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Relationship between carbon catabolite repression and the biosynthesis regulation of the prolidase PepQ from *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 397

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Abstract — *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 397 (*L. bulgaricus*) displays several enzymes specific of proline-containing peptides. We focused on the prolidase PepQ which specifically cleaves X-Pro dipeptides. PepQ biosynthesis was previously shown to be independent of the peptide concentration of the culture medium in contrast to the cell surface proteinase PrtB and several aminopeptidases. Regulation of PepQ biosynthesis can be explained by the genetic organization of the region *pepR1-cre-pepQ*. The *pepR1* gene encodes a CcpA-like regulator and its promoter harbors a *cre* site located immediately upstream of *pepQ*. Expression of fusions *cre-pepQ-lacZ* and *pepQ-lacZ* in *Bacillus subtilis* showed that, under glucose conditions, the regulator CcpA acts as a transcriptional activator of *pepQ* expression. Analysis of PepQ biosynthesis in *L. bulgaricus* cells grown in different media is in agreement with a regulation dependent on carbohydrates.

***Lactobacillus delbrueckii* ssp. *bulgaricus* / catabolite repression / PepQ / PepR1**

Résumé — Relation entre la répression catabolique et la régulation de la biosynthèse de la prolidase *PepQ* de *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 397. *L. bulgaricus* synthétise plusieurs enzymes spécifiques des peptides contenant de la proline, dont la prolidase PepQ qui hydrolyse spécifiquement les dipeptides de type X-Pro. Nous avions précédemment montré que, contrairement à certaines aminopeptidases et à la protéinase de surface PrtB, la biosynthèse de PepQ ne dépend pas de la concentration en peptides du milieu de culture. La régulation de la biosynthèse de PepQ peut s'expliquer par l'organisation génétique de la région *pepR1-cre-pepQ*. Le gène *pepR1* code pour une protéine de type CcpA et renferme une séquence cible *cre* qui se situe juste en amont du promoteur du gène *pepQ*. L'expression de fusions *cre-pepQ-lacZ* et *pepQ-lacZ* chez *Bacillus*

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subtilis a montré que, en présence de glucose, CcpA jouait un rôle d'activation de la transcription du gène *pepQ*. L'analyse de la biosynthèse de PepQ chez *L. bulgaricus* dans différentes conditions de culture est en accord avec une régulation dépendante des carbohydrates.

***Lactobacillus delbrueckii* ssp. *bulgaricus* / répression catabolique / PepQ / PepR1**

1. INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* CNRZ 397 (*L. bulgaricus*) is a thermophilic lactic acid bacterium widely used in fermented milk. Its growth in milk requires a complex proteolytic system which hydrolyzes the major milk proteins (proline-rich caseins) and which provides cells with essential amino acids. First of all, caseins are degraded by a cell surface endoprotease, PrtB [7]. Then, the resulting peptides containing proline are hydrolyzed by several specific peptidases that probably constitute two catabolic pathways leading to the release of the proline residue, essential to *L. bulgaricus*. One pathway involves an X-prolyl dipeptidyl aminopeptidase (EC 3.4.14.5, PepX) which catalyzes the release of X-Pro dipeptides from oligopeptides with a proline at a penultimate position [2]. X-Pro can be further hydrolyzed by a specific dipeptidase (EC 3.4.13.9, PepQ) [18]. An aminopeptidase P (PepP) and a prolyl aminopeptidase (PepI) are the components of the second pathway [3].

In contrast to lactococci and numerous lactobacilli, PepX and PepI activities of *L. bulgaricus* are constitutive. Surprisingly, the level of PepQ activity is dependent on the composition of the culture medium, but not on the peptide concentration [18]. A possible regulation mechanism may involve a well-conserved *cre* sequence ("catabolite responsive element") located immediately upstream of the *pepQ* promoter (25 nucleotides) and included in the promoter region of *pepR1* gene. The PepR1-deduced amino acid sequence is significantly identical (44%) to the regulatory CcpA protein ("carbon catabolite protein") that, in the presence of glucose, represses the expression of catabolic operons of Gram-positive bacteria (for a review see [19]). A seryl-phosphorylated Hpr and CcpA are able to form a complex that enables CcpA to bind a *cre* sequence.

In this paper, we investigate the regulation mechanism of PepQ biosynthesis via two approaches. On one hand, expression of *pepQ-lacZ* fusions in *Bacillus subtilis* pointed out the role of CcpA and its *cre* target in a regulatory mechanism dependent on glucose conditions. On the other hand, we followed PepQ biosynthesis in *L. bulgaricus* by western blot analysis and confirmed its dependence on the composition of the culture medium.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table I. *Escherichia coli* was grown aerobically at 37 °C in LB medium [16]. *Bacillus subtilis* was cultivated in LB medium or minimal medium (CSK) supplemented or not with 1% glucose [15]. *L. bulgaricus* was grown anaerobically at 40 °C in MRS [6] or reconstituted Gamma milk (10%) as previously described [18]. When required, the medium was supplemented with ampicillin (100 $\mu g \cdot mL^{-1}$), kanamycin (5 $\mu g \cdot mL^{-1}$) or erythromycin (5 $\mu g \cdot mL^{-1}$).

