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Development of an inducible system to control and easily monitor gene expression in *Lactococcus lactis*

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Abstract

This report describes the implementation and use of a maltose-inducible system for regulated gene expression in *Lactococcus lactis*. The system was established using Green Fluorescent Protein as reporter. The transcription of a gene of interest from the inducible promoter of pLS1RGFP plasmid vector can be easily monitored by fluorescence spectroscopy and microscopy. As an example, the lactococcal ribonuclease III was overproduced in an active form.

Keywords: *Lactococcus lactis*; Expression vector; Ribonuclease III; Maltose-induction system; Regulation of gene expression

1. Introduction

Lactococcus lactis is a lactic acid bacterium (LAB) that plays an important role in the manufacture of fermented foods. The end products of these lactococcal fermentations confer protection against spoilage, and some of them contribute to the flavor (diacetyl), and texture (exopolysaccharides) of the fermented products (Neves et al., 1999). The availability of tools for genetic manipulation (Renault, 2002) and the recent release of the genome sequences of strains IL1403 and MG1363 (Bolotin et al., 2001; Siezen et al., 2002), are additional incentives for the study of the physiology of this organism. Microarrays are also commercially available for lactococci and 232 proteins of IL1403 strain have been identified by 2D-analysis and peptide mass fingerprints (Guillot et al., 2003).

It has been recently demonstrated that the pLS1GFP plasmid can be used as a *gfp*-fusion vector using Green Fluorescent Protein (GFP) as a reporter for gene expression in *L. lactis* (Fernández de Palencia et al., 2000). This plasmid contains the *gfp* gene from *Aequorea victoria* cloned under the control of the pneumococcal P_M promoter. An inducible version of pLS1GFP plasmid was constructed by cloning of the pneumococcal *mal*R gene. The resulting plasmid, pLS1RGFP, allows the regulation of the transcription from P_M

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promoter depending on the presence of maltose in the growth medium. MalR protein negatively regulates the transcription from P_M promoter (Nieto et al., 2000). Addition of maltose to the culture releases the repression of P_M promoter by MalR repressor, resulting in the transcription of the genes under its control. The pLS1RGFP plasmid was shown to be a suitable inducible vector for gene expression in *Streptococcus pneumoniae* (Nieto et al., 2000). *L. lactis* is able to utilize maltose as carbon source and possesses a protein which shears 30% identity with the pneumococcal MalR repressor. This protein (previously called RliA) seems to be an activator of maltose transport (Andersson and Radstrom, 2002). However, expression from P_M of pLS1GFP was neither induced nor repressed by maltose in *L. lactis* (Fernández de Palencia et al., 2000). This could be due to the fact that the operators for the pneumococcal and lactococcal MalRs are similar but not identical (Andersson and Radstrom, 2002; Nieto et al., 1997). In this report, we have established the experimental conditions to use pLS1RGFP as a new maltose-inducible system for the induction of gene expression in *L. lactis*. We also demonstrate that the levels of induction can be easily followed in living cells in real time. Moreover, we tested the suitability of the system by cloning of the lactococcal *rnc* gene, that encodes the ribonuclease III (RNase III).

2. Materials and methods

2.1. Bacterial strains, growth media, and plasmids

The strains used in this study were *L. lactis* subsp. *lactis* biovar *cremoris* MG1363 (Gasson, 1983) for cloning and expression experiments and *L. lactis* subsp. *lactis* IL1403 (Bolotin et al., 2001) as source of the *rnc* gene. Lactococcal cells were grown, at 30 °C without shaking, in AGCH casein-hydrolysate-based medium (Lacks, 1966), AGCHY (AGCH medium supplemented with 0.25% yeast extract) or M17 broth (Oxoid). These media contained 1% of glucose (AGCHG, AGCHYG, and M17G) or different amounts of maltose (as referred in the figure legends). Bacterial strains carrying plasmids were grown under selective pressure for erythromycin (5 μg/ml). Plasmids used were pLS1RGFPrnc (this work), pLS1RGFP (Nieto et al., 2000), and pJFD4 (Mitra and Bechhofer, 1994). Plasmids were transferred to *L. lactis* MG1363 by electroporation as previously described (López de Felipe et al., 1995) and transformants were selected in agar medium supplemented with erytromycin at 5 μg/ml.

