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In Vivo Activity of Enzymatic and Regulatory Components of the Phosphoenolpyruvate:Sugar Phosphotransferase System in *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae **is a pathogenic bacterium that is highly adapted to life on mucosal surfaces. This adaptation is reflected by the very compact genome and the small number of regulatory proteins. However, *M. pneumoniae* possesses the HPr kinase/phosphorylase (HPrK/P), the key regulator of carbon metabolism in the *Firmicutes*. In contrast to the enzymes of other bacteria, the HPrK/P of *M. pneumoniae* is already active at very low ATP concentrations, suggesting a different mode of regulation. In this work, we studied the ability of *M. pneumoniae* to utilize different carbohydrates and their effects on the activity of the different phosphotransferase system (PTS) components. Glucose served as the best carbon source, with a generation time of about 30 h. Fructose and glycerol were also used but at lower rates and with lower yields. In contrast, *M. pneumoniae* is unable to use mannitol even though the bacterium is apparently equipped with all the genes required for mannitol catabolism. This observation is probably a reflection of the continuing and ongoing reduction of the *M. pneumoniae* genome. The general enzymatic and regulatory components of the PTS, i.e., enzyme I, HPr, and HPrK/P, were present under all growth conditions tested in this study. However, HPrK/P activity is strongly increased if the medium contains glycerol. Thus, the control of HPrK/P in vivo differs strongly between *M. pneumoniae* and the other *Firmicutes*. This difference may relate to the specific conditions on lipid-rich cell surfaces.**

Mycoplasma pneumoniae is a pathogen that lives on mucosal surfaces and causes diseases such as mild pneumonia and tracheobronchitis and also causes complications affecting the central nervous system, the skin, and mucosal surfaces (19, 24). The parasitic lifestyle of this bacterium is reflected by its small and highly compacted genome, its slow growth, and its reduced metabolic abilities. With only nine regulatory proteins, *M. pneumoniae* belongs to the organisms with the lowest number of regulators studied so far, suggesting a good adaptation to constant environments (5, 15, 37). In addition to regulatory proteins that are thought to act at the DNA level, we identified the key regulatory protein of carbon metabolism in gram-positive bacteria, HPr kinase/phosphorylase (HPrK/P), in *M. pneumoniae* (33, 42). Moreover, HPrK/P activity was detected in other *Mollicutes* such as *Mycoplasma capricolum*, *Mycoplasma genitalium*, and *Acholeplasma laidlawii* (17, 53).

HPrK/P controls the activity of the HPr protein of the bacterial phosphoenolpyruvate:sugar phosphotransferase system by phosphorylation at a regulatory site, Ser-46. In the gram-positive model organism *Bacillus subtilis*, this phosphorylation interferes with the phosphoenolpyruvate (PEP)- and enzyme I-dependent phosphorylation on His-15 of HPr, which is important for the phosphorylation of transported sugars (12, 38). In addition to its role in sugar transport, HPr is the major signal transducer in carbon metabolism in low-GC gram-positive bacteria (now referred to as *Firmicutes* [27]). In the absence of glucose, HPr is present, either nonphosphorylated or phosphorylated, at His-15. If glucose becomes available, a significant part of the cellular HPr pool is phosphorylated on Ser-46, and even some doubly phosphorylated HPr(His~P)(Ser-P) was detected (25, 32; for a review, see reference 44). HPr(His~P) is implicated in sugar transport and is moreover required for the activation of a class of transcription factors and of glycerol kinase in several bacteria (6, 43). In contrast, HPr(Ser-P) is not able to phosphorylate and thereby activate those enzymes and regulators but rather acts as a cofactor for the transcription regulator CcpA. The CcpA-HPr(Ser-P) complex binds to target sequences in the promoter regions of catabolic and certain anabolic operons to repress or activate their transcription (7, 26, 49). Thus, HPrK/P controls the phosphorylation state of HPr and thereby the regulatory activity of this protein. It is therefore crucial to study the activity of HPrK/P itself. In *B. subtilis*, the enzyme is active as a kinase under conditions of good nutrient supply which are indicated by high ATP and fructose-1,6-bisphosphate concentrations. In contrast, phosphorylase activity is triggered by high concentrations of inorganic phosphate, which indicate the absence of good carbon sources (12, 14, 21, 29, 38).

As stated above, several metabolic and regulatory features of the *Mollicutes* are in good agreement with their adaptation to their nutrient-rich mucosal habitats. This was also observed when we investigated the properties of *M. pneumoniae* HPrK/P. While the *B. subtilis* enzyme exhibits a phosphorylase activity by default, the *M. pneumoniae* protein is already active as a kinase at very low ATP concentrations and is barely reg-

