Biochemical Characterization of Phosphoryl Transfer Involving HPr of the Phosphoenolpyruvate-Dependent Phosphotransferase System in *Treponema denticola*, an Organism that Lacks PTS Permeases[†]

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ABSTRACT: *Treponema pallidum* and *Treponema denticola* encode within their genomes homologues of energy coupling and regulatory proteins of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) but no recognizable homologues of PTS permeases. These homologues include (1) Enzyme I, (2) HPr, (3) two IIA^{Ntr}-like proteins, and (4) HPr(Ser) kinase/phosphorylase (HprK). Because the Enzyme I-encoding gene in *T. pallidum* is an inactive pseudogene and because all other *pts* genes in both *T. pallidum* and *T. denticola* are actively expressed, the primary sensory transduction mechanism for signal detection and transmission appears to involve HprK rather than EI. We have overexpressed and purified to near homogeneity four of the five PTS proteins from *T. denticola*. Purified HprK phosphorylates HPr with ATP, probably on serine, while Enzyme I phosphorylates HPr with PEP, probably on histidine. Furthermore, HPr(His)-P can transfer its phosphoryl group to IIA^{Ntr}-1. Factors and conditions regulating phosphoryl transfer prove to differ from those described previously for *Bacillus subtilis*, but cross-enzymatic activities between the *Treponema*, *Salmonella*, and *Bacillus* phosphoryl-transfer systems could be demonstrated. Kinetic analyses revealed that the allosterically regulated HPr kinase/phosphorylase differs from its homologues in *Bacillus subtilis* and other low G+C Gram-positive bacteria in being primed for kinase activity rather than phosphorylase activity in the absence of allosteric effectors. The characteristics of this enzyme and the *Treponema* phosphoryl-transfer chain imply unique modes of signal detection and sensory transmission. This paper provides the first biochemical description of PTS phosphoryl-transfer chains in an organism that lacks PTS permeases.

Spirochetes are free-living or host-associated microorganisms, several of which are causative agents of animal and human diseases (*1*). The most important human diseases caused by spirochetes are syphilis (*Treponema pallidum*), periodontitis (*Treponema denticola*), Lyme disease (*Borrelia burgdorferi*), and leptospirosis (*Leptospira interrogans*). Nevertheless, our knowledge about the physiology of this group of bacteria is minimal. Poorly defined growth requirements and sparse genetic tools provide primary obstacles to *in vivo* experimentation (*2*−*4*).

In recent years, the availability of complete genome sequences has led to predictions of important and surprising physiological characteristics. For example, in *T. pallidum*, TCA cycle enzymes are lacking, and consequently, it seems likely that these organisms must obtain many biosynthetic precursors from exogenous sources (*5*). *T. pallidum* also lacks the enzymes necessary for fatty acid biosynthesis, although this bacterium is rich in lipids (*6*). These characteristics differ from those of free-living bacteria and impose a need for unique regulatory constraints.

Phosphoenolpyruvate-dependent phosphotransferase systems (PTSs) of spirochetes exhibit unique features (*7*, *8*). Although *Spirochaeta aurantia* has been shown to possess a functional mannitol-specific PTS (*9*, *10*) and a complete set of PTS proteins including PTS permeases has been identified in the fully sequenced genome of *Borrelia burgdorferi* (*11*, *12*), spirochetes with completely sequenced genomes in the genuses *Treponema* and *Leptospira* apparently lack PTS permeases (*5*, *8*, *13*). However, they do encode the ATP-dependent HPr kinase/phosphorylase (HprK) and homologues of several PTS energy-coupling proteins, Enzyme I (PtsI), the small phosphocarrier protein, HPr (PtsH), and either one or two Enzyme IIA proteins (*7*, *14*).

The PTS plays at least two important roles in many bacterial cells: (1) sugar uptake coupled to phosphorylation, and (2) regulation of transport, metabolism, and gene expression (*15*). The primary function is sugar uptake, which involves transfer of the phosphoryl group from phosphoenolpyruvate (PEP) to the substrate sugar via a PTS protein phosphoryl-transfer chain. The sequential phosphotransfer cascade includes the following enzymes: Enzyme I, HPr, IIA, IIB, and IIC. Only the IIC constituent is an integral membrane protein, and only this protein is not phosphorylated. It is the sugar permease/kinase.

The PTS protein homologues present in *T. denticola* are PtsI, HPr, and two homologous nitrogen-regulatory Enzyme IIA proteins, IIA^{Ntr}-1 and IIA^{Ntr}-2 (*7*, *16*). HprK is also present, but no recognizable IIB or IIC homologue has been identified (*7*, *8*). The absence of PTS permeases in bacteria

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Table 1: Strains and Plasmids Used in This Paper

strains	characteristics	reference
T. denticola		
ATCC35405		
E. coli		
DH5α	F$^-$, *ϕ80dlacZΔM15, endA1, recA1, hsdR17* (r_k^-, m_k^+), *supE44, thi-1, gyrA96, relA1,* Δ*(lacZYA-argF)U169,* λ^-	
Origami (DE3)	F$^-$*ompT [lon](trxB) (gor) hsdS$_B$* ($r_B^-m_B^-$) with DE3, λ T7 RNA polymerase gene	
CGSC 7319	*lacI22, dctB3,* λ^-*, glk-1, ptsI2, relA1, spoT1, thi-1*	
S. typhimurium		
SB-1477	*ptsI18*	*51*
B. subtilis		
BAL218	*trpC2*	*52*
GM273	*trpC2 ptsH1*	*46*
GP202	*trpC2 hprK::spc*	*53*
plasmids		
PET28(a)+		Novagen
PET-H	pET28(a)+ carrying *T. denticola ptsH*	this paper
PET-K	pET28(a)+ carrying *T. denticola hprK*	this paper
PET-EI	pET28(a)+ carrying *T. denticola ptsI*	this paper
PET-EII^{Ntr}-1	pET28(a)+ carrying *T. denticola ptsA1*	this paper
PET-EII^{Ntr}-2	pET28(a)+ carrying *T. denticola ptsA2*	this paper
PDG148-*stuI*		*27*
PDG-K	pDG148-*stuI* carrying *T. denticola hprK*	this paper
PDG-H	pDG148-*stuI* carrying *T. denticola ptsH*	this paper

that possess the PTS energy-coupling proteins is a characteristic of some related spirochetes such as *T. pallidum* and *L. interrogans*, as well as the distantly related green bacterium, *Chlorobium tepidum* (*17*) and the δ-proteobacterium, *Geobacter metallireducens* (*54*). The absence of membrane-integrated PTS permeases prompted us to speculate that the phosphotransferase system is not associated with sugar transport in these species and that it therefore serves a strictly regulatory role. Moreover, the absence of PTS permeases means that the sensor for regulation cannot be PTS-mediated sugar transport or an indirect consequence of such transport such as a change in PEP levels. This is not true for any other PTS regulatory system so far studied.

