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Intracellular Phosphorylation of Glucose Analogs via the Phosphoenolpyruvate:Mannose-Phosphotransferase System in *Streptococcus lactis*

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The bacterial phosphoenolpyruvate:sugar-phosphotransferase system (PTS) mediates the vectorial translocation and concomitant phosphorylation of sugars. The question arises of whether the PTS can also mediate the phosphorylation of intracellular sugars. To investigate this possibility in *Streptococcus lactis* 133, lactose derivatives have been prepared containing ^{14}C-labeled 2-deoxy-glucose (2DG), 2-deoxy-2-fluoro-D-glucose (2FG), or α-methylglucoside as the aglycon substituent of the disaccharide. Two of the compounds, β-*O*-D-galactopyranosyl-(1,4′)-2′-deoxy-D-glucopyranose (2′D-lactose) and β-*O*-D-galactopyranosyl-(1,4′)-2′-deoxy-2′-fluoro-D-glucopyranose (2′F-lactose), were high-affinity substrates of the lactose-PTS. After translocation, the radiolabeled 2′F-lactose 6-phosphate (2′F-lactose-6P) and 2′D-lactose-6P derivatives were hydrolyzed by *P*-β-galactoside-galactohydrolase to galactose-6P and either [^{14}C]2FG or [^{14}C]2DG, respectively. Thereafter, the glucose analogs appeared in the medium, but the rates of sugar exit from mannose-PTS-defective mutants were greater than those determined in the parent strain. Unexpectedly, the results of kinetic studies and quantitative analyses of intracellular products in *S. lactis* 133 showed that initially (and before exit) the glucose analogs existed primarily in phosphorylated form. Furthermore, the production of intracellular [^{14}C]2FG-6P and [^{14}C]2DG-6P (during uptake of the lactose analogs) continued when the possibility for reentry of [^{14}C]2FG and 2DG was precluded by addition of mannose-PTS inhibitors (*N*-acetylglucosamine or *N*-acetylmannosamine) to the medium. By contrast, (i) only [^{14}C]2DG, [^{14}C]2FG, and trace amounts of [^{14}C]2FG-6P were found in cells of a mannose-PTS-defective mutant, and (ii) only [^{14}C]2FG and [^{14}C]2DG were present in cells of a double mutant lacking both mannose-PTS and glucokinase activities. We conclude from these data that the mannose-PTS can effect the intracellular phosphorylation of glucose and its analogs in *S. lactis* 133.

In many anaerobic and facultatively anaerobic bacteria the transport and phosphorylation of sugars is mediated by the multi-component, phosphoenolpyruvate (PEP)-dependent sugar-phosphotransferase system (PTS; for reviews see references 9, 12, 30). It is currently believed that the transfer of the phosphoryl moiety from PEP to the sugar is obligatorily coupled to vectorial (inward) translocation of sugar phosphate (25, 35). Since the original discovery of this group translocation system by Kundig et al. (19), there has been controversy concerning the possibility of intracellular phosphorylation of sugars by the bacterial PTS. Several investigators, using intact cells or membrane vesicles, have presented evidence both for (5, 6, 8, 16) and against (8, 15, 17) this possibility. However, as emphasized by Hays (12) and by Postma and Roseman (30), many of the data are equivocal because the participation of intracellular kinases, PTS recapture, phosphate transfer by phosphohydrolases, or transphosphorylation via the enzyme II (EII) components of the PTS have not been rigorously excluded (26, 37).

The converse of PTS function involves the hydrolysis and subsequent expulsion of free (PTS) sugars. This process has received recent attention in studies with gram-positive organisms (11, 32, 33, 49). Dephosphorylation requires a hexose-6-phosphate (6P)-phosphohydrolase (47), and although the mechanism(s) for sugar exit has not been defined, in one instance the EII^{lac} appears to be involved (34). An important question has been posed: will efflux of PTS substrates also occur from cells lacking a functional PTS system? The resolution of this question has been precluded because no method was available for preloading PTS-defective cells with the appropriate PTS substrates.

In attempting to answer the two preceding questions, we have been helped by the following factors. First, glucokinase (GK)- and mannose-PTS-defective mutants (GK$^-$ and mannose-PTS^d, respectively) of *Streptococcus lactis* 133 have now been isolated. Second, we have prepared lactose derivatives containing ^{14}C-labeled, non-metabolizable glucose analogs (2-deoxy-2-fluoro-D-glucose [2FG], 2-deoxy-glucose [2DG], and α-methylglucoside [α-MG]) as the aglycon substituent. Third, because the lactose-PTS can be induced in wild-type and mannose-PTS^d mutants, the "illicit" transport of the lactose analogs provides a method for preloading mannose-PTS^d cells with high levels of mannose-PTS substrates.

In this communication we describe the preparation of three lactose analogs and show how these compounds have been used to (i) probe sugar exit mechanisms and (ii) demonstrate PTS-mediated phosphorylation of intracellular sugars in *S. lactis*.

MATERIALS AND METHODS

Organism. *S. lactis* 133 (ATCC 11454) was obtained from the culture collection of the New Zealand Dairy Research Institute, Palmerston North, New Zealand. The mannose-PTS^d mutant (*S. lactis* 133 mannose-PTS^d) was isolated by

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positive selection for resistance to 2DG (46, 49), and a GK^- mutant of this strain was obtained by UV mutagenesis. The double mutant (*S. lactis* 133 mannose-PTS^d GK^-) failed to grow on glucose (48) and was readily distinguished from the parental and mannose-PTS^d strains when grown on lactose agar plates by the formation of a deep red zone around the colonies after gentle spraying of the surface with glucose oxidase solution. Strains were maintained and grown in a complex medium containing 28 mM galactose as described previously (49).

Preparation of starved cells. Cells were harvested from 250 ml of culture (at mid-log phase of growth) by centrifugation at 13,000 × *g* for 1 min at 4°C. Supernatant fluid was removed by aspiration, and the cells were washed twice with 200-ml volumes of 50 mM potassium phosphate solution (pH 7) containing 1 mM $MgCl_2$ (KPM buffer). These freshly washed (starved) cells contained an intracellular PEP potential (ca. 30 mM) comprising 2- and 3-phosphoglyceric acids and PEP (23, 43, 50, 51).

Transport studies. In the standard transport procedure, starved cells were resuspended in KPM buffer to a final density of 200 μg (dry weight) of cells per ml. The cell suspension was gently agitated at 30°C in a water bath shaker, and radiolabeled sugars were added to a final concentration of 0.2 mM (specific activity, 0.2 to 0.5 μCi/μmol). In kinetic studies, the incubation period was 5 s. Accumulation of the analogs was monitored by membrane filtration and liquid scintillation procedures described previously (42, 50).

Time-course analysis of intracellular and extracellular products. The following procedures were used for the quantitative analysis of intracellular and extracellular products formed during the uptake of ^{14}C-labeled β-*O*-D-galactopyranosyl-(1,4')-2'-deoxy-D-glucopyranose (2'D-lactose) and β-*O*-D-galactopyranosyl-(1,4')-2'-deoxy-2'-fluoro-D-glucopyranose (2'F-lactose) by *S. lactis* 133 (see Fig. 6). The transport medium (5 ml) contained 0.1 M potassium phosphate buffer (pH 7), 1 mM $MgSO_4$, and 200 μM [^{14}C]lactose analog (specific activity, 0.5 μCi/μmol). A 1-ml portion of this solution (which served as the time-zero control) was frozen and lyophilized. To the remaining 4 ml of solution, 0.1 ml of a thick cell suspension was added to obtain a final density of 650 μg (dry weight) of cells ml^{-1}. At intervals of 15, 30, 60, and 90 s, 1-ml volumes of suspension were removed and quickly filtered through chilled (0°C) 25-mm-diameter membrane filters (type HA, 0.22-μm pore size, Millipore Corp., Bedford, Mass.). Cells retained on the filter were rinsed with 3 ml of distilled water at 0°C. The filtrates from each sample were collected, frozen, and lyophilized. Filters plus adhering cells were immediately transferred into 5 ml of boiling water for 5 min. After cooling, the cell extracts were clarified by centrifugation at 27,000 × *g* for 30 min (at 4°C). Supernatant fluids were removed and lyophilized, and the residues were reconstituted with 50 μl of distilled water. Radiolabeled sugars present in 10 μl of reconstituted filtrate were separated by chromatography (see below) and localized by autoradiography. The radioactive areas were cut out, and sugars were quantitatively determined by liquid scintillation counting. The lyophilized cell extracts were redissolved in 0.5 ml of distilled water, and each solution was transferred to a mini-column containing 1 ml of anion-exchange resin (AG1-X8, 100-200 mesh, acetate form; Bio-Rad Laboratories, Richmond, Calif.). Free and phosphorylated sugars were separated by sequential elution with 2.5 ml of distilled water and 3 ml of 0.2 M formic acid containing 0.5 M ammonium formate (43). Radioactive compounds were identified by paper and polyethyleneimine (PEI)–thin-layer chromatography and were quantitatively determined by liquid scintillation counting. Intracellular concentrations of metabolites were calculated on the basis that 1 g (dry weight) of cells was equivalent to 1.67 ml of intracellular fluid volume (42).

