Maltose metabolism of *Lactobacillus sanfranciscensis*: cloning and heterologous expression of the key enzymes, maltose phosphorylase and phosphoglucomutase

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Received 31 August 1998; received in revised form 5 October 1998; accepted 6 October 1998

Abstract

The maltose degradation operon containing genes encoding maltose phosphorylase *mapA* and phosphoglucomutase *pgmA* from *Lactobacillus sanfranciscensis DSM20451* were cloned and expressed in *Escherichia coli*. These genes represent the first genetic data available for this species beyond taxonomic classification. *MapA* encodes a 754-amino acid polypeptide representing maltose phosphorylase, MapA, with a calculated molecular mass of 85.7 kDa. Comparative sequence analysis showed that *mapA* is of a new type distinct from other α-glucosidase genes sequenced so far. Putatively, pyridoxal 5′-phosphate is required as cofactor. The deduced amino acid sequence of *pgmA* shows an overall similarity of 39% to the phosphoglucomutase of *Lactococcus lactis*. *pgmA* is separated by a single nucleotide from the preceding *mapA* gene indicating effective translation by translational coupling. Upon subcloning *mapA* was heterologously expressed in *E. coli*. Additionally, upstream of the maltose-degrading operon ORF1 and ORF2 are located in the opposite direction. These genes show homology to *fabZ* and *accB* from *E. coli* and *Bacillus subtilis*, respectively, both involved in fatty acids biosynthesis. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Lactobacillus sanfranciscensis*; Maltose phosphorylase; *mapA*; Phosphoglucomutase; *pgmA*; *accB*; *fabZ*

1. Introduction

The microbial flora of traditionally prepared sour-dough mainly consists of characteristic and highly competitive lactobacilli. Among the species sharing this environment *Lactobacillus sanfranciscensis*, formerly *L. sanfrancisco*, is the most adapted organism [1,2]. The effective metabolism of maltose, the predominant sugar in this environment, relies on its specific catabolic pathway. We describe the cloning and sequencing of the genes encoding two key enzymes of the maltose metabolism of *L. sanfranciscensis* catalysing the following reactions:

\[
\text{maltose + phosphate} \xrightarrow{\text{maltose phosphorylase}} \text{glucose + glucose 1-phosphate} \quad (1)
\]

\[
\text{glucose 1-phosphate} \xrightarrow{\text{phosphoglucomutase}} \text{glucose 6-phosphate} \quad (2)
\]
Phosphoglucomutase (PGM) catalyses the conversion of glucose 6-phosphate and glucose 1-phosphate, which represents a branch point in carbohydrate metabolism. Glucose 6-phosphate enters catabolic processes to yield energy and reducing power, whereas glucose 1-phosphate is the precursor of UDP-glucose which is used by the cells in the synthesis of various glucose-containing polysaccharides [3]. Maltose phosphorylase, known to need no pyridoxal-phosphate, has been previously isolated from Neisseria meningitidis and Lactobacillus brevis [4-6]. Due to their high substrate specificity they are useful for the determination of maltose or orthophosphate concentrations and amylase activity [7,6,8].

2. Methods

2.1. Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in Table 1. Lactobacilli were grown on modified MRS containing 20 g l⁻¹ maltose under anaerobic conditions at 30°C. The E. coli strain TSM90 mal-60::Tn5 carries an insertion of Tn5 in malP or malQ resulting in a maltose-negative phenotype. Screening for maltose-positive transformants was performed using a modified McConkey agar containing 100 μl containing 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3, 200 mM dNTP, 0.5 pmol of each primer, and Taq DNA polymerase (Boehringer, Germany).

2.2. PCR amplification

The sequence of the oligonucleotides used as specific primers for amplification and subcloning of a 2.56-kb fragment containing the MalA gene were P6: 5'-AGTTACTGATGCGAGTTTCG-3', and P32: 5'-GCGGTGTCCCGTGATGACG-3' (Fig. 1). PCR conditions were set as follows: one initial cycle 94°C 120 s, followed by 32 cycles 94°C 45 s, 56°C 90 s, 72°C 120 s. The PCR reactions were carried out in an Omnigene thermocycler (Hybaid, UK) in a total volume of 100 μl containing 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3, 200 mM dNTP, 0.5 pmol of each primer, and Taq DNA polymerase (Boehringer, Germany).