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype or relevant characteristic	Reference or source
Escherichia coli		
NM 522	F' *lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$ / *supE thi* Δ(*lac-proAB*) Δ(*hsdMS-mcrB*)5 (r_k^-, m_k^- McrBC$^-$)	
FM9003	RecA$^-$ and PepP$^-$ derivative of CM90	[18]
Bacillus subtilis		
168	*trpC*	Institut Pasteur, Paris
FMML	168 derivative, *amyE*:: ('*lacZ*), Kn^R	this work
FMML1	168 derivative, *amyE*::(*cre-pepQ'–'lacZ*), Kn^R	this work
FMML2	168 derivative, *amyE*::(*pepQ'*–'lacZ), Kn^R	this work
Lactobacillus delbrueckii ssp. *bulgaricus* CNRZ 397		[18]
Plasmid		
pAC7	10.6 kb, Ap^R, Kn^R, promoterless *lacZ*, *amyE'–'amyE*	[24]
pAQ1	pAC7 derivative, *amyE*::(*cre-pepQ' –'lacZ*)	this work
pAQ2	pAC7 derivative, *amyE*::(*pepQ' –'lacZ*)	this work
pQBB12	pLG339 derivative containing promoter and *pepQ* gene	[18]

2.2. Preparations of cell extracts

Bacterial cultures were grown in 50 mL medium and collected at A_{600nm} 0.8–1.0 (or as indicated), by centrifugation (12 000 *g*, 10 min, 4 °C). After washing with 10 mL of 200 mmol·L^{-1} Tris/HCl pH 7.0, bacterial cells were broken with a French Press (20 000 psi) and unbroken cells removed by centrifugation (20 000 *g*, 20 min, 4 °C).

2.3. Protein and β-galactosidase assay

Protein concentration was determined by the Bradford method [5] with bovine serum albumin as a standard. β-galactosidase activity was assayed by the Miller method [16]. One unit of β-galactosidase activity is defined as the amount of enzyme that produces 1 μmol of *o*-nitrophenol per min at 37 °C.

2.4. α-amylase activity in situ test

Bacterial colonies were grown overnight on LB medium supplemented with 50 g·L^{-1} agar and 50 g·L^{-1} starch. The addition of a 3 mmol·L^{-1} I_2 and 125 mmol·L^{-1} KI solution turns the starch to a violet background. Colonies secreting α-amylase are surrounded by a clear halo.

2.5. DNA procedures

Plasmid extraction and *E. coli* transformation were carried out as described by Sambrook et al. [20]. Spontaneous competence of *B. subtilis* was used for transformation as described by Kunst and Rapoport [11]. PCR was performed with the Advantage™ Genomic PCR kit (Clontech, Palo Alto, CA, USA) and primers were provided by Life Technologies-Gibco-BRL (Cergy-Pontoise, France). DNA fragments were

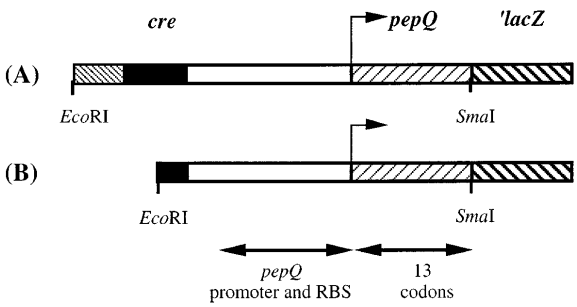

Figure 1. Fusions *cre-pepQ-lacZ* (**A**) and *pepQ-lacZ* (**B**).

purified with the UltraClean Gen-apex DNA purification kit (Prolabo-Merck Eurolab, Fontenay-sous-Bois, France). DNA sequencing was performed using the T7-sequencing kit (Amersham Pharmacia Biotech, Saclay, France).

2.6. Western blotting and immunodetection

Cell extracts equivalent to 7 μg of bacterial proteins were boiled for 4 min in the presence of 0.1% SDS and 0.1 mol·L^{-1} β-mercaptoethanol. Bacterial proteins were separated by SDS-PAGE [12] and electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Ecquevilly, France). Immunoblots were carried out by the Harlow and Lane method [10] using anti-PepQ antibodies and anti-rabbit IgG peroxidase conjugate (Sigma-Aldrich, L'isle-d'Abeau Chesnes, France) revealed by a chemiluminescent substrate (BM Chemiluminescence Western Blotting Substrate, Roche Molecular Biochemicals, Meylan, France). Each sample was tested at least three times.

