Genetic localization and regulation of the maltose phosphorylase gene, malP, in Lactococcus lactis

Ulrika Nilsson and Peter Rådström

Maltose phosphorylase (MP) from Lactococcus lactis was purified and the corresponding gene was cloned and expressed in Escherichia coli. The isoelectric point of the pure enzyme was determined to be 7.0. According to zymogram analysis and SDS-PAGE, the native MP was shown to be a monomeric enzyme with a molecular mass of 75 kDa. A polyclonal antiserum was produced to assess the regulation of the gene encoding MP in Lc. lactis. According to immunoblot analysis, synthesis of the enzyme was markedly repressed by both glucose and lactose in the growth medium. When the lactococci were cultivated in the presence of other sugars, including maltose, trehalose or galactose, there was a pronounced expression of the MP gene. In addition, when the cells were grown in media without any added sugar, there was also pronounced expression of the enzyme, according to immunoblot analysis and specific activity data. These results indicated that no particular sugar specifically induces the gene encoding MP. However, an effect of glucose on MP expression was demonstrated by performing fermentations in the presence of both maltose and glucose. When glucose was added to maltose-grown lactococci in the mid-exponential growth phase, both the specific activity and amount of MP per millilitre of cell extract decreased rapidly. The genetic locus for the MP gene was found to be in the vicinity of the region encoding a possible regulator belonging to the LacI-GalR family of transcriptional regulators. Furthermore, this genetic location was separated from the previously characterized maltose-inducible and glucose-repressible β-phosphoglucomutase (β-PGM) gene. The different genetic loci for the genes encoding MP and β-PGM explains the different gene regulation behaviour.

Keywords: lactic acid bacteria, disaccharide phosphorylase, β-phosphoglucomutase, glucose repression

INTRODUCTION

Maltose metabolism in Lactococcus lactis involves the reversible conversion of β-glucose 1-phosphate to glucose 6-phosphate. This reaction is catalysed by a β-phosphoglucomutase (β-PGM; EC 5.4.2.6) specific for the β-anomer of glucose 1-phosphate (Qian et al., 1994). The expression of the β-PGM gene has been found to be induced by maltose and other disaccharides sharing an α-D-glucopyranosyl structure, and to be repressed by glucose or lactose (Qian et al., 1997).

β-Glucose 1-phosphate is formed when maltose is degraded by the action of an inorganic-phosphate-dependent maltose phosphorylase (MP; EC 2.4.1.8) (Moustafa & Collins, 1968; Sjöberg et al., 1995; Sjöberg & Hahn-Hägerdal, 1989). This enzyme, or its activity, has been described in Neisseria meningitidis (Fitting & Doudoroff, 1952), Lactobacillus brevis (Hüwel et al., 1997; Kamogawa et al., 1973; Bacillus subtilis (Tangney et al., 1992) and Streptococcus bovis (Scott & Russell, 1987). Furthermore, recently a maltose operon, includ-
ing the genes mapA and pgmA encoding an MP and a β-PGM, respectively, was cloned from *Lactobacillus sanfranciscensis* (Ehrmann & Vogel, 1998).

In *Lc. lactis*, an inorganic-phosphate-dependent MP activity has been shown to be correlated with the activity of β-PGM (Sjöberg *et al.*, 1995). In maltose-grown cells, higher activities of both enzymes have been detected relative to those found in glucose-grown cells. Although the presence of MP in the bacteria has been reported, very little is known about the regulation of the expression of the gene encoding this enzyme, or about its genetic location in relation to other genes important in maltose metabolism.

In this study, purification and characterization of the MP of *Lc. lactis* subsp. *lactis* were performed. A polyclonal antiserum was produced in order to perform immunoblot analysis. Particular emphasis was laid on the expression of the MP gene in *Lc. lactis*, in the presence of different carbon sources, was investigated. In addition, the gene encoding the MP was cloned and its genetic location was mapped in *Lc. lactis*.

