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Cooperative binding of lactose and the phosphorylated phosphocarrier protein HPr(Ser-*P*) to the lactose/H^+ symport permease of *Lactobacillus brevis*

(sugar transport/allosteric control/protein phosphorylation/protein effector/phosphotransferase system)

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ABSTRACT ***Lactobacillus brevis* accumulates lactose and nonmetabolizable lactose analogues via sugar/H^+ symport, but addition of glucose to the extracellular medium results in rapid efflux of the free sugar from the cells due to the uncoupling of sugar transport from proton transport. By using vesicles of *L. brevis* cells, we recently showed that these regulatory effects could be attributed to the metabolite-activated ATP-dependent protein kinase-catalyzed phosphorylation of serine-46 in the phosphocarrier protein HPr [HPr(Ser-*P*)] of the phosphotransferase system and that a mutant form of HPr with the serine-46 → aspartate replacement ([S46D]HPr) is apparently locked in the seryl phosphorylated conformation. We here demonstrate that [S46D]HPr binds directly to inside-out membrane vesicles of *L. brevis* that contain the lactose permease. Sugar substrates of the permease markedly and specifically stimulate binding of [S46D]HPr to the membranes while certain transport inhibitors such as *N*-ethylmaleimide block binding. The pH dependency for binding follows that for transport. Wild-type HPr and the [S46A]HPr mutant protein did not appreciably compete with [S46D]HPr for binding to the permease. These results provide evidence for the direct interaction of HPr(Ser-*P*) with an allosteric site on the lactose/proton symporter of *L. brevis* for the purpose of regulating sugar accumulation in response to the metabolic needs of the cell.**

Bacteria can utilize a variety of carbon sources for growth, and studies conducted in the 1940s established that some of these are used preferentially over others (1, 2). For example, glucose is utilized by *Escherichia coli* in preference to lactose, maltose, or glycerol, a phenomenon termed "the glucose effect" or "diauxic growth" (3). In *E. coli* this phenomenon has been attributed to catabolite repression and inducer exclusion, both of which regulate transcription of target carbon catabolic genes (4, 5). The mechanisms accounting for both phenomena involve a specific protein constituent of the phospho*enol*pyruvate/sugar phosphotransferase system (PTS), the glucose-specific IIA protein (IIA^{glc}). IIA^{glc} binds to and allosterically inhibits a number of target enzymes and transport systems to control their activities (6, 7). These target systems include glycerol kinase, arabinose isomerase, and permeases specific for lactose, galactose, maltose, melibiose, and raffinose (refs. 6 and 8; C. Hoischen and M.H.S., unpublished results). IIA^{glc} can be phosphorylated on a histidyl residue by phospho*enol*pyruvate and the two energy-coupling proteins of the PTS, enzyme I and HPr, and it can be dephosphorylated in the presence of a PTS sugar substrate by HPr and an enzyme II complex specific for that sugar (6, 7, 9). The phosphorylation state of IIA^{glc} determines whether or not it binds to its target proteins.

In earlier publications from this laboratory, *E. coli* inside-out membrane vesicles containing the lactose permease were shown to bind IIA^{glc} in a process that was cooperative with binding of the sugar substrates of the permease (10, 11), and these observations were later confirmed (12). The recent x-ray structure of the complex of glycerol kinase with the IIA^{glc} protein (13) and mutagenic analyses of the binding of IIA^{glc} to the lactose, melibiose, and maltose permases (for a review, see ref. 6) leave little doubt that the allosteric mechanism originally proposed (5) is correct.

Gram-positive bacteria do not possess the cytoplasmic IIA^{glc} protein, and the regulatory mechanisms demonstrated in *E. coli* are apparently lacking. Instead, these organisms possess an ATP-dependent metabolite-activated protein kinase that phosphorylates a seryl residue in HPr (14, 15). Phosphorylation of serine-46 in HPr or conversion of that residue to a permanently charged aspartate due to the S46D mutation results in strong inhibition of the PTS phosphoryl transfer reaction *in vitro* (16). NMR analyses suggested that the S46D mutation induces the same conformational transitions as does phosphorylation of serine-46 (17).

