

Develop efficient gene expression systems in lactobacilli

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by

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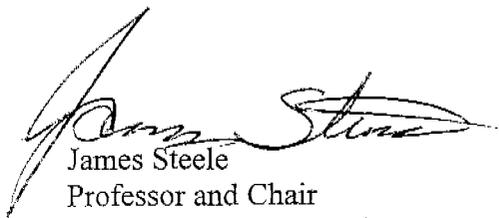
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## Abstract

Six putative constitutive promoter fragments and one putative inducible promoter fragment derived from the *Lactobacillus helveticus* CNRZ32 genes (*pepC*, *pepN*, *pepX*, *pepO*, *pepE*, *pepO2* and *hsp17*) were analyzed. These promoters were fused to a shuttle vector, pNZ272<sup>RBS□ATG□</sup>. The analysis was performed in three lactic acid bacteria, *Lb. helveticus* CNRZ32, *Lb. casei* ATCC334 and *Lactococcus lactis* MG1363. Except for the *P<sub>pepN</sub>::gusA* construct which could not be maintained in either of the *Lactobacillus* strains examined, all other promoters were active in all three lactic acid bacteria, as well as in *Escherichia coli* KW1. Studies of specific activity (S.A.) revealed that these promoters had higher activity in the exponential growth phase cultures than in the stationary phase cultures for both *Lactobacillus* strains, but similar activity was observed in both growth phases for *Lc. lactis* MG1363. In general, higher S.A. was observed in *Lb. casei* ATCC334. Activities of promoters varied in different organisms, with the most significant increase (480-fold) with *P<sub>pepO2</sub>::gusA* in *Lb. casei* ATCC334 when compared to *Lb. helveticus* CNRZ32. Less dramatic (2- to 242-fold) differences between the activities of individual promoters were also observed. Expression of *P<sub>hsp17</sub>::gusA* was well detected in all growth phases of the cultures examined. These results indicate that all seven promoters are well constitutively expressed and that the promoter strength is organism-dependent. Reduced expression derived from *P<sub>hsp17</sub>::gusA* at lower temperature and the presence of an open reading frame (*orfX*) upstream of *hsp17* in the opposite orientation had indicated the possible role of *orfX* in the regulation of *P<sub>hsp17</sub>* and the existence of a repressor gene. Sequence analysis of these promoters was performed. The putative ribosomal binding sites as well as the putative -10 hexamers were well conserved. Features such as UP element, TG motif, and -35 hexamers were observed in most promoters. However, these would only explain partially the sequence analogy of a given fragment being recognized as a promoter fragment. We believe what is more important in defining the strength of a promoter would be the overall context of the sequence and its interaction with the  $\sigma$ -factor of the RNA polymerase complexes.

## **Introduction**

This report addresses the request for proposals number RC1-9-29059 entitled "Develop efficient gene expression systems in lactobacilli".

Lactic acid bacteria (LAB) have been utilized in the manufacture of fermented foods, for their beneficial effect on health (Dunn et al., 1998; Palva, 1998), as well as for their ability to convert a wide range of substrates into industrially important products (Beshkova et al., 1998; Nakasaki et al., 1999). LAB modified by genetic engineering will lead to a better understand the importance of specific genetic traits and serve to optimize and improve the production of industrially important products (Gold et al., 1996; Klein et al., 1996). During the last decade, gene expression in LAB has received significant attention. For the construction of strains with enhanced industrial utility, especially applications which require expression of heterologous proteins, the development of efficient tightly regulated expression systems is required.

To construct an expression vector, components such as a selective marker, an origin of replication, and a promoter (inducible or constitutive) and a reporter gene are required (Wells and Schofield, 1996). To date, a number of selective markers, primarily antibiotic-resistance genes, which function in LAB have been identified. It has been demonstrated that the mode of plasmid replication plays a significant role in plasmid stability (Kiewiet et al., 1993). Many cloning vectors have been constructed using rolling circle replicons; however, these plasmids are typically relatively unstable. Significantly

fewer cloning vectors are based on the more stable theta-type replicons. Cloning vectors have been constructed utilizing theta replicons from pJW563 (Gravesen et al., 1995), pNP40 (Froseth and McKay, 1991), pAM $\beta$ 1 (Bruand et al., 1991), and pCI305 (Hayes et al., 1990). The published reporter genes which have been used in LAB include luciferase (Kahala and Palva, 1999),  $\beta$ -glucuronidase (GusA; Platteuw et al., 1994),  $\beta$ -galactosidase (LacZ; Lin et al., 1996) and alkaline phosphatase (PhoA; Franke et al., 1999).

During the last ten years, our laboratory has conducted research on *Lactobacillus helveticus* CNRZ32, with a primary focus on its proteolytic enzyme system. To date, fourteen genes have been cloned and characterized. Reported here is the cloning and analysis of seven promoter fragments ( $P_C$ ,  $P_N$ ,  $P_X$ ,  $P_O$ ,  $P_E$ ,  $P_{O2}$  and  $P_H$ ) derived from *Lb. helveticus* CNRZ32. These include aminopeptidase genes (*pepC*, *pepN*, and *pepX*), endopeptidase genes (*pepE*, *pepO* and *pepO2*) and a small heat-shock protein gene (*hsp17*).

