In vivo* effect of mutations in the antiterminator LacT in *Lactobacillus casei

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The antiterminator LacT regulates the expression of the lactose operon in *Lactobacillus casei* and its activity is controlled by EII^{Lac} and common PTS elements. LacT shows the two conserved domains (PRD-I and PRD-II) characteristic of the BglG antiterminator family that are implicated in the regulation of their activity, possibly by phosphorylation of conserved histidines. By site-directed mutagenesis of LacT, four histidines (His-101, His-159 in PRD-I and His-210, His-273 in PRD-II) were replaced by alanine or aspartate, mimicking non-phosphorylated and phosphorylated forms, respectively. These constructions were used to complement $\Delta lacT$ and $\Delta ccpA$ mutants. *L. casei* strains ($\Delta lacT$) carrying the replacement of His-101 or His-159 by Ala showed phospho- β -galactosidase activity in absence of the inducer (lactose), indicating that these amino acids, located in PRD-I, are essential for EII-dependent induction of the *lac* operon, possibly by dephosphorylation. Interestingly, these mutations rendered LacT thermosensitive. Moreover, expression of H210A and H273A (PRD-II) mutations in *L. casei* $\Delta ccpA$ showed that these two histidyl residues could have a role in LacT-dependent carbon catabolite repression (CCR) of this system. Overexpression of LacT in a *ccpA* background rendered the *lac* operon insensitive to CCR, but it was still sensitive to lactose induction. This suggests that the transfer of phosphate groups from PTS elements, which controls these two regulatory processes (CCR and substrate induction), could have different affinity for PRD-I and PRD-II histidines.

Keywords: *L. casei*, lactose induction, carbon catabolite repression, mutagenesis, phosphorylation

INTRODUCTION

In lactic acid bacteria, lactose transport and metabolism can be mediated by several pathways. A lactose-specific phosphoenol-pyruvate-dependent phosphotransferase system (PTS) and phospho- β -galactosidase (P- β -gal) have been described in the genera *Staphylococcus*, *Streptococcus*, *Lactococcus* and *Lactobacillus* (de Vos & Vaughan, 1994). In *Lactobacillus casei*, lactose genes are transcribed as an operon, *lacTEGF*, encoding an antiterminator (LacT), lactose-specific PTS proteins (LacE and LacF) and a P- β -gal (LacG) (Alpert & Chassy, 1988, 1990; Porter & Chassy, 1988; Alpert & Siebers,

1997; Gosalbes *et al.*, 1997). In this micro-organism, the *lac* operon is subject to induction by lactose through a mechanism of transcriptional antitermination in which LacT activity is negatively regulated by the lactose-specific enzymes, EII^{Lac} (Gosalbes *et al.*, 1999). Moreover, the expression of this operon is repressed by glucose and other rapidly metabolizable carbon sources, partly through CcpA-mediated carbon catabolite repression (CCR) (Monedero *et al.*, 1997; Viana *et al.*, 2000). However, it has been shown that LacT and PTS elements are involved in an additional CcpA-independent CCR mechanism (Gosalbes *et al.*, 1999).

Antitermination activity has been extensively studied in homologous proteins, such as BglG of *Escherichia coli* (Mahadevan & Wright, 1987; Mahadevan *et al.*, 1987; Schnetz *et al.*, 1987; Schnetz & Rak, 1988, 1990; Amster-Choder *et al.*, 1989; Houtman *et al.*, 1990;

Abbreviations: CCR, carbon catabolite repression; P- β -gal, phospho- β -galactosidase; PRD, PTS regulation domain; PTS, phosphoenol-pyruvate-dependent phosphotransferase system; RAT, ribonucleic antiterminator.