Ye *et al.* (18, 19) have demonstrated that in *Lactobacillus brevis*, an organism that possesses an anaerobically inducible fructose-specific PTS (S. Klinke and M.H.S., unpublished results), lactose/H^+ and glucose/H^+ symports (20) are regulated by HPr(Ser) phosphorylation. In another Gram-positive bacterium, *Lactococcus lactis*, the PTS itself and a cytoplasmic sugar-phosphate phosphatase appear to be regulated by HPr(Ser) phosphorylation (21, 22). In contrast, in *Bacillus subtilis*, HPr(Ser) phosphorylation apparently does not serve any of these functions but instead influences the phenomenon of catabolite repression (23). Phosphorylation of serine-46 in HPr thus appears to have different physiological consequences in different low guanine plus cytosine (GC) Gram-positive bacteria, although the ultimate purpose is always control of carbon utilization.

The mechanisms by which HPr(Ser) phosphorylation regulates the above-mentioned processes are not known. By analogy with regulation of corresponding processes by the IIA^{glc} protein in *E. coli*, we postulated that HPr(Ser-*P*) might directly bind to its target enzymes and permeases to allosterically control their activities (18, 19). In this communication, we apply a modification of the experimental approach developed by Osumi and Saier (10, 11) to provide direct evidence for this postulate.

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Abbreviations: PTS, phospho*enol*pyruvate:sugar phosphotransferase system; IIA^{glc}, glucose-specific IIA protein; TMG, methyl β-D-thiogalactoside; TDG, β-D-thiodigalactoside; IPTG, isopropyl β-D-thiogalactoside; NEM, *N*-ethylmaleimide; GC, guanine plus cytosine.
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MATERIALS AND METHODS

Purification of Proteins. *B. subtilis* HPr and its S46D and S46A mutant derivatives were purified to virtual homogeneity from *E. coli* cells after overproduction as described (16).

Preparation of Inside-Out Membrane Vesicles. Lactose permease-rich membranes were prepared from *L. brevis* ATCC367. Cultivation of the bacteria and induction of the lactose permease were carried out as described (18). For the preparation of the inside-out membrane vesicles, the bacteria were suspended in 50 mM Tris·HCl (pH 7.5) containing 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and deoxyribonuclease at 10 μg/ml, and the cells were broken by three passages through a French pressure cell [11,000 lb/in^2 (76 MPa)]. Cell debris was removed by centrifugation at 10,000 × *g* for 5 min, and the membranes were collected by centrifugation at 100,000 × *g* for 90 min. They were washed once with 50 mM Tris·HCl (pH 7.5) containing 1 mM dithiothreitol, resuspended in the same solution at a protein concentration of 6.5 mg/ml, and stored frozen at −70°C until used. Protein was estimated by the method of Lowry *et al.* (24).

Iodination of [S46D]HPr. HPr of *B. subtilis* contains two tyrosyl residues (25). Iodination of the mutant [S46D]HPr protein (120 μg) was performed essentially as described by Greenwood *et al.* (26). The ^{125}I-labeled [S46D]HPr was purified by gel filtration through a Sephadex G-25 column (1 × 10 cm). Elution was with a solution containing 1% bovine serum albumin in 50 mM sodium phosphate (pH 7.5). The iodinated [S46D]HPr protein (10^8 cpm; 80 μg) was collected and stored at −20°C until used. Control experiments showed that bovine serum albumin in the amounts present did not interfere with the binding reaction.

^{125}I-Labeled [S46D]HPr Binding Experiments. Incubations were carried out at room temperature in cellulose nitrate tubes (5 × 20 mm) designed for use in a Beckman Airfuge essentially as described by Osumi and Saier (11). In a typical experiment, the incubation mixture contained, in a total volume of 50 μl, 30 mM Tris·HCl (pH 7.5), 0.6 mM dithiothreitol, 65 μg of membrane protein from *L. brevis*, and 2.5 μl of ^{125}I-labeled [S46D]HPr (specific activity, 4.5 mCi/μmol; 1 Ci = 37 GBq). After 10 min of incubation at room temperature, the membranes were collected by centrifugation at 100,000 × *g* for 10 min in an Airfuge centrifuge. The supernatants were discarded, tubes were rinsed twice with 100 μl of ice-cold 50 mM Tris·HCl (pH 7.5) containing 1 mM dithiothreitol, and the membrane pellets were resuspended in 10 ml of Bio-Safe II scintillation fluid (Research Products International) for determination of membrane-bound radioactivity in a Beckman LS-230 scintillation counter.