METHODS

**Bacterial strains, culture media and culture conditions.** *Escherichia coli* DH5α (Life Technologies) was grown in Luria–Bertani liquid medium or on Luria–Bertani plates. For the selection of *E. coli* transformants, ampicillin, IPTG and X-Gal were added to the media at concentrations of 100, 32 and 32 µg ml⁻¹, respectively. *Lc. lactis* subsp. *lactis* 19435, obtained from the American Type Culture Collection (ATCC), and *Lc. lactis* subsp. *cremonis* MG 1363 (Gasson, 1983) were grown in a medium of the following composition (per litre): tryptone (Merck), 5 g; yeast extract (Merck), 5 g; Casamino acids (Difco), 1 g; K₂HPO₄, 2.5 g; KH₂PO₄, 2.5 g; MgSO₄·7 H₂O, 0.5 g (pH 6.8). For studies in which only one carbohydrate was used, the sugars required (10 g l⁻¹) were autoclaved and added to the medium separately. In experiments in which both maltose and glucose were used, the cells were first grown on one of the sugars (10 g l⁻¹) and the other was added during the mid-exponential growth phase. The lactococci were grown batchwise in standing flasks, under oxygen-limited conditions at 30 °C. For studies of MP regulation in the presence of maltose or glucose or both sugars, the bacteria were grown in batch fermenters, with a working volume of 800 ml, at 30 °C under pH-controlled conditions. Stirring was set at 300 r.p.m. The parent cultures were grown overnight in the same media as the respective experimental cultures, in standing batch cultures at 30 °C. The inoculum of 5% (v/v) was centrifuged, washed twice and resuspended in fresh culture medium without sugar, before being added to the experimental cultures. For the purification of MP, *Lc. lactis* 19435 was grown until the stationary phase was reached (OD₆₀₀ 1.2–1.5) in a 2 l standing batch culture, using maltose (10 g l⁻¹) as the carbon source. Cell growth was monitored by measuring the OD₆₀₀ on a spectrophotometer (U-2000; Hitachi). During fermentation, the pH was set at 7.0 and controlled by automatic addition of 3 M NaOH. The controlling device was a laboratory pH meter (Radiometer).

**Preparation of cell extract.** At determined times, samples (50–100 ml) were collected from the fermenter and the cells were harvested by centrifugation at 5000 g at 2 °C for 10 min in a centrifuge (Avanti J-25i; Beckman Instruments). The cell pellet was washed twice and resuspended in 1–10 ml 20 mM triethanolamine buffer (pH 7.2) containing 0.5 mM EDTA, 0.5 mM DTT and protease inhibitors (Complete Protease Inhibitor Cocktail tablets; Roche Molecular Biochemicals). Cells were disintegrated either by using an X-Press (Biox) or with glass beads (0.5 mm) (Keb Lab). Cell debris was removed by centrifugation at 19500 g at 2 °C for 10 min. The supernatants were collected and stored at −80 °C until used.

**MP assay and protein determination.** The specific activity of MP was estimated at 30 °C using a coupled assay detecting the formation of NADPH at 340 nm with a spectrophotometer (U-2000; Hitachi). The reaction mixture, consisting of 500 µl 0.2 M potassium phosphate buffer (pH 7.2) containing 10 mM MgCl₂, 20 µl 40 mM NADP⁺, 20 µl 150 mM ATP, 10 µl each of glucose 6-phosphate dehydrogenase (0.2 U µl⁻¹) and hexokinase (0.2 U µl⁻¹) and 50 µl 100 mM maltose, was first incubated for 5 min at 30 °C. This was to remove any traces of glucose present in the maltose. Then an additional 20 µl 40 mM NADP⁺ and 20 µl 150 mM ATP were added to ensure sufficient amounts of these components when the cell extract was finally added and the assay was started. A total of 50–100 µg protein was added to each assay mixture (final volume 1000 µl). The protein concentrations were determined according to the method of Bradford (1976).

**Purification protocol.** To prevent the inhibitory effect of EDTA from the cell extract preparation, the cell extract was pre-treated by adding MgCl₂ to a final concentration of 10 mM. The cell extract was then treated with DNase I (Appligene Oncor) to reduce the viscosity resulting from the presence of DNA. DNase I was added to a final concentration of 1 mg ml⁻¹, and the cell extract was incubated at 16 °C for 1 h. Degraded DNA was removed by centrifugation at 19500 g and 2 °C for 10 min. Solid ammonium sulphate was added to the supernatant, and the precipitate was collected at ammonium sulphate saturation in the range 20–70%. The precipitate was further dissolved in, and dialysed against, 20 mM triethanolamine buffer (pH 7.5) containing 30 mM KCl, 5% (w/v) glycerol, 0.5 mM EDTA and 0.5 mM DTT (buffer 1).

