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Structural Features of the *lac* Promoter Affecting *gus*A Expression in *Lactobacillus casei*

Isabel Pérez-Arellano, Gaspar Pérez-Martínez

Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos, (CSIC), Apartado de Correos 73, 46100 Burjassot, Valencia, Spain

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Abstract. With the aim of designing efficient expression systems for *Lactobacillus casei*, different factors affecting gene expression from the strong and highly modulated *lac* promoter have been systematically analyzed, by using the *Escherichia coli* β-glucuronidase gene (*gus*A) as reporter. The activity of this enzyme (GUS) was quantified when *plac*::*gus*A fusions were cloned in plasmids with different copy number or when *gus*A was inserted in the chromosomal lactose operon (single copy). Results showed a clear gene dosage effect and a positive influence of the native *lac* operon transcription and translation signals on GUS expression.

Lactobacillus casei is a lactic acid bacterium (LAB) used as a cheese starter and is also found in the human intestine; it is used in commercial probiotic preparations. A reduced number of heterologous genes have been expressed in this bacterium with different promoters, with or without secretion signals [10, 11, 14, 16, 25, 30]. In spite of the interest in this GRAS microorganism that can serve as a probiotic [7], there are as yet no efficient expression systems, and very little is known about the molecular factors directly involved in the expression of heterologous genes. In contrast, efficient expression systems have been developed for some time in *Lactococcus lactis* by using different marker genes and regulatory elements [6]. The *gus*A gene from *Escherichia coli* has already been used in this bacterium to study the efficiency of the nisin promoter, to select strong promoters, and as an intermediate stage in the construction of food-grade cloning vectors [13, 18, 20, 21].

In *Lactobacillus casei*, lactose genes are transcribed as an operon, *lacTEGF*, encoding an antiterminator (LacT), lactose-specific PTS proteins (LacE and LacF), and a phospho-β-galactosidase (LacG) [1–3, 8, 22]. This operon is induced by lactose through a transcriptional antitermination mechanism in which LacT activity is negatively regulated by the lactose-specific PTS elements, EII^{lac} [9]. In addition, this operon is repressed by glucose and other rapidly metabolized carbon sources. This effect is partly mediated through CcpA-dependent carbon catabolite repression (CCR) [17, 29] and by negative modulation through LacT and PTS elements, constituting an additional CcpA-independent CCR mechanism [9].

Similar systems of antiterminator-dependent regulation have been reported in other lactic acid bacteria, such as Bg1R from *Lactococcus lactis* and Bg1G from *Lactobacillus plantarum* [4, 15]; however, *L. casei lac* operon has been most thoroughly studied, and preliminary gene expression assays with the *lac* promoter have already been published [9, 11, 17]. Nevertheless, heterologous gene expression experiments often yield unexpected results, which can only be explained after the fine molecular scrutiny of the structures generated. In this work, we have compared already existing and new constructs in order to define some of the factors affecting expression of *E. coli gus*A gene from *L. casei lac* promoter, as a model for heterologous protein expression.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this work are listed in Table 1. Transformants of *E. coli* were selected on 300 μg ml^{-1} erythromycin, 5 μg ml^{-1} chloramphenicol, and 50 μg ml^{-1} ampicillin; and of *L. casei* on 5 μg ml^{-1} erythromycin or chloramphenicol. *L. casei* was grown in MRS (Oxoid) or MRS

