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Regulation of Methyl-β-D-Thiogalactopyranoside-6-Phosphate Accumulation in *Streptococcus lactis* by Exclusion and Expulsion Mechanisms

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Starved cells of *Streptococcus lactis* ML_3 (grown previously on galactose, lactose, or maltose) accumulated methyl-β-D-thiogalactopyranoside (TMG) by the lactose:phosphotransferase system. More than 98% of accumulated sugar was present as a phosphorylated derivative, TMG-6-phosphate (TMG-6P). When a phosphotransferase system sugar (glucose, mannose, 2-deoxyglucose, or lactose) was added to the medium simultaneously with TMG, the β-galactoside was excluded from the cells. Galactose enhanced the accumulation of TMG-6P. Glucose, mannose, lactose, or maltose plus arginine, when added to a suspension of TMG-6P-loaded cells of *S. lactis* ML_3, elicited rapid expulsion of intracellular solute. The material recovered in the medium was exclusively free TMG. Expulsion of galactoside required both entry and metabolism of an appropriate sugar, and intracellular dephosphorylation of TMG-6P preceded efflux of TMG. The rate of dephosphorylation of TMG-6P by permeabilized cells was increased two- to threefold by adenosine 5'-triphosphate but was strongly inhibited by fluoride. *S. lactis* ML_3 (DG^r) was derived from *S. lactis* ML_3 by positive selection for resistance to 2-deoxy-D-glucose and was defective in the enzyme II^{Man} component of the glucose:phosphotransferase system. Neither glucose nor mannose excluded TMG from cells of *S. lactis* ML_3 (DG^r), and these two sugars failed to elicit TMG expulsion from preloaded cells of the mutant strain. Accumulation of TMG-6P by *S. lactis* ML_3 can be regulated by two independent mechanisms whose activities promote exclusion or expulsion of galactoside from the cell.

The accumulation of glucose, lactose, and nonmetabolizable analogs 2-deoxy-D-glucose (2-DG) and methyl-β-D-thiogalactopyranoside (TMG) by *Streptococcus lactis* (18, 20, 21, 36, 37, 39) and other streptococci (2, 8, 9, 13, 33, 34) is mediated by the glucose:phosphotransferase system (PTS) and lactose:PTS. During transport via the PTS (for reviews see references 7, 25, and 29), incoming sugars are phosphorylated simultaneously with translocation, as follows:

$$\text{PEP} + \text{HPr} \xrightarrow{\text{EI, } Mg^{2+}} \text{HPr}\sim\text{P} + \text{pyruvate}$$

$$\text{HPr}\sim\text{P} + \begin{cases} \text{Glucose (2DG)} \xrightarrow{EII^{Man}} \text{HPr} + \text{glucose 6-phosphate (2DG-6P)} \\ \text{Lactose (TMG)} \xrightarrow{EII^{Lac}/EIII^{Lac}} \text{HPr} + \text{lactose 6-phosphate (TMG-6P)} \end{cases}$$

(where PEP is phosphoenolpyruvate; EI, EII^{Man}, EII^{Lac}, and $EIII^{Lac}$ are enzymes I, II^{Man}, II^{Lac}, and III^{Lac}, respectively; and 2-DG-6P and TMG-6P are 2-DG and TMG 6-phosphates, respectively). In this multicomponent system EI and a histidine-containing phospho carrier, HPr, initiate phosphoryl transfer from PEP. These soluble (cytoplasmic) proteins are the general energy coupling proteins. Enzyme $EIII^{Lac}$ is also a soluble component required for lactose transport, and EII^{Man} and EII^{Lac} are the integral membrane proteins which function as the sugar recognition components of the PTS. In a previous study (40) it was found that glucose, 2-DG, and lactose prevented uptake of TMG by *S. lactis* ML_3, and similar observations have since been reported with other streptococci (8, 9). Glucose and lac-

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tose have much greater affinity for EII^{Man} and EII^{Lac}, respectively, than TMG exhibits for EII^{Lac}, and it was proposed that: (i) inhibition of TMG accumulation by lactose was a consequence of competition and preferential binding of disaccharide to the common EII^{Lac} recognition protein, and (ii) that exclusion of TMG by glucose resulted from preferential utilization of the common phosphoryl donor (HPr~P) during translocation of glucose via the glucose-PTS. In the previous report (40) (because of a lack of suitable PTS mutants) we could not eliminate the possibility that TMG exclusion was a consequence of inhibition of EII^{Lac} by glucose. The isolation of *S. lactis* ML_3 (DG^r), an EII^{Man}-defective strain, has enabled us to study and to discount this possibility.

Reizer and Panos (28) recently described a novel mechanism for the regulation of TMG-6P accumulation by *Streptococcus pyogenes*. In this organism translocation of glucose via the PTS (or an intracellular derivative) promoted expulsion of free galactoside from the intracellular TMG-6P pool. We have found that a number of sugars, including glucose, elicit TMG-6P hydrolysis and rapid efflux of TMG from *S. lactis* ML_3. It has become apparent from this investigation that certain sugars (e.g., glucose and lactose) may regulate TMG accumulation in *S. lactis* ML_3 by both exclusion and expulsion mechanisms. In the case of glucose, these dual regulatory functions can be relieved by introduction of a lesion in the EII^{Man} complex of the glucose-PTS.

MATERIALS AND METHODS

Strains. *S. lactis* ML_3 was obtained from the culture collection of the New Zealand Dairy Research Institute. *S. lactis* ML_3 (DG^r) was derived from the wild type by positive selection for resistance against 2-DG by a modified method of Saier et al. (30).