All procedures for the purification of MP were carried out at 8 °C unless otherwise stated. The chromatography procedures were performed using an FPLC system (Pharmacia Biotech) containing two model P-500 high-precision pumps, a model LCC-501 Plus liquid chromatography controller, two motor valves (MV-7 and MV-8) and a model REC 102 recorder. Protein elution was monitored at 280 nm with a UV-M II control unit, and fractions were collected with a FRAC-200 fraction collector. Gel filtration chromatography was carried out on a HiLoad 16/60 Superdex 200 column (Pharmacia Biotech) that had been equilibrated with buffer 1. Proteins were eluted with the same buffer at a flow rate of 1 ml min⁻¹. Fractions showing the highest MP activities were pooled and dialysed against a 1 mM potassium phosphate buffer (pH 6.5) containing 0.5 mM DTT (buffer 2). The dialysed pool was applied to an equilibrated column (15 × 2 cm) of hydroxyapatite (20 µm, Macro-Prep Ceramic Hydroxyapatite; Bio-Rad). The column was washed with 50 ml buffer 2. Proteins were eluted at a flow rate of 2 ml min⁻¹ with a linear gradient of 0–100% 200 mM potassium phosphate buffer (pH 6.5), within a volume of 200 ml.

Fractions containing the highest MP activities were again pooled and dialysed against buffer 1. The pool was loaded onto a Pharmacia MonoQ HR 5/5 anion-exchange column (5 × 0.5 cm) equilibrated with buffer 1. Proteins were eluted
Maltose phosphorylase in *Lactococcus lactis*

Table 1. Primers used for the mapping of *malP*

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Primer sequence</th>
<th>GenBank accession no./reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>5'-GGCGCATGCTT(T/C)/GT(T/C)/A/G/GC(T/C)/A/G/GG(T/C)/A/G/TT(T/C)/AT-3'</td>
<td>U37409</td>
</tr>
<tr>
<td>2</td>
<td>5'-CAAACAAAAAGGATT/TGGG-3'</td>
<td>AF039028</td>
</tr>
<tr>
<td>3</td>
<td>5'-TGGCACAACCTTGACCTAAA-3'</td>
<td>AF039028</td>
</tr>
<tr>
<td>4†</td>
<td>5'-ATA(G/A)/AG(A/T)/A(C/G)/T/AT(T/A)/G(A)/C/G/CCTAC(T/A)/ACA-3'</td>
<td>AF039028, U28137, M85182, Q08511, S44187 (Bunte, 1998)</td>
</tr>
<tr>
<td>5‡</td>
<td>5'-CCCGGATGGAA(A/G)/CA(A/G)/AT(A/T)/C/AA(A/G)/C(A/C)/G(T/C)/G(A)/AT(T/C)/A</td>
<td>This work</td>
</tr>
</tbody>
</table>

* This primer is provided with a *SphI* restriction enzyme recognition site at the 5' end, and an additional GCC nucleotide sequence.
† This primer (reverse) was used for the cloning of *malP*.
‡ This primer (forward) was used for the cloning of *malP*. It is provided with a *SmaI* restriction enzyme recognition sequence at the 5' end.

with a linear gradient of 30–500 mM KCl formed by buffers 1 and 3, buffer 3 having the same composition and pH as buffer 1, but containing 500 mM KCl. The flow rate was set at 1 ml min⁻¹. MP-active fractions were pooled, dialysed against buffer 1, and anion-exchange chromatography was used once again, as described above, to achieve further purification. The resulting MP pool was checked for purity using SDS-PAGE and was finally concentrated using a centrifugal filter device with a molecular mass cut-off of 10 kDa (Centricon; Amicon Bioseparations).

**Molecular mass determination.** Molecular masses were determined using SDS-PAGE and native PAGE. A low-molecular-mass standard (Pharmacia Biotech) containing phosphorylase *b* (94.0 kDa), BSA (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) was used for SDS-PAGE. A high-molecular-mass standard (Pharmacia Biotech) including thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and BSA (67 kDa) was used for the native PAGE.

