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Regulation of Glycerol Uptake by the Phosphoenolpyruvate-Sugar Phosphotransferase System in *Bacillus subtilis*

JONATHAN REIZER, MICHAEL J. NOVOTNY, INGRID STUIVER, AND MILTON H. SAIER, JR.*
The Department of Biology, The John Muir College, The University of California at San Diego, La Jolla, California 92093

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Enteric bacteria have been previously shown to regulate the uptake of certain carbohydrates (lactose, maltose, and glycerol) by an allosteric mechanism involving the catalytic activities of the phosphoenolpyruvate-sugar phosphotransferase system. In the present studies, a *ptsI* mutant of *Bacillus subtilis*, possessing a thermosensitive enzyme I of the phosphotransferase system, was used to gain evidence for a similar regulatory mechanism in a gram-positive bacterium. Thermoinactivation of enzyme I resulted in the loss of methyl α-glucoside uptake activity and enhanced sensitivity of glycerol uptake to inhibition by sugar substrates of the phosphotransferase system. The concentration of the inhibiting sugar which half maximally blocked glycerol uptake was directly related to residual enzyme I activity. Each sugar substrate of the phosphotransferase system inhibited glycerol uptake provided that the enzyme II specific for that sugar was induced to a sufficiently high level. The results support the conclusion that the phosphotransferase system regulates glycerol uptake in *B. subtilis* and perhaps in other gram-positive bacteria.

Carbohydrate uptake in bacteria is regulated by at least five distinct mechanisms (10, 44; M. H. Saier, Jr., *Mechanisms and Regulation of Carbohydrate Transport in Bacteria*, in press). These distinct mechanisms reflect (i) the intracellular metabolite levels, (ii) the chemiosmotic energy level of the cell, and (iii) the extracellular concentrations of various carbon sources. In general, these regulatory mechanisms allow bacteria to take up exogenous sources of carbon at rates which are in accord with their energy needs. In any one bacterial species, several control mechanisms may simultaneously operate, rendering the identification and characterization of any one process difficult.

Genetic techniques have been of particular value in dissecting the different regulatory processes from one another. Thus, glycerol uptake in enteric bacteria is regulated by sugar substrates of the phosphoenolpyruvate-sugar phosphotransferase system (PTS), both as a result of the accumulation of intracellular metabolites derived from the sugars and by an allosteric mechanism involving the proteins of the PTS. This second mechanism is termed PTS-mediated regulation. Cells which possess low activity of either of the two energy-coupling proteins of the PTS (enzyme I or HPr) exhibit *enhanced* sensitivity to PTS-mediated regulation, but decreased sensitivity to metabolite-sensitive regulation. It was the use of a leaky enzyme I mutant (*ptsI17* of *Salmonella typhimurium*) which originally led to the identification of PTS-mediated regulation (40, 41, 48), and the use of a temperature-sensitive enzyme I mutant (*ptsI313* of *Escherichia coli*) which established the catalytic role of this protein in regulation (4). Subsequent biochemical studies (12, 29, 47; Saier, in press) established the veracity of the mechanism originally proposed on the basis of genetic and physiological studies (41).

Little is known concerning the regulation of carbohydrate utilization in bacteria other than *E. coli* and *S. typhimurium*. PTS-mediated regulation has been demonstrated only in these two organisms although metabolite-sensitive regulation of glycerol uptake has been demonstrated in the gram-positive bacterium, *Staphylococcus aureus* (51). Streptococcal species have been shown to possess protein kinase-mediated regulatory mechanisms for the control of both sugar uptake and sugar efflux (9, 32, 33, 35, 56; J. Deutscher, U. Kessler, C. A. Alpert and W. Hengstenberg, unpublished data).

Of the gram-positive bacteria, *Bacillus subtilis* is an ideal organism for studies concerned with carbohydrate metabolism. Both the pathway of glycerol metabolism (22, 26, 38) and the components of the phosphotransferase system (8, 17, 19, 21, 24, 25, 31) have been extensively studied. That tight *ptsI* mutants of *B. subtilis* do not utilize glycerol has suggested that glycerol uptake (or utilization) might be subject to regulation by the PTS (21, 28). An involvement of the proteins of the phosphotransferase system in the repression of other catabolic enzyme systems has also been suggested (23). The availability of a temperature-sensitive *ptsI* mutant of *B. subtilis* (28) rendered feasible a rigorous search for PTS-mediated regulation in this organism. In this communication, we describe the results of studies which establish that the PTS regulates glycerol uptake in *B. subtilis* in a fashion similar to that observed in enteric bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* 60015, requiring L-tryptophan and L-methionine, served as the parental (control) strain. *B. subtilis* 61652 is a nearly isogenic strain which requires L-tryptophan and carries the *ptsI1* mutation (28). Both strains were provided by E. Freese, National Institutes of Health, Bethesda, Md. Cultures were grown with shaking at 30°C in C mineral medium (30) which was supplemented with 50 μg of the auxotrophic requirements per ml, 0.1% yeast extract (Difco Laboratories, Detroit, Mich.), and 1% glycerol. For induction of the glucose phosphotransferase, glucose (0.5%) was added to logarithmic-phase cultures, and growth was allowed to continue for 2 h. Growth of cells was followed by measuring the optical density at 600 nm with a Hitachi 100-40 spectrophotometer.