Regarding PTS-mediated gene regulation, catabolite repression of gene expression in enteric bacteria depends on IIA^{Glc} (*15*), but in Gram-positive bacteria, catabolite repression depends on HPr phosphorylation by a specific ATP-dependent kinase/phosphorylase (HprK) (*18*−*20*). HPr phosphorylation by HprK occurs at Ser-46, and the resulting P-seryl HPr acts as a corepressor together with the mediator of catabolite repression, the catabolite control protein A, CcpA (*21*, *22*). The high-resolution three-dimensional structures of several Gram-positive HprKs are known (see ref *19*). Many Gram-negative bacteria possess homologues of the Gram-positive bacterial HPr kinases (*7*, *11*, *54*), but these bacteria lack CcpA and in no case is their physiological function known (*23*, *54*).

The aim of the work reported here is to provide a detailed description of the PTS protein phosphoryl-transfer chains in *T. denticola* and *T. pallidum*. We show that PTS proteins in *T. denticola* are active in catalyzing phosphoryl transfer with phosphoenolpyruvate as the phosphoryl donor but that in *T. pallidum* the PTS phosphoryl-transfer chain is inactivated because of the loss of functional Enzyme I. The presence of active *hprK*, *ptsH* (HPr), and *ptsN* (IIA) genes in both organisms leads to the probability that HprK rather than PtsI is the sensor of the metabolic state and that the spirochete regulatory system is therefore fundamentally different from the *Escherichia coli* regulatory systems described (*15*, *24*). We also show that the kinetic properties of the *T. denticola* HprK differ dramatically from those recognized in the well-characterized low G+C Gram-positive bacteria.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions. *T. denticola* ATCC 35405 (Table 1) was grown at 37 °C in liquid NOS broth (*25*) in a CO_2-enriched atmosphere using a GasPak-Plus system (*25*). *E. coli* strains were grown at 37 °C with agitation in LB broth culture media. Cultures were supplemented with an antibiotic (kanamycin, 30 μg/mL, or ampicillin, 100 μg/mL) when necessary. The same culture conditions were used for *Bacillus subtilis* with 5 μg/mL of kanamycin when necessary. *Bacillus* strains were also cultured in C broth (*26*) to evaluate sugar uptake and catabolite repression in response to the inclusion of plasmids encoding select PTS enzymes from *T. denticola*. The characteristics of all strains and plasmids used in this paper are presented in Table 1.

Cloning. Primers used for PCR amplification of *T. denticola* genes encoding PTS proteins were designed using the genome sequence available at http://hgsc.bcm.tmc.edu/microbial/Tdenticola/ according to the genome annotations (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3. spl?database=gtd). Primers were flanked by specific restriction sites to obtain directional cloning on the pET28a-(+) (Novagen) expression vector with an N-terminal His tag. The *T. denticola ptsI* and *ptsH* genes and the two genes encoding IIA^{Ntr} homologues, *ptsA1* and *ptsA2*, were cloned into *Nde*I and *Eco*RI restriction sites; A *Bam*HI restriction site was introduced into the reverse primer when cloning the *hprK* gene. The sequences of the primers used are shown in Table 2, and the restriction sites used in each case are underlined. Using T7 universal primers, the sequences of the fragments cloned were verified by sequencing. For expression of the *pts* genes in *B. subtilis*, the same primers used for cloning into pET28a(+) were modified as described by Joseph et al. (*27*), and the fragments were cloned into

Table 2: Primers Used for PCR Amplification of *T. denticola pts* Genes[a]

	forward primer	reverse primer
hprK (HprK)	TGATTGCACATATGGCTAACATAAGCTTTTC	CTAGGATCCGGTATTCCTGCCCGATTTTGA
PtsH (HPr)	TGATTGCACATATGATTTCAAAAACTATTAAGGTTCAAA	ACGAATTCCCGAAAAAGTTTATTCTTCTTCAAA
ptsI (EI)	TGATTGCACATATGAAAAAACTTAACGGACTCATA	ACGAATTCCAATAATTTCATCTTTAGTTTTCGGC
PtsA1 (EII^{Ntr}-1)	TGATTGCACATATGGAATTTAAAGATGCATTGAA	ACGAATTCTTACTCTTCGTCGCTTTCCA
PtsA2 (EII^{Ntr}-2)	TGATTGCATATGCCCGTTATTTGCGTGTTT	ACGAATTCGAGTTCATCGAACCCGTACC

[a] Restriction sites used for directional cloning are underlined.

the pDG148-*stuI* vector. Additionally, a FLAG sequence encoding 8 amino acids (DYKDDDDK) was introduced at the N termini of these proteins to follow expression of the encoding genes by Western blotting.

Protein Overexpression and Purification. The recombinant plasmids (pET-H, pET-K, pET-I, pET-IIA^{Ntr}-1, and pET-IIA^{Ntr}-2) carrying the different *T. denticola pts* genes were transferred to the expression host Origami (DE3). Gene expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the LB broth once the OD_{600} reached 0.5. Cells were collected at an OD_{600} of 0.6 by centrifugation, washed 3 times, and resuspended in 25 mM Tris-HCl at pH 8.0 before disruption in a French press. All five proteins that except IIA^{Ntr}-1, which was produced in a soluble form, were recovered from inclusion bodies. HprK, Enzyme I, and IIA^{Ntr}-2 were then solubilized *in vivo* using a sorbitol (1 M)-betaine (2.5 mM) solution (*28*). IIA^{Ntr}-1 was recovered without treatment. HPr was solubilized with guanidinium·HCl followed by dialysis. In this last case (HPr), the inclusion bodies were fully denatured in 6 M guanidinium·HCl (45 min at 50 °C) and HPr was refolded by dialysis in four steps. In the first step, the dialysis buffer consisted of 0.5 M Tris-HCl at pH 8.0, 10 mM dithiothreitol (DTT), and 3 M guanidinium·HCl. In the second and third steps, the guanidinium·HCl concentration was decreased from 3 to 1.5 M and then from 1.5 to 0.8 M. In the last step, the concentration of the Tris-HCl buffer in the dialysis solution was reduced to 0.05 M and DTT and guanidinium·HCl were omitted, allowing completion of the refolding process. Each dialysis step was carried out over 24 h, and two changes of buffer were used in the last two steps. All renatured proteins as well as IIA^{Ntr}-1 were purified by affinity chromatography using nickel columns (Novagen) following the protocol recommended by the manufacturer. The EII^{Ntr}-2 was also cloned and overexpressed; however, we were not able to obtain sufficient pure soluble protein to conduct the *in vitro* phosphorylation experiments.

Protein Phosphorylation Assays. Unless otherwise indicated, the standard assay mixture for *in vitro* phosphorylation of HPr by HprK contained 40 mM Tris-maleate buffer at pH 7.8, 1 mM DTT, 5 mM $MgCl_2$, 0.2 mM fructose-1,6-bisphosphate, 0.5 mM [γ^{32}P]ATP (specific activity of 10^9 cpm μmol^{-1}), and 2−5 μg HPr and HprK in a final volume of 40 μL. Reaction mixtures were incubated at 37 °C for 20−30 min, stopped by adding PAGE sample buffer (*29*), and analyzed by denaturing electrophoresis. After determination of kinetic parameters, reactions were terminated by protein precipitation with trichloroacetic acid (*30*). Radioactivity in sliced bands from dried polyacrylamide gels and precipitated proteins was quantitated by liquid scintillation counting. All experiments were performed 3 times. HPr phosphorylation by PtsI was performed at 37 °C for 15−20 min in a reaction mixture containing 15 mM $MgCl_2$ in 25 mM Tris-HCl buffer at pH 7.4. Phosphoenolpyruvate was radiolabeled using pyruvate kinase as described by Roosien et al. (*31*).

