The Phosphoproteome of the Minimal Bacterium *Mycoplasma pneumoniae*

ANALYSIS OF THE COMPLETE KNOWN SER/THR KINOME SUGGESTS THE EXISTENCE OF NOVEL KINASES*[S]

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Mycoplasma pneumoniae* belongs to the Mollicutes, the group of organisms with the smallest genomes that are capable of host-independent life. These bacteria show little regulation in gene expression, suggesting an important role for the control of protein activities. We have studied protein phosphorylation in *M. pneumoniae* to identify phosphorylated proteins. Two-dimensional gel electrophoresis and mass spectrometry allowed the detection of 63 phosphorylated proteins, many of them enzymes of central carbon metabolism and proteins related to host cell adhesion. We identified 16 phosphorylation sites, among them 8 serine and 8 threonine residues, respectively. A phosphoproteome analysis with mutants affected in the two annotated protein kinase genes or in the single known protein phosphatase gene suggested that only one protein (HPr) is phosphorylated by the HPr kinase, HPrK, whereas four adhesion-related or surface proteins were targets of the protein kinase C, PrkC. A comparison with the phosphoproteomes of other bacteria revealed that protein phosphorylation is evolutionarily only poorly conserved. Only one single protein with an identified phosphorylation site, a phosphosugar mutase (ManB in *M. pneumoniae*), is phosphorylated on a conserved serine residue in all studied organisms from archaea and bacteria to man. We demonstrate that this protein undergoes autophosphorylation. This explains the strong conservation of this phosphorylation event. For most other proteins, even if they are phosphorylated in different species, the actual phosphorylation sites are different. This suggests that protein phosphorylation is a form of adaptation of the bacteria to the specific needs of their particular ecological niche.** ***Molecular & Cellular Proteomics 9:1228–1242, 2010.

Bacteria of the group called Mollicutes are unique among all living organisms because of their small genome that nevertheless allows them to grow independently from any host cell. The two most intensively studied representatives of the Mollicutes are *Mycoplasma genitalium* and *Mycoplasma pneumoniae* with genome sizes of 580 and 816 kb, respectively. The approximately 475 protein-coding and 43 RNA-coding genes of *M. genitalium* define the lower limit of the genetic equipment that permits independent life. The small genomes of the Mollicutes reflect their adaptation to rarely changing ecosystems, such as lung epithelia for *M. pneumoniae* (1). Furthermore, *M. pneumoniae* has only limited metabolic capabilities: this bacterium can utilize only a few carbon sources (glucose, fructose, and glycerol) (2), and its only way to produce ATP is by substrate level phosphorylation in glycolysis. The citric acid cycle, respiration, and most anabolic reactions are not carried out by *M. pneumoniae* (3).

M. pneumoniae is a human pathogen that causes usually mild infections such as atypical pneumonia; however, the infections may be severe in children and elderly people. In addition, *M. pneumoniae* is involved in extrapulmonary complications, such as erythema multiforme and pediatric encephalitis (4–6).

The close adaptation of *M. pneumoniae* to human mucosal surfaces did not only result in a reductive evolution of metabolic capabilities but also affected the regulation of protein biosynthesis and activity: in contrast to versatile environmental bacteria such as *Pseudomonas aeruginosa* that reserve about 10% of their coding capacity for regulators of gene expression, *M. pneumoniae* encodes only three potential transcription regulators (less than 0.5% of all genes) (1). This absence of regulation at the level of gene expression suggests that the control of protein activities might play an important role in *M. pneumoniae* and other Mollicutes.

An important way to control the activity of a protein is post-translational modification with protein phosphorylation being the most prominent regulatory modification both in bacteria and in eukaryotes (7, 8). Studies on the phosphoproteome have been performed for several bacteria, including the model organisms *Escherichia coli* and *Bacillus subtilis.* In both bacteria, about 5% of all proteins can be phosphorylated on Ser, Thr, or Tyr residues (9–12). Interestingly, there is a high level of species specificity with respect to the phosphoproteome: a comparison of the phosphoproteomes of *B. subtilis*, *E. coli*, and *Lactococcus lactis* revealed only eight proteins that are phosphorylated in all three species. Six of these

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proteins are involved in sugar metabolism, and two are translation factors. Of these eight proteins, only one, phosphoglucosamine mutase, is phosphorylated at a conserved residue (13).

In *M. pneumoniae*, only the phosphorylation of the HPr protein of the phosphoenolpyruvate:sugar phosphotransferase system by the HPr kinase has been studied to some detail (2, 14, 15). Once phosphorylated, HPr can be dephosphorylated by a protein phosphatase, PrpC (16). The corresponding *prpC* gene is in many Gram-positive bacteria clustered with the gene for a protein kinase, *prkC*, suggesting that PrkC and PrpC form a functional couple with opposing activities. Evidence for this idea was provided by the observation that the two proteins from *B. subtilis* have identical targets *in vitro* (17). The *prpC*/*prkC* gene cluster also exists in *M. pneumoniae*; however, the targets of the corresponding proteins are so far unknown. Previous studies with *M. pneumoniae* demonstrated that several proteins implicated in adhesion to the host cell are subject to protein phosphorylation *in vivo* (18–20). These proteins, HMW1 and HMW2, are important for the virulence of *M. pneumoniae* because adhesion is the first step in the interaction with the host. A recent proteomics study resulted in the identification of 18 phosphorylated proteins in *M. pneumoniae*, among them adhesion and surface proteins and metabolic enzymes (21). Unfortunately, none of the previously known phosphoproteins were detected in this study. Moreover, Ser-46 in HPr is still the only known phosphorylation site in *M. pneumoniae* (with the exception of phosphorylated His and Cys residues in phosphotransferase system proteins), and the (de)phosphorylation of HPr is the only event for which the kinase and the phosphatase are known. Finally, nothing is known about protein tyrosine phosphorylation in *M. pneumoniae* and other Mollicutes.

We are interested in the elucidation of the molecular networks that allow life of *M. pneumoniae* as a minimal organism. The analysis of these bacteria is hampered by their slow growth and by the lack of efficient genetic systems that allow the elucidation of gene functions. We have established a technique, haystack mutagenesis, that can be used to isolate mutants of *M. pneumoniae* (16). In this work, we have studied the phosphoproteome of the *M. pneumoniae* wild type strain and of three isogenic mutants that are affected in the two protein kinases, HPr kinase (HPrK) and protein kinase C (PrkC), and in the protein phosphatase PrpC. We identified 63 phosphorylated proteins. However, most of these proteins are phosphorylated by so far unknown kinases. An in-depth analysis of the only universally conserved phosphoprotein, a phosphosugar mutase, revealed that this enzyme autophosphorylates.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The *M. pneumoniae* strains used in this study were *M. pneumoniae* M129 (ATCC 29342) in the 32nd broth passage and its isogenic mutant derivatives GPM11 (*prkC*::mini-Tn, Gm^R) (20), GPM51 (*hprK*::mini-Tn, Gm^R) (16), and GPM68 (*prpC*::mini-Tn, Gm^R) (16). *M. pneumoniae* was grown at 37 °C in 150-cm^2 tissue culture flasks containing 100 ml of modified Hayflick medium with glucose (1%, w/v) as the carbon source as described previously (2). Strains harboring transposon insertions were cultivated in the presence of 80 μg/ml gentamicin. *B. subtilis* 168 (*trpC2*; laboratory collection) was grown in LB and in minimal medium containing succinate and glutamate/ammonium as basic sources of carbon and nitrogen, respectively (22). The media were supplemented with tryptophan (at 50 mg/liter) and glucose (0.5%, w/v). *B. subtilis* was transformed with plasmid DNA or PCR products according to the two-step protocol (23). Transformants were selected on sporulation medium plates (22) containing erythromycin plus lincomycin (2 and 25 μg/ml, respectively) and/or spectinomycin (100 μg/ml).

Preparation of Cell Extracts—After 4 days of incubation at 37 °C, the culture medium was removed from the *M. pneumoniae* cell layer, and the cells were washed twice with 20 ml of cold PBS. After washing, cells were collected by scraping into 1.5 ml of PBS and subsequent centrifugation (5 min, 15,000 $\times$ *g*, 4 °C). The cell pellet was resuspended in 500 μl of 10 mM Tris/HCl, pH 7.5, and the cells were disrupted by sonication (3 $\times$ 10 s, 50 watts, 4 °C). Cell debris were sedimented by centrifugation (10 min, 15,000 $\times$ *g*, 4 °C), and the resulting supernatant was centrifuged again (30 min, 24,000 $\times$ *g*, 4 °C) to remove disturbing particles. The protein concentration of the supernatant was determined using the Bio-Rad dye binding assay with bovine serum albumin as the standard.