Transport studies. Cells from 50 to 100 ml of culture medium in the logarithmic phase of growth were harvested by centrifugation (12,000 × *g* for 5 min) at 4°C, washed twice

* Corresponding author.

with transport buffer (C mineral medium omitting ferric ammonium citrate, the carbon source, and the supplemented amino acids), and resuspended in the same buffer at a concentration of 1.0 absorbancy units at 600 nm. Cell suspensions containing 1% L-alanine were prewarmed to 30°C for 5 min before addition of [^{14}C]glycerol (0.5 mM; specific activity, 0.5 μCi/μmol) or [^{14}C]methyl α-glucoside (0.05 mM; specific activity, 2 μCi/μmol). The effect of heat inactivation of enzyme I on [^{14}C]glycerol or [^{14}C]methyl α-glucoside uptake was studied with cultures preincubated for various times (5 to 30 min) at 45°C and quickly chilled to 4°C. The transport reaction was terminated by withdrawing samples (0.5 ml) at intervals and collecting the cells by vacuum filtration on membrane filters (pore diameter, 0.45 μm; Millipore Corp., Bedford, Mass.) which were presoaked in a 5 mM concentration of the unlabeled test compound. The cells were washed with two 3-ml volumes of warm (30°C) transport buffer. Radioactivity was determined as described previously (34). An intracellular volume of 0.83 μl per absorbancy unit at 600 nm was used to determine the concentration of substrate accumulated (18).

Extraction and determination of intracellular [^{14}C]methyl α-glucoside and [^{14}C]methyl α-glucoside-6-phosphate. Intracellular [^{14}C]methyl α-glucoside, [^{14}C]methyl α-glucoside-6-phosphate and total ^{14}C-labeled glucoside were determined by a slight modification of our previously published procedures (32, 36). Duplicate samples were withdrawn from the transport reaction, and cells were collected and washed by filtration on membrane filters. One filter was used for determination of total intracellular label, [^{14}C]methyl α-

