The use of bacterial luciferase genes as reporter genes in *Lactococcus*: regulation of the *Lactococcus lactis* subsp. *lactis* lactose genes

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Lactose metabolism is an important industrial trait in dairy lactococci. In *Lactococcus lactis*, lactose is taken up via the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) and is subsequently metabolized via the glycolytic and tagatose 6-phosphate pathways. Genes for the lactose-specific PEP-PTS proteins, phospho-β-galactosidase and tagatose 6-phosphate pathway enzymes are encoded by a single 8 kb operon, *lac*ABCDEFGX, and there is a divergently transcribed *lacR* repressor gene. Transcriptional fusions of both the *lac* operon promoter and the *lacR* promoter to the *lux*AB genes of *Vibrio fischeri* were used to investigate the regulation of expression of both promoters. *In vivo* bioluminescence assays demonstrated that *lacR* negatively regulates the *lac* operon and also autoregulates itself. Induction of transcription occurred for both promoters during growth on lactose: sevenfold for *lacR* and fivefold for the *lac* operon. The *lacR* promoter was demonstrated to be a particularly strong promoter, being approximately four times more efficient than the *lac* operon promoter. Both promoters provide good potential for the inducible expression of foreign proteins in *Lactococcus*.

Introduction

Lactic acid bacteria are of major economic and nutritional importance, being used extensively in both the production and preservation of a wide variety of fermented food products. Genetic studies of these bacteria are aimed at the characterization of industrially relevant traits and the application of molecular techniques for strain improvement. The majority of industrially important traits are plasmid encoded and this has facilitated rapid analysis of genes for key catabolic pathways. The genetic determinants of several enzymes involved in protein degradation (Kok et al., 1988; Vos et al., 1989), citrate utilization (David et al., 1990; Verhue & Tjan, 1991) and sugar metabolism (De Vos & Gasson, 1989; De Vos et al., 1990; Van Rooijen et al., 1991) have been cloned and characterized. An important aspect of genetic technology is the analysis of gene expression signals and the characterization of regulatory elements. Strong controllable promoters are an essential requirement both for the efficient expression of heterologous genes and for the optimum exploitation of homologous genes. In many cases the analysis of promoters depends on their fusion to reporter genes which can then be assayed and used to monitor levels of transcription.

Bacterial luciferase genes have been used widely as *in vivo* reporters of gene expression in Gram-negative bacteria and there are a growing number of examples of *lux* as a monitor in Gram-positive species (Schauer et al., 1988; Guijarro et al., 1988; Carmi et al., 1987; Ahmad & Stewart, 1991; Sohaskey et al., 1992). Light emission has several advantages over alternative systems, providing rapid assays of superior sensitivity without the need for cell disruption and resulting in real-time expression data (for reviews, see Meighen, 1988, 1991; Stewart & Williams, 1992). This study describes the use of the *lux* genes of *Vibrio fischeri* as reporter genes to investigate the regulation of expression from the lactose operon promoters of *Lactococcus lactis* subsp. *lactis*.

In *L. lactis* lactose is taken up via the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS). Lactose becomes phosphorylated during entry into the cell and the lactose 6-phosphate is cleaved by phospho-β-galactosidase to yield glucose and galactose 6-phosphate, which are subsequently metabolized via the glycolytic and tagatose 6-phosphate pathways respectively (Blisset & Anderson, 1973). A single 8 kb operon, *lac*ABCDEFGX (hereafter the *lac* operon), encodes the genes for the lactose-specific PEP-
PTS enzymes, phospho-β-galactosidase and the tagatose 6-phosphate pathway (De Vos et al., 1990; Van Rooijen et al., 1991). Recent work has provided more information about the regulatory regions associated with the lactose operon and its possible mechanisms of control. However, a complete molecular model of _L. lactis_ lac operon regulation has yet to be determined. A repressor protein, produced by the divergently transcribed lacR gene, is known to regulate expression of the lac operon at the transcriptional level (Van Rooijen & De Vos, 1990; De Vos et al., 1990). During growth on glucose, expression of the lac operon is repressed at least fivefold and recent work (Van Rooijen et al., 1992) has indicated that the DNA sequences flanking the lac promoter are involved in transcriptional activity, regulation and possibly stability of the transcripts produced. Transcriptional fusion of the promoters for both the lac operon and lacR to the _luxAB_ reporter genes of _V. fischeri_ was used in this study to further characterize lactose gene expression. As well as demonstrating the value of luciferase as a reporter system in lactococci, the potential of the lactose promoter for control and high-level expression of heterologous genes is established. This study also demonstrates that the lactose operon provides a regulatory system, which can be exploited to provide a series of promoters producing different levels of inducible gene expression.

