

Physiological Role of β -Phosphoglucomutase in *Lactococcus lactis*

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A β -phosphoglucomutase (β -PGM) mutant of *Lactococcus lactis* subsp. *lactis* ATCC 19435 was constructed using a minimal integration vector and double-crossover recombination. The mutant and the wild-type strain were grown under controlled conditions with different sugars to elucidate the role of β -PGM in carbohydrate catabolism and anabolism. The mutation did not significantly affect growth, product formation, or cell composition when glucose or lactose was used as the carbon source. With maltose or trehalose as the carbon source the wild-type strain had a maximum specific growth rate of 0.5 h^{-1} , while the deletion of β -PGM resulted in a maximum specific growth rate of 0.05 h^{-1} on maltose and no growth at all on trehalose. Growth of the mutant strain on maltose resulted in smaller amounts of lactate but more formate, acetate, and ethanol, and approximately 1/10 of the maltose was found as β -glucose 1-phosphate in the medium. Furthermore, the β -PGM mutant cells grown on maltose were considerably larger and accumulated polysaccharides which consisted of α -1,4-bound glucose units. When the cells were grown at a low dilution rate in a glucose and maltose mixture, the wild-type strain exhibited a higher carbohydrate content than when grown at higher growth rates, but still this content was lower than that in the β -PGM mutant. In addition, significant differences in the initial metabolism of maltose and trehalose were found, and cell extracts did not digest free trehalose but only trehalose 6-phosphate, which yielded β -glucose 1-phosphate and glucose 6-phosphate. This demonstrates the presence of a novel enzymatic pathway for trehalose different from that of maltose metabolism in *L. lactis*.

In *Lactococcus lactis* maltose is split into glucose and β -glucose 1-phosphate (β -G1P) by a P_i -dependent reaction catalyzed by maltose phosphorylase (23). The glucose formed enters the glycolysis by glucokinase while β -G1P is converted to glucose 6-phosphate by β -phosphoglucomutase (β -PGM) before entering the glycolysis (29). β -PGM is repressed by glucose and lactose and induced by maltose and trehalose (28), suggesting that trehalose is also catabolized by a phosphorylase, analogous to observations in *Euglena gracilis* (2) and in *Micrococcus varians* (16). However, the initial catabolism of maltose and trehalose is still not well established in *L. lactis*, and the possibility of alternative pathways has not been investigated.

To our knowledge, maltose and trehalose phosphorylase, together with β -PGM, are the only known enzymes in any organism that catalyze reactions involving β -G1P. Although the presence of β -PGM and β -G1P in both bacteria and algae has been reported, information about the metabolic role of this enzyme and intermediate is scarce. There are, however, indications that β -G1P can be used as a precursor for cell wall material in *L. lactis* (31), and it has been found to be a component of glycan in *Enterococcus faecalis* (26).

In this study, we constructed a β -PGM mutant of *L. lactis* which was used to further elucidate the role of β -PGM in carbohydrate catabolism. It was also used to assess the ability of the cells to utilize β -G1P in anabolic reactions, as the β -G1P formed from maltose phosphorylase is prevented from entering the central metabolism. The mutant strain was compared

with the wild-type strain under controlled fermentation conditions with regard to growth and product formation on different sugars. Measurements of enzymatic activities and intracellular metabolites were used to further investigate the metabolism.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani medium at 37°C . Erythromycin ($250\text{ }\mu\text{g/ml}$ for *E. coli* and $1\text{ }\mu\text{g/ml}$ for *L. lactis*) or ampicillin ($100\text{ }\mu\text{g/ml}$) was added as required. 5-Bromo-4-chloro-3-indolyl- β -galactopyranoside (X-Gal) was used at a concentration of $160\text{ }\mu\text{g/ml}$ in agar plates. *L. lactis* was grown in M17 medium (35) from Difco Laboratories (Detroit, Mich.), where lactose was replaced by glucose (GM17) or maltose (MM17) at a concentration of 5 g/liter . For physiological characterization, *L. lactis* was grown in semidefined SD3 medium (37) with Casamino Acids (10 g/liter) and carbohydrate (10 g/liter). All components of the growth media were sterile filtered. Fermentation was carried out in Bioflo III fermentors (New Brunswick Scientific Co., Edison, N.J.) at 30°C at an initial volume of 1.5 liters . The pH was maintained at 6.50 by automatic base addition (1 N NaOH). Stirring was set at 250 rpm , and anaerobic conditions were maintained by continuous nitrogen flushing above the medium. Precultures for fermentation were inoculated at 1% (vol/vol) from fresh M17 cultures and grown in SD3 medium with double buffer concentration and with glucose (5 g/liter) as the carbon source. The cells were grown for 10 h and then harvested by centrifugation at $5,000 \times g$ for 10 min , washed once and resuspended in fresh SD3 medium without sugar, and finally inoculated into the experimental culture. Batch cultures with maltose or trehalose as carbon sources were performed in duplicate or triplicate, and the data presented are averages. Continuous cultures were run with a mixture of glucose, 2 g/liter , and maltose, 8 g/liter , at a dilution rate of 0.04 h^{-1} . The pH was set to 6.50 with automatic addition of 10 N NaOH .

