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Proteome

Use of *lac* regulatory elements for gene expression in *Lactobacillus casei*

María José GOSALBES, Isabel PÉREZ-ARELLANO, Carlos David ESTEBAN, José Luis GALÁN, Gaspar PÉREZ-MARTINEZ*

Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, 46100-Burjassot, Valencia, Spain

Abstract — The lactose operon, *lacTEGF*, of *Lactobacillus casei* ssp. *casei* ATCC393 [pLZ15⁻] is encoding an antiterminator protein (LacT), the elements (LacE and LacF) of the lactose-specific phosphotransferase system (PTS) and a phospho-β-galactosidase (LacG). The *lac* operon is repressed by glucose and fructose and is induced by lactose, through the PTS/CcpA signal transduction system and an antiterminator mechanism, respectively. Furthermore, the antiterminator activity of LacT is also negatively modulated possibly by a PTS-mediated phosphorylation event. These strong regulatory mechanisms have been used in this work for the design of expression systems. Hence, *Bacillus licheniformis* α-amylase has been efficiently expressed from pIAβ5lacamy on lactose grown cells. Furthermore, a food-grade mutant, expressing *Lactococcus lactis* acetohydroxy acid synthase genes (*ilvBN*), was obtained with an integrative system, developed using *lacG* and *lacF* as homologous sequences for recombination. As a result, *ilvBN* genes were integrated in tandem between *lacG* and *lacF* in the chromosome and were co-ordinately expressed with the genes of the lactose operon.

***Lactobacillus* / lactose expression system / replicative vector / integrative vector**

Résumé — Usage des éléments régulateurs de l'opéron lactose pour l'expression de gènes chez *Lactobacillus casei*. Les gènes du lactose *Lactobacillus casei* ssp. *casei* ATCC393 [pLZ15⁻] sont regroupés dans un même opéron, *lacTEGF*, codant pour un antiterminateur (LacT), pour les éléments spécifiques du transport du lactose par le système phosphotransferase dépendant du phosphoénolpyruvate (PTS) (LacE et LacF) et pour une phospho-β-galactosidase (LacG). L'opéron *lac* est soumis à une répression par le glucose et le fructose via le système de transduction de signal PTS/CcpA et à une induction par le lactose au moyen d'un mécanisme d'antiterminaison. De plus, l'activité de l'antiterminateur est aussi négativement modulée possiblement par un événement de phosphorylation étant impliqué le PTS. Dans ce travail, on a utilisé ces mécanismes régulateurs pour la construction de systèmes d'expression. Avec le vecteur pIAβ5lacamy, on a obtenu l'expression de l'α-amylase de *Bacillus licheniformis* à partir des éléments régulateurs de l'opéron lactose. On a aussi construit un vecteur intégratif utilisant les gènes *lacG* et *lacF* comme séquence homologue

* Correspondence and reprints
Tel.: (34) 96 3900022; fax: (34) 96 3636301; e-mail: gaspar.perez@iata.csic.es

pour la recombinaison. Les gènes *ilvBN* qui codent pour l'acétohydroxyacide synthétase de *Lactococcus lactis* ont été intégrés dans le chromosome de *L. casei* entre les gènes *lacG* et *lacF* et dans les mutants alimentaires obtenus, l'expression des gènes *ilvBN* est donc coordonnée avec ceux de l'opéron lactose.

***Lactobacillus* / système d'expression du lactose / vecteur replicatif / vecteur intégratif**

1. INTRODUCTION

1.1. Gene expression and food-grade systems in lactic acid bacteria

A number of heterologous genes have been expressed in lactobacilli with different constitutive promoters, with or without secretion signals [11, 15, 17, 22, 32, 37]. However, more efficient expression systems have been developed for *Lactococcus lactis* that used different marker genes and regulatory elements [9]. Recently, a very remarkable system has been designed based on the inducible promoter of the nisin operon from *L. lactis* [31], that could be efficiently transferred to other lactic acid bacteria (LAB), including *Lactobacillus* [18]. Its regulation required the activity of the gene products of *nisK* and *nisR*, elements of a two-component-regulatory system induced by the antimicrobial peptide, nisin. This fact could represent an operative difficulty because previous chromosomal integration of *nisK* and *nisR* or a two-plasmid system was required.