**Methods**

_Bacterial strains and media._ Escherichia coli MC1022 (Casabaudi & Cohen, 1980) was used in the plasmid cloning experiments. _L. lactis_ strains used were the plasmid-free, lactose negative strain MG1363 (Gasson, 1983) and MGS267, which contains chromosomally integrated lactose genes (S. R. Swindell & M. J. Gasson, unpublished). M17 medium was used for growth of _L. lactis_ at 30 °C. The carbon source was either 0.5% (w/v) lactose (LM17) (Terzaghi & Sandine, 1975) or 0.5% glucose (GM17) or a mixture of both sugars, each at 0.5% (GLM17). L broth (Lennox, 1955) was used for growth of _E. coli_ at 37 °C. Where appropriate, media were supplemented with ampicillin at 100 µg ml⁻¹ for _E. coli_, chloramphenicol at 15 µg ml⁻¹ and 5 µg ml⁻¹, and erythromycin at 500 µg ml⁻¹ and 10 µg ml⁻¹, for _E. coli_ and _L. lactis_ respectively.

_Plasmids._ Plasmid vector pUC18 (Yanisch-Perron et al., 1985) and promoter-probe plasmid pSB292 (Park et al., 1992) were used in the cloning experiments. pSB292 contains translational stop codons in all three reading frames and a multiple cloning site preceding the _lacR_ gene, is established. This study also demonstrates that the _lac_ operon provides a regulatory system, which can be exploited to provide a series of promoters producing different levels of inducible gene expression.

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_Molecular cloning, reagents and enzymes._ Plasmid DNA was isolated from _E. coli_ by the alkaline lysis method (Birnboim & Doly, 1979). _L. lactis_ plasmid DNA was extracted by the same method except that mutanolysin was added at the lysozyme treatment step (300 units ml⁻¹) and samples were incubated at 37 °C for 15 min. All in vitro DNA manipulations and _E. coli_ methods were performed as described by Maniatis et al. (1982). Transformation of _L. lactis_ was achieved by electroporation of glycerine-grown cells (Holo & Nes, 1989). Restriction enzymes, X-gal and IPTG were purchased from Life Technologies and enzymes were used according to the manufacturers' instructions. Taq polymerase was obtained from Promega and nonyl aldehyde from Aldrich.

_Luciferase assays._ In _vivo_ lux expression was determined at 30 °C in an assay cuvette containing 1 ml culture and 5 µl nonyl aldehyde. The cuvette was inverted sharply twice and light emission detected immediately using a Turner 20e luminometer. Samples were assayed for 10 s and the average light emission during the integrate period was recorded. Simultaneously the OD₆₀₀ of identical samples was measured. Time-course analyses were performed in one of two ways: (i) strains were inoculated directly into GM17 or LM17, or (ii) strains were grown initially in GM17, and the samples harvested by centrifugation at 3000 r.p.m. for 4 min and resuspended gently in the appropriate M17 medium (GM17, LM17 or GLM17). Samples (1 ml) were then removed at regular time intervals and assayed for luminescence. Bioluminescence was measured in light units (LU); 1 LU = 1.5 × 10⁶ quanta s⁻¹, based on calibration against an ATP luciferin/luciferase standard (Sigma).

**PCR amplifications.** Oligonucleotide primers for PCR were produced using an ABI DNA synthesizer. The primers used were: P1 (5'-GGATTCCGACAAAAACATACATAGGAACTGGAATGTT-3'), P2 (5'-GGGATCCGTTAAATTATTTAAGAGTAGTCCAAT-3') and P3 (5'-GGGATCCGAAATGCCTAGTGAAGTGAAATA-3'). Primer binding sites are indicated in Fig. 1. All primers incorporated a synthetic _BamHI_ site at their 5' end. Primers P1 and P3 were used to generate a fragment which contains both the _lac_ operon and _lacR_ promoters and the whole of the _lacR_ gene together with its putative transcription terminator. Primers P2 and P3 were used to create an equivalent fragment which lacks the transcription terminator. PCR was carried out on 40 ng pLP712 DNA in a 50 µl volume of PCR mixture (10 mM-Tris/HCl pH 8.3, 50 mM-KCl, 2 mM-MgCl₂, 0.01% gelatin, 100 mM each dNTP and 0.1% Triton X-100) containing 100 ng of each primer and 0.5 units Taq polymerase. Samples were overlaid with 50 µl mineral oil before being subjected to 25 cycles of 2 min denaturation at 92 °C, 2 min annealing at 61 °C and 2 min elongation at 72 °C. PCR products were purified using GeneClean as recommended by the manufacturers (Stratech Scientific) and subsequently treated with T4 polymerase and T4 polynucleotide kinase, before use in cloning experiments.

