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Physiological Studies on Regulation of Glycerol Utilization by the Phosphoenolpyruvate:Sugar Phosphotransferase System in *Enterococcus faecalis*

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In vitro studies with purified glycerol kinase from *Enterococcus faecalis* have established that this enzyme is activated by phosphorylation of a histidyl residue in the protein, catalyzed by the phosphoenolpyruvate-dependent phosphotransferase system (PTS), but the physiological significance of this observation is not known. In the present study, the regulation of glycerol uptake was examined in a wild-type strain of *E. faecalis* as well as in tight and leaky *ptsI* mutants, altered with respect to their levels of enzyme I of the PTS. Glycerol kinase was shown to be weakly repressible by lactose and strongly repressible by glucose in the wild-type strain. Greatly reduced levels of glycerol kinase activity were also observed in the *ptsI* mutants. Uptake of glycerol into intact wild-type and mutant cells paralleled the glycerol kinase activities in extracts. Glycerol uptake in the leaky *ptsI* mutant was hypersensitive to inhibition by low concentrations of 2-deoxyglucose or glucose even though the rates and extent of 2-deoxyglucose uptake were greatly reduced. These observations provide strong support for the involvement of reversible PTS-mediated phosphorylation of glycerol kinase in the regulation of glycerol uptake in response to the presence or absence of a sugar substrate of the PTS in the medium. Glucose and 2-deoxyglucose were shown to elicit rapid efflux of cytoplasmic [^{14}C]lactate derived from [^{14}C]glycerol. This phenomenon was distinct from the inhibition of glycerol uptake and was due to phosphorylation of the incoming sugar by cytoplasmic phosphoenolpyruvate. Lactate appeared to be generated by sequential dephosphorylation and reduction of cytoplasmic phosphoenolpyruvate present in high concentrations in resting cells. The relevance of these findings to regulatory phenomena in other bacteria is discussed.

Several mechanisms by which the uptake of carbohydrates is regulated in both gram-negative and gram-positive bacteria are known (27–29). One such mechanism, studied in detail in gram-negative enteric bacteria, involves the allosteric regulation of a rate-limiting permease or catabolic enzyme by the glucose-specific enzyme III (III^{Glc}) of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) (30). With regard to glycerol uptake, this non-PTS carbohydrate rapidly enters the cytoplasm via a nonspecific proteinaceous pore known as the glycerol facilitator (12) and is then phosphorylated at the expense of ATP in the rate-limiting glycerol kinase-catalyzed reaction (29). Glycerol kinase is subject to negative allosteric regulation by both the intracellular metabolite, fructose-1,6-diphosphate, and the central cytoplasmic regulatory protein of the PTS, III^{Glc} (3, 22, 24, 30).

Physiological studies with a temperature-sensitive mutant of *Bacillus subtilis* defective for the energy-coupling protein of the PTS, enzyme I, led to the conclusion that the PTS regulates glycerol uptake in this gram-positive bacterium by a superficially similar process (26). However, the regulatory and target proteins were not identified. Subsequent biochemical studies with homogeneous glycerol kinase from *Enterococcus* (formerly *Streptococcus*) *faecalis* (32) revealed that this enzyme can be phosphorylated on position N-3 of a histidyl residue in a reaction involving reversible phosphoryl transfer from HPr-P of the PTS (5, 6). PTS-mediated phosphorylation of glycerol kinase from *B. stearothermophilus* has also been demonstrated (27). In neither case have physiological or molecular genetic experiments been conducted to establish the physiological significance of this reaction (30).

In the present work, we provide physiological evidence for the regulation of glycerol uptake by a PTS-mediated phosphorylation mechanism in *E. faecalis*. Both tight and leaky *ptsI* mutants were isolated, characterized, and examined for their regulatory properties. The use of these strains allowed us to establish that the PTS regulates the uptake of glycerol in this gram-positive bacterium. Also, regulatory characteristics of glycerol kinase synthesis and of the efflux of metabolites derived from glycerol are described.

MATERIALS AND METHODS

Organisms and growth conditions. The following organisms were used: *E.* (*Streptococcus*) *faecalis* ATCC 19433, *Staphylococcus aureus* S710A (*ptsI*), and *S. aureus* S797A (*ptsH*) (13). *E. faecalis* 19433 (wild-type strain) and enzyme I-deficient (*ptsI*) mutants derived from it (see below) were grown for 14 h at 30°C on a rotary shaker (250 rpm) in TYEA medium containing the following (grams per liter): tryptone, 15; proteose peptone, 5; yeast extract, 2 (all from Difco Laboratories, Detroit, Mich.); NaCl, 5; L-arginine, 10. Specified sugars were sterilized separately and added to a final concentration of 50 mM. While the cell yield of the wild-type strain in TYEA plus glycerol was three times greater than that of the tight *ptsI* mutant strain, growth rates were not markedly different (wild type, 0.7 doubling per h; *ptsI* mutant, 0.5 doubling per h) because of the presence of an alternate energy source, L-arginine. Cells were harvested by centrifugation at 12,000 × *g* for 10 min, washed, and

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resuspended in appropriate buffer solutions for uptake measurements or for cell extract preparation.