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ulated by fructose-1,6-bisphosphate (14, 42). These differences were attributed to the different affinities of *B. subtilis* and *M. pneumoniae* HPrK/P for ATP. While the former has a K_d value of about 100 to 300 μM, the latter has a K_d value of about 5 μM, indicating an at least 20-fold-increased affinity (21, 30, 36). The high affinity of *M. pneumoniae* HPrK/P for ATP results in a kinase activity as the apparent default state of this protein in vitro (42). Since the *M. pneumoniae* HPrK/P is the only known enzyme of its class with the inversed default activity, we wondered whether the aberrant regulation was reflected by the structure of the protein. The determination of the crystal structure revealed that the enzyme is composed of six identical subunits that are arranged as bilayered trimers. Each subunit is made up of a C-terminal domain that contains the ATP-binding P-loop motif and an N-terminal domain of so-far-unknown function (1, 41). The structure of the *M. pneumoniae* HPrK/P is very similar to those of HPrK/Ps from *Lactobacillus casei* and *Staphylococcus xylosus*, suggesting that subtle differences must be responsible for the differential activity patterns (9, 28, 33). Although HPrK/P is one of the very few regulatory proteins of *M. pneumoniae*, it is not essential as revealed by an analysis of randomly generated transposon mutants (18).

According to the genome sequence of *M. pneumoniae* and the biochemical evidence, these bacteria are able to utilize sugars as carbon sources by glycolysis (5, 15, 31). As observed for other *Firmicutes*, the concentration of fructose-1,6-bisphosphate is increased in glycolytically active cells of *M. gallisepticum* (8, 29). Moreover, enzymes of carbon metabolism seem to be important for other metabolic pathways as well. This is illustrated by the finding that the glycolytic kinases of several *Mollicutes* are moonlighting in nucleoside metabolism (35).

So far, very few studies concerning the regulation of carbon metabolism in *Mollicutes* have been reported. However, this problem is important not only for a better understanding of the biology of these interesting bacteria but also to improve our knowledge of virulence mechanisms of the mycoplasmas. Recently, the implication of proteins of the phosphotransferase system in *M. pneumoniae* pathogenicity was demonstrated (54). While the regulatory output of the phosphotransferase system (PTS) is well understood in *Escherichia coli* and in the *Firmicutes* related to *B. subtilis*, nothing is known about regulatory pathways in *M. pneumoniae*. Among the proteins interacting with the different forms of HPr in *B. subtilis*, only the glycerol kinase is present in *M. pneumoniae*, whereas transcription regulators potentially phosphorylated by HPr(His~P) are not found. Similarly, the transcription factor CcpA that interacts with HPr(Ser-P) has no counterpart in the *Mollicutes* (16). Thus, the mechanisms of carbon regulation, if present, must differ drastically from those studied in *B. subtilis* and its close relatives.

In this work, we studied the utilization of different carbohydrates by *M. pneumoniae* and found that glucose was the carbon source allowing the fastest growth. To address the relevance of the results obtained with *M. pneumoniae* HPrK/P in vitro, we analyzed the HPr phosphorylation state in vivo. Surprisingly, the enzyme did not exhibit constitutive kinase activity but required the presence of glycerol for HPr phosphorylation. The proteins acting on HPr, i.e., enzyme I of the PTS and HPrK/P, were constitutively present in cell extracts of *M. pneumoniae*. Thus, a novel mode of control seems to modulate the *M. pneumoniae* HPrK/P activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α and BL21(DE3)/pLysS (40) were used for overexpression of recombinant proteins. The cells were grown in Luria Bertani medium containing ampicillin (100 μg ml^{-1}).

The *M. pneumoniae* strain used in this study was *M. pneumoniae* M129 (ATCC 29342) in the 31st broth passage. *M. pneumoniae* was grown at 37°C in 150-cm^2 tissue culture flasks containing 100 ml of modified Hayflick medium with the following composition. The basic medium consisted of 18.4 g of PPLO broth (Difco), 29.8 g of HEPES, 5 ml of 0.5% phenol red, and 35 ml of 2 N NaOH per liter. Horse serum (Gibco) and penicillin were included to a final concentration of 20% and 1,000 U/ml, respectively. Carbon sources were added as indicated. For each sugar, several individual culture flasks were inoculated with a biomass of 5 mg (wet weight), and one flask for each condition was harvested at the indicated time points and used to determine the fresh weight. For wet weight measurements, cells were washed twice with cold phosphate-buffered saline (PBS), scraped into 1.5 ml of PBS, and collected by centrifugation (5 min, 15,000 × *g*, 4°C) in a 2.0-ml microcentrifuge tube. Supernatants were discarded, and the pellets were recentrifuged to get rid of all excess liquid. The wet weight of the obtained cell pellet was determined by subtraction of the weight of the tube containing the pellet from that of the empty tube prior to cell collection.

Protein purification. His_6-tagged HPr (*M. pneumoniae*), His_6-tagged enzyme I (*B. subtilis*), and *Strep*-tagged HPrK/P (*M. pneumoniae*) were overexpressed by using the expression plasmids pGP217 (42), pAG3 (11), and pGP611 (30), respectively. Expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (final concentration, 1 mM) to exponentially growing cultures (optical density at 600 nm of 0.8). Cells were lysed by using a French press. After lysis, the crude extracts were centrifuged at 15,000 × *g* for 30 min. For purification of His-tagged proteins, the resulting supernatants were passed over an Ni^{2+} NTA superflow column (5-ml bed volume; QIAGEN) followed by elution with an imidazole gradient (from 0 to 500 mM imidazole in a buffer containing 10 mM Tris-HCl [pH 7.5], 600 mM NaCl, and 10 mM β-mercaptoethanol). For HPrK/P carrying an N-terminal *Strep* tag, the crude extract was passed over a Streptactin column (IBA, Göttingen, Germany). The recombinant protein was eluted with desthiobiotin (final concentration, 2.5 mM; Sigma). For the recombinant HPr protein, the overproduced protein was purified from the pellet fraction of the lysate by urea extraction and renatured as described previously (42).