**Results**

_Transcriptional fusion of lux to the lac promoter._

Details of the construction of plasmids pFI400, pFI402, pFI517, pFI518 and pFI530 are shown in Fig. 1. The PCR fragment generated by primers P1 and P3 was obtained as a pUC18 clone and the 1.34 kb _BamHI_ fragment from this plasmid was cloned into the _BamHI_ site of pSB292 in both orientations to produce pFI400 and pFI402. Transcription of the _lux_ genes is initiated from the _lac_ operon promoter in pFI402 and from the _lacR_ promoter in pFI400. This fragment included a putative terminator sequence downstream of _lacR_. This terminator sequence was absent in the 1-25 kb PCR fragment from this plasmid was cloned into the _SmaI_ site of pUC18. The same region was excised as a _BamHI_ fragment and cloned into the _BamHI_ site of pSB292, resulting in a transcriptional fusion between the _lacR_ and _lux_ genes in pFI517. In this
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harbouring pFI400, pFI402, pFI517 or pSB292 were taken at regular intervals during growth at 30°C. The untransformed strains *L. lactis* MG1363 and MG5267, and control strains harbouring pSB292, produced only very low levels of luminescence (< 50 LU ml⁻¹). Varying levels of luminescence were obtained from MG1363 and MG5267 cells harbouring pFI400, pFI402 and pFI517. Fig. 2(a) shows a representative growth curve for MG5267 and MG1363 harbouring pFI517 during growth in GM17 and LM17. Equivalent strains harbouring pFI400 and pFI402 produced comparable growth curves. Figs 2(b), (c) and (d) show plots of bioluminescence vs growth for strains MG5267 and MG1363 harbouring pFI400, pFI402 and pFI517, respectively. Results in GM17 and in LM17 are compared for the MG5267 strains, but only low levels of activity were recorded for MG1363 strains during growth on LM17 and the data are not shown. In order to illustrate the induction of the *lac* promoters by lactose, transformed MG5267 cells were grown to mid-exponential phase in GM17 before addition or substitution of 0.5% lactose as the carbohydrate source (Fig. 3a, b). Extremely high levels of luminescence were obtained with *L. lactis* MG5267 (pFI517), making it necessary to dilute samples in order to obtain on-scale luminometer readings. Bioluminescence of established colonies of this strain could be easily visualized within a few seconds with the dark-adapted eye, even in the repressed state during growth on glucose.

MG1363 cells transformed with pFI400 and pFI517 produced similar luminescence levels to the corresponding MG5267 strains when grown in GM17. However, MG1363 transformed with pFI402 produced significantly lower levels of luminescence during growth in GM17 than the corresponding MG5267 strain.

Light emission characteristically reached a peak just before the culture entered stationary phase and thereafter decreased rapidly. The pH of a growing culture of *L. lactis* drops from pH 6.2 to 5.4 within a few hours. To investigate whether the accumulation of an inhibitory concentration of lactic acid limited the luminescent reaction, the assays were repeated using a fermenter with pH controlled at 6.8. Comparable values of luminescence were obtained.

**Induction of the *lac* operon and *lacR* transcription occurs during growth on lactose**

Induction levels in plasmid-carrying MG5267 strains were calculated by dividing the peak luminescence value during growth in LM17 by the value in GM17, at the equivalent optical density. Induction during growth on lactose was approximately fivefold for the *lac* operon.
Fig. 2. (a) Growth curve of MG1363(pFI517) during growth on GM17 (○) and LM17 (●) and MG5267(pFI517) during growth on GM17 (□) and LM17 (■). (b), (c) and (d) Bioluminescence of growing cultures of MG5267(pFI400), MG5267(pFI402) and MG5267(pFI517), respectively, during growth in GM17 (□) and LM17 (■) and of equivalent MG1363 strains in GM17 (○).

Fig. 3. Induction of bioluminescence in growing cultures of (a) MG5267(pFI402) and (b) MG5267(pFI517) during growth in GM17 (○), after shift to LM17 (■) and after shift to GLM17 (▲).