Amster-Choder & Wright, 1993; Görke & Rak, 1999) and SacT, SacY, LicT and GlcT of *Bacillus subtilis* (Zukowski *et al.*, 1990; Crutz *et al.*, 1990; Débarbouillé *et al.*, 1990; Arnaud *et al.*, 1992, 1996; Crutz & Steinmetz, 1992; Le Coq *et al.*, 1995; Krüger & Hecker, 1995; Schnetz *et al.*, 1996; Rutberg, 1997; Stülke *et al.*, 1997; Tortosa *et al.*, 1997; Bachem & Stülke, 1998; Idelson & Amster-Choder, 1998). In lactic acid bacteria, only two other antiterminator proteins have been reported: BglR in *Lactococcus lactis* and, recently, BglG in *Lactobacillus plantarum* (Bardowski *et al.*, 1994; Marasco *et al.*, 2000). Antitermination activity requires binding of these proteins to a ribonucleic antiterminator (RAT) sequence in the mRNA, stabilizing a stem-loop structure that prevents the formation of the transcriptional terminator located in the leader fragment preceding the coding regions (Aymerich & Steinmetz, 1992). The antiterminators show a modular organization, with an RNA-binding region and two conserved PTS regulation domains (PRD-I and PRD-II), which, as demonstrated for BglG, LicT, SacT and SacY, contain phosphorylatable histidyl residues (two in each domain) that render them susceptible to regulation by PTS elements (Stülke *et al.*, 1998; Tortosa *et al.*, 1997). It has been proposed that PRD-I is negatively regulated by the sugar-specific PTS elements, EII, and PRD-II could be subject to a positive control by the general PTS protein, HPr (Stülke *et al.*, 1998). Biochemical and genetic studies allowed the phosphorylation sites in different regulators to be identified. However, despite the fact that they are well conserved, they have different regulatory roles. The antiterminators SacT, GlcT and LicT from *Bacillus subtilis* are negatively controlled by EII-dependent phosphorylation of one histidyl residue in PRD-I (His-97, His-104 and His-100, respectively) (Arnaud *et al.*, 1992, 1996; Le Coq *et al.*, 1995; Bachem & Stülke, 1998). However, in the PRD-containing activators LicR and LevR of *B. subtilis*, and MtlR of *Bacillus stearothermophilus*, the phosphorylation sites involved in this regulatory mechanism are located in the EIIA-like domain and PRD-II (Martin-Verstraete *et al.*, 1998; Tobisch *et al.*, 1999; Henstra *et al.*, 2000). In addition, some of these regulators (SacT, LicT and LevR) are activated by HPr-dependent phosphorylation at different sites (Martin-Verstraete *et al.*, 1995, 1998; Arnaud *et al.*, 1996; Lindner *et al.*, 1999). In *E. coli*, the antiterminator BglG regulates the expression of the *bgl* operon involved in β -glucoside transport. EII^{Bgl} transports and phosphorylates β -glucosides but it also modulates BglG activity by phosphorylation. The His-208 of this antiterminator, located in PRD-II, was identified as the phosphorylation site (Chen *et al.*, 1997, 2000). On the other hand, it has been recently shown that HPr also phosphorylates BglG, but the presumptive sites remain unidentified (Görke & Rak, 1999).

The aim of this work was to investigate the role of the conserved histidyl residues (His-101, His-159, His-210 and His-273) of LacT in the mechanisms that control antiterminator activity in *Lactobacillus casei*. For this purpose, the potential phosphorylation sites of LacT

were replaced by alanine or aspartate by site-directed mutagenesis. The effect of these mutations on the expression of the *lac* operon was evaluated by determining P- β -gal activity from cultures grown under different induction/repression conditions.

METHODS

Plasmids, bacterial strains and growth conditions. *Lactobacillus casei* strains and plasmids used in this work are listed in Table 1. *L. casei* cells were grown in MRS medium (Oxoid) and MRS fermentation broth (Adsa-Micro) plus 0.5% (w/v) of the different carbohydrates at 37 °C and 30 °C under static conditions. *E. coli* DH5 α was grown with shaking at 37 °C in Luria-Bertani (LB) medium. Plating of bacteria was performed on the respective media solidified with 1.5% agar. When required, the concentrations of antibiotics used were 100 μ g ampicillin and 300 μ g erythromycin ml⁻¹ to select *E. coli* transformants and 5 μ g erythromycin ml⁻¹ for *L. casei*.

