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Involvement of Lactose Enzyme II of the Phosphotransferase System in Rapid Expulsion of Free Galactosides from *Streptococcus pyogenes*

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Streptococcus pyogenes accumulated thiomethyl-β-galactoside as the 6-phosphate ester due to the action of the phosphoenolpyruvate:lactose phosphotransferase system. Subsequent addition of glucose resulted in rapid efflux of the free galactoside after intracellular dephosphorylation (inducer expulsion). Efflux was shown to occur in the apparent absence of the galactose permease, but was inhibited by substrate analogs of the lactose enzyme II and could not be demonstrated in a mutant of *S. lactis* ML_3 which lacked this enzyme. The results suggest that the enzymes II of the phosphotransferase system can catalyze the rapid efflux of free sugar under appropriate physiological conditions.

Accumulation and concomitant phosphorylation of lactose or thiomethyl-β-galactoside (TMG) in *Streptococcus pyogenes* or *Steptococcus lactis* ML_3 occur via a phosphoenolpyruvate (PEP)-dependent lactose phosphotransferase system (PTS) (23, 39). Similar lactose PTSs have been characterized in other species of streptococci (3, 7, 9, 10, 18) and in *Staphylococcus aureus* (32). Addition of a metabolizable hexose such as glucose, mannose, or glucosamine to cultures of *S. pyogenes* or *S. lactis* ML_3 preloaded with TMG-phosphate (TMG-P) results in rapid disappearance of the galactoside-P from the cytoplasm and the appearance of free TMG in the medium (22, 23, 38). This process, termed inducer expulsion (23), occurs in *S. pyogenes* by a two-step mechanism; the phosphate ester is first hydrolyzed intracellularly to the free sugar in a process apparently activated by an ATP-dependent mechanism, and subsequently the free sugar is expelled from the cell (22). The enzymatic machinery responsible for these two steps has not been characterized.

In this report we survey the galactoside transport systems present in wild-type and mutant strains of *S. pyogenes* and *S. lactis* ML_3 to ascertain which permease is responsible for galactoside efflux in the presence of glucose. It is shown that whereas *S. lactis* strains possess an ATP-dependent galactose permease (12, 13, 36) which is strongly inhibitable by thiodigalactoside (TDG), this permease is apparently lacking in *S. pyogenes*, and the glucose-elicited efflux of TMG from the latter species is not subject to TDG inhibition. We therefore suggest that glucose does not promote TMG efflux via the galactose permease.

In further studies, an *S. lactis* ML_3 mutant which lacks the plasmid-associated lactose enzyme II of the PTS (2, 17) was shown to be incapable of effecting accumulation of TMG-P or a glucose-elicited efflux of TMG. Moreover, specific inhibitors of the lactose enzyme II were shown to inhibit both TMG uptake and expulsion in *S. pyogenes*. The evidence therefore leads to the suggestion that the lactose enzyme II catalyzes the rapid expulsion of free TMG from the cells.

MATERIALS AND METHODS

Organisms and growth conditions. *S. lactis* ML_3 *lac gal*$^+$ (kindly obtained from J. Thompson) and *S. pyogenes* type 12 were grown at 30 and 37°C, respectively, as described in previous communications (23, 38).

Transport studies. Midlogarithmic cultures, washed and suspended in 0.05 M Tris-maleate buffer (pH 7.2) containing 5 mM $MgCl_2$, were used for transport studies. Transport of sugars by *S. pyogenes* or *S. lactis* was measured at 37 and 30°C, respectively. Preloading of cells with [^{14}C]TMG or [^{14}C]TMG-P and expulsion of intracellular TMG-P were performed as described before (22, 38). Starved cultures were used to measure uptake of [^{14}C]galactose or β-galactosides via the corresponding PEP-dependent PTS of *S. pyogenes* or *S. lactis*. Uptake of ^{14}C-labeled sugars via the putative ATP-dependent permease of *S. pyogenes* was studied with PEP-depleted cells (36) or with cultures treated (5 min at 37°C) with iodoacetate (IAA) (10 mM) or fluoride (NaF; 15 mM) before measuring uptake in the presence of 5 mM arginine, a nonglycolytic source of ATP. Uptake of [^{14}C]galactose or galactosides via the ATP-dependent permease of *S. lactis* ML_3 *lac gal*$^+$

was measured in the presence of glucose (20 mM). The following concentrations of radioactive sugars were used: [^{14}C]lactose, TMG, or isopropylthio-β-galactoside (IPTG; specific activity, 1 μCi/μmol), 0.5 mM; [^{3}H]TDG (specific activity, 3.65 μCi/μmol), 1 mM. A cytoplasmic volume of 1.67 or 2.04 ml/g (dry weight) was used to calculate the intracellular concentration of accumulated sugar in *S. lactis* (35) or *S. pyogenes* (24), respectively.