Correspondence to: G. Pérez-Martínez; *email:* gaspar.perez@iata.csie.es

Table 1. Strains and plasmids used in this work

Strain or plasmid	Relevant features	Reference
Escherichia coli DH5α	*F, sup-E44, hsd-R17, dlac-U169 (f80 lacZ DM15)–rec-A1, end-A1, gyr-A96, thi1, rel-A1*	
Lactobacillus casei CECT 5275	Formerly *Lactobacillus casei* ATCC 393 [pLZ15$^-$]	[10]
L. casei CECT 5290	*Lactobacillus casei* CECT 5276 with *gus*A gene integrated	[10]
pIAβ5	Broad host range vector, 7,25 kb, Er^R, *lacZ*, T1T2 terminators	[19]
pIAlac	pIAβ5 derivative with *plac* from *L. casei* cloned in *Sma*I	This work
pIAlacgus1	pIAβ5 derivative with *plac*::*gus*A (Figs. 1 and 2)	This work
pIAlacgus2	pIAβ5 derivative with *plac*::*gus*A (Figs. 1 and 2)	This work
pNZ272	Promoter probe vector carrying *gus*A from *E. coli*	[20]
pNZlac	pNZ272 with Δplac (without RAT-terminator sequences) from *L. casei*	[17]
pNZRAT	pNZ272 with *plac* from *L. casei*	[9]

fermentation broth (Scharlau) containing 0.5% of ribose, lactose, and/or glucose.

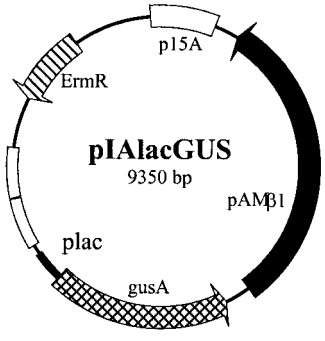
Recombinant DNA procedures. Plasmid DNA from *E. coli* was isolated by the alkaline lysis method [24] and from *L. casei* as described previously [23]. Restriction and modification enzymes were used according to recommendations of manufacturers. General cloning procedures were performed as described by Sambrook et al. [24]. The proof-reading TaqPlus Precision™ PCR system (Stratagene, La Jolla, CA), which renders blunt-ended PCR fragments, was used for all the cloning experiments. Electroporation of *L. casei* was carried out as previously described [5] with a Gene-Pulser apparatus (BioRad Laboratories, Richmond, CA). DNA sequencing was carried out with the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase and run on an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA).

Enzymatic assays. β-glucuronidase activities were assayed in permeabilized *L. casei* cells as previously described [17].

Construction of plasmids. The promoter of the *lac* operon (*plac*) of *L. casei* was cloned into *Sma*I-digested pIAβ5 [19] by using primers lac11 (5′TAGCACTGATCATTAAA3′) and lac33 (5′TTGCACTGGGAGGGGAT3′). The resulting plasmid was named pIAlac. Two vectors were constructed to achieve inducible expression of *gus*A. For the first one, *gus*A was amplified by PCR from pNZ272 with primers gus1 (5′AAAACTGCAGTATTATTATCTTAATGAGG3′) that includes a newly created *Pst*I restriction site, and gus2 (5′CGGAATTCTCATTGTTTGCCTCCC3′). Then, the amplified fragment was cloned in *Sma*I-digested pUC19, and the orientation of the insert was checked by PCR. One clone with the appropriate orientation was digested with *Pst*I, and the resulting fragment was cloned into *Pst*I-digested pIAlac vector. The orientation of this insert was again checked by PCR, and the plasmid obtained was named pIAlacgus1 (Fig. 1). Another vector was made by using pNZRAT (Fig. 1) as a template to amplify a fragment with primers lac11 and gus2, containing a previously reported fusion of the *lac* promoter to the complete *gus*A gene [17], and it was cloned into *Sma*I-digested pIAβ5. The resulting plasmid was named pIAlacgus2 (Fig. 1).

Results and Discussion

Genetic structure of *plac::gus*A fusions. During the preliminary characterization of catabolite repression (CR) on the promoter of the lactose operon (*plac*) in *L. casei* [17], different constructions were obtained with the *gus*A gene of *E. coli* as reporter. Vector pNZRAT contained the complete *plac* and included the start codon of the first gene of the *lac* operon (*lacT*) (Fig. 2A). A similar fusion was made to study the importance of the RNA antiterminator recognition site (RAT)/terminator region of this promoter. In this vector, named pNZlac (Figs. 1 and 2A), the transcription initiation region cloned upstream from *gus*A comprised a deleted promoter (Δplac) lacking the RAT/terminator region. In this report, a significant difference was observed between the maximal activity obtained with both constructs, which was attrib-



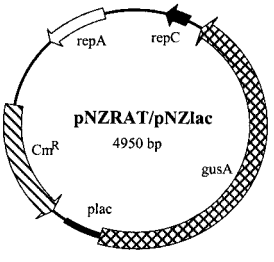


Fig. 1. Schematic representation of the plasmids constructed to test *gus*A expression from *L. casei plac*. Differences between pIAlacgus1 and pIAlacgus2 were at the nucleotide level; therefore, the plasmid map named pIAlacgus represents both of them.

