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Two-Component Systems of *Corynebacterium glutamicum*: Deletion Analysis and Involvement of the PhoS-PhoR System in the Phosphate Starvation Response§

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Corynebacterium glutamicum **contains genes for 13 two-component signal transduction systems. In order to test for their essentiality and involvement in the adaptive response to phosphate (P_i) starvation, a set of 12 deletion mutants was constructed. One of the mutants was specifically impaired in its ability to grow under P_i limitation, and therefore the genes lacking in this strain were named *phoS* (encoding the sensor kinase) and *phoR* (encoding the response regulator). DNA microarray analyses with the *C. glutamicum* wild type and the Δ*phoRS* mutant supported a role for the PhoRS system in the adaptation to P_i starvation. In contrast to the wild type, the Δ*phoRS* mutant did not induce the known P_i starvation-inducible (*psi*) genes within 1 hour after a shift from P_i excess to P_i limitation, except for the *pstSCAB* operon, which was still partially induced. This indicates an activator function for PhoR and the existence of at least one additional regulator of the *pst* operon. Primer extension analysis of selected *psi* genes (*pstS*, *ugpA*, *phoR*, *ushA*, and *nucH*) confirmed the microarray data and provided evidence for positive autoregulation of the *phoRS* genes.**

Phosphorus (P) is an essential nutrient for all cells and required, e.g., for the biosynthesis of nucleotides, DNA, and RNA and in addition for the functional regulation of protein activity by phosphorylation. The common phosphorus source is inorganic phosphate (P_i), and cells have developed mechanisms for the acquisition, assimilation, and storage of phosphate. Under phosphate starvation, many bacteria induce the synthesis of proteins that enable them to use the limiting phosphate resources more efficiently and to make alternative phosphorus sources accessible. The corresponding genes are collectively named P_i starvation-inducible genes, or *psi* genes. The phosphate starvation response, in particular its regulation, has been most carefully studied in *Escherichia coli* (33) and *Bacillus subtilis* (11). In both species, two-component signal transduction systems consisting of a histidine kinase and a response regulator play a prominent role.

In *E. coli*, induction of the P_i starvation genes is dependent on the PhoR-PhoB two-component system. Under P_i limitation, the histidine kinase PhoR phosphorylates the response regulator PhoB, and PhoB~P in turn activates transcription of at least 31 genes, which form the Pho regulon (33). The genes include the *phoBR* operon; the *pstSCAB-phoU* operon, encoding an ABC transporter for high-affinity P_i uptake and a regulatory protein; the *ugpBAECQ* operon, encoding an *sn*-glycerol 3-phosphate ABC uptake system and glycerophosphoryl diester phosphodiesterase; the *phoA-psiF* operon, encoding alkaline phosphatase and a protein of unknown function; *phoE*, encoding an anion-specific porin; *phoH*, encoding an ATP-binding protein of unknown function; and the *phnCDEFGHI JKLMNOP* operon, encoding proteins involved in the uptake of phosphonates and their degradation via the C-P lyase pathway. Thus, when P_i is scarce, *E. coli* takes up P_i by an ATP-driven high-affinity transport system, mobilizes P_i outside the cytoplasm by phosphatases and esterases, and forms proteins for the uptake and degradation of organophosphates and phosphonates.

In *B. subtilis*, the response to P_i starvation is more complex, since it involves the induction both of a specific Pho regulon and of the σ^B-dependent general stress response. The Pho regulon is controlled by a two-component system composed of the histidine kinase PhoR and the response regulator PhoP. It includes the *phoPR* operon; *phoA* and *phoB* for two alkaline phosphatases; *phoD* for an alkaline phosphatase/phosphodiesterase with a putative role in cell wall teichoic-acid turnover; the *pstSACB1B2* operon; the *tuaABCDEFGH* operon, encoding proteins which are responsible for the synthesis of teichuronic acid, which replaces the teichoic acid in the cell walls of phosphate-starved cells; *glpQ*, encoding glycerophosphoryl diester phosphodiesterase; and *ydhF*, encoding a lipoprotein (2, 11). The *tagAB* and *tagDEF* operons, which encode protein involved in teichoic acid biosynthesis, are repressed by PhoP under P_i starvation (20). Besides PhoR-PhoP, the ResD-ResE two-component system, the response regulator Spo0A, and the AbrB regulator are also involved in the control of Pho regulon genes (11).

Corynebacterium glutamicum is a gram-positive bacterium belonging to the order *Actinomycetales* and has gained considerable interest because of its use in the large-scale biotechnological production of L-glutamate and L-lysine (6). Phosphorus constitutes 1.5% to 2.1% of the cell dry weight of *C. glutami-*

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§ Supplemental material for this article may be found at http://jb.asm.org/.

cum (18), part of which is present as polyphosphate (16, 24). In a previous study, we analyzed the P_i starvation response of C. *glutamicum* by using whole-genome DNA microarrays (12). The comparison of the mRNA profiles before and at different times after a shift from P_i excess to P_i starvation led to the identification of a group of genes that are specifically required to cope with a limited P_i supply. This group includes the *pstSCAB* operon, encoding an ABC transporter for high-affinity P_i uptake; the *ugpAEBC* operon, encoding an ABC transporter for the uptake of glycerol 3-phosphate; *glpQ1*, encoding a glycerophosphoryl diester phosphodiesterase; *ushA*, encoding a secreted enzyme with UDP sugar hydrolase and 5′-nucleotidase activity (27); *nucH*, encoding a putative secreted nuclease which possibly serves a role in liberating P_i from extracellular nucleic acids; NCgl2959/Cg3393, which may encode a cell wall-associated phosphatase (34); *phoH1*, encoding an ATPase of unknown function; and the *pctABCD* operon, encoding an ABC transport system which might be involved in the uptake of a yet-unknown phosphorus-containing compound (12).