2.3. Molecular cloning procedures

A genomic library of L. sanfranciscensis partially digested Sau3A fragments was prepared in E. coli TSM90 using the plasmid pSU1 [9]. Transformation were performed via electroporation. PCR products were cloned in the vector pGEM-T according to the manufacturer’s instructions (Promega).

2.4. DNA sequence analysis

DNA sequence was determined by the chain-termination method using ABI Prism® Dye Terminator cycle Sequencing kit (Perkin Elmer) on an ABI...
373 stretch sequencing system. The accession number is AJ224340 (EMBL).

2.5. Preparation of crude cell extracts

Exponentially growing cultures (10 ml) were harvested and washed twice with Tris buffer (100 mM, pH 7.0). After resuspension cells were subjected to a ultrasonic treatment in 500 W Tris buffer for 1 min. After centrifugation at 14 000 g for 10 min at 4°C the clear supernatant was immediately used for enzyme assays.

2.6. Enzyme assays

Maltose phosphorylase activities of crude extracts were assayed either by a spectrophotometric method or by HPLC analysis. Spectrophotometrically, the formation of glucose, liberated from maltose via maltose phosphorylase, was determined in a reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 1 mM NADP+, 5 units each of hexokinase and glucose 6-phosphate dehydrogenase (Boehringer, Germany), 20 mM maltose and 5 µl of crude extract.

HPLC analysis was performed by using a Poly-}

spher® OAKC column (Merck, Germany). The mobile phase consisted of H2SO4 (50 mM). Degradation of maltose, maltotriose, maltotetraose and formation of glucose were detected by their refractive indices (RI detector Gynkotek, Germany). Reaction assays were performed in a final volume of 500 µl containing 20 mM maltose, 10 µl crude extract in potassium phosphate buffer (100 mM, pH 6.5), and Tris buffer (100 mM, pH 6.5), respectively. Reactions were incubated at 30°C for 3 h and stopped by boiling for 10 min. After addition of 25 µl HClO4, precipitated proteins were separated by centrifugation, and 50 µl of the clear supernatants were subjected to HPLC analysis.

2.7. Polyacrylamide gel electrophoresis

Denatured crude cell extracts were separated on SDS-PAGE using 10% Tris-glycine gels. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 (Sigma, Germany). Samples for gels subjected to analysis of enzymatic activities were not heated prior to loading the gel. After electrophoresis gels were washed overnight in potassium phosphate buffer (50 mM, pH 6.5) at 4°C with several changes of buffer. Reaction solution for activity staining con-
tained 20 mM maltose, 1 mM NADP⁺; 1 unit each of hexokinase and glucose 6-phosphate dehydrogenase; 1 mg ml⁻¹ nitroblue tetrazolium chloride (NBT), 0.05 mg ml⁻¹ phenazonium methosulfate (PMS).

3. Results and discussion

E. coli TSM90 was transformed with the plasmid library of partially Sau3A-digested chromosomal DNA of L. sanfranciscensis in plasmid pSU1. Maltose-positive transformants were identified by acid production during growth on media containing maltose as sole carbon source. The GC content of the 4.2-kb insert (44.7 mol%) is slightly higher than the overall GC content of L. sanfranciscensis total DNA (36–38 mol%) [10]. Four complete open reading frames (nucleotide positions: ORF1, 48–459; ORF2, 461–881; ORF3, 1181–3442; ORF4, 3444–4115) were identified, all beginning with an ATG start codon preceded by a potential ribosome-binding site.

ORF1 encodes a polypeptide of 137 amino acids with a molecular mass of 15 248 Da. Based on the similarity of its deduced peptide sequence to previously published dehydrase primary structures, ORF1 was identified as the coding region for a β-hydroxymyristoyl ACP dehydrase. It exhibits 37% amino acid sequence similarity to the E. coli fabZ gene product, which encodes a dehydrase, active on hydroxymyristoyl ACP published by Mohan et al. [11]. This enzyme functions in the dissociated, type II fatty acid synthase system of E. coli [12] and is involved in the synthesis of unsaturated fatty acids.

ORF3 is recognised as responsible for complementation of the maltose deficiency in E. coli TSM90 via phosphorolytic cleavage of maltose. This gene, designated mapA, encodes a polypeptide of 754 amino acids and its calculated molecular mass of 85 723 Da agreed reasonably well with the size determined by SDS-PAGE for previously isolated maltose phosphorylases of L. brevis ranging from 75 kDa to 86 kDa [5,6]. After PAGE under denaturing conditions and subsequent reconstitution in phosphate buffer MapA retained enzymatic activity.