Isolation of enzyme I-deficient mutants. The selection and screening procedures designed to yield *E. faecalis* mutants defective in the general components of the PTS (enzyme I or HPr) were based on the resistance of prospective mutants to streptozotocin (14, 17, 18) and the inability to ferment either of two PTS sugars, mannitol or lactose, when presented together in a suitable indicator medium. Thus, the procedure was carried out in two steps: selection for streptozotocin resistance, and screening for loss of fermentation capacity. In the initial selection procedure, 0.1 ml of an overnight culture of *E. faecalis* ATCC 19433 in a modified MRS broth (4) containing 50 mM D-glucosamine (instead of glucose) and 0.2 g of sodium azide per liter, adjusted to pH 6.5, was used to inoculate 10 ml of the same medium containing, in addition, 50 μg of streptozotocin per ml. (Note that sodium azide was added to all media used for mutant isolation to minimize risk of contamination.) Cultures containing streptozotocin showed a much reduced turbidity in comparison to control cultures not containing the antibiotic after incubation at 37°C for 24 to 40 h. Portions (0.1 ml) of the streptozotocin-containing cultures were spread on plates containing the following selection medium (per liter): peptone, 10 g; beef extract, 10 g; yeast extract, 5 g; Tween 80, 1 ml (all Difco); mannitol, 10 g; lactose, 10 g; K_2HPO_4, 2 g; sodium acetate, 5 g; tri-ammonium citrate, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4$, 0.2 g; sodium azide, 0.2 g; agar, 15 g; pH adjusted to 6.5. Streptozotocin (50 μg/ml) and 2,3,5-triphenyltetrazolium chloride (100 μg/ml) were added separately from stock solutions after sterilization and cooling of the medium to 50°C immediately before pouring plates. After incubation at 32°C for 40 h, wild-type colonies were large and deep maroon, which on examination with a dissecting microscope showed banded deep-red crystal-like deposits of insoluble reduced 2,3,5-tri-phenyltetrazolium chloride. Much smaller colonies with little or no color were picked with sterile toothpicks onto the screening indicator medium that was a modified MacConkey agar adapted from Morse and Alire (21). It contained the following (per liter), added in order: Tris, 1.3 g; bile salts (BBL, Cockeysville, Md.), 1.0 g (allowed to dissolve completely before adding other ingredients); beef extract, 4.5 g; tryptose, 15 g; NaCl, 7.5 g; sodium azide, 0.2 g; mannitol, 10 g; lactose, 10 g; L-arginine, 5.3 g; pH adjusted to 7.2; neutral red, 0.075 g; agar, 15 g. Wild-type colonies were large and red on this medium; presumptive mutants that could ferment neither mannitol nor lactose were small and yellow (alkaline reaction due to deamination of L-arginine). Presumptive mutant colonies were purified by streaking on the same medium and then were assayed for 2-deoxy-D-[^{14}C]glucose uptake, enzyme I, and HPr activities. Of 32 presumptive mutants showing the described colony phenotype on repeated subculture, 9 were identified as enzyme I-deficient mutants. Two of these, designated ML3 and ML14, that showed a leaky and tight enzyme I-deficient phenotype, respectively, were selected for this study (see Results). It is of interest to note that this procedure yielded only one leaky *ptsH* mutant that showed 20% of wild-type HPr activity and no tight HPr mutants. The low incidence of HPr mutants may reflect the smaller size of the gene encoding HPr.

It is worth noting that the neutral red indicator medium described above was superior to 2,3,5-triphenyltetrazolium chloride as an indicator of sugar fermentation in our experience. However, direct streptozotocin resistance selection in this medium was not possible because necessary components of the bile salts in the neutral red medium are not soluble at the slightly acid (6.2 to 6.5) pH required to maintain activity or stability of streptozotocin. The two-step plating procedure described above was advantageous for this reason.

Uptake measurements. Cells were suspended in 0.05 M Tris hydrochloride (pH 7.2) containing 10 mM $MgSO_4 \cdot 7H_2O$ (T buffer) at a cell density of 1 to 2 mg (dry weight)/ml (cell density in each experiment was determined from a standard curve relating optical density to cell dry weight. Radiolabeled transport substrates and other additions specified in Results were added to 0.6 to 1.0 ml of the cell suspensions preincubated at 37°C in a water bath. Samples of 0.1 ml were taken at appropriate intervals, filtered on 25-mm membrane filters (0.45-μm pore size; type HAWP; Millipore Corp., Bedford, Mass.) to stop uptake, and washed with 0.9% NaCl. Filters with cells thereon were dried under an infrared lamp and placed in a toluene-based scintillation fluid for counting.

Preparation of cell extracts. Cells suspended in 0.02 M Tris maleate buffer (pH 7.2) containing 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride were ruptured by freeze-thawing (three times) followed by passage five times through a French pressure cell at 10,000 lbs/in^2. Extracts were centrifuged at 12,000 × *g* for 10 min to remove unbroken cells and cell debris. Protein content was determined by the method of Lowry et al. (20).