Determination of intracellular free [^{14}C]TMG and [^{14}C]TMG plus [^{14}C]TMG-P. Intracellular free [^{14}C]TMG and the total concentration of intracellular [^{14}C]galactoside (phosphorylated ester plus the free form) were determined as follows: duplicate samples were withdrawn at intervals from the transport reaction, and cells were collected on membrane filters. One filter was used for determination of total intracellular label, and the other filter was immediately suspended in 3 ml of boiling water. Extraction proceeded for 10 min. Cell debris was removed by centrifugation (12,000 × *g*, 10 min), and free [^{14}C]TMG was determined by ion-exchange chromatography on AG1-X2 resin (50 to 100 mesh; Bio-Rad Laboratories, Richmond, Calif.) as previously described (15).

Chemicals. D-[*U*-^{14}C]galactose (specific activity, 318 mCi/mmol) and D-glucose-[1-^{14}C]lactose (specific activity, 59 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. Methyl β-D-[^{14}C]thiogalactopyranoside (specific activity, 54.7 mCi/mmol) was purchased from New England Nuclear, Boston, Mass. Isopropyl β-D-[^{14}C]thiogalactoside (specific activity, 20 mCi/mmol) was from Research Products International Corp., Mount Prospect, Ill. [^{3}H]β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (specific activity, 3.65 mCi/mmol) was kindly obtained from H. R. Kaback. The 6-*O*-tosylgalactosides were a gift from J. B. Hays. All other materials were obtained from commercial sources and were of the highest purity available.

RESULTS

Lack of accelerative TMG exchange transport. The presence of unlabeled external TMG during expulsion might stimulate efflux of [^{14}C]TMG by blocking recapture of labeled molecules or by eliciting an exchange reaction if a mobile carrier recycles across the membrane during efflux (34). However, the rates of expulsion of labeled TMG were not significantly different when preloaded cells were exposed to glucose alone or simultaneously to glucose and unlabeled galactoside at an external concentration similar to that present intracellularly (21 nmol/mg, dry weight). This lack of homoexchange between the internally formed labeled TMG and the external unlabeled analog suggests that expulsion is not mediated by a mobile carrier which exhibits the phenomenon of accelerative exchange transport (34). A slower rate of expulsion was observed at a high concentration of external galactoside (100 mM). This was probably due to uptake of unlabeled TMG and competition for efflux (22). Cells preloaded with [^{14}C]TMG-P and exposed to a high concentration of unlabeled TMG (0.1 M) but not to glucose did not expel the labeled sugar, confirming the lack of significant turnover of intracellular TMG-P (23). This experiment distinguishes TMG expulsion activity from that of 2-deoxyglucose since *S. lactis* cells preloaded with [^{14}C]2-deoxyglucose-6-P and exposed to unlabeled free 2-deoxyglucose rapidly expelled the intracellular ^{14}C-labeled sugar (37).

Apparent lack of an ATP-dependent permease for galactosides in *S. pyogenes*. In contrast to the results obtained with *S. lactis* 7962 (12, 13), *S. lactis* ML_3 (36), or a *lac gal*$^+$ mutant of *S. lactis* ML_3 (see below), we were unable to observe the permease-mediated accumulation of β-galactosides in *S. pyogenes*. Thus, arginine did not promote significant accumulation of TMG in the presence of NaF or in PEP-depleted *S. pyogenes* cells which had been treated with IAA (Fig. 1). In addition, a similar extent of TMG accumulation was observed in the presence or absence of arginine with cultures poisoned with IAA (see Fig. 1). Similar results were obtained with *S. pyogenes* when IPTG was used as a substrate (data not shown).