Discussion

Previous studies of gene expression in lactococci have relied on the use of chloramphenicol acetyltransferase (CAT) or β-galactosidase as reporters of gene expression. The homologous phospho-β-galactosidase gene of L. lactis has been proposed as an alternative reporter gene (Simons et al., 1990) and more recently the E. coli β-glucuronidase (GUS) gene has been successfully used in Lactococcus (W. M. De Vos, personal communication). The lux assay is 10–100 times more sensitive than the CAT or β-galactosidase assays. The lux genes have been demonstrated to be suitable monitors of gene expression in vivo and they are particularly useful for the analysis of
physiologically and environmentally controlled promoters. For the study of temperature-responsive genes and their promoters, however, the luciferases of *V. harveyi* and *Xenorhabdus luminescens* might be advantageous, being stable at 37 °C and 42 °C, respectively (Seitzner & Meighen, 1990). *V. fischeri* luciferase is rapidly inactivated at 37 °C (Friedland & Hastings, 1967; Sakharov et al., 1988; Ahmad & Stewart, 1991). Levels of light emission obtained from Gram-positive bacteria are generally 100-fold less than those obtained from Gram-negative bacteria (Karp, 1989). Active luciferase depends on the translation of two co-transcribed genes, *luxA* and *luxB*, and the correct folding of the protein subunits. In Gram-positive bacteria poor expression of luciferase activity could be caused by inefficient translation of the Gram-negative *lux* genes. Although translational fusion could overcome this for the *luxA* gene it cannot be applied to *luxB* whilst both genes are in an operon. One solution to this problem, which is also relevant to *lux* gene expression in eukaryotes, is to construct *luxAB* fusion genes, thereby eliminating the need for translation initiation in front of the *luxB* gene. Several groups have reported the construction of *luxAB* fusion genes from *V. harveyi* (Boylan et al., 1989a, b; Kirchner et al., 1989; Olsson et al., 1989; Escher et al., 1989). In *E. coli* the luciferase activity expressed by these genes was invariably less than that from the equivalent unfused genes. The luciferase produced from fused genes also tended to be more sensitive to high temperature than enzyme consisting of separate subunits. However, recent work in *Bacillus subtilis* has shown that a *V. harveyi luxAB* gene fusion expressed from a strong *Bacillus* promoter with translational coupling produced an equivalent amount of light to one of the most luminescent constructs of *E. coli* (Jacobs et al., 1991). In this case the equivalent luciferase expressed from unfused *luxAB* genes produced dramatically reduced light yields in *B. subtilis*. It is therefore possible that a *luxAB* gene fusion will facilitate the optimization of the luminescent reaction in lactococci.

Light emission in *Lactococcus* expressing *luxAB* reached a peak in late exponential phase and rapidly decreased as cells entered stationary phase. A number of factors may influence the luminescent reaction. On entering stationary phase, cells undergo a number of changes in both intracellular biochemistry and DNA topology (Siegel & Kotler, 1992). Their overall metabolic rates decrease to a very low level but the ability to transport substrates into the cell is unaffected. Entry of aldehyde and oxygen is therefore not a limiting factor. It has been previously suggested that Gram-positive cells have inadequate capacity to regenerate reduced flavin mononucleotide, which is an essential substrate of the luminescent reaction (Karp, 1989), and this may be more apparent during stationary phase. Equally, it may be that stationary-phase shut-off is a natural property of the *lac* promoters.

Both the *lacR* and *lac* operon promoters were induced during growth on lactose; approximately sevenfold and fivefold respectively. Expression of both promoters was repressed during growth on glucose. These data suggest that the *lacR* repressor acts as a negative regulator of the lactose operon during growth on glucose and that it also autoregulates itself. The *lacR* promoter proved to be a very efficient lactococcal promoter, being at least four times stronger than the *lac* operon promoter. It was also induced to a greater extent than the *lac* operon promoter during growth on lactose. A model of negative regulation of the *lac* operon promoter by the *lacR* repressor has been proposed (Van Rooijen & De Vos, 1990) and this is confirmed by the luciferase data presented here. Van Rooijen & De Vos (1990) used Northern blot analyses to show the presence of a 1·2 kb *lacR* transcript whose synthesis was apparently repressed fivefold during growth on lactose, indicating activation of the *lacR* gene during growth on glucose. However, the luciferase data presented here clearly demonstrate that the *lacR* promoter was repressed by growth on glucose, implying that *lacR* acts to repress transcription initiated from the promoters of both the *lac* operon and the *lacR* gene. Varying the level of repressor in response to lactose availability could provide a fine control of gene expression. The elevated level of repressor during induction would not reduce transcription of the *lac* operon as the repressor would be inactive in the presence of the inducer. Increased amounts of inactive repressor during induction could however serve to effect more rapid repression of transcription once a cell exhausts its supply of lactose. As the amount of inducer decreases, active repressor molecules would be formed more rapidly and in the continued absence of inducer a reduced level of repressor would maintain repression of the lactose operon. A cell with a repressed lactose operon that encountered lactose would require less inducer to be formed before full induction of the operon took place. An analogous explanation for the autoregulation of the repressor in fine-tuning gene regulation has been proposed for the *E. coli* tryptophan operon (YanoFSky & Crawford, 1987).