Growth of cells. The organisms were grown in complex medium containing, per liter: beef extract, 2 g; yeast extract, 2 g; peptone (Difco), 5 g; tryptone (Difco), 2 g; ascorbic acid, 0.5 g; arginine, 0.25 g; Na_2HPO_4, 8.5 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; and sugar, 5 g.

Accumulation of sugars by starved cells. Starved cells containing an endogenous PEP potential (36, 39) were prepared as described previously (35). In the standard procedure approximately 80 to 100 μl of starved cell suspension was added to 9.8 ml of 0.1 M Tris-maleate buffer (pH 7.0) (containing, when required, 10 mM iodoacetate [IAA]) to obtain a cell density of 200 μg (dry weight) of cells per ml. After 2 min of incubation (or 10 min when IAA was present to block glycolysis) ^{14}C-labeled sugar (specific activity, 0.2 μCi/μmol) was added to a final concentration of 0.2 mM for glucose analogs and 0.4 mM for TMG. Accumulation of sugars was followed by using previously described procedures (36).

P-β-galactosidase assay. Phospho-β-galactosidase (P-β-galactosidase) activity was determined by the method of Heller and Roschenthaler (8).

Preparation of [^{14}C]TMG-6P-loaded cells for expulsion studies. The preloading medium (20 ml) contained 0.1 M Tris-maleate buffer (pH 7.0), 0.2 mM [^{14}C]TMG (specific activity, 0.4 μCi/μmol), 10 mM IAA (when required), and 16 mg (dry weight) of starved cells. After exactly 2 min or 8 min (using galactose- or maltose-grown cells, respectively) of incubation at 30°C, by which time maximum accumulation of [^{14}C]TMG-6P had occurred, the suspension was chilled rapidly to 0°C. The cells were collected by centrifugation (12,000 × *g*, 1 min), supernatant fluid was removed by aspiration, and excess liquid was removed by using cotton buds. The [^{14}C]TMG-6P-loaded cell pellet was suspended by rapid mixing on a Vortex stirrer with 8 ml of ice-cold 0.1 M Tris-maleate buffer (pH 7.0) containing, if necessary, 10 mM IAA. In the expulsion experiments 1.0 ml of this suspension (equivalent to 2 mg [dry weight] of cells) was transferred to 9.0 ml of incubation medium (at 30°C) containing 0.1 M Tris-maleate buffer (pH 7.0) and appropriate additions.

Preparation of [^{14}C]TMG-6P. Starved cells of *S. lactis* ML_3 readily accumulated [^{14}C]TMG as [^{14}C]-TMG-6P via the lactose-PTS (36, 39), and this provided the method for preparation of substrate amounts of the phosphorylated derivative. In the standard procedure three 20-ml volumes of uptake solution (at 30°C) were prepared containing 0.1 M Tris-maleate buffer (pH 7.0) and 0.5 mM [^{14}C]TMG (specific activity, 0.4 μCi/μmol). Starved cells of *S. lactis* ML_3 (0.1 g [dry weight] of cells) were added to each flask, and incubation was continued for 2 min, by which time maximum accumulation of [^{14}C]TMG-6P had occurred. The suspensions were chilled to 0°C, and the preloaded cells were collected by centrifugation (12,000 × *g*, 1 min). Supernatant fluid was removed by aspiration, and the sides of the tube were swabbed free of adhering liquid. Each pellet was suspended with 10 ml of 0.05 M Tris-maleate buffer (pH 7.0) at 0°C. The cells were collected by centrifugation, and 10 ml of boiling water was added to each pellet. The suspensions were boiled in a water bath (100°C) for 7 min, and after cooling to 0°C cell debris was removed by centrifugation (27,000 × *g*, 15 min). The clarified supernatant fluids were collected by Pasteur pipette, pooled, and freeze-dried overnight. The [^{14}C]TMG-6P was isolated by ion-exchange chromatography by the method of Kashket and Wilson (14). The freeze-dried residue was reconstituted with 1.5 ml of distilled water, and the solution was transferred to a column (0.8 by 6.0 cm) of AG1-X2 resin (50 to 100 mesh; bicarbonate form; Bio-Rad Laboratories). Free [^{14}C]TMG was eluted from the resin with 7 ml of distilled water, and [^{14}C]TMG-6P was subsequently eluted with 6 ml of a saturated solution of ammonium bicarbonate. All traces of NH_4HCO_3 were removed by repeated rotary evaporation (7 × 5-ml volumes of water) of the preparation. The residual material was redissolved in 1.5 ml of distilled water. This final solution usually contained ca. 14 μmol of [^{14}C]TMG-6P (equivalent to a total of 12×10^6 cpm) and was maintained frozen until required. The preparation consisted of ca. 99%

[^{14}C]TMG-6P as determined by ion-exchange (Cl^-) chromatography, and the derivative was converted to free [^{14}C]TMG after treatment with alkaline phosphatase.

PEP depletion and permeabilization procedures. For studies of [^{14}C]TMG-6P hydrolysis by intact or permeabilized cells, the cells had to be first depleted of endogenous PEP potential in order to eliminate the possibility of subsequent re-phosphorylation of free TMG by the lactose-PTS. In this procedure, two samples of 40 mg (dry weight) of cells were each suspended in 20 ml of 0.1 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA. After 2 min of incubation (at 30°C), 0.1 ml of 0.1 M 2-DG solution was added to each system, and incubation was continued for 30 min. (The endogenous PEP potential was consumed within ca. 30 s during uptake of 2-DG [as 2-DG-6P], and thereafter, after dephosphorylation in vivo, free 2-DG exited the cell [see Fig. 5 of reference 36].) The suspensions were cooled and centrifuged (12,000 × *g*, 1 min), and the two cell pellets (80 mg, total dry weight) were combined in a total volume of 1 ml of 0.1 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA at 0°C. The cells were permeabilized by addition of three glass beads and 25 μl of a toluene-acetone (1:4) mixture, followed by 2 min of vigorous agitation (with intermittent cooling in ice water) using a Vortex mixer. The process was repeated.