Recombinant DNA procedures. Genomic DNA from *L. casei* was purified using a Purogene DNA Isolation Kit (Gentra Systems), following the procedure described by the manufacturer. Restriction and modifying enzymes were purchased commercially and used according to the recommendations of the manufacturers. General cloning procedures were performed as described by Sambrook *et al.* (1989). *L. casei* strains were transformed by electroporation with a Gene-pulser apparatus (Bio-Rad) as described elsewhere (Posno *et al.*, 1991). PCR was performed using the Expand High-Fidelity PCR system (Roche Molecular Biochemicals), containing 200 μ M of each deoxynucleoside triphosphate and 10 pmol of each primer. After agarose gel electrophoresis, the amplified DNA was recovered with a GFX PCR Kit (Amersham Pharmacia Biotech).

Construction of a Δ *lacT* mutant. Deletion of *lacT* was obtained by Campbell-like recombination using the integrative plasmid pRV300 (Leloup *et al.*, 1997). Two fragments of 500 and 543 bp spanning the regions upstream and downstream of *lacT*, respectively, were obtained by PCR using total DNA of *L. casei* as template. The primers used were lac50 (5'-GAGCGGTACCATCAGTAGA-3') and lac51 (5'-GTTGTCATCCCCCTCCCAG-3') for one fragment and lac52 (5'-CGCGCTCGCAGTCGTAGA-3') and lac25 (5'-CGATATGAGCTCAGATC-3') for the other fragment. The two PCR products were ligated and the ligation mix used as template in a PCR with lac50 and lac25. These two oligonucleotides carried *KpnI* and *SacI* sites (underlined), respectively, allowing the cloning of the last PCR product into *KpnI/SacI*-digested pRV300, giving pRV Δ lacT. This plasmid was used to transform *L. casei*. A Lac⁺ and Er⁺ transformant was grown for 200 generations without antibiotic in order to allow the second recombination event, which would excise the vector, giving Lac⁻ Er⁻ colonies. The correct first and second recombination events were confirmed by PCR. One of the *lacT* mutants was selected and the strain named BL195. In this strain the *lacT* gene was totally deleted, while keeping the *lac* promoter, including all the initiation signals and regulatory sites unaltered upstream of the remaining genes of the *lac* operon.

Plasmid constructions and site-directed mutagenesis. To obtain the plasmid pLacT, *lacT* was amplified with primers lac53 (5'-GCACTGGGAGGGGATGACAA-3') and lac54 (5'-TTGGAAGCTTGCTTCAAAGCC-3') using *L. casei* DNA as template, and the PCR product was cloned into the *SmaI* site of pUC18 using a Sureclone kit (Amersham Pharmacia Biotech). The lac54 primer had two substitutions (boldface) to