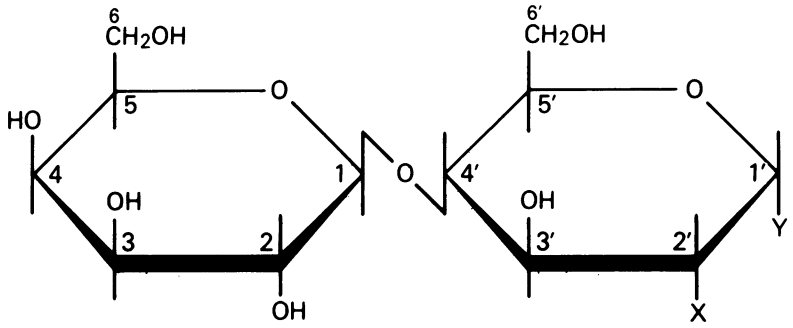
Intracellular products from transport of lactose analogs by wild-type and mutant strains. For the experiments to identify the intracellular products (see Fig. 7) the basal medium contained 0.1 M potassium phosphate buffer (pH 7), 5 mM $MgCl_2$, 100 μM [^{14}C]2'D-lactose or [^{14}C]2'F-lactose (specific activity, 0.5 μCi/μmol), and, when necessary, 5 mM *N*-acetylmannosamine. The appropriate cells (6 mg [total dry weight]) were added to the medium (30°C), and after 60 s the cells were collected by rapid filtration through a precooled 47-mm membrane filter (pore size, 0.45 μm). The extracellular sugars were removed by rinsing the filter with 20 ml of distilled water (0°C), and intracellular metabolites were extracted with boiling water. After lyophilization, the residues were reconstituted with 30 μl of distilled water. Samples of 5 μl (8 μl for the system in which *N*-acetylmannosamine was used) were applied to PEI–thin-layer plates, and radiolabeled compounds were separated and identified by fluorography (43, 51).

Partial purification of GK. Cells of *S. lactis* 133 were harvested by centrifugation (ca. 48 g [wet weight]) from 14 liters of medium that contained 14 mM lactose as energy source. A cell extract was prepared by high-speed centrifugation (47), and after dialysis the enzyme was partially purified by using the same anion-exchange (DEAE-Sephacel) and gel filtration (Ultrogel AcA-54) columns and chromatographic procedures as described in a previous communication (47). GK activity was eluted from the DEAE-Sephacel column (fractions 136–156) at a KCl concentration of ca. 0.25 M. After concentration and passage through the Ultrogel AcA-54 column, the enzyme ($M_r \approx 60{,}000$) was recovered in fractions 67 through 77. These fractions were pooled and concentrated by Amicon ultrafiltration, and this preparation (specific activity, 125 μmol of glucose-6P formed mg of $protein^{-1}$ min^{-1}) was used for substrate specificity studies. Disc gel electrophoresis (7) of this preparation revealed two major and several minor protein bands after staining with Coomassie blue R250. GK activity in the upper major band in the gel was localized enzymatically by precipitation of reduced Nitro Blue Tetrazolium (27).

GK assays. During purification, GK activity was assayed spectrophotometrically by the glucose 6-phosphate dehydrogenase (glucose-6P dehydrogenase) (EC 1.1.1.49):NADP-coupled assay of Porter et al. (27), and protein was determined by the method of Bradford (4). For substrate specificity and inhibitor studies a lactate dehydrogenase (EC 1.1.1.27)-pyruvate kinase (EC 2.7.1.40)-coupled enzyme assay was used (27). The ATP-dependent phosphorylation of specific ^{14}C-labeled sugars was also monitored by binding to Whatman DE81 filter disks, and the identities of the phosphorylated products were confirmed by paper chromatography (27) and autoradiography.

Preparation of ^{14}C-labeled lactose analogs. The lactose analogs were prepared enzymatically by modification of procedures described by Schanbacher and Ebner (38), Zemek et al. (52), and Babad and Hassid (2). In the presence of α-lactalbumin and Mn^{2+} ion, the enzyme UDP-galactose:D-glucose 1-galactosyltransferase (EC 2.4.1.22, lactose synthetase) catalyzes the biosynthesis of lactose (4-*O*-β-D-galactopyranosyl-α-D-glucopyranose) as follows.

$$\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP} \quad (1)$$



Lactose: X = OH, Y = OH
2'D - Lactose: X = H, Y = OH
2'F - Lactose: X = F, Y = OH
1'-α-methyl - Lactose: X = OH, Y = 0.CH_3

FIG. 1. Structures of lactose analogs used in this study.

Glucose is the usual and most effective galactosyl acceptor from UDP-galactose, but other glucose analogs, including 2DG and 2FG (this study), may serve as relatively poor substitutes for glucose in reaction 1. When the analogs are radiolabeled, appropriate [^{14}C]lactose derivatives can be obtained.

UDP-galactose + [^{14}C]2DG → [^{14}C]2'D-lactose + UDP (2)

In our procedure, each reaction system (final volume, 1 ml) contained 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5); 15 mM $MnSO_4$; 0.2 mg of α-lactalbumin; 3 mg of galactosyltransferase; 40 μmol of UDP-galactose; and 40 μmol of ^{14}C-labeled 2DG, 2FG, or α-MG (final specific activity, 0.5 μCi/μmol). The reaction mixtures were incubated for 24 h at 37°C. Each solution was then transferred to a mixed-bed ion-exchange column (0.5 by 12 cm) containing equivalent amounts of Dowex 50 and Dowex 1 resins. The radiolabeled sugars (i.e., lactose derivatives and unreacted glucose analogs) were collected from the column by elution with ca. 10 ml of water. The solutions were frozen in dry ice, lyophilized, and reconstituted with 0.5 ml of water. Radiolabeled sugars were separated by descending paper chromatography (Whatman 3M; running time ca. 18 h) in a solvent containing 1-butanol–concentrated acetic acid–water (5:2:3, vol/vol). The positions of the ^{14}C-labeled compounds were determined by autoradiography.

In each case only two radioactive regions were detected. The faster-migrating band corresponded to the original glu-

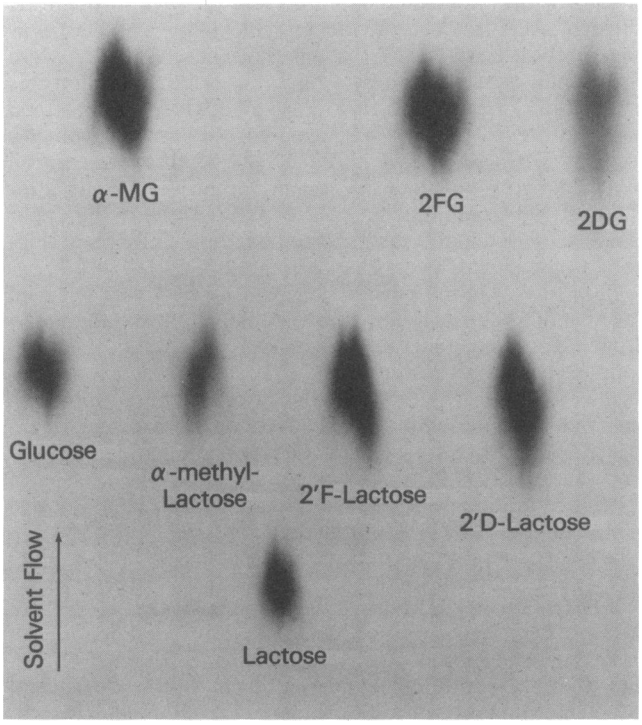


FIG. 2. Fluorographic characterization and relative migration of the lactose analogs with respect to glucose and lactose standards. Radiolabeled α-MG, 2FG, and 2DG were formed by incubation of α-methyllactose, 2'F-lactose, and 2'D-lactose with β-galactosidase. Arrow indicates direction of solvent flow.