Hydrolysis of [^{14}C]TMG-6P by intact and permeabilized cells. The usual assay system consisted of: 100 μl of permeabilized (or intact) cell suspension (ca. 8 mg total [dry weight] of cells); 15 μl of [^{14}C]-TMG-6P solution (0.14 μmol, ca. 10^5 cpm); 50 μl of 0.2 M Tris-maleate buffer (pH 7.0); and appropriate addition of substrates to a final volume of 200 μl contained in 1.5-ml disposable polypropylene tubes. After incubation for 60 min at 30°C the assays were stopped by heating in boiling water for 7 min, and cell debris was removed by centrifugation for 2 min on an Eppendorf Microcentrifuge model 5412 (Brinkmann Instruments Inc., Westbury, N.Y.). Free and phosphorylated sugars present in the clarified supernatant fluid were determined by ion-exchange chromatography and liquid scintillation as previously described (16).

Reagents. Chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Radioactive sugars were obtained from the Radiochemical Centre, Amersham, England, and New England Nuclear Corp., Boston, Mass.

RESULTS

Accumulation of PTS sugars by *S. lactis* ML_3 and *S. lactis* ML_3 (DG^r). Starved cells of *S. lactis* ML_3 (grown previously on galactose) accumulated glucose, mannose, 2-DG, and fructose to a maximum intracellular concentration of ca. 20 mM (Fig. 1A). By contrast, galactose-grown cells of *S. lactis* ML_3 (DG^r) were unable to accumulate significant levels of any of these PTS sugars (Fig. 1B). Kinetic analyses conducted with intact cells (Table 1) and in vitro assays using cell-free extracts (data not shown) revealed that glucose-PTS activity of the 2-DG-resistant strain was <5% that of the wild-type organism. Complementation studies showed that the lesion had occurred in the membrane-bound EII^{Man} complex of the PTS (J. Thompson, B. Feucht, and M. Saier, unpublished data). Both *S. lactis* ML_3 and *S. lactis* ML_3 (DG^r) readily accumulated TMG via the lactose-PTS (Fig. 1A and B, respectively).

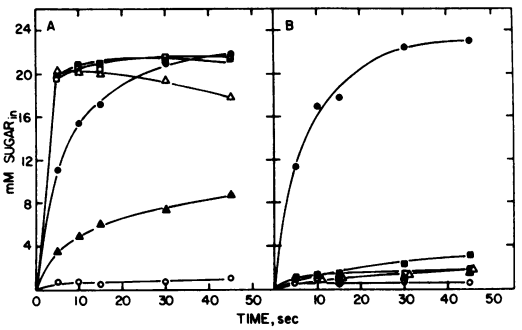

FIG. 1. *PEP-dependent sugar uptake by starved cells of (A) S. lactis ML_3 and (B) S. lactis ML_3 (DG^r). The cells, grown on galactose, were suspended at 200 μg (dry weight) per ml in 0.1 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA and 0.2 mM radiolabeled sugar (specific activity, 0.2 μCi/μmol). Symbols: ●, TMG; ○, α-MG; ▲, fructose; △, 2-DG; ■, glucose; and □, mannose. $SUGAR_{in}$, Intracellular sugar concentration.*

TABLE 1. *Transport of PTS sugars by S. lactis ML_3 and S. lactis ML_3 (DG^r)*[a]

Sugar	Initial rate of sugar transport[b]	
	S. lactis ML_3	*S. lactis* ML_3 (DG^r)
Glucose[c]	405	16
2-DG	418	16
Mannose	398	23
Fructose	68	20
α-MG	8	6
TMG	233	228

[a] Both strains were grown in complex medium containing 0.5% (wt/vol) galactose. All sugars except TMG (0.4 mM) were present at a final concentration of 0.2 mM, and other conditions were as described in the text.

[b] Expressed as micromoles of sugar accumulated per gram (dry weight) of cells per minute.

[c] In this study the EII complex of the glucose-PTS present in *S. lactis* and *S. mutans* (33) has been designated EII^{Man}. The complex is similar to a system in *S. typhimurium* and *E. coli* which catalyzes the transport and phosphorylation of sugars with affinities in the order: glucose > mannose > 2-DG > fructose. This nomenclature avoids confusion with a second glucose-specific PTS whose EII complex (designated EII^{Glc}) exhibits specificity toward glucose and α-MG (see reference 25).

Accumulation and exclusion of TMG by wild-type and mutant strains. Starved cells of *S. lactis* ML_3 contain an endogenous PEP potential (36, 39), and TMG was accumulated to a maximum concentration of ca. 25 to 30 mM within 5 min of incubation (Fig. 2A). Approximately 98% of the total intracellular material was present in the phosphorylated form (TMG-6P), and the maximum rate of TMG uptake via the lactose-PTS occurred at pH 6.5 to 7. When glucose, mannose, or lactose was added to the medium simultaneously with the radiolabeled β-galactoside, the TMG was excluded from the cells (Fig. 2A). Exclusion was not dependent upon metabolism of the PTS sugars since the effect was produced by 2-DG and by glucose, mannose, and lactose in IAA-inhibited cells (data not shown, but see references 36 and 40). Galactose enhanced uptake of TMG, but the stimulation was abolished by IAA. In contrast to the parental strain, only lactose caused exclusion of TMG from *S. lactis* ML_3 (DG^r), and this result showed that TMG exclusion in the wild type was not due to glucose inhibition of EII^{Lac}. Glucose, mannose, and galactose stimulated TMG accumulation in the mutant (Fig. 2B), but in the presence of IAA, accumulation was reduced to control levels (data not shown).