For construction of pLS1RGFPrnc plasmid the lactococcal *rnc* gene was obtained by PCR amplification of the chromosomal DNA from IL1403 strain, using rnc1 (5′-ACTAAAAAT**CT**AGAGAA GTTTTAAGTGAAGGAGAT-3′) and rnc2 (5′-C AATATTTGATTT**T**C**T**AGAACCATTTGGAC-3′) primers containing *Xba*I restriction sites (mismatches in bold). The resulting 845 bp fragment included the complete coding sequence of the *rnc* gene, its putative ribosomal binding site (RBS) and its translation termination signal but not a transcriptional terminator (see Drider et al., 2002). The PCR product and the pLS1RGFP plasmid were digested with *Xba*I and ligated, and *L. lactis* MG1363 was electrotransformed with the ligation mixture. The resulting plasmid was named pLS1RGFPrnc.

2.2. Induction of gene expression from P_M promoter

Induction of gene expression from P_M promoter was performed by growth of lactococcal strains in maltose containing media. In all analysis performed, cultures of *L. lactis* were grown in AGCHYG or M17G medium up to an A_{650} of 0.2. The cultures were sedimented by centrifugation at 6000*g* for 10 min, washed with PBS buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, pH 8.0), and resuspended in the growth medium supplemented with maltose (induction) or 1% of glucose (non-induction). The strains, concentration of maltose, time, and conditions of induction used in the different analysis are indicated in the respective section.

2.3. Measurement of GFP fluorescence

The expression of GFP by lactococci was evaluated by measuring the fluorescence of the cells on

an LS-50B spectrophotometer (Perkin–Elmer) by excitation at 488 nm with a slit of 2.5, and detection of emission at 511 nm with slit of 10, as previously described (Fernández de Palencia et al., 2000). For this purpose, cells were grown in M17G or AGCHG media and induced as indicated in Results. Detection of fluorescence in AGCH-grown cultures was performed in real time, during growth at 30 °C without shaking in microplates located in the spectrophotometer. For M17-grown cultures, aliquots of 1 ml were sedimented, washed and resuspended in the original volume with PBS buffer, prior to measurement of the fluorescence. The fluorescence obtained in the AGCH and M17 media takes in consideration the background of AGCH and PBS.

2.4. Detection of GFP expression by fluorescence microscopy

Lactococcal cells were grown in M17 medium and induced with maltose 5% until A_{650} of 0.5, as described above. Cultures were then sedimented, washed, and resuspended in the original volume with PBS buffer. Cells were directly analyzed, without fixing, by phase-contrast or fluorescence microscopy, as previously described (Fernández de Palencia et al., 2000).

2.5. RNA isolation

Cells were grown in M17G medium to an A_{650} of 0.2 and induced (maltose 5%), until A_{650} of 0.5. Total cellular RNA was extracted as previously described (Santos et al., 2002), with the exception that cells were treated with lysozyme at 10 mg/ml. RNA was always quantified by UV spectroscopy and its integrity verified in agarose gels.

2.6. Dot-blot hybridization

Samples containing 10 μg of total RNA were blotted onto a nitrocellulose membrane (Schleicher and Schuell) and fixed by heat (80 °C) for 2 h. Nitrocellulose membranes were hybridized with a [α-^{32}P]dATP labeled probe. The *rnc* gene amplified with primers rnc1 and rnc2 was used as substrate to synthesize the probe, using the Multiprime DNA labeling system from Amersham. The procedure of hybridization and washing was performed as previously described (Santos et al., 2002). The membranes were scanned with the PhosphorImager System (Molecular Dynamics) and the intensity of the dots was quantified using ImageQuant software.