Table 1. *L. casei* strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<i>L. casei</i>		
CECT 5275*	Wild-type	B. Chassy
PL20	CECT 5275(pGAL9)	This work
BL195	$\Delta lacT$	This work
BL190	$\Delta ccpA$	C. D. Esteban
Plasmids		
pRV300	Er ^r	Leloup <i>et al.</i> (1997)
pRV $\Delta lacT$	pRV300 with two fragments spanning the regions downstream and upstream of <i>lacT</i>	This work
pUC18	Am ^r	Amersham Pharmacia Biotech
pLacT	pUC18 with <i>lacT</i>	This work
pLacTH101A	pLacT derivative (codon 101 of <i>lacT</i> is GCT for Ala)	This work
pLacTH101D	pLacT derivative (codon 101 of <i>lacT</i> is GAT for Asp)	This work
pLacTH159A	pLacT derivative (codon 159 of <i>lacT</i> is GCC for Ala)	This work
pLacTH210A	pLacT derivative (codon 210 of <i>lacT</i> is GCT for Ala)	This work
pLacTH210D	pLacT derivative (codon 210 of <i>lacT</i> is GAT for Asp)	This work
pLacTH273A	pLacT derivative (codon 273 of <i>lacT</i> is GCC for Ala)	This work
pGAL9	Er ^r	Pérez-Martínez <i>et al.</i> (1992)
pGALT	pGAL9 derivative expressing LacT	This work
pGALTH101A	pGAL9 derivative expressing LacTH101A	This work
pGALTH101D	pGAL9 derivative expressing LacTH101D	This work
pGALTH159A	pGAL9 derivative expressing LacTH159A	This work
pGALTH210A	pGAL9 derivative expressing LacTH210A	This work
pGALTH210D	pGAL9 derivative expressing LacTH210D	This work
pGALTH273A	pGAL9 derivative expressing LacTH273A	This work

* CECT, Colección Española de Cultivos Tipo.

Fig. 1. Site-directed mutagenesis and strategy of plasmid construction. The bar represents the LacT protein, with the two PRDs (in black) and four putative phosphorylation sites, His-101, His-159, His-210 and His-273. Arrows indicate oligonucleotides that were used in the PCRs as primers. A point mutation (*) is indicated as example. The resulting PCR fragments containing the mutations are indicated as H101A (His-101 replaced by alanine), H101D (His-101 replaced by aspartate), H159A (His-159 replaced by alanine), H210A (His-210 replaced by alanine), H210D (His-210 replaced by aspartate) and H273A (His-273 replaced by alanine). These fragments were cloned into pUC18 and subcloned into pGAL9 vector, giving pGALT, pGALTH101A, pGALTH101D, pGALTH159A, pGALTH210A, pGALTH210D and pGALTH273A, which contained wild-type *lacT* and the mutated genes, respectively (see Table 1). These plasmids were used to transform *L. casei* CECT5275, the $\Delta lacT$ strain (BL195) and the *ccpA* mutant (BL190).

introduce a new *Hind*III site (underlined). pLacT was used as template in the site-directed mutagenesis to replace the triplets encoding histidyl residues of LacT by alanine or aspartate (H101A, H101D, H159A, H210A, H210D and H273A). These amino acids (Ala and Asp) mimic non-phosphorylated and phosphorylated forms of the residue. The overlap extension method applied allows introduction of site-specific mutations and generation of a mutant gene in two PCR steps (Ho *et al.*, 1989). In the first PCR step, two fragments of *lacT* were independently amplified using outside primers (lac53 or lac54) and the appropriate oligonucleotide of the following mutagenic primers (lac59 5'-TGAGTGATGCTATTTACGA and lac60 5'-TCGTAAATAGCATCACTCA, lac61 5'-TGAGTGATGATATTTACGA and lac62 5'-TCGTAAATATCATCATCA, lac63 5'-TGGCGTTGGCCTTTATCAA and lac64 5'-TTGATAAAGGCCAACGCCA, lac65 5'-TTATGATTGCTCTCCAGTA and lac66 5'-TACTGGAGAGCAATCA-TAA, lac67 5'-TTATGATTGATCTCCAGTA and lac68 5'-TACTGGAGATCAATCATAA, lac69 5'-TCATCATCGC-CATTACGCG and lac70 5'-CGCTGAATGGCGATGA-TGA) (Fig. 1). The mutated triplets are in boldface. These pairs of primers are complementary, hence the strands of the fragments obtained overlapped and they could be extended in a subsequent reaction using lac53 and lac54 primers. The products of these second PCRs were cloned into pUC18/*Sma*I. The resulting plasmids were named pLacH101A, pLacH101D, pLacH159A, pLacH210A and pLacH210D (Fig. 1). To replace His-273 by an Ala residue, lac2 (5'-CAACGATATAAGCG-CAGATC) was used as external primer. The strategy was followed as described above and the plasmid obtained was designated pLacH273A (Fig. 1). The orientation of the inserts in pUC18 vector was checked by digestion with the endonuclease *Hind*III. The fragments were subsequently sequenced to exclude PCR artifacts using a Perkin-Elmer Abi Prism 310 automated sequencer. Plasmids carrying wild-type and mutated genes were digested with *Bam*HI and *Hind*III and the resulting fragment cloned into *Bam*HI/*Hind*III-digested pGAL9 (Pérez-Martínez *et al.*, 1992), generating pGALT, pGALTH101A, pGALTH101D, pGALTH159A, pGALTH210A, pGALTH210D and pGALTH273A. In these pGAL9-derived plasmids *lacT* variants are under control of the constitutive SPO2/AL9 promoter. These plasmids were used to transform *L. casei* CECT5275, the Δ *lacT* strain (BL195) and the *ccpA* mutant (C. D. Esteban, unpublished data) (Fig. 1). The *L. casei* CECT5275(pGAL9) transformant was named PL20.