TMG expulsion from *S. lactis* ML_3. Maximum uptake of TMG-6P (ca. 5 min) was followed by a slow efflux of accumulated material (Fig. 3A). However, the addition of glucose or lactose (or mannose; data not shown) induced rapid loss of accumulated material from *S. lactis* ML_3. Galactose, by contrast, stimulated uptake

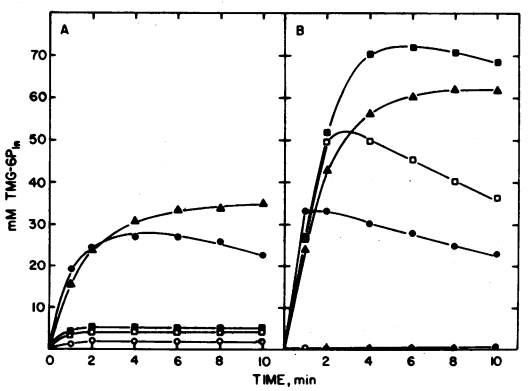

FIG. 2. *Accumulation of TMG-6P by (A) S. lactis ML_3 and (B) S. lactis ML_3 (DG^r) in the presence of various sugars: ●, control (no added sugar); ▲, galactose; ○, lactose; □, mannose; and ■, glucose. The concentrations of [^{14}C]TMG and metabolizable sugars were 0.2 mM (specific activity, 0.2 μCi/μmol) and 1 mM, respectively. Both strains were grown on galactose. TMG-6P$_{in}$, intracellular TMG-6P concentration.*

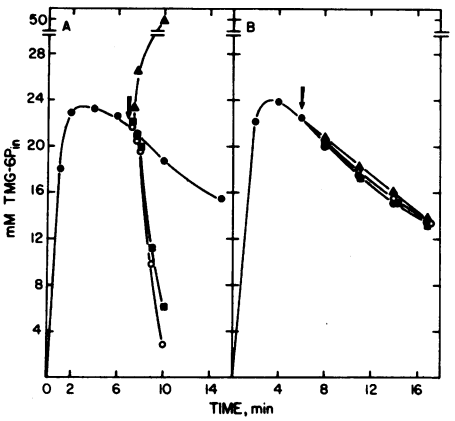

FIG. 3. *Effects of added sugars on TMG-6P accumulation by cells of S. lactis ML_3 in the absence (A) and presence (B) of IAA. The starved cells, grown previously on galactose, were suspended at a concentration of 200 μg (dry weight) per ml in buffered medium containing 0.2 mM [^{14}C]TMG (specific activity, 0.2 μCi/μmol) and, when appropriate, 10 mM IAA. After maximal accumulation of galactoside, sugars were added (arrow) to a final concentration of 1 mM. Symbols: ●, control (no sugar added); ▲, galactose; ○, lactose; ■, glucose.*

of the galactoside. The effects of added sugars were abolished by previous exposure of the cells to IAA (Fig. 3B). The analytical data (Table 2) showed that in control cells, and in cells metabolizing galactose, approximately 97 to 98% of the total material present was in the form of TMG-6P. During expulsion elicited by glucose or lactose there was a marked increase in intracellular levels of free TMG, and TMG-6P was not detected in the medium (data not shown). These two observations suggested that dephosphorylation of TMG-6P preceded expulsion of the free galactoside.

Expulsion of TMG from *S. lactis* ML_3 (DG^r). The loss of intracellular TMG observed upon addition of glucose, mannose, or lactose (Fig. 3A) could have been a consequence of two separate mechanisms, as follows. (i) If accumulation of TMG-6P at any instant represented a steady-state condition (between uptake via the lactose-PTS and subsequent exit of free galactoside), then inhibition of TMG entry by glucose, mannose, or lactose would cause the unidirectional efflux of β-galactoside. Alternatively, (ii) the sugars (or derivatives formed within the cells) could promote loss of accumulated solute via specific activation of a dephosphorylation/expulsion mechanism. Experiments described in Fig. 4 established the operation of the second mechanism. When [^{14}C]TMG-6P-preloaded cells of *S. lactis* ML_3 were suspended in buffered medium (lacking [^{14}C]TMG) a slow efflux of β-

galactoside was observed. However, in the presence of glucose, mannose, or lactose (but not galactose) the intracellular concentration of TMG-6P decreased by >90% within 2 min of incubation (Fig. 4A). After introduction of the EII^{Man} lesion, only lactose induced rapid expulsion of the galactoside from *S. lactis* ML_3 (DG^r) (Fig. 4B). In separate experiments it was found that (i) expulsion of TMG was abolished by previous exposure of the cells to IAA, and (ii) non-metabolizable sugar analogs (including 2-DG, 6-deoxy-D-glucose, 2-deoxy-D-galactose, and α-methylglucoside [α-MG]) failed to elicit significant expulsion of TMG. The slight response observed in some instances was most probably due to glucose present as contaminant.