Enzyme assays. Enzyme I and HPr activities in *E. faecalis* extracts were determined by complementation assays with extracts of *S. aureus* S710A (*ptsI*) and S797A (*ptsH*) (13). The standard assay mixture contained the following (final concentration in 0.1 ml): K^+ phosphate buffer (pH 7.4), 50 mM; $MgCl_2$, 12.5 mM; KF, 25 mM; dithiothreitol, 2.5 mM; phosphoenolpyruvate, 10 mM; β-D-[*methyl*-^{14}C]thiogalactopyranoside ([^{14}C]TMG), 1 mM (0.5 mCi/mmol); and cell extracts containing 0.5 to 1 mg of protein. Reaction mixtures were incubated in a water bath at 37°C for 30 min. Reactions were stopped by addition of 1 ml of ice-cold water. Phosphorylated sugar ([^{14}C]TMG-P) was determined essentially as described by Kundig and Roseman (16). The diluted reaction mixtures were applied to columns (0.8 by 4 cm; Bio-Rad Econocolumns) containing 1 ml of Bio-Rad AG1-X2 anion-exchange resin (analytical grade, 50/100 mesh, chloride form). Unbound [^{14}C]TMG was washed from the column with 15 ml of distilled water; [^{14}C]TMG-P was subsequently eluted with 3 ml of 1 M LiCl into scintillation vials. The vials were then filled with Aqua Mix (ICN Biochemicals, Inc., Costa Mesa, Calif.) for counting.

Glycerol kinase was assayed by a modification of the method described by Deutscher and Sauerwald (6). The standard assay mixture contained the following (final concentration in 0.1 ml): Tris hydrochloride (pH 7.2), 30 mM; $MgCl_2$, 5 mM; ATP, 2.5 mM; [^{14}C]glycerol, 0.2 mM (2.5 mCi/mmol); cell extract containing 0.015 to 0.25 mg of protein. Reaction mixtures were incubated in a water bath at 37°C. Samples of 30 μl taken at 2, 5, and 10 min were added to 1 ml of ice-cold water to stop the reaction. Diluted reaction mixtures were immediately applied to Bio-Rad AG1-X2 anion-exchange resin (chloride form) and processed as described above to determine [^{14}C]glycerol phosphate formed. Activity was essentially linear over the 10-min period of the assay.

Radiolabeled substrates and chemicals. [U-^{14}C]glycerol and 2-deoxy-D-[U-^{14}C]glucose ([^{14}C]2-DOG) were from ICN Biochemicals; [^{14}C]TMG was from DuPont New England Nuclear Products, Boston, Mass. Sugars and other chemi-

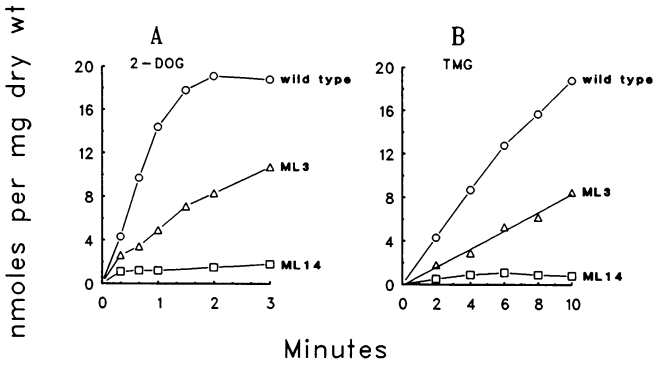

FIG. 1. Uptake of [^{14}C]2-DOG (A) and [^{14}C]TMG (B) by *E. faecalis* 19433 (wild type) and *ptsI* mutants derived from it. Cells grown on TYEA plus 50 mM glucose (A) or TYEA plus 50 mM lactose (B) were incubated with 0.2 mM [^{14}C]2-DOG (1 mCi/mmol) (A) or 0.5 mM [^{14}C]TMG (2 mCi/mmol) (B).

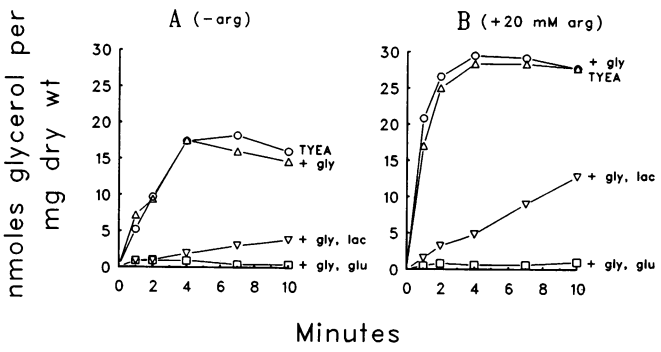

FIG. 2. Effect of carbohydrate present during growth on [^{14}C] glycerol uptake by *E. faecalis* 19433. Cells were grown in TYEA without further addition (○), with 50 mM glycerol (△), with 50 mM glycerol and 50 mM lactose (▽), or with 50 mM glycerol and 50 mM glucose (□). Uptake was carried out in T buffer containing 0.2 mM [^{14}C]glycerol (2.5 mCi/mmol) without further addition (A) or with 20 mM L-arginine (B).

cals, obtained from Sigma Chemical Co., St. Louis, Mo., were of analytical grade or the highest purity available.