Methyl α*-Glucoside Uptake*. *B. subtilis* strains were grown in C broth in the presence of glucose (0.5% w/v) to an OD_{600} of 0.5. Sugar uptake assays were performed as described previously (*32*). Exponentially growing cells were washed 3 times and temperature-equilibrated before the addition of labeled sugar (final concentration of 0.1 mM). Aliquots were periodically removed, and cells were filtered on 0.54 μm Millipore filters and washed 3 times with TM buffer before radioactivity was determined by scintillation counting (*32*). Values represent the averages of three independent determinations.

Mannitol Phosphorylation. PTS assays were conducted using cell-free extracts of *ptsI* mutant strains to evaluate the ability of *T. denticola* PtsI to complement the mutations. Extracts were obtained by passage of the bacterial cells through a French press at 10 000 psi. Samples were centrifuged for 10 min at 10 000 rpm, and the supernatant was used as the crude PTS enzyme preparation. The reaction mixture contained 36 μg of extract (total protein), 1−16 μg of pure *T. denticola* PtsI, 5 mM $MgCl_2$, 10 mM KF, 10 mM phosphoenolpyruvate, 1 mM DTT, 20 μM [^{14}C]mannitol, and 25 mM phosphate buffer at pH 7.0. The assay mixture was incubated at 37 °C for 1−30 min. The same conditions were used in the control experiments, but *T. denticola* PtsI was omitted. The resin used to separate [^{14}C]sugar from [^{14}C]-sugar−phosphate was AG1-X2, 50−100 mesh, chloride form (BioRad).

Enzymatic Determinations. For determination of myo-inositol or glucitol dehydrogenase activities, the *B. subtilis* wild-type strain and the isogenic *hprK* mutant were transformed with the pDG-HprK plasmid or the empty plasmid and cultivated in medium C (*26*), in the presence of 0.5% myoinositol or 0.5% sorbitol, respectively, with or without 0.5% glucose. For determination of gluconate kinase activity, cells were grown in LB in the presence of 0.5% gluconate with or without 0.5% glucose. The cells were disrupted by passage through a French press, and the enzymatic activities of myoinositol dehydrogenase (*33*), glucitol dehydrogenase (*34*) or gluconate kinase (*35*) were determined. Protein concentration was estimated using the BioRad protein assay kit.

RESULTS

Sequence Alignments and Comparisons. Table 3 presents the properties of *T. denticola* PTS proteins and compares them with those present in two other spirochetes. When the five *T. denticola* protein sequences were compared with those from *T. pallidum*, four of them showed high degrees of conservation. Percent identity values for the orthologous pairs

Table 3: Comparison of *T. denticola* PTS Proteins with the Homologous Enzymes Present in Other Spirochetes

		PtsI (42525603)[a]	HPr (42526803)	HprK (48374381)	IIA^{Ntr}-1 (42525581)	IIA^{Ntr}-2 (42526589)
T. denticola	length	595	88	329	163	201
T. pallidum	length	684	88	319	143	180
	% identity[b]	32	54	66	72	51
L. interrogans	length	610	87	321	160	not present
	% identity[b]	30	38	47	32	

[a] Genbank Index number of the *T. denticola* homologue. [b] Percent identity compared to the orthologous *T. denticola* protein.

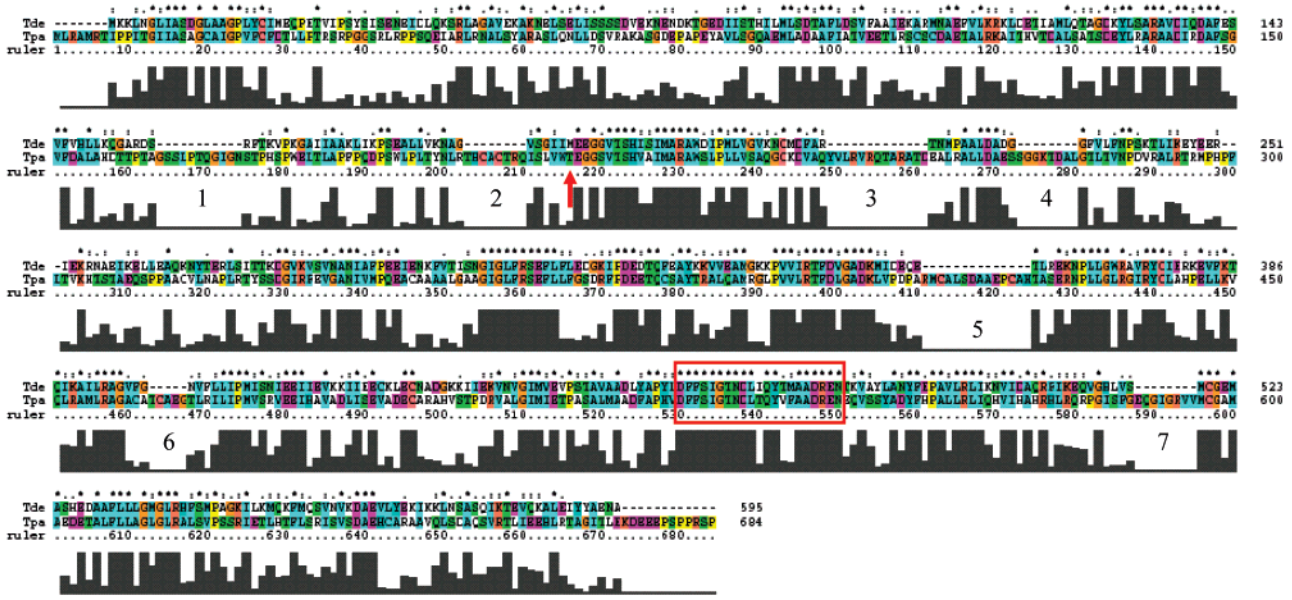

FIGURE 1: Binary alignment of the sequences of the *T. denticola* (Tde) and *T. pallidum* (Tpa) Enzyme I. The rectangle shows the enzyme signature present in both proteins in a region of high sequence identity. Alignment positions (ruler) are presented underneath the alignment. Residue number in each protein is presented at the end of each line. The bar graph beneath the alignment indicates the degree of conservation at each position. *, identity; :, close similarity; ., distant similarity. The arrow indicates the position of the frameshift mutation in the *T. pallidum* PtsI. The seven insertions in the *T. pallidum* Enzyme I are numbered 1−7.

proved to be higher than 50% for HPr, IIA^{Ntr}-1, IIA^{Ntr}-2 and HprK, but the percent identity observed for the Enzyme I orthologues was much lower (Table 3).