3. RESULTS AND DISCUSSION

3.1. Construction of fusions between *pepQ* and *'lacZ* genes

In order to determine if the *cre* site located upstream of the *pepQ* promoter is involved in the *pepQ* expression, we constructed two fusions between *pepQ* and the reporter *lacZ* gene, devoid of its own promoter. Two fragments were amplified by PCR introducing *Eco*RI and *Sma*I restriction sites at the 5' and 3' ends, respectively. The fragment A (Fig. 1), containing the *cre* sequence and the adjacent *pepQ* and *pepR1* promoters, was obtained with the primers Q2 (5'-CTAATTACTGAATTCATGATACCATGA-3') and Q1 (5'-GTCCATCCCGGGTTCCTGCAGCC-3') (restriction sites are underlined). PCR with the primers Q3 (5'-GGTGCAATCGAATTCAGCTATTTTTTC-3') and Q1 allowed the amplification of the fragment B with a truncated *cre* site. These PCR fragments were cloned between the *Eco*RI and *Sma*I sites of plasmid pAC7 (Fig. 2). The resulting plasmids pAQ1 and pAQ2, harboring *cre-pepQ-lacZ* and *pepQ-lacZ* fusions, respectively, were used to transform *E. coli* NM522. The Ap^R transformants obtained

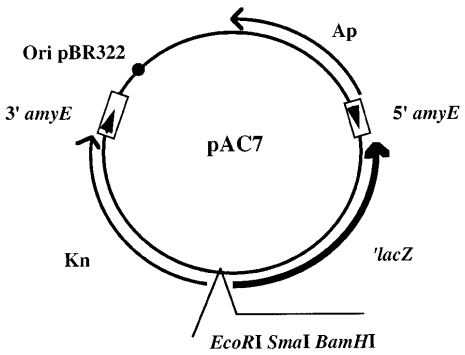

Figure 2. Plasmid pAC7 [24].

with pAQ1 or pAQ2 were able to degrade the chromogen substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in contrast to transformants harboring pAC7. This reflects the presence of a β-galactosidase activity expressed from *cre-pepQ-lacZ* and *pepQ-lacZ* fusions, whose nucleotide sequences were controlled by sequencing. Then, *B. subtilis* 168 was transformed with pAC7, pAQ1 and pAQ2, resulting in strains FMML, FMML1 and FMML2, respectively, with *Kn-lacZ* regions inserted at the chromosomal *amyE* locus. The three strains are resistant to kanamycin and deficient in α-amylase activity, as shown by an in situ enzymatic test. The presence of chromosomal fusions were also controlled by Southern blot experiments with an appropriate probe labeled with digoxigenin (data not shown).

3.2. Effect of the *cre* site on the expression of *pepQ-lacZ* fusions in *B. subtilis*

Strains FMML, FMML1 and FMML2 were cultivated in LB medium supplemented, or not, with 1% glucose. No significant β-galactosidase activity was detected in cell extracts from FMML (data not shown). The presence of glucose does not significantly affect the β-galactosidase activity of strain FMML2 (Fig. 3). By contrast, the strain FMML1 displays a two-fold higher β-galactosidase activity, after cellular growth with glucose. This data suggests that, in vivo and under glucose conditions, the *cre* site upstream of the *pepQ-lacZ* fusion is recognized by the CcpA regulator from *B. subtilis* and results in an activation of the fusion expression.

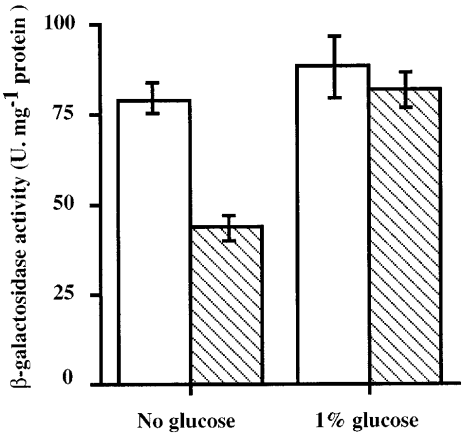

Figure 3. β-galactosidase activities assayed in cellular extracts from exponential- or stationary-phase cultures of strains FMML1 (hatched bars) and FMML2 (blank bars) grown in LB medium with or without glucose. Enzyme activities were calculated from three independent experiments.

The comparison of strains FMML1 and FMML2 points out that, in the absence of glucose, the presence of the *cre* site is correlated to a lower expression of the fusion.