DNA manipulation. Plasmid DNA was isolated from *E. coli* using Quantum (Bio-Rad Laboratories AB, Sundbyberg, Sweden) or Qiagen (Qiagen Inc., Santa Clarita, Calif.) kits. For *L. lactis* plasmid preparations the Quantum miniprep kit was used, with the modification that the cell pellets were dissolved in $140\text{ }\mu\text{l}$ of cell resuspension solution plus $60\text{ }\mu\text{l}$ of 100-mg/ml lysozyme solution and incubated at 37°C for 15 min . DNA digestion, dephosphorylation, agarose gel electrophoresis, and ligation were performed according to standard methods (1). All DNA enzymes were obtained from Roche (Roche Diagnostics Scandinavia AB, Bromma, Sweden) or MBI Fermentas (Vilnius, Lithuania). Gel fragments were

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s) ^a	Reference or source
Strains		
<i>E. coli</i> DH5 α	Cloning host	Life Technologies inc.
<i>L. lactis</i> subsp. <i>lactis</i>		
19435	Lactococcal type strain; Lac ⁺	ATCC ^b
TMB 5001	Single-crossover <i>pgmB</i> mutant; Em ^r Lac ⁺	This study
TMB 5002	Double-crossover <i>pgmB</i> mutant; Lac ⁺	This study
Plasmids		
pSMA500	Integration vector with promoterless β -galactosidase; Em ^r	20
pUC18	Cloning vector; Amp ^r <i>lacZ'</i>	24
pGh5 ⁺ host	Temperature-sensitive shuttle vector; Em ^r	4
pNQ3	3.8-kb genomic <i>L. lactis</i> DNA fragment in pUC19; Amp ^r	28
pFL1	pNQ3 with deletion in <i>pgmB</i>	This study
pFL2	pGh5 ⁺ host with fragment from pFL1	This study
pFL4	pSMA500 with internal fragment of <i>pgmB</i>	This study
pFL20	pUC18 derivative; Em ^r	This study
pFL23	pFL20 with fragment from pFL2	This study

^a Lac⁺, harbours lactose plasmid; Em^r, erythromycin resistant; Amp^r, ampicillin resistant.

^b ATCC, American Type Culture Collection.

purified using Qiaquick kits (Qiagen). Ultracompetent *E. coli* cells were prepared and transformed as previously described (15). *L. lactis* was transformed by electroporation according to the method of Holo and Nes (14). PCR was performed using *Taq* DNA polymerase with standard buffer concentrations. For screening with PCR, a bacterial colony was touched with a toothpick and the cell material was dissolved in 20 μ l of water. A 2.5- μ l aliquot of this solution was used as the template in a total PCR volume of 25 μ l.

Constructs contained lactococcal chromosomal DNA according to Fig. 1. pFL4 was constructed by cloning an internal *Hind*III-*Sau*3A fragment of the β -PGM gene, *pgmB*, from pNQ3 (28) into pSMA500 (20). The construction of the integration plasmid pFL20 was performed by ligating the 1,525-bp pUC18 *Hind*III-*Psp*1406I fragment containing the replicon to the pSMA500 *Hind*III-*Cla*I erythromycin resistance fragment. pFL1 was constructed by making an internal *Vsp*I deletion in the *pgmB* gene in pNQ3 by partial digestion and religation (Fig. 1). The *Psp*1406I fragment containing the deletion was cloned into pGh5⁺ host (4), cut out again as a *Kpn*I-*Pst*I fragment, and further cloned into pFL20, yielding pFL23.

Construction of a β -PGM mutant. A single-crossover deletion mutant, TMB 5001, was constructed by transforming 19435 with the vector pFL4 (Fig. 1). In

order to study the stability of TMB 5001 under nonselective conditions, the mutant was propagated for 20 generations without erythromycin in GM17 and MM17. When grown in GM17, no revertants were detected. However, growth in MM17 showed that about 75% of the colony-forming cells had lost the integration vector, which resulted in large white colonies on MM17 X-Gal. Furthermore, erythromycin was observed to reduce the growth of TMB 5001 on glucose. Therefore, a double-crossover *pgmB* deletion mutant, TMB 5002, was constructed in order to study the role of *pgmB* deletion under nonselective conditions. A minimal integration vector, pFL20, was constructed, as pSMA500-based constructs with *pgmB* fragments frequently integrated in the β -galactosidase gene instead of *pgmB*. A derivative containing the *pgmB* gene with an internal 576-bp deletion, pFL23 (Fig. 1), was electroporated into 19435. A mutant with a single-crossover in the 800-bp fragment upstream of the deletion was chosen. This strain was grown in GM17 without erythromycin for 45 generations and was then plated on MM17. Small colonies were chosen and checked for erythromycin sensitivity. Three colonies that had lost their erythromycin resistance were analyzed by PCR and plasmid preparations. All exhibited the expected deletion in the *pgmB* gene, and no changes were observed in the plasmid content compared with the wild-type strain. One mutant, which showed no detectable β -PGM activity when grown on maltose, was named TMB 5002 and selected for further studies.