T. denticola PtsI is 595 amino acyl residues long and shows more than 35% identity with several Gram-positive Enzymes I such as PtsI from *B. halodurans*. When *T. denticola* PtsI was compared with the putative *T. pallidum* PtsI, the latter protein proved to be 89 residues longer and the two proteins exhibited only 32% sequence identity. These two proteins had the highest percent identity in the region of the PtsI signature sequence (DFFSIGTNDLiQYtmAA-DREN) corresponding to residues 461−481 in the *T. denticola* protein (rectangle in Figure 1). Seven gaps were found to interrupt the alignment as a result of short insertions (6−14 residues each) in the *T. pallidum* sequence. Because (1) these insertions are not present in other Enzymes I, (2) all *pts* genes in both *T. denticola* and *T. pallidum* exhibit strong promoters in front of their structural genes with the sole exception of the *T. pallidum ptsI* gene, and (3) only the *T. pallidum ptsI* gene exhibits a documented frameshift mutation (position 216; see arrow in Figure 1), we conclude that *ptsI* of *T. pallidum* is an inactive pseudogene. As shown below, the *ptsI* gene product of *T. denticola* is enzymatically active.

Purification of PTS Proteins from T. denticola. As noted above, the *T. denticola* genome encodes five PTS-related proteins: Enzyme I, HPr, two IIA^{Ntr} proteins, and HprK (*7*). All five genes encoding these proteins were PCR-amplified using the primers shown in Table 2 and cloned into pET-28A^{+} (Novagen, Inc.) for overproduction of the His-tagged proteins in the *E. coli* Origami strain (Table 1) (see the Experimental Procedures). All native and refolded proteins were applied to a nickel-affinity column and eluted with 1 M imidazole as described by Novagen Inc. Figure 2 shows the results of SDS−PAGE analyses for all of these purified His-tagged proteins. The proteins migrated with apparent molecular weights of roughly 70, 12, 38, and 22 kDa for EI, HPr, HprK, and IIA^{Ntr}-1, respectively. No contamination was detected in any of these preparations.

Phosphorylation of HPr with [^{32}P]ATP and HprK. Using [^{32}P]ATP as the phosphoryl donor, phosphorylation of *T. denticola* and *B. subtilis* HPr proteins by both the *T. denticola* and *B. subtilis* HprKs could be demonstrated (Figure 3). In lane 1, phosphorylation of the wild-type *B. subtilis* HPr by *T. denticola* HprK is shown. Inclusion of the same concentration of the S46D mutant *B. subtilis* HPr protein did not give rise to a radioactive band (lane 2), suggesting that, as expected, serine-46 in the wild-type protein is phosphorylated (*20*).

When the *B. subtilis* Crh protein was the substrate with the *T. denticola* HprK, phosphorylation was also demonstrated (lane 3). The *E. coli* HPr, which cannot be phosphorylated by HprKs from Gram-positive bacteria, was not phosphorylated by the *T. denticola* HprK (lane 4). When the

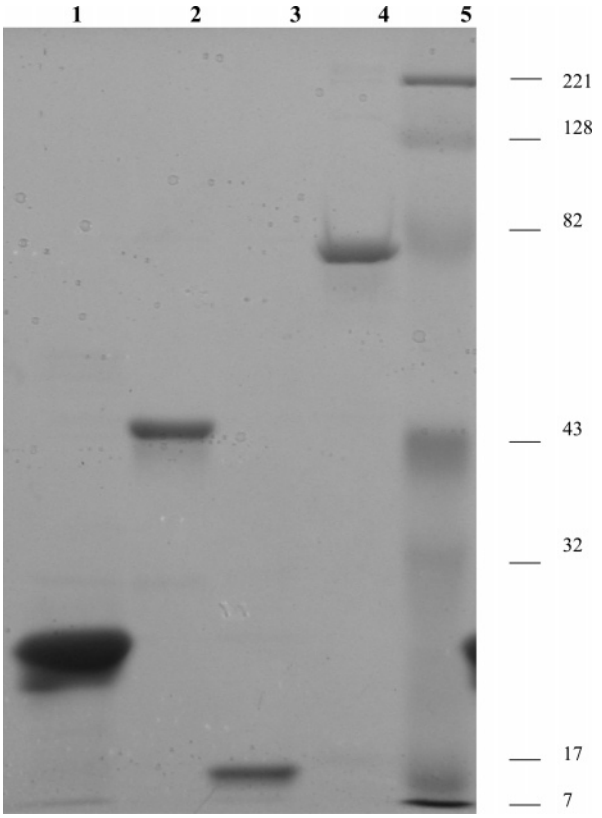

FIGURE 2: PAGE of purified *T. denticola* proteins. Lanes (1) IIA^{Ntr}-1, (2) HprK, (3) HPr, (4) PtsI, (5) kaleidoscope prestained molecular weight markers (BioRad). IIA^{Ntr}-2 was not purified to homogeneity but runs together with IIA^{Ntr}-1 (data not shown). The gel was stained using Coomassie Tablets, PhastGel Blue R-350.

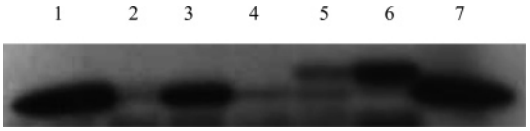

FIGURE 3: Autoradiogram of an SDS gel showing the phosphorylation of heterologous HPr proteins by the HprK (2 μg) purified from *T. denticola* as follows: lane (1) *B. subtilis* HPr (1 μg), (2) *B. subtilis* HPr S46D (2 μg), (3) *B. subtilis* Crh (2 μg), and (4) *E. coli* HPr (2 μg). The reciprocal assay is shown in lane 5 using *B. subtilis* PtsK (2 μg) with *T. denticola* HPr (3 μg). Lane 6 shows the positive control using *T. denticola* HprK (2 μg) and *T. denticola* HPr (3 μg). Lane 7 shows the positive control using *B. subtilis* HprK (0.5 μg) and *B. subtilis* HPr (0.5 μg). The phosphorylation assays were conducted as described under the Experimental Procedures.

reciprocal experiment was conducted with *B. subtilis* HprK and *T. denticola* HPr, a low level of HPr phosphorylation was detected (lane 5). Finally, as expected, HprKs from both *T. denticola* and *B. subtilis* could phosphorylate their own HPr's efficiently (lanes 6 and 7, respectively). These experiments demonstrate that HprK from *T. denticola* catalyzes HPr phosphorylation, as do typical HprKs from low G+C Gram-positive bacteria, and that the *T. denticola* and *B. subtilis* systems exhibit enzymatic cross reactivities.

T. denticola HprK activity was measured as a function of pH (Figure 4). The enzyme was essentially inactive below pH 6, but activity increased dramatically above pH 6.5 until maximal activity was observed at pH 8.5. Different behavior has been reported for Gram-positive bacterial HprKs. For example, the *Streptococcus pyogenes* HprK shows a pH optimum at 7.0 (*30*).