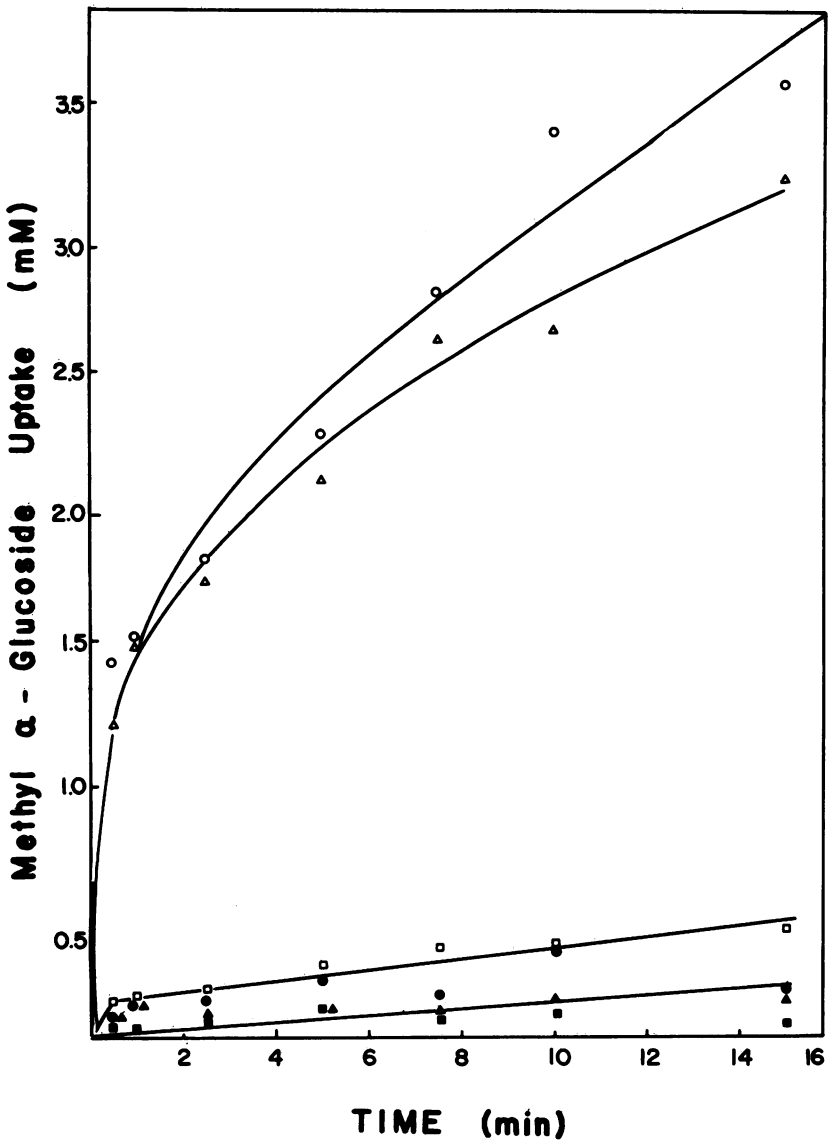

FIG. 1. Temperature sensitivity of methyl α-glucoside uptake in *Bacillus subtilis* strain *ptsH*. Uptake of [^{14}C]methyl α-glucoside as well as the extraction and determination of the intracellular sugar and sugar phosphate pools were performed as described in the text. The washed bacteria were resuspended in transport buffer by using the standard conditions and either incubated at 45°C for 30 min (heated; closed symbols) or left at 0°C (nonheated; open symbols). Uptake of 50 μM [^{14}C]methyl α-glucoside was conducted at 37°C. Total [^{14}C]methyl α-glucoside (free sugar plus sugar phosphate) (circles); methyl α-glucoside-6-phosphate (triangles) and nonesterified methyl α-glucoside (squares) are shown. A 30 min preincubation of the parental strain at 45°C decreased the rate and extent of methyl α-glucoside uptake by 15%.

glucoside plus [^{14}C]methyl α-glucoside-phosphate, whereas cells retained on the other filter were immediately submerged in 3 ml of boiling water and extracted at 100°C for 10 min. Cell debris was removed by centrifugation (12,000 × *g*, 10 min), and free as well as phosphorylated glucosides were determined in the clarified extract by ion-exchange chromatography (AG1-X2 resin, 50 to 100 mesh; Bio-Rad Laboratories, Richmond, Calif.) as previously described (20).

Chemicals. Radioactive compounds were obtained from Amersham Corp., Arlington Heights, Ill., or New England Nuclear Corp., Boston, Mass. Nonradioactive sugars and amino acids were purchased from Sigma Chemical Co., St. Louis, Mo., and from Nutritional Biochemical Corp., Cleveland, Ohio. All chemicals used were of analytical grade and from standard sources.

RESULTS

The effect of heat inactivation of the thermolabile enzyme I in *B. subtilis ptsI1* on the uptake of [^{14}C]methyl α-glucoside is shown in Fig. 1. Both the wild-type and *ptsI* mutant bacteria grown at 30°C accumulated methyl α-glucoside primarily as the phosphate ester. Heat inactivation of enzyme I virtually abolished uptake and phosphorylation of the glucoside (Fig. 1). A similar incubation of the parental strain at 45°C resulted in an insignificant loss of uptake activity.

The effect of a 10-min incubation at 45°C on the regulation of glycerol uptake by methyl α-glucoside is illustrated in Fig. 2. As shown in Fig. 2A, the parental and *ptsI* mutant strains took up [^{14}C]glycerol at comparable rates after growth at 30°C. Uptake was inhibited by methyl α-glucoside to a similar degree in the two strains. Incubation at 45°C for 10 min did not alter the response of the parental strain to 50 μM methyl α-glucoside, but it resulted in increased sensitivity of the temperature-sensitive mutant to inhibition by this glucoside (Fig. 2B). The inhibition of glycerol uptake elicited by addition of methyl α-glucoside was immediate and complete under the conditions used (Fig. 2B, inset).

Comparable studies were conducted employing glucose as the inhibiting sugar (Fig. 3). In contrast to methyl α-glucoside, glucose is readily metabolized under the conditions of the experiment. In the wild-type parental strain, 50 μM glucose inhibited glycerol uptake less than 20% regardless of whether the cells had been preincubated at 45°C (data not shown). This fact suggests that the inhibitory response of the parental strain to methyl α-glucoside observed in Fig. 2 may have been due either to energy depletion, resulting from energy expenditure during methyl α-glucoside accumulation, or to a direct inhibitory response to intracellular methyl α-glucoside-6-phosphate (Fig. 1).

Quite different results were observed when the temperature-sensitive *ptsI* mutant was studied. 50 μM glucose inhibited uptake of [^{14}C]glycerol about 40% before heat treatment, and a 10-min incubation at 45°C increased the degree of inhibition to about 90% (Fig. 3).