2.7. Western blot hybridization

For the Western blot analysis, 5 ml of each culture were grown in M17G medium to A_{650} of 0.2 and induced (maltose 5%) up to A_{650} of 0.5. The cultures were sedimented, washed with buffer A (30 mM Tris, 1 M pH 7.5, 10 mM $MgCl_2$, 130 mM KCl, and 5% glycerol), centrifuged, and concentrated 10-fold by suspension in lysis buffer (buffer A plus 0.4 mg/ml lysozyme). Samples were incubated for 30 min at 37 °C. Then, the cells were disrupted by sonication (3×10 s with 20 s cooling intervals, 40% output), and centrifuged at 16,000*g* for 5 min to remove cell debris. Protein concentration of the cellular extracts was quantified by the Lowry method (Lowry et al., 1951). Seventeen microgram of total protein were fractionated by 10% SDS–PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell) by electroblotting. Polyclonal antibodies against GFP (purchased by Clontech) were diluted 1:6000 and anti-rabbit IgG conjugated with Horseradish Peroxidase (Sigma) was diluted 1:10,000 and used as secondary antibody. Horseradish Peroxidase was used for detection with chemiluminescence (ECL detection kit, Amersham). The bands were visualized by autoradiography using Amersham Hyperfilm MP. The bands were quantified by densitometry of the autoradiograms.

2.8. Determination of RNase III activity

The RNase III activity of lactococcal extracts was tested. For this purpose, lactococcal cells were induced, and crude extracts were prepared as described for Western-blot analysis. The transcript used as substrate for the enzymatic assays was obtained from pJFD4 plasmid, a specific substrate for *Bacillus subtilis* RNase III (Mitra and Bechhofer, 1994; Wang and Bechhofer, 1997). The

transcription reaction from pJFD4 plasmid, previously digested with *Hpa*I, was performed using T7 RNA polymerase and Riboprobe kit from Promega. The activity assay was carried out as described before for *B. subtilis* (Mitra and Bechhofer, 1994). The reaction was carried out at 37 °C for 5 min, using 5000 cpm of substrate and 1 μg of total protein per reaction. The reactions were stopped by adding formamide containing dye and analyzed in 6% polyacrylamide–7 M urea gels.

3. Results and discussion

3.1. Inducible expression of *gfp* in *L. lactis*

To establish a maltose-inducible system for *L. lactis*, we have adapted and optimized the usage of the pneumococcal expression vector pLS1RGFP (Nieto et al., 2000) in *L. lactis*. This plasmid carries the P_M-*gfp* fusion and encodes the pneumococcal MalR regulator, which in the absence of maltose represses the transcription from P_M promoter by interaction with its operator (Nieto et al., 2001). The pLS1RGFP was transferred to *L. lactis* MG1363 strain and *gfp* expression was analyzed by measuring the fluorescence of the cultures. To determine the optimal concentration of maltose for the induction, exponential cultures of *L. lactis* MG1363[pLS1RGFP] were grown in AGCHYG up to A_{650} of 0.2 and induced in AGCH, as described in Materials and methods, using different amounts of maltose or 1% of glucose (Fig. 1). The fluorescence emission was directly measured during growth in microplates. An increase of the fluorescence was observed, as a function of time, in cultures treated with maltose at concentrations higher than 0.001%. The maximum GFP activity was detected with 5% of maltose, where an average of 15-fold increase of fluorescence was observed. The use of higher amounts of inducer (10%) produced cellular lysis (data not shown).

Due to the intrinsic fluorescence of the M17 medium, it could not be used for the continuous detection of fluorescence during growth of *L. lactis*. It was required to wash and resuspend the cells in PBS buffer prior measurement. We have shown that AGCH is an appropriate growth medium for the fluorescence detection in real time. However, given that M17 broth is the general medium used for growth of lactococcal cells, we wanted to assure that the results of GFP production were similar in both media.

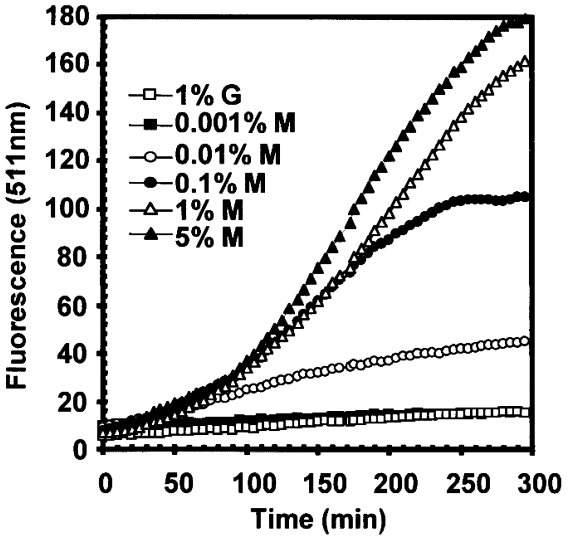

Fig. 1. Response of *gfp* expression to increasing amounts of maltose concentration in *L. lactis* MG1363[pLS1RGFP]. Real time measurements of fluorescence emission during growth in AGCH medium, at the maltose or the glucose concentrations indicated. A representative experiment is depicted.