## Materials and methods

**Bacterial strains, plasmids and media.** *Lb. helveticus* CNRZ32 and *Lb. casei* ATCC334 were typically propagated in MRS broth (Difco Laboratories, Detroit, Mich.) (DeMan et al, 1960) at 37°C. For studies on temperature effects on expression of  $\beta$ -glucuronidase from specific promoters, exponential phase cultures were shifted to 30° or 42°C for 30 minutes (min) and heat shocked at 52°C for 0, 5, 10, 15 and 20 min. *Lactococcus lactis* MG1363 was propagated in GM17 broth (Difco Laboratories) (Terzaghi and Sandine, 1975) at 30°C. When needed, exponential phase cultures were shifted to 37° or 42°C for 30 min. Heat shocked cultures were recovered at their normal growing temperatures for 30 min, and then on ice for 5 min before harvested. *Escherichia coli* KW1 was grown in Luria broth (Difco Laboratories) (Sambrook et al, 1989) at 37°C with aeration. Agar plates were prepared by adding 1.5% (wt/vol) granulated agar to broth. Chloramphenicol was used for screening strains harboring derivatives of pNZ272<sup>RBS</sup>ΔMG (Fenster and Steele, unpublished) at a concentration of 10 µg/ml for LAB and 25 µg/ml for *E. coli*. GusA-positive clones were identified by use of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (X-Gluc) (Clontech Lab. Inc., Palo Alto, Calif.) at a concentration of 0.5 mM.

**Molecular manipulation and cloning.** Plasmid isolation from *E. coli* and chromosomal DNA isolation from LAB were performed as described by Sambrook et al.(1989). Restriction enzymes and T4-DNA ligase were purchased from GICO-BRL Life Technologies Inc., and used as recommended by the manufacturer. Electroporations were conducted with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). Electroporation of *E. coli* was performed as recommended by the manufacturer (Bio-Rad) and procedures followed for LAB were as described for *Lb. helveticus* CNRZ32 by Chen and Steele (1998), *Lb. casei* ATCC334 by Ahmé et al. (1992) and *Lc. lactis* MG1363 by Holo and Nes (1989). Total RNA isolation was done by using the Qiagen RNeasy kit (Qiagen Inc., Valencia, Calif.).

Primers used to generate promoter regions were gene specific, and gave extended fragment (up to 200 bp) of promoter regions, including the ribosomal binding sites and start codon of each individual gene. Promoters were synthesized by polymerase chain reaction (PCR) and then ligated to *PvuI* site of pNZ272<sup>RBS</sup>ATG□.

**Sequencing and promoter sequence analysis.** PCR and DNA sequencing reactions were performed in a Perkin-Elmer (Norwalk, Conn.) model 480 thermal cycler. DNA sequencing reactions were conducted with the Prism Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.). DNA templates were purified with the Qiagen PCR cleaning kit. Synthesis of primers was performed by GIBCO-BRL Custom Primers (Grand Island, N.Y.). DNA sequence determination was conducted by the Nucleic Acid and Protein Facility of the University of Wisconsin-Madison Biotechnology Center, using an ABI model 370/3 automated sequencer. Mapping of the 5' end of each gene was conducted by using the 5' rapid amplification of cDNA (5' RACE) kit from GIBCO-BRL.

**β-glucuronidase assays.** Harvested cultures were washed and resuspended in 20 mM phosphate buffer at pH 6.8. Cell-free extracts were obtained by vortexing the samples with glass beads as described previously by Chen and Steele (1998). The protein content of cell-free extracts were determined by the method of Lowry et al. (1951) with the Sigma Total protein Kit (Sigma Chemical Co. St. Louis) using bovine serum albumin as the standard. β-glucuronidase activity was analyzed by using 1 mM substrate *p*-nitro-β-D-glucuronic acid (Sigma) and measured the absorbance at 420 nm by using a Beckman DU-65 spectrophotometer. Enzyme assays were conducted with cell-free extracts pre-equilibrated at 37°C and stopped by the addition of 800 μl of 2 mM Na<sub>2</sub>CO<sub>3</sub> to 100 μl of reaction mixtures as described by Platteeuw et al. (1994). Reaction rates were verified to be linear based on duplicate sample analysis and were quantified on the basis of release of *p*-nitrophenol (extinction coefficient of 18380 at OD401 nm) (Bowers et al, 1980). Enzymes assays were performed in duplicate

on two independently prepared cell-free extracts.  $\beta$ -glucuronidase activity was expressed as micromoles of *p*-nitrophenol released per minute per milligram of protein.

## Results and Discussion

The development of an efficient and a tightly regulated expression system for lactic acid bacteria (LAB) has industrial significance. It will not only improve the properties and performances of these industrial important microorganisms, but also allow heterologous protein expression on an industrial scale. During the last decade, gene expression in LAB has received attention for their beneficial effect on health and food fermentations. However, much effort has been focusing on developing a gene expression system that either requires addition of chemicals as inducers of gene expression or requires introducing synthetic components into the expression system. In either case, the gene expression system would likely have limited application in the food industry due to concerns related to its regulatory status. Therefore, the intent of this study was to develop a food-grade gene expression system starting with a series of promoters derived from *Lb. helveticus* CNRZ32, a LAB commonly used as a starter adjunct in cheese industry. We analyzed and characterized seven promoters with the aid of a readily detectable reporter gene,  $\beta$ -glucuronidase (*gusA*) (Platteeuw et al., 1994), and their possible usage in different bacterial strains.

### $\beta$ -glucuronidase gene expression system

Seven promoters ( $P_C$ ,  $P_N$ ,  $P_X$ ,  $P_O$ ,  $P_E$ ,  $P_{O2}$ , and  $P_H$ ) of *Lb. helveticus* CNRZ32 genes (*pepC*, *pepN*, *pepX*, *pepO*, *pepE*, *pepO2* and *hsp17*) along with their ATG start sites were fused to the *PvuI* site of the multiple cloning site of vector pNZ272<sup>RBS<sup>-</sup>ATG<sup>-</sup></sup> (Fenster and Steele, unpublished). The use of vector pNZ272<sup>RBS<sup>-</sup>ATG<sup>-</sup></sup>, a ribosome binding site-deficient and start codon ATG-deficient derivative of plasmid pNZ272 allows the study of expression without the interference of the  $\beta$ -glucuronidase (GusA) background activity derived from the promoterless *gusA* reporter gene harboring in pNZ272 (Platteeuw

et al., 1994). pNZ272 is an *Escherichia coli*–*Lactobacillus* shuttle vector. It is readily introduced and maintained without apparent structural changes in different LAB strains. Meanwhile, within a single species the copy number was relatively constant (Platteeuw et al., 1994). In order to perform expression studies under defined and comparable conditions, we have selected *E. coli*  $\beta$ -glucuronidase as a reporter gene. There are many features that make  $\beta$ -glucuronidase attractive as a reporter gene (Jefferson et al., 1986). It is a well-studied system and the assays are relatively easy. The expression can be easily detected by using a commercially available chromogenic substrate (Wilson et al., 1992). Most importantly, with few exceptions, LAB lack appreciable GusA activity.