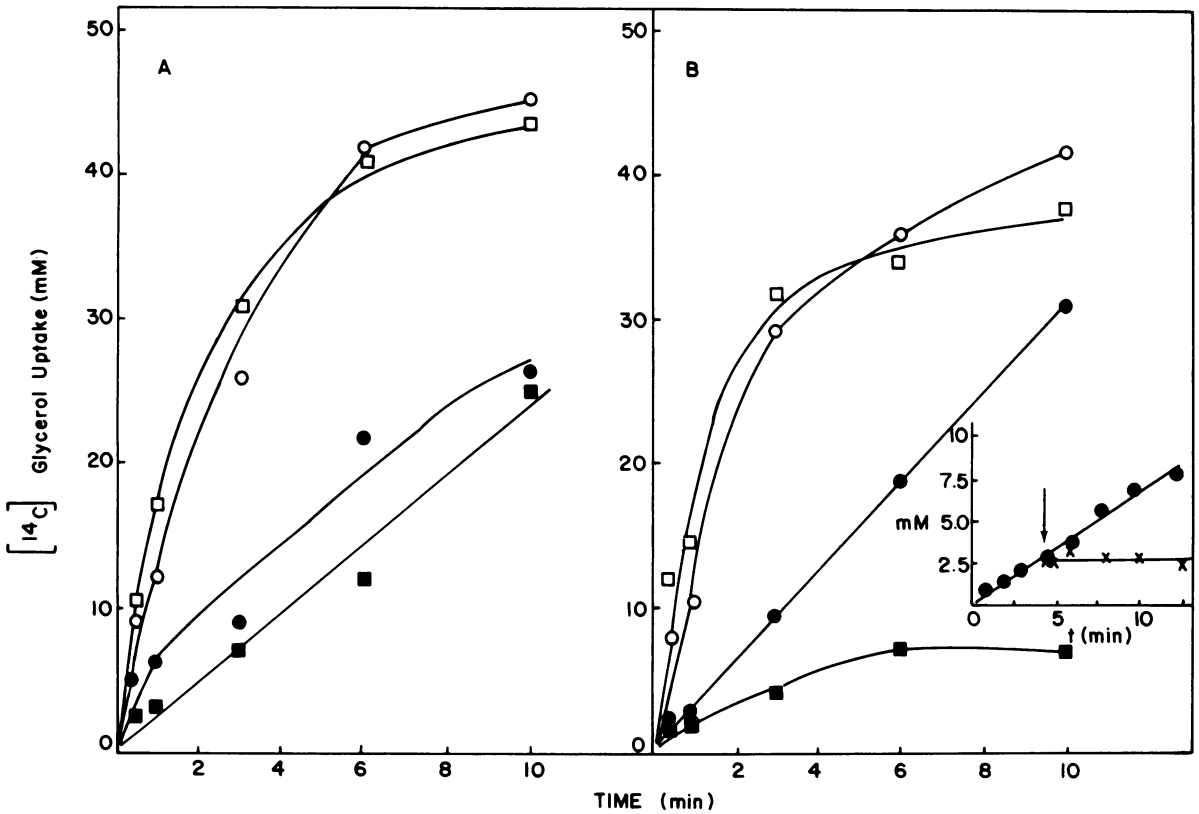

FIG. 2. Effect methyl α-glucoside and enzyme I inactivation on glycerol uptake. Uptake of [^{14}C]glycerol by cultures of *ptsI1* (squares) or the parental strain (circles) was measured in the absence (open symbols) or presence (closed symbols) of [^{14}C]methyl α-glucoside (50 μM). Uptake measurements were conducted at 30°C with cells which were not incubated at 45°C (A) or with cells preincubated at 45°C for 10 min (B). Experimental procedures were as described in the text. Inset to Fig. 2B: cells preincubated at 45°C for 30 min were allowed to accumulate metabolites from [^{14}C]glycerol. At t = 4 min (as indicated by the arrow), methyl α-glucoside was added to a final concentration of 50 μM. The results show that inhibition of glycerol uptake was immediate and complete under these conditions.