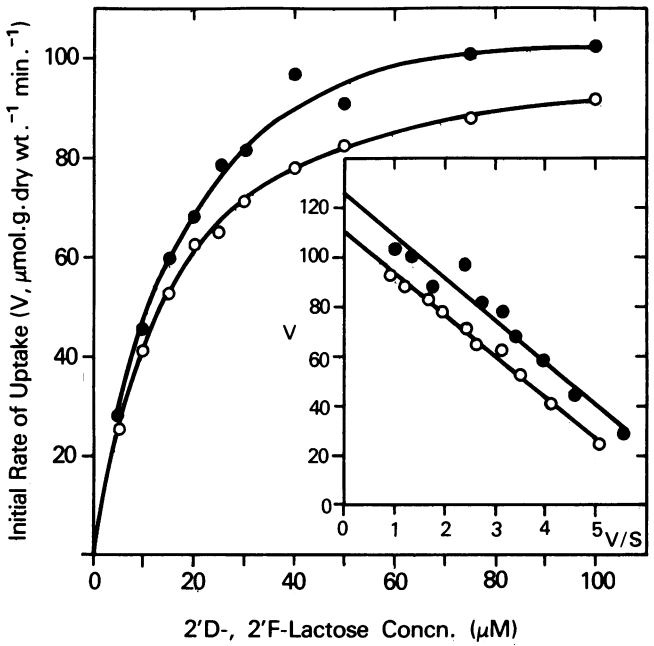


FIG. 3. Kinetic analyses of (○) 2'F-lactose and (●) 2'D-lactose uptake via the lactose-PTS in starved cells of *S. lactis* 133. Inset shows Hofstee transformation plots of initial rate data.

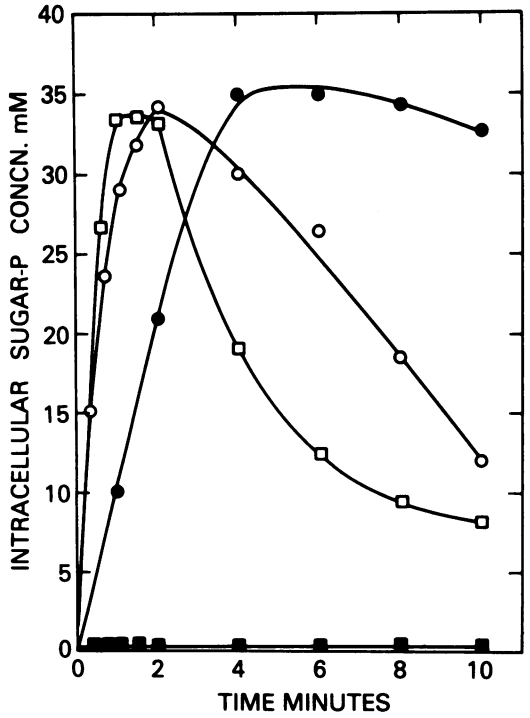


FIG. 4. PEP-dependent accumulation of lactose analogs by *S. lactis* 133. Starved cells (grown previously on galactose) were suspended at a density of 200 μg (dry weight) of cells ml^{-1} in KPM buffer (pH 7), and lactose analogs were added to a final concentration of 0.1 mM (specific activity, 0.5 μCi/μmol). [^{14}C]TMG concentration was 0.2 mM (specific activity, 0.2 μCi/μmol), and accumulation of substrates was followed as described in the text. Symbols: ■, α-methyllactose; ○, 2'D-lactose; □, 2'F-lactose; and ●, TMG.

cose analog, and the slower band corresponded to the disaccharide derivative. The putative [^{14}C]lactose analogs were extracted from the paper by centrifugal elution with distilled water. The eluents were lyophilized, reconstituted with 1 ml of water, and stored at −20°C until required for transport experiments. From the 40 μmol of 2DG, 2FG, and α-MG present initially in the reaction, this procedure yielded 15.5 μmol of [^{14}C]2'D-lactose (39% conversion), 16.6 μmol of [^{14}C]2'F-lactose (42%), and 1.4 μmol of [^{14}C]1-*O*-methyl lactose (3% conversion).

Reagents. Galactosyltransferase, α-lactalbumin, other enzymes, and sugars were obtained from Sigma Chemical Co., St. Louis, Mo. 2DG and 2FG were obtained from Calbiochem-Behring, La Jolla, Calif., and 6-amino-6-deoxy-D-glucopyranose was purchased from Vega Biochemicals, Tucson, Ariz. Radiolabeled sugars and sugar analogs were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Structure and composition of lactose analogs. The structures of the three lactose analogs are presented in Fig. 1. The compounds were purified chromatographically, and all migrated significantly faster than lactose (Fig. 2). In each case, treatment with β-galactosidase yielded galactose and the corresponding ^{14}C-labeled glucose analog (Fig. 2).

Transport and kinetic analyses. Starved cells of *S. lactis* contain an endogenous PEP pool (23, 50, 51) which permits PTS-mediated sugar transport to the followed in intact cells. Kinetic studies (Fig. 3) showed that the lactose-PTS exhibited high affinity for both 2'F-lactose and 2'D-lactose ($K_m \approx$ 16 μM; $V_{max} \approx$ 120 μmol of disaccharide accumulated g [dry weight] of cells $^{-1}$ min^{-1}). The parameters are similar to those determined previously for the transport of lactose (K_m = 21 μM; V_{max} = 150 μmol accumulated g [dry weight] of cells $^{-1}$ min^{-1}; 44). When starved cells of *S. lactis* 133 were

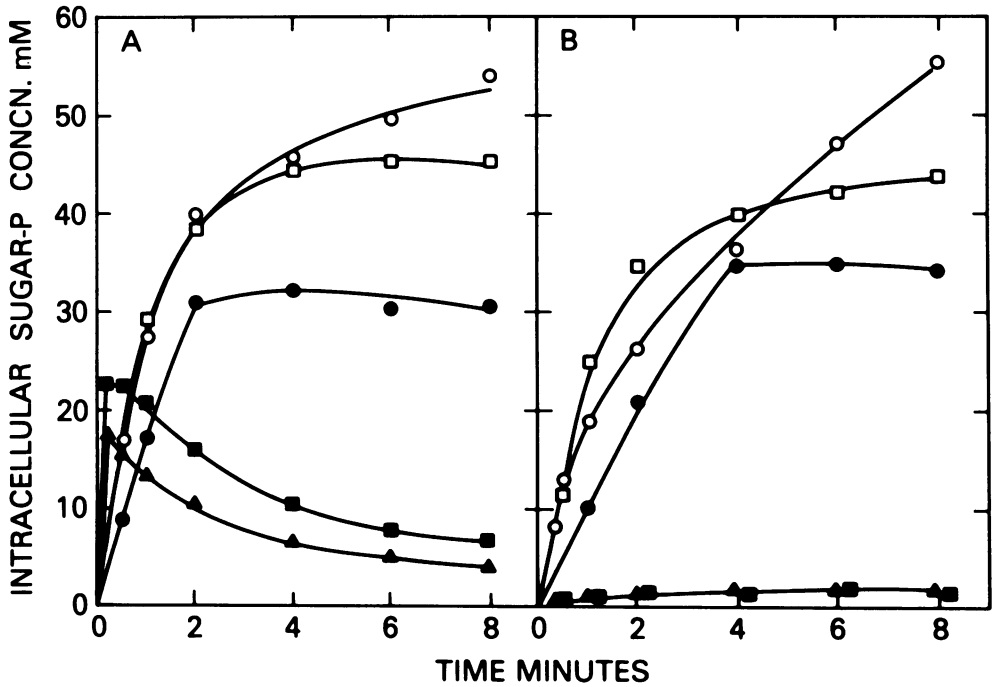


FIG. 5. Accumulation of sugar analogs by starved cells of (A) *S. lactis* 133 (wild type) and (B) *S. lactis* 133 mannose-PTS^{d}. Sugars were present at a final concentration of 0.4 mM, and other experimental conditions were as described in the text. Symbols: ▲, 2DG; ■, 2FG; ●, TMG; □, 2'F-lactose; ○, 2'D-lactose.