All promoter constructs were confirmed by sequencing and were stably maintained in *E. coli* KW1. Constructs were introduced into *Lb. helveticus* CNRZ32, *Lb. casei* ATCC334 and *Lactococcus lactis* MG1363 via electroporation procedures as described previously (Chen and Steele 1998; Ahrne et al., 1992; Holo and Nes 1989). GusA activities were detected for each bacterium only in the presence of these promoters. This result indicates that these promoters are functional in these microorganisms. All the promoters examined, except  $P_N$  were successfully introduced and maintained. This failure to maintain  $P_N::gusA$  in lactobacilli is consistent with  $\beta$ -glucuronidase being toxic when highly expressed in these organisms,  $\beta$ -glucuronidase toxicity has been previously reported (McCracken and Timms, 1999; McCracken et al., unpublished).

### **Activities of the promoters**

Previously, Northern hybridization was performed for *pepN*, *pepO*, *pepO2* and *pepE*. The results have shown that *pepN* is expressed highly and constitutively throughout all growth phases, while *pepO*, *pepO2* and *pepE* were expressed at a relatively lower level throughout all growth phases (Chen and Steele 1998; Chen and Steele, in preparation). The expression of *hsp17* was also examined by Northern

hybridization under different growth temperature with a digoxigenin (DIG)-labelled 314-bp *hsp17* PCR fragment (Fig. 1). The identification of *hsp17* transcript under heat shock indicates that  $P_H$  is a heat-inducible promoter.

### **Analysis of $\beta$ -glucuronidase production**

Specific activity (S.A.) of  $\beta$ -glucuronidase derived from the peptidase-derived promoters was determined for both exponential growth phase and stationary growth phase in three strains of LAB, *Lb. helveticus* CNRZ32, *Lb. casei* ATCC334 and *Lc. lactis* MG1363 (Table 1 and Table 2). In both *Lactobacillus* strains, the S.A. of  $\beta$ -glucuronidase was at least two-fold higher in the exponential phase cultures than in the stationary phase cultures. However, these promoters yielded relatively consistent expression in *Lc. lactis* MG1363 in both growth phases (Table 1 and Table 2, Fig. 3a). Surprisingly, most of these promoters had greater absolute activities in *Lb. casei* ATCC334 than in their original host, *Lb. helveticus* CNRZ32. The increase of activities can be as high as 480-fold (Fig. 2). The strengths of these promoters were somewhat correlated between these two organisms (Fig. 3b and 3c). In *Lb. helveticus* CNRZ32, these promoters ranged in activity from 0.002 to 0.165 U/min/mg, which represents an 80-fold difference in activity. In *Lb. casei* ATCC334, these promoters had an even larger range in activity, from 0.007 to 1.708 U/min/mg which is a 242- fold difference in relative activity. These results indicate that promoter strength is organism-dependent (Fig. 2). In addition, these promoters could offer desired and constitutive expression levels for genes of interest in different strains.

Previously, we have identified the transcription of *hsp17* at a lower level when growing *Lb. helveticus* CNRZ32 at 30°C and a significant increase in transcription of *hsp17* when the temperature was increased to 52°C (data not shown). Therefore, it is not surprising to see the expression of GusA derived from the activity of  $P_H$  constitutively (Table 1 and 2). However, that GusA was expressed at a

higher level at lower temperature in all three LAB examined was unexpected (Fig. 4). The effect of heat on  $P_H$  was also examined for all three LAB strains (Fig. 5). We did not detect any significant heat-induced expression. On the contrary, there was a slight drop in absolute value of GusA for both *Lb. helveticus* CNRZ32 and *Lc. lactis* MG1363. We then examined the stability of GusA by heating the enzyme at 52°C for 0, 5, 10, 15, and 20 minutes and determined that GusA is heat stable at 52°C for up to 20 minutes (data not shown). Temperature-mediated regulation occurs at the level of transcription and translation, supercoiling, and changes in mRNA conformation and protein conformation are all implicated in thermosensing (Hurme and Rhen, 1998). Thus, increased expression under a given condition is not a proof that expression of a given gene is induced (Rallu et al, 1996). Therefore, what we have seen with the heat induction effect on *hsp17* might be far more complicated than what we had expected. Subject to an often suboptimal growth condition, bacteria acquired both inducible resistance and constitutive resistance to stresses, such as glucose starvation, acidity, temperature, salt, and ethanol, imposed by cell growth as well as by the environment. Transcription of many stress response genes is transiently increased after a temperature upshift as a consequence of enhanced translation, stability of mRNA/protein and/or titration of regulatory proteins (Narberhaous 1999). Thus, our data implies that *hsp17* is regulated at a different level. Simply examining  $P_H$  with the aid of GusA at different temperatures is not sufficient enough to determine the role of  $P_H$  under heat shock. Reduced expression of GusA after heat induction could mean an elevated activity of protease as a result of heat shock. Mediated by the enhanced activity of many Hsps to ensure maintenance of protein integrity; recognition of GusA as a foreign protein in the bacteria systems examined could explain the results seen here as well as shown previously for reduced activity of fused  $\beta$ -galactosidase in *Bacillus subtilis* (Zuber and Schumann 1994). In addition, many Hsps are regulated by a repressor, which provides a simple and economical way of controlling gene expression from what would normally be a constitutive promoter (Narberhaus 1999). If a repressor is indeed involved in the regulation of *hsp17*, the repressor would