**SDS-PAGE, native PAGE and isoelectric focusing.** All of the reagents used for SDS-PAGE and pre-cast native gels were purchased from Bio-Rad. SDS-PAGE was performed according to the Laemmli (1970) method, using an acrylamide concentration of 12%. The acrylamide concentration gradient was 4–15% in the native gels. Cell extract and purified MP fractions were denatured by heating with an SDS buffer containing 2-mercaptoethanol and were then separated using SDS-PAGE. The protein bands in the gels were visualized by staining with Coomassie brilliant blue R-250 or by silver staining (Silver Stain Plus Kit; Bio-Rad). In the Western blot studies, the protein bands were visualized by immunoblotting. In native PAGE, the samples were not denatured but separated directly, and proteins were detected using an assay mixture (see below). Native PAGE was carried out at 8 °C, using precooled gels and running buffer. Isoelectric focusing was performed using a Phast System and Phast Gel IEF 3-9 (Pharmacia Biotech). After separation, the gels were either silver-stained or used for assay staining as in zymogram analysis.

**Zymogram analysis.** Native PAGE gels and gels from isoelectric focusing were subjected to zymogram analysis performed at room temperature. Gels were first equilibrated in 100 mM potassium phosphate buffer (pH 7.2). They were then placed in an assay mixture with the same composition as that used for the MP activity assay. Phenazine methosulphate and nitroblue tetrazolium chloride were also added to the mixture to final concentrations of 0.1 mg ml⁻¹ and 2 mg ml⁻¹, respectively, to detect MP-active bands. The procedure was terminated by rinsing the gels in water.

**N-terminal and peptide sequencing.** The pure MP fraction was denatured and subjected to SDS-PAGE. The protein was transferred to a PVDF membrane (Sequi-Blot PVDF Membrane, 0.2 μm; Bio-Rad) using a Trans-Blot Semi-Dry transfer cell (Bio-Rad), according to the manufacturer’s instructions. The protein band was cut out from the membrane, and the N-terminal amino acid sequence was determined by Edman degradation. Both N-terminal and internal peptide sequencing were performed at the Department of Cell Research, Uppsala University (Uppsala, Sweden).

**MP antiserum production.** Seventy-five micrograms (in a volume of 500 μl) purified MP was mixed with an equal amount of Freund’s complete adjuvant and injected into a rabbit. Twenty-eight days after the first injection, a booster injection was given. Seventy-five micrograms purified MP was mixed with an equal amount of Freund’s incomplete adjuvant and again injected into the rabbit. Twenty millilitres antiserum was collected from the rabbit 28 d after the booster injection, to check for antibody specificity using Western blot analysis. Finally, another 60 ml antiserum was collected after checking of the antibody specificity.

**Western blot analysis.** Proteins were denatured and separated using SDS-PAGE and were thereafter transferred to a PVDF membrane (Trans-Blot Transfer Medium, PVDF membrane, 0.2 μm; Bio-Rad) using a Trans-Blot Semi-Dry transfer cell (Bio-Rad) set at 0.60 A for 65 min. The membranes were further treated according to Ausubel et al. (1996), and proteins were detected by immunostaining using rabbit polyclonal antiserum and a goat anti-rabbit alkaline phosphatase immunoblot kit (Bio-Rad).

**DNA techniques.** Plasmid DNA was purified from *E. coli* by using a Bio-Rad Quantum miniprep kit. Chromosomal DNA from the lactococcal strains was isolated essentially as described by Sambrook et al. (1989). Digestion using restriction enzymes, ligations and agarose gel electrophoresis
was performed according to Sambrook et al. (1989), Ausubel et al. (1996), or the manufacturer’s instructions. All DNA-modifying enzymes were purchased from Roche Molecular Biochemicals. DNA was extracted from gel fragments obtained by agarose gel electrophoresis using a Qiaquick Gel Extraction kit (Qiagen). Competent E. coli cells, prepared according to the method of Inoue et al. (1990), were transformed as described by Sambrook et al. (1989). PCR was used for the amplification of the MP gene using Expand High Fidelity DNA polymerase with standard buffer concentrations. The forward degenerate primer was synthesized according to the N-terminal amino acid sequence of MP, determined by Edman degradation (Table 1). The reverse primer for the amplification was selected from the deduced amino acid sequence of part of the chromosomal DNA sequence of Lc. lactis subsp. cremoris MG 1363 (GenBank accession no. AF03982) and an alignment of regulatory proteins of the LacI-GalR family (GenBank accession nos S44187, U28137, Q08511 and M85182). This degenerate primer has previously been used to demonstrate the presence of a putative regulatory protein for maltose utilization in Lc. lactis subsp. lactis 19435 (Bunte, 1998). The PCR product was cloned into a cloning vector, pUC18 (Vieira & Messing, 1982), and the resulting construct was designated pTMB2000. The junction regions of pTMB2000 were sequenced to check if the MP gene had been successfully cloned into pUC18. For this purpose, primers specific to the DNA sequence of pTUC18 (Vieira & Messing, 1982) were used, and sequencing was performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (PE Applied Biosystems). Mapping of the genetic location of the MP gene was performed using PCR. For amplifications, Taq DNA polymerase was used with standard buffer concentrations; the primers used are listed in Table 1 (see also Fig. 5).