Measurement of growth, substrate consumption and product formation. Cell growth was monitored by measuring the optical density at 620 nm (OD₆₂₀) after appropriate dilutions. Dry weight was measured in all cultures, and separate OD-dry weight calibrations were made. Samples for substrate and product determination were filtered immediately through 0.2- μ m-pore-size filters after sampling and analyzed immediately or kept at -20°C until analysis. Sugars, lactate, formate, acetate, and ethanol were separated at 65°C on a cation-exchange column (Aminex HPX-87H; Bio-Rad) and quantified using a refractive index detector (RID 6A, Shimadzu Co.). The mobile phase was 5 mM H₂SO₄ at a flow rate of 0.6 ml/min. Sugar phosphates and oligosaccharides were separated at room temperature using high-performance anion-exchange chromatography (HPAEC) on a Carbowac PA-10 column (Dionex, Sunnyvale, Calif.) with a sodium acetate gradient. The mobile phase consisted of 160 mM NaOH at a flow rate of 1.0 ml/min. A linear sodium acetate gradient from 0 to 500 mM was applied from 12 to 30 min after sample injection. For sugar phosphate analysis the supernatant samples were diluted 100 times and the injection volume was 50 μ l. The phosphorylated sugars were quantified by pulsed amperometric detection with an ED40 detector from Dionex.

Cell composition analysis. Culture samples were collected at two different times in the late exponential phase. The samples were stored at -20°C until analysis. A 1.6-ml aliquot of sample was centrifuged at 4°C, and the cell pellets were washed three times in a buffer containing KH₂PO₄, 3 g/liter; K₂HPO₄, 3 g/liter; and NaCl, 9 g/liter, pH 6.6. The pellets were dissolved in 1,300 μ l of water, and three 100- μ l samples were analyzed for total carbohydrate content, while two 500- μ l samples were used for total protein analysis. The total cell carbohydrate content was determined using the anthrone method (22), with a glucose standard. The total protein content was determined using the biuret method as described previously (13) with a bovine serum albumin standard. In order to isolate intracellular polysaccharides and cell wall polysaccharides, cell samples were washed three times in 50 mM KPO₄, pH 7.0. Cells were ruptured in an X-press (type X5; AB Biox, Göteborg, Sweden) at a pressure of 6 MPa.

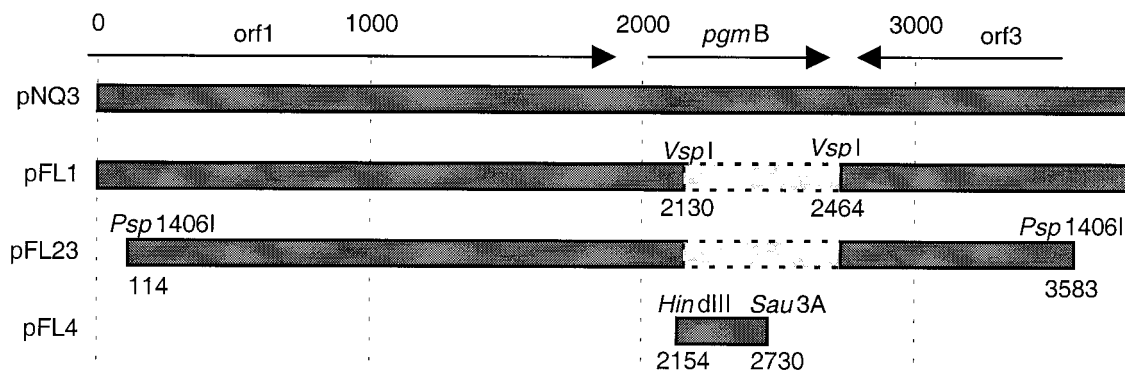


FIG. 1. DNA fragments in various plasmids and the corresponding genes in *L. lactis*. Numbering is according to a previously published sequence (28). Arrows indicate the coding directions of open reading frames.

TABLE 2. Maximum specific growth rates and yields^a

Sugar and strain	$\mu_{\max}$ (h ⁻¹)	Yield								$Y_{s,x}$
		Lactate	Formate	Acetate	Ethanol	Acids	β -GIP	EPS	Total	
Glucose										
19435	0.81	0.803	0.024	0.022	0.020	0.870	ND	0.8	0.871	4.6
TMB 5002	0.79	0.799	0.024	0.021	0.020	0.864	ND	0.6	0.865	5.3
Lactose										
19435	0.72	0.792	0.021	0.016	0.020	0.850	ND	0.6	0.850	4.7
TMB 5002	0.73	0.795	0.025	0.018	0.020	0.859	ND	0.7	0.859	5.5
Maltose										
19435	0.54	0.607	0.082	0.070	0.070	0.829	<0.005	0.5	0.812	6.7
TMB 5002	0.05	0.354	0.115	0.111	0.116	0.697	0.087	3.0	0.787	6.2
Trehalose										
19435	0.52	0.603	0.071	0.066	0.063	0.804	ND	0.6	0.804	6.3
TMB 5002	0.004	ND	ND	ND	ND	ND	ND	ND	ND	ND

^a The strains were grown in batch cultures in SD3 medium, as described in the text. Yields are given in Cmol per Cmol at the time of total sugar consumption, except for the supernatant polysaccharide yield (EPS), which is expressed as mCmol per Cmol, and the biomass yield ($Y_{s,x}$), which is given in grams per Cmol. Acids indicates the total lactate, formate, acetate, and ethanol yield. Total is the value for Acids plus the β -GIP and EPS yields. ND, not determined.