P- β -gal assay. Activity was assayed as previously described (Chassy & Thompson, 1983). *L. casei* cells were permeabilized by vortexing for 5 min with 10 mM deoxycholic acid (Taranto, 1999).

RESULTS

Effect of mutations in His-101 and His-159 on LacT activity

LacT activity is negatively regulated by EII^{Lac}, as *L. casei* mutants in these elements displayed an inducer-independent antiterminator activity (Gosalbes *et al.*, 1999). The conserved histidyl residues of PRD-I were replaced by Ala (H101A and H159A) and by Asp (H101D) to study their role in the induction mechanism of the *lac* operon. These *lacT* mutant genes were cloned under a constitutive promoter SPO2/AL9 in pGAL9 (Table 1, Fig. 1) and were used to complement a mutant with a chromosomal deletion of *lacT* (BL195), obtained

by Campbell-like recombination. This Δ *lacT* mutant could not be grown on lactose, since it lacked the anti-terminator. BL195 was transformed with pGALT, pGALTH101A, pGALTH101D and pGALTH159A, and its complementation was initially tested on MRS fermentation plates with lactose. While BL195(pGALT), expressing wild-type *lacT* from the SPO2/AL9 promoter, and the wild-type strain (PL20) fermented this sugar, the same strain transformed with plasmids bearing mutated *lacT* [BL195(pGALTH101A), BL195(pGALTH101D) and BL195(pGALTH159A)] did not utilize lactose at 37 °C. However, surprisingly, when grown at 30 °C BL195(pGALTH101A) and BL195(pGALTH159A) showed a Lac⁺ phenotype. In order to confirm this finding, Δ *lacT* transformants carrying one of these two mutations (H101A or H159A) were grown at 37 °C and 30 °C on MRS fermentation broth supplemented with lactose and the P- β -gal activity was determined, as a measure of the *lac* operon expression. Neither BL195(pGALTH101A) nor BL195(pGALTH159A) showed activity when grown at 37 °C [4.66 ± 0.30 and 1.18 ± 0.64 nmol min⁻¹ (mg dry weight)⁻¹, respectively], but a significant activity was detected at 30 °C [22.55 ± 3.37 and 23.45 ± 5.48 nmol min⁻¹ (mg dry weight)⁻¹, respectively].