TABLE 1. Sugar PTS[a] and GK activities in strains of *S. lactis* 133

S. lactis strain[b]	PTS activity (μmol of sugar accumulated g [dry wt] of cells^{-1} min^{-1}) for:						GK activity (μmol of G6P formed mg of protein^{-1} min^{-1})
	Glucose	Mannose	Glucosamine	2FG	2DG	2'D-lactose	
133 (wild type)	256.1	347.7	141.9	180.9	98.4	82.0	0.92
133 mannose-PTS^d	9.1	ND[c]	1.7	2.2	0.3	89.6	1.03
133 mannose-PTS^d GK$^-$	ND	ND	ND	ND	ND	86.5	0.00

[a] Glucose, mannose, glucosamine, 2FG, and 2DG are substrates for the mannose-PTS, and 2'D-lactose is substrate for the lactose-PTS.
[b] All strains were grown in complex medium containing 28 mM galactose.
[c] ND, No detectable accumulation.

incubated with the lactose analogs (0.1 mM), maximum accumulation of the phosphorylated compounds was attained within 1 to 2 min. The two analogs contain one metabolizable (galactose) and one non-metabolizable hexose moiety. After depletion of these substrates from the medium, a rapid decrease in the level of intracellular radiolabeled material was observed (Fig. 4). The rate of uptake of the non-metabolizable lactose analog methyl-β-D-thiogalactopyranoside (TMG) by the cells was considerably slower than that of 2'F-or 2'D-lactose, and no accumulation of α-methyllactose occurred.

Accumulation of glucose and lactose analogs by wild-type and mannose-PTS^d strains. Starved cells of *S. lactis* 133 rapidly accumulated the non-metabolizable glucose analogs (2FG, 2DG) via the mannose-PTS system (Fig. 5A), and maximum concentrations (ca. 20 mM) of intracellular hexose phosphates were attained within 15 to 20 s. After dephosphorylation by an intracellular hexose-6P-phosphohydrolase (47, 49), a first-order efflux of free sugar was observed. By contrast, cells of the mannose-PTS^d strain (*S. lactis* 133 mannose-PTS^d; Table 1) accumulated the glucose analogs at a rate <5% of that observed for the parental strain and to a maximum concentration of ca. 2 mM (Fig. 5B; Table 1). No accumulation of glucose or its non-metabolizable analogs occurred in starved cells of a double mutant lacking both mannose-PTS and GK activities. (*S. lactis* 133 mannose-PTS^d GK$^-$; see Table 1). However, all three strains rapidly accumulated 2'F-lactose, 2'D-lactose, and TMG via the lactose-PTS. The inability of *S. lactis* 133 mannose-PTS^d and the double mutant to take up the glucose analogs was therefore not due to a deficiency in the general PTS components (i.e., heat-stable phosphoryl carrier protein [HPr], EI) or to a reduced intracellular PEP potential. As suggested previously (49), it appears to be a consequence of a defect in the membrane-bound EII^{man} component of the PTS.

Products of 2'D-lactose transport by *S. lactis* 133. The intracellular and extracellular products of [^{14}C]2'D-lactose transport were quantitatively determined in the time-course experiment presented in Fig. 6. Cells of *S. lactis* 133 were incubated with the disaccharide, and at intervals cells were collected by membrane filtration and immediately extracted with boiling water. Filtrates were also collected and lyophilized, and the identities and concentrations of ^{14}C-labeled sugars present extracellularly were determined by paper chromatography (Fig. 6A). Before addition of cells (Fig. 6A, time zero) only [^{14}C]2'D-lactose was present in the medium and no traces of contaminating sugars, e.g., [^{14}C]2DG, were detected by autoradiography. After 15 s from cell addition, only trace amounts of [^{14}C]2DG (<5 μM) were found in the medium, but thereafter the concentration of the glucose

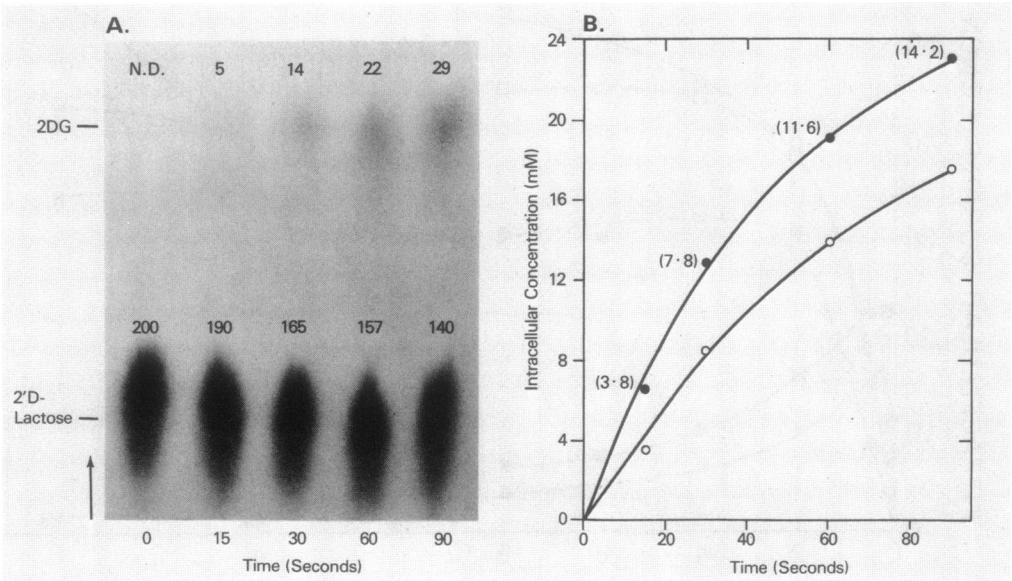


FIG. 6. Time-course kinetics and quantitative analyses of products formed during uptake of [^{14}C]2'D-lactose by cells of *S. lactis* 133. (A) Demonstration by paper chromatography of extracellular 2'D-lactose utilization and 2DG formation; numerical values indicate micromolar concentrations of the sugars in the medium. (B) Corresponding time course of (●) total concentration of intracellular products (2DG plus 2DG-6P) and (○) concentration of 2DG-6P alone. Numbers in parentheses indicate percentage of the total [^{14}C]2'D-lactose initially present in the medium which was accumulated by the cells.