Two-dimensional SDS-Polyacrylamide Gel Electrophoresis—Two-dimensional separation of proteins was performed as described previously (24). For separation in the first dimension, IPG strips with a linear pH range of 4–7, 4.5–5.5, or 6–11 were used (Immobiline™ DryStrip, Amersham Biosciences). The gels were subsequently stained with Pro-Q Diamond (Invitrogen) and Flamingo™ fluorescent dye (Bio-Rad) to visualize the phosphoproteins and all proteins, respectively. Image analysis, spot quantification, and determination of putative phosphorylated protein spots were performed as described previously (9). The determination of phosphorylated proteins was based on the Pro-Q/Flamingo log ratio. Phosphoprotein spots were cut from the gel and identified by mass spectroscopy.

Protein Identification by Mass Spectrometry—Gel pieces were washed twice with 200 μl of 20 mM NH_4HCO_3, 30% (v/v) ACN for 30 min at 37 °C and dried in a vacuum centrifuge (Concentrator 5301, Eppendorf). Trypsin solution (10 ng/μl trypsin in 20 mM ammonium bicarbonate) was added until gel pieces stopped swelling, and digestion was allowed to proceed for 16–18 h at 37 °C. Peptides were extracted from gel pieces by incubation in an ultrasonic bath for 15 min in 20 μl of HPLC grade water and transferred into microvials for mass spectrometric analysis. Peptides were separated by liquid chromatography and measured on line by ESI-mass spectrometry using a nanoACQUITY UPLC™ system (Waters) coupled to an LTQ Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were desalted onto a trap column (Symmetry® C_{18}, Waters). Elution was performed onto an analytical column (BEH130 C_{18}, Waters) by a binary gradient of buffer A (0.1% (v/v) acetic acid) and B (100% (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 50 min with a flow rate of 400 nl/min. The LTQ Orbitrap was operated in data-dependent MS/MS mode using multistage activation for phosphorelevant masses. Proteins were identified by searching all MS/MS spectra in .dta format against all 689 *M. pneumoniae* proteins (extracted from the NCBI database) using Sorcerer™-SEQUEST® (SEQUEST version 2.7 revision 11, Thermo Electron) including Scaffold_2_05_02 (Proteome Software Inc., Portland, OR). SEQUEST was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10 ppm. Up to two missed tryptic cleavages were allowed. Methionine oxidation (+15.99492 Da), cysteine carbam-

idomethylation (+57.021465 Da), and phosphorylation (+79.966331 Da) were set as variable modifications. For protein identification, a stringent SEQUEST filter for peptides was used (Xcorr *versus* charge state: 1.90 for singly, 2.2 for doubly, and 3.3 for triply charged ions and ΔCn value greater than 0.10), and at least two peptides per proteins were required. With this method, all identified proteins had a protein probability score greater than 99.9%. Protein probabilities were assigned by the Protein Prophet algorithm (25). Phosphorylated peptides that had a peptide probability score greater than 95.0% as specified by the Peptide Prophet algorithm (26) were examined manually and accepted only when b- or y-ions confirmed the identification. All spectra with Scaffold-annotated b- and y-ions series are provided as screen shots in the supplemental material (see supplemental Fig. S3).

Analysis of Protein Conservation and Structure Prediction—To address the potential conservation of phosphorylation sites, multiple sequence alignments were performed using the EXPRESSO alignment server that takes available structures into account (27). Structural models of the phosphoproteins were created using SWISS-MODEL in the automated mode and Swiss-PdbViewer v4.0.1 (28, 29). Ordered and disordered regions of proteins were predicted using the DisEMBL v1.5 web server (30).

Cloning Procedures—*E. coli* DH5α (31) was used for cloning experiments and protein expression. *E. coli* was grown in LB medium. LB plates were prepared by addition of 17 g of Bacto agar/liter (Difco) to LB (31). Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (31). Restriction enzymes, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using the QIAquick Gel Extraction kit (Qiagen). *Pfu* DNA polymerase was used for the polymerase chain reaction as recommended by the manufacturer. DNA sequences were determined using the dideoxy chain termination method (31). Standard procedures were used to transform *E. coli* (31), and transformants were selected on LB plates containing ampicillin (100 μg/ml).

Plasmid Constructions—Plasmids for the overexpression and purification of the phosphosugar mutases GlmM and ManB from *B. subtilis* and *M. pneumoniae*, respectively, were constructed as follows. The coding sequence of the genes was amplified by PCR with gene-specific primers (listed in supplemental Table S1) using chromosomal DNA of *B. subtilis* 168 and *M. pneumoniae* M129 as the template. The PCR products were digested as shown in supplemental Table S2 and cloned into the appropriately linearized expression vector pWH844 (32). These plasmids allowed the expression of the phosphosugar mutases carrying an N-terminal strep tag. The resulting plasmids pGP1401 and pGP656 are listed in supplemental Table S2. In *M. pneumoniae*, the UGA specifies a tryptophan; however, in *E. coli*, it is a stop codon. The *manB* gene contains three UGA codons that were replaced by the multiple mutation reaction (33) using the phosphorylated mutagenic oligonucleotides SS109, SS110, and SS111 (see supplemental Table S1) and plasmid pGP656 as the template. The resulting plasmid was pGP657 (see supplemental Table S2). The phosphorylation sites of GlmM and ManB were replaced by alanine residues. For this purpose, the combined chain reaction (34) was applied with the mutagenic primers NP116 and SS112 (see supplemental Table S1) and plasmids pGP1401 and pGP657. The resulting plasmids are pGP1405 (*glmM*) and pGP658 (*manB*) (see supplemental Table S2). All plasmid inserts were verified by DNA sequencing.

The plasmids pGP400 and pGP1403 were used to express the wild type and S100A mutant forms of GlmM, respectively, in *B. subtilis*. For the construction of pGP400, the *glmM* gene was amplified using the oligonucleotides NP108/NP109. The PCR product was digested with BamHI and SalI and cloned into pBQ200 (35) digested with the same enzymes. For the construction of the plasmid encoding the S100A GlmM variant, mutagenesis was performed using the combined chain reaction with NP116 as the mutagenic primer and NP108/NP109. The resulting PCR product was cloned into pBQ200 as described for the wild type allele. The resulting plasmid was pGP1403.

Overexpression and Purification of Recombinant Proteins—*E. coli* DH5α transformed with the appropriate expression vector was used as host for the overexpression of recombinant proteins. Expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (final concentration, 1 mM) to exponentially growing cultures (A_{600} of 0.6). Cells were lysed using a French press (20,000 p.s.i., 138,000 kilopascals; Spectronic Instruments). After lysis, the crude extracts were centrifuged at 15,000 × *g* for 1 h. For purification of His-tagged proteins, the resulting supernatants were passed over a Ni^{2+} HiTrap chelating column (4-ml bed volume; GE Healthcare) 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, 10 mM β-mercaptoethanol) over 30 ml at a flow rate of 0.5 ml/min. After elution, the fractions were tested for the desired protein using 12.5% SDS-PAGE. The relevant fractions were combined and dialyzed overnight. Protein concentration was determined using the Bio-Rad dye binding assay where bovine serum albumin served as the standard.

Assays for Protein Phosphorylation—Protein phosphorylation assays were carried out with purified proteins in assay buffer (25 mM Tris/HCl, pH 7.6, 1 mM dithiothreitol, 0.4 mM [γ-^{32}P]ATP (480 Ci/mmol), divalent cations as indicated) using purified target proteins. The assays were carried out at 37 °C for 30 min followed by thermal inactivation of the protein (10 min at 95 °C). The assay mixtures were analyzed on 16% SDS-polyacrylamide gels. Proteins were visualized by Coomassie staining. Radioactive protein spots were detected using a phosphorimaging system (Storm 860, GE Healthcare).

Allelic Replacement of B. subtilis glmM Gene—To delete the chromosomal copy of the *glmM* gene, the long flanking homology PCR technique was used (36). Briefly, a cassette carrying the *spc* resistance gene was amplified from plasmid pDG1726 (37). DNA fragments of about 1,000 bp flanking the *glmM* gene at its 5′- and 3′-ends were amplified. The 3′-end of the upstream fragment as well as the 5′-end of the downstream fragment extended into *glmM* in a way that all expression signals of genes up- and downstream of the *glmM* remained intact. The joining of the two fragments to the resistance cassette was performed in a second PCR as described previously (38). In this reaction, we used the primer pair spec-fwd/spec-rev (38) for the amplification and joining of the *spc* cassette. The PCR product was directly used to transform *B. subtilis* carrying plasmids that express different alleles of *glmM*. The integrity of the regions flanking the integrated resistance cassette was verified by sequencing PCR products of about 1,000 bp amplified from chromosomal DNA of the resulting mutants.