3.3. PepQ biosynthesis in *L. bulgaricus* CNRZ 397

PepQ biosynthesis was followed in *L. bulgaricus* cells during growth in MRS medium (rich in dextrose, i.e. glucose) or milk (rich in lactose). Western blot analysis of cell extracts from *L. bulgaricus* (Fig. 4) was performed with antibodies raised against the purified PepQ protein from *L. bulgaricus* CNRZ 397 [18]. The major band (48 kg·mol^{-1}) corresponds to PepQ: its concentration is slightly higher in extracts obtained from MRS cultures than those from milk (at an equivalent step of growth) (Fig. 4A). The ratio of PepQ to total cellular proteins does seem modified during the exponential phase in MRS medium (Figs. 4A and 4B). Taken together, these results support the hypothesis that a factor of MRS medium could induce an activation mechanism of the PepQ biosynthesis. Previous results have shown that the addition of peptides from a casein hydrolysate has no effect on the PepQ biosynthesis [18]. Therefore, the presence of D-glucose (dextrose) could be a factor implied in the regulation mechanism.

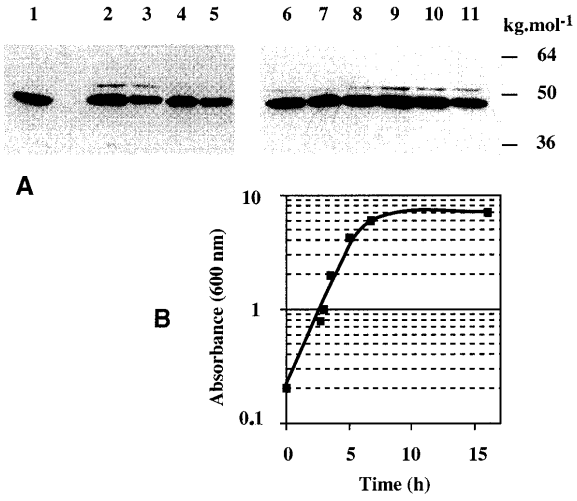

Figure 4. PepQ immunodetection from total cellular extracts after SDS-PAGE (**A**) and growth kinetics of *L. delbrueckii* ssp. *bulgaricus* in MRS medium (**B**). Lane 1: *E. coli* strain harboring pQBB12 plasmid; lane 2 to 11: *L. delbrueckii* ssp. *bulgaricus* after growth in milk (lanes 3 and 5: A_{600} 0.7 and 1.0, respectively) or in MRS (the lanes 2 and 4 are identical to lanes 6 and 7: A_{600} 0.8 and 1.0, respectively; lanes 8 to 11: A_{600} 2.0, 4.2, 6.0 and 7.0, respectively). Western blots of each sample were repeated at least three times.

4. CONCLUSION

In *L. delbrueckii* ssp. *bulgaricus*, the genetic organization *pepR1* (or *ccpA* homologous)-*pepQ* is identical to that found in *Streptococcus mutans* (*regM*) [23] and several lactic acid bacteria: *L. delbrueckii* ssp. *lactis* (*pepR1*) [21], *L. pentosus* [14], *L. casei* [17] and *Lactococcus lactis* [4]. Surprisingly, the PepQ biosynthesis appears differently regulated among these strains. In *Streptococcus mutans*, PepQ biosynthesis is shown to be constitutive [23]. By contrast, PepQ activity in *L. delbrueckii* ssp. *lactis* was shown to be 1.7 to 2.0-fold higher in cells grown in the presence of glucose compared to a culture with lactose [21].

Our results showed that the presence of the *cre* site upstream of the *pepQ* promoter leads to a regulation mechanism of *pepQ* transcription mediated by carbon catabolite repression. Actually, under catabolite repression conditions (presence of glucose in the culture medium), the complex CcpA-HPr~P of *B. subtilis* appears to be able to bind the *cre* target and this results in an activation of the *pepQ* promoter. The activator function of CcpA has already been pointed out for two genes of *B. subtilis* encoding acetate kinase or phosphotransacetylase [9, 22]. A two-fold increase of pyruvate kinase and lactate deshydrogenase activities of *Lactococcus lactis* has also been observed under glucose conditions [13]. The genes encoding these enzymes belong to the *las* operon, the transcription of which is activated by the binding of CcpA to a *cre* site located immediately upstream of the –35 box of the *las* promoter. It is tempting to speculate that in *L. bulgaricus* cells, the higher amount of PepQ protein obtained in MRS culture could be the result of a transcriptional activation involving the CcpA-like regulator, PepR1. The analysis of the regulation of PepR1 biosynthesis is in process.

The proteolytic system of *L. bulgaricus* is not submitted to a single global regulation but depends on different signals. Actually, biosynthesis of some proteolytic enzymes are constitutive (PepN, PepX, PepIP) or dependent on the concentration of peptides (PrtB, Aminopeptidases I and III) or glucose (PepQ) [1, 8].

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