Treatment of Membranes with *N*-Ethylmaleimide (NEM). For examination of the effect of NEM on the binding of ^{125}I-labeled [S46D]HPr to membranes containing the lactose permease, the membranes (prepared without dithiothreitol) were incubated at room temperature for 10 min with 0.5 mM NEM in 45 mM sodium phosphate (pH 7.0). After this incubation period, dithiothreitol was added to 2 mM. Membranes were collected by centrifugation in an Airfuge and washed twice with 50 mM Tris·HCl (pH 7.5) containing 1 mM dithiothreitol.

Transport in Intact Cells. Uptake of radioactive sugars was conducted as described (18, 19).

RESULTS

Table 1 summarizes the association of ^{125}I-labeled [S46D]HPr with inside-out membrane vesicles from galactose-grown *L. brevis* cells. These cells contain high levels of the lactose/H$^+$ symporter (18–20). Low association of ^{125}I-labeled [S46D]HPr with the pelleted membranes was observed after pretreatment of the membranes with NEM. When excess [S46D]HPr was added, values were also reduced to near background levels (see below). Similarly, when cells were grown in glucose-containing medium, the β-D-thiodigalactoside (TDG)-stimulated binding of ^{125}I-labeled [S46D]HPr to the membranes was reduced to ≈10% of the value observed with membranes from galactose-grown cells (see Table 1).

Table 1. Binding of ^{125}I-labeled [S46D]HPr to *L. brevis* membranes containing the lactose permease

Addition	Membrane protein, mg	^{125}I-labeled [S46D]HPr binding, ng
NEM (0.5 mM)	0.065	0.0
None	0	1.3 ± 0.7
	0.065	3.9 ± 1.3
	0.195	11.0 ± 5
	0.325	15.0 ± 9
TDG	0	3.3 ± 0.7
	0.065	44.0 ± 1
	0.195	81.0 ± 8
	0.325	97.0 ± 14
Lactose	0.065	38.0 ± 5
IPTG	0.065	42.0 ± 7
TMG	0.065	34.0 ± 7
Melibiose	0.065	46.0 ± 2
Galactose	0.065	30.0 ± 5
Maltose	0.065	6.5 ± 1.3
Mannose	0.065	22.0 ± 1
*P-e*Prv	0.065	5.9 ± 0.6

For ^{125}I-labeled [S46D]HPr binding, the value obtained in the presence of NEM was 0.29 mg of [S46D]HPr. This background value was subtracted from all experimentally obtained values as recorded in this table and the figures to provide an estimate of the amount of [S46D]HPr bound specifically to the permease. When present, sugar or phospho*enol*pyruvate (*P-e*Prv) was added to a final concentration of 10 mM. When cells were grown in glucose instead of galactose medium, the amount of [S46D]HPr bound to 0.065 mg of membrane protein was 3.5 ± 0.1 ng in the presence of TDG or IPTG (background subtracted).

The binding of ^{125}I-labeled [S46D]HPr to membranes isolated from cells grown in galactose-containing medium was studied in the presence and absence of 10 mM TDG as a function of membrane protein (Table 1). In the presence of TDG, associated counts increased nearly linearly up to about 0.1 mg of membrane protein per 50 μl of assay solution, but nonlinearity was observed thereafter (Table 1). In the absence of TDG, the amount of radioactive protein bound was much lower. In all subsequent experiments, 65 μg of membrane protein was used per 50 μl of assay solution.

When any one of the several substrates of the lactose permease was added to the incubation mixture, the amount of radioactive protein associated with the membrane pellet increased dramatically (Table 1). Thus, when excess melibiose, TDG, or isopropyl β-D-thiogalactoside (IPTG) was added, the amount of [S46D]HPr bound specifically to the lactose permease-containing membranes increased over 10-fold. Lactose and methyl β-D-thiogalactoside (TMG) promoted binding to a somewhat lesser degree. Poor substrates such as galactose and nonsubstrates such as mannose, maltose, and phospho*enol*pyruvate exerted a lesser effect that in the latter cases was probably of negligible significance. The stimulatory effects of glucose and mannose were shown to be due to the presence of the glucose/H$^+$ symporter, which is also regulated by HPr(Ser-*P*) (ref. 19 and unpublished results).