only be titrated away by introduction of extra copies of  $P_H$  and therefore, no heat induction effect could be seen. Finally, a repressor gene *rheA* (formerly *orfY*) was recently found to be a thermosensor of the *hsp18* in *Streptomyces albus* (Servant et al., 2000). Located 150bp upstream of *hsp18* in the opposite orientation, *rheA* played a role in the transcriptional regulation of the *hsp18*. Disruption of *rheA* resulted in elevated *hsp18* transcript level at low temperature (Servant and Mazodier 1996). We have also found an open reading frame upstream (*orfX*) of *hsp17* in the opposite orientation (see next section). It is possible that interruption of *orfX* leads to the elevated expression of  $P_H::gusA$ . Further investigations are required to clarify the mechanism of *hsp17* regulation.

### Sequence analysis of the promoters

The transcriptional start site for each promoter was mapped by using the 5' rapid amplification of cDNA (5' RACE) kit with gene specific primer sets. In most cases, a purine residue was identified for the initiation of transcription (Table 3). Putative -10 and -35 hexamers were identified by resemblance with the previously defined *Lactobacillus* consensus sequences (Pouwels and Leer 1993). The -10 sequence, or Pribnow box, TATAAT is well conserved for these promoters, with a lower degree of consensus at the -35 sequence TTGACA. Putative ribosomal binding sites were well conserved as AAGGAGG, complementary to the 3'-OH-end of the 16S rRNA from *Lb. plantarum* (Hols et al., 1994). The TG motif, often found 1 bp upstream of the -10 sequence, was observed in 4 of the 7 promoters studied. The AT-rich region upstream of the -10 and -35 hexamers also resembled the UP element generally described in other bacteria (Table 3) (Estrem et al. 1998). The distance between -10 and -35 hexamers ranged from 15 to 22bp, with an average of 19.3bp. The average distance between -10 region and the transcription initiation site was 6bp (Table 4). Many studies have shown that the consensus identities at the -10 and/or -35 hexamers are important, but not ultimate factors affecting promoter strength (McCracken et al., 2000). The efficiency of transcription is both strain and context dependent,

where the  $\sigma$  factors of RNA polymerase complex that recognize these promoters have different structures or context requirements in different microorganisms due to differences in amino acid sequences (McCracken and Timms 1999). Therefore, factors other than consensus sequences, such as spacers, TG motif, UP elements and other factors present in the promoter sequence would only partially explain the strength of a given promoter. What would be more significant in defining the strength of a promoter could be the overall three-dimensional structure that affects efficiency and interaction between the RNA polymerase holoenzyme and the promoter sequence (Jensen and Hammer 1998).

Two inverted repeat sequences, also known as the CIRCE element (controlling inverted repeat of chaperone expression), were identified in  $P_H$  (Fig. 6). One is located upstream of  $-35$  hexamer, and the other immediately downstream of the start point of transcription. CIRCE element functions as an operator site to which a repressor binds (Yuan and Wong 1995). The presence of CIRCE indicated that  $P_H$  is associated with heat shock genes and suggested the presence of a repressor gene in *Lb. helveticus* CNRZ32. Interestingly, an open reading frame (*orfX*) located 232bp upstream of *hsp17* in the opposite orientation was also mapped. Along with the fact that  $P_H$  had a higher expression level at lower temperature, the same observation as seen in *hsp18* in *S. albus* G with a disrupted *rheA*, which is located 150bp upstream of *hsp18* in the opposite orientation (Servant and Mazodier 1996). We suspect that *orfX* is related to the repressor gene. The presence of two copies of CIRCE elements in the  $P_H$  region illustrates the remarkable diversity in gene regulation (Segal and Ron 1996). When placed upstream of  $-35$  hexamer, CIRCE elements serve to regulate transcription on the DNA level, where binding of repressor or RNA polymerase would be mutual exclusive. This permits a basal level of Hsps expression once RNA polymerase gained access to the promoter and proceeded with transcription initiation. While located downstream of the transcription start site, CIRCE also provides fine-tuning during heat shock response. As an RNA element, CIRCE promotes the rapid turnover of the mRNA under normal growth conditions (Narberhaus 1999).

## Conclusions:

In this study, we demonstrated seven promoters from *Lb. helveticus* CNRZ32 were also recognized in two other LAB, *Lb. casei* ATCC334 and *Lc. lactis* MG1363, as well as *E. coli*. The activity of each individual promoter fragment in different organisms illustrates that discrete but notable differences among promoters exist in different strains. It also implies that  $\sigma$  factor of RNA polymerase complexes that recognize these promoters have different structures/context requirements in these microorganisms. The objective was to establish an efficient expression system in LAB, with the ultimate goal of constructing a food-grade vector that allows steady expression of industrial significant genes. This work provides the first step towards that purpose.

To improve the properties and performances of industrial microorganisms, in many cases, optimization is far more important than massive overexpression and/or gene inactivation. A mechanism for fine-tuning gene expression that's applicable to food-grade organisms would have significant utility.. Analysis of promoter strengths in different microorganisms would not only offer a powerful tool for achieving steady and various levels of heterologous protein production without compromising the quality of the final products (flavor of cheese, for example), but also allow the studies of regulation of cloned genes in different organisms.

Seven promoters afford constitutive expressions of heterologous protein GusA at different levels in different microorganisms. To our surprises,  $P_H$ , the promoter fragment that was originally recognized as a heat shock promoter, did not permit heat-induced overexpression of GusA. Further research on identifying the repressor that might be associated with  $P_H$  and/or the *orfX* is required.