In parallel to the elucidation of the P_i starvation stimulon of *C. glutamicum*, we initiated studies aimed at the identification of the regulators controlling the P_i starvation response. Evidence derived from other bacteria showed that two-component regulatory systems are prime candidates for this function. Here we present evidence that one of the 13 two-component systems of *C. glutamicum*, named PhoS-PhoR, is involved in the adaptation of *C. glutamicum* to limiting P_i concentrations.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains, plasmids, and oligonucleotides used or constructed for this work are listed in Tables S1, S2, and S3, respectively (see the supplemental material). For the construction of plasmids, *E. coli* DH5α was used as a host and routinely grown aerobically at 37°C on a rotary shaker (120 rpm) in LB medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) or on LB agar plates (LB medium with 1.5% [wt/vol] agar). *C. glutamicum* ATCC 13032 (1) and its derivatives were cultivated aerobically on a rotary shaker (120 rpm) at 30°C either in LB medium, in brain heart infusion medium supplemented with 0.5 M sucrose, in CGIII medium (21), or in CGXII minimal medium (15) containing 30 mg/liter protocatechuic acid as an iron chelator and 40 g/liter glucose as a carbon and energy source. CGXII medium contains 13 mM phosphate in the forms of KH_2PO_4 and K_2HPO_4 as its sole phosphorus source. In order to obtain phosphate-limited conditions (0.065 mM), the concentration of these two compounds was reduced from 1 g/liter to 0.005 g/liter. If appropriate, kanamycin was added to a final concentration of 25 μg/ml (*C. glutamicum*) or 50 μg/ml (*E. coli*).

For an analysis of the influence of different phosphate concentrations on the growth of *C. glutamicum* strains, they were first grown overnight in CGIII medium. After being washed, the cells were cultured for 24 h in CGXII medium under P_i-limiting conditions (0.065 mM) and then inoculated into CGXII medium containing either 0.065 mM phosphate (P_i limitation) or 13 mM phosphate (P_i excess). Growth was followed by a measurement of the optical density at 600 nm (OD_{600}).

For an analysis of the response of *C. glutamicum* to a shift from P_i excess to P_i limitation by DNA microarray or primer extension analysis, cells of the wild type or the Δ*phoRS* mutant were first precultured in CGIII medium and then grown for 24 h in CGXII medium under P_i-sufficient conditions and finally inoculated into the same medium to an initial OD_{600} of 0.6. Exponentially growing cells from this P_i-sufficient culture (OD_{600}, 4 to 5) were harvested and washed. RNA was prepared from one aliquot, whereas the other aliquot was used to inoculate cultures with a medium containing a limiting P_i concentration (0.065 mM). RNA was prepared 10, 30, 60, or 90 min after the P_i downshift.

Construction of *C. glutamicum* deletion mutants. Deletion mutants of *C. glutamicum* lacking *citAB*, *cgtSR1*, *cgtRS2*, *cgtRS3*, *cgtRS5*, *cgtRS6*, *cgtSR7*, *cgtSR8*, *cgtRS9*, *cgtSR10*, or *cgtSR11* were constructed via a two-step homologous-recombination procedure involving crossover PCR (19) and the suicide vector pK19*mobsacB* (28) as described previously (23). The deletions were verified by PCR using primers annealing outside the regions involved in recombination (e.g., oligonucleotides cgtRS3-out-fw and cgtRS3-out-rv). In the cases of the Δ*phoRS* (=Δ*cgtRS3*), Δ*citAB*, and Δ*cgtRS9* mutants, Southern blot analysis was also performed (23) and confirmed the deletions (data not shown).

Construction of plasmid pEKEx2-*phoRS*. The *phoRS* coding sequence was amplified from the chromosomal DNA of the *C. glutamicum* wild type by PCR using the oligonucleotides phoRS-SbfI-fw and phoRS-KpnI-rv (Table S3 in the supplemental material) and the Expand High Fidelity PCR system (Roche Diagnostics, Mannheim, Germany). The primer phoRS-SbfI-fw introduces an SbfI restriction site 40 bp upstream of the *phoR* start codon. The primer phoRS-KpnI-rv introduces a KpnI restriction site after the stop codon of *phoS* and adds eight codons (WSHPQFEK) before the stop codon, leading to a PhoS protein with a carboxy-terminal StrepTag-II (29). The resulting PCR product (2,263 bp) was cleaved with SbfI and KpnI, gel purified using the QIAEX kit (QIAGEN, Hilden, Germany), and cloned into the *E. coli-C. glutamicum* shuttle vector pEKEx2 (7) cut with the same enzymes, resulting in plasmid pEKEx2-*phoRS*. DNA sequence analysis confirmed that the cloned *phoRS* genes were identical to the published wild-type sequence (13).

Preparation of total RNA for DNA microarray and primer extension experiments. RNA from *C. glutamicum* was prepared from 20-ml culture aliquots using the RNeasy kit (QIAGEN, Hilden, Germany) with on-column DNase I treatment as described previously (22). Isolated RNA samples were checked for purity by denaturing formaldehyde agarose gel electrophoresis, quantified by UV spectrophotometry, and stored at −70°C until use.

DNA microarray analysis. Whole-genome DNA microarrays for *C. glutamicum* were generated as described previously (12, 17). Identical amounts (20 to 25 μg) of total RNA were used for random hexamer-primed synthesis of fluorescently labeled cDNA by reverse transcription with Superscript II (Invitrogen, Karlsruhe, Germany) and the fluorescent nucleotide analogues FluoroLink Cy3-dUTP (green) and Cy5-dUTP (red) (Amersham Pharmacia, Little Chalfont, United Kingdom) as described previously (17). The labeled cDNA probes were purified and concentrated by using Microcon YM-30 filter units (Millipore, Bedford, Mass.). Subsequently, the mixed Cy3- and Cy5-labeled cDNAs containing 1.2 μg poly(A)/ml (Sigma, Munich, Germany) as a competitor, 30 mM HEPES, and 0.3% sodium dodecyl sulfate in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were hybridized to whole-genome arrays in a humid chamber for 5 to 16 h at 65°C. After hybridization, the arrays were washed in a mixture of 1× SSC and 0.03% sodium dodecyl sulfate and finally in 0.05× SSC. Immediately after stringent washing, the fluorescence intensities at 635 and 532 nm were determined using a GenePix 4000 laser scanner (Axon, Inc., Union City, Calif.), and the images were processed by using GenePix 3.0 software. Data were normalized to the average ratio for *C. glutamicum* genomic DNA. The normalized ratio of median fluorescence was taken to reflect the relative RNA abundance for spots with green or red fluorescent signals that were at least threefold greater than the median fluorescence background signal. For statistical analysis of the gene expression data, *P* values for the independent replicate experiments were calculated based on the Student *t* test by using log-transformed fluorescence ratios for individual genes on the one hand and for genomic DNA on the other hand. In a first series of experiments (performed in triplicate), the mRNA levels of the Δ*phoRS* mutant 10, 30, and 60 min after a shift from P_i excess (13 mM) to P_i limitation (0.065 mM) were compared to the mRNA levels before the shift (zero time). In a second series of experiments, the mRNA levels of the wild type were compared with the mRNA levels of the Δ*phoRS* mutant before and 60 min after a shift from P_i excess to P_i limitation.