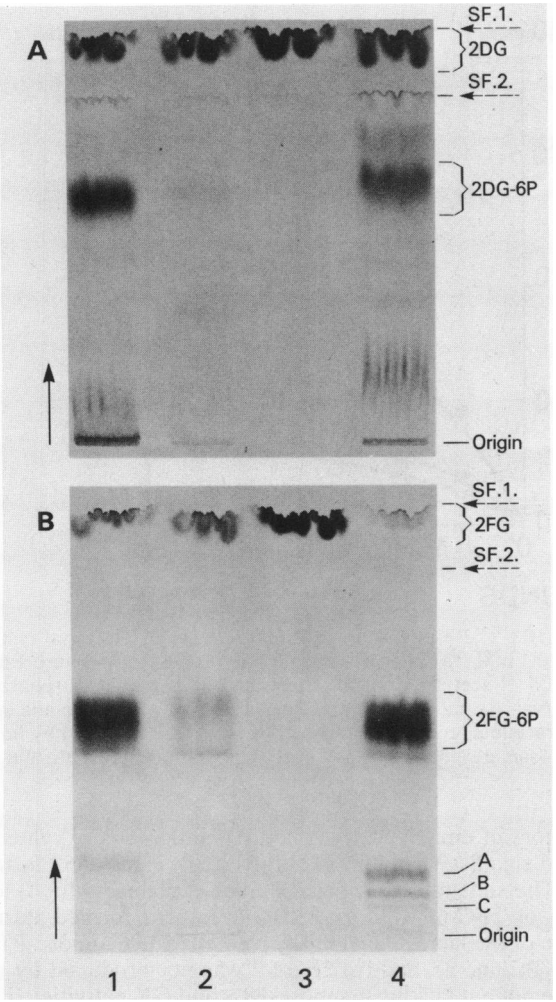


FIG. 7. Fluorographic identification of metabolites present in cell extracts from wild-type and mutant strains of *S. lactis* 133 after uptake of (A) [^{14}C]2′D-lactose and (B) [^{14}C]2′F-lactose. Transport protocols, methods of extraction, and PEI–thin-layer chromatographic procedures are given in the text. SF.1. and SF.2. refer to the water front and LiCl-formic acid solvent front, respectively. Arrow indicates direction of solvent flow. The extracts were prepared after accumulation of the lactose analogs by: lane 1, *S. lactis* 133 (wild type); lane 2, *S. lactis* 133 mannose-PTS^d; lane 3, *S. lactis* 133 mannose-PTS^d GK$^-$; and lane 4, *S. lactis* 133 (in the presence of 5 mM *N*-acetylmannosamine).

analog increased (29 μM, t = 90 s) with a concomitant decrease in [^{14}C]2′D-lactose concentration. Intracellular products (free and phosphorylated sugars) formed throughout the time course were quantitatively determined in cell extracts by ion-exchange chromatography (Fig. 6B). Within 15 s, the cells had accumulated 3.8% of the [^{14}C]2′D-lactose initially present in the medium. Of this intracellular radioactive material, 45% was recovered as free [^{14}C]2DG (2.9 mM), but unexpectedly 55% was recovered as a phosphorylated derivative (3.5 mM). The latter compound co-chromatographed with authentic [^{14}C]2DG-6P (see Fig. 7A, lane 1) and after treatment with alkaline phosphatase produced [^{14}C]2DG. The intracellular concentration and the relative proportion of [^{14}C]2DG-6P increased with time, and by 90 s the hexose-6P (17.6 mM) made up 76% of the total radioactive material in the cell. Similar results were obtained with [^{14}C]2′F-lactose (data not shown), but in neither case were the initial products of transport (2′F-lactose-6P, 2′D-lactose-6P) detected in any of the extracts.

Probable mechanisms for intracellular 2DG-6P and 2FG-6P formation. Because hydrolysis of phosphorylated lactose analogs by *P*-β-galactosidase would yield metabolizable galactose-6P and free [^{14}C]2DG or [^{14}C]2FG, at least five mechanisms must be considered for formation of intracellular 2DG-6P: (i) transport by the mannose-PTS of [^{14}C]2DG present as contaminant in [^{14}C]2′D-lactose, (ii) transphosphorylation (14, 37) from intracellular galactose-6P to [^{14}C]2DG mediated via EII of the PTS, (iii) exit of intracellular [^{14}C]2DG and subsequent reentry via the mannose-PTS, (iv) phosphorylation of the glucose analog by ATP-dependent GK, and (v) direct phosphorylation of intracellular [^{14}C]2DG by the mannose-PTS. Mechanism (i) can be eliminated because the [^{14}C]2′D-lactose was chromatographically pure (Fig. 6A; time zero), and data presented in the accompanying paper (48) preclude mechanism (ii). Data in Fig. 6 (t = 15 through 30 s) show that high levels of intracellular [^{14}C]2DG-6P appeared *before* significant concentrations of free [^{14}C]2DG were detectable in the medium. Even after 90 s, the extracellular concentration of the glucose analog (29 μM) was still far below the K_m of 2DG for the mannose-PTS (80 μM; 43). It is kinetically unlikely that the intracellular [^{14}C]2DG-6P formed during the 15- to 30-s period can arise via exit and subsequent reentry of sugar [proposal (iii)] via the mannose-PTS, although at later times this must occur.

Products of 2′D- and 2′F-lactose transport by GK$^-$ and mannose-PTS^d mutants of *S. lactis* 133. Of the five mechanisms presented, only participation of GK (iv) and the mannose-PTS [proposal (v)] seemed plausible, and these mechanisms were investigated in experiments described in Fig. 7. Cells of *S. lactis* 133 (wild type), *S. lactis* 133 mannose-PTS^d, and *S. lactis* 133 mannose-PTS^d GK$^-$ were

TABLE 2. Substrate specificity and potential inhibitors of GK from *S. lactis* 133[a]

Substrate[b] or potential inhibitor[c]	Sp act (μmol of sugar phosphorylated mg of protein^{-1} min^{-1}	Rate of sugar phosphorylation relative to glucose (100%)
Substrate		
Glucose	125.2	100
N-Acetylglucosamine	101.1	81
Glucosamine	75.8	61
2FG	18.8	15
2DG	ND	ND
Potential inhibitor		
Glucose (control, no inhibitors)	72.3	100
3-Acetamido-3-deoxy-D-glucose	79.2	110
2DG	79.2	110
3-*O*-methyl-D-glucopyranose	79.2	110
2FG	72.3	100
β-MG	71.2	98
α-MG	59.7	83
6-Chloro-6-deoxy-glucose	57.4	79
6-Deoxy-D-glucose	50.5	70
6-Amino-6-deoxy-glucose	3.4	5

[a] Determined by the pyrate kinase-lactate dehydrogenase-coupled enzyme assay.
[b] Substrate concentration, 10 mM.
[c] Glucose and potential inhibitor concentrations, 0.5 and 10 mM, respectively.

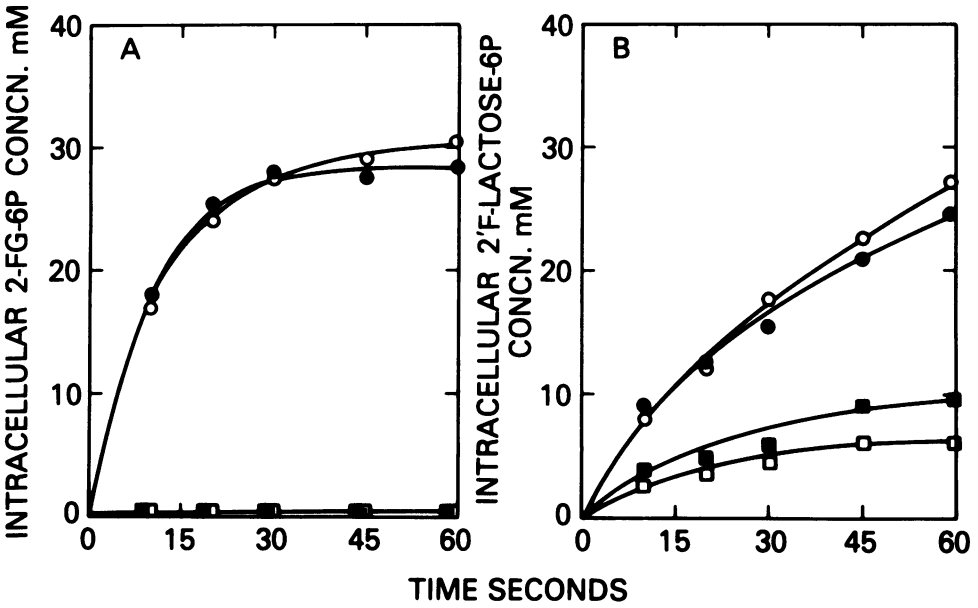


FIG. 8. Effects of *N*-acetylated sugars on accumulation of (A) [^{14}C]2FG and (B) [^{14}C]2'F-lactose by cells of *S. lactis* 133. The basal system (2 ml) contained 0.1 M potassium phosphate buffer (pH 7); 5 mM $MgCl_2$; either 0.1 mM [^{14}C]2FG (specific activity, 0.2 μCi/μmol) or 0.1 mM [^{14}C]2'F-lactose (specific activity, 0.5 μCi/μmol), and when appropriate 5 mM *N*-acetylated sugar. Starved cells (45 μl of suspension, equivalent to 1 mg [dry weight] of cells) were added to the medium, and at intervals 0.3-ml volumes of suspension were withdrawn and cells were collected by membrane filtration. After rinsing, filters were dried, and intracellular concentrations of sugars were determined by liquid scintillation counting. Symbols: ●, control (no addition); ○, *N*-acetylgalactosamine present; □, *N*-acetylglucosamine present; and ■, *N*-acetylmannosamine present.