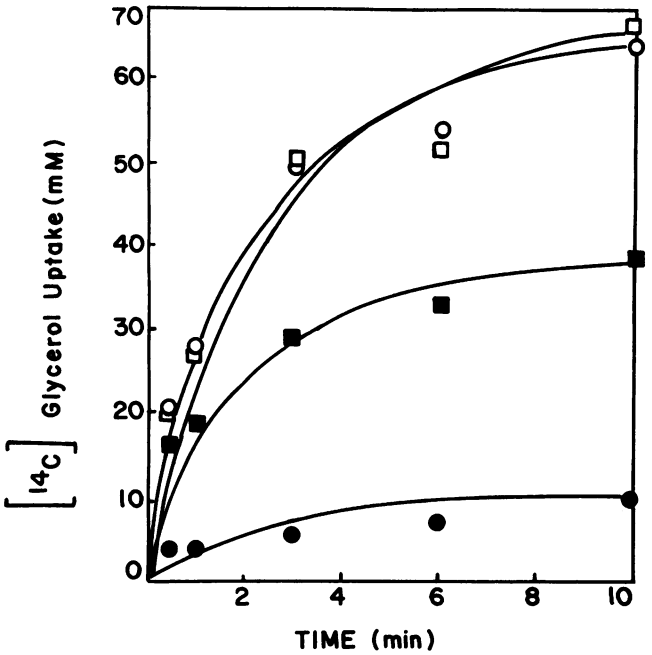

FIG. 3. Effect of D-glucose and enzyme I inactivation on glycerol uptake. Uptake of [^{14}C]glycerol by *B. subtilis* strain *ptsII* was measured at 30°C as described in the text before (squares) or after (circles) a 30-min preincubation at 45°C. Uptake was measured in the absence (open symbols) or presence (closed symbols) of 50 μM glucose.

Figure 4 shows the effects of the duration of preincubation at 45°C on both glycerol and methyl α-glucoside uptakes (measured at 30°C) as well as the inhibitory response of glycerol uptake to methyl α-glucoside by using the temperature-sensitive mutant, *ptsII*. Preincubation at 45°C resulted in an almost linear decrease in the methyl α-glucoside uptake rate until after 20 min when the rate was reduced to 20% of the original value. Glycerol uptake by cells preincubated at 45° for 20 min was reduced by ca. 15%. By contrast, sensitivity of glycerol uptake to inhibition by methyl α-glucoside increased dramatically over the first 10 min of preincubation, approaching the maximal degree of inhibition at that time. As had been concluded from studies with *E. coli*, the results clearly indicate that enzyme I performs a catalytic role in relieving inhibition of glycerol uptake by a PTS-mediated mechanism.

Figure 5 illustrates the inhibitory response of glycerol uptake to various concentrations of methyl α-glucoside. As shown in Fig. 5A, glycerol uptake in the parental strain was inhibited only at high concentrations of the glucoside, and this response was not appreciably altered by a 10-min preincubation at 45°C. By contrast, glycerol uptake in the *ptsII* mutant showed sensitivity to a 100-fold-lower concentration of the sugar after a 10-min preincubation at 45°C (Fig. 5B). This dramatic response resembles that previously observed in a temperature-sensitive *ptsI* mutant of *E. coli* (4). It provides convincing evidence for a PTS-mediated regulatory mechanism similar to that established in enteric bacteria (Saier, in press).

If the inhibitory responses of glycerol uptake to glucose and methyl α-glucoside are truly reflective of a PTS-mediated mechanism analogous to that characterized in *E. coli*, then all sugar substrates of the PTS should inhibit in cells with low enzyme I, provided that the enzyme II specific for the inhibitory sugar is induced. The results establish the veracity of this prediction (Table 1). Thus, strong inhibition by glucose, methyl α-glucoside, or 5-thioglucose required that the cells be grown in the presence of glucose, mannose, sucrose, or fructose. Strong inhibition by fructose was observed with cells grown in the presence of fructose, mannose, mannitol, or sucrose, but not glucose. Strong inhibition by sucrose was observed by cultures grown on sucrose or mannitol. Sensitivity to inhibition by mannose or mannitol was only observed after the cells were grown in the presence of mannose or mannitol, respectively. Growth of cells in the presence of mannitol also rendered glycerol uptake partially sensitive to inhibition by glucitol. From these results, we conclude that *B. subtilis* possesses at least five enzymes II specific for (i) glucose, methyl α-glucoside, and 5-thioglucose; (ii) mannose and possibly glucose; (iii) fructose; (iv) sucrose; and (v) mannitol. Like the *E. coli* mannitol enzyme II, the *B. subtilis* mannitol enzyme II can translocate glucitol slowly, although this may not normally be of physiological significance (5). Although the mannose and mannitol enzymes II are specifically induced by growth in the presence of their respective sugars, synthesis of the glucose enzyme II is induced when cells are grown in the presence of glucose, mannose, sucrose, or, to some extent, fructose. Synthesis of the fructose enzyme II is induced when cells are grown in the presence of fructose, mannose, mannitol, or sucrose, and synthesis of the sucrose enzyme II is induced when cells are grown in the presence of sucrose or mannitol (Table 1). It should be noted that hydrolysis of sucrose gives the two inductive monosaccharides, glucose