The involvement of His-101 and His-159 in the induction mechanism of the *lac* operon was studied *in vivo* by determining P- β -gal activity in BL195(pGALT), BL195(pGALTH101A), BL195(pGALTH101D), BL195(pGALTH159A) and wild-type strains, when grown on ribose (non-inducing sugar), lactose (inducer) and glucose plus lactose (CCR) (Table 2). In BL195(pGALT), the expression of *lac* operon was restored in lactose-grown cells, although P- β -gal activity was lower than that found in the wild-type strain (PL20). Both strains, BL195(pGALT) and PL20, showed the same pattern of P- β -gal expression: induction by lactose and repression by glucose. This suggested that PRD-I from LacT could be dephosphorylated by EII^{Lac} even when it is overexpressed. In the absence of lactose (ribose-grown cells), transformants with Ala replacements [BL195(pGALTH101A) and BL195(pGALTH159A)] showed about half the activity detected on lactose, suggesting an additive effect of these mutations. Alternatively, if each of the Ala replacements in PRD-I could render a fully active LacT, the structural instability provoked by these mutations may be the cause for the reduced activity detected. In contrast, no, or negligible, activity was detected in BL195(pGALTH101D) grown on any of the three sugars used (Table 2), indicating that the phosphorylation of this residue may inactivate LacT, which would complement the results obtained with the Ala replacements.

Characterization of putative phosphorylation sites in the PRD-II of LacT

Sequence alignment of proteins belonging to the BglG family indicated His-210 and His-273 of LacT as potential phosphorylation sites. Thus, strains

Table 2. P- β -gal activity of different strains

Strain	P- β -gal activity* of cells grown on:		
	Lactose	Glucose + lactose	Ribose
PL20	36 $\pm$ 2.56	ND	0.84 $\pm$ 0.43
BL195(pGAL9)	ND	ND	1.29 $\pm$ 0.15
BL195(pGALT)	19.34 $\pm$ 5.86	2.02 $\pm$ 0.73	1.88 $\pm$ 0.25
BL195(pGALTH101A)†	22.55 $\pm$ 3.37	1.20 $\pm$ 0.44	13.07 $\pm$ 4.02
BL195(pGALTH101D)	ND	1.40 $\pm$ 0.66	1.34 $\pm$ 0.60
BL195(pGALTH159A)†	23.45 $\pm$ 5.48	1.94 $\pm$ 0.45	12.35 $\pm$ 3.70
BL195(pGALTH210A)	ND	2.3 $\pm$ 0.34	2.15 $\pm$ 1.06
BL195(pGALTH210D)	ND	1.66 $\pm$ 0.70	0.74 $\pm$ 0.22
BL195(pGALTH273A)	ND	0.60 $\pm$ 0.13	0.97 $\pm$ 0.32
BL190(pGAL9)	44.60 $\pm$ 3.65	ND	ND
BL190(pGALT)	44.86 $\pm$ 3.22	40.90 $\pm$ 0.58	ND
BL190(pGALTH210A)	49.49 $\pm$ 2.5	ND	ND
BL190(pGALTH210D)	46.63 $\pm$ 1.45	16.26 $\pm$ 0.96	ND
BL190(pGALTH273A)	48.42 $\pm$ 3.25	ND	ND

* P- β -gal activity is expressed as nmol *o*-nitrophenyl β -D-galactopyranoside 6-phosphate hydrolysed min⁻¹ (mg dry weight)⁻¹. The values and standard deviations are from at least three independent experiments. ND, Not detected.

† P- β -gal activity from BL195(pGALTH101A) and BL195(pGALTH159A) corresponds to the cultures grown at 30 °C (all other values are from cultures grown at 37 °C).

[BL195(pGALTH210A), BL195(pGALTH210D) and BL195(pGALTH273A)] were constructed with the His-210 and His-273 residues replaced by Ala or Asp, mimicking non-phosphorylated and phosphorylated forms, respectively. In this case, thermosensitivity was also tested on MRS fermentation plates supplemented with lactose at 37 °C and 30 °C. None of the mutants grew on lactose at either temperature. In fact, no, or negligible, P- β -gal activity was detected on ribose- or glucose-plus-lactose-grown cells in these mutants (Table 2).