The effects of various concentrations of nonradioactive wild-type and mutant forms of HPr on the binding of ^{125}I-labeled [S46D]HPr to the membranes in the presence of a saturating concentration of TDG was examined (Fig. 1). [S46D]HPr effectively competed with the iodinated protein at low concentration. In contrast, wild-type HPr and the S46A mutant protein did not effectively compete at low concentra-

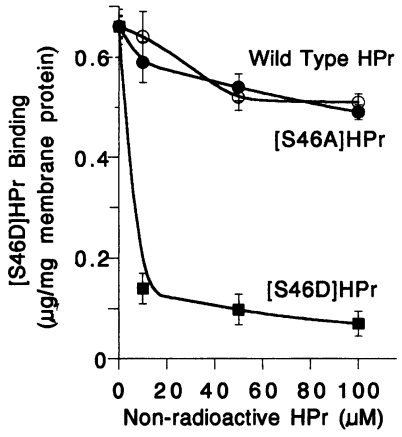

FIG. 1. Competition for ^{125}I-labeled [S46D]HPr binding to membranes prepared from galactose-grown *L. brevis* cells by wild-type and mutant HPr proteins. TDG at 10 mM was present in all tubes. Radioactive and nonradioactive proteins were added simultaneously to the membrane preparations at $t = 0$. Wild-type HPr (○), the mutant [S46A]HPr protein (●), or the mutant [S46D]HPr protein (■) was added as indicated.

tions, and even at high concentrations, their inhibitory effects were minimal. These results indicate that of these three forms of HPr, only [S46D]HPr exhibits appreciable affinity for the lactose permease of *L. brevis*. Only this protein displays activity in the PTS phosphoryl transfer reaction and conformational features resembling those of HPr(Ser-*P*) (16, 17).

Fig. 2*A* shows the dependency of ^{125}I-labeled [S46D]HPr binding to membranes on the concentrations of TDG, melibiose, and IPTG. The three curves exhibit half-maximal binding enhancement at a concentration of about 35 μM sugar. These sugars at 100 μM concentrations gave nearly maximal binding, and a 10-fold increase did not further enhance the radioactivity associated with the membranes. These concentration dependencies correlated with those observed in transport experiments (Fig. 2*B*). Radioactive IPTG uptake exhibited a K_m of about 35 μM, and equimolar amounts of either TDG or melibiose inhibited IPTG uptake about 50%. The three sugars, therefore, bind to the permease with comparable affinities.

The binding of ^{125}I-labeled [S46D]HPr was examined as a function of pH (Fig. 3). A sharp peak with maximal binding at neutral pH was observed. Almost no binding was observed below pH 5 or above pH 9. This pH curve for *in vitro* binding was similar to but sharper than the pH curve of the uptake of [^{14}C]TMG by intact cells (Fig. 3).

DISCUSSION

The results presented in this communication provide compelling evidence for the suggestion that [S46D]HPr [and therefore HPr(Ser-*P*)] binds directly and specifically to the allosteric binding site(s) on the lactose/proton symport permease of *L. brevis*. This binding reaction is clearly cooperative with the binding of a transportable sugar substrate of the permease. All known effective sugar substrates of the permease enhanced binding, but poor substrates or nonsubstrates of the permease exerted little or no effect. The appreciable binding of [S46D]HPr in the presence of glucose or mannose to these membrane preparations reflects the presence of the glucose/H$^+$ symporter of this bacterium (ref. 19; J.J.Y. and M.H.S., unpublished results).

The binding of ^{125}I-labeled [S46D]HPr was abolished by treatment of the membranes with NEM, a treatment that completely blocked lactose permease activity in whole cells (unpublished observations). Further, the pH optimum for binding was similar to that for transport (Fig. 3), and the