RESULTS

Enzyme characterization

MP was purified using size exclusion, affinity, and anion-exchange chromatography. The overall procedure resulted in 8% recovery of the MP activity and 130-fold purification compared with the crude extract (Table 2). The MP activity was dependent on inorganic phosphate (data not shown), as previously demonstrated by Sjöberg et al. (1995). The final fraction containing MP activity showed a band in SDS-PAGE corresponding to a molecular mass of 75 kDa under denaturing conditions (Fig. 1, lane 2). Zymogram analysis revealed a single protein band at the same position as that in SDS-PAGE, indicating that native MP is composed of a single monomer (Fig. 1, lane 4). In isoelectric focusing, MP exhibited a single band at pl 7.0 (Fig. 1, lane 6).

Purified MP was also used to determine the N-terminal amino acid sequence and an internal polypeptide sequence (Fig. 2). Its first N-terminal amino acids showed significant identity with the first 20 amino acid residues of the Lb. brevis MP determined by Hüwel et al. (1997), as well as with deduced amino acid sequences from the MP enzymes of Lb. sanfranciscensis (Ehrmann & Vogel, 1998), a Bacillus sp. (GenBank accession no. E17377) and N. meningitidis (GenBank accession no. AAF40830), respectively. Database homology searches showed that part of the amino acid sequence internal to the peptide is highly similar to part of a deduced amino acid sequence of a truncated ORF from Lc. lactis subsp. cremoris MG 1363 (GenBank accession no. AF03982) (Fig. 2). This predicted truncated 595 aa polypeptide, in turn, shows an overall similarity of 69% to the MP of Lb. sanfranciscensis.

Table 2. Purification of MP from Lc. lactis subsp. lactis

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume of cell extract (ml)</th>
<th>Total amount of protein (mg)</th>
<th>Total MP activity (mU)</th>
<th>Specific activity of MP (mU mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>10</td>
<td>110</td>
<td>10.53</td>
<td>0.096</td>
<td>1-5</td>
<td>70</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>5</td>
<td>55</td>
<td>7.25</td>
<td>0.13</td>
<td>1-5</td>
<td>70</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>12</td>
<td>4</td>
<td>2.42</td>
<td>0.58</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>Affinity</td>
<td>10</td>
<td>0.17</td>
<td>1.85</td>
<td>10.88</td>
<td>115</td>
<td>18</td>
</tr>
<tr>
<td>Anion-exchange, step 1</td>
<td>4</td>
<td>0.12</td>
<td>1.41</td>
<td>11.75</td>
<td>120</td>
<td>13</td>
</tr>
<tr>
<td>Anion-exchange, step 2</td>
<td>2</td>
<td>0.07</td>
<td>0.89</td>
<td>12.71</td>
<td>130</td>
<td>8</td>
</tr>
</tbody>
</table>

*One unit of activity corresponds to 1 µmol substrate converted per min.
The purified preparation of MP was used to produce rabbit antiserum against the enzyme. Immunoblot analysis of cell extracts showed that the antiserum had cross-reactivity with other proteins under denaturing conditions. However, these cross-reactions did not affect the analysis of the presence of MP in cell extracts (Fig. 3).

**Regulation of MP gene expression in the presence of different sugars**

*L. lactis* was grown in rich media containing glucose, lactose, galactose, maltose or trehalose to investigate the effects of these sugars on the specific activity of MP. The cells were also grown in media without any added sugar. The specific activity of MP was found to be dependent on the carbon source chosen for cultivation. When lactococci were grown on glucose or lactose, the MP specific activity detected was lower than that in the other cultures. When lactococci were grown on glucose or lactose, the MP specific activity detected was lower than that in the other cultures (0.004 U (mg protein)^{-1}). These results were also supported by immunoblot analysis (Fig. 3), showing that less enzyme was present in the lactococci when grown on glucose or lactose. Maltose- or galactose-grown cells showed an MP specific activity of 0.1 U (mg protein)^{-1}, whereas cells grown on trehalose or in medium without any added sugar gave an activity of 0.14 or 0.18 U (mg protein)^{-1}, respectively.