To study the involvement of PRD-II (His-210 and His-273) in CCR, a $\Delta ccpA$ mutant (BL190) was transformed with pGAL9, pGALT, pGALTH210A, pGALTH210D and pGALTH273A. All the transformants showed P- β -gal activity in lactose-grown cells due to the normal expression of the chromosomal *lac* operon in the host strain BL190. However, any effect on the expression of the *lac* operon in BL190 on glucose-plus-lactose medium would be exclusively due to LacT-mediated CCR. Expression of LacT from a multicopy plasmid (pGALT) in the *ccpA* mutant conferred glucose insensitivity, possibly due to dephosphorylation of PRD-II when the inducer was present and in conditions of LacT molar excess. Expression of the mutation H210D in BL190 resulted in the activation of the operon in the presence of glucose and the inducer, lactose, indicating that this mutation was partially active and insensitive to glucose repression in the *ccpA* mutant. However, Ala replacements did not give an active form of antiterminator protein, since the repressing effect of glucose on

P- β -gal activity remained in BL190(pGALTH210A) and BL190(pGALTH273A) (Table 2).

DISCUSSION

The antiterminator LacT regulates lactose operon (*lacTEGF*) expression in *L. casei*. The activity of this protein is controlled by EII^{Lac} and PTS elements in response to the availability of lactose or the presence of glucose (Gosalbes *et al.*, 1999; Monedero *et al.*, 1997; Viana *et al.*, 2000). Sequence analysis of the members of the BglG family revealed a modular organization, with an RNA-binding domain and PTS-regulated domains (PRD-I and PRD-II) with usually four well-conserved potential phosphorylation sites (two in each domain). PRD-containing regulators could be subject to a dual regulation by phosphorylation: negative control by EII exerted on PRD-I and positive control by HPr-His-P generally on PRD-II. Therefore, His-101, His-159 in PRD-I and His-210, His-273 in PRD-II of LacT were mutated to investigate their role in the phosphorylation-dependent regulatory mechanisms.

BL195 ($\Delta lacT$) had a complete deletion of *lacT*, and therefore it had a Lac⁻ phenotype. The operator, RAT sequence and terminator structure remained functional, as demonstrated by the fact that constitutive expression of LacT from pGALT partially restored growth on lactose and gave the same expression pattern as the wild-type (glucose repression and induction by lactose). However, in other works reporting overproduction of antiterminators, such as LicT, substrate induction was

eliminated (Krüger & Hecker, 1995; Krüger *et al.*, 1996).

Antiterminator activity of all members of the BglG family of antiterminators has been shown to be subject to substrate induction through phosphate exchange with the sugar-specific EII PTS elements (Stülke *et al.*, 1998). In *L. casei*, transcriptional studies of the *lac* operon in *lacE* and *lacF* mutants showed an inducer-independent antiterminator activity, indicating a negative control of these elements (Gosalbes *et al.*, 1999). According to the model of PTS-mediated regulation of antiterminators (Stülke *et al.*, 1998), EII^{Lac} would dephosphorylate LacT PRD-I in the presence of lactose to yield its active form. EII-dependent negative control has been demonstrated to involve at least one of two conserved histidines of PRD-I in LicT, SacY, SacT and GlcT (His-101, His-99, His-97 and His-104, respectively) (Schnetz *et al.*, 1987; Débarbouillé *et al.*, 1990; Arnaud *et al.*, 1992, 1996; Stülke *et al.*, 1997; Bachem & Stülke, 1998). PRD-I alignment of different antiterminators located highly conserved histidines at positions His-101 and His-159 in LacT. In this work, the replacement of His-101 or His-159 by Ala rendered LacT active in the absence of lactose (ribose-grown cells), although the P- β -gal activity observed in these strains [BL195(pGALTH101A) and BL195(pGALTH159A)] was lower than that detected on lactose-grown cells. It could be proposed that both residues were involved in the lactose-induction process and one or both of them should be dephosphorylated to obtain full LacT activity. These replacements of His-101 and His-159 by Ala yielded the same phenotype of thermosensitive LacT, suggesting that these histidyl residues may interact and be playing a role in the conformational stability of PRD-I in LacT, as has recently been proposed from the resolved structure of PRDs in LicT (van Tilbeurgh *et al.*, 2001).