After elution, the fractions were tested for the desired protein by using sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (PAGE). The relevant fractions were combined and dialysed overnight. Protein concentration was determined according to the method of Bradford (3) with a Bio-Rad dye-binding assay for which bovine serum albumin served as the standard.

Western blot analysis. Purified His_6-tagged HPr was used to generate rabbit polyclonal antibodies (SeqLab, Göttingen, Germany). For Western blot analysis, *M. pneumoniae* crude cell extracts were separated on sodium dodecyl sulfate–12.5% polyacrylamide gels. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad) by electroblotting. HPr was detected with polyclonal antibodies raised against HPr of *M. pneumoniae*. Antibodies were visualized by using anti-rabbit immunoglobulin G-alkaline phosphatase secondary antibodies (Promega) and the CDP* detection system (Roche Diagnostics).

In vivo HPr phosphorylation was assayed by Western blot analysis as follows. Bacteria were cultivated for 96 h. Cells were washed twice with cold PBS and harvested as described above for wet weight measurements. Subsequently, cells were resuspended in 500 μl of a solution containing 10 mM Tris-HCl (pH 7.5) and 600 mM NaCl and disrupted with sonication (3 × 10 s, 4°C, 50 W). Cell debris was pelleted by centrifugation (10 min, 15,000 × *g*, 4°C), and the obtained supernatant served as crude extract. Proteins were separated on nondenaturing 10% polyacrylamide gels. On these gels, phosphorylated HPr migrates faster than the nonphosphorylated protein. HPr(His~P) was dephosphorylated by incubation of the crude extract for 10 min at 70°C. After electrophoresis, the proteins were blotted onto a polyvinylidene difluoride membrane. The different forms of HPr were detected by using antibodies directed against *M. pneumoniae* HPr.

In vitro activity assays of HPrK/P and enzyme I. HPrK/P activity assays were carried out with 5 μg of freshly prepared cell extracts in 20 μl of assay buffer (25 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol) with purified His_6-tagged HPr (final concentration, 20 μM). The concentration of ATP was 0.5

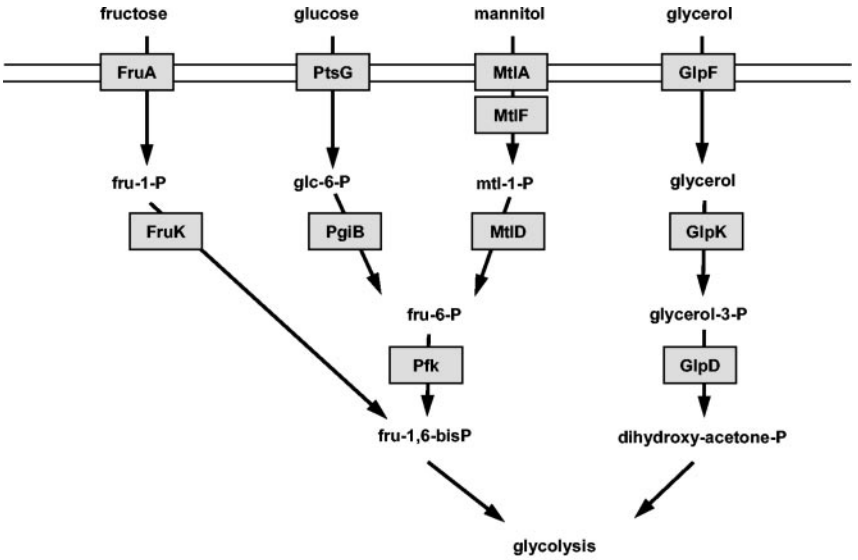

FIG. 1. Systems for the uptake and catabolism of carbohydrates in *M. pneumoniae* as predicted from the genome sequence (15). FruA (MPN078) is the EIIABC component specific for fructose, and PtsG (MPN207) is the EIICBA component for the uptake of glucose. MtlA (MPN651) and MtlF (MPN653) are the putative EIIBC and EIIA proteins for the transport of mannitol, whereas GlpF (MPN043) is the glycerol uptake facilitator. The glucose-6-phosphate isomerase PgiB (MPN250) and phosphofructokinase Pfk (MPN302) transform glucose-6-phosphate to fructose-1,6-bisphosphate. The 1-phosphofructokinase FruK (MPN079) and the mannitol-1-phosphate dehydrogenase MtlD (MPN652) are necessary for the conversion of fructose and mannitol to intermediates of glycolysis. The glycerol kinase GlpK (MPN050) and the glycerol-3-phosphate dehydrogenase GlpD (MPN051) metabolize glycerol to dihydroxyacetone phosphate.

mM. The assay mixtures were incubated at 37°C for 120 min followed by thermal inactivation of the enzyme (10 min at 95°C).