Primer extension analysis. Nonradioactive primer extension analysis of the genes *pstS*, *ugpA*, *phoR*, *ushA*, and *nucH* was performed as described previously (8, 9) using IRD800-labeled oligonucleotides (Table S3 in the supplemental material) and 20 μg isolated RNA as a template. The lengths of the primer extension products were determined by running the four lanes of a DNA-sequencing reaction mixture set up using the same oligonucleotide as that used for reverse transcription alongside the primer extension products. The templates for DNA sequencing were obtained by PCR using the oligonucleotide pairs pstS_promreg_fw/pstS_promreg_rv, ugpA_promreg_ fw/ugpA_promreg_rv, phoR_promreg_fw/phoR_promreg_rv, ushA1/ushA2, and nucH1/nucH2 (for sequences, see Table S3 in the supplemental material).

RESULTS

In silico and deletion analyses of *C. glutamicum* two-component systems. During the annotation of the *C. glutamicum* genome sequence (13), genes encoding 13 sensor kinases and 13 response regulators were identified. In Fig. 1, maps of the

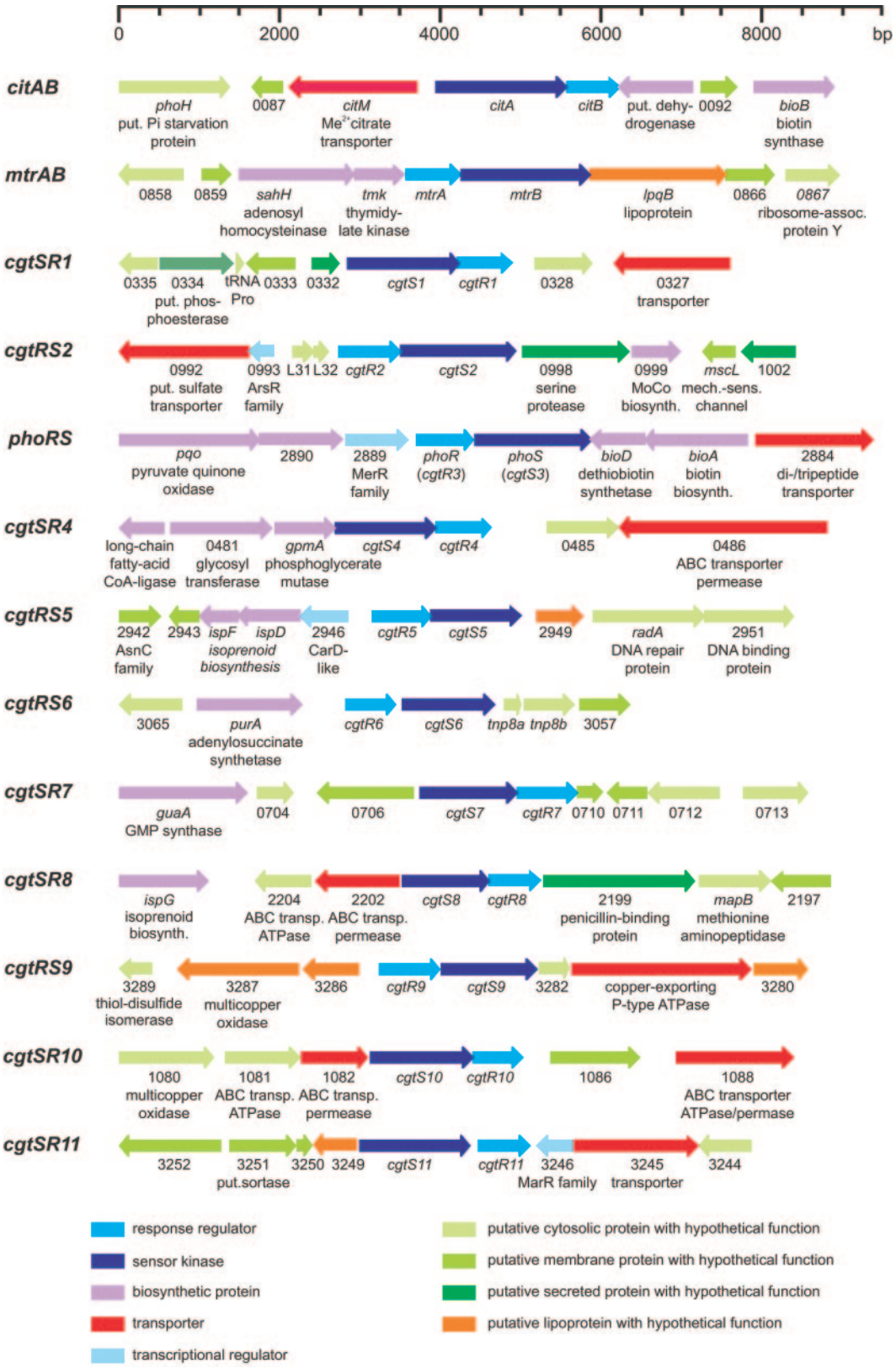

FIG. 1. Maps of the 13 *C. glutamicum* genome loci containing genes for two-component signal transduction systems. The different colors indicate different functions of the encoded proteins or different localizations of proteins with unknown functions. The numbers are derived from GenBank accession no. BX927147 (13). put., putative; Me, metal; assoc., associated; biosynth., biosynthesis; mech.-sens., mechano-sensitive; CoA, coenzyme A; transp., transporter.

corresponding genomic loci and the designations given to the corresponding genes are shown. As is evident from this figure, each sensor kinase gene is paired with a response regulator gene, suggesting that the corresponding proteins form the cognate partners of a typical two-component signal transduction system. No orphan sensor kinase or response regulator genes are present in the genome. Bioinformatic analysis indicated that each of the 13 sensor kinases possesses at least one transmembrane helix, indicating that all are integral membrane proteins (Table S4 in the supplemental material). All sensor kinases contained the two domains that are characteristic for these proteins, i.e., the histidine kinase acceptor domain containing the phosphorylated histidine residue and the ATP-binding domain. Analysis of the response regulators predicted that all contain the characteristic amino-terminal receiver domain and a carboxy-terminal DNA-binding domain. Thus, all two-component systems of *C. glutamicum* appear to have roles in gene regulation.