Ideally gene expression systems for LAB should also consider the use of food-grade markers. There is a reduced number of interesting examples, such as the use of the sucrose utilisation genes as dominant marker in an integrative vector to achieve amplification of the desired foreign genes in the chromosome of *Lactococcus lactis* [19]. Other food-grade selection mechanisms have been developed where specific mutations in the host chromosome were complemented. An example of this complementation could be the system where an ochre supressor tRNA rescued a nonsense mutation in the chromosomal purine genes that are required for the growth on milk of *L. lactis* [8]. Also, lactose utilisation genes were chosen for this purpose and different vectors have been constructed using *lacF* and the *lac* promoter from the lactose operon of *L. lactis* [23, 28].

Genetic integration of desired genes can also provide safe food-grade expression systems. A direct way to achieve it is the use of randomly cloned chromosomal fragments or specific genes, such as *cdh*, as integration target [16, 21, 33]. In a more sophisticated mechanism of integration, a phage integrase-mediated site-specific insertion has been used [4, 24]. In the case of *Streptococcus thermophilus*, a system has been developed to integrate reporter genes between *lacS* and *lacZ* and they were consequently induced by lactose [25]. However, there are no examples of studies that used natural regulatory mechanisms in lactobacilli for the expression of heterologous proteins.

1.2. Lactose operon of *Lactobacillus casei*

In *Lactobacillus casei* ATCC393 [pLZ15^{-}], the lactose genes are grouped in a cluster transcribed as single operon, but it does not include the genes of the tagatose-6-P pathway, as occurs in other *lac* operons described [1–3, 10, 13, 29]. The cluster *lacTEGF* encodes an antiterminator protein (LacT), lactose-specific elements (LacE and LacF) of the phosphotransferase system (PTS) and a phospho-β-galactosidase (LacG) (Fig. 1). The promoter region contains a

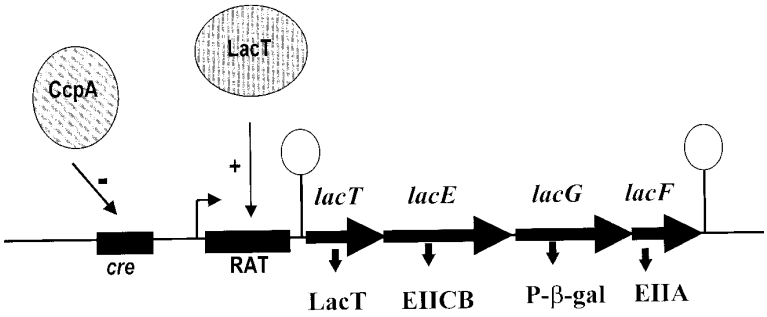

Figure 1. Genetic organisation of the lactose operon in *Lactobacillus casei* and proteins involved in its regulation (LacT and CcpA).

cre element (catabolite responsive element) overlapping the –35 region, which is followed by a highly conserved sequence, the ribonucleic antiterminator (RAT) sequence, and a terminator structure. In fact, it has been shown that the expression of the *lac* operon in *L. casei* ATCC393 [pLZ15^{-}] is subject to dual regulation: carbon catabolite repression (CR) and induction by lactose through transcriptional antitermination [14, 26]. This induction mechanism is remarkably different from the system found in the *lac* operon from *L. lactis*, where gene expression is controlled by the repressor LacR, with tagatose-6-phosphate as the likely inducer [10]. The above-mentioned CR of the *lac* operon is mediated by the general regulatory protein, CcpA, possibly by binding to the *cre* element at the lactose promoter (*plac*). Furthermore, the antiterminator activity of LacT is also negatively controlled by glucose, possibly by PTS-mediated phosphorylation as explained below [14]. Antiterminator proteins of the BglG family, such as LacT, share three common domains: an RNA binding domain and two PTS regulated domains (PRD-I and PRD-II) [30, 35, 36]. According to the proposed model of PTS-mediated control of PRD-containing antiterminators, LacT would exist in three forms: (i) active (inducing *lac* operon), when PRD-I is dephosphorylated and PRD-II phosphorylated; (ii) inactive (non-induced), when both domains are phosphorylated; and (iii) inactive, when PRD-II is dephosphorylated, possibly by HPr, which occurs in the presence of glucose and could be considered as a secondary CR mechanism.