For detection of enzyme I contained in mycoplasmal cell extracts, His_6-tagged HPr (20 μM), PEP (0.5 mM), and 1 μg of cell extract were incubated in 20 μl of assay buffer for 30 min at 37°C. When indicated, the assay mixture was subjected to an additional incubation step at 70°C for 10 min to hydrolyze HPr(His~P). The assay mixtures were analyzed on 10% native polyacrylamide gels as described previously (14). Proteins were visualized by Coomassie staining.

Northern blot analysis. Preparation of total RNA of *M. pneumoniae* was carried out as described previously by Weiner et al. (51). Northern blot analysis was performed according to the protocol of Wetzstein et al. (52). The *ptsH* digoxigenin (DIG) RNA probe was obtained by in vitro transcription with T7 RNA polymerase (Roche Diagnostics) using a PCR-generated fragment obtained with the primer pair SH1 (5′-AGAAGATTCAAGTAGTCGTTAAAG)-SH2 (5′-CTAATACGACTCACTATAGGGAGATGCTTTAATAGCATTTAGTGCCTC). The reverse primer contained a T7 RNA polymerase recognition sequence (underlined in SH2). In vitro RNA labeling, hybridization, and signal detection were carried out according to the manufacturer's instructions (DIG RNA labeling kit and detection chemicals; Roche Diagnostics).

RESULTS

Utilization of different carbon sources by *M. pneumoniae*. The inspection of the genome sequence of *M. pneumoniae* suggested that the bacteria are able to transport and utilize glucose, fructose, mannitol, and glycerol as sources of carbon and energy. The genes encoding the general components of the PTS, *ptsI* and *ptsH*, are present, as are the genes for permeases specific for glucose, fructose, and mannitol. The glucose and fructose permeases are three-domain enzymes with the domain order CBA and ABC, respectively. In contrast, the putative mannitol permease is composed of a CB and a separate A protein encoded by *mtlA* and *mtlF*, respectively. The GlpF protein is a glycerol facilitator. Moreover, *M. pneumoniae* possesses the enzymes to convert the primary phosphorylation products to intermediates of glycolysis (5, 15) (Fig. 1). Since the growth properties of *M. pneumoniae* in the presence of different carbon sources have not been studied previously, we decided to analyze whether *M. pneumoniae* can use these carbon sources.

Precultures were obtained with modified Hayflick medium supplemented with glucose. Cells isolated from these cultures were used to inoculate fresh medium containing the different carbon sources. A culture without added sugar served as a control. As shown in Fig. 2, only slight initial growth resulting from residual glucose was observed in the control culture, and growth ceased after 2 days of incubation. In contrast, cultures incubated in the presence of glucose immediately started to grow, and growth continued until a biomass of about 50 mg (wet weight) per 100 ml of medium was reached on a surface of 150 cm^2. The minimal generation time of *M. pneumoniae* in glucose-supplemented medium was determined to be about 30 h. With fructose, the bacteria grew as well; however, the yield was significantly lower (about 15 mg [wet weight] per 100 ml of medium on a surface of 150 cm^2). In the presence of both glucose and fructose, the growth characteristics were similar to those observed with glucose. With mannitol, no growth was observed, suggesting that *M. pneumoniae* is not able to use this carbohydrate, at least under the conditions employed in this study (Fig. 2A). Glycerol was metabolized by *M. pneumoniae*, although it seems to be a poor substrate as observed for fructose. Again, the addition of glucose and glycerol resulted in higher biomass yields. Thus, among the candidate substrates, glucose was clearly the most efficient, and fructose and glycerol were utilized, whereas mannitol did not serve as a carbon source.

Detection of HPr in *M. pneumoniae* cells. The growth assays demonstrated that *M. pneumoniae* is able to use sugars that are