RESULTS

Properties of enzyme I mutants. The capacities of two mutant strains, derived from *E. faecalis* 19433 as described in Materials and Methods, to take up [^{14}C]2-DOG and [^{14}C]TMG are compared with the wild-type parent strain in Fig. 1. The rates of [^{14}C]DOG and [^{14}C]TMG uptake in strain ML3 were approximately one-third those of the wild-type parent. Strain ML14 showed >90% reduction in the rates of uptake of these two sugars analogs. Results of complementation assays for enzyme I and HPr, using cell extracts of the wild-type or mutant strains of *E. faecalis* and *S. aureus* extracts lacking either enzyme I (*ptsI*) or HPr (*ptsH*), are shown in Table 1. The extract of strain ML3 was able to complement the *S. aureus ptsI* extract to an extent that was about one-sixth of that of the wild-type parent extract, but there was no significant complementation of *S. aureus ptsI* by the extract from strain ML14. Extracts of both strains ML3 and ML14 showed strong complementation of *S. aureus ptsH*, comparable to the wild-type parent, indicating the presence of normal levels of HPr. Thus, on the basis of these complementation assays and the capacity to transport two distinct PTS sugars, strain ML3 was characterized as a leaky enzyme I (*ptsI*) mutant, and strain ML14 was characterized as a much tighter *ptsI* mutant.

TABLE 1. Complementation assays for enzyme I and HPr showing phosphoenolpyruvate-dependent phosphorylation of TMG by cell extracts

Extract[a]	[^{14}C]TMG-P (nmol/30 min)
E. faecalis 19433	38
E. faecalis ML3	1
E. faecalis ML14	<1
S. aureus ptsI	<1
S. aureus ptsH	1
S. aureus ptsI + *S. aureus ptsH*	112
S. aureus ptsI + *E. faecalis* 19433	83
S. aureus ptsI + *E. faecalis* ML3	14
S. aureus ptsI + *E. faecalis* ML14	1
S. aureus ptsH + *E. faecalis* 19433	106
S. aureus ptsH + *E. faecalis* ML3	114
S. aureus ptsH + *E. faecalis* ML14	109

[a] Cell extracts of *E. faecalis* strains were prepared from cells grown on TYEA plus 50 mM lactose. Extract protein present in a 0.1-ml reaction mixture: *E. faecalis* 19433, 0.9 mg; *E. faecalis* ML3, 0.8 mg; *E. faecalis* ML14, 0.9 mg; *S. aureus ptsI*, 0.5 mg; *S. aureus ptsH*, 0.7 mg.

Glycerol uptake by *E. faecalis* ATCC 19433. Figure 2 shows the capacity of wild-type cells, grown in the presence or absence of added sugars, to take up [^{14}C]glycerol when incubated in the absence (Fig. 2A) or presence (Fig. 2B) of an exogenous energy source, L-arginine. The glycerol uptake system appeared to be constitutive, since cells grown on TYEA without glycerol showed the same capacity to take up [^{14}C]glycerol as cells grown on TYEA plus glycerol; however, the possibility of induction by glycerol that may be present in peptone cannot be excluded. The presence of sugars in the growth medium repressed the formation of the glycerol uptake system. Glucose completely repressed glycerol uptake; lactose also repressed the glycerol system, though less strongly than glucose. The provision of L-arginine in the uptake buffer stimulated the rate and extent of [^{14}C]glycerol uptake more than twofold in all cases, except in glucose-grown cells, in which the system was strongly repressed. All further glycerol uptake measurements were carried out in the presence of 20 mM L-arginine unless specified otherwise.

Glycerol uptake by enzyme I-deficient mutants. The capacities of *E. faecalis* strains to take up glycerol were proportional to their enzyme I activities (Fig. 3; cf. Fig. 1). Thus, the rate of glycerol uptake by strain ML3, the leaky *ptsI* mutant, was approximately one-fourth the rate of the wild-type parent, while strain ML14, the tighter *ptsI* mutant, showed a 90% decrease in initial rate of glycerol uptake in comparison to its parent. These results establish a relationship between the PTS and the glycerol uptake system of *E. faecalis*.

Effect of PTS sugars on glycerol uptake by wild-type cells. Addition of glucose to *E. faecalis* 19433 cells simultaneously with [^{14}C]glycerol brought about an initial increase in the rate of glycerol uptake, occasioned by the provision of energy, followed by strong inhibition. At concentrations below 0.5 mM, the inhibition was reversed as the result of glucose depletion (Fig. 4A). The inhibitory effect of sugars transported by the PTS was more effectively studied by the use of nonmetabolizable analogs that would not provide energy and would not be depleted in the course of the

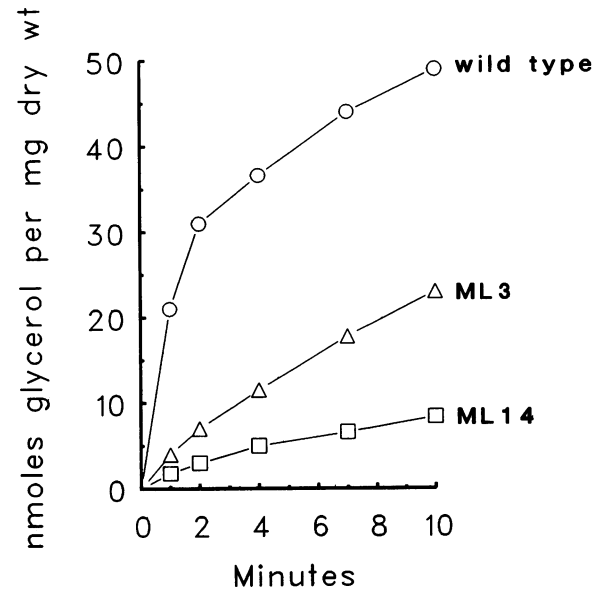

FIG. 3. Uptake of [^{14}C]glycerol by *E. faecalis* 19433 (wild type) and *ptsI* mutants derived from it. Cells grown in TYEA plus 50 mM glycerol were incubated with 0.2 mM [^{14}C]glycerol (2.5 mCi/mmol) in T buffer containing 20 mM L-arginine.