Except for the CitAB system, which belongs to a family of two-component systems controlling the uptake and metabolism of citrate or dicarboxylates (5, 10, 14, 26), obvious predictions of the stimuli recognized by the sensor kinases and of the target genes regulated by the response regulators were not possible. In a recent analysis of the MtrAB system, we provided experimental evidence that this system is involved in the regulation of genes involved in cell wall metabolism and osmoregulation (22).

For functional analysis of the two-component systems, deletion mutants, each lacking one pair of sensor kinase/response regulator genes, were constructed using a previously established method (23). In these deletion mutants, only the first six codons of the promoter-proximal gene and the last 12 codons of the promoter-distal gene are still present, whereas the intervening region is replaced by a 21-bp sequence tag. For 12 two-component systems, the corresponding deletion strains could be obtained, showing that these genes are not essential (Table S1 in the supplemental material). In the case of the *cgtSR4* genes, however, no deletion mutants were obtained, suggesting that *cgtS4* and/or *cgtR4* is essential.

Screening of two-component deletion mutants for growth under P_i limitation. In order to determine whether one of the *C. glutamicum* two-component systems is involved in the phosphate starvation response, the set of 12 deletion mutants was tested for growth in glucose minimal medium containing different P_i concentrations. One deletion strain (Δ*cgtRS3* mutant) showed a growth defect under P_i-limiting conditions (0.065 mM) but not under P_i excess (13 mM), indicating that the *cgtRS3* genes might have a specific function in the P_i starvation response. When the Δ*cgtRS3* mutant was transformed with a plasmid containing the *cgtRS3* genes (pEXEx2-phoRS), the growth defect under P_i limitation was abolished and the complemented strain grew almost like the wild type (Fig. 2). This experiment confirmed that the growth phenotype of the mutant was caused by the deletion of the *cgtRS3* genes. In this work the corresponding genes were renamed *phoR* for *pho*sphate *r*esponse regulator and *phoS* for *pho*sphate *s*ensor kinase and the mutant was renamed the Δ*phoRS* mutant. The coding regions of *phoR* and *phoS* are separated by 8 bp, suggesting cotranscription of the two genes.

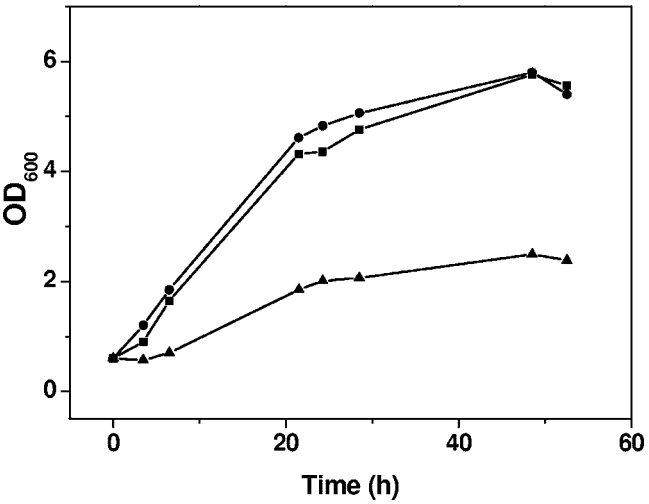

FIG. 2. Growth of the *C. glutamicum* 13032/pEKEx2 (■), Δ*phoRS*/pEKEx2 (▲), and Δ*phoRS*/pEKEx2-*phoRS* (●) strains in CGXII minimum medium with 40 g/liter glucose and 0.065 mM phosphate. Cells were precultured in CGXII medium with 0.065 mM phosphate for 24 h.

The sensor kinase encoded by PhoS is composed of 485 amino acids (52,365 Da). It contains two putative transmembrane helices extending from residues 44 to 64 and 184 to 200, enclosing an extracytoplasmic domain of 120 amino acid residues. The carboxy-terminal cytoplasmic portion of PhoS contains a HAMP domain (3) extending from residues 185 to 255, a histidine kinase A acceptor domain extending from residues 266 to 330, and a DNA gyrase B/Hsp90-like ATPase domain extending from residues 373 to 485. The histidine residue which presumably is phosphorylated is located at position 276. The response regulator encoded by PhoR is composed of 235 amino acids (26,350 Da) and contains two conserved domains, i.e., an amino-terminal receiver domain extending from residues 9 to 128 and a carboxy-terminal DNA binding domain extending from residues 156 to 230. The aspartate residue which presumably is phosphorylated by PhoS is located at position 59.

Gene expression changes in the Δ*phoRS* mutant after a shift from P_i-sufficient to P_i-limiting conditions. The growth defect under P_i-limiting conditions indicated that the Δ*phoRS* mutant is impaired in its ability to induce the phosphate starvation genes (see the introduction). Therefore, the changes in gene expression occurring 10, 30, and 60 min after a shift from P_i excess to P_i starvation were analyzed as described previously for the wild type (12) and as outlined in Materials and Methods. Overall, 34 and 47 genes of the *C. glutamicum* Δ*phoRS* mutant showed a ≥2-fold-increased and a ≥2-fold-decreased mRNA level, respectively, at least at one time point after the shift (Table 1). Except for the *pst* genes, none of the prominent phosphate starvation-inducible genes, e.g., the *ugpAEBC* operon, *glpQ1*, *phoH1*, *ushA*, or *nucH*, were induced either 10, 30, or 60 min after the shift (Fig. 3B). This is in marked contrast to the situation in the wild type (Fig. 3A) and supports a critical role for the PhoRS two-component system in the phosphate starvation response of *C. glutamicum*. In the case of the *pstSCAB* operon, a twofold- to fivefold-increased mRNA level was observed within 1 hour after the shift. Since this induction level is lower than in the

TABLE 1. Genes showing ≥2-fold-altered mRNA ratios in the *C. glutamicum* Δ*phoRS* mutant 10, 30, or 60 min after a shift from P_i excess to P_i limitation