TMG expulsion in the presence of galactose. Galactose metabolism stimulated TMG-6P accumulation by wild-type and glucose-PTS-defective strains (Fig. 2A and B, respectively). It was of interest to determine whether glucose or lactose addition would also induce TMG expulsion from cells metabolizing galactose. In medium containing galactose, cells of *S. lactis* ML_3 accumulated TMG-6P to a maximum concentration of 35 to 40 mM within 6 min of incubation (Fig. 5A). The addition of glucose, mannose, or lactose (and to a lesser degree, 2-DG) caused the immediate expulsion of galactoside. The effect of 2-DG may be due to inhibition of galactose metabolism rather than expulsion. The accumulation of TMG-6P by *S. lactis* ML_3 (DG^r) was considerably greater than observed in the wild type, attaining a maximal concentration of ca. 55 to 60 mM within 10 min of incubation (Fig. 5B). Addition of the glucose-PTS substrates elicited only a slight loss of intracellular material, but as with *S. lactis* ML_3 the presence of lactose promoted immediate expulsion of TMG from the EII^{Man} mutant.

TMG transport by glucose-grown cells of *S. lactis* ML_3 and *S. lactis* ML_3 (DG^r). Starved cells of *S. lactis* ML_3 (grown previously on glucose) possessed low levels of the lactose-PTS enzymes, and TMG uptake was correspondingly slow (Fig. 6A; 1.7 μmol of TMG-6P accumulated g^{-1} [dry weight] of cells min^{-1}). The galactoside was excluded by glucose, mannose, and lactose. These cells contained low levels of P-β-galactosidase activity (28 μmol of *o*-nitrophenyl-β-D-galactopyranoside 6-phosphate hydrolyzed g^{-1} [dry weight] of cells min^{-1}). Glucose-grown cells of *S. lactis* ML_3 (DG^r) transported TMG at a rate ca. eightfold greater than the wild type (14.7 μmol of TMG-6P accumulated g^{-1} [dry weight] of cells min^{-1}) and possessed four- to fivefold higher levels of P-β-galactosidase (122 μmol of *o*-nitrophenyl-β-D-galactopyranoside 6-phosphate hydrolyzed g^{-1} [dry weight] of cells min^{-1}). As with *S. lactis* ML_3, lactose caused TMG exclusion from *S. lactis* ML_3 (DG^r), but other

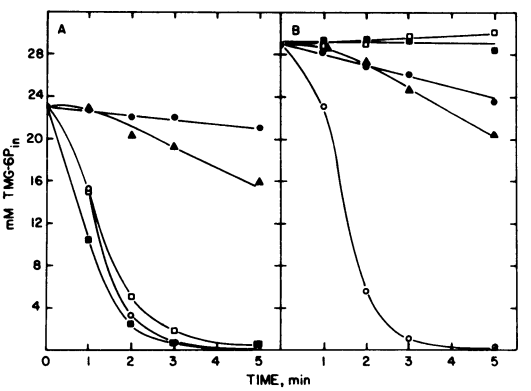

FIG. 4. *Expulsion of TMG from TMG-6P-loaded cells of (A) S. lactis ML_3 and (B) S. lactis ML_3 (DG^r). Both strains were grown on galactose and preloaded with TMG-6P as described in the text. The cells were suspended in buffered medium containing 1 mM concentrations of the appropriate sugar. Symbols: ●, control (no sugar); ▲, galactose; ○, lactose; □, mannose; and ■, glucose.*

TABLE 2. *Intracellular concentrations of TMG and TMG-6P in starved cells of S. lactis ML_3 after addition of glucose, lactose, or galactose to the medium*

Sugar added[a]	Galactoside concn (mM) at time after sugar addition (min):									
	~0.3		~0.6		1		2		3	
	TMG	TMGP	TMG	TMGP	TMG	TMGP	TMG	TMGP	TMG	TMGP
Glucose	0.2	22.5	4.5	21.9	5.8	14.2	3.4	7.9	1.8	4.5
Lactose	0.4	21.3	0.7	19.9	3.8	15.8	4.2	5.7	1.8	1.1
Galactose	0.7	22.7	0.4	26.1	0.3	31.3	1.2	41.7	1.4	48.9

[a] The experimental conditions were as described for Fig. 3A, and sugars were added after 7 min to a final concentration of 1 mM. Samples (1 ml) were removed at intervals from each system, and cells were collected by rapid (<10 s) membrane filtration and immediately transferred to 2 ml of boiling water. Free and phosphorylated galactosides were determined by ion-exchange chromatography. The intracellular concentrations (millimolar) of [^{14}C]TMG and [^{14}C]TMG-6P were calculated on the basis that 1 g (dry weight) of cells of *S. lactis* ML_3 contained the equivalent of 1.67 ml of intracellular (protoplast) volume (35).

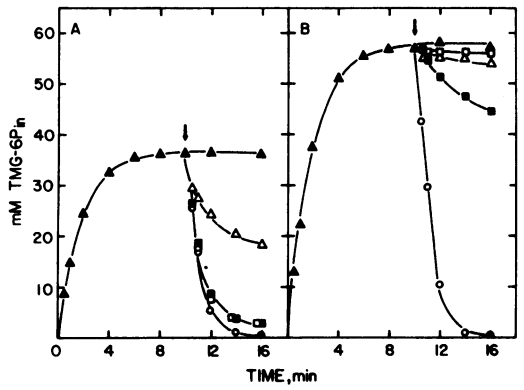

FIG. 5. *Accumulation of TMG-6P by (A) S. lactis ML$_3$ and (B) S. lactis ML$_3$ (DG^r) in the presence of galactose, and capacity of subsequent addition of sugars to promote TMG expulsion. Cells, grown previously on galactose, were suspended in buffered medium containing 0.2 mM [^{14}C]TMG (specific activity, 0.2 μCi/μmol) and 1 mM galactose. After attainment of steady state, the appropriate sugars were added (arrow) to a concentration of 1 mM. TMG-6P uptake in the presence of: ▲, galactose (control); △, 2-DG; ○, lactose; □, mannose; and ■, glucose.*