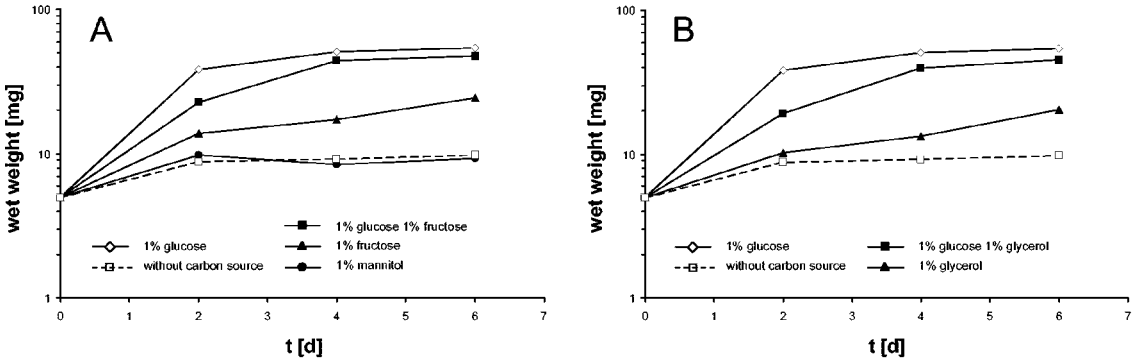

FIG. 2. Growth of *M. pneumoniae* in modified Hayflick medium containing different carbon sources. One hundred milliliters of medium was inoculated with 5 mg of cells and incubated for 2, 4, or 6 days at 37°C in 150-cm^2 cell culture flasks. Glucose, fructose, mannitol (A), and glycerol (B) were added to a final concentration of 1% (wt/vol). Attached cells were collected by scraping, and growth was monitored by determination of the wet weight of the cell pellets. Medium without any additional carbon source served as a negative control. All measurements were done at least twice.

transported by the PTS. Moreover, the functionality and important role of PTS components for glucose and fructose utilization were already demonstrated in a global mutagenesis study (18). In gram-positive bacteria, the HPr protein links sugar transport and different regulatory pathways and is thus the key protein of the PTS. To study the regulation of HPr synthesis and its modifications in *M. pneumoniae*, we raised rabbit polyclonal antibodies against the His_6-tagged *M. pneumoniae* HPr.

The amounts of HPr present in the cells after growth with different carbon sources were determined by Western blot analysis using crude cell extracts. The antibody reacted with a single protein band that corresponded to the size of the native HPr protein (9.5 kDa [Fig. 3]). The His_6-tagged HPr used as a control was larger and migrated somewhat slower. As judged from these experiments, HPr is constitutively synthesized in *M. pneumoniae*. The cellular amount did not depend on the presence or absence of PTS substrates such as glucose or fructose. This finding suggests that HPr may be required not only for sugar transport but also for regulatory purposes.

In contrast to most other bacteria, the *ptsH* and *ptsI* genes encoding HPr and enzyme I, respectively, are not clustered in *M. pneumoniae*. The transcription of *ptsH* was studied by Northern blot analysis (Fig. 4). The major transcript corresponded to a 0.32-kb mRNA. In addition, two larger minor signals were detected. The 0.32-kb mRNA has the size expected for the monocistronic *ptsH* gene, for which promoter and terminator sequences were predicted in silico (15, 50). The minor signals may result from cross-hybridization with 16S rRNA and a very abundant 550-bp RNA. The nature of this RNA is so far unknown.

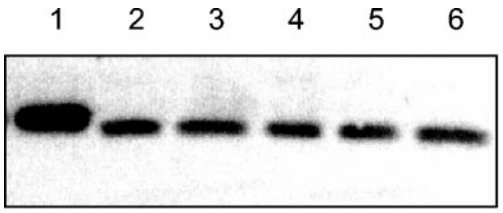

FIG. 3. Western blot analysis of HPr synthesis in *M. pneumoniae*. Antibodies raised against *M. pneumoniae* HPr were used to determine the total amounts of HPr in cells grown in the presence of glucose (lane 2), glucose and fructose (lane 3), fructose (lane 4), glucose and glycerol (lane 5), or glycerol (lane 6). The concentrations of the carbon sources were 1% (wt/vol). A total of 200 ng of recombinant His_6-tagged HPr served as a control (lane 1). His_6-tagged HPr is somewhat retarded due to its slightly higher molecular weight.

Taken together, our results demonstrate that *ptsH* is a constitutively expressed monocistronic transcription unit. This finding is in good agreement with the previous observation that *ptsH* is one of the highly expressed *M. pneumoniae* genes (51).

In vivo phosphorylation pattern of *M. pneumoniae* HPr. *M. pneumoniae* HPr is the target of two distinct phosphorylation events. However, the in vivo activity profile of the two phosphorylating enzymes, HPrK/P and enzyme I, has so far not been investigated in any *Mollicute*.

To study the in vivo phosphorylation pattern of HPr, we made use of the different migration behaviors of HPr carrying no phosphates or one or two phosphates in native acrylamide gels. Protein extracts from *M. pneumoniae* cells grown in modified Hayflick medium with different carbon sources were prepared as described in Materials and Methods and subjected to native gel electrophoresis. The different forms of HPr were detected by Western blot analysis, and the site of phosphorylation was determined by incubation of an aliquot of the cell extract at 70°C prior to electrophoresis. While phosphorylation on His-15 is heat labile, phosphorylation at Ser-46 is not (Fig. 5). In the presence of glucose, essentially all HPr was phosphorylated at His-15 as judged from the complete loss of phosphorylation upon heat exposure. Similar results were obtained with fructose and a mixture of glucose and fructose. Thus, HPr is exclusively phosphorylated by enzyme I in the presence of glucose or fructose, whereas HPrK/P has no kinase activity under these conditions. If glycerol was present as a carbon source, two phosphorylated forms of HPr, which correspond to singly and doubly phosphorylated forms of the protein, were observed. As expected, the doubly phosphorylated form disappeared completely after incubation at 70°C due to the heat lability of the His phosphate. Only a small fraction of total HPr was unphosphorylated after heat exposure. These observations indicate that HPr was present to about one third as HPr(His~P), HPr(Ser-P), and HPr(His~P)(Ser-P), respec-