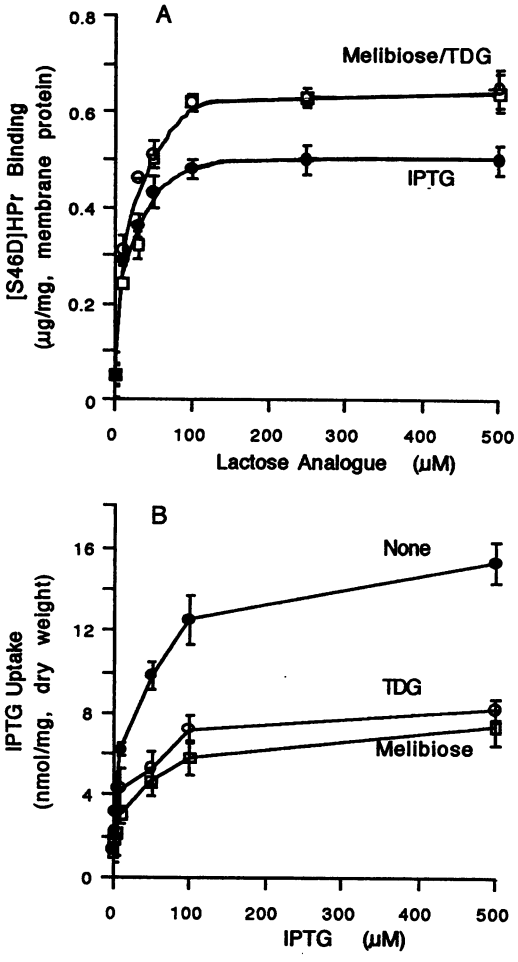

FIG. 2. (*A*) Effects of TDG (○), IPTG (●), and melibiose (□) concentrations on the binding of ^{125}I-labeled [S46D]HPr to *L. brevis* membranes containing the lactose/H$^+$ symport permease. (*B*) IPTG uptake as a function of IPTG concentration in the absence (●) or in the presence of equimolar concentrations of either TDG (○) or melibiose (□). Uptake experiments were conducted as described (18, 19) with [^{14}C]IPTG at a specific activity of 28 mCi/mmol. Measurements were made after a 10-min incubation at 37°C.

affinities of the permease for its various substrates correlated roughly with the capacity of low concentrations of these

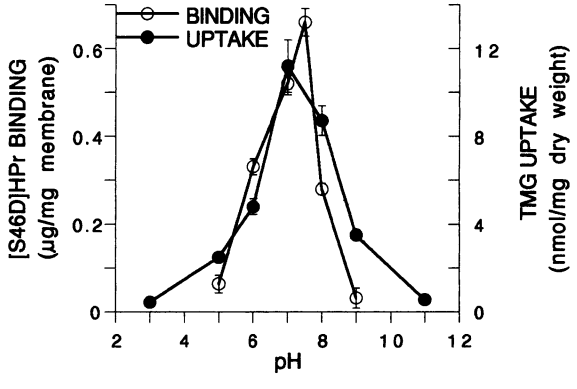

FIG. 3. Effect of pH on [^{14}C]TMG uptake and the binding of ^{125}I-labeled [S46D]HPr to *L. brevis* membranes containing the lactose permease. For the binding experiments, TDG was uniformly present at a concentration of 10 mM. The buffers used were 40 mM sodium acetate (pH 3–5), 40 mM potassium phosphate (pH 6–8), or 40 mM sodium borate (pH 9–11). Transport was measured by employing intact galactose-grown cells as described (18, 19).