experiment. The effect of 2-DOG is shown in Fig. 4B. Inhibition of glycerol uptake by 2-DOG was not immediate when added simultaneously with glycerol; rates of glycerol uptake were the same for the first minute in control cells and in cells receiving 0.1 to 0.5 mM 2-DOG. At a lower 2-DOG concentration (0.05 mM), inhibition was not manifest for 4 min (not shown). When cells were preincubated with 2-DOG for 5 min before commencement of [^{14}C]glycerol uptake, there was a decrease in initial rate and extent of glycerol uptake that was proportional to 2-DOG concentration over a range of 0.02 to 0.5 mM (Fig. 5A).

TMG inhibited [^{14}C]glycerol uptake by *E. faecalis* 19433 cells that had been induced for β-galactoside uptake by addition of 25 mM lactose during the last 3 h of their growth in glycerol-containing TYEA medium (Fig. 5B). TMG was considerably less effective than 2-DOG, however. The relative inhibitory activities of 2-DOG and TMG probably reflect the relative affinities and efficiencies of their respective

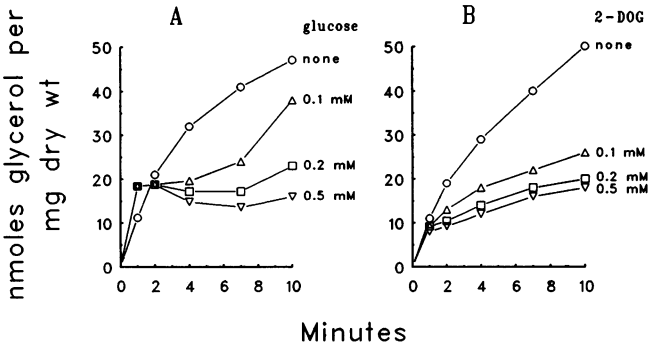

FIG. 4. Effect of glucose (A) or 2-DOG (B) on [^{14}C]glycerol uptake by *E. faecalis* 19433. Cells were grown in TYEA plus 50 mM glycerol, incubated in T buffer containing 20 mM L-arginine, and received 0.2 mM [^{14}C]glycerol (2.5 mCi/mmol) and the indicated concentrations of glucose (A) or 2-DOG (B) at zero time.

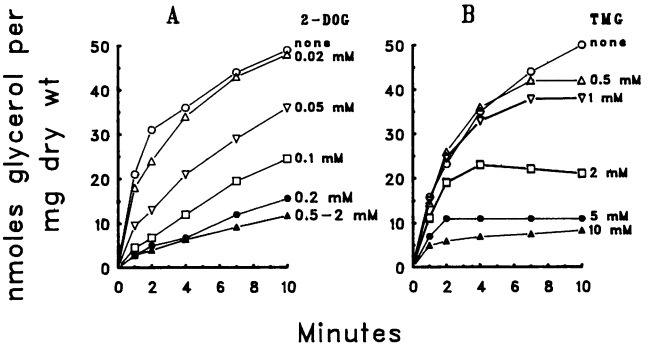

FIG. 5. Effect of 2-DOG (A) or TMG (B) on [^{14}C]glycerol uptake by *E. faecalis* 19433. Cells grown in TYEA plus 50 mM glycerol (A) or TYEA plus 50 mM glycerol plus 50 mM lactose (B) were preincubated in T buffer containing 20 mM arginine for 5 min with 2-DOG (A) or for 10 min with TMG (B) at the concentrations indicated before addition of 0.2 mM [^{14}C]glycerol (2.5 mCi/mmol).

transport systems. The measured apparent K_ms for 2-DOG and TMG uptake were 0.14 and 1.4 mM, respectively (data not shown).

Effect of PTS sugars on glycerol uptake by enzyme I-deficient mutants. Figures 6A and B show the effect of 2-DOG on [^{14}C]glycerol uptake by *E. faecalis* ML3 (leaky *ptsI*) and ML14 (tight *ptsI*), respectively. (Note the difference in scale between Fig. 5 and 6.) Though 2-DOG uptake was lower in these two mutant strains compared with the wild-type strain (Fig. 1), a stronger inhibitory effect was exerted by lower 2-DOG concentrations in the mutants than in the wild-type strain.

A summary of the effects of 2-DOG and TMG on glycerol uptake by the wild-type strain and the two enzyme I-deficient strains over a 2,000-fold concentration range is shown in Fig. 7. Unlike 2-DOG, which was more inhibitory at low concentrations in the leaky *ptsI* mutant strain ML3 than in the wild-type strain, TMG was less inhibitory in ML3 and inactive as an inhibitor in the tight *ptsI* mutant strain ML14. This is probably due to the restricted capacity of the mutants to take up β-galactosides (Fig. 1) that consequently limited the induction of galactoside-specific components of the PTS.