incubated with [^{14}C]2'D-lactose (Fig. 7A) or [^{14}C]2'F-lactose (Fig. 7B), and intracellular products were extracted and identified by PEI–thin-layer fluorography. Analysis of the extracts from *S. lactis* 133 confirmed the results obtained previously by ion-exchange chromatography: (i) neither [^{14}C]2'D-lactose-6P nor [^{14}C]2'F-lactose-6P was detected, and these derivatives (like lactose-6P; 44) must be rapidly hydrolyzed by *P*-β-galactosidase; and (ii) intracellular glucose analogs were recovered predominantly in phosphorylated form (Fig. 7A and B, lane 1). By contrast, when cells of *S. lactis* 133 mannose-PTS^d or *S. lactis* 133 mannose-PTS^d GK$^-$ were incubated with [^{14}C]2'D-lactose, only the free sugar [^{14}C]2DG was detected in the cell extracts (Fig. 7A, lanes 2 and 3). The absence of [^{14}C]2DG-6P in the mutants confirmed participation of the mannose-PTS in phosphorylation of 2DG in the parental strain. After accumulation of [^{14}C]2'F-lactose by *S. lactis* 133 mannose-PTS^d, cell extracts contained predominantly [^{14}C]2FG. In addition, a small amount of [^{14}C]2FG-6P (ca. 15% of the concentration found in the wild type) was also detected by fluorography (Fig. 7B, lane 2), but [^{14}C]2FG-6P was not present in extracts prepared from *S. lactis* 133 mannose-PTS^d GK$^-$ (Fig. 7B, lane 3). Again we conclude that the mannose-PTS system is primarily responsible for intracellular 2FG phosphorylation, but in addition the 2-fluoro analog may also be slowly phosphorylated via ATP-dependent GK.

Substrate specificity of GK. Of a number of potential substrates tested, only glucose (K_m = 0.5 mM), *N*-acetylglucosamine, glucosamine, and 2FG were phosphorylated by GK prepared from *S. lactis* 133 (Table 2). Conversion of these [^{14}C]-labeled sugars to the corresponding hexose-6-phosphates were confirmed by paper chromatography and autoradiography (data not shown). Mannose, galactose, fructose, and 2DG were not phosphorylated by the ATP-dependent enzyme. Of the glucose analogs tested as potential inhibitors of enzyme activity, only 6-amino-6-deoxyglucose caused significant inhibition of the rate of glucose-6P formation. The substrate specificity studies explained why (i) low levels of 2FG-6P (but not 2DG-6P) were formed during uptake of the lactose analogs by *S. lactis* 133 mannose-PTS^d (Fig. 7B, lane 2), and (ii) 2FG-6P was not produced by the double mutant lacking mannose-PTS and GK activities (Fig. 7B, lane 3).

Intracellular phosphorylation of glucose analogs via the mannose-PTS system. From the kinetic studies (Fig. 6) it seemed improbable that 2DG-6P and 2FG-6P (produced during the initial 15 to 30 s of lactose analog transport) were formed by exit and subsequent reentry of the glucose analogs via the mannose-PTS. However, a more direct demonstration was required to confirm PTS-mediated phosphorylation of intracellular sugars. To distinguish between the two modes of PTS-mediated phosphorylation, it was necessary to find conditions which would prevent reentry of the glucose analogs without at the same time abolishing the translocation of [^{14}C]2'F- and [^{14}C]2'D-lactose. These conditions were satisfied by inclusion of 5 mM *N*-acetylglucosamine or *N*-acetylmannosamine (but not *N*-acetylgalactosamine) in the medium. The two low-affinity substrates of the mannose-PTS (1, 41, 46) completely inhibited uptake of exogenous 2FG by *S. lactis* 133 (Fig. 8A) but only partially inhibited uptake of 2'F-lactose (Fig. 8B). Similar results were obtained in transport studies with 2DG and 2'D-lactose (data not shown). To identify intracellular products, cells of *S. lactis* 133 were suspended in medium containing [^{14}C]2'F-lactose plus *N*-acetylmannosamine, and after 1 min of incubation cells were collected by membrane filtration and extracted with boiling water. The experiment was repeated with [^{14}C]2'D-lactose plus *N*-acetylmannosamine, and when the extracts were analyzed by PEI–thin-layer chromatography both [^{14}C]2DG-6P and [^{14}C]2FG-6P were

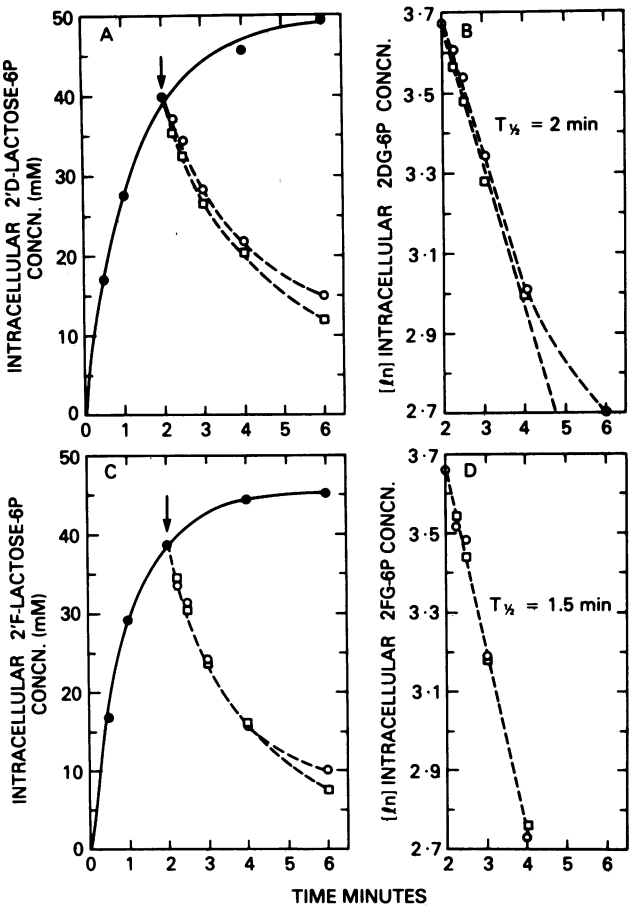


FIG. 9. Accumulation of lactose analogs by *S. lactis* 133 and exit of intracellular material elicited by addition of unlabeled lactose or iodoacetate. (A) Cells were suspended at a density of 0.4 mg (dry weight) ml^{-1} in 5 ml of 0.1 M potassium phosphate buffer (pH 7) containing 5 mM $MgCl_2$; [^{14}C]2'D-lactose was added to a concentration of 0.2 mM (specific activity, 0.5 μCi/μmol), and accumulation (●) of sugar (i.e., intracellular [^{14}C]2DG-6P) was determined. To duplicate systems, 10 mM iodoacetate or 10 mM lactose was added (arrow, 2 min), and the loss of intracellular sugar phosphate was monitored (□ and ○, respectively). (B) First-order decrease in intracellular [^{14}C]2DG-6P concentration (which is equivalent to the appearance of [^{14}C]2DG in the medium). These experiments were repeated with [^{14}C]2'F-lactose as substrate, and the corresponding data are presented in (C) and (D), respectively.

detected (Fig. 7A and B, lane 4, respectively). These compounds could have been formed only by *intracellular* phosphorylation mechanisms, i.e., via the mannose-PTS system (for [^{14}C]2DG-6P) or by the combined activities of the mannose-PTS system plus GK (for [^{14}C]2FG-6P). The three minor products designated A, B, and C in Fig. 7B, lane 4, have not been identified, but the migration distances are similar to those of sugar diphosphates and certain sugar nucleotides, e.g., UDP-2-fluoroglucose, which has previously been detected in yeast and chicken embryo cells (39).