For this purpose, the production of GFP upon maltose induction in M17 and AGCH was analyzed over a 300 min time period (Fig. 2A). The optimal 5% maltose concentration was used for the induction of MG1363[pLS1RGFP] cells. Fluorescence emission of induced cells growing in AGCH or M17 was monitored and compared with the fluorescence produced with 1% of glucose. The maximum fluorescence obtained upon maltose induction was about 2-fold higher in M17 medium than in AGCH (Fig. 2A). However, since the growth rate was higher in M17 than in AGCH (Fig. 2B), the highest specific activity of GFP upon induction was similar in both media (Fig. 2C). The specific activity of GFP decreased during the stationary phase probably due to acidification of the internal pH of the cells that could affect irreversibly the fluorescence of the protein (Sullivan and Kay, 1999). Fluorescence produced in cultures grown with glucose was mainly due to the intrinsic

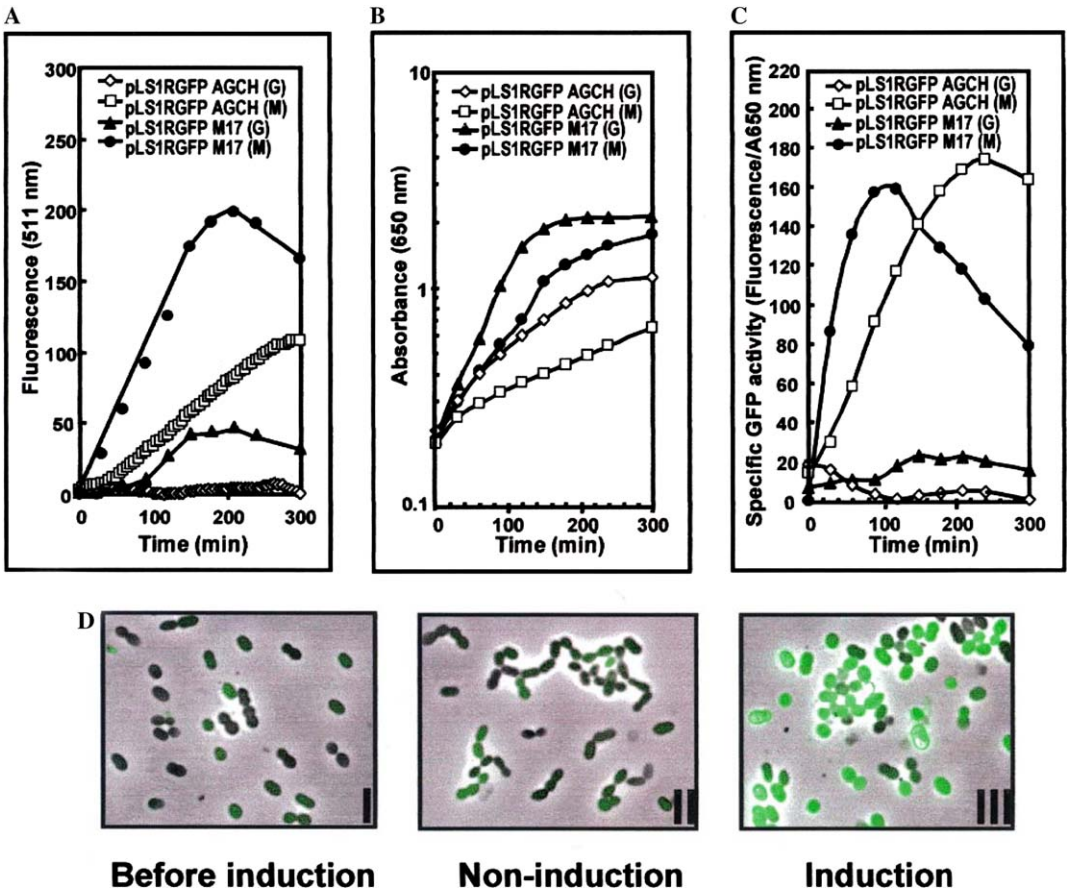

Fig. 2. Analysis of induction of *gfp* expression from pLS1RGFP plasmid. Cultures of *L. lactis* MG1363[pLS1RGFP] were grown in AGCH or M17 media with 1% glucose (G) or 5% maltose (M) as indicated in the text. (A) Detection of fluorescence by spectrofluorometry. The fluorescence emission of cells was measured and fluorescence of AGCH or PBS buffer (AGCH- or M17-grown cultures, respectively) was subtracted as background. (B) The absorbance of the cultures was measured during growth at the times indicated. (C) Specific GFP activity of the cultures. The ratio between the fluorescence values and the absorbances obtained is depicted. (D) Detection by fluorescence microscopy of the fluorescence of the cells grown and induced in M17 medium (see details in the text). The microscopy amplification was of 125×.

fluorescence of the growing cells. Thus, cells can be grown and induced in M17 or AGCH medium, depending on the requirements of the user, without affecting GFP production.