Effect of glucose and 2-DOG on cellular pools of metabolites

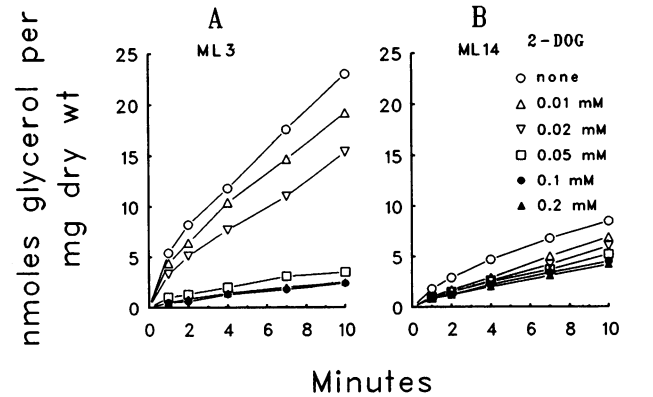

FIG. 6. Effect of 2-DOG on [^{14}C]glycerol uptake by enzyme I-deficient strains *E. faecalis* ML3 (A) and *E. faecalis* ML14 (B). Cells grown in TYEA plus 50 mM glycerol were preincubated for 5 min in T buffer containing 20 mM arginine and 2-DOG at the concentrations indicated before addition of 0.2 mM [^{14}C]glycerol (2.5 mCi/mmol).

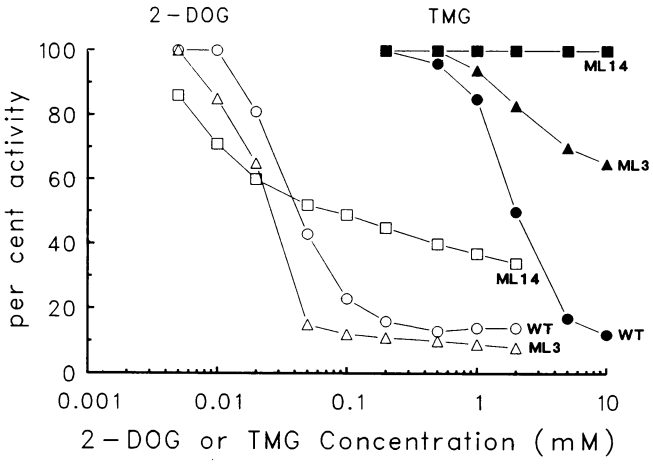

FIG. 7. Effect of 2-DOG and TMG concentration on glycerol uptake activity by *E. faecalis* 19433 (wild type [WT]), *E. faecalis* ML3, and *E. faecalis* ML14. Open symbols, 2-DOG; closed symbols, TMG. Note: Initial uptake rates in the absence of sugar analogs were much lower in the *ptsI* mutants than in the wild-type strain. Control initial uptake rates (100% activity) were as follows: *E. faecalis* 19433, 20.5; *E. faecalis* ML3, 5.2; and *E. faecalis* ML14, 1.8 nmol/mg (dry weight)/min.

derived from glycerol. The addition of glucose or 2-DOG to an *E. faecalis* 19433 cell suspension that had been allowed to take up [^{14}C]glycerol to a maximal level provoked immediate and rapid efflux of cellular radioactivity (Fig. 8). This effect was particularly striking in cells that had taken up [^{14}C]glycerol in the absence of added L-arginine. Two questions posed as the result of this observation were (i) Was the inhibition of accumulation of radioactivity from [^{14}C]glycerol by PTS sugars, described in previous sections, due to inhibition of glycerol uptake, or was it due to accelerated efflux of glycerol or metabolites derived from it? (ii) What was the identity of the radioactive substance(s) expelled

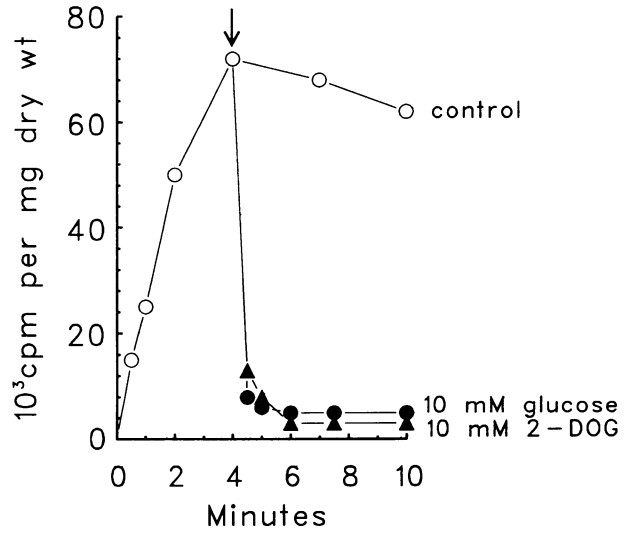

FIG. 8. Glucose or 2-DOG-induced efflux of cellular radioactivity derived from [^{14}C]glycerol. Cells grown in TYEA plus 50 mM glycerol were incubated in T buffer containing 0.2 mM [^{14}C]glycerol (2.5 mCi/mmol). Arrow denotes time of addition (4 min) of 10 mM glucose or 10 mM 2-DOG.