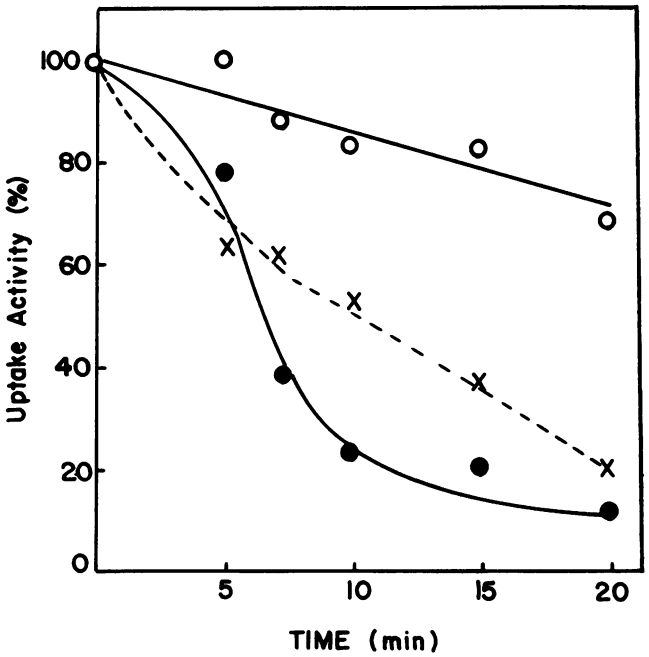

FIG. 4. Time course for the effect of enzyme I inactivation on glycerol and methyl α-glucoside uptake rates. Uptake of [^{14}C]glycerol in the absence (open circles) or presence (closed circles) of 50 μM methyl α-glucoside, or uptake of [^{14}C]methyl α-glucoside (×) was measured as described in the text by using *B. subtilis* strain *ptsII*. Uptake values are expressed as percent of the activity observed before heating. The duration of the preincubation period at 45°C is plotted on the abscissa.

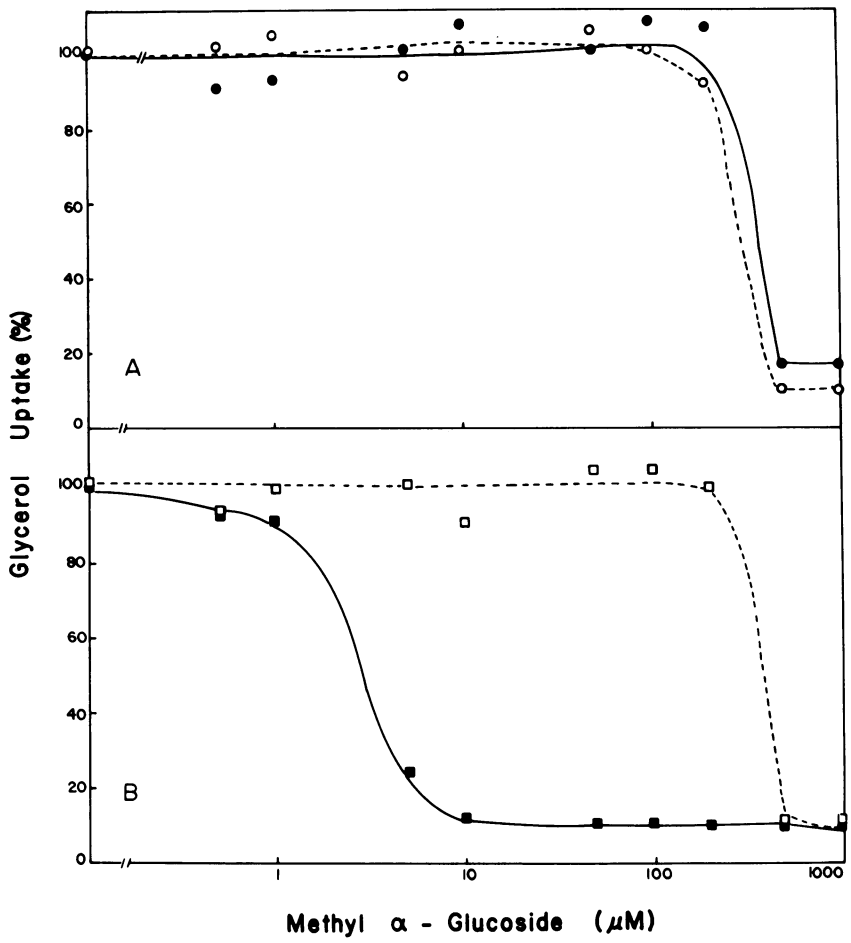

FIG. 5. Effect of methyl α-glucoside concentration and enzyme I inactivation on glycerol uptake. Uptake of [^{14}C]glycerol by the parental *B. subtilis* strain (A) or the *ptsI1* mutant (B) was measured in heated (45°C, 10 min; closed symbols) or nonheated (open symbols) cultures as described in the text. The apparent difference in the response of glycerol uptake to 50 μM methyl α-glucoside observed with different preparations of the wild-type organism (compare Fig. 2 with Fig. 5) presumably represents differences in enzyme II^{Glc} induction levels. Uptake by unheated wild-type and mutant bacteria gave a calculated internal glycerol concentration of about 15 mM in one minute in the absence of methyl α-glucoside. The heated bacteria accumulated glycerol at 80% of the rate of the unheated control in the absence of methyl α-glucoside.