The results in Table 1 show the uptake activity of galactose, lactose, IPTG, and TMG via the PEP-dependent PTS in *S. pyogenes* or via the ATP-dependent permease in a *lac gal*$^+$ mutant of *S. lactis* ML_3; the inhibitory effect of TDG is also shown. The results clearly demonstrate that

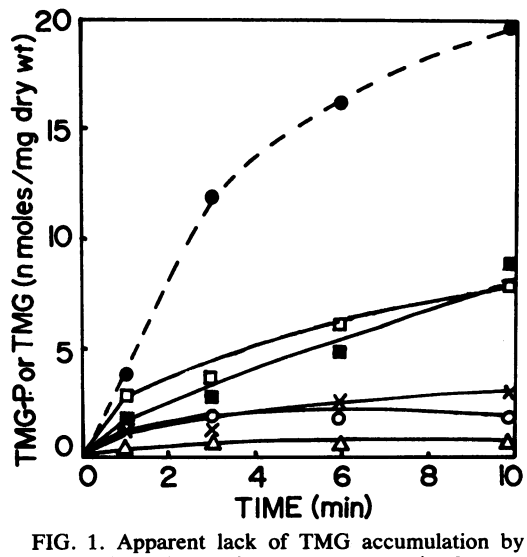

FIG. 1. Apparent lack of TMG accumulation by the ATP-dependent galactose permease in *S. pyogenes*. The uptake of [^{14}C]TMG (5 × 10^{-4} M) was measured as described in the text in the absence (●; control) or presence of (○) NaF, (×) NaF plus arginine, (□) IAA, (■) IAA plus arginine, or with (△) PEP-depleted cultures treated with IAA and arginine. Total uptake measured included the sum of the free TMG and TMG-P pools.

TABLE 1. Uptake of galactose or β-galactosides by *S. pyogenes* and an *S. lactis lac* mutant

Sugar	Route of uptake[a]	TDG (10 mM)	Uptake[b]	
			S. lactis ML_3 *lac*	*S. pyogenes*
Galactose	PTS	−	22.8	6.7
		+	18.9 (17%)	6.1 (9%)
Galactose	Permease	−	30.3	
		+	8.4 (73%)	
TMG	PTS	−	0.29	6.4
		+		5.2 (19%)
TMG	Permease	−	51.2[c]	0.4[d]
		+	6.3 (87%)	
Lactose	PTS	−	0.44	11.7
		+		13.8 (+17%)
IPTG	PTS	−	0.8	12.6
		+		12.1 (4%)
IPTG	Permease	−	35.8	2.4[d]
TDG	PTS	−	2.7	1.4[d]
TDG	Permease	−	29	0.8[d]

[a] The route of uptake is not meant to unequivocally define the transport system involved; it refers to the method used for measuring uptake (see text) and to the mechanisms which apparently are responsible for the indicated sugar uptakes.

[b] Expressed in nanomoles of sugar accumulated per milligram (dry weight) of cells per minute. Numbers in parentheses are percent inhibition.

[c] It was confirmed that only the free form rather than TMG-P was present intracellularly.

[d] These values do not represent initial rates since similar values were obtained at the steady state.

an ATP-dependent permease rather than a PEP-dependent PTS mediates the transport of TDG in the *S. lactis* mutant. This conclusion was substantiated by the fact that TDG strongly inhibited the ATP-dependent uptake of galactose and galactosides but not the PTS-mediated uptake of these sugars. In contrast, none of the sugars examined was accumulated to a significant extent by *S. pyogenes* treated with NaF (or IAA) regardless of the presence of arginine (see also Fig. 1). In addition, TDG was not accumulated by *S. pyogenes*, and it only slightly affected the uptake of galactose, TMG, lactose, and IPTG.

We previously demonstrated that *S. pyogenes* cells reaccumulate external TMG after glucose-promoted expulsion, provided that the cells were not poisoned with NaF (22). Similar reaccumulation was observed with *S. lactis* ML_3, and it was presumably mediated by the lactose-PTS since it was completely resistant to inhibition by trichlorosalicylate (Fig. 2) or dicyclohexylcarbodiimide (not shown). Interestingly, expelled TMG was also reaccumulated by fluoride-treated *S. lactis* ML_3 when the expulsion was elicited by glucose and arginine. This reaccumulation was most likely mediated by an ATP-dependent permease as it was completely prevented by trichlorosalicylate or dicyclohexylcarbodiimide (Fig. 2) and was severely inhibited by various substrates (TDG, fucose) of the galactose permease (data not shown). These findings confirm the presence of an ATP-dependent galactose permease in *S. lactis* and show that this permease is lacking in *S. pyogenes*.