RESULTS

Phosphoproteome of Growing M. pneumoniae Cells—In a previous study on protein phosphorylation in *M. pneumoniae*, no phosphorylation sites were determined, and no mutants affected in the players of protein phosphorylation were studied (21). To get a more comprehensive insight into protein phosphorylation in *M. pneumoniae*, we decided to analyze the phosphoproteome of the wild type strain M129 and of three isogenic mutants defective in the two annotated protein kinases and the single annotated protein phosphatase of *M. pneumoniae*.

The wild type strain M129 was grown in modified Hayflick medium with glucose as the principal carbon source, and

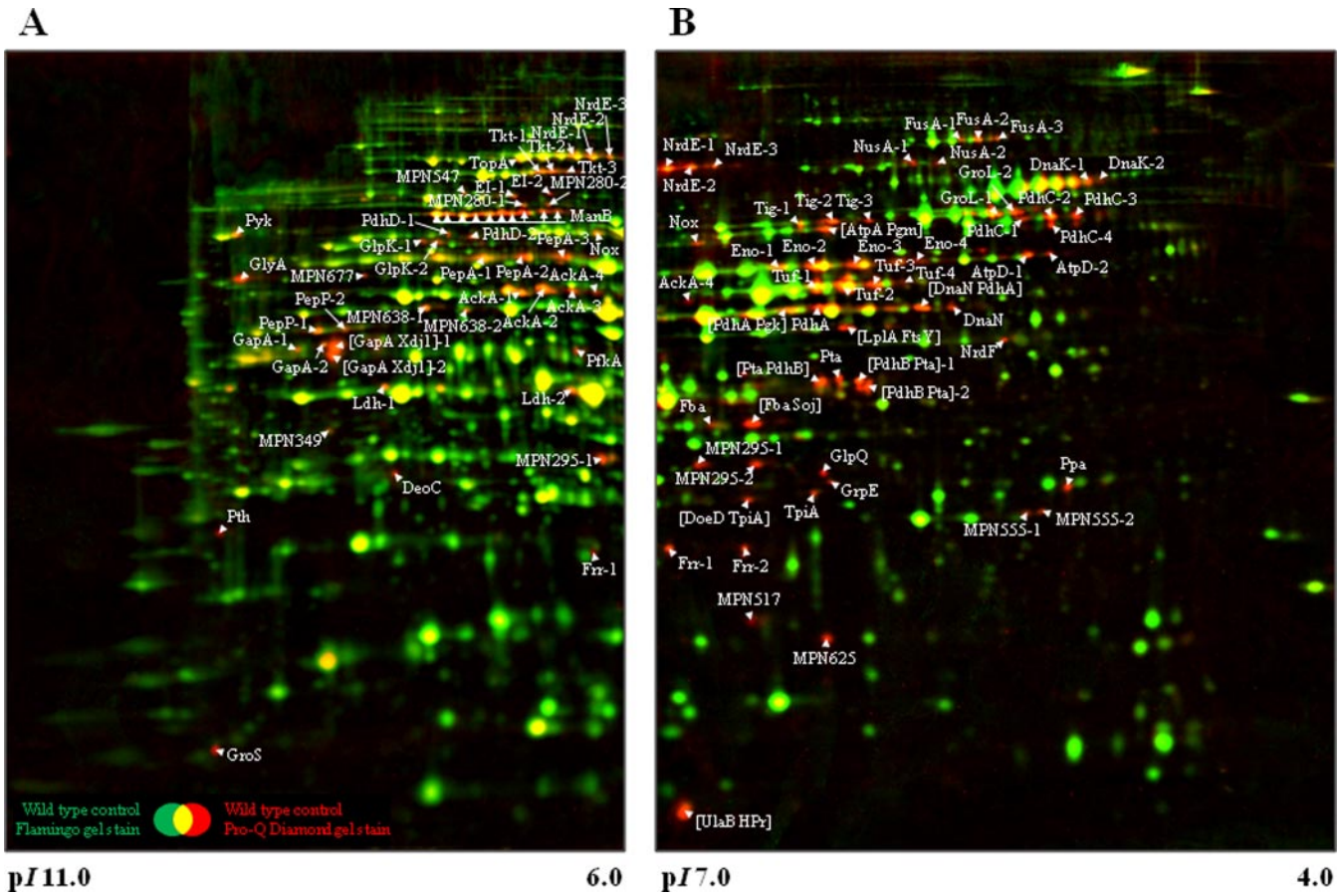

FIG. 1. **Dual channel image of total protein amount (Flamingo fluorescent dye-stained; *green*) and phosphorylated proteins (Pro-Q Diamond-stained; *red*) of stationary phase *M. pneumoniae* cells.** About 125 μg of a total protein extract of *M. pneumoniae* grown in modified Hayflick medium supplemented with glucose were subjected to two-dimensional gel electrophoresis using either an 18-cm IPG strip with a linear pH gradient of pI 6–11 (*A*) or pI 4–7 (*B*) in the first dimension. Dual channel images were obtained using the software DECODON Delta2D 3.6. Spots with high Pro-Q Diamond/Flamingo log ratios representing phosphorylated proteins (9) are indicated on the gel. Multiple protein spots corresponding to the same original protein are distinguished by appended digits (*e.g.* Tuf-1 and Tuf-2). Protein spots were cut from the gel and identified by MS/MS (see Table I and supplemental Table S3).

cells were harvested in the late logarithmic phase. Protein extracts were prepared as described under "Experimental Procedures," and the proteins were separated by two-dimensional gel electrophoresis (see Fig. 1). Traditional two-dimensional gels cover only the neutral and acidic proteins (pH range, 4–7). However, the theoretical proteome of *M. pneumoniae* shows a high number of more basic proteins that might escape detection (see supplemental Fig. S1). To include these basic proteins, we performed the isoelectric focusing in two pH ranges, 4–7 and 6–11. The gels were stained with Pro-Q Diamond and Flamingo fluorescent dye to detect the phosphorylated proteins and the all proteins, respectively. The presence of more than 700 protein spots on the gels revealed that the proteome of *M. pneumoniae* was completely covered by our system (39, 40). The identification of phosphorylated proteins revealed 58 phosphoproteins (see Table I). We observed 36 phosphorylated proteins in the pH range from 4 to 7 and 27 phosphoproteins in the pH range from 6 to 11 with only five proteins (*i.e.* AckA, Frr, MPN295, Nox, and NrdE) detected in both gel systems. Of the proteins previously known to be phosphorylated, we detected HPr and, with the exception of the ribosomal protein S2 and the adhesin-related protein P65, all 17 phosphoproteins reported by Su *et al.* (21).

In a next step, we tried to identify the phosphorylation sites in all phosphorylated peptides that had a peptide probability score greater than 95.0% as specified by the Peptide Prophet algorithm (see "Experimental Procedures"). This analysis identified 12 phosphorylation sites in 10 phosphoproteins (see Table I). These phosphorylations occurred on serine (eight sites) and threonine (four sites). Two proteins possessing two phosphorylation sites, NrdE and PepA, were phosphorylated on both a serine and a threonine residue. It is interesting to note that the individual NrdE molecules were phosphorylated only on one site: the two phosphopeptides for this protein were identified in different protein spots. In contrast, both PepA phosphopeptides were present in the protein from one spot, suggesting that this molecule was indeed doubly phosphorylated.

TABLE I
Phosphoproteins of M. pneumoniae

For detailed MS/MS data, see supplemental Tables S3 and S4 and Fig. S3. pS, phosphoserine; pT, phosphothreonine.