The *gfp* expression was also examined by fluorescence microscopy (Fig. 2D). Cells previously grown in M17G were induced and visualized under the microscope (see details in Materials and methods). Cultures at A_{650} of 0.2 (before induction), grown until 0.5 with 1% glucose (non-induction) or with 5% maltose (induction from A_{650} of 0.2 to 0.5) were analyzed. The results showed that there was a significant difference between the induced and non-induced cells. Only the maltose-induced bacteria were visualized as bright green cells (Fig. 2D, III versus II).

3.2. Cloning of rnc gene and induction of its expression using pLS1RGFPrnc

To confirm that the maltose-induction can be used for overexpression of a particular gene in *L. lactis*, we cloned the *rnc* gene into the pLS1RGFP plasmid and tested the system. The *L. lactis*

chromosomal *rnc* gene was amplified by PCR and cloned into the pLS1RGFP vector as described in Materials and methods. The resulting plasmid pLS1RGFPrnc, represented in Fig. 3, contains the P_M-*rnc*-*gfp* transcriptional but not translational fusion. The correct nucleotide sequence of the lactococcal chromosomal insert of pLS1RGFPrnc was confirmed by DNA sequencing.

The expression of P_M in cells harboring pLS1RGFPrnc was analyzed using the *gfp* reporter gene. The measurements of GFP levels and cell growth were carried out in cultures grown in either AGCH or M17 media, as described above. The results showed that upon addition of maltose there was a considerable increase of fluorescence in both media (data not shown). The maximum GFP specific activity obtained was similar in AGCH (88 after 240 min of induction) and M17 (112 after 120 min of induction) media. These values were only slightly lower than those obtained with pLS1RGFP (Fig. 2C). Therefore, the cloning procedure did not impair the fluorescence emission from pLS1RGFPrnc plasmid.

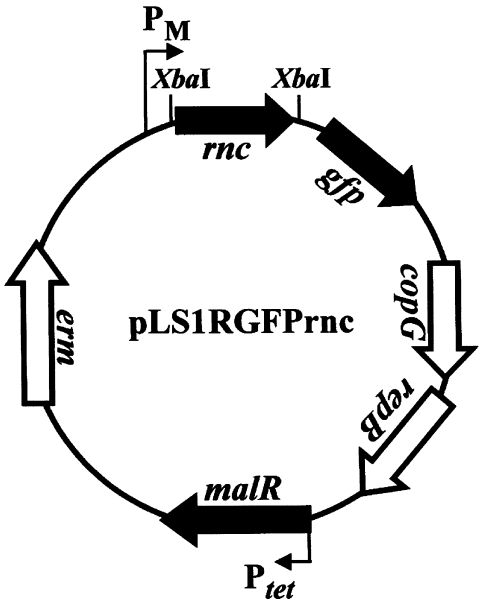

Fig. 3. Physical map of pLS1RGFPrnc plasmid. Plasmid-encoded genes are indicated with boxes pointing to the direction of transcription. The relevant genes, *malR*, *gfp*, and *rnc*, are represented with black boxes. The P_M and P_{tet} promoters directing transcription of *rnc* and *malR* are indicated with arrows.