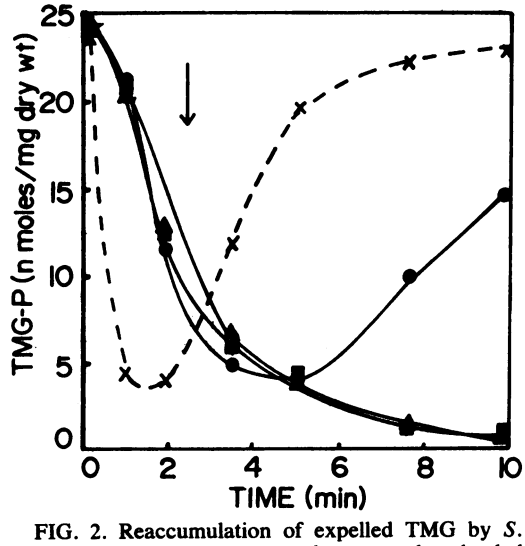

FIG. 2. Reaccumulation of expelled TMG by *S. lactis* ML_3. Cells, grown on galactose and preloaded with [^{14}C]TMG-P, were collected by centrifugation and suspended in 0.05 M Tris-maleate (pH 7.3) containing $MgCl_2$ (5 mM). After preincubation (5 min, 37°C) in the absence (×; broken line) or presence (●, ▲, ■; solid lines) of NaF (15 mM) and arginine (5 mM), glucose (1.5 mM) was added to elicit expulsion. At 2.5 min (arrows), the following compounds were added: (●) none; (X, ■) trichlorosalicylate (0.01 mM); (▲) dicyclohexylcarbodiimide (0.5 mM).

Lack of TMG expulsion in a *lac gal*$^+$ strain of *S. lactis*. No significant inhibition of TMG efflux was observed when *S. pyogenes* cells preloaded with the labeled TMG were preincubated in the presence of unlabeled TDG (0.1 M) before inducing expulsion (Fig. 3). In contrast, strong inhibition of [^{14}C]TMG efflux was observed when the expulsion was similarly elicited in cells pre-equilibrated with unlabeled IPTG (Fig. 3) or TMG (22). Since TDG is a good substrate of the galactose permease in *S. lactis* (13, 36; Table 1), this result further substantiates the conclusion that this permease does not mediate expulsion. The apparent lack of permease-mediated uptake of TMG in *S. pyogenes* and the inability to stimulate expulsion under conditions of exchange (see above) suggested that a carrier-mediated permease was not available to effect the expulsion of preaccumulated TMG-P in *S. pyogenes*. We further examined this possibility by using an *S. lactis* ML_3 mutant (*lac gal*$^+$). This mutant lacks the ability to grow on lactose, takes up TMG by an ATP-dependent permease, and does not hydrolyze *O*-nitrophenyl β-galactoside phosphate or phosphorylate TMG in the presence of PEP. Since dephosphorylation of intracellular TMG-P and the subsequent efflux of TMG from the parental cells involved independent processes (22), we reasoned that if TMG efflux were effected by a non-PTS permease, then glucose might elicit efflux of preaccumulated TMG from this mutant. Unexpectedly, the apparent loss of enzyme II^{lac} also resulted in the failure of glucose to elicit expulsion of preaccumulated TMG. Moreover, utilization and exhaustion of glucose from the medium or dissipation of the electrochemical gradient of protons with carbonylcyanide-*m*-chlorophenylhydrazone resulted in efflux of intracellular TMG from this *lac* mutant. Thus, in the parental strain, provision of energy by glucose metabolism elicited rapid efflux of intracellularly formed TMG, and proton conductors did not prevent this expulsion (22). In contrast, in the *lac* strains, provision of energy by glucose was essential for accumulation and maintenance of the intracellular pool of TMG, and addition of proton conductors effected a rapid loss of the preaccumulated galactoside (13; Reizer and Saier, data not shown). That the *lac* mutant and its parental strain both possessed the galactose permease, but only the latter strain contained enzyme II^{lac} and expelled TMG when exposed to glucose, suggested that efflux of TMG is mediated by enzyme II^{lac}. However, because the mutant apparently lacked the plasmid which encodes the lactose catabolic enzyme system, the possibility that a plasmid-encoded permease other than the enzyme II^{lac} was responsible for efflux had to be considered.