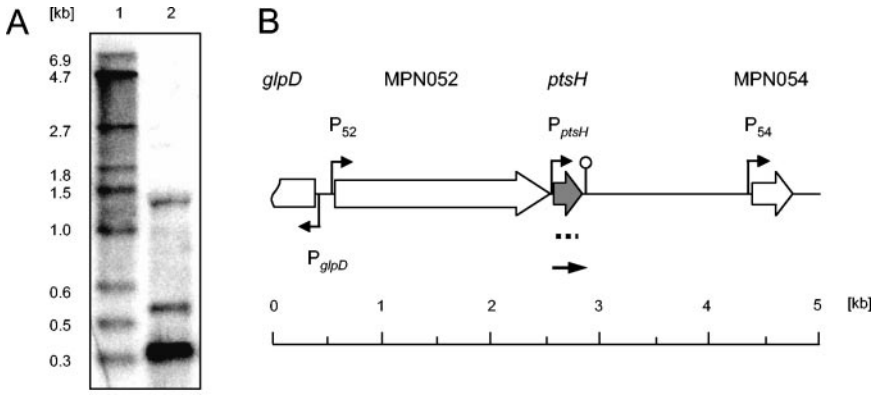

FIG. 4. Transcriptional organization of the *ptsH* locus (MPN053) of *M. pneumoniae*. (A) Northern blot. Ten micrograms of total RNA prepared from cells grown in modified Hayflick medium containing 1% (wt/vol) glucose was separated by using a 1.5% agarose gel containing 6% formaldehyde. After electrophoresis, the RNA was transferred onto a nylon membrane, and the *ptsH* mRNA was detected with a DIG-labeled riboprobe specific for *ptsH* (lane 2). DIG-labeled RNA molecular weight marker I (Roche Diagnostics) served as a standard (lane 1). (B) Genomic region surrounding the *ptsH* gene in *M. pneumoniae*. Indicated promoters are experimentally demonstrated (P_{52}) or predicted in silico (50). The position of the riboprobe is indicated by the dotted line. The detected *ptsH* mRNA is schematically shown as a solid arrow.

tively. The addition of glucose to glycerol-growing cells did not significantly affect the in vivo phosphorylation pattern of HPr (Fig. 5). Thus, we may conclude that HPrK/P kinase activity is triggered in the presence of glycerol in vivo and that it is not affected by glucose.

Detection of HPr phosphorylating enzymes in cell extracts of *M. pneumoniae*. The in vivo phosphorylation experiment suggests that enzyme I was active under all conditions studied here, whereas HPrK/P kinase activity was detectable only in glycerol-grown cells. Therefore, the synthesis or the activity of HPrK/P might be controlled by carbon source availability. To differentiate between these two possibilities, we investigated the presence of enzymatic activity of HPrK/P in *M. pneumoniae* cells after growth in modified Hayflick medium with different carbon sources. Crude extracts were incubated with HPr and with or without ATP, and the reaction mixture was analyzed by native gel electrophoresis (Fig. 6). None of the extracts was able to phosphorylate HPr in the absence of ATP. In contrast, all extracts contained HPrK/P resulting in the formation of HPr(Ser-P). Judging from these results, HPrK/P was present under all conditions. Thus, enzymatic activity rather than expression seems to be regulated.

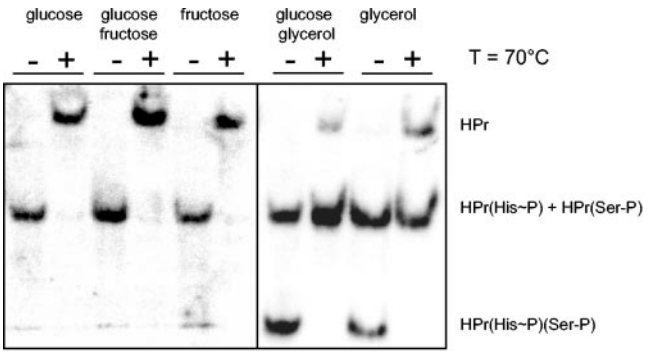

FIG. 5. Western blot for the detection of the different phosphorylation forms of HPr. Crude extracts of *M. pneumoniae* grown in the presence of different carbon sources (1% final concentration) were separated by using native gels. For each condition tested, a parallel aliquot was incubated for 10 min at 70°C to hydrolyze the heat-labile HPr(His~P). The different HPr species [HPr, HPr(His~P), HPr(Ser-P), and HPr(His~P)(Ser-P)] were detected by using polyclonal rabbit antibodies raised against *M. pneumoniae* His_6-tagged HPr. Ten micrograms of extract was applied to each lane.