Whole cells were removed by centrifugation ($3,000 \times g$, 10 min, 4°C) before cell walls and intracellular metabolites were separated by centrifugation ($20,000 \times g$, 20 min, 4°C). Cell wall polysaccharides were recovered as described by Looijesteijn et al. (18). To isolate extracellular polysaccharides, culture samples were centrifuged ($15,000 \times g$, 20 min, 4°C) and polysaccharides in the supernatant were precipitated with 3 volumes of absolute ethanol at 4°C for 24 h. The pellets were dissolved in water and dialyzed against water for 3 days (membrane cutoff, 12 to 14 kDa). The extracellular polysaccharides were quantified as total carbohydrate using the anthrone method and converted to moles of carbon (Cmol) as 30 g/Cmol. Polysaccharide composition was determined after hydrolysis in 4 M HCl for 30 min at 100°C using HPAEC with pulsed amperometric detection. Intracellular sugar phosphates were determined in cells 4 h after inoculation into maltose- or trehalose-containing medium. The cells were washed three times in 100 mM triethanolamine (TEA), pH 7.0, at 4°C and diluted to an OD₆₂₀ of 0.35 before passage through an X-press. Cell debris was removed by centrifugation, and the supernatants were analyzed by HPAEC. The intracellular metabolite concentrations were calculated using 1.7 ml/g as the average intracellular volume (31).

Polysaccharide linkage analysis. Intracellular samples were deproteinized by Carrez treatment as explained in *Methods of Enzymatic BioAnalysis and Food Analysis* (Boehringer Mannheim, Mannheim, Germany) and precipitated with 3 volumes of absolute ethanol. For cellulase treatment, the polysaccharides and *Trichoderma* cellulase were incubated in 100 mM sodium acetate, pH 4.6, at 37°C. Samples were analyzed using HPAEC before and after incubation. For amylase digestion the polysaccharides were incubated with soy bean amylase in 50 mM KPO₄, pH 7.0, at 37°C.

Trehalose and maltose characterization. Intracellular cell samples of TMB 5002 and maltose, trehalose, or trehalose 6-phosphate (T6P) at 100 μ M in 50 mM KPO₄, pH 7.0, were analyzed with HPAEC before and after incubation at 30°C. The samples were kept at 0°C to limit further enzymatic reactions between injections.

β -PGM activity measurements. Cells were washed and dissolved in 50 mM TEA buffer, pH 7.2, containing 5 mM MgCl₂, and broken using 0.5-mm-diameter glass beads (Kebo Lab, Stockholm, Sweden). The cell suspension and glass beads were vortexed three times for 5 min at 4°C, and the cell debris was removed by centrifugation. Total protein was measured using the method of Bradford (7) and compared with a bovine serum albumin standard. β -PGM activity was measured by the method described by Ben-Zvi and Schramm (3) and modified by Qian et al. (29).

RESULTS

Growth and mixed-acid formation. *L. lactis* 19435 and the β -PGM mutant TMB 5002 were grown in pH-controlled batch cultures to evaluate the effect of β -PGM in carbohydrate metabolism. When glucose or lactose was used as the carbon

source, no difference could be detected between the two strains (Table 2). The maximum specific growth rate ($\mu_{\max}$) was 0.8 h⁻¹ on glucose and 0.7 h⁻¹ on lactose for both strains. Product formation was homolactic. The similarity in the behavior of the two strains indicates that the genetic manipulation had no major effect on the cells when grown on glucose or lactose, which was expected as there is no detectable β -PGM activity in glucose- or lactose-cultured wild-type cells (28).

When the cells were grown on maltose the *pgmB* deletion had a pronounced effect, with a $\mu_{\max}$ of 0.05 h⁻¹ for TMB 5002 compared with 0.54 h⁻¹ for 19435. While 19435 showed exponential growth up to total sugar consumption, the specific growth rate of TMB 5002 decreased significantly during fermentation (Fig. 2). An initial 20-h exponential growth phase with a specific growth rate of 0.05 h⁻¹ could be distinguished, after which growth gradually decreased to a specific growth rate of less than 0.01 h⁻¹. The stationary phase was reached when a concentration of maltose higher than 5 mM remained in the medium. While the product pattern was constant throughout fermentation for 19435, a change from biomass to lactate formation could be seen in TMB 5002.

Regarding the overall fermentation yields with maltose as the substrate, higher portions of formate, acetate, and ethanol were observed than were observed with growth on glucose and lactose (Table 2). These portions were even larger for the mutant than for the wild-type strain. Both strains produced equimolar amounts of acetate and ethanol, and the molar amount of formate formed was roughly the sum of the other two.

As the identified carbon yield was considerably lower in maltose-grown TMB 5002 (Table 2), the growth medium was analyzed for phosphorylated sugars. Growth-associated formation of β -GIP was observed, with a final concentration of 5 mM, corresponding to 9% of the consumed maltose.