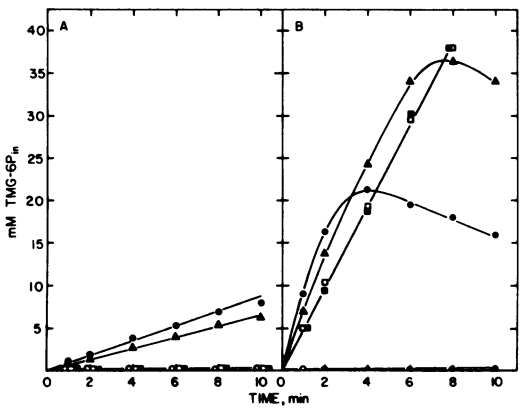

FIG. 6. *Accumulation of TMG-6P by glucose-grown cells of (A) S. lactis ML$_3$ and (B) S. lactis ML$_3$ (DG^r). The cells were suspended in 0.1 M Tris-maleate buffer (pH 7.0) containing 0.2 mM [^{14}C]TMG and 1 mM sugar. Symbols: ●, control (no added sugar); ▲, galactose; ○, lactose; □, mannose; and ■, glucose.*

sugars enhanced TMG-6P accumulation by the glucose-PTS mutant (Fig. 6B). When glucose-grown cells of *S. lactis* ML$_3$ (DG^r) were preloaded with TMG-6P, only lactose promoted expulsion of TMG (data not shown; see Fig. 4B).

Expulsion of TMG from maltose-grown cells of *S. lactis* ML$_3$ (DG^r). Glucose, mannose, and lactose are all transported via the PTS, and it was of interest to determine whether non-PTS sugars would also promote TMG expulsion. Maltose-grown cells were particularly suitable for such a study: (i) such cells possessed high lactose-PTS activity and could be preloaded with TMG-6P; (ii) ATP could be generated by catabolism of arginine; and (iii) maltose is a non-PTS sugar. When maltose-grown cells of *S. lactis* ML$_3$ (DG^r) were preloaded with TMG, 98% of the total intracellular material was present as TMG-6P (Table 3), and neither glucose, mannose, galactose, maltose, nor arginine (Fig. 7A) caused expulsion of TMG. Lactose, or the combination of maltose plus arginine, induced rapid expulsion of the β-galactoside, and during efflux there was a marked increase in the intracellular concentration of free TMG (Table 3). IAA did not prevent TMG efflux elicited by maltose plus arginine (Fig. 7B), although the effect of lactose was abolished by this glycolytic inhibitor.

Dephosphorylation of [^{14}C]TMG-6P by permeabilized cells. Intact and permeabilized cells of *S. lactis* ML$_3$ (maltose grown) were incubated with [^{14}C]TMG-6P, and formation of free TMG was monitored. Intact cells possessed only a limited capacity to hydrolyze the derivative (Table 4), and addition of arginine, maltose, or maltose plus arginine to the assay medium was without effect. When the permeability barrier of the cytoplasmic membrane was disrupted by toluene-acetone treatment, the rate of [^{14}C]TMG-6P hydrolysis was increased ca. eightfold (Table 4) and optimal activity occurred at pH 7.5 to 8. Similar results were obtained by using intact and permeabilized cells of *S. lactis* ML$_3$ grown previously on galactose. Permeabilized cells of *S. lactis* ML$_3$ (DG^r) (Table 4) and *S. lactis* 133 (Lac$^-$) (data not shown) also liberated TMG from TMG-6P, demonstrating that the hydrolytic activity was not dependent upon either the glucose- or lactose-PTS. The rate of [^{14}C]TMG-6P hydrolysis by permeabilized cells was increased approximately twofold upon addition of ATP or arginine plus maltose to the assay system (Table 4). Glycolytic intermediates (e.g., glucose-6P, fructose-6P, and fructose 1,6-diphosphate) did not significantly enhance the rate of TMG-6P hydrolysis above the level observed with ATP alone, and 10 mM fluoride caused marked inhibition (Table 5).

DISCUSSION

Mechanism of TMG exclusion. In the past decade, five general mechanisms have been described for the regulation of sugar transport in bacteria (for reviews see references 4 and 32). Two of these mechanisms, (i) competition of two substrates (lactose, TMG) for a common binding component, EII^{Lac}, and (ii) competition of two PTS's (lactose- and glucose-PTS) for a common

TABLE 3. *Intracellular concentrations of TMG and TMG-6P when preloaded cells of S. lactis ML₃ (DG⁺) were incubated with various exogenous substrates*[a]

Substrate	Galactoside concn (mM) after incubation period (min):											
	0		0.5		1		1.5		2		3	
	TMG	TMGP	TMG	TMGP	TMG	TMGP	TMG	TMGP	TMG	TMGP	TMG	TMGP
None (control)	0.5	21.7	0.4	21.9	0.4	21.2	0.5	21.9	0.5	21.5	0.3	15.9
Arginine	0.3	21.7	0.2	21.7	0.3	20.7	0.3	20.1	0.4	19.1	0.3	17.0
Maltose	0.1	21.7	0.2	21.9	0.2	21.2	0.3	18.9	0.2	20.1	0.3	18.9
Arginine + maltose	0.2	21.7	0.8	22.4	2.3	17.9	2.8	9.3	0.9	5.8	0.4	1.9

[a] *S. lactis* ML₃ (DG⁺) was grown in complex medium containing 0.5% (wt/vol) maltose as the energy source. Starved cells were preloaded with [^{14}C]TMG-6P and suspended at a concentration of 200 μg (dry weight) of cells per ml in buffered medium containing 10 mM IAA and either 1 mM maltose or 2 mM arginine. Sampling procedures and calculation of intracellular solute concentrations (millimolar) were as described in Table 2.