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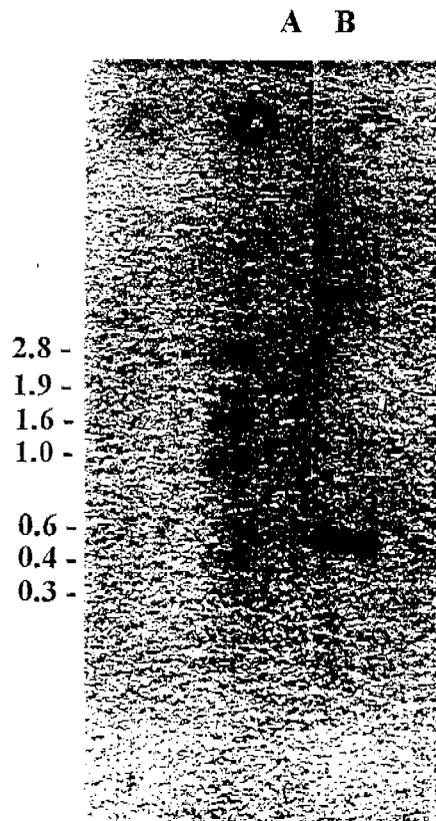


Fig. 1. Northern hybridization to identify *hsp17* transcript in *Lactobacillus helveticus* CNRZ32 growing in MRS to optical density at 600nm of 0.6 at 37°C. Lanes: A. Without heat shock. B. With heat shock at 52°C for 20 min. 30 µg of total RNAs was loaded in each lane and hybridized with an *hsp17*-specific probe.

(A)

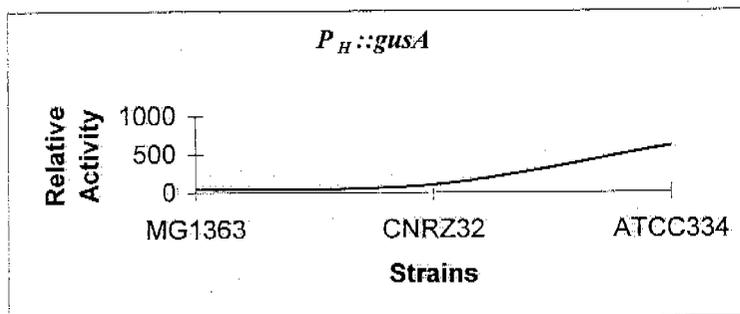
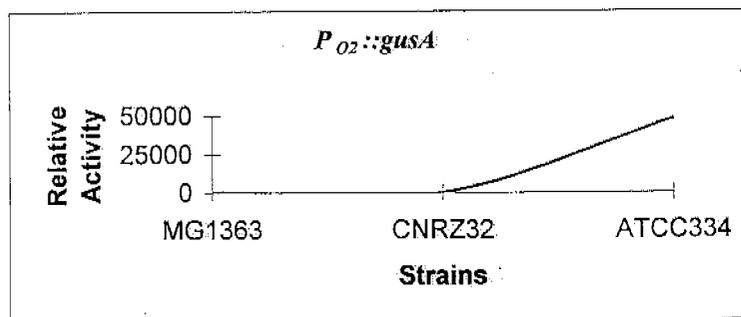
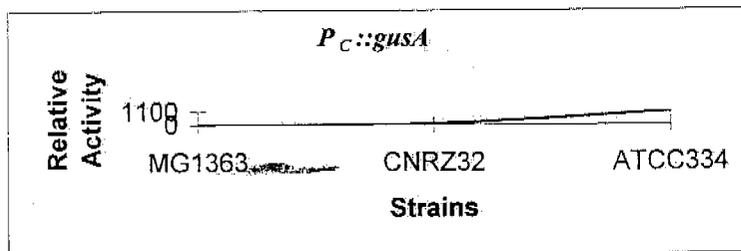
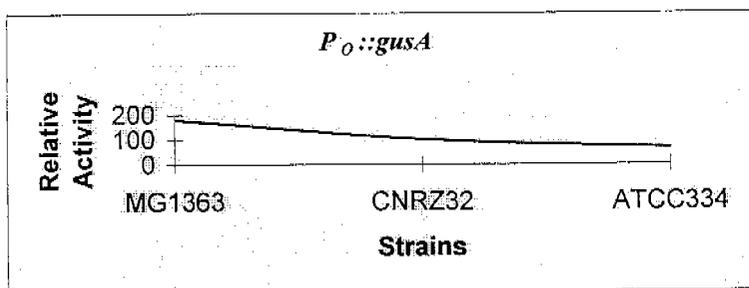


Fig. 2. Relative activities of  $P::gusA$  constructs in three lactic acid bacteria strains, *Lactococcus lactis* MG1363, *Lactobacillus helveticus* CNRZ32 and *Lb. casei* ATCC334, conducted with cell free extracts obtained from (A) mid-logarithmic phase, and (B) stationary phase growing cultures. The specific activity of a given  $P::gusA$  construct in *Lb. helveticus* CNRZ32 was arbitrarily defined as 100%, and the relative activity in two other strains were calculated accordingly.