To assess whether the considerable difference in product formation and cell composition was a primary effect of the mutation or was due to the slow growth of the mutant on maltose, chemostat experiments were carried out. The cells were grown at the lowest dilution rate technically possible and,

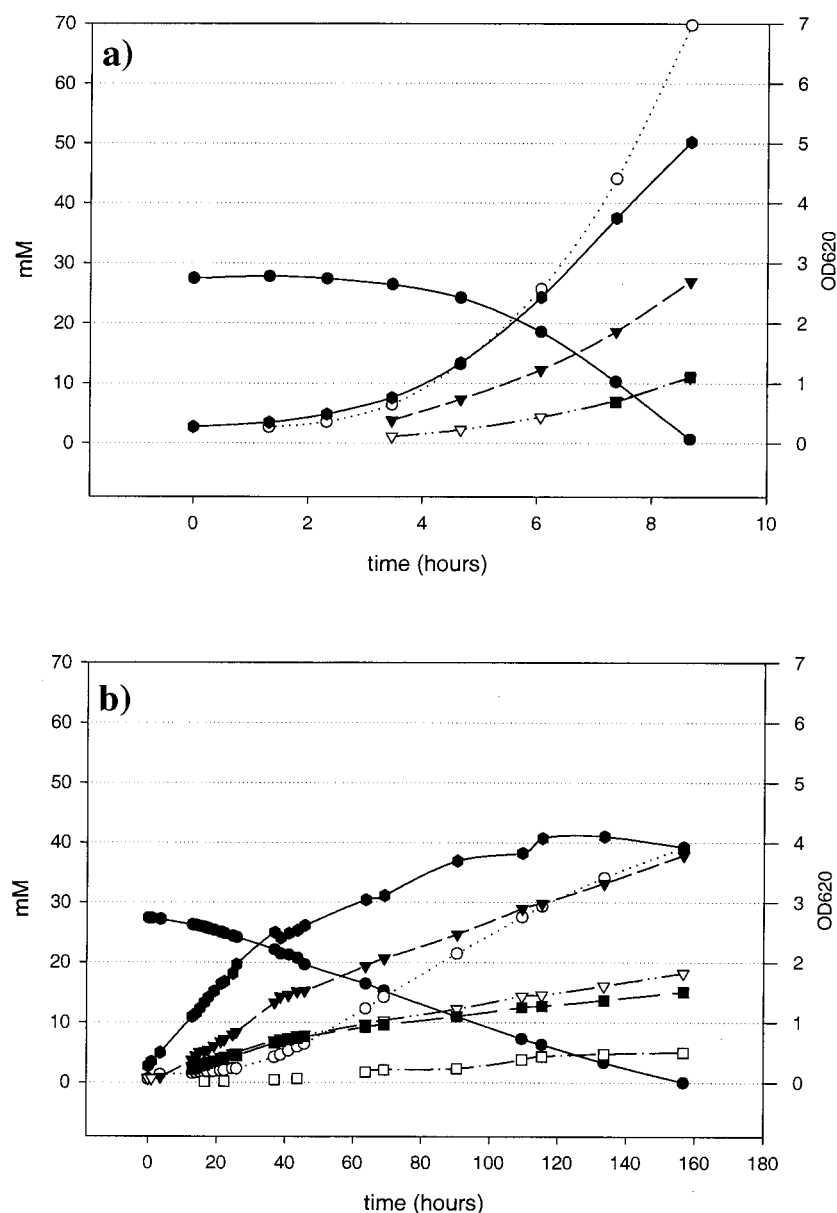


FIG. 2. Substrate consumption, product formation, and growth on maltose. (a) 19435; (b) TMB 5002. Symbols: ●, maltose; ○, lactate; ▽, acetate; ▼, formate; ■, ethanol; □, β -GIP; ●, OD₆₂₀.

apart from maltose, were also supplemented with glucose to ensure adequate cell density. At steady state the residual maltose concentration was 0.4 mM in the 19435 culture and 12.4 mM in the TMB 5002 culture. The difference in product formation observed in the batch cultures on maltose was also seen in the chemostat with a more homolactic product distribution for 19435 (Table 3).

When trehalose was used as the carbon source, the growth of the original strain was similar to the growth on maltose. The product formation was also approximately the same. The β -PGM mutant was, however, unable to grow on trehalose. One week after inoculation a negligible amount of trehalose had been consumed, and the slight OD increase (from 0.28 to 0.38) was more likely to be due to growth on amino acids as it was associated with an increase in pH.

Cell morphology and polysaccharides. When cell samples from batch fermentation experiments were analyzed using phase-contrast microscopy, no differences were seen between the strains when they were grown on glucose or lactose. On maltose, however, TMB 5002 cells were considerably larger

TABLE 3. Yields in chemostat cultures^a

Strain	Yield						Total yield
	Lactate	Formate	Acetate	Ethanol	β -GIP	EPS	
ATCC 19435	0.869	0.025	0.027	0.014	<0.005	0.4	0.935
TMB 5002	0.632	0.094	0.092	0.083	0.086	3.4	0.991

^a Yields are given in Cmol per Cmol, except for EPS, which is given in mCmol per Cmol.