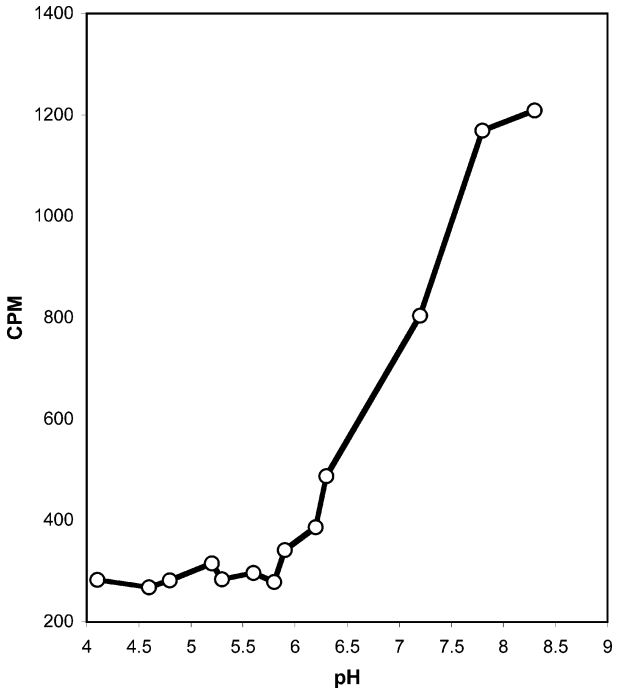

FIGURE 4: pH curve for *T. denticola* HPr phosphorylation by *T. denticola* HprK. The assays were performed using the standard reaction mixture. Different pH values were obtained using overlapping range buffers, with a 40 mM final concentration, citrate-citric acid from pH 4 to 6.2 and Tris-maleate from pH 5.2 to 8.3.

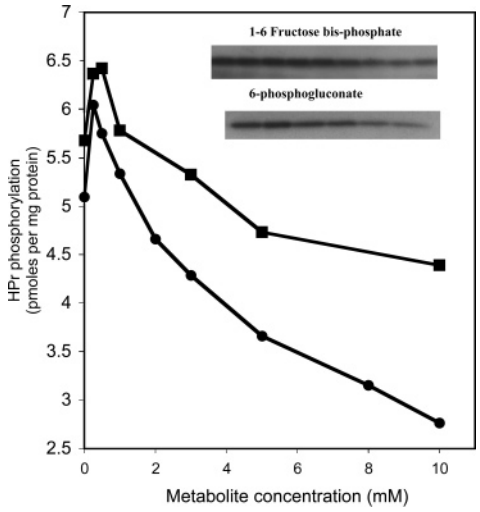

FIGURE 5: Concentration-dependent modulation of HprK activity by fructose-1,6-bisphosphate (●) and 6-phosphogluconate (■). The standard reaction mixture was as described under the Experimental Procedures. The varying concentrations of the intermediary metabolites were as indicated. Incubation was for 20 min at 37 °C. [^{32}P]HPr was quantified from bands sliced from the gel following SDS−PAGE (see insets).

In low G+C Gram-positive bacteria, HprK activity is allosterically activated by fructose-1,6-bisphosphate (FBP) and less effectively by gluconate-6-P (Gnt-P) (*30*, *36*−*38*). The *T. denticola* kinase was similarly activated by these two phosphorylated compounds, but unexpectedly, activity was very substantial in their absence. Moreover, FBP and Gnt-P exhibited maximal activation at concentrations of 0.5 and 0.2 mM, respectively, while inhibiting strongly at higher concentrations (Figure 5). Under the conditions used with 5 mM $MgCl_2$ present in Tris-maleate buffer at pH 7.8, FBP inhibited much more strongly than did Gnt-P, demonstrating

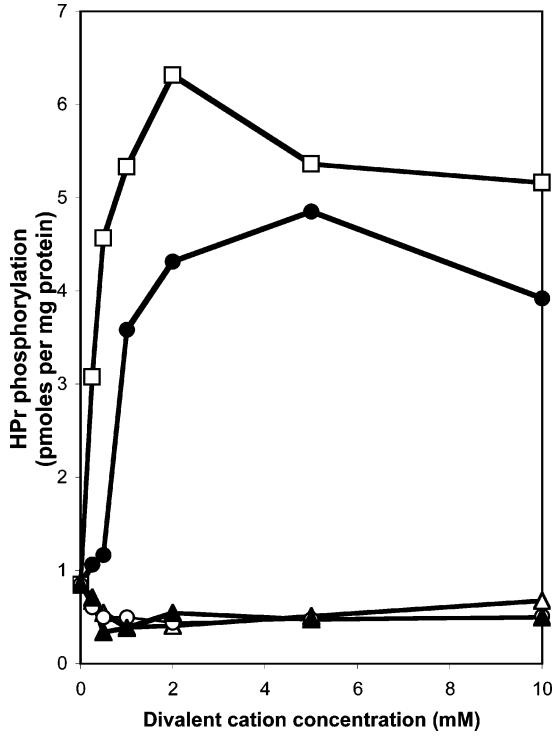

FIGURE 6: Concentration-dependent modulation of HprK activity by divalent cations added to the reaction mixture in the chloride form. Mg (●), Mn (□), Zn (△), Co (○), and Ca (▲). The pH was controlled using 40 mM Tris-maleate buffer at pH 8.3. Fructose-1,6-bisphosphate was used at the optimal concentration (200 μM) for the *T. denticola* HprK. The assays were performed for 20 min at 37 °C using 2 μg of the purified HprK and 2 μg of HPr. Samples were processed and quantified using the technique described by Reizer et al. (*30*).

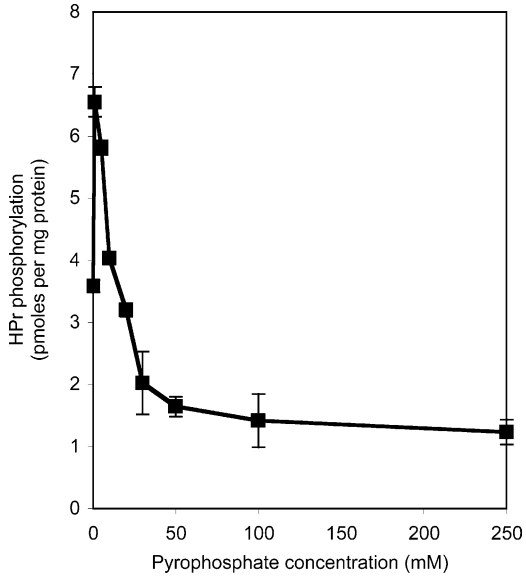

FIGURE 7: Effects of different concentrations of sodium pyrophosphate on HprK activity. The reaction mixture contained 2 μg each of HPr and HprK, fructose-1,6-bisphosphate (200 μM), $MgCl_2$ (5 mM), DTT (1 mM), and [γ^{32}P]ATP (0.5 mM) in 40 mM Tris-maleate buffer at pH 8.3. The reaction mixture was incubated at 37 °C for 20 min, and the product was quantified as described by Reizer et al. (*30*).

specificity of the interaction. The optimal concentrations of FBP and Gnt-P for activation of *T. denticola* HprK are approximately 20-fold lower than for the *B. subtilis* enzyme (*37*, *39*). No significant activation of HPr phosphorylation was observed when several other metabolic intermediates were added. These included glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1-phosphate, 2-phosphoglycerate, 3-phosphoglycerate, phosphoenolpyruvate, 2-deoxyglucose-6-phosphate, mannose-6-phosphate, and glucitol-6-phosphate, all tested at a concentration of 0.5 mM. HPr phosphorylation proved to be strongly inhibited by 0.5 mM inorganic phosphate (data not shown).

The dependency of *T. denticola* HprK activity on divalent cations was next investigated (Figure 6). Only Mg^{2+} and Mn^{2+} activated the enzyme for HPr phosphorylation, and their optimal concentrations were 5 and 2 mM, respectively. Zn^{2+}, Co^{2+}, and Ca^{2+} were not effective activators and instead seemed to be inhibitory.