Locus name	Protein name	Protein function	COG[a]	Phospho spots in two-dimensional gel	Phosphosite	Phosphopeptide sequence
MPN001	DnaN	DNA polymerase III subunit β	L	2		
MPN002	Xdj1	DnaJ-like protein	O	2		
MPN024[b]	RpoE	RNA polymerase subunit δ	K	1	[Ser-87 or Thr-93]	l**p**[**S**QAMFV**T**]KEIFEEGYEDLSNK
MPN025	Fba	Fructose-bisphosphate aldolase	G	2		
MPN050	GlpK	Glycerol kinase	C	2		
MPN053[c]	HPr	Phosphocarrier protein	G	1		
MPN062	DeoD	Purine-nucleoside phosphorylase	F	1		
MPN063	DeoC	Deoxyribose-phosphate aldolase	F	1		
MPN066	ManB	Phosphomannomutase/phosphoglucomutase	G	10	Ser-149	(K)YHFDGGVNVTA**pS**HNPK
MPN082	Tkt	Transketolase	G	3		
MPN120	GrpE	Heat-shock protein	O	1		
MPN154	NusA	Transcription elongation factor	K	2	Ser-503	PVVKPK**pS**VFSITVEADDSK
MPN221	Pth	Peptidyl-tRNA hydrolase	J	1		
MPN227	FusA	Elongation factor G	J	3		
MPN256[b]	MPN256	Uncharacterized protein	S	2	Thr-200	FIDELDQI**pT**K
MPN261	TopA	DNA topoisomerase I	L	1	Ser-426	TVA**pS**LMADCKK
MPN280	MPN280	Ribonuclease J	A	2		
MPN295	MPN295	Uncharacterized protein	S	2	Ser-63	YGPECEKSFLSLQ**pS**K
MPN302	PfkA	Phosphofructokinase	G	1		
MPN303	Pyk	Pyruvate kinase	G	1		
MPN311[b]	P41	Adhesin-related protein	M	1	Thr-223	TNNSIQQLEAEIQIP**pT**THIK
MPN322	NrdF	Ribonucleoside-diphosphate reductase subunit β	F			
MPN324	NrdE	Ribonucleoside-diphosphate reductase subunit α	F	3	Thr-159, Ser-412	FQPA**pT**PTFLNAGR, VGNDI**pS**CNLGSLNIAK
MPN331	Tig	Trigger factor	O	3		
MPN349	MPN349	Uncharacterized protein	S	1		
MPN389	LplA	Lipoate-protein ligase A	H	1		
MPN390	PdhD	Pyruvate dehydrogenase E3 component	C	2		
MPN391	PdhC	Pyruvate dehydrogenase E2 component	C	4		
MPN392	PdhB	Pyruvate dehydrogenase E1 component subunit β	C	3		
MPN393	PdhA	Pyruvate dehydrogenase E1 component subunit α	C	3	Ser-205	TKLE**pS**AVSDLSTK
MPN394	Nox	NADH oxidase	H	1		
MPN420	GlpQ	Glycerophosphoryldiester phosphodiesterase	C	1		
MPN425	FtsY	Cell division protein homolog	U	1		
MPN428	Pta	Phosphotransacetylase	C	4		
MPN429	Pgk	Phosphoglycerate kinase	G	1		
MPN430	GapA	Glyceraldehyde-3-phosphate dehydrogenase	G	4	Ser-245	VPVLTG**pS**IVELCVALEK
MPN434	DnaK	Chaperone protein	O	2		
MPN452[b]	HMW3	Cytadherence high molecular weight protein 3	M	1		
MPN470	PepP	Xaa-Pro aminopeptidase	E	2	Thr-317	LLCENAVI**pT**IEPGIYIPSVGGIR
MPN474[b]	MPN474	Coiled coil surface protein	M	1	Thr-773	QNNEEL**pT**DKCSNIQNELHDLNR
MPN495	UlaB	Ascorbate-specific phosphotransferase enzyme IIB component	G	1		
MPN517	MPN517	Reductase homolog	C	1		
MPN528	Ppa	Inorganic pyrophosphatase	P	1		
MPN533	AckA	Acetate kinase	C	4		

TABLE I—*continued*

Locus name	Protein name	Protein function	COG[a]	Phospho spots in two-dimensional gel	Phosphosite	Phosphopeptide sequence
MPN547	MPN547	Dihydroxyacetone kinase	C	1		
MPN555	MPN555	Uncharacterized protein	S	2		
MPN572	PepA	Cytosol aminopeptidase	E	3	Ser-249, Thr-420	YDMSGAAIVC**pS**TVLALAK, EGVPLIHCDIAS**pT**ASIQDLGQGV LVR
MPN573	GroL	60-kDa chaperonin	O	2		
MPN574	GroS	10-kDa chaperonin	O	1		
MPN576	GlyA	Serine hydroxymethyltransferase	E	1		
MPN598	AtpD	ATP synthase subunit β	C	2		
MPN600	AtpA	ATP synthase subunit α	C	1		
MPN606	Eno	Enolase	G	4		
MPN625	MPN625	Osmotically inducible protein C-like protein	O	1		
MPN627	EI	Phosphotransferase system enzyme I	G	2		
MPN628	Pgm	Phosphoglycerate mutase	G	1		
MPN629	TpiA	Triose-phosphate isomerase	G	2		
MPN636	Frr	Ribosome-recycling factor	J	2		
MPN638	MPN638	Putative type I restriction enzyme specificity protein	V	2		
MPN665	Tuf	Elongation factor TU	J	4	Thr-383	EGGR**pT**VGAGSVTEVLE
MPN674	Ldh	L-Lactate dehydrogenase	C	2		
MPN677	MPN677	Uncharacterized protein	S	1		
MPN688	Soj	ParA family protein	D	1		

[a] COG, cluster of orthologous groups of proteins; A, RNA processing and modification; C, energy production and conversion; D, cell cycle control, cell division, and chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; J, translation, ribosomal structure, and biogenesis; K, transcription; L, replication, recombination, and repair; M, cell wall, membrane, and envelope biogenesis; O, post-translational modification, protein turnover, and chaperones; P, inorganic ion transport and metabolism; S, function unknown; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms.

[b] Phosphoproteins only detectable in a *prpC*::Tn mutant (GPM68).

[c] HPr (MPN053) not detectable in a *hprK*::Tn mutant (GPM51).

Phosphoproteome in prpC Mutant—The *M. pneumoniae* genome encodes one protein phosphatase, PrpC. Previously, we have shown that PrpC dephosphorylates HPr(Ser-P) (16). We considered that the analysis of the phosphoproteome in a *prpC* mutant might allow the identification of additional phosphorylated proteins that may have escaped our attention in the experiments with the wild type strain. Therefore, we cultivated the *prpC* mutant strain GPM68 as described above for the wild type and analyzed the protein phosphorylation pattern by two-dimensional gel electrophoresis. A comparison of the phosphoproteomes of the two strains revealed that all phosphoproteins detected in the wild type were also present in the *prpC* mutant. In addition, a few additional phosphoprotein spots were detected in the *prpC* mutant. These proteins had isoelectric points between 4 and 5 (data not shown). Therefore, we increased the resolution for these proteins by performing the isoelectric focusing in the pH range from 4.5 to 5.5. In the *prpC* mutant, we observed five phosphoprotein spots that were not present in the wild type strain. The corresponding proteins were RpoE, the δ subunit of RNA polymerase, the adhesin-related protein P41, the high molecular weight cytadherence protein HMW3, the uncharacterized protein MPN256, and the cell surface protein MPN474 (see Table I and Fig. 2). With the exception of RpoE, phosphorylation of these proteins seemed likely in the wild type strain but was at the detection limit.

The analysis of the phosphorylated peptides that occur exclusively in the *prpC* mutant led to the identification of four additional phosphorylation sites. For P41, MPN256, and MPN474, phosphorylation occurred on a threonine residue. In addition, two different phosphorylation sites (Ser-87 and Thr-93) could be validated independently on the same phosphopeptide in the case of RpoE. However, the exact site could not be determined unambiguously, so it could only be assumed that phosphorylation occurred most likely on the threonine residue.

Effect of Inactivation of Ser/Thr Kinases on Phosphoproteome—*M. pneumoniae* encodes two known serine/threonine kinases, *i.e.* the HPr kinase HPrK and the protein kinase C PrkC. To analyze the impact of these kinases on protein phosphorylation on a global level, we analyzed the phosphoproteome in the corresponding mutant strains. As described for the wild type strain M129, the isogenic mutants GPM51 (*hprK*) and GPM11 (*prkC*) were grown in modified Hayflick medium, and cell extracts separated by two-dimensional gel electrophoresis.

In the *hprK* mutant, only one phosphoprotein spot appeared to be less intense as compared with the wild type (see Fig. 3). In the wild type strain, this spot contains two phosphorylated proteins, HPr and UlaB, a putative EIIB component of the phosphotransferase system. In the *hprK* mutant GPM51, this spot contained only the phosphorylated UlaB protein, whereas HPr was absent. This observation is in good agreement with the previous report that HprK is the only kinase for HPr (16). On the other hand, HPr is the only substrate that is phosphorylated by HprK.

For the *prkC* mutant, we observed that the majority of phosphoproteins were not affected by the inactivation of this kinase. However, the phosphorylation or the accumulation of a few proteins was different in the *prkC* mutant as compared with the isogenic wild type. The large surface protein MPN474 was weakly phosphorylated in the wild type, and this protein was not at all phosphorylated in the *prkC* mutant, suggesting that MPN474 is a substrate for PrkC. This hypothesis is reinforced by the finding that MPN474 phosphorylation is strongly enhanced in the *prpC* phosphatase mutant (see Fig. 2*A*). Similarly, the so far uncharacterized protein MPN256 and the cytadherence proteins HMW3 and P41 were phosphorylated in the wild type but not in the *prkC* mutant strain GPM11. As observed with MPN474, the intensity of the phosphoprotein spot of these proteins was strongly increased in the *prpC* mutant (see Fig. 2, *A* and *B*). Thus, MPN256, HMW3, and P41 are novel targets of PrkC-dependent phosphorylation in *M. pneumoniae*.