Exit of glucose analogs from wild-type and mannose-PTS^d cells. Starved cells of *S. lactis* accumulate glucose analogs via the mannose-PTS to an intracellular concentration of 20 to 30 mM (43, 44; Fig. 5A). The sugar phosphates are then hydrolyzed by an intracellular hexose-6P-phosphohydrolase, and efflux of free sugar occurs with a half-time ($T_{1/2}$) of exit of ca. 2 to 3 min. Because cells of *S. lactis* 133 mannose-PTS^d accumulated glucose and its analogs at <5% of the rate of the parental strain (Fig. 5B; Table 1), it was of interest to determine whether the rates of sugar exit from these cells would also be correspondingly reduced. The standard method for preloading cells of *S. lactis* with 2DG-6P or 2FG-6P before efflux studies simply requires incubation of starved organisms with the required analog, but this procedure could not be used to preload mannose-PTS^d mutants. However, the translocation of 2'D-lactose and 2'F-lactose (via the lactose-PTS) provided a method for preloading mannose-PTS^d strains with the intracellular substrates of this system. To determine the rates of sugar exit, cells of *S. lactis* 133 were incubated with either [^{14}C]2'D-lactose (Fig. 9A) or 2'F-lactose (Fig. 9C), and after 2 min, unlabeled lactose (a competitive inhibitor of transport) or iodoacetate (an inhibitor of glycolysis) was added to the cell suspension. The inhibitors prevented further uptake of the radiolabeled lactose analogs by the cells, and rapid exit of [^{14}C]2DG (Fig. 9B; $T_{1/2}$ = 2 min) and [^{14}C]2FG (Fig. 9D; $T_{1/2}$ = 1.5 min) was observed. When these experiments were repeated with *S. lactis* 133 mannose-PTS^d GK$^-$ it was found that the rates of exit of the glucose analogs from the mutant (Fig. 10; $T_{1/2}$ = 0.9 to 1.2 min) were somewhat greater than those determined in cells of the wild type.

DISCUSSION

The lactose derivatives described in this report are useful probes of lactose-PTS and glucose-PTS activities in *S. lactis* (Fig. 11). The availability of these compounds has enabled us to study the question of intracellular phosphorylation of 2DG and 2FG by the mannose-PTS system and to determine the rates of exit of these glucose analogs from mannose-PTS^d cells. Two of the three lactose derivatives (2'F-lactose, 2'D-lactose) differ from lactose only at C-2' of the glucose moiety (see Fig. 1), and both are accumulated via the lactose-PTS at a rate comparable to that of the natural disaccharide. The removal of oxygen and the substitution of a fluorine atom for the hydroxyl group at C-2' can be tolerated without loss of affinity for the lactose-PTS, and both of the phosphorylated products were hydrolyzed by *P*-β-galactosidase. The anomeric center may play a more important role in binding between potential substrates and EII^{lac}, because the compound formed by methylation at C-1' (α-methyllactose) was not accumulated by cells of *S. lactis* 133.

In this study we wished to know whether the rates of exit of mannose-PTS substrates (2DG, 2FG) from mannose-PTS^d organisms would differ from the rates determined in the wild-type cells. Initially we encountered the practical difficulty of preloading the mutant cells with the desired substrates, but a strategy used previously by Solomon et al. (40) enabled us to circumvent this problem. Solomon et al. found that the transport and subsequent hydrolysis of 1-*O*-β-D-galactopyranosyl-D-mannitol (by lactose-permease and β-galactosidase, respectively) provided a means of preloading cells of *Escherichia coli* with mannitol to study the exit of this compound from cells uninduced for mannitol EII. In our investigation, the translocation of the lactose analogs (via the lactose-PTS and hydrolysis by *P*-β-galactosidase) effectively bypassed the inoperative mannose-PTS, and this "Trojan horse" procedure permitted preloading of mutant cells of *S. lactis* with substrates (2DG, 2FG) of this PTS. Although mannose-PTS^d cells were unable to accumulate significant levels of the glucose analogs, data obtained from kinetic studies showed that the rates of efflux of 2DG and 2FG from such cells were faster than the rates determined in

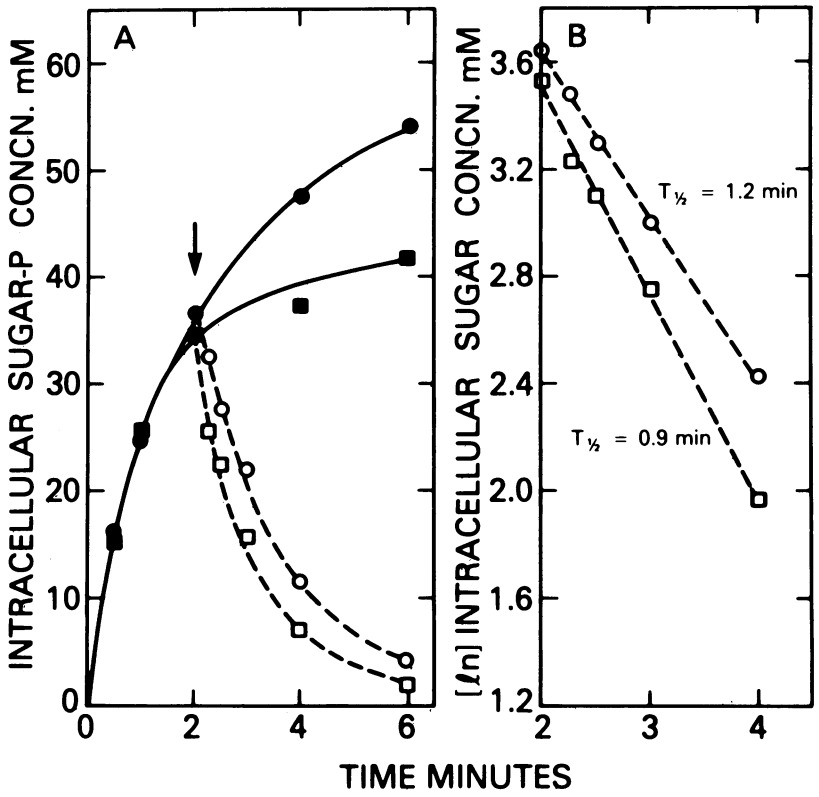


FIG. 10. (A) Accumulation of [^{14}C]2'D-lactose (●) and [^{14}C]2'F-lactose (■) by cells of *S. lactis* 133 mannose-PTS^{d} GK^{-} and efflux of intracellular [^{14}C]2DG (○) and [^{14}C]2FG (□) elicited by addition of 10 mM lactose (arrow, 2 min) to the suspension. (B) First-order decrease in intracellular concentrations of the glucose analogs on addition of lactose.