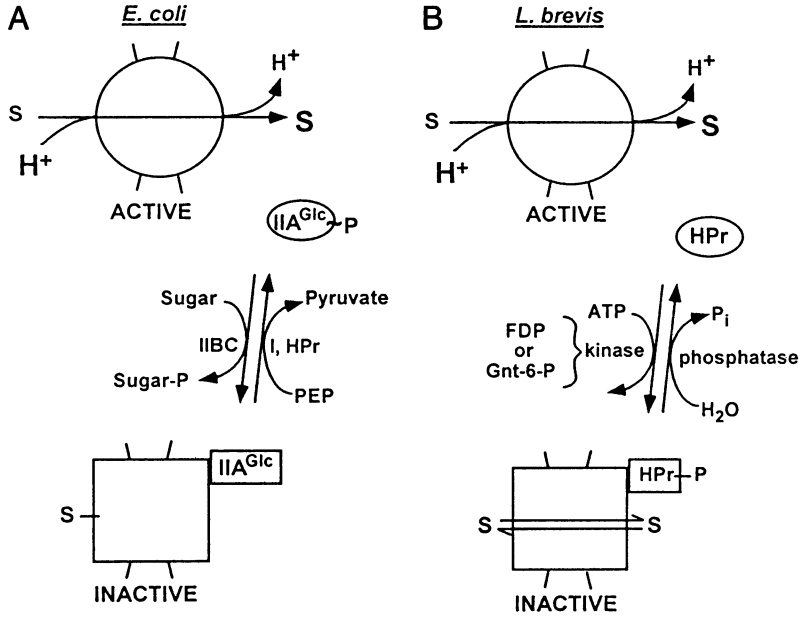

FIG. 4. Model for the regulation of lactose/H^+ symport in *E. coli* (*A*) compared with the analogous process in *L. brevis* (*B*). S, sugar substrate; IIA and IIBC, the sugar-specific constituents of a PTS enzyme II complex; IIA^{Glc}~P, enzyme IIA^{Glc}(His-*P*); FDP, fructose 1,6-bisphosphate; Gnt-6-P, gluconate 6-phosphate; HPr-P, HPr(Ser-*P*). The model illustrates the cooperative binding of sugar substrate and regulatory protein in both systems.

substrates to promote binding (Fig. 2). Although our studies clearly suggest direct binding of [S46D]HPr to the lactose permease, it should be noted that crude membranes were used, and consequently, it remains possible that another as yet unidentified protein mediates the regulatory interaction between the permease and the PTS protein. Reconstitution of TMG transport regulation in an artificial membrane with purified permease protein will be required to eliminate this possibility.

Fig. 4*B* presents our model for the regulation of lactose permease activity in *L. brevis* and Fig. 4*A* compares it with that for the regulation of lactose permease activity in *E. coli*. In the absence of an activating metabolite (i.e., gluconate 6-phosphate or fructose 1,6-bisphosphate), the HPr(Ser) kinase is not active and does not phosphorylate serine-46 in HPr. HPr is then capable of functioning in the PTS phosphoryl transfer reaction (ref. 9; S. Klinke and M.H.S., unpublished results; see Introduction), but it does not bind to or inhibit the lactose/H^+ symporter. Sugar substrates of the latter permease are, therefore, actively accumulated by a proton symport mechanism, and the elevated cytoplasmic concentration of the galactoside allows rapid hydrolysis by β-galactosidase followed by metabolism of the constituent monosaccharides.

When appropriate metabolites accumulate in the cell cytoplasm, the kinase is activated, and HPr is phosphorylated on serine. This phosphorylated protein then binds to the lactose permease (18) and presumably to other permeases, including the glucose/H^+ symport permease (19), provided that their sugar substrates are present in the extracellular medium. Binding of HPr(Ser-*P*) converts these permeases to simple facilitators, uncoupled from proton symport. As a result, sugars do not accumulate in the cytoplasm, and the phenomena of inducer exclusion and inducer expulsion (27) are observed.

This mechanism contrasts with the analogous regulatory mechanism established for *E. coli* (Fig. 4*A*) (6, 7) in three respects. In the latter process, (*i*) the availability of extracellular PTS sugars is sensed rather than the concentration of intracellular metabolites, (*ii*) sugar transport activity is blocked rather than being uncoupled from proton symport, and (*iii*) the free form of IIA^{glc} is the allosteric effector rather than the seryl phosphorylated form of HPr.

In spite of these striking differences, four parallels can be drawn. Thus, in both cases, (*i*) carbon availability is sensed, (*ii*) a PTS protein, the conformation of which is controlled by phosphorylation, is the effector, (*iii*) the target permeases exhibit cooperativity for PTS protein binding with respect to sugar substrate binding, and (*iv*) the net physiological consequence of regulation is a reduction in cytoplasmic inducer levels. The physiological consequences of these two mechanistically dissimilar processes are, therefore, the same even though the compounds sensed, the proteins involved, and transmission mechanisms utilized are different. These two regulatory mechanisms undoubtedly evolved independently of each other in two major prokaryotic phyla, yet they converged to provide a common essential function to the bacteria.

It is likely that other mechanisms are used in other groups of bacteria. For example, we have recently found that species of *Streptomyces* (high GC Gram-positive bacteria) possess a fructose-specific PTS as does *L. brevis* but they do not possess the HPr(Ser) kinase (refs. 28 and 29; S. Klinke and M.H.S., unpublished results). There is no evidence that the mechanisms of catabolite repression in high GC Gram-positive bacteria resemble those in low GC Gram-positive bacteria (30). These observations suggest that entirely different mechanisms will prove to be operative in these organisms. Further studies will be required to reveal the intricacies of these processes.

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