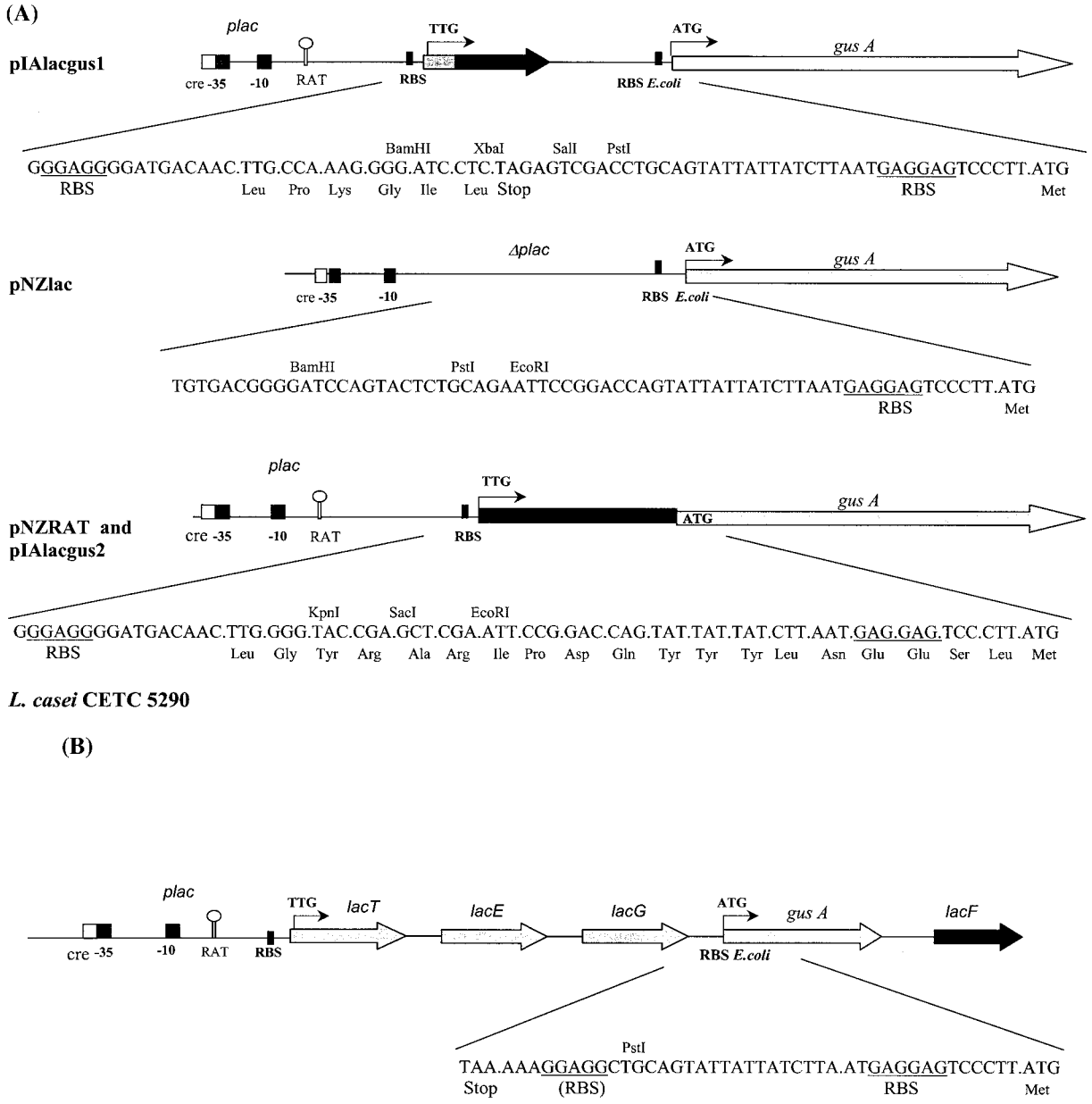


Fig. 2. (**A**) Structure of the different transcription and translation initiation regions cloned upstream *E. coli gus*A in *L. casei* in the plasmids described in the text. (**B**) Structure resulting from the integration of *gus*A in the chromosomal lactose operon of *L. casei*.

uted to a stimulatory effect of LacT binding. The present study began with the detailed sequence analysis of the cloned fragments and the resulting fusions with *gus*A. It showed a translational fusion created in the construction of pNZRAT, starting at the first codon (TTG) of LacT, which added 19 amino acids to the amino terminus of GUS, which had no significant effect on its activity. Hence, translation initiation was occurring from the *lac*T ribosomal binding site (RBS). In contrast, in pNZlac, translation initiation was presumably taking place from the *E. coli* RBS located upstream *gus*A (Fig. 2A). In order to test the cause of the inefficient expression of *gus*A in pNZlac, two new vectors were constructed with the complete *plac* upstream *gus*A in a highly stable cloning vector for *L. casei*, pIAβ5 [19]. In the first plasmid (pIAlacgus1), a stop codon was introduced that avoided the translational fusion between the initial codons of *lac*T and *gus*A; therefore, *gus*A translation was probably starting from the *E. coli* RBS. In the second one (pIAlacgus2), the same fusion obtained in

Table 2. Expression of different transcriptional fusions in *L. casei* β-glucuronidase activity (nmol/min mg [dry wt])[a]

	Lactose	Ribose	Glucose + Lactose
pIAlacgus1	87.2 ± 10.1	25.2 ± 6.2	n.d.[b]
pNZlac	146.5 ± 35.8	172.5 ± 42.1	1.2 ± 0.1
pIAlacgus2	632.0 ± 63.7	183.5 ± 36	7.8 ± 0.9
pNZRAT	4566.8 ± 884.4	1206.6 ± 571.3	14.9 ± 2.2
L. casei CECT 5290	189.1 ± 12.4	43.6 ± 2.8	1.6 ± 0.3

[a] Measured with *p*-nitrophenyl-β-D-glucuronic acid in permeabilized cells grown with 0.5% sugar to an optical density of 0.8. The values (means and standard deviations) are from three independent experiments.
