Inducible expression of green fluorescence protein in *Lactococcus lactis*

*Lactococcus* are lactic acid bacteria widely used in various foods and fermentation processes. A great deal of interest has been shown to genetically modify these economically important organisms to improve their traits by introducing specific genes through cloning techniques. In addition, since the lactic acid bacteria are considered GRAS (generally regarded as safe) organisms, considerable interest exists in the development of genetic tools that allow production of important proteins in lactic acid bacteria.

Due to the lack of commercially available expression vectors for *Lactococcus*, it is important to construct expression vectors to enable the expression of genes of interest in them. Several available vectors such as pMG36e have a constitutive promoter and therefore do not allow for controlled expression of the recombinant proteins. An improved expression vector can be constructed by replacing the constitutive promoter with those that are inducible. The nisin-based promoter has been shown to be a useful promoter for expression of genes in *Lactococcus*.

Nisin-inducible gene expression system is based on the autoregulatory properties of the *L. lactis* nisin gene cluster. Nisin is a small, cationic, hydrophobic peptide of 32 amino acids that belongs to the lanthibiotic class of bacteriocins. Two genes in the cluster, nisA and nisF, are induced by nisin via a two-component signal transduction pathway containing a histidine protein kinase, NisK, and a response regulator, NisR. Expression of both nisR and nisK is driven by the constitutive promoter of nisR. Nisin acts as an inducer on the outside of the cell and is sensed by NisK. Recently, it has been reported that two-plasmid systems in which the nisA promoter and the regulatory genes nisR and nisK are used, allow efficient control of gene expression by nisin in a variety of lactic acid bacteria.

The green fluorescence protein (GFP) used in this study was isolated from the Pacific jellyfish, *Aequorea victoria*. GFP is a protein of 238 amino acids, which spontaneously emits green light at 508 nm when excited with blue light at 395 nm in the presence of O₂. GFP has the advantage of being an auto-fluorescent protein that does not require a substrate. This fact allows its detection in living cells and in real time. Measurement of GFP activity requires a post-translational oxidation of the protein.

Here we constructed an inducible expression vector for *L. lactis* based on the nisA promoter and studied its functionality using GFP as the reporter protein. The inducibility of the promoter using galactose as the alternative inducing agent was also studied. This simple expression system would contribute to the pool of available expression systems for *Lactococcus* and could be utilized as a useful tool for studying the expression of genes of interest in *Lactococcus*. We also showed that gfp could be used as a reporter gene for analysis of promoters in *L. lactis*.

*Lactococcal* cells were grown at 30°C in M17 broth containing 0.5% glucose as standing culture. *Escherichia coli* cells were grown at 37°C with vigorous agitation in LB broth. Whenever required, a total concentration of 5 µg/ml of erythromycin was used for *Lactococcus*, while 150 µg/ml of erythromycin was used for *E. coli* cultures.