and fructose, and that induction of the glucose and fructose enzymes II by sucrose is probably a response to the hydrolyzed monosaccharides and not to the disaccharide. Induction of the fructose and sucrose enzymes II by mannitol may be due to the metabolism of this hexitol to fructose 6-phosphate.

DISCUSSION

Considerable effort has been devoted to the elucidation of the mechanism by which the phosphotransferase system regulates the activities of adenylate cyclase, a variety of carbohydrate permeases, and glycerokinase in *E. coli* and *S. typhimurium* (see above). However, no previous studies had addressed the important question of whether a similar regulatory mechanism exists in bacterial species of a divergent evolutionary background. No such regulatory mechanism would be expected for bacteria possessing the more primitive form of phosphotransferase systems such as those present in photosynthetic, nitrogen fixing, and heterotrophic bacteria which transport only fructose via a phosphoenolpyruvate-dependent process (7, 13, 43, 54). Similarly, spirochetes, which transport and phosphorylate only mannitol via the PTS (45, 46), would not be expected to exhibit PTS-mediated regulation of non-PTS carbohydrate permeases. Since the enzyme III^{Glc} has been shown to be the central regulatory protein controlling permease function in *E. coli* and *S. typhimurium* (27, 29; Saier, in press), only those bacteria which possess a glucose phosphotransferase (or remnants of it) could be expected to exhibit PTS-mediated regulation.

A cursory examination of bacteria possessing complex phosphotransferase systems with glucose as one of its several substrates (10) reveals that in addition to the gram-negative bacteria within Bergey's group 8 (2), the PTS has been described in mycoplasma (group 19) (6) and two splinter gram-negative genera, *Ancalomicrobium* (in group 4) (53) and *Megasphaera* (in group 11) (11). Complex phosphotransferase systems are found in a large number of gram-positive bacteria within Bergey's groups 14 to 16. Major gram-positive genera for which complex phosphotransferase systems have been demonstrated include *Staphylococcus*, *Streptococcus*, *Bacillus*, *Clostridium*, *Brochotrix*, and *Lactobacillus* (10). In only two gram-positive species (*S. aureus*

TABLE 1. Effect of carbon source and heat inactivation of enzyme I on sensitivity of glycerol uptake to inhibition by various sugar substances of the PTS[a]

Carbon source	Glycerol Uptake (% residual activity) in presence of inhibitor:															
	Glucose		Methyl α-glucoside		5-Thioglucose		Fructose		Mannose		Sucrose		Mannitol		Glucitol	
	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C
Glycerol	76	96	24	78	22	76	27	90	57	91	100	87	80	91	80	95
Glucose	20	74	18	26	23	22	65	100	66	94	77	90	90	95	93	95
Fructose	22	101	22	84	28	84	21	104	61	84	75	87	96	101	92	80
Mannose	17	105	17	30	15	44	15	110	28	91	61	81	89	108	106	97
Sucrose	36	95	17	16	21	31	17	98	63	72	14	65	74	84	81	76
Mannitol	75	102	18	59	38	75	19	102	53	96	13	84	11	104	26	83

[a] A mid-logarithmic-phase culture of *ptsI1* cells grown on glycerol was used to inoculate media containing the above carbon sources (0.5%). After growth for 2 h, cells were harvested, washed, and resuspended in transport buffer. Initial rates of [^{14}C]glycerol uptake were measured in heated (45°C, 10 min) (H) or nonheated (C) cultures in the absence or presence of the indicated sugars at a final concentration of 50 μM. Data are expressed as percentage of initial rates as compared to those obtained in the absence of sugar. Glycerol uptake in the presence of no inhibitor was set at 100% for all carbon sources.

[14–16, 55] and *B. subtilis* [19, 21, 28] have extensive genetic analyses of the PTS been performed. Examination of the available *ptsI* mutants of *S. aureus* has revealed that none were leaky or temperature sensitive (51; unpublished data). Consequently, appropriate mutant derivatives of *S. aureus* were not available for detailed scrutiny of a possible PTS-mediated regulatory mechanism in this organism.