LacT activity has also been shown to be sensitive to the presence of glucose, which caused the already reported CcpA-independent CCR of the *L. casei lac* operon (Monedero *et al.*, 1997; Gosalbes *et al.*, 1999; Viana *et al.*, 2000). In homologous antiterminators, this event apparently required phosphorylation of PRD-II by HPr-His-P, although this effect varied slightly among them. Whereas SacT and LicT are subject to a positive control by HPr-dependent phosphorylation, SacY and GlcT activities do not require it. LicT can be phosphorylated by HPr at three sites, one in PRD-I (His-159) and the other two in PRD-II (His-207 and His-269). Furthermore, His-207 is preferentially phosphorylated, indicating a fine modulation of LicT activity (Lindner *et al.*, 1999). In contrast, HPr-dependent phosphorylation at the equivalent sites (His-207 and His-269) in SacY does not affect its activity (Tortosa *et al.*, 1997). The total loss of activity in BL195(pGALTH210A) and BL195(pGALTH273A) when grown on lactose suggested that both His residues (His-210 and His-273) should be phosphorylated to obtain the active form of LacT. All the mutants and the wild-type showed a remarkable glucose repression effect, as expression of the *lac* operon is also repressed by CcpA-dependent

CCR in this genetic background ($\Delta lacT$). Thus, we studied the involvement of His-210 and His-273 in LacT-mediated CCR using a *ccpA* mutant. Strikingly, a derepressed phenotype was observed in BL190(pGALT), possibly due to the overexpression of LacT from a constitutive promoter in a high-copy-number plasmid. Also, a likely expression of the *lac* operon was found when the mutant protein H210D was expressed in a *ccpA* background, although the activity was lower than expected if the operon in this mutant was fully activated or insensitive to the glucose effect, as observed in LicT (van Tilbeurgh *et al.*, 2001). Although this mutation was not temperature sensitive, it may introduce conformational changes or difficulties in dimer formation, which is apparently required for activity in *E. coli* BglG (Amster-Choder & Wright, 1992, 1993; Boss *et al.*, 1999). In *E. coli* it has recently been proposed that HPr-dependent phosphorylation of PRD-II is necessary for formation of the dimer (active) form of BglG (Görke & Rak, 1999), and a similar model was initially suggested for LicT (Krüger & Hecker, 1995; Krüger *et al.*, 1996). However, recent data obtained from the crystallized regulatory domains of LicT (van Tilbeurgh *et al.*, 2001) showed that phosphorylated histidines in PRDs face inwards, hidden from interactions with the paired molecule in the dimer. Hence, the negative charge introduced in LacT(H210D) might introduce structural changes that indirectly alter the dimer interface. In BL190(pGALTH210D), the overproduced LacT(H210D) may be forming heterodimers with the chromosomally encoded LacT with weak antiterminator activity. In contrast, in BL195 ($\Delta lacT$) only an unstable homodimer would be formed from pGALTH210D. Nevertheless, the role of the PRD-II histidines in glucose inactivation of LacT has been confirmed with the H210A and H273A mutants as they lack activity in any background.

Further studies are required to clarify the mechanism by which phosphorylation regulates dimerization and activity of LacT. Furthermore, *in vitro* phosphorylation experiments with the different purified forms of LacT will be necessary to confirm the proposed phosphorylation processes.

In conclusion, we have determined the *in vivo* effect of site-directed mutations of the LacT antiterminator on *lac* operon expression in *L. casei*. Dephosphorylated PRD-I histidyl residues, His-101 and His-159, are indispensable for full induction of *lac* operon, whilst His-210 and His-273 of PRD-II play a role in CcpA-independent carbon catabolite repression. The results obtained suggest that there could be differences in the affinity of different phosphotransfer elements for PRD-I and PRD-II histidines.

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