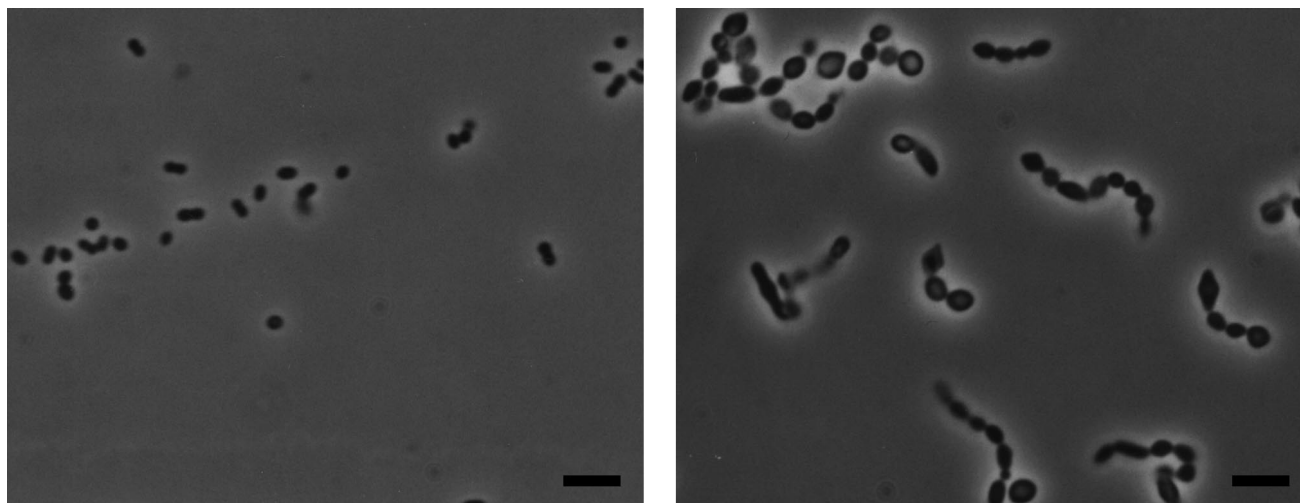


FIG. 3. Phase-contrast photographs of maltose-grown cells in the late exponential phase. (Left) 19435; (Right) TMB 5002. Bar, 5 μ m.

than those of the wild-type strain (Fig. 3a and b). TMB 5002 cells also tended to form chains, while the parent strain was found mostly as single cells or as diplococci. Cells of TMB 5002 that had been kept in the trehalose medium had a cell shape similar to that seen for the maltose-grown cells.

The protein and carbohydrate contents of the cells were also analyzed in the different cultivations, and the carbohydrate/protein ratios of the different washed cell samples were compared. The carbohydrate/protein ratios of the glucose- and lactose-grown cells were 0.10 and 0.11 g/g, respectively. The maltose-grown cells had a considerably higher carbohydrate content, with a carbohydrate/protein ratio of 0.38 g/g for the wild-type strain and 1.46 g/g for TMB 5002. On trehalose, the wild-type strain had a carbohydrate/protein ratio of 0.15 g/g.

The supernatants from batch fermentations were also analyzed for polysaccharides, and with glucose and lactose as carbon sources both strains produced equal amounts of polysaccharides (Table 2). On maltose, however, there was a clear difference between the strains, with 5 mg of glucose equivalents per liter for 19435 and 30 mg/liter for TMB 5002. This was more than four times more polysaccharide than the strains produced on any substrate tested but still constituted only a small amount of the total carbon yield (Table 2).

The cells from the two chemostats were examined by phase-contrast microscopy, and the difference between the two strains observed in the maltose batch experiments was still evident, although less pronounced. The carbohydrate/protein ratios of the cells were now 0.99 g/g for 19435 and 1.38 g/g for TMB 5002. The same analysis was also performed on the intracellular content of the cells, and the ratios were 0.90 g/g for 19435 and 1.17 g/g for TMB 5002.

The intracellular, cell wall, and extracellular polysaccharides were isolated from the chemostats and analyzed for sugar monomer content. In the intracellular fraction only glucose and rhamnose were found, and the fraction of glucose was 93% in 19435 and 99% in TMB 5002. The cell wall polysaccharides had the same composition in the two strains and contained 36% rhamnose, 26% glucosamine, 16% glucose, 14% galactose, and 8% galactosamine. Hydrolysis of the extracellular fraction from 19435 gave the same monomer distribution as

the cell wall polysaccharides. The extracellular fraction from TMB 5002 exhibited the same monomer pattern, except for a large increase in the glucose content.

To investigate the nature of the intracellular polysaccharide it was digested with amylase and cellulase. Digestion with amylase liberated maltose and oligosaccharides, indicating the presence of α -1,4-glucose linkages. Incubation with cellulase liberated no oligosaccharides.

Trehalose catabolic pathways. As the absence of β -PGM had a more severe effect on growth on trehalose than on maltose, differences in the enzymatic degradation of these substrates were sought. Analysis of enzyme assays using HPAEC enabled quantification and separation of sugars and phosphorylated sugars, with distinction between α -G1P and β -G1P. Incubation of cell extracts of TMB 5002 with maltose led to equimolar formation of glucose and β -G1P, as expected for maltose phosphorylase activity. When trehalose was used instead, no enzymatic degradation was observed. T6P, however, was degraded and yielded β -G1P and glucose 6-phosphate, but no glucose or trehalose.