If *M. pneumoniae* cells grow on glucose or fructose, HPr is quantitatively phosphorylated by enzyme I. In contrast, only a portion of HPr is phosphorylated on His-15 if glycerol is present in the medium (Fig. 5). We asked, therefore, whether enzyme I was present in lower amounts in glycerol-grown cells. To address this question, we used the cell extracts from cultures grown with the different carbohydrates and studied the presence of enzyme I. This was performed by incubating the cell extracts with HPr and PEP as the phosphate donor. To control the reaction, we incubated enzyme I of *B. subtilis* with *M. pneumoniae* HPr. As shown in Fig. 7, this process resulted in heat-labile phosphorylation of HPr. All cell extracts converted HPr to HPr(His~P) in the presence of PEP. In contrast, no HPr phosphorylation occurred in the absence of the phosphate donor (Fig. 7). Thus, enzyme I was present in all cell extracts tested. We may therefore conclude that the partial phosphorylation of HPr by enzyme I in glycerol-grown cells might result from the competition of HPrK/P and enzyme I for their common target, HPr.

DISCUSSION

For growth in artificial medium, *M. pneumoniae* requires the presence of an added carbohydrate. Among the carbohydrates tested in this study, glucose allowed the most rapid growth. In contrast, fructose and glycerol are poor carbon sources for *M. pneumoniae*. Interestingly, mannitol did not serve as a single carbon source even though the genetic information to use this carbohydrate is complete (Fig. 1). Two possible explanations for this finding can be envisioned: the *mtlA*, *mtlF*, or *mtlD* gene required for mannitol transport and conversion to fructose-6-phosphate might be poorly expressed. This argument is supported by the observation that MtlA, the mannitol-specific protein IIBC of the PTS, is not expressed in growing cultures

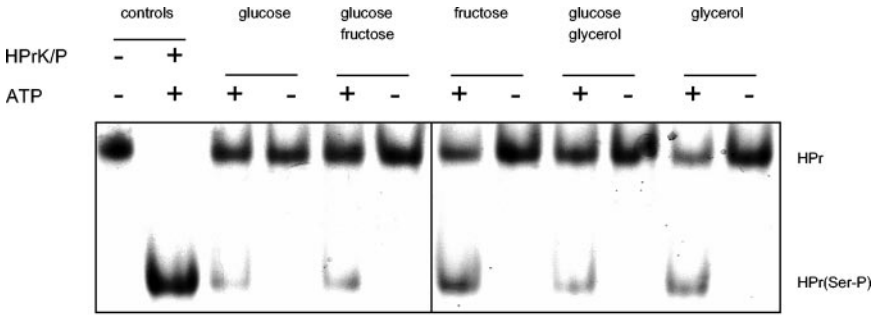

FIG. 6. In vitro phosphorylation assay to detect HPrK/P (MPN223) in *M. pneumoniae* crude extracts. *M. pneumoniae* His_6-tagged HPr (20 μM) was incubated with 5 μg of crude extract and 0.5 mM ATP in assay buffer in a final volume of 20 μl at 37°C for 120 min. Subsequently, the HPrK/P was heat inactivated by boiling for 10 min. The proteins were analyzed by using 10% native PAGE. *M. pneumoniae* crude extracts were from cells that had been cultivated in the presence of different sugars as indicated. The first lanes are positive controls with *M. pneumoniae* His_6-tagged HPr (first lane) and His_6-tagged HPr that had been phosphorylated at Ser-46 in vitro (second lane).

of *M. pneumoniae* (20). Alternatively, one of the genes necessary for mannitol utilization might harbor a mutation that results in the loss of the pathway. However, the loss of both expression and enzymatic function would result from a mutation(s) that may affect the promoter and the structural genes, respectively. Thus, we may be witnesses of a further step in the reductive evolution of the *M. pneumoniae* genome. There are several indications supporting this idea: *M. pneumoniae* possesses the genes for a putative ABC transporter for glycerol-3-phosphate with the notable exception of a binding protein. This finding may explain the inability of *M. pneumoniae* to use glycerol-3-phosphate as the single carbon source (data not shown). Moreover, in *M. genitalium*, the genes for mannitol transport are completely lost. With only 580 kb, *M. genitalium* may be a step ahead in the path of genome reduction (10, 16). Genes that are not expressed or encode nonfunctional proteins are also found in other bacteria. In *E. coli*, the *bgl* operon encoding the genes for the transport and utilization of aromatic β-glucosides is cryptic and requires mutations that activate the promoter (13, 39). The *B. subtilis gudB* gene encoding a cryptic glutamate dehydrogenase is an example of an enzyme that is inactive due to a mutation of the structural gene (2). Massive gene decay is also observed in the obligately pathogenic bacterium *Mycobacterium leprae* (48).

The need for an external carbon source seen in this study is in good agreement with the results of a global transposon mutagenesis approach with *M. pneumoniae* and *M. genitalium*; mutants affecting the fructose permease of the PTS were obtained only if glucose was provided. On the other hand, no mutations affecting the general components of the PTS, i.e., enzyme I and HPr, were observed (18). The general importance of the PTS for *M. pneumoniae* is also underlined by the observation that enzyme I and HPr are synthesized under all tested conditions (this study and reference 20). Constitutive expression of the general PTS components was also detected in *E. coli* and *B. subtilis*. This expression allows the general PTS proteins to fulfill their different regulatory functions in both the absence and the presence of PTS sugars (46).