The *prkC* mutation did not only affect protein phosphorylation but also the accumulation of some proteins. MPN256, a substrate of PrkC, was much less abundant in the *prkC* mutant as compared with the wild type. Similarly, the adhesion proteins P41 and HMW3 were present in reduced amounts in the *prkC* mutant. In addition, the adhesin-related protein P65 was less abundant in the *prkC* mutant. However, the phosphorylation signals for this protein in the wild type and the *prpC* mutant strains were below the threshold to conclude phosphorylation.

Classes of Phosphorylated Proteins—To get an impression of which cellular processes are affected by protein phosphorylation, we grouped the proteins according to their functional categories (cluster of orthologous groups of proteins) (see Table I). This analysis revealed that most phosphoproteins (36 proteins) are metabolic enzymes or transporters. 13 phosphorylated proteins are involved in cellular processes such as protein folding or envelope biogenesis. Nine of the phosphorylated proteins are required for the storage and processing of the genetic information, and the functions of five proteins are unknown.

Among the phosphorylated enzymes and transporters, a large number are involved in the central carbon metabolism (see Fig. 4). Of the glycolytic enzymes, all but the phosphoglucose isomerase are phosphorylated in *M. pneumoniae*. Moreover, all subunits of the pyruvate dehydrogenase and all three enzymes of overflow metabolism (Ldh, AckA, and Pta) are phosphoproteins. Of the enzymes of the pentose phosphate pathway, only the transketolase (Tkt) is phosphorylated in *M. pneumoniae*. In addition to glucose, *M. pneumoniae* can utilize glycerol and fructose. Although the enzymes that introduce fructose in the central metabolism are not phosphorylated, two glycerol 3-phosphate-forming enzymes are phosphoproteins.

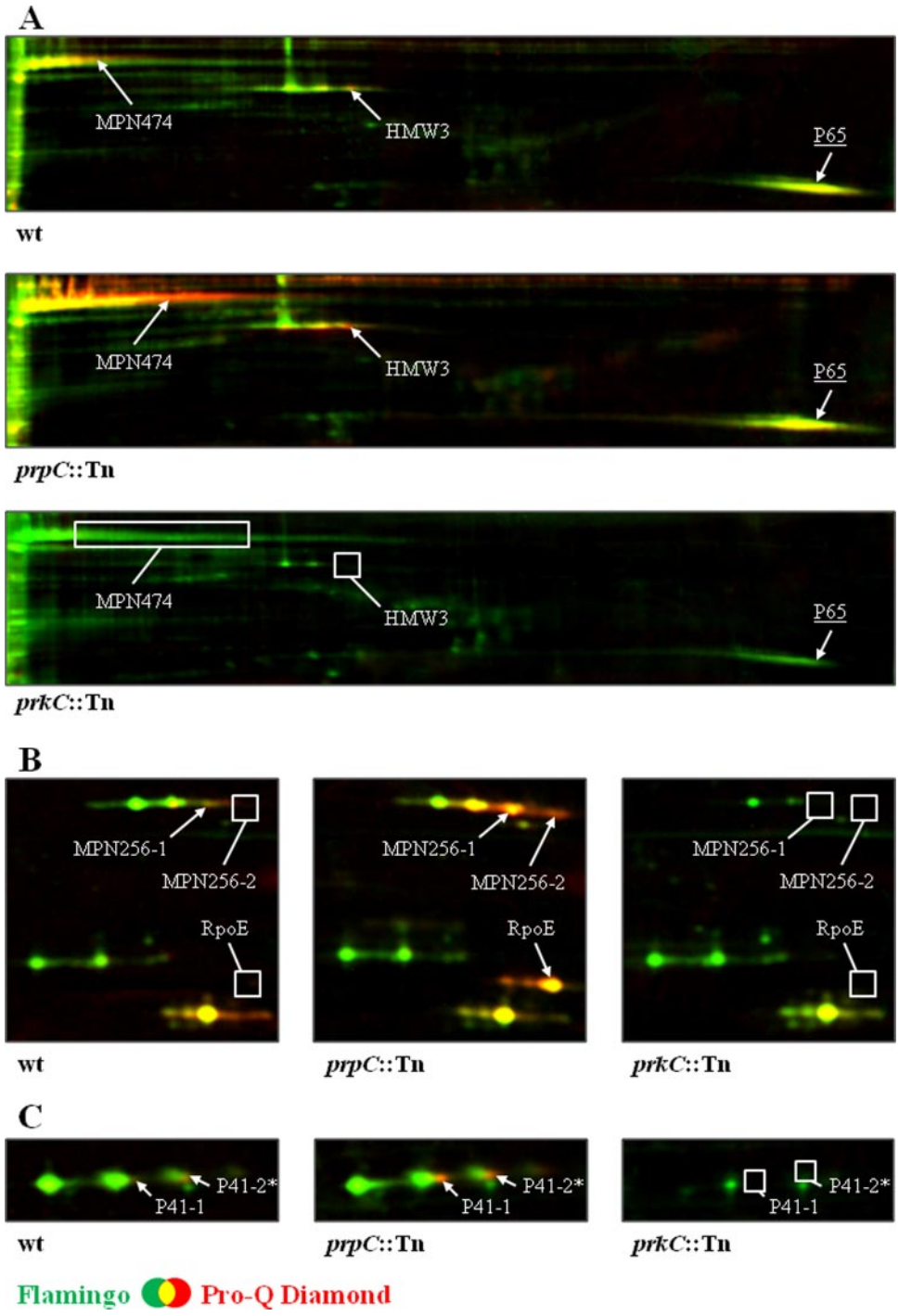

FIG. 2. **PrpC/PrkC-dependent modification of cytadherence proteins in *M. pneumoniae*.** Shown are sections of dual channel images of Flamingo fluorescent dye- (*green*) and Pro-Q Diamond-stained (*red*) two-dimensional gels representing selected proteins in the *M. pneumoniae* wild type, *prpC*::Tn mutant, and *prkC*::Tn mutant. Proteins were separated on an 18-cm IPG strip with a linear pH gradient of pI 4.5–5.5 in the first dimension. The proteins HMW3, MPN474, P65 (*A*), MPN256, RpoE (*B*), and P41 (*C*) are shown. Putative phosphorylated proteins in the *M. pneumoniae* wild type as well as proteins with increased or new phosphorylation spots in the *prpC*::Tn mutant are indicated. Missing phosphorylation spots in the *M. pneumoniae* wild type and the *prkC*::Tn mutant are highlighted by a *box*. Protein amounts of HMW3, MPN256, and P41 (all represented by a triple protein spot) seem to be reduced as well as for the P65 protein (*underlined*) in the *prkC*::Tn mutant. Note that the P41 spot "2*" is only putative because of a low Pro-Q Diamond/Flamingo log ratio in the analyzed strains. Protein spots were cut from the gel and identified by MS/MS (see Table I and supplemental Table S3).

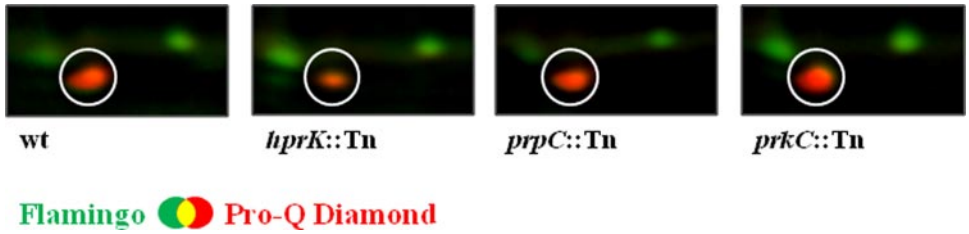

FIG. 3. **Comparison of HPr phosphorylation pattern between different *M. pneumoniae* strains.** Section of dual channel images of Flamingo fluorescent dye- (*green*) and Pro-Q Diamond-stained (*red*) two-dimensional gels displaying the phosphorylated spot of HPr in the *M. pneumoniae* wild type and different mutant strains. Proteins were separated on an 18-cm IPG strip with a linear pH gradient of pI 4–7 in the first dimension. Note that the HPr spot represents a multiprotein spot containing HPr and UlaB, whereas only UlaB could be detected in the *hprK*::Tn mutant (much lesser intensity of the phosphorylation spot).