Inorganic pyrophosphate, the product of the phosphorolysis reaction (*18*) activated HprK activity at a very low concentration (1 μM), but higher concentrations were strongly inhibitory (Figure 7). Surprisingly, inhibition never reached 100%, leading to the suggestion that inhibition is noncompetitive. Pyrophosphate may be capable of phosphorylating HPr(Ser), in a reaction that constitutes reversal of the phosphorolysis reaction (*19*).

The kinetics of HPr phosphorylation as a function of both ATP and HPr concentrations was studied as shown in parts A and B of Figure 8, respectively. In these two experiments, the concentrations of HPr and ATP were maintained constant at 200 μM (A) and 500 μM (B), respectively. Consequently, the K_m values calculated (18 μM for ATP and 29 μM for HPr) were determined in the presence of essentially saturating concentrations of the second substrate. These K_m values should therefore be close to the absolute K_m values. Corresponding values reported for the *B. subtilis* and *S. pyogenes* enzymes are roughly 60−80 μM for both ATP and HPr (*20*, *40*). Thus, the *T. denticola* enzyme shows a 2−3-fold higher affinity for its substrates than has been reported for the low G+C Gram-positive bacterial enzymes. Its kinetic properties more closely resemble those of HprK from *Mycoplasma pneumonia*, which appears to be in the default kinase mode rather than the default phosphorylase mode, as observed for the *B. subtilis* and *S. pyogenes* HprKs (*41*) (see the Discussion).

Phosphorylation of HPr and IIA^{Ntr}-1 with Enzyme I. Using [^{32}P]phosphoenolpyruvate, the purified *T. denticola* Enzyme I was used to demonstrate phosphorylation of itself, HPr, and IIA^{Ntr}-1 (Figure 9). On SDS−PAGE, a weak band for Enzyme I (PtsI; approximate molecular weight of 70 kDa) was clearly visible when [^{32}P]phosphoenolpyruvate was incubated with pure Enzyme I. A stronger band for HPr (approximate molecular weight of 10 kDa) could be demonstrated by autoradiography when only these two proteins were present in the reaction mixture (data not shown). When these proteins plus IIA^{Ntr}-1 were incubated under the same conditions, phosphorylated bands corresponding to all three of these proteins could be observed (Figure 9). No phosphorylated band was observed when HPr or IIA^{Ntr}-1 was incubated with [^{32}P]phosphoenolpyruvate either separately or together. It therefore appears that Enzyme I∼P phosphorylates HPr and HPr∼P phosphorylates IIA^{Ntr}-1 in a typical sequence of PTS phosphoryl-transfer reactions. We conclude that *T. denticola* Enzyme I, HPr, and IIA^{Ntr}-1 are catalytically active and comprise a single phosphoryl-transfer

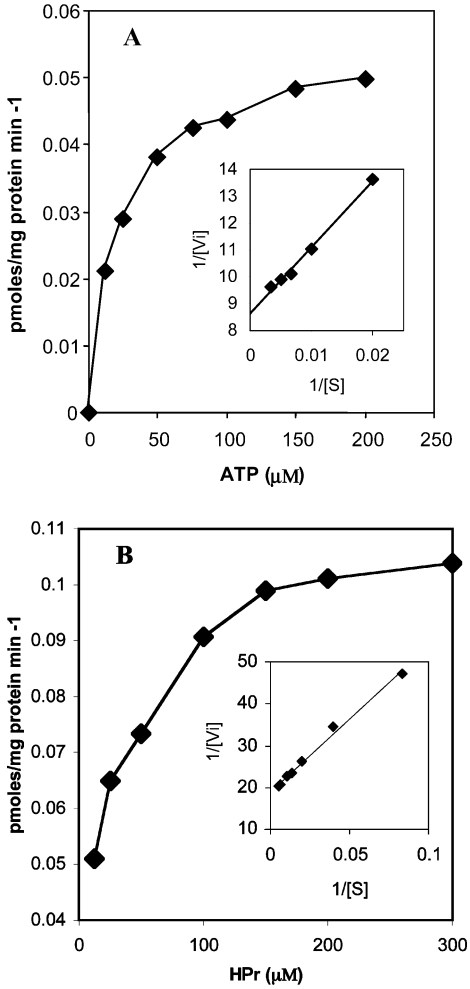

FIGURE 8: Kinetic analyses of HPr phosphorylation by HprK. (A) ATP dependency of HPr phosphorylation using an excess of purified HPr (200 μM). (B) HPr dependency of HPr phosphorylation using an excess of [γ^{32}P]ATP (500 μM). The 40 μL of reaction mixture contained 5 mM $MgCl_2$, 200 μM fructose-1,6-bisphosphate, and 1 mM DTT in 40 mM Tris-maleate buffer at pH 7.8, with the variable concentrations of [γ^{32}P]ATP and HPr indicated in the figure. The apparent kinetic parameters (K_m values) determined were 18 and 28 μM for ATP and HPr, respectively. Insets, Lineweaver−Burk regression plots. Assays were carried out at 37 °C for 30 min in triplicate, and all samples were quantified using the method of Reizer et al. (*30*).

chain comparable to the nitrogen regulatory PTS phosphoryl-transfer chain in *E. coli* (*24*).

When IIA^{Ntr}-2 was overexpressed, inclusion bodies resulted that were not easily solubilized. The protein obtained after affinity chromatography was in a low concentration and was contaminated with several other proteins. Consequently, we could not demonstrate its capacity to accept a phosphoryl group from HPr(His)-P.

Using a *ptsI* mutant of *Salmonella typhimurium*, we could show that Enzyme I of *T. denticola* could replace the mutated enzyme from this enteric bacterium using a sugar phosphorylation complementation assay (Figure 10A). [^{14}C]Mannitol phosphorylation was linear with time in the presence of the purified *T. denticola* Enzyme I (Figure 10B). This finding is of considerable interest, because *T. denticola* appears to lack all of the known sugar-phosphorylating Enzyme II complexes. The results suggest that *T. denticola* Enzyme I can slowly phosphorylate HPr from enteric bacteria. This

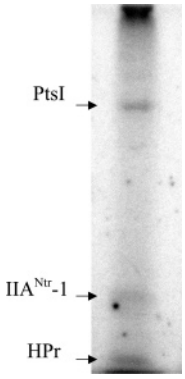

FIGURE 9: *In vitro* phosphorylation of *T. denticola* PTS proteins PtsI, HPr, and IIA^{Ntr}-1 using [^{32}P]phosphoenolpyruvate as the phosphoryl donor. The phosphorylation experiment was carried out using 3 μg of each purified PTS enzyme. A final reaction mixture of 40 μL contained 50 mM Tris-HCl at pH 7.4, 15 mM $MgCl_2$, and 0.5 mM [γ^{32}P]phosphoenolpyruvate. The reaction was performed at 37 °C for 20 min and stopped by addition of Laemmli buffer. After separation by PAGE, the radioactive bands were visualized with a phosphoimager.

situation may be analogous to the *E. coli* Enzyme I paralogue, Enzyme I^{Ntr}, which can phosphorylate HPr and PTS permease proteins at rates that are less than 1% of the rates at which these proteins are phosphorylated by Enzyme I (*24*).