The finding that glucose is the best carbon source for *M. pneumoniae* is in agreement with the fact that this sugar is preferred in many bacteria, including *E. coli* and *B. subtilis*. Moreover, glucose was detected in nasal secretions of compromised patients but was not detected in those of healthy patients (34). Thus, this sugar is available for *M. pneumoniae* in its natural habitats. Similarly, we would expect that glycerol resulting from the degradation of phospholipids is present on mucosal surfaces. Indeed, our results indicate that both glucose and glycerol are of special importance for *M. pneumoniae*. Glucose is the best carbon source, but glycerol is the one that

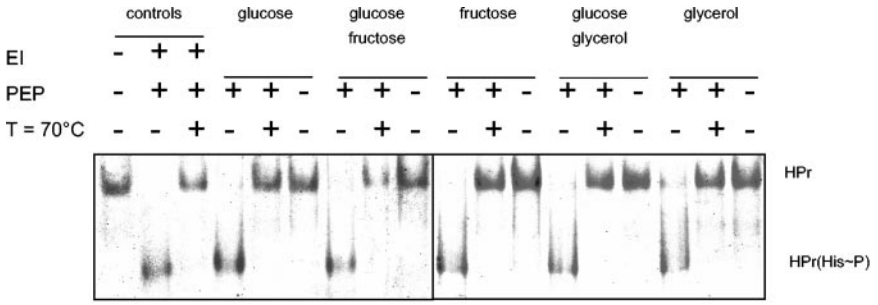

FIG. 7. In vitro phosphorylation assay to detect enzyme I (MPN627) in *M. pneumoniae* crude extracts. *M. pneumoniae* His_6-tagged HPr (20 μM) was incubated with 1 μg of crude extract and 0.5 mM PEP in assay buffer in a final volume of 20 μl at 37°C for 30 min. Assay mixtures that had been incubated for an additional 10 min at 70°C to hydrolyze the heat-labile HPr(His~P) and samples where PEP had been omitted served as negative controls. The proteins were analyzed by using 10% native PAGE. The crude extracts were prepared from cells that had been cultivated in the presence of different sugars as indicated. The first three lanes are positive controls with *M. pneumoniae* His_6-tagged HPr (first lane), in vitro phosphorylated His_6-tagged HPr(His~P) using *B. subtilis* enzyme I (second lane), and the same assay mixture after 10 min at 70°C (third lane).

provoked a regulatory output as determined by in vivo HPr phosphorylation assays.

In all organisms studied so far, the kinase activity of HPrK/P is maximal if the bacteria grow in the presence of glucose, i.e., under conditions that cause carbon catabolite repression (22, 25, 32, 45). Moreover, with the exception of the *M. pneumoniae* HPrK/P, the kinase activity of all these enzymes requires high ATP concentrations due to a low affinity for ATP (21, 33, 42). The results presented in this work indicate that the *M. pneumoniae* HPrK/P is unique not only in its high affinity for ATP (30) but also in its unusual mode of in vivo activity. The enzyme does not respond to the presence of the best carbon source, glucose, but its kinase activity is highest if the cells grow in the presence of glycerol. Interestingly, this activity is not affected by glucose as long as glycerol is available. This finding suggests a specific need for regulation in the presence of glycerol. The availability of glycerol might be an indication for the bacteria that they found their preferred ecological niche, the lipid-rich mucosal surface. If this was the case, one would expect significant changes in the global gene expression pattern in *M. pneumoniae* in response to the presence or absence of glycerol. In *Mycoplasma mycoides*, induction of cytotoxic H_2O_2 production requires the availability of glycerol (47). The use of a sugar as an indication of the nature of the habitat is not unprecedented in bacteria; in *Listeria monocytogenes*, the availability of the β-glucoside salicin is an indication that the bacteria are living in soil rather than in the human body. Accordingly, the activity of the regulatory protein BvrA, which responds to salicin, is mutually exclusive with that of the key activator of *L. monocytogenes* virulence gene expression, PrfA (4). Moreover, HPr phosphorylation by HPrK/P might be important for triggering glycerol catabolism; in *Firmicutes* such as *Enterococcus faecalis*, *Enterococcus casseliflavus*, and *B. subtilis*, glycerol utilization requires a functional PTS even though this substrate is not transported by the PTS. The glycerol kinases of these organisms require HPr-dependent phosphorylation for activity (6). It has been previously demonstrated that the doubly phosphorylated HPr(His~P)(Ser-P) can serve as a phosphate donor for the lactose permease of *Streptococcus salivarius* (23). Thus, it is possible that double phosphorylation of HPr in the presence of glycerol is required for phosphorylation and concomitant activation of the glycerol kinase of *M. pneumoniae*.

It will be interesting to study the global changes of gene expression in *M. pneumoniae* in response to the carbohydrate availability and the mechanisms that control the utilization of individual substrates such as glycerol. This work will undoubtedly be helpful in understanding the biology of *M. pneumoniae* as well as the role of carbon metabolism in virulence and pathogenicity.

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