C. glutamicum locus tag no.	NCBI no.	Annotation[c]	Gene	mRNA ratio (after shift/before shift to P_i-limiting conditions)[a]		
				10 min	30 min	60 min
cg0160	NCgl0123	Hypothetical protein		0.43[b]	0.34	0.65
cg0229	NCgl0181	Glutamine 2-oxoglutarate aminotransferase, large subunit	*gltB*	1.28	1.95	2.59[b]
cg0230	NCgl0182	Glutamine 2-oxoglutarate aminotransferase, small subunit	*gltD*	6.41[b]	5.83[b]	6.24[b]
cg0310	NCgl0251	Catalase	*katA*	3.49[b]	3.24[b]	1.55[b]
cg0464	NCgl0375	Copper-exporting P-type ATPase	*ctpA*	10.32[b]	21.51	5.16[b]
cg0467	NCgl0378	Put. hemin ABC transporter, periplasmic binding protein	*hmuT*	0.42[b]	0.40	0.63[b]
cg0468	NCgl0379	Put. hemin ABC transporter, permease	*hmuU*	0.33		0.83[b]
cg0469	NCgl0380	Put. hemin ABC transporter, ATPase	*hmuV*	0.41[b]	0.50	0.69
cg0470	NCgl0381	Conserved secreted protein		0.16[b]	0.22[b]	0.58
cg0500	NCgl0405	Transcriptional regulator, LysR family		2.07[b]	2.74	2.21[b]
cg0569	NCgl0465	Cation-transporting P-type ATPase		8.60[b]	4.77	2.65[b]
cg0589	NCgl0482	Put. Fe^{3+} siderophore ABC transporter, ATPase		0.06[b]	0.10[b]	0.44
cg0590	NCgl0483	Put. Fe^{3+} siderophore ABC transporter, permease		0.22[b]	0.12	0.51
cg0591	NCgl0484	Put. Fe^{3+} siderophore ABC transporter, permease		0.15[b]	0.21	0.26
cg0759	NCgl0628	Methylcitrate dehydratase	*prpD2*	0.33[b]	0.47[b]	0.55[b]
cg0760	NCgl0629	Methylisocitrate lyase	*prpB2*	0.37[b]	0.53[b]	0.49[b]
cg0762	NCgl0630	Methylcitrate synthase	*prpC2*	0.22[b]	0.39	0.40
cg0767	NCgl0635	Put. siderophore-interacting protein		0.16[b]	0.18	0.62
cg0768	NCgl0636	Put. Fe^{3+} siderophore ABC transporter, ATPase		0.25[b]	0.09	0.36
cg0769	NCgl0637	Put. Fe^{3+} siderophore ABC transporter, permease		0.16[b]	0.09	0.23
cg0771	NCgl0639	Put. Fe^{3+} siderophore ABC transporter, periplasmic binding protein		0.14[b]	0.10	0.29
cg0812	NCgl0678	Acetyl/propionyl-CoA carboxylase, beta subunit	*dtsR1*	0.26[b]	0.24[b]	0.48[b]
cg0921	NCgl0773	Put. siderophore-interacting protein		0.13[b]	0.12[b]	0.51[b]
cg0922	NCgl0774	Put. Fe^{3+} siderophore ABC transporter, periplasmic binding protein		0.11[b]	0.12[b]	0.27[b]
cg0924	NCgl0776	Put. Fe^{3+} siderophore ABC transporter, periplasmic-binding protein		0.07[b]	0.10[b]	0.18[b]
cg0926	NCgl0777	Put. Fe^{3+} siderophore ABC transporter, permease		1.24	0.17	
cg0927	NCgl0778	Put. Fe^{3+} siderophore ABC transporter, permease		0.24[b]	0.28[b]	0.38[b]
cg0928	NCgl0779	Put. Fe^{3+} siderophore ABC transporter, ATPase		0.65[b]	0.12	0.82
cg0957	NCgl0802	Fatty acid synthase	*fas-1B*	0.46	0.16	0.27[b]
cg1120	NCgl0943	Transcriptional regulator, AraC-family	*ripA*	0.21[b]	0.20	0.42[b]
cg1121	NCgl0944	Conserved membrane protein		0.49[b]	0.92	1.26
cg1343	NCgl1141	Nitrate reductase, beta subunit	*narH*	4.40[b]	1.91	1.08[b]
cg1344	NCgl1142	Nitrate reductase, alpha subunit	*narG*	2.26[b]	1.87	1.13
cg1405	NCgl1200	Put. siderophore-interacting protein		0.34[b]	0.17	0.46
cg1412	NCgl1205	Sugar ABC transporter, permease		0.48[b]	0.79	0.91
cg1413	NCgl1206	Sugar ABC transporter, periplasmic binding protein		0.40[b]	0.78	0.54
cg1418	NCgl1209	Put. Fe^{3+} siderophore periplasmic binding protein		0.22[b]	0.25	0.50[b]
cg1419	NCgl1210	Transporter, bile acid:Na^+ symporter family (TC 2.A.28)		0.39[b]	0.35	0.48
cg1447	NCgl1232	Put. Co/Zn/Cd exporter, cation diffusion facilitator family (TC 2.A.4)		7.16	6.08	7.18[b]
cg1695	NCgl1444	Put. DNA binding protein		3.17[b]	2.18	2.05[b]
cg1737	NCgl1482	Aconitase	*acn*	1.87	3.69[b]	2.25[b]
cg1785	NCgl1521	Ammonia permease	*amt*	1.77	2.07[b]	4.46
cg1930	NCgl1646	Put. secreted trypsin-like serine protease		0.14[b]	0.21[b]	0.51[b]
cg2118	NCgl1859	Transcriptional regulator of sugar metabolism, DeoR family		4.08[b]	0.93	0.69[b]
cg2120	NCgl1861	Enzyme II of fructose phosphotransferase system	*ptsF*	3.64[b]	0.66	0.41[b]
cg2181	NCgl1915	Oligopeptide ABC transporter, periplasmic binding protein	*oppA*	0.68[b]	0.53[b]	0.31[b]
cg2183	NCgl1917	Oligopeptide ABC transporter, permease	*oppC*	0.71	0.58	0.44[b]
cg2234	NCgl1959	Put. Fe^{3+} siderophore binding protein		0.26[b]	0.24	0.55[b]
cg2283	NCgl2001	Conserved hypothetical protein		0.33[b]	0.36	0.62
cg2422	NCgl2127	Lipoate-protein ligase	*lipB*	1.99[b]	2.17	1.46[b]
cg2423	NCgl2128	Lipoic acid synthase	*lipA*	1.65[b]	2.12[b]	1.26[b]
cg2726	NCgl2393	Put. membrane protein		15.60[b]	3.46	4.46[b]
cg2743	NCgl2409	Fatty acid synthase	*fas-IA*	0.42[b]	0.39	0.46[b]
cg2777	NCgl2434	Put. membrane protein		0.47[b]	0.37	0.55[b]
cg2782	NCgl2439	Ferritin	*ftn*	18.82[b]	17.89	12.14[b]
cg2843	NCgl2483	Phosphate ABC transporter, ATPase	*pstB*	1.02[b]	1.19	1.94[b]
cg2844	NCgl2484	Phosphate ABC transporter, permease	*pstA*	1.06[b]	1.93	2.43[b]
cg2845	NCgl2485	Phosphate ABC transporter, permease	*pstC*	1.39[b]	3.55	5.21[b]
cg2846	NCgl2486	Phosphate ABC transporter, binding protein	*pstS*	1.74[b]	2.55	4.10[b]