The GFP autofluorescence is affected by physicochemical factors especially pH and oxygen (reviewed in Sullivan and Kay, 1999). For this reason, GFP maltose induction was also confirmed by Western blot analysis. Cells harboring pLS1RGFP or pLS1RGFPrnc were grown in M17G to an A_{650} of 0.2 (before induction). Then, the cultures were further grown to an A_{650} of 0.5 either in M17G (non-induction) or in M17 supplemented with 5% maltose (induction). Protein extracts were prepared from the cultures, fractionated by SDS–PAGE, and blotted onto a nitrocellulose membrane. Protein extract from induced MG1363 strain was also used as control. Incubation of the membrane with antibodies anti-GFP revealed the presence of a band with the expected size of GFP (relative electrophoretic mobility of $M_r \cong 28{,}000$ Da) (see Nieto et al., 2000). As shown in Fig. 4, the amount of GFP increased upon maltose induction in plasmid containing cells. The intensity of GFP bands was quantified and compared to fluorescence of the cultures (Table 1). After induction, cells carrying either pLS1RGFPrnc or pLS1RGFP showed a fluorescence induction of 12- or 20-fold, respectively. Moreover, the levels of GFP synthesis in induced cells were similar with both plasmids, confirming that the cloning of *rnc* does not affect the GFP synthesis.

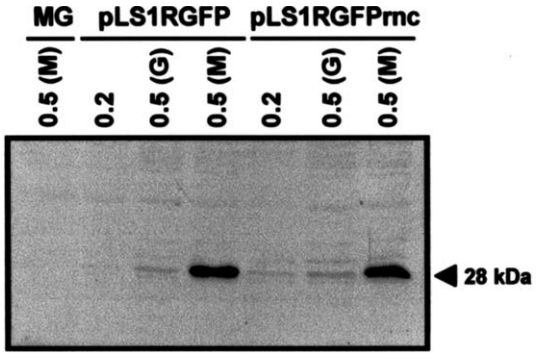

Fig. 4. Detection of GFP by Western blot analysis. Cultures of *L. lactis* MG1363 (MG), MG1363[pLS1RGFP] (pLS1RGFP), and MG1363[pLS1RGFPrnc] (pLS1RGFPrnc) were grown in M17 glucose (G) or maltose (M) until the A_{650} indicated. 0.2 (before induction), 0.5 G (non-induction), 0.5 M (induction from A_{650} of 0.2 to 0.5), (see details in the text). Protein extracts were prepared and analyzed as described in the text.

Table 1
Effect of maltose induction on GFP synthesis and activity in *L. lactis*

Plasmid[a]	Media	GFP levels[b]	GFP fluorescence
pLS1RGFPrnc	M17M	584	57
pLS1RGFPrnc	M17G	49	8
pLS1RGFP	M17M	606	81
pLS1RGFP	M17G	30	5

[a] The values presented were determined in *L. lactis* MG1363 strain carrying the indicated plasmid.

[b] GFP levels were inferred from quantification of the intensities of the bands depicted in Fig. 4.

To estimate the levels of transcription of *rnc* after maltose induction, a dot-blot analysis was performed using a probe specific for this gene (Fig. 5A). The amount of *rnc* transcript was analyzed in strains MG1363, MG1363[pLS1RGFP], and MG1363[pLS1RGFPrnc]. A comparison of the values obtained for induced or non-induced cells is shown in Fig. 5A. The quantification of the dots by densitometry showed that the expression of *rnc* gene from pLS1RGFPrnc was induced approximately 4.5-fold after growth in maltose. The basal levels of *rnc* mRNA detected in the pLS1RGFP and MG samples, corresponded to the transcription from the chromosomal copy of the gene.

RNase III of *L. lactis* is still not characterized and there are no antibodies available against this protein. For this reason, to prove that the induced *rnc* message is translated into an active protein, the endoribonuclease activity of lactococcal crude extracts on a specific substrate, was analyzed upon maltose induction.