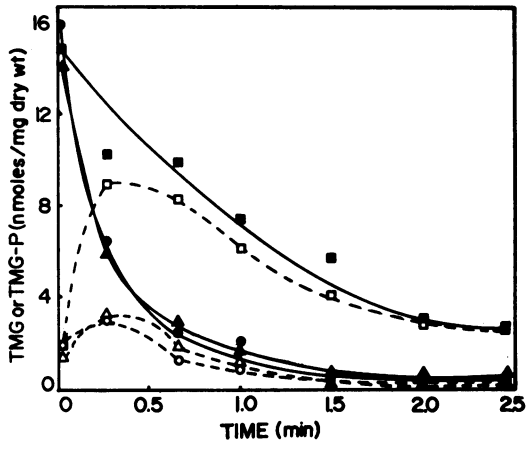

FIG. 3. Effect of unlabeled IPTG or TDG on efflux of [^{14}C]TMG from *S. pyogenes*. *S. pyogenes* cells preloaded with [^{14}C]TMG were suspended in buffered medium containing NaF (10 mM), arginine (5 mM), and IPTG (100 mM; squares) or TDG (100 mM; triangles). A control culture (circles) was not exposed to unlabeled galactoside. After preincubation (5 min) at 37°C, the expulsion was elicited by addition of glucose (10 mM). Duplicate samples were removed at intervals for determination of the total concentration of intracellular sugar ([^{14}C]TMG-P plus [^{14}C]TMG; solid symbols) and free [^{14}C]TMG (open symbols).

Effect of tosylgalactosides on expulsion. Previous studies (8, 29) have shown that 6-*O*-tosylgalactose and 6-*O*-tosylmethyl-β-galactoside, but not 6-*O*-tosylmethyl-α-galactoside, are potent and selective inhibitors of enzyme II^{lac} in *Staphylococcus aureus*. These analogs also severely inhibited the uptake of TMG in *S. pyogenes* via the PEP-dependent PTS (Fig. 4). In accordance with the inhibition of TMG accumulation in *Staphylococcus aureus* (8), the uptake of this sugar by *S. pyogenes* was more effectively inhibited by 6-*O*-tosylmethyl-β-galactoside than by 6-*O*-tosylgalactose. Also, no significant inhibition of the rate or extent of TMG uptake in *S. pyogenes* was observed with cells exposed to 6-*O*-tosylmethyl-α-galactoside. Similar specificity toward the tosyl derivatives was observed when the expulsion of preaccumulated TMG-P was measured in their presence (Fig. 5): 6-*O*-tosylmethyl-α-galactoside did not significantly affect expulsion, whereas an approximately threefold-lower rate of expulsion ($t_{1/2}$ = 1 min) was obtained in the presence of 6-*O*-tosylmethyl-β-galactoside or 6-*O*-tosylgalactose. It is noteworthy that uptake or efflux of galactose or TMG via the galactose permease in streptococci is similarly inhibited by α- and β-methyl galactosides (36), whereas both the uptake of TMG via enzyme II^{lac} and the expulsion were completely resistant to the α-anomer of the tosylmethylga-

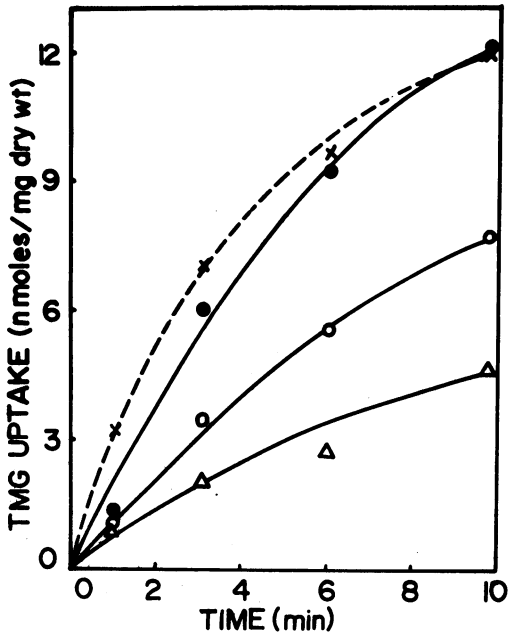

FIG. 4. Effect of tosylgalactosides on TMG uptake by *S. pyogenes*. The uptake of TMG was measured in the absence (×) or presence of 6-*O*-tosylmethyl-α-galactoside (●; 15 mM), 6-*O*-tosylgalactose (○; 15 mM), or 6-*O*-tosylmethyl-β-galactoside (△; 15 mM). Under the conditions used, >90% of the intracellular radioactive sugar was present as the phosphate ester.

lactosides (see Fig. 4 and 5). These observations suggest that the enzyme II^{lac} catalyzes the rapid efflux of TMG from *S. pyogenes*.