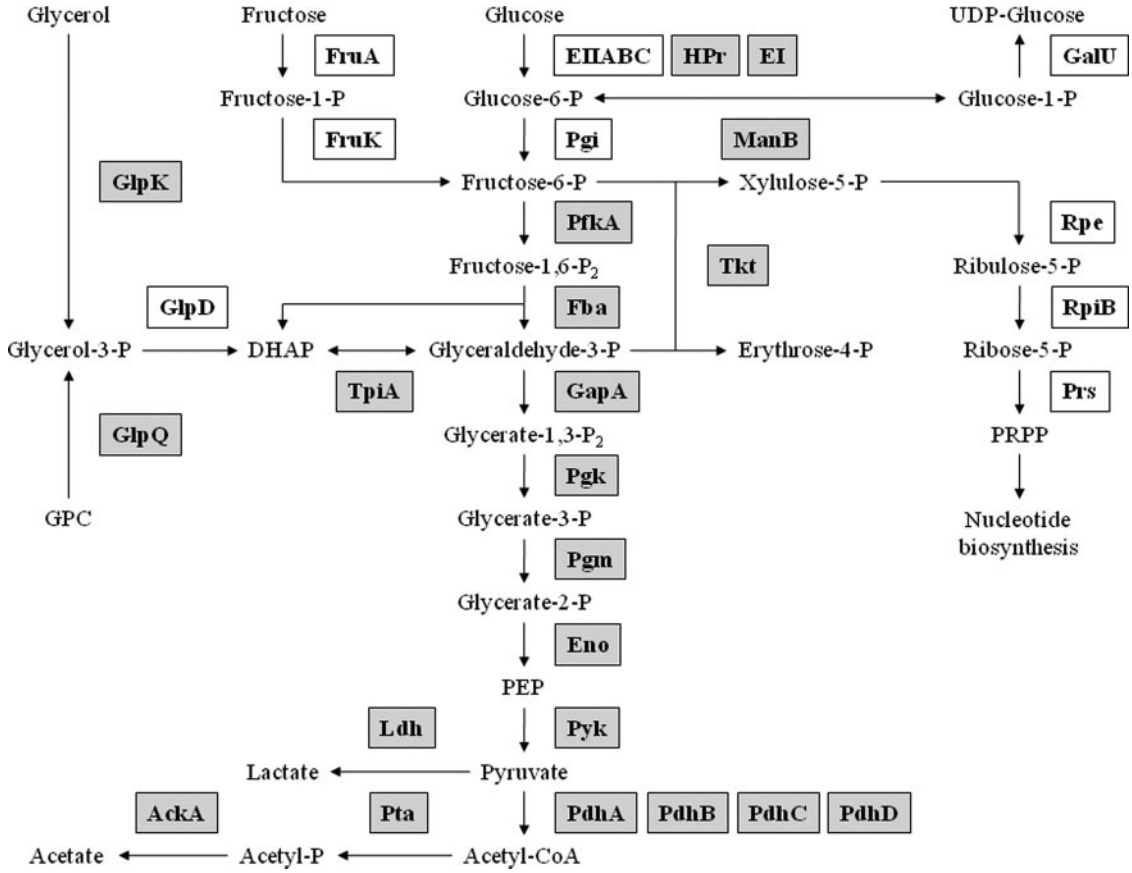

FIG. 4. **Schematic illustration of phosphorylation events in central metabolic pathways of *M. pneumoniae*.** Of the central metabolic pathways, only glycolysis is operative in *M. pneumoniae*, and a few carbon sources such as glucose, fructose, and glycerol can be utilized. Pyruvate can be oxidized to acetate or reduced to lactate. An incomplete pentose phosphate pathway serves to generate phosphoribosyl pyrophosphate for nucleotide biosynthesis. Phosphorylated enzymes are highlighted in *gray*. *DHAP*, dihydroxyacetone phosphate; *GPC*, glycerophosphorylcholine; *PEP*, phosphoenolpyruvate; *PRPP*, phosphoribosyl pyrophosphate; *P*, phosphate.

Glycolytic enzymes are phosphorylated in many bacteria (see Table II). A comparison of the phosphorylation pattern of these enzymes in different bacteria, however, revealed specific sets of phosphorylated glycolytic enzymes in each species. None of the glycolytic enzymes are phosphorylated in all species, and the phosphofructokinase is only phosphorylated in *M. pneumoniae* but not in any other bacterium studied so far. For some of the glycolytic phosphoproteins, the phosphorylation sites have been determined in multiple species. It is interesting to note that even if the same enzyme is phosphorylated in different species, the phosphorylation sites are not conserved. An exception is the pyruvate kinase that is phosphorylated on Ser-36 in a Gram-positive and in a Gram-negative bacterium (*B. subtilis* and *E. coli*, respectively) (11, 12). However, phosphorylation of this enzyme was not detected in *L. lactis* (13), a close relative of both *B. subtilis* and *M. pneumoniae* (see "Discussion").

Another interesting group of phosphoproteins is involved in cytadherence. These three proteins (P41, HMW3, and

TABLE II

Phosphorylation events in glycolytic pathway of bacteria

Bracketed letters indicate putative phosphorylation sites due to identification of the phosphopeptide.

Protein	Organism				
	M. pneumoniae	*B. subtilis*	*L. lactis*	*E. coli*	*C. jejuni*
Glucose-6-phosphate isomerase	No	Yes (Thr-39)	Yes (Ser-143)	Yes ([Ser-105], Thr-107)	No
Phosphofructokinase	Yes	No	No	No	No[a]
Fructose-bisphosphate aldolase	Yes	Yes (Thr-212, Thr-234)	Yes ([Thr-49, Ser-50], Ser-216)	No	Yes
Triose-phosphate isomerase	Yes	Yes (Ser-213)	No	No	Yes
Glyceraldehyde-3-phosphate dehydrogenase	Yes (Ser-245)	Yes ([Ser-148, Ser-151, Thr-153, Thr-154])	Yes (Ser-126, Ser-211, [Thr-212], Thr-321)	No	No
Phosphoglycerate kinase	Yes	Yes (Ser-183, Thr-299)	Yes (Ser-217)	Yes (Ser-192, Thr-196, Thr-199)	No
Phosphoglycerate mutase	Yes	Yes (Ser-62)	Yes (Thr-94, Ser-144)	Yes (Ser-146)	No
Enolase	Yes	Yes (Thr-141, Ser-259, Tyr-281, Ser-325)	No	Yes ([Ser-372, Thr-375, Thr-379])	Yes ([Ser-180, Tyr-185])
Pyruvate kinase	Yes	Yes (Ser-36)	No	Yes (Ser-36)	Yes

[a] *C. jejuni* does not encode for the glycolytic enzyme 6-phosphofructokinase.

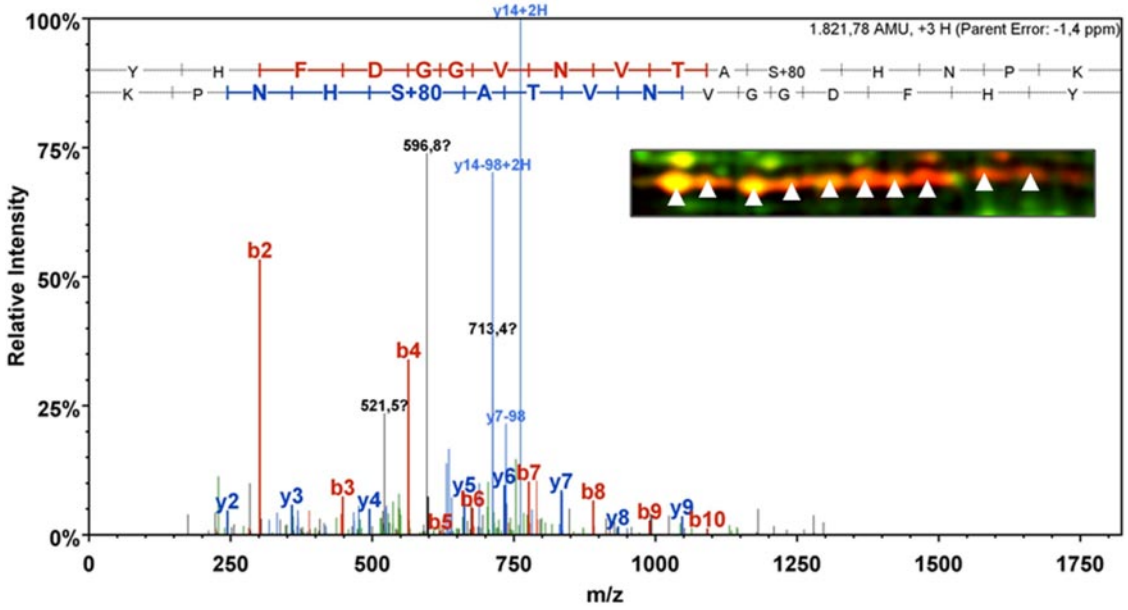

FIG. 5. **MS/MS spectrum of serine-phosphorylated peptide from phosphomannomutase/phosphoglucomutase ManB (MPN066).** The peptide was measured on line by ESI-mass spectrometry using a nanoACQUITY UPLC system coupled to an LTQ Orbitrap mass spectrometer (see "Experimental Procedures"). The phosphorylation site (Ser-149) is located in the conserved phosphoserine signature of phosphosugar mutases. Detection of the same phosphorylated peptide in all 10 spots of ManB (enhanced image section) suggests further post-translational modifications that affect the isoelectric point of the ManB protein.