Molecular cloning techniques were performed essentially as described by Sambrook *et al.* Restriction enzymes, T4 DNA ligase and deoxynucleotides, were used according to the instructions of the supplier. Electrotransformations of *L. lactis* were performed using a gene pulser. *E. coli* was used as an intermediate host for cloning and plasmid DNA was isolated from *E. coli* using the alkaline lysis method. DNA fragments were isolated and purified with agarose gel (Qiagen, USA) before being subjected to PCR. PCR was performed in 25 µl reaction volumes in the presence of 1X PCR buffer with 1.5 mM MgCl₂ (Fermentas, USA), 2.5 U Pfu DNA polymerase (Fermentas, USA), 5 mM dNTP (Fermentas, USA), 0.4 µM of each primers, 1 µl (0.1 µg) of template (genomic DNA of *L. lactis* ATCC 11454). Samples were denatured at 95°C for 1 min, 60°C for 1 min, 72°C for 45 s, and after the last cycle the reaction was held at 72°C for 10 min. Genomic DNA of *L. lactis* strain ATCC 11454 was isolated using the method of Engelke *et al.*, with minor modifications. PCR technique was applied to amplify the promoter region of nisin A gene. This is a 215 bp fragment from positions −156 to +3 with respect to the nisA promoter transcription start site, which includes the −35 and −10 sequences and the ribosomal binding site of Lactococcus. Primers were designed based on the published sequence of nisA gene. The forward primer was designed to carry EcoRI site and reverse primer was designed to carry Xmal site. PS1 is a 30-mer forward primer with a sequence of 5′-GAATTCCGAGTCTTAG ACATACTCTGAATGAC-3′ and Vnis is a 27-mer reverse primer with a sequence of 5′-CCCGGGCTACTCATTTTGAGTGCCTCC-3′. The EcoRI and Xmal recognition sites are underlined.
The PCR-amplified fragment was double-digested with EcoRI and XmaI and ligated to the expression vector pMG36e, which has been digested with EcoRI and XmaI. This resulted in the replacement of the constitutive p32 promoter with the inducible nisA promoter. The expression vector with the new nisA inducible promoter was then transformed into competent *E. coli* XL1-blue MRF cells by heat shock. The transformants were selected on LB-agar plates supplemented with 150 µg/ml erythromycin at 37°C for a minimum of 16 h. The recombinant plasmid pRnis was introduced into *L. lactis* by electroporation and transformants were selected on M17 agar supplemented with 5 µg/ml erythromycin.

The gfp gene was amplified using the plasmid vector pGFP (Clontech) as template. Primers GPF2 and GFPFR2 were designed based on published sequence (gene bank accession number U17997). The forward primer was designed to include a *PstI* site and reverse primer was designed to include a *HindIII* site. GPF1 is a 38-mer forward primer with a sequence of 5’-CTGCAGCATTGGTAAAGGAGAAGACTTTCAGGAT-3’. GFPFR1 is a 36-mer reverse primer with a sequence of 5’-AACCTTATACCGGACTTGTATAGTCCAATAGGCC-3’. *PstI* and *HindIII* recognition sites are underlined. The amplified gfp gene was then digested with *PstI* and *HindIII* and cloned into the newly constructed pRnis vector. The constructed plasmid was transformed into *E. coli* XL1-Blue (Stratagene, USA) by heat shock12 and later electro-transformed into *L. lactis* MG1363. Selection of transformants was carried out as described earlier.

*L. lactis* cells harbouring pRnisGFP and pRnis were grown in M17 medium until an OΔ660 of 0.6 to 0.8 was reached, after which the culture was aseptically dispensed into several sterile tubes and induced with 5 mM galactose for 6–8 h. The induced cells were centrifuged, washed and re-suspended in PBS buffer. The cells were directly analysed by fluorescent microscopy with Leica DMRA Fluorescence Microscope (Leica, Heerbrugg, Switzerland) using a 40X oil immersion objective and the FTC (excitation filter 470–490 nm; dichroic mirror 505 nm; emission filter 515–550 nm) filter cube.

The inducible promoter region of nisA was amplified from the chromosomal DNA of *L. lactis* and cloned into plasmid pMG36e in place of the constitutive p32 promoter. The recombinant plasmid was verified by restriction digestion analysis with EcoRI and XmaI and PCR amplification of the new promoter region.

Plasmid pRnis contains the pWV01 origin of replication from *L. lactis* subsp. cremoris Wg2. The erythromycin resistance gene is from *S. aureus* plasmid pE194 and the multiple cloning sites (MCS) originated from plasmid pUC18 (Figure 1). The newly constructed plasmid vector retained the ability to replicate in *E. coli* as well as in *L. lactis*. This feature facilitates the cloning of foreign genes because *E. coli* can also be used as an intermediate host for transformation of recombinant plasmids. The MCS downstream of the translation initiation signal allows the insertion of genes of interest in correct reading frames, thereby creating an in-frame fusion with the transcription start site present in the vector. The availability of common restriction sites such as those for restriction enzymes *XmaI*, *PstI* and *HindIII* are especially useful in this respect.