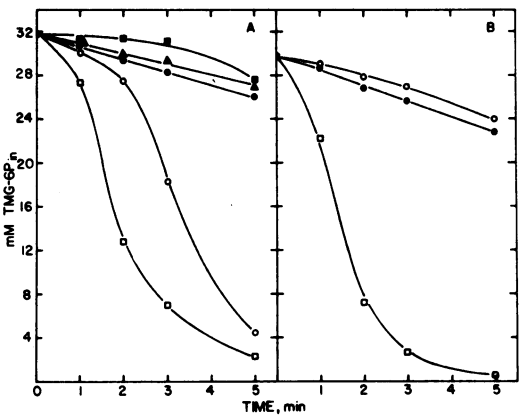

FIG. 7. *Expulsion of TMG from TMG-6P-loaded cells of S. lactis ML₃ (DG⁺) in the absence (A) and presence (B) of IAA. Starved cells (grown previously on maltose) were preloaded with TMG-6P as described previously. The cells were suspended in buffered medium containing, when required, 10 mM IAA and the following additions: ●, control (no addition); ■, 1 mM maltose; ▲, 2 mM arginine; ○, 1 mM lactose; and □, 1 mM maltose plus 2 mM arginine.*

TABLE 4. *[^{14}C]TMG-6P hydrolysis by intact and permeabilized cells*[a]

Substrate added	*S. lactis* ML₃, intact	*S. lactis* ML₃, permeabilized	*S. lactis* ML₃ (DG⁺), permeabilized
Cells only	3.0	26.8	23.2
Arginine	4.6	37.6	30.9
Maltose	2.9	28.9	34.2
Arginine + maltose	3.4	47.4	49.3
Glucose	4.4	30.0	30.9
ATP	2.5	51.8	46.1
ATP + glucose	2.8	50.3	51.9

[a] Expressed as nanomoles of TMG liberated per gram (dry weight) of cells per minute. Experimental conditions were as described in the text. Total volume of assay, 200 μl, containing where necessary: 0.1 M Tris-maleate buffer (pH 7.0); 0.7 mM [^{14}C]TMG-6P; 10 mM arginine; 10 mM IAA; 5 mM glucose, maltose, or ATP; and 8 mg total (dry weight) of cells. All strains were grown in complex medium containing maltose.

high-energy intermediate, HPr~P, provide satisfactory explanations for TMG exclusion by lactose and glucose, respectively. From mechanism (ii) one would expect that deletion or inactivation of the glucose-PTS would relieve the inhibition of TMG entry effected by glucose and its analogs. This prediction was verified by the observation that neither glucose, mannose, nor 2-DG caused exclusion of the galactoside from the EII^{Man} mutant. On the contrary, glucose and mannose enhanced the accumulation of TMG-6P. It should be noted that *S. lactis* ML₃ (DG⁺) grew well on glucose, though it possessed <5% the glucose-PTS activity of the parent strain. The mechanism of glucose uptake by the mutant is not known, but may be mediated by a non-PTS mechanism. Metabolism of glucose (and mannose) by *S. lactis* ML₃ (DG⁺) would yield PEP which, in the absence of an efficient glucose-PTS, could be utilized for group translocation of TMG (see Fig. 2B). Galactose did not exclude TMG from either the *S. lactis* strains or from *S. pyogenes* (J. Reizer and C. Panos, personal communication), presumably because the two sugars have comparable (low) affinities for EII^{Lac}. In addition, the metabolism of galactose by the galactose permease-Leloir-glycolytic pathway (38) would generate the PEP required for enhanced accumulation of TMG-6P noted in Fig. 2B and 6B.

Mechanism of TMG expulsion. The studies reported here, and those of Reizer and Panos (28), have demonstrated a novel system for regulating TMG-6P accumulation in the streptococci. Expulsion of TMG from *S. pyogenes* was elicited specifically by glucose, prompting Reizer and Panos to suggest a functional role for the glucose-PTS in dephosphorylation and expulsion of galactoside. However, in *S. lactis* ML₃

TABLE 5. *Effects of ATP and glycolytic intermediates on rate of [^{14}C]TMG-6P hydrolysis by permeabilized cells of S. lactis ML$_3$*[a]

Addition	[^{14}C] TMG-6P hydrolyzed[b]
Permeabilized cells (no addition)	11.6
Glucose-6P	10.3
Fructose-6P	11.2
Fructose diphosphate	12.7
Glucose-6P + ATP (5 mM)	31.0
Fructose-6P + ATP (5 mM)	30.2
Fructose diphosphate + ATP (5 mM)	26.2
ATP (5 mM)	28.9
ATP (10 mM)	23.5
ATP (5 mM) + fluoride	0.8

[a] Experimental conditions were as described in the text. Total volume of assay, 200 μl, containing: 0.1 M Tris-maleate buffer (pH 7.0); 0.7 mM [^{14}C]TMG-6P; 5 mM sugar phosphates; 5 mM and 10 mM ATP; 20 mM NaF; and 8 mg total (dry weight) of permeabilized cells.

[b] Expressed as nanomoles of [^{14}C]TMG-6P hydrolyzed per gram (dry weight) of cells per minute.

several metabolizable PTS and non-PTS sugars promoted rapid expulsion of TMG, and by using an EII^{Man} mutant, expulsion was shown to be independent of the glucose-PTS.

The molecular events of expulsion are not known, but most likely the process occurs in two stages requiring the initial dephosphorylation and subsequent efflux of free sugar. The following observations showed that in *S. lactis* ML$_3$ dephosphorylation occurred within the cell: (i) the intracellular concentration of free TMG increased during expulsion; (ii) intact cells were unable to hydrolyze exogenous [^{14}C]TMG-6P but (iii) [^{14}C]TMG was liberated after permeabilization of the cells; and (iv) fluoride, a potent inhibitor of many phosphatases, essentially abolished dephosphorylation of [^{14}C]TMG-6P by permeabilized cells.