The results presented in Fig. 5B show that the substrate was considerably cleaved only after maltose induction of pLS1RGFPrnc plasmid, leading to the accumulation of an intense processed product. Thus, it seems that an active lactococcal RNase III is produced upon induction with maltose.

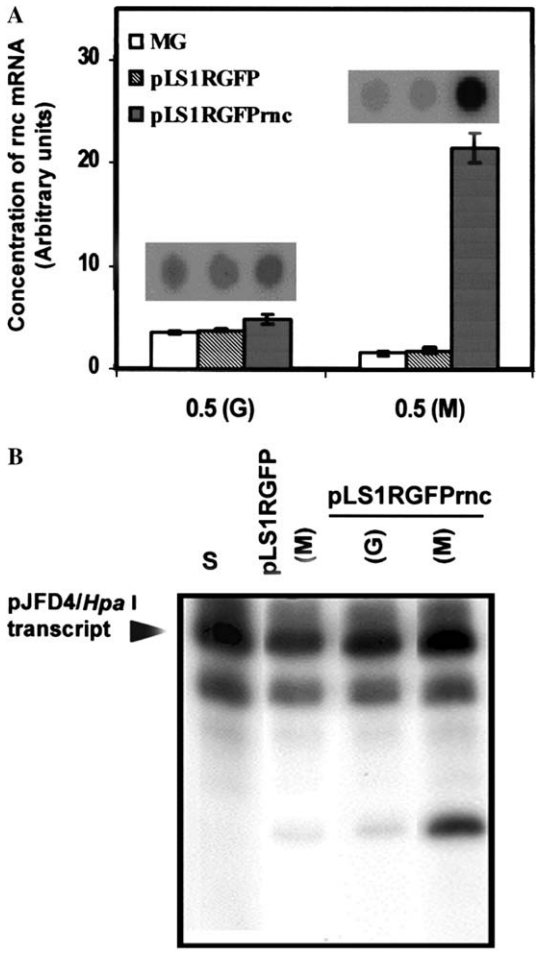

Fig. 5. Maltose induction of RNase III in *L. lactis* MG1363[pLS1RGFPrnc]. The indicated lactococcal strains were grown at A_{650} of 0.5 with (M) or without (G) maltose induction. (A) Dot-blot analysis of *rnc* mRNA. The dots and their inferred levels of message from the *L. lactis* strains indicated are depicted. MG corresponds to the MG1363 strain without plasmid. (B) RNase III activity assay in lactococcal crude extracts from *L. lactis* MG1363 containing pLS1RGFP or pLS1RGFPrnc plasmids. S, untreated substrate.

4. Conclusions

From all results presented in this report, we can conclude that pLS1RGFP is a suitable vector for regulated gene expression in LAB. The cloning of *rnc* shows that the transcriptional fusion of a particular gene with the *gfp* reporter enables the production of an independent protein, the initial expression of which can be estimated by the direct monitorization of GFP. Moreover, the levels of controlled induction by maltose can be quantified in real time during growth by fluorescence

spectroscopy or visualized in individual cells by fluorescence microscopy, facilitating the optimization of the induction conditions for each gene. However, attention should be paid to growth conditions and pH, since they affect GFP activity and consequently could hinder the use of this protein as reporter of gene expression. Maltose, in addition to be an economically favorable inducer, produces a clear, but moderate induction. We have tested this vector by cloning the *rnc* gene that encodes the lactococcal ribonuclease III (RNase III). Ribonucleases are enzymes involved in RNA maturation, turnover and degradation (Deutcher, 1993), and regulate the gene expression in virtually all organisms from bacteria to mammals. In *Escherichia coli* it is widely accepted that mRNA decay is initiated by a series of endonucleolytic cleavages catalyzed by RNase E or RNase III (reviewed in Régnier and Arraiano, 2000). The uncontrolled overproduction of some of these ribonucleases could be lethal for the cell. Similarly, there exist some essential genes in the cell that could not be highly overproduced because of their toxicity. Here, we present a system that can be used for inducible expression in lactococcal cells. This can be useful for genes that require moderate levels of induction.

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