The repression of MP gene expression was studied by using glucose and maltose as carbon sources (Fig. 4). *L. lactis* was first grown on one of the sugars, and the other was added during the mid-exponential growth phase. Cells were withdrawn and MP-specific activity was measured in cell extracts throughout fermentation. Immunoblot analysis was also performed using these cell extracts. When cells were grown on glucose, the MP specific activity was low but approximately constant during fermentation (Fig. 4a). After the addition of maltose, there was only a slight increase in the specific activity, and slightly darker bands corresponding to MP could be detected on the immunoblot membrane (Fig. 4b). The effect was more pronounced when the cells were first cultivated on maltose, and glucose was added during the mid-exponential growth phase (Fig. 4c). The specific MP activity increased until the point of glucose addition, after which the activity decreased. The immunoblot analysis also clearly showed a decrease in the intensity of the MP protein band after glucose addition. This indicates that the amount of MP in the cell extract followed the same pattern as the specific activity of the enzyme.

**Genetic locus of the MP gene**

The gene presumed to encode MP in *L. lactis* subsp. *lactis* 19435 was amplified using degenerate primers. The 2.3 kb PCR product was cloned into a vector and the resulting construct, pTMB2000, was propagated in *E. coli*. Cell extracts of *E. coli* cells harbouring the pTMB2000 construct showed an MP specific activity of 0.07 U (mg protein)^{-1}, which was twice as high as that in cell extracts from the host strain without the construct. The deduced amino acid sequence from DNA sequencing of the junctions of pTMB2000 confirmed the N-terminal amino acid sequence of the purified MP (Fig.
The identity of the cloned PCR product was thereby proved to be the gene encoding MP of *Lc. lactis* subsp. *lactis* 19435, henceforth called *malP*. Fig. 5 shows the genetic organization, based on published nucleotide sequences and PCR analysis, of the *malP* region in *Lc. lactis*. The PCR product of 0·65 kb was verified for *Lc. lactis* subsp. *cremoris* but not for *Lc. lactis* subsp. *lactis* 19435. The amplicon of 1·10 kb was detected for both lactococci. Thus, *malP* was shown to be proximal to a gene encoding the presumed regulator of the maltose operon, *malR* (Egeter & Bruckner, 1995; Puyet et al., 1993). Furthermore, PCR analysis also revealed that *malP* is located in the vicinity of a gene designated *malK*, which encodes the putative ATP-dependent protein of the maltose permease complex in *Lc. lactis* subsp. *cremoris* MG 1363 (Law et al., 1995). This proximity of *malP* to *malK* could not be confirmed for *Lc. lactis* subsp. *lactis* 19435. The location of *pgmB* has been described in a previous publication by Qian et al. (1997). The nucleotide sequence upstream of *pgmB* (4·5 kb) shows strong similarities to trehalose-degrading enzymes, and part of the sequence probably encodes the putative trehalose-specific phosphotransferase system in *Lc. lactis* subsp. *lactis* (GenBank accession no. Y18267). Furthermore, the sequence downstream of *pgmB* (3·0 kb) contains a putative terminator followed by three unidentified ORFs (GenBank accession no. Z70730). In conclusion, our results prove that *malP* and *pgmB*, involved in maltose metabolism in *Lc. lactis*, are situated in two different operons.