As a consequence of the studies on its genetic regulation, the lactose operon is the best-characterised gene cluster in *L. casei* [1–3, 13, 14, 26, 29]. Due to the mentioned regulatory mechanisms controlling the lactose operon of *L. casei*, this system is highly inducible on lactose, but altogether, it is totally turned down when glucose is added as carbon source. Thus, it has been used to construct replicative and integrative vectors in *Lactobacillus casei* that allowed the achievement of the expression of heterologous proteins from the lactose promoter. This system has provided a useful tool that can be used for strain improvement of *Lactobacillus casei* in fermentation processes and a variety of new applications.

2. RESULTS

2.1. Lactose induction from a shuttle plasmid: expression of *Bacillus licheniformis* α-amylase in *Lactobacillus casei*

The *lac* promoter region (EMBL accession number Z80834) which includes the regulatory elements (*cre*, RAT and terminator

structure) could be amplified with primers lac11 (5'-TAGCACTGATCATTAAA-3') and lac33 (5'-TTGCACTGGGAGGGGAT-3') using the chromosomal DNA of *Lactobacillus casei* ATCC393 [pLZ15$^-$] as template.

The structural part of *Bacillus licheniformis* α-amylase gene and a *L. lactis* secretion signal sequence were amplified by PCR from pGAL9 plasmid using AL93 (5'AATGAGAGCATTAATGTTTC) and AMIR2 (5'TCAACTTAAGTCGACGTCCTTCCTGAGGGC) [27, 38]. This DNA fragment

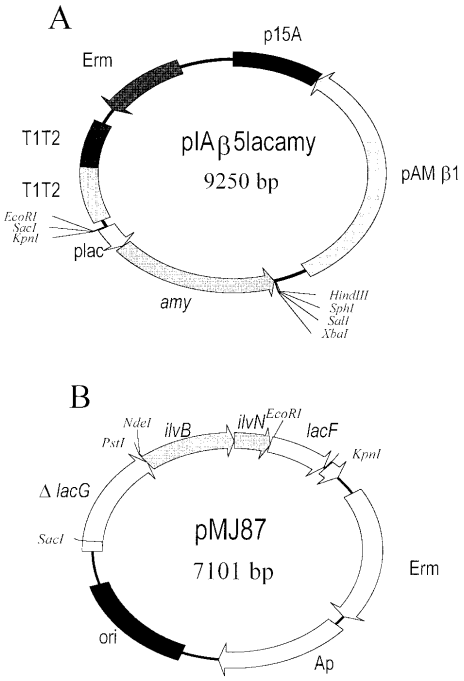

Figure 2. Restriction maps of replicative and integrative vectors. (**A**) Replicative vector: *amy* gene encodes α-amylase from *Bacillus licheniformis*, Erm represents erythromycin-resistance gene, the lactose promoter is indicated as *plac*, T1T2 represent transcriptional terminators, p15A and pAMβ1 correspond to *E. coli* and *Enterococcus* replicons. (**B**) Integrative vector: *ilvBN* genes encode the catalytic and regulatory subunits of acetohydroxy acid synthase, respectively, Erm and Ap are erythromycin and ampicillin-resistance genes, *ori* represents *E. coli* replicon.

was cloned in plasmid pIAβ5 under the promoter of the *lac* operon from *L. casei* (Fig. 2A). The shuttle vector pIAβ5 is derived from pIL253 [34] and contains p15A replicon from pACYC184 [5] and the *lacZ*-T1T2 terminators cassette from pJDC9 [6]. The resulting plasmid pIAβ5lacamy was used to transform *L. casei* and one transformant was selected for further studies. Then, *L. casei* [pIAβ5lacamy] was grown on ribose, lactose or glucose + lactose, and the production of α-amylase was quantified from the supernatants with Phadebas amylase test (Pharmacia and Upjohn). The results obtained indicated that α-amylase expression was clearly induced by lactose and repressed when glucose was present, following the expected expression pattern of the *lac* operon (Tab. I).

The genes coding for other proteins, such as GFP and β-glucuronidase, have also been cloned under *lac* promoter and displayed the same pattern of expression (data not shown).