To assess if the slow growth on maltose and trehalose could be due to accumulation of intermediate sugar phosphates, cell samples were analyzed 4 h after inoculation into trehalose- or maltose-containing medium. In 19435 no sugar phosphates were found at levels above 40 mM. In TMB 5002 the intracellular concentrations of β -G1P and T6P were approximately 0.7 and 2.7 M, respectively, independently of the carbon source.

DISCUSSION

In this study, we constructed a stable β -PGM mutant of *L. lactis* in order to study the role of this enzyme during growth on different sugars under nonselective conditions. The deletion of *pgmB* showed that the encoded enzyme is essential in the catabolism of trehalose and has a major effect on cell growth, product formation, and morphology for maltose-utilizing cells (Table 2; Fig. 3). The metabolic pathways of maltose and trehalose have been extensively studied in *Bacillus subtilis* and *E. coli*. In these bacteria several transport and metabolic pathways are involved. Less is known about the transport and

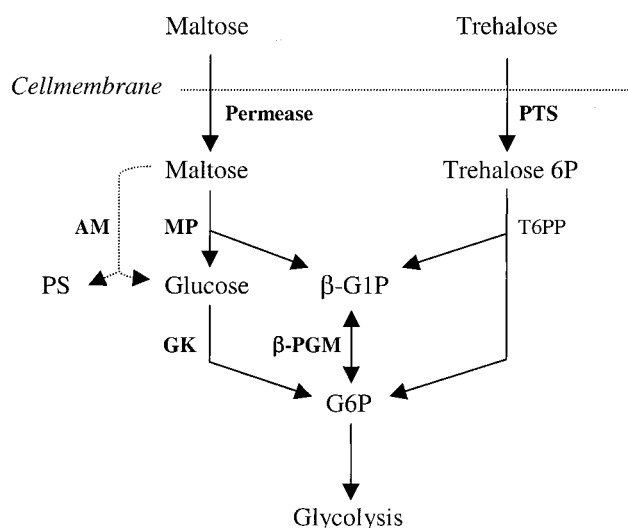


FIG. 4. Proposed pathways for maltose and trehalose assimilation in *L. lactis* 19435. Enzymes are shown in boldface type. Abbreviations: AM, amylomaltase; MP, maltose phosphorylase; GK, glucokinase; T6PP, T6P phosphorylating activity; PS, polysaccharides; G6P, glucose 6-phosphate; PTS, phosphotransferase system.

metabolism of these sugars in *L. lactis*. Recently, the maltose phosphorylase was purified and genetically characterized (23), and it has also been shown that a presumed maltose permease (*malk*) mutant is unable to grow on maltose, indicating that there is only one maltose transport system in *L. lactis* (17). From the results in the present study, one can conclude that the maltose phosphorylase- β -PGM pathway dominates in maltose degradation in *L. lactis*.

Furthermore, the inability of the mutant to grow on trehalose indicates important differences in the catabolism of maltose and trehalose. As the wild-type strain grew equally well on both substrates, one may have expected a similar decrease in growth of the mutant on the two substrates. It is obvious from the growth results that β -G1P must be an intermediate in trehalose degradation, and trehalose phosphorylase is the only known trehalose-degrading enzyme yielding this metabolite. Trehalase and amylorehalase would bypass the β -PGM deletion as they yield glucose or glucose and α -G1P, respectively (6). However, in our hands, no cell extracts of *L. lactis* showed any trehalose-degrading activity (data not shown). One may also suspect differences in the initial transport of maltose and trehalose. Little is known about trehalose transport in lactococci, but a trehalose phosphoenolpyruvate phosphotransferase transporter has been identified in *Streptococcus mutans* (27), and the corresponding genes are present in *L. lactis* (5, 23). We therefore incubated cell extracts with T6P, and the results show a novel enzymatic reaction that phosphorylates T6P and yields β -G1P and glucose 6-phosphate. The previously characterized phospho- α -glucosidases TreA and GlvA of *B. subtilis* yield glucose and glucose 6-phosphate (12, 36), while T6P phosphatase liberates trehalose (6). None of these enzymes were used for T6P assimilation in *L. lactis*, and our results thus show that trehalose is metabolized by a new pathway in *L. lactis* (Fig. 4). Work is in progress to characterize the enzymatic pathway for T6P phosphorylation on a biochemical and genetic level.

An accumulation of β -G1P could favor synthesis of maltose and T6P instead of phosphorolysis by the phosphorylases, due to the equilibria of the reactions. As a consequence, the intracellular glucose level might also be so low that the whole metabolic flux is slowed down. Indeed, it has been shown that *E. coli* strains missing glucokinase activity and thus only being able to metabolize half of the disaccharides—lactose, maltose, and trehalose—were unable to grow on maltose or trehalose, and these strains did only grow slowly on lactose (25, 30). The only explanation presented for this was the buildup of a product for the enzyme that splits the disaccharide, which makes the enzymatic reaction inefficient. To verify if such accumulation could be the case in the β -PGM mutant, we estimated the levels of intracellular sugar phosphates in cells grown on maltose or trehalose. The only phosphorylated sugars found at high concentrations in TMB 5002 were β -G1P and T6P. Similar concentrations were found in both maltose- and trehalose-grown cells, indicating that T6P was synthesized in maltose-grown cells. In 19435 neither of these metabolites were detected. It is thus likely that TMB 5002 is unable to grow on trehalose as a consequence of an unfavorable equilibrium in the breakdown of the disaccharide and that growth on maltose is slowed down due to β -G1P accumulation.