Enzymatic analyses using crude cell extracts of L. sanfranciscensis and E. coli subclones carrying the mapA containing plasmids showed a strong requirement for inorganic phosphate. No maltose degrading activity could be detected after incubation in phosphate-free buffer systems. The inability to cleave either maltotriose or maltotetraose (data not shown) was consistent with previously published findings in L. brevis confirming an extremely narrow substrate specificity.

Searching the EMBL database with the nucleotide sequence of mapA resulted in a significant sequence similarity to yvdK of Bacillus subtilis (EMBL O06993) located within an operon, containing ORFs showing similarity to maltose-binding proteins, maltose permease and other carbohydrate-degrading enzymes. MapA is devoid of all essential structural requirements characteristic of the catalytic mechanism of maltodextrin phosphorylases including MalP of E. coli. The narrow substrate specificity, the difference in cofactor requirements as well as the different primary structure support the status of MapA as a new class of maltose-degrading enzyme.

ORF4 encodes a polypeptide of 224 amino acids with a molecular mass of 23 794 Da. A sequence homology search showed that it shares 41% similarity with PgmB (EMBL Z70730) of Lactococcus lactis and 42% with YvdM of B. subtilis (EMBL O06993) [13]. Therefore, ORF4 was identified as the coding region of a phosphoglucomutase (PGM) and designated pgmA. As is the case for the lactococcal β-PGM, the deduced amino acid sequence of the pgmA gene does not contain any of the previously reported amino acid regions that are typical of the catalytic domain and a Mn²⁺- and Mg²⁺-binding motif of α-phosphoglucomutases [14]. Despite the lack of enzymatic studies on substrate specificity PgmA is regarded as a phosphoglucomutase specific for the β-anomer [15]. A highly conserved sequence -FDLDGV- near the N-terminus was found in PgmA, the lactococcal β-PGM as well in the YvdM gene product from B. subtilis resembling -DGDGD- of the α-phosphoglucomutases. This sequence was already recognised as a metal-binding site by Qian et al. [13] (Fig. 2). A second conserved amino acid region -KPDP- located near the carboxy termini was also found in all three genes.

All four ORFs are preceded by translation initiation sites resembling a Shine-Dalgarno motif at distances of 6 or 7 nucleotides 5’ to the start codon. A
different degree of complementarity to the 3′ end of 16S RNA sequence of *L. sanfranciscensis* which has the sequence 5′-UAUUUCCUCCACU-3′ resulted in different values for free energy ranging from −6.9 to −17.8 kcal mol⁻¹ [16].

The *mapA* gene encoding the maltose phosphorylase (ORF3) and the downstream located *pgmA* gene encoding the phosphoglucomutase (ORF4) were separated only by one nucleotide (guanine). As has been previously demonstrated for other lactic acid bacteria, the expression of a distal gene gradually increased as the stop and start codons were placed in closer proximity [17].

A significant intra-species heterogeneity of codon usage bias was observed between genes involved in fatty acid metabolism (ORF1 and ORF2) and maltose metabolism (*pgmA* and *mapA*), respectively. For example, UUG is the preferred codon for Leu in preference to UUA in *mapA* and *pgmA*, while this codon is avoided in ORF1 and 2. A strong tendency is also visible to use NNG over NNA in the codons for Thr and Lys, and NNC codons over NNU in *malA* as well as in *pgmA*.

According to the general rule found for *E. coli*, that NNC codons are preferentially used in highly expressed genes [18], *mapA* and *pgmA* should be much more efficiently expressed in *L. sanfranciscensis* than is the case for ORF1 and 2.

A 2.56-kb fragment harbouring the entire coding sequence of *mapA* was amplified using primers P6 and P32 and subcloned in pGEM-T resulting in plasmid pMAP3.1.19. Subsequently, a SacI/SphI fragment was cloned into the SacI/SphI site downstream of the T7 promoter of vector pSP73 retaining the correct orientation. Transformation of the resulting plasmid pMAP5.1 into *E. coli* JM109DE3 led to high level expression under the control of the T7 promoter of the maltose phosphorylase (MapA) with an appropriate molecular mass (Fig. 3). In crude cell extracts full enzymatic activity was observed, which remained even after SDS-PAGE, if a renaturation in phosphate buffer was performed (data not shown).

**Acknowledgments**

Part of this work was supported by the Commission of the European Communities within FAIR CT96 116. We thank Monika Hadek for outstanding technical assistance.
References