In Vivo HprK Complementation Studies in B. subtilis. The loss of HprK in *B. subtilis* results in depressed methyl-α-glucoside uptake rates, probably because of poor expression (inefficient antitermination) of the *ptsG* gene (*42*, *43*, and unpublished results). The results shown in Figure 11 reveal that inclusion of a high-level expression plasmid (pDG148-*stuI*) bearing the *hprK* gene of *T. denticola* partially restored methyl-α-glucoside uptake rates. The uptake rate observed for the complemented mutant strain was 3−4-fold higher than for the *B. subtilis hprK* mutant. However, it was still substantially lower than for the wild-type strain.

Complementation of a B. subtilis ptsH Mutant with the T. denticola ptsH Gene. HPr is required for catabolite repression of many operons in *B. subtilis* (*44*, *45*). A *ptsHI* deletion mutant of *B. subtilis* was therefore employed to determine if the *T. denticola* HPr could replace the *B. subtilis* homologue in promoting catabolite repression in a *ptsH* deletion mutant. The *T. denticola ptsH* gene was expressed using the pDG148 plasmid. As shown in Table 4, expression of the *ptsH* gene from *T. denticola* in the *ptsHI* deletion mutant of *B. subtilis* resulted in a 30−40% glucose-promoted decrease in the activity levels of three enzymes encoded within operons that are known to be subject to HPr(Ser)-P-dependent catabolite repression. These enzymes are inositol dehydrogenase, glucitol dehydrogenase, and gluconate kinase (*46*). The results are in accordance with the conclusion reported above (see Figure 3) that *T. denticola* HPr can be phosphorylated by the *B. subtilis* HprK. It appears that this phosphorylated protein can interact with *B. subtilis* CcpA to mediate weak catabolite repression of several operons although *T. denticola* lacks CcpA (*23*).

DISCUSSION

Several bacteria including three pathogenic spirochetes with fully sequenced genomes, *T. pallidum*, *T. denticola*, and *L. interrogans*, as well as the green bacterium, *C. tepidum*, and the δ-proteobacterium, *G. metallireducens*, encode within

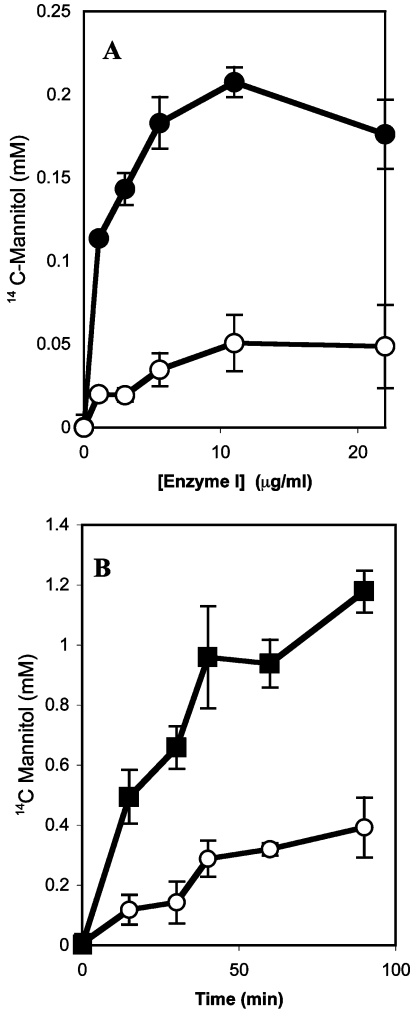

FIGURE 10: *In vitro* PTS complementation of an *S. typhimurium* strain SB1477 (*ptsI18*) cell-free extract using pure *T. denticola* PtsI. Sugar phosphorylation was measured using [^{14}C]mannitol, which is phosphorylated only by the PTS. (A) Reaction mixture consisted of an *S. typhimurium* SB1477 cell-free extract (180 μg/mL total protein) plus increasing amounts of pure *T. denticola* PtsI. (○) Control experiment with bovine serum albumin. (●) Phosphorylated [^{14}C]mannitol recovered after ion-exchange chromatography when *T. denticola* PtsI was added to the reaction mixture. Incubation was at 37 °C for 15 min. (B) Time course, [^{14}C]mannitol phosphorylation by cell-free extracts (180 μg/mL total protein) of *S. typhimurium* SB1477 (*ptsI18*) (○) supplemented with pure *T. denticola* PtsI (10 μg/mL) (■).

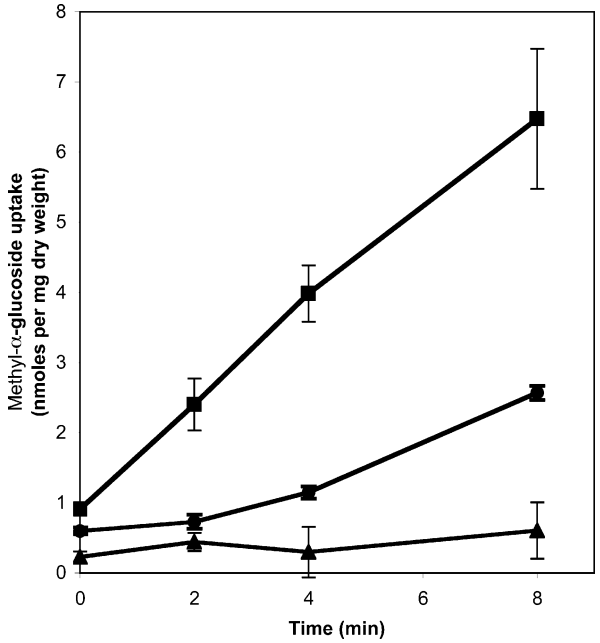

FIGURE 11: Restoration of [^{14}C]methyl-α-glucoside transport in a *B. subtilis* HprK-negative mutant. *B. subtilis* BAL218 (■), *B. subtilis* GM202 carrying plasmid pDG148-*hprK* bearing the *hprK* gene of *T. denticola* (●), *B. subtilis* GM202 carrying control plasmid pDG148-*stuI* (empty plasmid) (▲).