In enteric bacteria, tight *ptsI* mutants can not utilize non-PTS carbohydrates such as glycerol, maltose, lactose, and melibiose in the absence of exogenous cyclic AMP or a *crrA* mutation (49, 50, 52). This fact reflects the operation of PTS-mediated regulation (39). The same might be expected for tight *ptsI* mutants of gram-positive bacteria if an analogous regulatory mechanism is operative. In *S. aureus*, glycerol (37) and maltose (3) are non-PTS carbohydrates. An examination of tight *ptsI* mutants of *S. aureus* revealed that these mutants are glycerol positive but maltose negative (3, 14; M. H. Saier, Jr., unpublished data). Poor maltose utilization could not be explained exclusively on the basis of deficient glucose phosphorylation since exogenous glucose was utilized more rapidly than exogenous maltose (14). This fact suggested that PTS-mediated regulation of maltose uptake was operative in *S. aureus*. This observation appeared to contradict the fact that these same mutants could utilize glycerol at normal rates. However, the glycerol-catabolic enzyme system in *S. aureus* was shown to be synthesized constitutively (51), and *E. coli* double mutants lacking enzyme I (*ptsI*) and constitutive for synthesis of the glycerol-catabolic enzyme system (*glpR*) had previously been shown to utilize glycerol at normal rates (1). These observations were therefore consistent with (but did not prove) the presence of a PTS-mediated regulatory mechanism.

The isolation and characterization of tight *B. subtilis ptsI* mutants (19, 28) revealed that these mutants could not utilize glycerol, even though glycerol transport and phosphorylation in *Bacillus* was known not to involve the PTS (22, 26, 38). This was proven to be in accordance with our expectations since glycerol uptake in wild-type *B. subtilis* was found to be inducible (I. Stuiver and M. H. Saier, Jr., unpublished data). Thus, the preliminary genetic evidence was consistent with the conclusion that a PTS-mediated regulatory mechanism was operative both in *S. aureus* and *B. subtilis*.

The availability of a *Bacillus* spp. temperature-sensitive *ptsI* mutant (28) has rendered a detailed examination of this possibility feasible. In this report, we tested the various ramifications of PTS-mediated regulation on sensitivity of glycerol uptake to inhibition by sugar substrates of the PTS. The following observations are consistent with the mechanism established for *E. coli* and herein proposed for *B. subtilis* and other gram-positive bacteria. (i) Thermoinactivation of enzyme I results in rapid loss of the methyl α-glucoside phosphorylating and uptake activities in the intact bacterial cell (Fig. 1 and 4). (ii) Partial loss of enzyme I enhances the degree of inhibition of glycerol uptake by methyl α-glucoside without appreciably affecting the rate of glycerol uptake in the absence of this glucoside (Fig. 2 and 4). (iii) Inhibition of glycerol uptake by methyl α-glucoside is virtually instantaneous, and the degree of inhibition is dependent on the balance of the enzyme I and enzyme II activities (inset to Fig. 2; Table 1). (iv) Both metabolizable sugars (glucose) and non-metabolizable sugar substrates of the PTS (methyl α-glucoside and 5-thioglucose) can effect regulation (Fig. 3; Table 1). (v) The concentration of the inhibiting sugar which half maximally blocks glycerol uptake is inversely related to the amount of the accumulated inhibiting sugar (Fig. 1 and 5), and directly related to the residual enzyme I activity (Fig. 4). (vi) If the enzyme II specific for the inhibiting sugar is induced to a high level, the residual concentration of cellular enzyme I does not determine the maximal degree of inhibition (Fig. 5). (vii) Any sugar substrate of the PTS can inhibit glycerol uptake provided that the enzyme II specific for that sugar is induced to a sufficiently high level (Table 1).

The results of our studies serve also to define the sugar-specific constituents of the *Bacillus* PTS and their induction properties. Thus, *Bacillus* was shown to possess at least five distinct PTS permeases (enzymes II), specific for glucose, mannose, fructose, sucrose, and mannitol. With the exception of the sucrose system, which is lacking in *E. coli*, these enzymes II appear to resemble those in enteric bacteria with respect to both their inducer and their substrate specificities (42). A single exception is the mannose enzyme II which is apparently induced specifically by mannose in *B. subtilis* (Table 1) but by both mannose and glucose in *E. coli* (42). If the regulatory mechanism characterized in *Bacillus* is analogous to that characterized in enteric bacteria, one would predict that the gram-positive bacteria possess a soluble Enzyme III^{Glc} which functions as a central regulatory protein. Such a protein has been functionally identified in *B. subtilis* (M. J. Novotny, J. Reizer and M. H. Saier, Jr., unpublished data). This protein stimulated the phosphorylation of methyl α-glucoside by butanol-urea-extracted membranes in the presence of excess enzyme I and HPr ca. 10-fold. To gain evidence for the involvement of this protein in

the regulation of glycerol uptake it will be necessary to isolate and characterize *crrA* (enzyme III^{Glc}-negative) mutants. These studies are presently in progress.

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