Continued on following page

TABLE 1—*Continued*

C. glutamicum locus tag no.	NCBI no.	Annotation[c]	Gene	mRNA ratio (after shift/before shift to P_i-limiting conditions)[a]		
				10 min	30 min	60 min
cg2925	NCgl2553	Enzyme II of sucrose phosphotransferase system	*ptsS*	3.83[b]	0.93[b]	0.56[b]
cg3116	NCgl2717	Phosphoadenosine phosphosulfate reductase	*cysH*	2.53[b]		0.95
cg3118	NCgl2718	Sulfite reductase (hemoprotein)	*cysI*	3.17[b]	1.41	0.79
cg3132	NCgl2731	Put. membrane protein		2.53[b]	1.00	1.13[b]
cg3195	NCgl2787	Flavin-containing monooxygenase		0.40[b]	0.53	0.88
cg3227	NCgl2817	Put. L-lactate dehydrogenase (quinone)	*lldD*	18.31[b]	11.82	1.32
cg3281	NCgl2859	Cation-transporting P-type ATPase		3.77[b]	2.03	1.47[b]
cg3282	NCgl2860	Put. secreted lipoprotein		14.95[b]	4.27	4.54
cg3286	NCgl2864	Put. secreted lipoprotein		3.54[b]	2.44	2.67[b]
cg3287	NCgl2865	Put. secreted multicopper oxidase		4.13[b]	2.81	1.57
cg3303	NCgl2877	Transcriptional regulator, PadR-like family		7.37[b]	4.16	4.18[b]
cg3327	NCgl2897	Starvation-induced DNA protection protein	*dps*	3.76[b]	3.51	2.44[b]
cg3335	NCgl2904	Malic enzyme	*mez*	0.46[b]	0.45	0.38[b]
cg3386	NCgl2952	Put. maleylacetate reductase	*tcbF*	0.41[b]	0.37	0.49
cg3387	NCgl2953	Put. myo-inositol:H^+ symporter (TC 2.A.1.1.26)		0.32[b]	0.32	0.50
cg3390	NCgl2956	Put. sugar phosphate isomerase/epimerase		0.38[b]	0.58	1.15[b]
cg3391	NCgl2957	Myo-inositol 2-dehydrogenase	*idhA1*	0.41[b]	0.43	0.38
cg3404	NCgl2970	Put. Fe^{3+} siderophore periplasmic binding protein		0.06[b]	0.07[b]	0.18

[a] The mRNA ratios are averages from at least two of three experiments. Only values for open reading frames whose mRNA ratio was altered at least twofold either 10, 30, or 60 min after the P_i downshift are shown.
[b] $P < 0.05$ as determined by a *t* test.
[c] Put., putative; CoA, coenzyme A.

wild type, the data suggest also that the *pst* operon is controlled by the PhoRS system.

In the hierarchical cluster analysis of genes showing altered expression in the wild type after a shift from P_i excess to P_i limitation, five subgroups were identified (12). Whereas subgroup 1 harbored genes characteristic of phosphate starvation, subgroups 2 to 5 contained genes of which most are apparently not directly related to phosphate metabolism, i.e., genes putatively involved in copper metabolism (subgroup 2, with transiently increased expression after the shift), in protocatechuate degradation (subgroup 3, with transiently increased expression after the shift), in protein synthesis (subgroup 4, with continuously decreasing expression after the shift), or in iron uptake (subgroup 5, with transiently decreased expression after the shift). The changed expression of these genes is presumably due to the exchange of the culture medium in the course of the experiment (subgroups 2, 3, and 5) or to the reduced growth rate of the cells after the shift to P_i limitation (subgroup 4). Whereas the mRNA levels of the majority of the genes belonging to subgroup 1 were unaltered after the shift in the Δ*phoRS* strain, many of the genes belonging to the other subgroups, in particular subgroups 2 and 5, showed alterations in expression profiles similar to those in the wild type. This result supports the assumption that the *phoRS* deletion specifically affects the expression of the phosphate starvation-inducible genes.

In a second set of DNA microarray experiments, the mRNA levels of the wild type and the Δ*phoRS* mutant were directly compared before and 60 min after a shift from P_i excess (13 mM) to P_i limitation (0.065 mM). As expected from the previous experiment, the majority of phosphate starvation-inducible genes (*pstS*, *pstA*, *pstB*, *ugpA*, *ugpE*, *ugpB*, *ugpC*, *glpQ1*, *phoH1*, *ushA*, *nucH*, *pctB*, *pctC*, and cg3393) showed higher mRNA levels in the wild type than in the Δ*phoRS* mutant after the shift (data not shown).