(B)

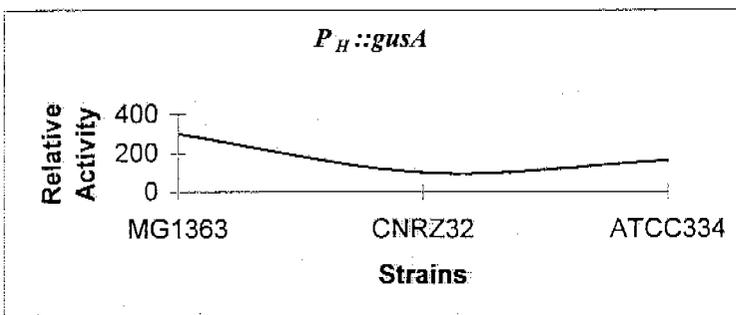
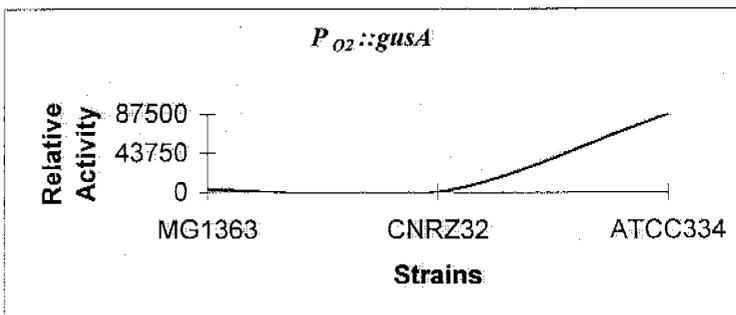
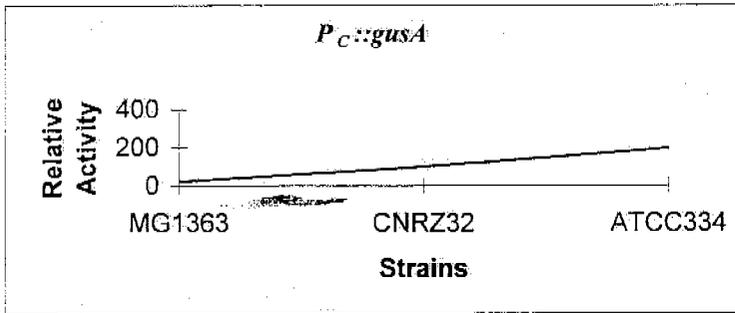
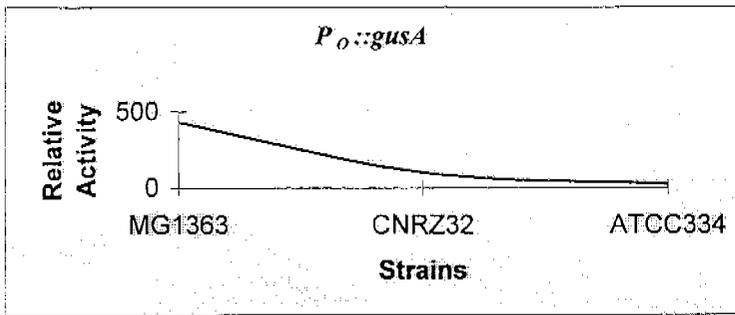
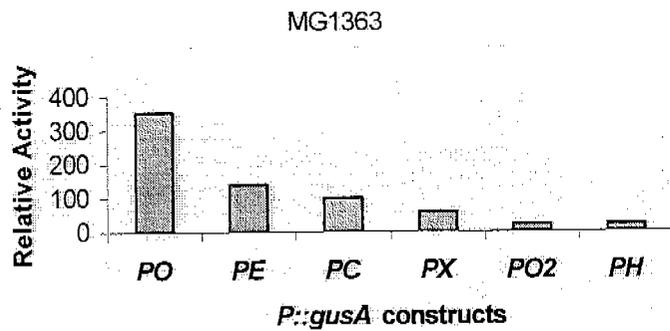
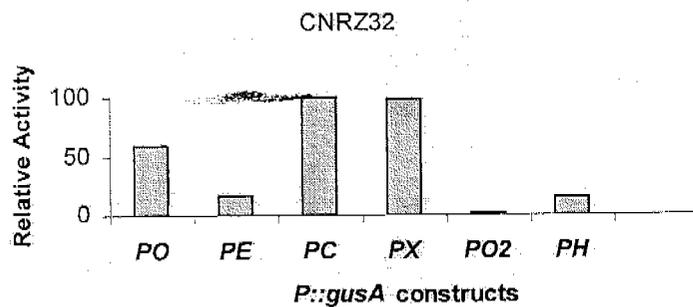


Fig. 2. Relative activities of *P::gusA* constructs in three lactic acid bacteria strains, *Lactococcus lactis* MG1363, *Lactobacillus helveticus* CNRZ32 and *Lb. casei* ATCC334, conducted with cell free extracts obtained from (A) mid-logarithmic phase, and (B) stationary phase growing cultures. The specific activity of a given *P::gusA* construct in *Lb. helveticus* CNRZ32 was arbitrarily defined as 100%, and the relative activity in two other strains were calculated accordingly.

(A)



(B)



(C)

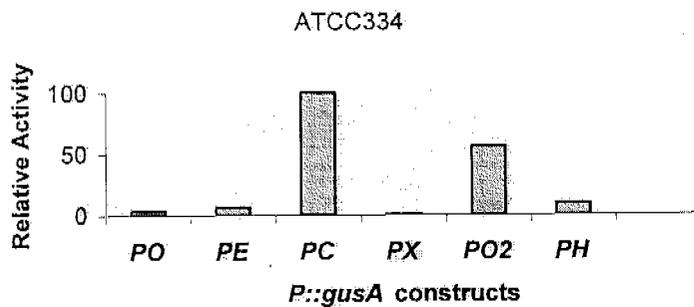
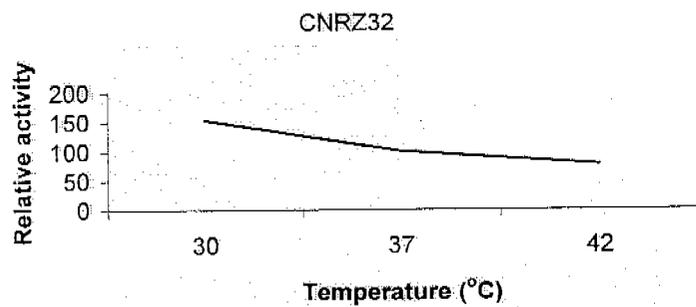
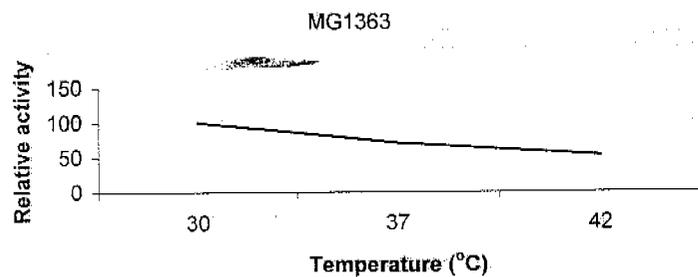