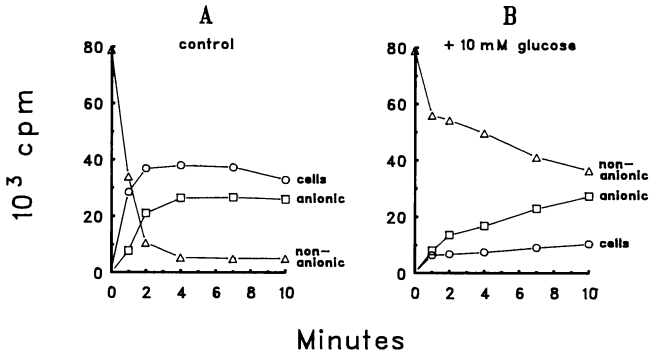

FIG. 9. Effect of glucose on [^{14}C]glycerol utilization and excretion of anionic products. *E. faecalis* 19433 cells (density, 4 mg [dry weight]/ml) grown in TYEA plus 50 mM glycerol were incubated in T buffer plus 20 mM L-arginine and 0.2 mM [^{14}C]glycerol (2.5 mCi/mmol) in the absence (A) or presence (B) of 10 mM glucose. Radioactivity was measured in cells, in the nonanionic fraction of the suspending buffer, and in the anionic fraction of the suspending buffer as described in the text.

from the cells upon glucose or 2-DOG addition? The first question was investigated by collecting samples of both cells and suspending buffer (filtrates) during uptake of [^{14}C]glycerol in the presence or absence of glucose or 2-DOG. Filtrates of samples taken at each time point were chromatographed on Bio-Rad AG1-X2 anion-exchange resin to separate nonanionic fractions (taken as a measure of residual [^{14}C]glycerol in the suspending buffer) and anionic fractions (taken as a measure of acidic metabolites derived from [^{14}C]glycerol). The results confirmed that the presence of glucose (Fig. 9B) strongly inhibited both the disappearance of nonanionic radioactivity (glycerol) from the suspending buffer and the accumulation of radioactivity in cells, in comparison to control cells (Fig. 9A). Glucose also decreased the rate of excretion of anionic metabolites, but the decrease in this parameter was not as great as glycerol disappearance from the medium and its accumulation in cells. These results confirm that glucose in fact inhibited the uptake and utilization of glycerol. Similar results were obtained with 2-DOG (results not shown).

The identity of the material expelled from the cells upon glucose or 2-DOG addition was determined as follows. Cells allowed to take up [^{14}C]glycerol for 6 min while suspended in T buffer in the absence of an added energy source were recovered by centrifugation at 16,000 × *g* for 2 min, washed quickly with cold water, and resuspended in aqueous 10 mM 2-DOG for 5 min at 37°C to allow expulsion of radioactivity. After centrifugation to remove cells, a portion of the supernatant was chromatographed on Bio-Rad AG1-X2 resin to determine whether the expelled material was anionic or nonanionic; the remaining supernatant was concentrated to dryness on a rotary vacuum evaporator, redissolved in a small volume of water, and subjected to thin-layer chromatography and autoradiography. Essentially all radioactivity applied to the anionic exchange resin was adsorbed to the column, demonstrating that the expelled material was not glycerol but an anionic product derived from it. Thin-layer chromatography with two different solvent systems (solvent A, *n*-butanol–pyridine–water [10:3:3, vol/vol/vol]; solvent B, 95% ethanol-ammonia [77:23, vol/vol]) showed a major radioactive spot (solvent A, R_f 0.40; solvent B, R_f 0.72) that corresponded to lactic acid and a minor spot (solvent A, R_f 0.59; solvent B, R_f 0.62) that corresponded to glycerol,

TABLE 2. Effect on glycerol kinase activity of carbohydrate present during growth

E. faecalis strain	Carbohydrate present[a]	Glycerol kinase (nmol/min/mg of protein)
19433	Glycerol	113
19433	Glucose	0.4
19433	Lactose	43
ML14	Glycerol	5.5

[a] Added to TYEA medium at 50 mM.

probably representing material that was not washed from the cells (not shown). Our interpretation of these results is that glycerol was metabolized to phosphoenolpyruvate which accumulated in the cells under the energy-limiting conditions that prevailed (34). Operation of the PTS triggered by the addition of glucose or 2-DOG resulted in the rapid conversion of phosphoenolpyruvate to pyruvate, which was reduced to lactate and excreted.

Glycerol kinase activity. Since the uptake of glycerol has been proposed to be regulated by the PTS at the level of glycerol phosphorylation in *E. faecalis* (5, 6) and in other organisms studied (3, 22, 24, 30), the activity of glycerol kinase in cell extracts of wild-type and enzyme I-deficient strains was determined. Results shown in Table 2 indicate the following. Glycerol kinase activity correlated closely with the [^{14}C]glycerol uptake activities reported in previous sections. Thus, *E. faecalis* ML14 showed drastically reduced glycerol kinase activity in comparison with the wild-type strain. Also, glucose strongly repressed glycerol kinase in the wild-type strain, while lactose caused a weaker but significant repression.

DISCUSSION

Previous studies have described mutants of *Streptococcus* or *Lactobacillus* species, selected most frequently by resistance to toxic sugar analogs such as 2-DOG, that were defective in the PTS-mediated transport of specific sugars (1, 7, 9, 19, 33, 35, 36). Nevertheless, the present work describes for the first time the isolation of pleiotropic sugar uptake mutants of a lactic acid bacterium specifically defective in one of the general energy-coupling components of the PTS. In this paper, we characterize and examine the physiological properties of tight and leaky enzyme I (*ptsI*) mutant strains of *E. faecalis* and use them to study the regulation of the uptake of a non-PTS substrate, glycerol. These mutants exhibited defective 2-DOG and TMG uptake activities in vivo (Fig. 1) and defective enzyme I activities in vitro (Table 1).

