Physiology, metabolism

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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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 endopeptidase, 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 μg mL⁻¹), kanamycin (5 μg mL⁻¹) or erythromycin (5 μg mL⁻¹).
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2.4. a-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 I₂ and 125 mmol·L⁻¹ KI solution turns the starch to a violet background. Colonies secreting a-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

<p>| Table 1. Bacterial strains and plasmids. |</p>
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristic</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM 522</td>
<td>F' lacIΔ(lacZ)M15 proA'B' supE thi Δ(lac-proAB) Δ(hsdMS-mcrB5) (rE-, mE- McrBC+)</td>
<td>[18]</td>
</tr>
<tr>
<td>FM9003</td>
<td>RecA⁺ and PepP⁺ derivative of CM90</td>
<td></td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC</td>
<td>Institut Pasteur, Paris</td>
</tr>
<tr>
<td>FMML</td>
<td>168 derivative, amyE::(lacZ), KnR</td>
<td>this work</td>
</tr>
<tr>
<td>FMML1</td>
<td>168 derivative, amyE::(cre-pepQ'–lacZ), KnR</td>
<td>this work</td>
</tr>
<tr>
<td>FMML2</td>
<td>168 derivative, amyE::(pepQ'–lacZ), KnR</td>
<td>this work</td>
</tr>
<tr>
<td><strong>Lactobacillus delbrueckii</strong></td>
<td></td>
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<tr>
<td>ssp. bulgaricus CNRZ 397</td>
<td></td>
<td>[18]</td>
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<tr>
<td><strong>Plasmid</strong></td>
<td></td>
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<tr>
<td>pAQ1</td>
<td>pAC7 derivative, amyE::(cre-pepQ'–lacZ)</td>
<td>this work</td>
</tr>
<tr>
<td>pAQ2</td>
<td>pAC7 derivative, amyE::(pepQ'–lacZ)</td>
<td>this work</td>
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<tr>
<td>pQBB12</td>
<td>pLG339 derivative containing promoter and pepQ gene</td>
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</tbody>
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2.2. Preparations of cell extracts

Bacterial cultures were grown in 50 mL medium and collected at A₆₀₀ₙₘₜ 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 I₂ 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
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 electro-transferred 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 EcoRI and SmaI restriction sites at the 5' and 3' ends, respectively. The fragment A (Fig. 1), containing the cre sequence and the adjacent pepQ and pepR promoters, was obtained with the primers Q2 (5'-CTAATTACTGAATTCATGATACCATGA-3') and Q1 (5'-GTCCATCCCGGTTCCTGCAGCC-3') (restriction sites are underlined). PCR with the primers Q3 (5'-GGTGCAATCGAATTCAGC-TATTTTTTC-3') and Q1 allowed the amplification of the fragment B with a truncated cre site. These PCR fragments were cloned between the EcoRI and SmaI 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⁺ transformants obtained
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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.

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.

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

![Figure 3](image)

**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.

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.
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 dehydrogenase 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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