MPN474) were all only weakly phosphorylated in the wild type strain, but the intensity of the phosphorylation signals was strongly increased in the *prpC* phosphatase mutant, whereas these phosphorylation events were completely abolished in the *prkC* mutant. As outlined above, these observations suggest that PrkC phosphorylates these proteins. In addition to HPr, the well known target of the HprK, these adhesion proteins and the unknown protein MPN256 are the only phosphoproteins for which the kinase could be identified.

Autophosphorylation of Single Universally Conserved Phosphoprotein—As mentioned above, there is little conservation of the phosphorylation sites even if the same proteins are phosphorylated in fairly closely related organisms. There is only one exception to this notion, the phosphosugar mutase ManB (according to the nomenclature for *M. pneumoniae*). Phosphosugar mutases are phosphorylated in all domains of life from the archaeon *Halobacterium salinarum* and bacteria such as *M. pneumoniae* and *E. coli* to eukaryotes such as yeast, *Drosophila melanogaster*, and humans (11, 41–44).

The phosphoproteome analysis showed the presence of 10 phosphospots that do all correspond to ManB. The analysis of the phosphorylation site revealed that Ser-149 was phosphorylated in ManB of *M. pneumoniae* (see Fig. 5). This site was detected in all 10 spots, suggesting that ManB is subject to different post-translational modifications that affect the isoelectric point of the protein. An alignment of the regions

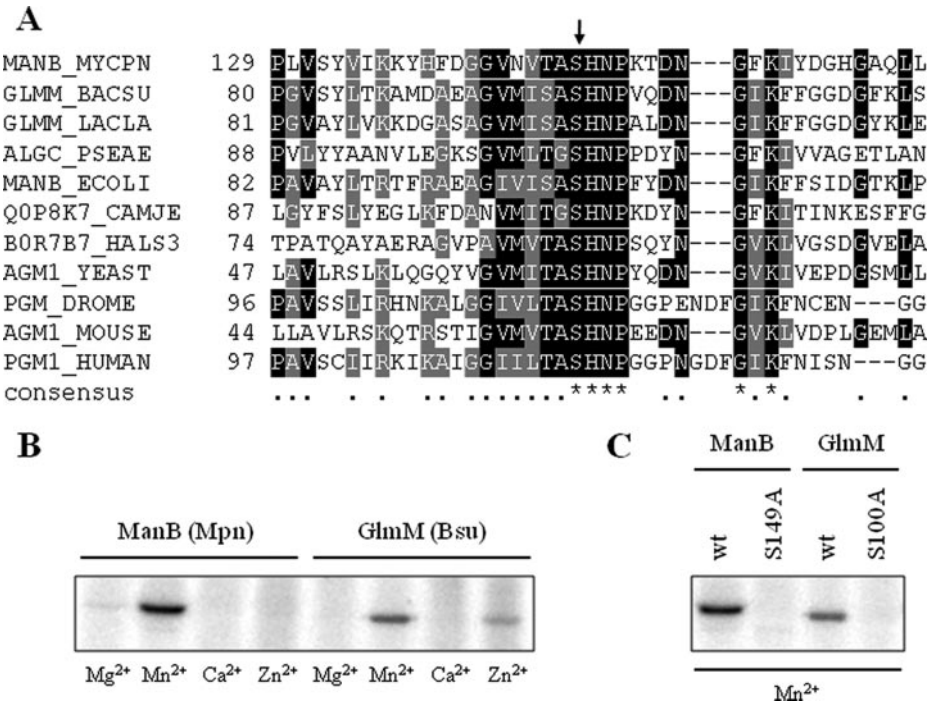

FIG. 6. **Autophosphorylation on universally conserved serine residue in active site of phosphosugar mutases.** *A*, multiple alignment of the conserved phosphoserine signature of phosphosugar mutases from all domains of life. Amino acids with similarities in at least two of the sequences are highlighted in *gray*, whereas amino acids that are identical in at least two of the sequences are depicted in a *black background*. The UniProtKB entry names of the aligned sequences are MANB_MYCPN (ManB, *M. pneumoniae*), GLMM_BACSU (GlmM; *B. subtilis*), GLMM_LACLA (GlmM; *L. lactis*), ALGC_PSEAE (AlgC; *P. aeruginosa*), MANB_ECOLI (ManB; *E. coli*), Q0P8K7_CAMJE (ManB; *C. jejuni*), B0R7B7_HALS3 (PMM1; *H. salinarum*), AGM1_YEAST (AGM1; *Saccharomyces cerevisiae*), PGM_DROME (PGM; *D. melanogaster*), AGM1_MOUSE (AGM1; *Mus musculus*), and PGM1_HUMAN (PGM1; *Homo sapiens*). The conserved active site phosphoserine is indicated by an *arrow*. *B*, *M. pneumoniae* ManB and *B. subtilis* GlmM autophosphorylation assay in the presence of various divalent cations. About 5 μg of purified His-tagged ManB (*lanes 1–4*) or GlmM (*lanes 4–8*) were incubated in the presence of [γ^{32}P]ATP in an autophosphorylation assay (see "Experimental Procedures"). Each reaction mixture was analyzed by SDS-PAGE and phosphorimaging analysis. Divalent cations (5 mM) used in the assays are indicated *below* the *lanes*. *Bsu*, *B. subtilis*; *Mpn*, *M. pneumoniae. C*, autophosphorylation assay of *M. pneumoniae* ManB (wild type (*wt*) and S149A) and *B. subtilis* GlmM (wild type and S100A) recombinant proteins. The four recombinant proteins were checked for autophosphorylation as described in *B*. Autophosphorylation reactions were conducted in the presence of Mn^{2+} (5 mM) as divalent cation as it showed the strongest signal in *B*.

surrounding the phosphorylation site of these proteins revealed a very strong conservation of the region around the phosphorylated serine residue (see Fig. 6*A*). The motif (T/S)ASHN(P/R) is present in all proteins. This strong similarity suggests phylogenetic conservation of the phosphorylation site and also implies conservation of the phosphorylation mechanism (the kinase) and of the biological function of the phosphorylation event.

Because there are no conserved protein kinases in the three domains of life, we considered the possibility that ManB might autophosphorylate as it had been shown for the phosphoglucosamine mutase from *E. coli* (45). To test this idea, we purified ManB carrying an N-terminal His tag and performed phosphorylation assays. As shown in Fig. 6*B*, the protein did indeed autophosphorylate in the presence of manganese. Only faint phosphorylation signals were observed in the presence of other divalent cations. A protein in which the phosphorylated serine residue was replaced by a non-phosphorylatable alanine did not autophosphorylate (Fig. 6*C*). This result demonstrates that ManB is phosphorylated on the same site *in vivo* and *in vitro* and supports the hypothesis of autophosphorylation. To demonstrate that autophosphorylation is not limited to the enzyme of *M. pneumoniae*, we also purified the phosphosugar mutase GlmM from *B. subtilis* and observed autophosphorylation for this protein as well. Again, no phosphorylation was detectable if the phosphorylated serine residue (Ser-100 (9, 12)) was replaced by an alanine. Thus, autophosphorylation is the common mechanism of phosphorylation of these enzymes. This explains the unique conservation of a phosphorylation site.

The phosphosugar mutases play a key role in metabolism as shown by the fact that they are often essential. This is true for GlmM from *E. coli* and *B. subtilis* (46, 47). To test whether ManB of *M. pneumoniae* is also encoded by an essential gene, we attempted to isolate a *manB* mutant from an ordered collection of transposon insertions. In this collection, each viable mutant is included with a probability of 99.999% (16, 48). No transposon insertion in *manB* was identified, suggest-

ing that the gene is indeed essential. This finding is in good agreement with previous reports that no transposon insertion in *manB* could be isolated in *M. pneumoniae* and *M. genitalium* (49, 50).

The universal conservation of the phosphorylation site in phosphosugar mutases led us to conclude that this phosphorylation event might be essential. This hypothesis could not be addressed in *M. pneumoniae* because of the lack of genetictools(48).Therefore,westudiedtheroleofthisphosphorylation site for GlmM *B. subtilis*. The *glmM* gene and its variant encoding the S100A protein were established on multicopy plasmids in *B. subtilis*. In a strain expressing the wild type *glmM* gene from a plasmid, the chromosomal copy could be deleted by homologous recombination using a PCR fragment obtained by long flanking homology PCR. In contrast, a resistance cassette could not replace the chromosomal copy of *glmM* when the *glmM*-S100A allele was expressed from the plasmid. Thus, the phosphorylation of GlmM on Ser-100 seems to be essential for its function and thus for the survival of the bacteria.