E. faecalis ML14, the tight *ptsI* mutant, did not accumulate 2-DOG significantly above the external concentration in the presence of an added energy source, L-arginine (results not shown). Thus, an active transport system for glucose in this organism is not indicated. A proton motive force-driven glucose transporter in *Streptococcus mutans* has been proposed (9), but a more recent study has failed to provide support for this proposal (2).

Glycerol uptake, assayed in vivo (Fig. 2), and glycerol kinase, assayed in vitro (Table 2), were shown to be weakly repressible by inclusion of lactose in the growth medium of the wild-type parental strain and strongly repressible by inclusion of glucose in the growth medium. Surprisingly, the rates of glycerol uptake correlated with enzyme I activities and uptake rates for PTS sugars (Fig. 1 and 3). The low glycerol uptake activity of the mutants was found to be attributable to the reduced glycerol kinase activity in the mutant bacteria (Table 2). Similar results have been obtained previously when tight (but not leaky) *pts* mutants of enteric bacteria were studied (29). The repressibility of glycerol kinase by glucose in *E. faecalis* 19433 (Table 2) differs from results reported in a previous communication with a different strain of *E. faecalis* (6).

Most relevant to a consideration of PTS-mediated regulation was the observation that glucose and 2-DOG exhibited similar degrees of inhibition when uptake of glycerol was examined in wild-type cells (Fig. 4). Significantly, 2-DOG was more strongly inhibitory in the leaky *ptsI* mutant (Fig. 6A) than in the wild-type parent (Fig. 5A; see Fig. 7 for quantitative comparisons) even though the inhibitory sugar was accumulated in the cytoplasm to a greatly reduced extent (Fig. 1). These results are in accord with biochemical studies implicating HPr(His-P) as a reversible phosphoryl donor that activates glycerol kinase (6). It would be expected that provision of high-affinity PTS substrates such as glucose or 2-DOG would drain phosphate groups from HPr(His-P), resulting in dephosphorylation and a consequent decrease in glycerol kinase activity. The impaired enzyme I activity in the leaky *ptsI* mutant would result in decreased regeneration of HPr(His-P) and thus an increased sensitivity to high-affinity PTS substrates. However, the results are also in accord with expectation assuming that a PTS catalytic regulatory process is operative which involves phosphorylation of a III^{Glc}-like protein. The sensitivity of residual glycerol uptake in the *ptsI* mutants to inhibition by 2-DOG is in accord with observations with *ptsI* mutants of *Escherichia coli* and *S. typhimurium* (31). Thus, almost all reported observations with 2-DOG are analogous to those reported previously with enteric bacteria (30, 31) and *B. subtilis* (26). It appears, therefore, that the PTS regulates glycerol uptake in *E. faecalis* by processes that are mechanistically related to those of these other two groups of organisms. III^{Glc} has not been found as a distinct molecular species in gram-positive bacteria. However, the demonstration of III^{Glc}-like domains as covalent parts of enzymes or permeases in *B. subtilis* (8; S. L. Sutrina, P. Reddy, M. H. Saier, Jr., and J. Reizer, J. Biol. Chem., in press), *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* (23) may indicate a widespread occurrence of such domains as regulatory elements in gram-positive bacteria.

While the sensitivity of glycerol uptake in *E. faecalis* to inhibition by PTS sugars (and the hypersensitivity in the leaky *ptsI* mutant) may reflect effects of unphosphorylated components of the PTS (HPr and/or a III^{Glc}-like protein) on the activity of glycerol kinase as described in the foregoing paragraph, the mechanisms determining the reduced levels of glycerol kinase in the *ptsI* mutant and the apparent repressive effect of PTS sugars (Table 2) are less clear. A possible explanation is that reduced activity of glycerol kinase occasioned by unphosphorylated PTS proteins that are the result of either enzyme I deficiency in the *ptsI* mutant or PTS sugar addition in the wild-type strain may limit the intracellular concentration of glycerol phosphate, which, in turn, serves as the inducer of glycerol kinase synthesis, as it does in the *Enterobacteriaceae* (11).

Glucose and 2-DOG were found to elicit rapid efflux of [^{14}C]glycerol-derived radioactive metabolites from intact *E. faecalis* cells (Fig. 8). PTS-mediated inducer expulsion has been demonstrated in several species of lactic acid bacteria (1, 25, 28, 35), including *E. faecalis* (unpublished results). The expelled species in the latter process was shown to be

the preaccumulated sugar. By contrast, the species which exited the bacterial cell upon addition of a PTS sugar in the present study was shown to be lactate. A similar observation was reported for *Streptococcus mutans* by Dills and Seno in a study of hexitol uptake regulation (7). These investigators did not provide an explanation for their observations. More recent studies have shown that resting streptococci generate and maintain high cytoplasmic phosphoenolpyruvate equivalents (up to 50 mM), but that addition of a metabolizable or nonmetabolizable PTS sugar results in the consumption of this phosphoenolpyruvate pool with the initial generation of pyruvate (for a review, see reference 34). Reducing equivalents generated during glycolysis apparently then reduce the pyruvate to lactate, and this compound exits the cell via a previously characterized lactate permease (10, 15). The stimulation of metabolite efflux therefore appears to be an indirect consequence of PTS function rather than a direct catalytic consequence of the functioning of this system.

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