Table 4: Restoration of Catabolite Repression by Inclusion of the *T. denticola ptsH* Gene in a *ptsHI* Mutant of *B. subtilis* Strain GM273[a]

	wild type	GM273 pDG-148-*stul*	GM273 pDG-HprK
inositol dehydrogenase			
glucose−inositol	<0.2 ± 0.1	6.3 ± 0.1	4.1 ± 0.2
inositol	6.4 ± 0.3	6.7 ± 0.3	6.2 ± 0.4
gluconate kinase			
glucose−gluconate	0.2 ± 0.1	2.1 ± 0.1	1.0 ± 0.1
gluconate	2.1 ± 0.2	2.2 ± 0.1	1.9 ± 0.1
glucitol dehydrogenase			
glucose−glucitol	<0.2 ± 0.1	2.1 ± 0.2	1.1 ± 0.1
glucitol	2.0 ± 0.3	2.4 ± 0.2	1.9 ± 0.1

[a] The *T. denticola ptsH* gene was cloned into the pDG-148-*stuI* expression vector and expressed in the *B. subtilis* GM273Δ*ptsHI* mutant. Expression of *T. denticola ptsH* was achieved by addition of 1 mM IPTG to a synthetic medium (medium C) for inositol or glucitol dehydrogenase measurements or LB medium for gluconate kinase measurements supplemented with the sugars listed in the first column. Cells were recovered by centrifugation after the culture reached an optical density of 0.7 at 600 nm. After the cells were washed and crude extracts were prepared, enzyme activities were measured as described under the Experimental Procedures. Results are expressed in nanomoles min^{-1} mg of protein^{-1}.

their genomes Enzyme I, HPr, the HPr kinase/phosphorylase, and either one or two IIA^{Ntr} homologues of the phosphoenolpyruvate-dependent phosphotransferase system (*7*, *54*). However, these same bacteria do not encode homologues of any other recognized PTS proteins including PTS permeases (Enzyme II complexes) (*47*). This fact leads to the possibility that these organisms do not use the PTS energy coupling proteins for sugar phosphorylation but instead use these proteins for strictly regulatory purposes. This suggestion is in agreement with published work in *E. coli* showing that this enteric bacterium possesses at least two distinct phosphoryl-transfer chains, one involving Enzyme I, HPr, and sugar-transporting Enzyme II complexes for the phosphorylation of sugars, and a second one involving Enzyme I^{Ntr}, NPr, and IIA^{Ntr} for the coordination of nitrogen metabolism with carbon metabolism (*16*, *24*). Although several publications are in agreement with this suggestion (*48*, *49*), the detailed mechanism of this nitrogen regulatory process in *E. coli* is still poorly understood. Because the *T. pallidum ptsI* gene is an inactive pseudogene and because *E. coli* lacks an *hprK* gene, it is unlikely that the regulatory phosphoryl-transfer mechanism operative in the spirochetes is mechanistically at all similar to those in *E. coli*.

Kinetic analyses of the HprK-catalyzed phosphorylation of HPr in *T. denticola* revealed several characteristics that differed from the *B. subtilis* and *S. pyogenes* enzymes (*20*, *40*). Thus, (1) the high kinase activity in the absence of a glycolytic metabolite, (2) high affinities for ATP and HPr, (3) a minimal stimulatory effect of fructose-1,6-bisphosphate, and (4) the strong inhibitory effects of inorganic phosphate and pyrophosphate all suggest that HprK of *T. denticola* is

active as a kinase in the absence of metabolic intermediates as has been suggested for the *Mycoplasma pneumoniae* HprK (*41*). HprKs from low G+C Gram-positive bacteria appear to exhibit phosphorylase activity in the absence of allosteric regulatory metabolites (*18*, *20*, *40*, *41*). In this respect, it is interesting to note that, while the low G+C Gram-positive bacteria use HPr(Ser)-P as a CcpA-dependent catabolite corepressor, the *Mycoplasma* and Gram-negative bacteria appear to lack CcpA (*19*, *23*). Perhaps the default mode of HprK (kinase or phosphorylase) reflects the mode of action of and the target proteins regulated by its product of phosphorylation, HPr(Ser)-P.

As noted above, a target of *E. coli* Enzyme I^{Ntr} and NPr phosphorylation is IIA^{Ntr} (*24*). IIA^{Ntr} and NPr are encoded within an operon that also encodes the RNA polymerase sigma factor, sigma-54 (*16*), but Enzyme I^{Ntr} is encoded within a distinct operon (*50*). Sigma-54 plays a primary role in the transcription of nitrogen metabolic genes in *E. coli*. However, the mechanism by which this putative nitrogen regulatory PTS phosphoryl-transfer chain senses carbon availability is largely unknown. Until recently, it had been assumed that the carbon-sensing device in *E. coli* requires the participation of the sugar-transporting Enzyme II complexes of the PTS. This could, for example, occur by a mechanism that involves competition by the two PTS phosphoryl-transfer chains for the energy-rich phosphoryl donor of both chains, phosphoenolpyruvate (*24*). The work reported here, however, sheds doubt on this postulate.

The discovery that certain spirochetes and various other bacteria have nitrogen-type PTS phosphoryl-transfer chains but lack PTS enzymes involved in sugar transport leads to two possibilities. (1) If the mechanism of carbon sensing by this nitrogen-related phosphoryl-transfer chain in *E. coli does* involve the sugar-specific Enzyme II, then the mechanism of action of the corresponding proteins in *Treponema* species must differ from that in *E. coli*. Alternatively, (2) it is possible that both the *E. coli* and *T. denticola* phosphoryl-transfer chains use a mechanism that is independent of PTS-mediated sugar phosphorylation. These two alternatives represent hypotheses to guide future studies in both organisms.

We showed that both *T. pallidum* and *T. denticola* have genomes encoding the same PTS proteins. All evidence suggests that these genes are orthologous. Sequence analyses, however, clearly indicate that, in contrast to Enzyme I in *T. denticola*, that in *T. pallidum* is inactive and that the encoding *ptsI* gene is a pseudogene. Evidence for this conclusion is as follows. (1) Seven insertions are found in the *T. pallidum ptsI* gene that are not present in the *T. denticola ptsI* gene or in any other functionally characterized bacterial *ptsI* gene (Figure 1). (2) This *T. pallidum* gene contains a frameshift mutation not present in a functionally active *ptsI* gene. (3) The *T. pallidum ptsI* gene has undergone sequence divergence much more rapidly that expected for a functional *ptsI* gene and more rapidly than for the other four *pts* genes in *T. pallidum*. (4) Of all of the *pts* genes in *T. pallidum* and *T. denticola*, only the *T. pallidum ptsI* gene lacks a good promoter. These four independent lines of evidence clearly suggest that PtsI of *T. pallidum* is nonfunctional.

The facts that the genes for all others PTS proteins in *T. pallidum* are (1) apparently intact, (2) have undergone slow sequence divergence, and (3) have good promoters all suggest that these genes are actively transcribed and encode functional proteins. If it is assumed that the functions of these orthologous PTS phosphoryl-transfer chains are essentially the same in these two *Treponema* species, then it follows that Enzyme I is not essential for the regulatory function(s) that these chains perform. We therefore put forth the following postulates. (1) The target(s) of regulation interact with and are primarily controlled by HPr or HPr(Ser)-P. (2) HPr(Ser) phosphorylation is catalyzed exclusively by HprK in the presence of ATP. (3) IIA^{Ntr}-1 and IIA^{Ntr}-2 modulate HPr(Ser) phosphorylation and/or the interaction of either HPr or HPr(Ser)-P with one or more protein targets that bind one of these HPr forms, either to HPr or HPr(Ser)-P. (4) In *T. denticola*, but not in *T. pallidum*, Enzyme I plays a secondary regulatory role by phosphorylating the active-site histidyl residue in HPr, thereby inhibiting phosphorylation of the seryl residue (*17*, *19*, *30*). It is possible that HPr or HPr(Ser)-P has a primary function of regulating a transcriptional event. However, it is equally possible that the phosphorylation state of this protein controls a metabolic target, e.g., a transport protein or catabolic enzyme. Further studies are in progress to determine the biochemical targets and the physiological functions of this unique phosphoryl-transfer chain in *T. denticola*.

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