As the conversion of β -G1P to glucose 6-phosphate was blocked in TMB 5002, one may expect that only the glucose moiety from maltose phosphorolysis would finally be found as primary end products (lactate, formate, acetate, and ethanol). However, the recovery as lactate, formate, acetate, and ethanol was about 0.7 cmol/cmol of consumed maltose, which demonstrates that some of the β -G1P could enter the glycolysis or that there are alternative pathways for maltose degradation. Qian et al. found indications of β -PGM activity in a purified fraction of α -PGM (29). This low β -PGM activity of α -PGM, or other phosphotransferase activities, could explain some of the leakage of β -G1P into the glycolysis seen in the present study (33). An alternative way for maltose degradation such as via amylomaltase or maltase could also be active, but this system would be expressed at a low level and would not be able to replace the maltose phosphorylase- β -PGM pathway (Fig. 4).

The product analysis showed that β -G1P was accumulated in the medium at a rate proportional to the maltose consumption, indicating that it may also be transported out of the cells. A hexose phosphate transporter, UhpT, which antiports hexose phosphates in exchange for inorganic phosphate, has been characterized in *E. coli* (10, 32). Although the primary function of UhpT is thought to be the uptake of sugar phosphates, it can also work in the opposite direction. The first hexose phosphate-inorganic phosphate antiporter was found in *L. lactis* (21), and the nucleotide sequence coding for a *L. lactis* protein with 31% identity to the *E. coli* UhpT has been deduced (11).

The ability of the cells to use β -G1P as a precursor for polysaccharides was also investigated. Although some β -G1P could enter the glycolysis and some was excreted into the medium, the accumulation of this intermediate metabolite would favor anabolic reactions with it as a precursor. Measurements of the carbohydrate and protein contents of the cells showed that maltose-grown 19435 cells had a four-times-higher carbohydrate/protein ratio than the glucose- and lactose-grown cells. In TMB 5002 the carbohydrate/protein ratio was further elevated, suggesting that the incorporated carbohydrate was de-

rived from β -G1P. In the chemostat culture at low dilution rate, the carbohydrate content was elevated in 19435 compared with the batch cultures but was still lower than that in TMB 5002. The measurements of the ratios in the whole cells, as well as in the intracellular fractions, indicated the accumulation of an intracellular polysaccharide. The results also suggest that the synthesis of intracellular polysaccharide is upregulated at low growth rates. The polysaccharide consisted of glucose units linked by 1 $\rightarrow$ 4 bonds as in maltodextrins or glycogen. It has previously been shown that *L. lactis* 65.1 accumulates radioactively labeled maltose in the cell wall four times faster than glucose (31). Additionally, growth on maltose was also associated with spherical cells, while glucose-grown cells were elongated. In the present study, cells of TMB 5002 were considerably larger than 19435 cells when grown on maltose. A similar change in cell shape has been seen in α -PGM mutants of *E. coli* (19), where it was considered to be associated with the accumulation of maltodextrins, synthesized by the incorporation of α -G1P by the reversed maltodextrin phosphorylase reaction. A similar incorporation of β -G1P into polysaccharides could explain the accumulation of α -glucan seen in the present study. Another explanation could be that some maltose is assimilated by an amylomaltase like in *E. coli*, which liberates glucose and builds up oligosaccharides at the same time (9). This alternative pathway would also explain why TMB 5002 grows on maltose but not on trehalose. Indeed, incubation of a cell extract of TMB 5002 with maltodextrin released glucose and maltose and to some extent larger oligosaccharides (data not shown). Both the *malP* gene encoding maltodextrin phosphorylase of the *E. coli* maltodextrin system and the *malQ* gene encoding amylomaltase have homologues in *L. lactis* (5).

Under the conditions employed, there was a considerable increase in extracellular polysaccharide production by TMB 5002 when it was cultivated on maltose. The 30 mg produced per liter is more than four times more than that produced by any of the strains on the substrates tested. This increase in polysaccharide production was independent of growth rate, indicating that it was a direct effect of the β -PGM deletion. Compositional analysis of the extracellular polysaccharide showed that it was probably a combination of intracellular and cell wall polysaccharides, and not of the exopolysaccharide type produced by some ropy strains of lactic acid bacteria (8).

In conclusion, the pathways for maltose and trehalose utilization have been characterized and shown to differ in *E. coli* and in *B. subtilis*. While maltose catabolism in *B. subtilis* could involve β -PGM (34), this enzyme does not have an apparent role in the metabolism of *E. coli*. In the present study, we found evidence that β -PGM is a central enzyme in the maltose and trehalose catabolic pathways of *L. lactis* and also that trehalose is assimilated by a novel pathway in this bacterium.

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