**DISCUSSION**

Recently, MPs from two lactobacilli, *Lb. brevis* (Hüwel et al., 1997) and *Lb. sanfranciscensis* (Ehrmann & Vogel, 1998), isolated from sourdough, were described. The MP from *Lb. brevis* was shown to consist of two similar subunits of 88 kDa each, whilst the calculated molecular mass of the enzyme of *Lb. sanfranciscensis* was 85·7 kDa. No indication of the conformation of the latter was given. Some reports have been published concerning other microbial phosphorylases. A dimeric sucrose phosphorylase has been found in *Pseudomonas saccharophila* (Silverstein et al., 1967), whilst another monomeric sucrose phosphorylase has been characterized in *Leuconostoc mesenteroides* (Koga et al., 1991). In recent years, some trehalase phosphorylases have been shown to be tetrameric enzymes—f, for example, those from *Agaricus bisporus* (Wannet et al., 1998) and *Catellatospora ferruginea* (Aisaka et al., 1998). However, the trehalase phosphorylase from *Schizophyllum commune* was identified as a monomeric protein (Eis & Niedetzky, 1999). There are further reports concerning monomeric microbial enzymes with phosphorolytic action, including the cellobiose and cellodextrin phosphorylases from the thermophile *Clostridium stercoreum* (Reichenbecher et al., 1997). This study also presents a monomeric phosphorylase responsible for the degradation of maltose into glucose and β-glucose 1-phosphate. The pI of *Lc. lactis* MP was estimated to be 7·0, which is quite different from that of *Lb. brevis* MP, whose subunits have pI values of 4·2 and 4·6, respectively (Hüwel et al., 1997). Furthermore, the isoelectric points of the presumed MP enzymes of both *N. meningitidis* and a *Batillus* sp. were calculated, on the basis of their deduced amino acid sequences, to be 5·36 and 5·63.
glucose was also found when maltose was present in the culture medium. When *L. lactis* was initially cultivated on maltose, and glucose was added after a certain period of time, the glucose effect was obvious. The expression of *malP* increased during fermentation, but after the addition of glucose there was a considerable decrease in MP in the cell extracts. This could be seen in both immunoblot analysis and measurements of MP specific activity (Fig. 4).

Concerning the induction of *malP*, considerable expression could be detected in lactococci grown in media without any added sugar. Thus, it is highly likely that there is no pronounced induction of the gene while the existence of repression is definite. This differs from the induction/repression pattern of the gene encoding β-PGM in *L. lactis* (Qian et al., 1997). No β-PGM activity could be detected in cells grown in rich media without any added sugar (data not shown).

The genetic location of *malP* in *L. lactis* subsp. *lactis* 19435 and in *L. lactis* subsp. *cremoris* MG 1363 could be determined by PCR analysis. A possible regulator of the maltose operon, *malR*, was found downstream of *malP* in both strains (Fig. 5). *malR* shows strong similarity to the LacI-GalR family of transcriptional regulators (Egeter & Bruckner, 1995; Puyet et al., 1993). Furthermore, *malR*, encoding the energy-dependent protein of the maltose permease complex, could be found directly upstream of *malP* in *L. lactis* subsp.
cremoris MG 1363 (Law et al., 1995). This organization of \textit{maltP} and \textit{malK} could not be confirmed in \textit{Lc. lactis} subsp. \textit{lactis} 19435; this could be due to sequence divergence of \textit{malK}. When another set of PCR primers designed from the MG 1363 \textit{malK} sequence was used, there was still no success in establishing the location of \textit{malK} in \textit{Lc. lactis} subsp. \textit{lactis} 19435. However, it remains possible that \textit{malK} is located in the neighbourhood of \textit{maltP}. During our own sequencing studies (GenBank accession no. Y18267), we have been able to prove, with information from the ongoing genome sequencing project of \textit{Lc. lactis} subsp. \textit{lactis} IL1403 (Bolotin et al., 1999), that the gene encoding the \textit{β}-PGM (\textit{pgmB}) is linked to the putative trehalose operon in \textit{Lc. lactis} subsp. \textit{lactis}. The fact that \textit{maltP} belongs to an operon different from that of \textit{pgmB} is interesting in comparison with \textit{Lb. sanfranciscensis} (Ehrmann & Vogel, 1998). In this strain, the gene encoding MP has been found to be located directly upstream of the gene encoding \textit{β}-PGM. In addition, according to the sequence of the genome of \textit{N. meningitidis} (GenBank accession no. AE002395), the organization of the genes encoding MP and \textit{β}-PGM is similar in this organism to that in \textit{Lb. sanfranciscensis}. Earlier research concerning \textit{β}-PGM showed a notable induction of \textit{pgmB} when \textit{Lc. lactis} was grown on trehalose (Qian et al., 1997). In this case, it was suggested that \textit{β}-PGM served not only to degrade maltose but also to degrade trehalose. In conclusion, these results strongly suggest that there are marked differences in the organization of the maltose operon among different bacteria, which might also influence its regulation.

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