DISCUSSION

The analysis of the phosphoproteome of *M. pneumoniae* revealed substantial protein serine/threonine/tyrosine phosphorylation even in this minimal organism. We identified 63 phosphoproteins that account for nearly 10% of all proteins encoded by *M. pneumoniae*. This exceeds the percentage of phosphorylated proteins in other bacteria by far. For example, only 2.5% of all proteins of *B. subtilis* are known to be phosphorylated on a serine, threonine, or tyrosine residue (9, 10, 12). This raises the question whether protein phosphorylation is more common in *M. pneumoniae* than in other bacteria. It is tempting to speculate that the high rate of protein phosphorylation compensates for the obvious lack of transcriptional regulation in *M. pneumoniae*. However, this lack of regulation of gene expression allows the detection of the complete phosphoproteome in a single (or a few) experiment(s). Indeed, we detected about 500 CHAPS-soluble proteins that correspond to 80% of all 635 proteins that are in the pI range covered in this study (see supplemental Fig. S1). In other species with complex transcriptional regulation, only about 1,000 proteins are detectable under any specific condition, whereas the rest are required under specific growth conditions (51). For *B. subtilis* or *E. coli*, this corresponds to only 25% of all proteins. Thus, the higher coverage in our study is most probably due to the lack of transcription regulation in *M. pneumoniae* but does not seem to reflect a higher impact of protein phosphorylation.

A striking feature of the phosphoproteomes of different bacteria is the phosphorylation of enzymes of the central metabolic pathways. In *M. pneumoniae*, metabolism is strongly reduced, and the major pathways are depicted in Fig. 4. The citric acid cycle and pathways of lipid, amino acid, and nucleotide metabolisms are absent in these bacteria. As in the other Firmicutes, most glycolytic enzymes are phosphorylated in *M. pneumoniae*. Unfortunately, the functions of these phosphorylations as well as the responsible kinases are so far unknown. It is interesting that all four subunits of the pyruvate dehydrogenase are phosphorylated in *M. pneumoniae*. In *B. subtilis*, there are different results concerning the pyruvate dehydrogenase. One study reports phosphorylation of PdhB (12), whereas PdhC and PdhD were found to be phosphoproteins in another work (9). In contrast, none of the subunits is phosphorylated in *E. coli*, *Campylobacter jejuni*, and *L. lactis* (11, 13, 52).

This work is the first global analysis of the effects of mutations in the complete set of annotated protein kinases on the phosphoproteome. We have shown that the HPr kinase has only one substrate, HPr. The phosphorylation of HPr on Ser-46 is a major signal to trigger carbon catabolite repression of catabolic genes and operons in *B. subtilis* and other Firmicutes (53). If HPr(Ser-P) had the same function in *M. pneumoniae*, one would have expected major changes in the proteome of the *hprK* mutant as compared with the wild type strain. However, with the exception of HPr phosphorylation (see Fig. 3), no differences were detectable. This suggests that HPr phosphorylation on Ser-46 has a function different from that in the other bacteria. This conclusion is supported by three observations. First, HPr(Ser-P) acts as the cofactor for the pleiotropic transcription factor CcpA to cause catabolite repression. No protein similar to CcpA is encoded in the genome of any *Mycoplasma* species. Second, the phosphorylation of HPr on Ser-46 interferes with its participation in sugar transport due to the inhibition of enzyme I-dependent phosphorylation of His-15. This inhibition is relevant even in Gram-negative bacteria that also do not possess an equivalent of CcpA (54). However, in *M. pneumoniae*, these two phosphorylation events are not mutually exclusive (55). Finally, although the activity of the HPr kinase of *B. subtilis* and most other bacteria is strongly controlled by the ATP and fructose 1,6-bisphosphate concentrations, the enzyme of *M. pneumoniae* is constitutively active due to its high affinity to ATP (56). Thus, the phosphorylation of HPr on Ser-46 seems to serve other purposes in *M. pneumoniae*.

In addition to the HPr kinase, *M. pneumoniae* encodes only one protein kinase, PrkC. This kinase is implicated in a multitude of cellular functions in different bacteria, among them spore germination, virulence, and control of glycolysis (57–59). We found that PrkC has at least four targets in *M. pneumoniae*, the cell adhesion proteins HMW3 and P41, the cell surface protein MPN474, and the uncharacterized protein MPN256. These proteins are not phosphorylated in a *prkC* mutant, but their phosphorylation is enhanced if the protein phosphatase PrpC, the antagonist of PrkC, is not expressed (see Fig. 2). Moreover, HMW1 and P1, two very large proteins that were out of the detection window applied in this study, are likely to be phosphorylated by PrkC (20). This suggests that PrkC might be required for cell adhesion in *M. pneu-*

moniae. Indeed, a *prkC* mutant strain is not capable of adherent growth and has lost toxicity to human host cells, most likely due to the defect in cell adhesion (20). Thus, PrkC has a specific function in *M. pneumoniae* and phosphorylates a specific set of proteins.

As stated above, HPrK and PrkC are the only two annotated protein kinases in *M. pneumoniae*. However, they account for only five of 63 identified protein phosphorylation events. This suggests the existence of other, yet to be discovered protein kinases in *M. pneumoniae*. Alternatively, autophosphorylation might be more relevant than previously anticipated. Indeed, the only phosphorylation site that is universally conserved from archaea via bacteria such as *M. pneumoniae* to eukaryotes is the result of an autophosphorylation event in the catalytic site of phosphosugar mutases such as GlmM and ManB (Refs. 45 and 47 and this work). This autophosphorylation is essential for the enzymatic activity of these enzymes, thus providing an explanation for the universal but highly exceptional conservation of the phosphorylation event at a conserved site. Interestingly, another ubiquitous protein, the heat-shock protein DnaK (also called Hsp70) is also present as a phosphoprotein in all organisms that were studied so far. In *E. coli* and mycobacteria, DnaK is capable of autophosphorylating at a strongly conserved threonine residue (60–63). This site is also present in the DnaK protein of *M. pneumoniae* (Thr-182), but it is not conserved in the archaeal DnaK of *H. salinarum* (41). It is therefore possible that DnaK autophosphorylates at a conserved site in bacteria and eukaryotes, whereas another phosphorylation mechanism occurs in archaea.

In this study, we unambiguously identified 16 phosphorylation sites. Phosphorylation on serine or threonine residues is equally distributed (eight phosphorylation sites each). Serine and threonine are also the predominant phosphorylation sites in other bacteria, and they are used with similar frequency as well (11–13). For eukaryotic phosphoproteins, it was shown that phosphorylations occur often in disordered regions and that the actual phosphorylation sites are not completely fixed in these regions (64, 65). Indeed, seven of the 16 phosphorylation sites identified in this study are predicted to be localized in disordered regions (data not shown). An analysis of the regions surrounding the phosphorylation sites revealed the absence of defined amino acid sequence motifs. Rapid evolution and a corresponding lack of conservation of phosphorylation sites have recently also been observed for protein phosphorylation in yeast and other eukaryotes (66–68). Taken together, these findings suggest that either the responsible protein kinases are quite nonspecific or that a large number of so far unidentified protein kinases exist in *M. pneumoniae*. An alternative explanation is offered by an analysis of the positions of the phosphorylation sites: structural information is available for seven of the phosphoproteins with identified phosphorylation sites (see supplemental Fig. S2). All nine phosphorylation sites in these proteins are buried in the protein and are thus poorly accessible for protein kinases. This would suggest that these phosphorylation events are autophosphorylations as observed for ManB. Based on the structure of the *P. aeruginosa* phosphomannomutase/phosphoglucomutase (69) (see Fig. 6), the site of autophosphorylation of ManB and GlmM is also buried in the inside of the protein.

The lack of conservation of phosphorylation sites was also observed in similar phosphoproteins from different bacteria. Only two strongly conserved phosphorylation sites were detected, Ser-46 in HPr and Ser-149 in ManB. Although HPr is phosphorylated by the ATP-dependent HPr kinase that is conserved in many bacteria (Ref. 15 and see Fig. 3), ManB is subject to autophosphorylation. Even among the glycolytic enzymes, only one identified phosphorylation site was conserved between two species (Ser-36 in the pyruvate kinases from *B. subtilis* and *E. coli*). Because this latter site is conserved in *M. pneumoniae*, it is tempting to speculate that it is also phosphorylated in the Mollicutes.

The observations reported in this work have important implications for the evolution of protein phosphorylation in bacteria: protein phosphorylation events seem to be highly specific for each individual bacterial species; even among members of one bacterial phylum such as the Firmicutes, there is little conservation of protein phosphorylation. Moreover, one protein kinase may phosphorylate distinct sets of proteins in the different species. Finally, there may be a large number of protein kinases that have so far escaped discovery, and these kinases are likely to be specific for small groups of bacteria. In conclusion, protein phosphorylation may be one of the major features in the adaptation of bacteria to their individual ecological niche.

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S This article contains supplemental Figs. S1–S3 and Tables S1–S4.

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