Fig. 3. Relative activities of *P::gusA* constructs in three lactic acid bacteria, (A) *Lactococcus lactis* MG1363, (B) *Lactobacillus helveticus* CNRZ32 and (C) *Lb. casei* ATCC334 at mid-logarithmic phase. The specific activities of *P<sub>C</sub>::gusA* constructs were arbitrarily defined as 100% in each organism examined.

(A)



(B)



(C)

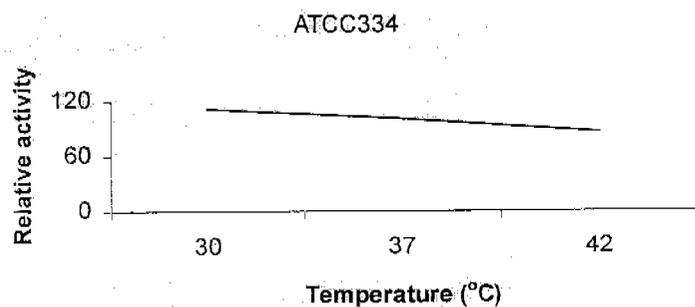
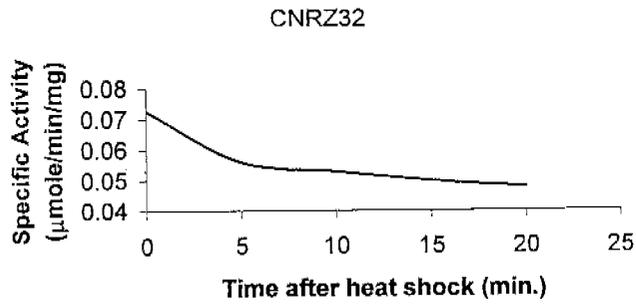
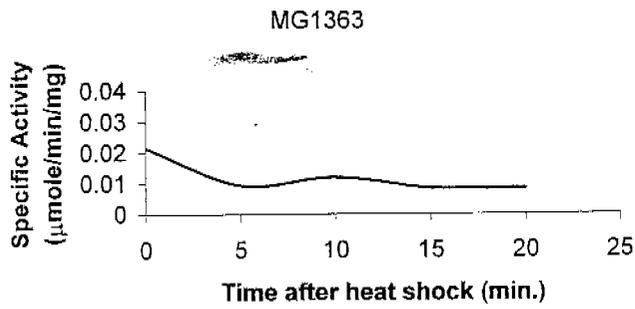


Fig. 4. Relative activities of  $P_H::gusA$  construct at three different growth temperatures (30, 37 and 42°C). Specific activities measured at normal growth temperature, (A) 37°C for *Lactobacillus helveticus* CNRZ32, (B) 30°C for *Lactococcus lactis* MG1363, and (C) 37°C for *Lb. casei* ATCC334, were determined to be 100%, and relative activities of  $P_H::gusA$  construct at different temperatures for each bacterium was calculated accordingly.

(A)



(B)



(C)

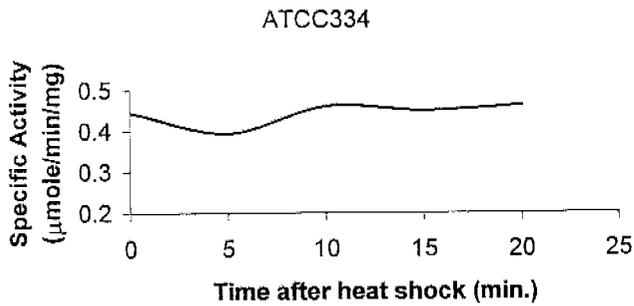


Fig. 5. Heat shock effect on expression of  $P_H::gusA$  in (A) *Lactobacillus helveticus* CNRZ32, (B) *Lactococcus lactis* MG1363, and (C) *Lb. casei* ATCC334.

<<< *orfX*

**ATTT**CATCTTCAATTTGTTTGTAGCCTTCCGGCGTCATTTTTT**GAAAA**TATACCATAATT 60  
 TAACACCTGATTAACAGTTCATTCATTTTTTCTTTCTAATTATACAAAAGTTATCCACAG 120  
 GTGTTAAGCATTGATCTAGCAAAGTTTTTCTACAAAAATTAGCACTATATGCTTTT**GAA**T 180  
→ IR-1 ←  
**GCTAA**ATAGT**TTAACT**TATTTAAAAAACGACT**TATAAT**AATCAG**TGTAAGG**AAAAAGAACG 240  
-35 -10 ↑ → IR-2  
 AGACCTTACAGATTA**AGCAAG**CAAAAAAATTGAA**AGCAAG**TTTTCAAT**ATGGCAACGAT** 300  
← RBS *hsp17* >>>  
**ATGATGAATCGTCGTAATGATATGATGGAT** 330

Fig.6. The 5' untranslated region of *hsp17*. The putative ribosomal binding site (RBS), -10 and -35 hexamers are in boldface and as labeled. Transcriptional start site of *hsp17* is in boldface type and marked with a vertical arrow. Two inverted repeats (IR-1 and IR-2) are underlined and indicated with two inverted arrows. An open reading frame *orfX* is located 232bp upstream of *hsp17* in the opposite orientation.