The nisA promoter has been recognized to be a strong inducible promoter15. This is extremely useful for the expression of genes in *L. lactis*, because in many cases the uncontrolled overexpression of foreign genes can be deleterious to the host cells. The nisA promoter is subjected to autoregulation by a two-component regulatory system consisting of the sensor kinase NisK and the response regulator NisR, which respond to extracellular nisin. This regulatory system belongs to quorum-sensing systems that are widely distributed in Gram-positive bacteria17. This allows the system to be controlled by nisin as the inducer.

To evaluate the use of the new expression vector for cloning and regulated expression, the promoter-less gfp gene was cloned downstream of the nisA promoter of vector pRnis and transformed into *L. lactis* MG1363. Restriction digestion analysis confirmed that the newly constructed plasmid contained the gfp gene (data not shown), and that the recombinant was able to replicate and was found to be stably maintained in *L. lactis*.

In this study, the nisA promoter of *L. lactis* was utilized. However, induction by nisin can only be carried out if the system includes the nisK and nisR operon as part of the host chromosome or included in the plasmid construct. Since we used MG1363 as host and did not clone.

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**Figure 1.** Map of expression vector pRnis which contains nisA promoter, ribosomal binding site, start of an open reading frame, pWV01 origin of replication, MCS, and erythromycin resistance gene.
the nisK and nisR genes together with nisA, alternatively, galactose can be used as an inducer for nisA promoter\(^8\). To determine the optimum concentration of galactose needed for induction, \emph{L. lactis} transformants harbouring the pRnisGFP construct were grown to an OD\(_{600}\) of 0.5–0.9 in M17 medium containing various concentrations of galactose ranging from 2 to 20 mM. The cells showed high levels of GFP expression upon galactose induction, with a maximum effective galactose concentration of 5 mM. Since expression of GFP was not detected in the absence of galactose, this indicates that the functionality of the nisA promoter in our construct is dependent on the presence of galactose.

To test for GFP expression, \emph{Lactococcal} cells harbouring pRnisGFP plasmid were grown in M17 medium and induced with galactose. Induced cells were centrifuged, washed and resuspended in PBS buffer; the cells were then observed under a fluorescence microscope. Our results showed that the \emph{L. lactis} transformants harbouring plasmid construct pRnisGFP fluoresced significantly, while the plasmid-less \emph{Lactococcus} MG1363 strain and \emph{Lactococcus} cells with pRnis plasmid did not show any production of green fluorescence (Figure 2). In addition, \emph{L. lactis} cells harbouring pRnisGFP vector did not show any green fluorescence without induction with galactose. Results from this study therefore showed that the nis-

\begin{itemize}
  \item inA promoter was capable of driving the expression of genes under the control of either nisin or galactose inducer. These results confirmed the effectiveness of the nisA promoter and the use of gfp as a reporter gene in \emph{L. lactis}.
\end{itemize}

1. Oria-Jensen, S., \emph{The Lactic Acid Bacteria, Host and Son, Copenhagen, Denmark, 1919.}

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1\textsuperscript{Department of Biotechnology, Faculty of Biotechnology and Biomolecular Sciences, }
2\textsuperscript{Laboratory of Microbial and Enzyme Technology, Institute Bioscience, }
3\textsuperscript{Faculty of Medicine and Health Science, Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia }
4\textsuperscript{For correspondence. e-mail: raha@putra.upm.edu.my}

\begin{flushright}
N. R. S. VARMA\textsuperscript{1} \\
A. R. RAHA\textsuperscript{1,2,*} \\
E. ROSSI\textsuperscript{1} \\
N. S. MARIANA\textsuperscript{3} \\
K. YUSOFF\textsuperscript{4}
\end{flushright}

\textsuperscript{1}Department of Biotechnology, Faculty of Biotechnology and Biomolecular Sciences, \textsuperscript{2}Laboratory of Microbial and Enzyme Technology, Institute Bioscience, \textsuperscript{3}Faculty of Medicine and Health Science, Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia \textsuperscript{*For correspondence. e-mail: raha@putra.upm.edu.my}