[b] n.d. = not detectable.

pNZRAT was cloned in pIAβ5 in order to prevent expression variations due to the host vector, such as those related to copy number (Fig. 2A).

These vectors were compared with an integrant strain, *L. casei* CETC 5290, which had the *gus*A integrated between *lac*G and *lac*F in the lactose chromosomal operon, and was created using an integrative food-grade expression system [10]. There, the *E. coli gus*A gene with its ribosomal binding sequence was cloned in the multicloning site of this integrative vector after a typical RBS for *Lactobacillus* (Fig. 2B).

Gene dosage effect. When comparing β-glucuronidase activity (Table 2), from the strain *L. casei* CETC 5290 with *gus*A integrated in the lactose chromosomal operon, with that of *L. casei* carrying plasmids with the native *plac* structure, pNZRAT, and pIAlacgus2, a progressive increase was observed as a function of the copy number, although there was no directly proportional effect.

pNZRAT and pIAlacgus2 are harboring the same construction (Fig. 2A), but their host vectors are completely different. pNZRAT contains the pSH71 replication origin [6], which is very homologous to pWV01; they have almost identical features, and they are the rolling circle replication (RCR) plasmid origins most commonly used in LAB [6]. Despite the fact that these vectors are frequently used, copy number estimations in *L. lactis* vary broadly [6, 12]. Recently, with real-time quantitative PCR, the copy number of pWV01 plasmids in *L. casei* was estimated to be 162 to 170 copies per genome [19]. On the other hand, pIAlacgus2 is a theta-type replicating plasmid, carrying pAMβ1 origin, derived from pIAβ5, which was estimated to have 30 copies per genome in *L. casei* [19]. When GUS activities from pNZRAT and pIAlacgus2 were compared, the ratio obtained (6.6–7.2) was almost equivalent to that of their copy number (5.7).