Primer extension analysis. In order to verify the DNA microarray data and to determine the transcriptional start sites, primer extension analyses were performed for prominent *psi* genes, i.e., the *pstSCAB* operon, the *upgAEBC* operon, the *phoRS* operon, *ushA*, and *nucH* (Fig. 4). In these experiments, RNA of the wild type and of the Δ*phoRS* mutant grown either under P_i excess (13 mM) or under P_i limitation (0.065 mM) for 10 min (*phoRS*), 60 min (*pst*, *ugp*), or 90 min (*ushA*, *nucH*) were used. The growth conditions and the preparation of RNA were identical to those in DNA microarray experiments. In the case of the wild type, primer extension products were observed for all five tested genes with RNA from cells grown under P_i limitation but not with RNA from cells grown under P_i excess, confirming that the expression of these genes is induced under P_i limitation. In the case of the Δ*phoRS* mutant, primer extension products for *ugpA*, *phoR*, *ushA*, and *nucH* were observed neither with RNA from cells grown under P_i excess nor with RNA from cells grown under P_i limitation. Again, this confirms the results of the microarray data, which indicate that induction of these genes under P_i starvation is dependent on the PhoRS two-component system. In the case of *pstS*, a primer extension product was obtained with RNA from Δ*phoRS* cells grown under P_i limitation but not with RNA from Δ*phoRS* cells grown under P_i excess. The signal intensity of the *pstS* primer extension product was lower in the Δ*phoRS* mutant than in the wild type (Fig. 4A), which is in agreement with the DNA microarray data, where the induction ratio was lower in the Δ*phoRS* strain than in the wild type. The primer extension data thus support the assumption that induction of the *pstSCAB* operon by P_i starvation is dependent on PhoRS and one or several additional regulators which are not yet known. The fact that no primer extension product was obtained for *phoR* with RNA from Δ*phoRS* cells grown under P_i limitation suggests a positive autoregulation of the *phoRS* genes.

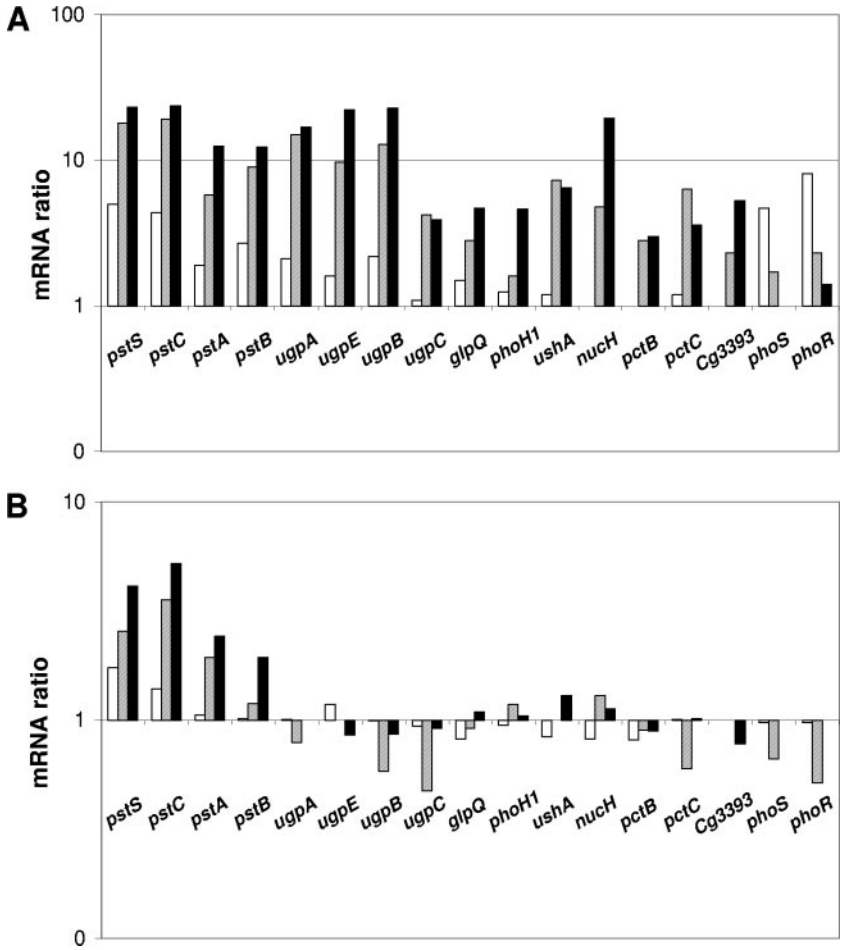

FIG. 3. Relative mRNA levels of phosphate starvation-inducible genes in the *C. glutamicum* wild type (A) and the Δ*phoRS* strain (B). The ratios represent the mRNA levels 10 min (white bars), 30 min (gray bars), and 60 min (black bars) after the onset of P_i starvation versus the mRNA level immediately before the onset of P_i starvation. The experiment was performed in triplicate, and average mRNA ratios were calculated. The criterion used for selection of RNA ratios was a signal-to-noise ratio of ≥3 for either Cy3 or Cy5 fluorescence. The data for the wild type (A) were taken from our previous studies (12).

The transcriptional start points identified by the primer extension experiments were located 81 bp upstream of the GTG start codon of *pstS*, 28 bp upstream of the proposed GTG start codon of *ugpA*, 44 bp upstream of the ATG start codon of *phoR*, 60 bp upstream of the ATG start codon of *ushA*, and 35 bp upstream of the ATG start codon of *nucH*. Analysis of the promoter sequences (Fig. 4F) revealed motifs with similarity to the proposed −10 region of *C. glutamicum* (25) but no obvious PhoR binding site.

DISCUSSION

In many bacteria whose phosphate starvation response has been studied in some detail, e.g., *E. coli*, *B. subtilis*, *Synechocystis* spp. (32), or *Streptomyces* spp. (30, 31), two-component signal transduction systems play a central regulatory role in this process. In the work presented here, phenotypic and genetic evidence that this is also the case in *C. glutamicum* was obtained. Screening of 12 *C. glutamicum* deletion mutants, each lacking one specific two-component system, revealed that one of them, the Δ*phoRS* strain, is specifically impaired in its ability to grow under P_i limitation. Complementation showed that the *phoRS* deletion is responsible for the growth impairment of this strain. Independent evidence for an involvement of the *phoRS* genes in the P_i starvation response was obtained previously during the elucidation of the phosphate starvation stimulon of *C. glutamicum* by DNA microarrays (12). In these studies, the mRNA levels of *phoS* and *phoR* were strongly increased (fivefold and eightfold, respectively) 10 min after a shift from P_i excess to P_i limitation but reached preshift levels within 60 to 90 min. Of the other 24 genes encoding proteins of two-component systems, only *cgtR9* also showed a >4-fold-altered expression after the shift. Its mRNA level was sevenfold increased 30 min after the shift and remained increased up to 120 min after the shift (12). Preliminary results suggest that the CgtSR9 two-component system is involved in copper metabolism, since the Δ*cgtSR9* mutant has a lower copper resistance than the wild type (M. Brocker and M. Bott, unpublished data). It therefore seems likely that the CgtSR9 two-component system is responsible for the induction of the genes involved in copper metabolism (subcluster 2) that were identified in the hierarchical cluster analysis (12). Consequently, PhoRS is the only two-com-