Glycolytic inhibitors, including fluoride and arsenate, prevented glucose-stimulated expulsion of TMG from *S. pyogenes* (28). In *S. lactis* ML$_3$ a similar effect was produced by IAA, and after introduction of the EII^{Man} lesion neither glucose nor mannose induced expulsion of TMG from *S. lactis* ML$_3$ (DG^r). Non-metabolizable sugar analogs failed to stimulate expulsion in *S. lactis* ML$_3$ and *S. pyogenes*, showing that TMG efflux was not the result of vectorial transphosphorylation via the EIIs of the PTS (31). The preceding observations showed that entry and metabolism of sugar were required for TMG expulsion. Energy coupling (or a high-energy intermediate) may accelerate the rate of dephosphorylation of TMG-6P. Studies with permeabilized cells (Tables 4 and 5) showed that ATP increased the rate of TMG-6P hydrolysis. However, the role of ATP in expulsion must remain speculative since the postulate cannot (at present) be reconciled with the following data: (i) glucose and mannose did not promote TMG expulsion from *S. lactis* ML$_3$ (DG^r) although both sugars were metabolized by the organism; and (ii) three ATP-generating sources (arginine, maltose, galactose) failed to stimulate dephosphorylation or induce TMG expulsion. Surprisingly, when arginine and maltose were present together, there was immediate dephosphorylation and expulsion of β-galactoside, and this process was not inhibited by iodoacetate (Table 3, Fig. 7B). These observations suggested that perhaps both ATP and a glycolytic intermediate (formed prior to the glyceraldehyde 3-phosphate dehydrogenase reaction) were required for optimal rates of dephosphorylation. However, the addition of various glycolytic intermediates caused no greater increase in the rate of dephosphorylation than occurred in the presence of ATP alone (Table 5).

Route of TMG exit. In *S. lactis* ML$_3$ there are at least two possible routes for TMG exit: (i) via EII^{Lac} and (ii) via the galactose permease system (15, 38). Postma and Stock (26) have shown that EIIs are unable to carry out rapid facilitated diffusion of some sugars (for discussion, see reference 25), and it is unlikely that EII^{Lac} would mediate exit of TMG from *S. lactis* ML$_3$. The galactose permease system exhibits a high affinity for the β-galactoside (15, 38), and we suggest that this system (possibly via H$^+$-coupled symport) facilitates the efflux of TMG from *S. lactis* ML$_3$.

Expulsion of TMG in the streptococci has a parallel in the exit of 2-DG from yeast cells (22), and also in the respiration induced expulsion of α-MG from *Escherichia coli* and *Salmonella typhimurium* (6, 11, 12). In these organisms accumulation of α-MG (as α-MG-6P) was mediated via the glucose-PTS, but after dephosphorylation (by an as yet unidentified phosphatase) free α-MG exited the cell by a route which was probably independent of the glucose EII (11). In *E. coli*, respiratory activity rather than sugar metabolism accelerated dephosphorylation of α-MG-6P and expulsion of α-MG (11). In *S. aureus* the disappearance of intracellular sugar phosphate occurred with concomitant appearance of free sugar in the medium (10). The expulsion systems described in the streptococci, though novel, may therefore represent a specific aspect of a more general regulatory phenomenon in microorganisms. At the physiological level,

exclusion and expulsion mechanisms provide the cell with the capacity to detoxify (1) and to regulate the intracellular concentration of non-metabolizable (and hence potentially lethal) sugar phosphates.

Regulation of the lactose operon in *S. lactis*. Galactose-6P is believed to be the inducer of the lactose-PTS and P-β-galactosidase in *Staphylococcus aureus* (24). In *S. lactis* the levels of these enzymes are considerably higher in lactose- or galactose-grown cells than in cells previously grown on glucose (18, 23). It has been inferred, largely from such circumstantial evidence, that galactose-6P may function as the inducer for these plasmid-coded enzymes (3, 5, 18, 19) in the group N streptococci. However, data obtained in this and other studies showed that the enzymes required for lactose transport and metabolism may be expressed in the (apparent) absence of galactose-6P. For example: (i) *S. lactis* ML_3, grown previously on maltose, readily accumulated TMG, contained high levels of P-β-galactosidase (3, 37), and metabolized lactose (Fig. 7A); (ii) ribose-grown cells of *S. lactis* accumulated TMG (Thompson, unpublished data) and contained high levels of P-β-galactosidase (18); and (iii) glucose-grown cells of *S. lactis* ML_3 (DG^r) transported TMG at a rate ca. eightfold greater than the glucose-grown wild type while possessing four- to fivefold greater activity of P-β-galactosidase. It should be noted that under the conditions described (and during growth of cells on galactose and lactose) the glucose-PTS was inoperative. This suggests that expression of the *lac* operon in *S. lactis* may be partially constitutive, but subject to negative control by a component (or a derivative, e.g., glucose-6P) of the glucose-PTS. Cords and McKay (3) had previously considered competitive interaction between glucose and galactose-6P for control of the *lac* operon. More recently, Ratliff et al. (27) and Heller and Roschenthaler (8) have demonstrated repression of P-β-galactosidase synthesis by glucose in *S. lactis* C2 and *Streptococcus faecalis*, respectively. Our data neither confirm nor refute an inductive role for galactose-6P, but do suggest that regulation of the *lac* operon in the industrially important group N streptococci (17) may be more complex than currently envisaged.

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