In *L. casei* CETC 5290, where *gus*A gene was in monocopy, β-glucuronidase activity was three times lower than in pIAlacgus2, and 24 times lower than in pNZRAT. However, it was higher than in the multicopy vectors pNZlac and pIAlacgus1. In this strain, the phospho-β-galactosidase activity was about twice as high as in the wild type [10], meaning that the operon was being translated at a higher rate, possibly owing to a polar effect that produced an inefficient translation of *lac*F and, thus, LacT became phosphorylated (inactivated) at a slower rate. This would be supported by the fact that *lac*F-deficient mutants displayed a constitutive expression of the *lac* operon [9].

Expression of *gus*A in the different plasmid constructs. β-Glucuronidase activities of all constructs (Table 2) showed induction by lactose as well as repression by glucose, even in the presence of the inducer (lactose). When cells were grown on ribose, there was no carbon catabolite repression nor lactose induction, and the activity observed was possibly due to a small escape, which was almost proportional to the amount expressed under full induction. This should not account for pNZlac, where the *lac* promoter is deprived of the RAT and terminator sequences and, thus, in ribose-grown cells would be active, as corroborated by the data.

pIAlacgus1 is harboring the complete *lac* promoter, and translation of a small oligopeptide of six amino acids seems to start at the first three codons of LacT. Then, 40 nt downstream the stop codon, translation of *gus*A, would start from the *E. coli* RBS. Here, GUS activity was similar to that of pNZlac. Considering the difference in copy number, as seen with pNZRAT and pIAlacgus2, pNZlac should be expected to have between six- and sevenfold higher activity than pIAlacgus1, but it scarcely doubled it. This could be explained by the presence in pIAlacgus1 of the complete *lac* promoter, including the RAT/terminator sequences. As shown previously [27], secondary structures can stabilize mRNA in *L. lactis*, suggesting that the terminator stem loop in *lac* mRNA might improve its half-life. Besides, translation initiation efficiency might be improved by the binding of LacT to the RAT sequence, which is missing in pNZlac.

pIAlacgus1 also had considerably lower activity than pIAlacgus2. The difference between them basically was that in pIAlacgus1 there was a longer mRNA fragment upstream *gus*A encoding a small oligopeptide, and then *gus*A was possibly translated from its native *E. coli* RBS. pNZRAT and pIAlacgus2 were the most efficient constructs for the expression of GUS. Both showed a pronounced lactose induction of three to five times in relation to non-induced conditions (ribose). They were

carrying the native promoter structure, and GUS translation started from *L. casei lac*T RBS.

The results presented in this study clearly show that a high level of expression can be achieved in *L. casei* by using the highly modulated promoter from the *lac* operon. It has been demonstrated that the copy number of the vector has great importance in improving protein expression. However, some aspects are still to be controlled and understood regarding heterologous protein expression. A number of species-specific molecular factors influencing expression have been proposed [26, 28], such as the spacing optima from the RBS to the start codon, the distance between the transcription and translation sites, the preferred initiation codon, and events like translational coupling or interference, or the complementarity between the Shine–Dalgarno sequence and the 3′ end of the 16S rRNA of the host. Considering the present knowledge available and the results obtained here, it can be suggested that a useful and rapid strategy to assure good expression of heterologous genes in *L. casei* would be to imitate the native structures in efficiently expressed genes or operons, replacing a homologous gene for the one of interest, but keeping the spacing and key structures, especially the RBS, as was suggested in general terms for Gram-positives from the Clostridiaceae family [28].

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