2.2. Food-grade integration system in *Lactobacillus casei*: expression of *Lactococcus lactis ilvBN* genes

In *Lactobacillus*, chromosomal integration can be achieved by Campbell-like recombination events through DNA cloned in a non-replicating plasmid, which is homologous to the integration target, normally a chromosomal gene. An integrative

Table I. α-amylase activity (U·L^{-1}) detected in *L. casei* transformed with pIAβ5 and pIAβ5lacamy.

	pIAβ5	**pIAβ5lacamy**
Ribose	ND	0.65 ± 0.077
Lactose	ND	13.244 ± 0.577
Glucose + lactose	ND	ND

ND, not detectable. The values and standard deviations are from at least 3 independent experiments.

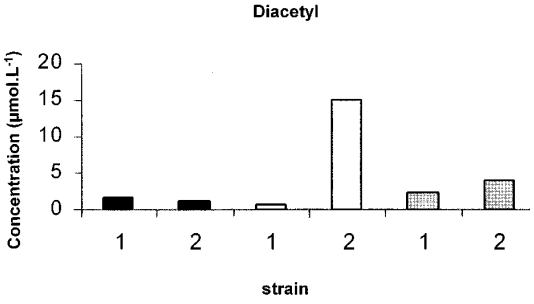

Figure 3. Diacetyl production by the wild-type (1) and *ilvBN* integrant (2) from glucose + lactose (black), lactose (white) and ribose (grey) grown cells.

vector, pMJ67, was constructed by ligation of two fragments of the *lac* operon, corresponding to the 3'end of *lacG* and the complete *lacF* gene, into pRV300 [20]. The intergenic region was modified by introducing different recognition sites for endonucleases and a ribosome-binding site.

Lactococcus lactis ilvBN genes are encoding the catalytic and regulatory subunits of acetohydroxy acid synthase [12]. This enzyme is involved in the biosynthesis of branched-chain amino acids (isoleucine and valine) and also catalyses the conversion of pyruvate to α-acetolactate, with high affinity for pyruvate. Then, α-acetolactate can be decarboxylated to diacetyl, an important compound providing a characteristic flavour of many fermented milk products. In order to increase the production of α-acetolactate and then to obtain a high yield of diacetyl, *ilvBN* genes were integrated between *lacG* and *lacF* in the chromosome of *L. casei*. For this purpose, a PCR fragment was amplified from *L. lactis* MG1363 (kindly provided by J. Kok) genomic DNA and cloned into pMJ67 digested with *Nde*I/*Eco*RI. The resulting plasmid pMJ87 (Fig. 2B) was used to transform *L. casei* CECT5276. This host strain carried a frameshift mutation in *lacF* that conferred it a Lac^- phenotype, which facilitated the selection of integrants after the double recombination event as Lac^+ colonies. Initial integration of pMJ87 in *L. casei* chromosome occurred through a single crossover event. One colony with Erm^R Lac^- phenotype (plasmid integration occurred in *lacF*) was selected and grown for two hundred generations in liquid medium without antibiotic. Colonies that had undergone a second recombination event (through the cloned *lacG* fragment) suffered the excision of the vector giving rise to Erm^s Lac^+ colonies which had integrated the *ilvBN* genes in the *lac* operon. A double recombinant (Erm^s and Lac^+) of *L. casei*, designated as MJ146, was selected for further analysis. This strain showed an expression pattern of P-β-gal activity similar to that of the wild-type strain, it was induced by lactose and repressed by glucose (data not shown). Therefore, the insertion of *ilvBN* did not interfere with the normal expression of the *lac* operon. Diacetyl production by the wild-type and mutant strains has been analysed as described previously [7] in a resting cell system, using bacterial cells that had been grown on ribose, lactose and glucose + lactose. A very high production of this metabolite could be detected when *ilvBN* integrant was grown on lactose, if compared with the other carbon sources (Fig. 3). This indicated that a new and very efficient system has been accomplished for the production of diacetyl in *L. casei*, where *ilvBN* genes from *L. lactis* are coordinately expressed with the *lac* genes.

3. CONCLUSION

A useful tool has been designed using the regulatory elements of the *lac* operon in a multicopy plasmid to express heterologous

proteins in *L. casei* in a controllable way, induced by lactose and strongly repressed by glucose. In addition, a chromosomal insertion system has been developed to facilitate the integration of any gene in the lactose operon, that would be expressed following its regulation pattern. This system could easily be applied to industrial settings, since its inducer, lactose, is the main sugar in milk and milk whey. This integration system can be considered a self-cloning procedure, for which, depending on the country, genetically modified lactobacilli obtained through this process could be more easily accepted for industrial trials.

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