The luminescence data show that the transcription terminator downstream of *lacR* reduces transcription by a factor of 20. Previous studies using β-galactosidase as the reporter gene indicated a fivefold reduction (Van Rooijen et al., 1992), perhaps illustrating the superior sensitivity of the *lux* assay.

MG1363 cells lack the *lac* operon genes and are therefore unable to ferment lactose via the PEP-PTS
pathway. Although there is evidence for the presence of a lactose permease gene (De Vos & Simons, 1988; Kim & Batt, 1988), MG1363 appears to have no β-galactosidase activity and internalized lactose cannot be metabolized. Despite this, slow growth (71% of the growth rate on glucose) does occur on LM17 medium, which contains lactose as the only added sugar, presumably due to the presence of other metabolizable components in M17 medium. As expected, luminescence levels of plasmid-carrying MG1363 cells grown on LM17 are much lower than those of the equivalent MG5267 strains. For MG1363 cells transformed with pFI400, pFI402 and pFI517 and grown in glucose, transcription of both the lacR gene and the lac operon will occur. The lacR repressor will associate with the operator sites (Van Rooijen et al., 1992), permitting only the repressed levels of transcription. The in vitro inducer of the lactose operon is tagatose 6-phosphate (W. M. De Vos, personal communication), which should not be produced by the lactose-negative MG1363 strain; hence no induction of the operon is expected. The levels of lux gene expression might therefore be expected to be similar for MG5267 and MG1363 strains grown on glucose. Whilst this was true for strains carrying pFI400 or pFI517 significantly greater expression of lux was found for MG5267 carrying pFI402 compared to MG1363 carrying the same plasmid. Analogous experiments utilizing the β-galactosidase gene as the reporter gene produce the same results (Griffin & Gasson, 1993). This difference appears to be specific for expression from the lac operon promoter, and the presence of chromosomal lactose genes in this case appeared to elevate lac operon expression even though no lactose was present in the growth medium. Background expression of one or more chromosomal lactose genes in MG5267 could generate low levels of a metabolite with inducer activity, causing higher levels of expression due to partial induction of the lac operon. Alternatively, the lacX gene, which has yet to be assigned a function, may act as a positive regulator in MG5267.

In order to investigate the role of the lacR repressor further, plasmids pFI530 and pFI518 were constructed. Transformation of MG5267 with these plasmids proved difficult and only unstable transformants carrying pFI530 were recovered. In MG5267 strains harbouring these plasmids, only one copy of the lacR gene would be present compared to the multiple copies of the lac operator regions (Van Rooijen et al., 1992). Although some repression of the lac operon and lacR gene would occur during growth on glucose the repressor molecules are effectively titrated out. An increased level of transcription would be expected compared to the MG5267 strains carrying pFI400, pFI402 and pFI517, which contain an equivalent number of repressor molecules and operator regions. Attempts to transform pFI518 and pFI530 into L. lactis MG1363 also proved unsuccessful. In MG1363 the lactose promoter on these plasmids will be expressed in the absence of lacR and they are therefore not subject to repression whether grown in GM17 or LM17. In these circumstances it is possible that excessive transcription of the lux genes could occur, resulting in host cell lethality, or structural instability of the plasmids. Another possible explanation may be the occurrence of a ‘dark’ reaction associated with the luciferase which takes place in the absence of aldehyde and produces H2O2 (Hastings & Nealson, 1977). At high levels of luxAB expression this could be lethal.

Luminescence assays were also performed on cultures grown in a mixture of glucose and lactose (Fig. 3). Intermediate levels of luminescence between the high values for lactose and the lower levels for glucose were obtained, supporting the possibility of catabolite repression of the L. lactis lac operon (De Vos & Simons, 1988). Strains of Lactococcus have been shown to have intermediate levels of phospho-β-galactosidase activities under the same conditions (J. Smart, personal communication).

Whilst further work is required to fully determine the regulation of lac operon expression, this work demonstrates that the lactose promoters can be used to generate a range of promoter efficiencies coupled to regulation by carbon source. This provides a valuable approach to the more precise control of gene expression levels which should find application in the control of both heterologous and homologous genes in lactococci. Also the luciferase assay has clearly been shown to have considerable potential as a reporter for fine analyses of gene expression in lactococci.

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