DISCUSSION

Bacteria regulate the rates at which catabolic enzyme synthesis occurs by several mechanisms. One such mechanism involves regulation of the cytoplasmic concentration of inducer. Inducers are usually taken up from the external medium via specific carbohydrate permeases, and these permeases are regulated by a diversity of mechanisms. Five such mechanisms have been identified in bacteria (4), and all permit bacteria to efficiently satisfy their energy needs. Thus, external sources of carbohydrates (27), intracellular metabolites (14), and chemiosmotic energy (11) all regulate the activities of carbohydrate permeases (30) which normally catalyze inducer uptake. All such regulatory processes which result in the inhibition of inducer uptake have been collectively termed "inducer exclusion" (16).

Recently a process which results in the rapid efflux of β-galactosides from the cell has been described in species of *Streptococcus* (22, 23, 38). Like inducer exclusion, this novel process, termed inducer expulsion (23), occurs in response to energy availability. Only when a metabolizable hexose such as glucose is presented to the streptococcal cell is the rapid expulsion of galactosides from the cytoplasm observed (23). Attempts to characterize the expulsion process have clearly shown that two steps are involved: first, galactoside phosphate is hydrolyzed with release of free galactoside in the cytoplasm; and second, the free sugar is transported across the membrane via a specific permease (22). An analogous process has been shown to occur in *Escherichia coli* (5, 6), but in neither organism have the enzymatic components responsible been characterized.

The studies reported here provide evidence that the lactose enzyme II is the permease which catalyzes rapid efflux of TMG from the cell in the presence of glucose. This conclusion was unexpected because the enzymes II normally catalyze group translocation in which the vectorial process is tightly coupled to sugar phosphorylation. Considerable evidence suggests that the enzymes II are essentially incapable of catalyzing facilitated uptake of sugars without concomitant phosphorylation (21, 28, 31). Uptake of free sugars by the enzymes II has been demonstrated only under certain abnormal physiological conditions (33) and in mutants with genetically altered enzymes II (20). Enzyme II^{mtl}-mediated efflux of free mannitol has been demonstrated under certain abnormal conditions where the sugar accumulates to very high con-

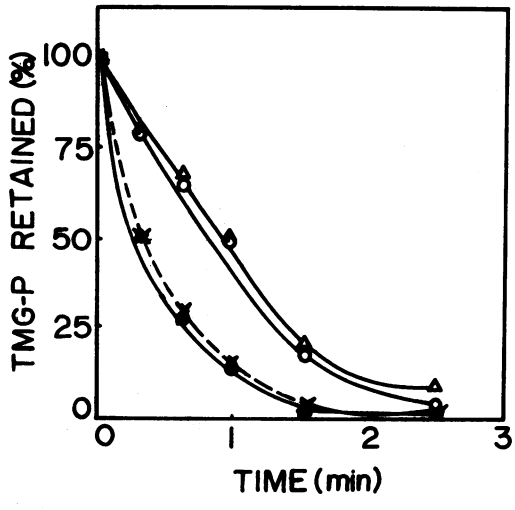

FIG. 5. Effect of tosylgalactosides on expulsion. Expulsion of [^{14}C]TMG from preloaded *S. pyogenes* cells was measured in the absence (×) or presence of 6-*O*-tosylmethyl-α-galactoside (10 mM; ●), 6-*O*-tosylgalactose (20 mM; ○) or 6-*O*-tosylmethyl-β-galactoside (10 mM; △).

centrations within the *E. coli* cell (33). In unpublished work, it has also been suggested that the glucose enzyme II in *E. coli* may be capable of catalyzing methyl α-glucoside efflux (33). The suggestion that the lactose enzyme II of streptococcal species catalyzes TMG efflux is therefore not unprecedented. It should be noted, however, that the rates of TMG efflux reported here far exceed those reported in other studies. It would therefore be reasonable to propose that two alternative states of the lactose enzyme II catalyze facilitated diffusion and group translocation and that the state of the enzyme is subject to regulatory control.

A variety of studies have shown that the enzymes II of the PTS are multifunctional proteins, catalyzing both unidirectional and bidirectional group translocation (25, 26), playing a role in chemoreception (1, 19), and regulating the rate of transcription of certain carbohydrate catabolic operons (29a). This report suggests that these complex enzymes also catalyze an additional process: transmembrane sugar transport, uncoupled from sugar phosphorylation, probably by facilitated diffusion (30) under normal physiological conditions. Further studies will be required to ascertain the mechanism by which the enzymes II of the PTS catalyze this transport process.

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