the wild-type strain. In *S. lactis* 133, intracellular 2DG-6P and 2FG-6P must first be hydrolyzed by a hexose-6P-specific phosphohydrolase (47; Fig. 11, step 3), but in cells of *S. lactis* 133 mannose-PTS^{d} and the double mutant, the glucose analogs were primarily in the free form. The elimination of the dephosphorylation step and the greater concentration of free sugar may contribute to the increased rate of exit (i.e., smaller $T_{1/2}$) from the mutant cells. We do not know the mechanism(s) for sugar exit (Fig. 11, step 5) from either the wild-type or mutant organisms, but evidently a functional mannose-PTS is not a prerequisite. In terms of substrate specificity, the mannose-PTS in *S. lactis* (43, 49) is similar to one (EIIA-EIIB) of the two glucose-PTS systems described for *Salmonella typhimurium* and *E. coli* (12, 30). The membrane component of this low-affinity system designated EII^{man} (3, 6, 41) consists of two integral membrane proteins EII^{A} and EII^{B} (20). Previous studies by Thompson and Saier (49) indicated that the biochemical lesion in the mannose-PTS^{d} isolates of *S. lactis* was confined to the membrane component. However, as for EII^{man} mutants of *Salmonella typhimurium* (41), it is not known whether the mutational locus is in the genes coding for the EII^{A} or EII^{B} domains. It is conceivable, although controversial (12, 22, 30, 31, 34, 40), that one of these two membrane proteins may serve as a facilitator for the exit of glucose (and its analogs) from *S. lactis*. The lesion in the mannose-PTS^{d} isolates may result from uncoupling (29) between sugar translocation and phosphorylation because (i) EII^{A} cannot accept the phosphoryl group from P~HPr or (ii) EII^{A} is unable to transfer this group during transmembrane passage of the incoming sugar (18). Alternative mechanisms for 2FG and 2DG exit may involve a separate non-PTS glucose permease (10) or the participation of the galactose permease. The latter active transport system is present in galactose-grown cells of *S. lactis* (45), and this system is known to transport glucose and 2DG in *E. coli* and *Salmonella typhimurium* (13, 28, 36).

The vectorial translocation and concomitant phosphorylation of sugar is an accepted feature of the bacterial PEP:PTS (25, 30, 35), but the question of PTS-mediated phosphorylation of sugar produced intracellularly (i.e., without membrane translocation) has been controversial. In a recent study, Beneski et al. addressed this topic by using membrane vesicles prepared from *Salmonella typhimurium* (3). It was found that the vesicles, in either the right-side-out or the inside-out orientation, catalyzed phosphorylation of 2DG when incubated with PEP and the soluble components (HPr, EI) of the PTS. The accessability of EII^{man} to these components (and sugar) at both inner and outer surfaces of the cytoplasmic membrane indicated that EII^{man} was a symmetrical transmembrane complex. By extrapolation from the vesicle to the whole cell, Beneski et al. suggested that the EII^{man} system should be capable of phosphorylating its sugar substrates when these compounds are formed intracellularly. Our studies with the lactose analogs (summarized schematically in Fig. 11) have provided evidence for such phosphorylation by the mannose-PTS system in physiologically intact cells of *S. lactis*: (i) after hydrolysis of intracellular 2'D-lactose-6P (Fig. 11, step 1) the aglycon moiety (2DG) was recovered predominantly as 2DG-6P and not as the anticipated free sugar; (ii) 2DG-6P formation continued even under conditions where the possibility of 2DG efflux and reentry via the mannose-PTS (Fig. 11, step 6) had been eliminated by the presence of exogenous *N*-acetylmannosamine; (iii) 2DG was not a substrate for ATP-dependent GK;

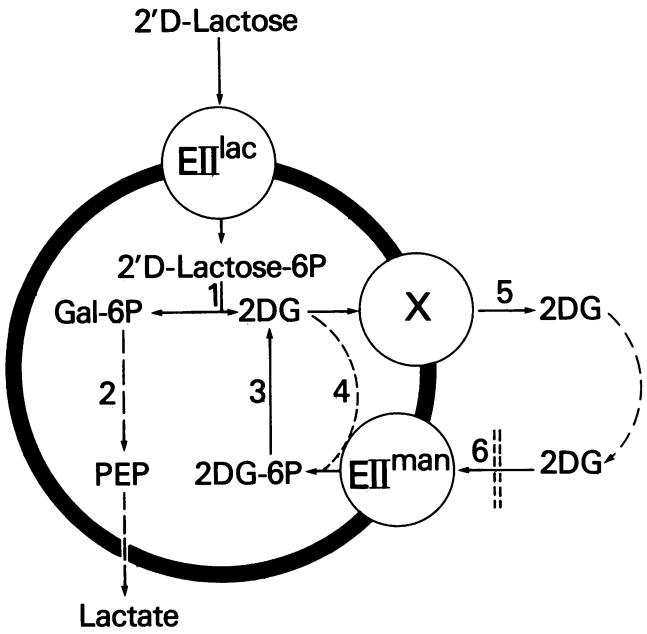


FIG. 11. Schematic representation of pathways involved in the transport and metabolism of lactose analogs by cells of *S. lactis* 133. EII^{lac} and EII^{man} are lactose-enzyme II and mannose-enzyme II of the PTS, and X represents the unidentified exit system for glucose and its non-metabolizable analogs. Numbered stages: 1, *P*-β-galactosidase; 2, *D*-tagatose-6P pathway; 3, hexose-6P-phosphohydrolase; 4, phosphorylation of intracellular glucose analogs via the mannose-PTS; 5, efflux of glucose analogs; and 6, uptake or reentry of glucose analogs via the mannose-PTS. Dashed double lines indicate inhibition by *N*-acetylglucosamine and *N*-acetylmannosamine.

and (iv) 2DG-6P was not formed during uptake of 2'D-lactose by *S. lactis* 133 mannose-PTS^d. Similar results were obtained when 2'F-lactose was used as substrate for the lactose-PTS, but in this case 2FG (in contrast to 2DG) was also slowly phosphorylated by intracellular GK. It may be noted parenthetically that α-MG is not an efficient substrate for EII^{man} in *S. lactis* (43, 49), *E. coli*, or *Salmonella typhimurium* and we anticipated (i) that intracellular α-MG (generated from transport of α-methyllactose) would not undergo internal phosphorylation via the mannose-PTS and (ii) that free α-MG might remain within the cell because of a lack of a suitable exit system. Unfortunately, α-methyllactose was not translocated via the lactose-PTS (Fig. 4), and a test of these postulates was precluded.

The conclusions we have drawn from intact cell studies with *S. lactis* are in agreement with those reached by Beneski et al. using membrane vesicles of *Salmonella typhimurium* (3). We recognize that the whole-cell approach, although physiologically more relevant, is also more complex, and there exists the potential for ambiguity (and criticism) of mechanisms assigned to product formation. However, we have no evidence to indicate significant phosphotransfer (i.e., from galactose-6P to glucose and its analogs) via EII^{lac}, the defective EII^{man}, or the hexose-6P-phosphohydrolase. Furthermore, a study of the metabolism of [^{14}C-*glucose*]lactose by *S. lactis* 133 mannose-PTS^d GK^- (48) showed that this organism was unable to phosphorylate and metabolize [^{14}C]glucose generated intracellularly during growth on the disaccharide. It is unlikely that inactivation of all potential mechanisms for glucose phosphorylation could have occurred during selection of this mutant.

Data presented in this communication are consistent with intracellular phosphorylation of glucose analogs by the mannose-PTS system in *S. lactis* 133 (Fig. 11, step 4). This conclusion has physiological significance for group N streptococci used as starters in the dairy industry (21, 24) and also provides an explanation for the unexpected findings in a previous investigation (44). Earlier we reported a 1:1 ratio for PEP utilized/monosaccharide accumulated via the PTS in iodoacetate-treated starved cells of *S. lactis*. However, under the same conditions, the apparent ratio for lactose accumulation was approximately 2:1. Enzymatic analysis of intracellular products revealed galactose-6P, but in addition to free glucose, the cells contained unexpectedly high concentrations of glucose-6P. Because the glycolytic inhibitor (iodoacetate) prevented ATP generation and phosphorylation of glucose by GK, the mechanism for glucose-6P formation remained unclear. The PEP-dependent phosphorylation of intracellular glucose via the mannose-PTS could account for both the anomalous stoichiometry and glucose-6P formation. Finally, we note that rapid homolactic fermentation of lactose by starter organisms requires the cometabolism of galactose-6P and glucose (44). It has been generally assumed that phosphorylation of the glucose moiety of the disaccharide was mediated exclusively via ATP-dependent GK. From our studies, it is likely that the mannose-PTS operates in concert with GK to ensure the efficient phosphorylation of intracellular glucose before its metabolism by the glycolytic pathway.

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