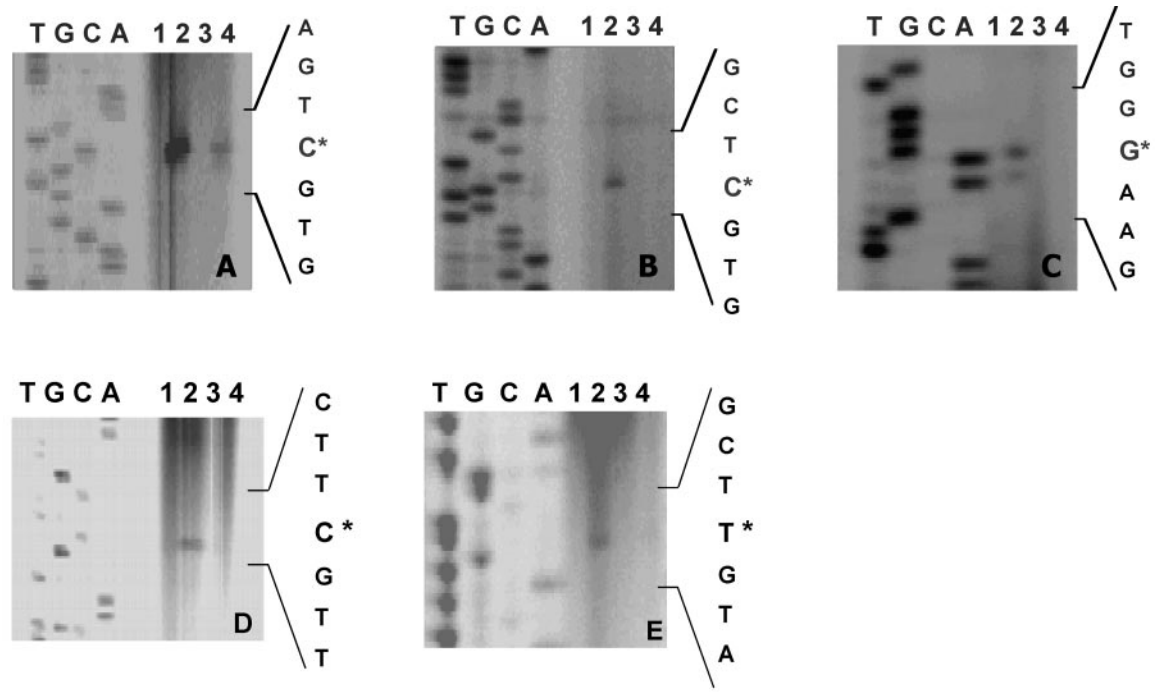

FIG. 4. Comparison of mRNA levels and determination of the transcriptional start sites of the *C. glutamicum* genes *pstS* (A), *ugpA* (B), *phoR* (C), *ushA* (D), and *nucH* (E) by primer extension analysis. The reverse transcriptase reactions were performed with the oligonucleotides pstS_prext2, ugpA_prext2, phoR_prext1b, ushA80prext, and nucH90prext for these four genes, respectively, and 20 μg of total RNA was isolated from the following strains: the wild type grown under phosphate excess (lane 1); the wild type 60 min (A and B), 10 min (C), or 90 min (D and E) after a shift from 13 mM P_i to 0.065 mM P_i (lane 2); the Δ*phoRS* mutant grown under P_i excess (lane 3); and the Δ*phoRS* mutant 60 min (A and B), 10 min (C), or 90 min (D and E) after a shift from 13 mM P_i to 0.065 mM P_i (lane 4). The transcriptional start sites are indicated by asterisks. The corresponding sequencing reactions were generated by using the same IRD-800-labeled oligonucleotide as in the primer extension reactions as well as PCR products which cover the region of the respective transcriptional start site as the template DNA. In panel F, the promoter regions derived from the primer extension studies were aligned. The transcriptional start points are shown in bold and underlined, the proposed start codons are indicated in bold, and putative −10 regions are shaded in gray.

ponent system of *C. glutamicum* whose synthesis is rapidly and transiently induced in response to a shift to P_i limitation.

Transcriptome comparisons with DNA microarrays of the Δ*phoRS* mutant before and after a shift from P_i excess to P_i limitation showed that in contrast to what occurs in the wild type, none of the characteristic *psi* genes except *pstSCAB* (e.g., *ugpAEBC*, *glpQ1*, *phoH*, *ushA*, or *nucH*) was induced within 60 min after the shift (Fig. 3). The results of the microarrays regarding the expression pattern of the *pst*, *ugp*, and *phoRS* genes were confirmed independently by primer extension analyses (Fig. 4), which also provided evidence for positive autoregulation of the *phoRS* genes. The induction of the *pstSCAB* genes in the Δ*phoRS* mutant suggests the presence of one or more additional regulators which are not yet known. A situation where more than one regulator is involved in the phosphate starvation response has also been found in *B. subtilis* (4).

Bioinformatic analyses revealed that the PhoS and PhoR proteins of *C. glutamicum* are highly conserved within the suborder *Corynebacterineae*. The sensor kinase PhoS orthologs from *Corynebacterium efficiens* (locus tag CE2493), *Corynebacterium diphtheriae* (DIP1935), *Mycobacterium avium* subsp. *paratuberculosis* (MAP0592), and *Mycobacterium tuberculosis* (Rv0758) possess 73%, 51%, 44%, and 44% sequence identity, respectively. The response regulator PhoR orthologs from *C. efficiens* (CE494), *C. diphtheriae* (DIP1936), *Mycobacterium avium* subsp. *paratuberculosis* (MAP0591), and *Mycobacterium tuberculosis* (Rv0757) possess 91%, 81%, 65%, and 65% sequence identity, respectively. However, an involvement of these orthologs in phosphate regulation has not been shown. The results provided here for *C. glutamicum* certainly support such a function.

In summary, our results show that 12 of the 13 two-component systems of *C. glutamicum* are not essential, and they provide clear evidence for an involvement of the *C. glutamicum* PhoRS two-component system in the adaptation to phosphate-limiting conditions. Our future studies will focus on the iden-

tification of the direct target genes and the binding site of the response regulator PhoR.

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