost, as induction of cultures can take place by simply adding small amounts of nisin [typically between 0.05 and 5 μg(nisin) l^{-1}, or less than 0.1% of a nisin-producing *L. lactis* fermentation in milk]. This means that other

starter culture bacteria will not suffer any damage from these subinhibitory amounts of nisin. With some nisin mutants even lower concentrations are required for induction than with wild-type nisin[19].

- It is a versatile and flexible system because several different expression strains and plasmids, containing convenient multiple cloning sites, are currently available[21].
- It seems feasible to implement this system in LAB other than *L. lactis*, by introducing *nisRK* genes on a plasmid and other plasmids containing the *nisA* promoter and the gene(s) of interest.
- The expression is extremely tightly controlled, leading to essentially no detectable production of the protein of interest in the uninduced state, enabling production of lethal proteins.
- A controllable level of expression can be obtained, depending on the amount of nisin added, leading ultimately to very high protein production, of up to 60% of total intracellular protein. The cells will in most cases not lyse, so it can be anticipated that secretion of proteins using suitable signal sequences can be achieved, facilitating downstream processing.
- A fully food-grade system has been obtained by use of *lacF*-deficient lactococcal strains[5] that have *nisRK* genes integrated on the chromosome, complemented by plasmids containing the *nisA* promoter controlling expression of the gene of interest, the *lacF* gene as a selectable marker and nisin as an inducer[21].

Examples and prospects of application of controlled expression systems

For industrial enzyme production the ϕ31-promoter/*ori*- and *nisA*-promoter based systems probably hold the most potential in view of their ability to produce very high amounts of protein. The ϕ31 based system has the additional advantage that it reaches high production in less than an hour after phage infection. For *in situ* production of enzymes by starter cultures or for metabolic pathway studies, the nisin-inducible system is ideally suited since any desired production level less than maximum can also be obtained. Another interesting application of the nisin-inducible system could be the regulated production of antisense RNA, enabling modulation of enzyme activities during growth of the bacterium.

Recently it has been reported that non-modified peptides encoded in some bacteriocin gene clusters of LAB other than lactococci can also have a signalling function and activate transcription of specific genes via signal transduction[30–32]. Although no examples of controlled overproduction of proteins using these inducing peptides, putative signal transduction proteins and promoter elements have been reported yet, systems based on these elements certainly hold potential for future application.

Finally, food-grade inducible expression systems have a high potential for developing autolytic starter cultures, which could accelerate cheese ripening. An example of an inducible autolytic bacterium was reported by Shearman *et al.*, who used induced expres-

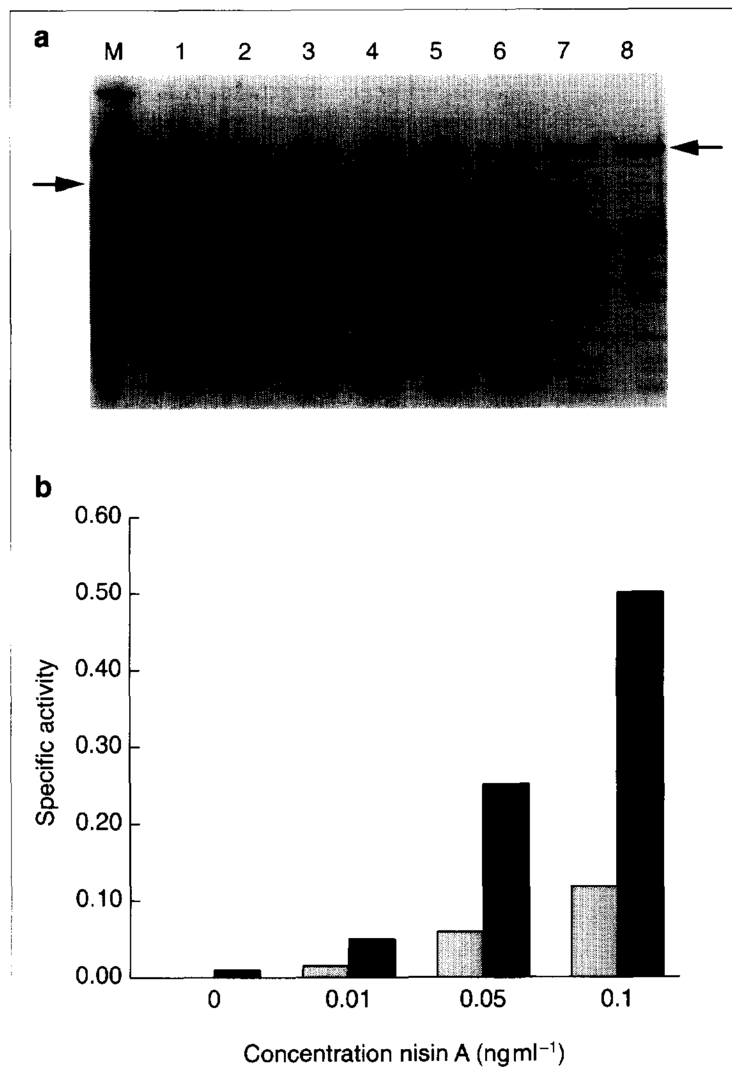

Figure 3

(**a**) Coomassie blue-stained gel after SDS–PAGE of extracts of *L. lactis* containing the *PnisA-gusA* and the *PnisA-pepN* fusion plasmids producing β-glucuronidase (lanes 1–4), or aminopeptidase N (lanes 5–8), after induction with nisin. Lanes 1–4: 0, 0.1, 1 and 5 ng(nisin A) ml^{-1} added, respectively. Lanes 5–8: 0, 0.05, 0.1 and 1 ng(nisin A) ml^{-1} added, respectively. (**b**) β-Glucuronidase (light grey) and aminopeptidase N (dark grey) specific activities shown in AU per $A_{600\ nm}$.

sion of two lytic genes of ϕvML3 by putting them under the control of a T7-promoter fragment from bacteriophage T7 of *E. coli*, yielding a 34-fold increase in lysin activity in *E. coli* after induction with IPTG (Ref. 33). However, no controlled expression in LAB was achieved due to the lack of a suitable controlled expression system. Recently, de Ruyter *et al.*[21] fused the holin-lysin encoding cassette *lytHA* of ϕUS3 to the *nisA* promoter fragment, which yielded a stable construct in *L. lactis*. The lytic muramidase LytA can degrade cell walls of LAB and is thought to be exported by passive diffusion through pores formed by the holin protein LytH. Upon induction of the construct by addition of nisin to the growing culture a rapid and efficient lysis of the cells occurred. This result illustrates the applicability of tightly controlled expression systems for the expression of lethal genes. An attractive possibility for application of this system is to

combine expression of the lytic genes with that of genes involved in flavour formation, e.g. peptidases, lipases and amino-acid convertases. Autolytic strains can also conveniently be used for controlled over-production of desired proteins in the culture medium as an alternative to *sec*-dependent secretion systems. Future work will focus on assessing the effects of controlled lysis of LAB, combined with production of desirable enzymes, on the maturation of fermented products.

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