Table 1. Specific activity of  $\beta$ -glucuronidase derived from different promoters in *Lactobacillus helveticus* CNRZ32, *Lb. casei* ATCC334 and *Lactococcus lactis* MG1363 at exponential growth phase.

Promoters	Specific activity <sup>a</sup>		
	MG1363 <sup>b</sup>	CNRZ32	ATCC334
$P_O$	0.173 $\pm$ 0.009	0.097 $\pm$ 0.015	0.0625 $\pm$ 0.0023
$P_E$	0.068 $\pm$ 0.018	0.027 $\pm$ 0.008	0.109 $\pm$ 0.007
$P_C$	0.049 $\pm$ 0.010	0.165 $\pm$ 0.011	1.708 $\pm$ 0.056
$P_X$	0.029 $\pm$ 0.007	0.163 $\pm$ 0.037	0.007 $\pm$ 0.001
$P_{O2}$	0.011 $\pm$ 0.002	0.0018 $\pm$ 0.0001	0.96 $\pm$ 0.25
$P_N$	0.0009 $\pm$ 0.0002	N.D.	N.D.
$P_H$	0.011 $\pm$ 0.001	0.026 $\pm$ 0.002	0.158 $\pm$ 0.001

<sup>a</sup> Specific activity expressed as mean (micromoles per minute per milligram of protein)  $\pm$  standard deviation.

<sup>b</sup> Exponential growth phase cultures were harvested from media of GM17 (*Lc. lactis* MG1363) or MRS (*Lb. helveticus* CNRZ32 and *Lb. casei* ATCC334) at 30, 37 and 37°C, respectively.

Table 2. Specific activity of  $\beta$ -glucuronidase derived from different promoters in *Lactobacillus helveticus* CNRZ32, *Lb. casei* ATCC334 and *Lactococcus lactis* MG1363 at stationary growth phase.

Promoters	Specific activity <sup>a</sup>		
	MG1363 <sup>b</sup>	CNRZ32	ATCC334
$P_O$	0.133 $\pm$ 0.073	0.031 $\pm$ 0.002	0.0079 $\pm$ 0.0005
$P_E$	0.056 $\pm$ 0.003	0.0054 $\pm$ 0.0006	0.014 $\pm$ 0.003
$P_C$	0.014 $\pm$ 0.001	0.073 $\pm$ 0.008	0.143 $\pm$ 0.075
$P_X$	0.017 $\pm$ 0.007	0.067 $\pm$ 0.010	0.002 $\pm$ 0.001
$P_{O2}$	0.013 $\pm$ 0.004	0.0004 $\pm$ 0.0004	0.351 $\pm$ 0.006
$P_N$	0.0011 $\pm$ 0.0001	N.D.	N.D.
$P_H$	0.015 $\pm$ 0.002	0.005 $\pm$ 0.002	0.0076 $\pm$ 0.0005

<sup>a</sup> Specific activity expressed as mean (micromoles per minute per milligram of protein)  $\pm$  standard deviation.

<sup>b</sup> Stationary growth phase cultures were harvested from media of GM17 (*Lc. lactis* MG1363) or MRS (*Lb. helveticus* CNRZ32 and *Lb. casei* ATCC334) at 30, 37 and 37°C, respectively.

Table 3. Alignment of the sequences of the *Lactobacillus helveticus* CNRZ32 promoters used in this study<sup>a</sup>.

Promoters	-59/-39	-35	TG	-10	+1	RBS
P <sub>X</sub>	TTCT <b>TTATTGATTTC</b> ATTTT	<b>TTGGTT</b>	<b>GG</b>	<b>TAAAAT</b>	A	<b>AAGGAG</b>
P <sub>E</sub>	<b>AATTCTCTGAAGCGAGTTCA</b>	<b>TTGAAC</b>	GT	<b>TAAAAT</b>	A	<b>AAGGAG</b>
P <sub>C</sub>	GTTGG <b>TTATTTTCCCAGACA</b>	<b>TAGACT</b>	AT	<b>TAAAAT</b>	G	<b>AAGGAG</b>
P <sub>N</sub>	<b>ATCAGAAAGAAATTGCCTTT</b>	TTAAGC	<b>TG</b>	<b>TAAAAT</b>	T	<b>AGGAGG</b>
P <sub>O2</sub>	<b>AAAATTAATAAGCAA</b> AATT	<b>TTTTCA</b>	<b>TG</b>	<b>TATGAT</b>	C	<b>AAGGAG</b>
P <sub>O</sub>	<b>ATTAATGCGGAAGA</b> ACGTTT	<b>TTGGCT</b>	<b>TG</b>	<b>TATAAT</b>	A	<b>AAGGAG</b>
P <sub>H</sub>	CT <b>ATATGCTTTTGAAT</b> GCTA	<b>TTAACT</b>	CG	<b>TATAAT</b>	G	<b>AGGAAG</b>
Consensus	AAAWWTWTTTnAAAAAnnn <sup>b</sup>	TTGACA		TATAAT		AAGGAGG

a Promoters were aligned with features defined in promoter regions of bacteria. The UP element (-59 to -39) described by Estrem et al., (estrem et al., 1998), -35/-10 regions as defined in *Lactobacillus* consensus sequences (Pouwels and Leer 1993), and the ribosomal binding site as described in most Gram-positive and Gram-negative bacteria. The bases that are the same as the bases in consensus sequences are indicated as in boldface.

b W=A or T

Table 4. Analysis of promoters of the *Lactobacillus helveticus* CNRZ32.

Promoters	Spacing (bp)	
	-10 vs. -35	-10 vs. +1
$P_H$	15	5
$P_E$	17	6
$P_O$	22	6
$P_{O2}$	18	5
$P_N$	19	6
$P_C$	17	7
$P_X$	17	7
Average	19.3	6