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The Phosphate Starvation Stimulon of *Corynebacterium glutamicum* Determined by DNA Microarray Analyses

Takeru Ishige, Malgorzata Krause, Michael Bott, Volker F. Wendisch,* and Hermann Sahm

Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany

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The phosphate (P_i) starvation stimulon of *Corynebacterium glutamicum* was characterized by global gene expression analysis by using DNA microarrays. Hierarchical cluster analysis of the genes showing altered expression 10 to 180 min after a shift from P_i-sufficient to P_i-limiting conditions led to identification of five groups comprising 92 genes. Four of these groups included genes which are not directly involved in P metabolism and changed expression presumably due to the reduced growth rate observed after the shift or to the exchange of medium. One group, however, comprised 25 genes, most of which are obviously related to phosphorus (P) uptake and metabolism and exhibited 4- to >30-fold-greater expression after the shift to P_i limitation. Among these genes, the RNA levels of the *pstSCAB* (ABC-type P_i uptake system), *glpQ* (glycerophosphoryldiester phosphodiesterase), *ugpAEBC* (ABC-type *sn*-glycerol 3-phosphate uptake system), *phoH* (unknown function), *nucH* (extracellular nuclease), and Cgl0328 (5′-nucleotidase or related esterase) genes were increased, and *pstSCAB* exhibited a faster response than the other genes. Transcriptional fusion analyses revealed that elevated expression of *pstSCAB* and *ugpAEBC* was primarily due to transcriptional regulation. Several genes also involved in P uptake and metabolism were not affected by P_i starvation; these included the genes encoding a PitA-like P_i uptake system and a putative Na^+-dependent P_i transporter and the genes involved in the metabolism of pyrophosphate and polyphosphate. In summary, a global, time-resolved picture of the response of *C. glutamicum* to P_i starvation was obtained.

Phosphorus (P) is an indispensable component of all cells in living organisms. In bacteria, P typically is assimilated as inorganic orthophosphate (P_i), which is transported into the cell by specific uptake systems. Alternatively, organophosphates and phosphonates may serve as sole P sources; either these compounds are imported by specific uptake systems and degraded intracellularly or P_i that is liberated by extracellular degradation of the compounds is taken up into the cell. For several bacteria, particularly *Escherichia coli* and *Bacillus subtilis*, P metabolism and the regulatory mechanisms that permit adaptation to varying P availability have been well studied (22, 47).

E. coli possesses three P_i uptake systems (19). PitA is expressed constitutively and transports phosphate in a proton motive force-dependent manner (47). When the extracellular P_i concentration falls below about 4 μM, P_i is taken up primarily by the Pst system at the expense of ATP. The Pst system is an ABC transport system encoded by the *pstSCAB* genes of the *pstSCAB-phoU* operon, which is induced under P_i starvation conditions (47). When formed, the PitB transporter encoded by a cryptic homolog of *pitA* is able to transport P_i in a manner similar to the manner used by PitA (19). The genes constituting the P_i starvation stimulon were identified in screening analyses based on transcriptional *lacZ* fusions (32) and by proteome analysis (44). Induction of the P_i starvation genes is dependent on the PhoR-PhoB two-component regulatory system. Under P_i starvation conditions the sensor kinase PhoR phosphorylates PhoB, and PhoB~P in turn activates transcription of at least 31 genes, which form the PhoB regulon (47). These genes include the *phoBR* operon, the *pstSCAB-phoU* operon encoding the high-affinity P_i ABC transport system 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 a polyanion porin, *phoH* encoding an ATP-binding protein, *psiE* (function unknown), and *phnC DEFGHIJKLMNOP* (uptake of phosphonates and degradation via the C-P lyase pathway) (47). Thus, when P_i is scarce, *E. coli* takes up P_i by an ATP-driven high-affinity transport system, mobilizes P_i from extracellular sources by phosphatases, and induces systems for the uptake and degradation of organophosphates, such as glycerol 3-phosphate, or of phosphonates, such as ethylphosphonate (47). The response of *B. subtilis* to P_i starvation involves increased expression of two alkaline phosphatases (*phoA* and *phoB*), of the high-affinity P_i uptake system encoded by $pstSCAB_1B_2$, of the *phoPR* and *resABCDE* operons encoding the PhoP-PhoR and ResD-ResE two-component regulatory systems as well as proteins involved in cytochrome *c* synthesis, of *glpQ* coding for glycerolphosphoryl phosphodiesterase, of 13 hypothetical genes (*ydhF*, *yfhM*, *yhaX*, *yhbH*, *yheK*, *yjbC*, *ykoL*, *ykzA*, *ysnF*, *yttB*, *yvgO*, *yxiE*, and *csbD*), of the phosphodiesterase gene *phoD* having a role in teichoic acid turnover, and of the *tuaABCDEFGH* operon coding for teichuronic acid biosynthesis (4, 25, 35). Reduced expression was observed for the *tagAB* and *tagDEF* teichoic acid biosynthesis operons (22). Thus, upon P_i starvation *B. subtilis* takes up P_i by an ATP-driven high-affinity uptake system, mobilizes phosphate extracellularly by using phosphatases, and replaces teichoic acid in the cell wall with the non-phosphate-containing compound teichuronic acid. In *B. subtilis* P_i starvation activates

* Corresponding author. Mailing address: Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany. Phone: 49 2461 61 5169. Fax: 49 2461 61 2710. E-mail: v.wendisch@fz-juelich.de.

a specific response involving PhoPR, Spo0A, and ResDE (7, 22, 42), as well as the general stress response (22). Upon P_i starvation the sensor kinase PhoR phosphorylates the response regulator PhoP, and PhoP~P activates transcription of *phoPR* and *resABCDE* and activates or represses transcription of other Pho regulon genes. ResD-ResE is required for full induction of the Pho regulon genes, and Spo0A is required for termination of the P_i response and subsequent initiation of sporulation. The general stress response is mediated by σ^B, and genes of the σ^B regulon code for proteins that protect DNA, membranes, and proteins against oxidative stress and are required for survival under extreme environmental conditions, such as those imposed by heat, acid or osmotic stress (18, 36).

In *Mycobacterium tuberculosis*, a pathogenic member of the *Corynebacterineae* and the causative agent of tuberculosis, it was recognized that the most immunogenic antigen, Pab, is present at higher levels under P_i starvation conditions (3) and is a phosphate-binding protein similar to *E. coli* PstS (11). It was then realized that the genome of *M. tuberculosis* harbors three different phosphate-binding protein genes in three operons (*pstBS1C1A2*, *pstS2*, and *pstS3C2A1*) at one locus (12, 27) and that the phosphate-binding proteins are surface attached (27). By using translational fusions, two P_i starvation-responsive promoters were identified for the *pstBS1C1A2* operon (43). In the related organism *Mycobacterium smegmatis*, alkaline phosphatase activity was shown to increase under P_i starvation conditions (43).

Corynebacterium glutamicum is a gram-positive bacterium, and its genome has a high G+C content. *C. glutamicum* is used for biotechnological production of more than 10^6 tons of amino acids, especially L-glutamate and L-lysine, per year (38). Amino acids are derived from intermediates of the central carbon metabolism. As P assimilation occurs mainly in the form of P_i in the energy and carbon metabolism reactions, P metabolism is closely intertwined with energy and central carbon metabolism. Thus, the interplay between P metabolism and C metabolism is of particular interest in amino acid-producing *C. glutamicum* strains. However, very little is known about P metabolism or its regulation in *C. glutamicum*. In this study, the P_i starvation stimulon of *C. glutamicum* was determined by monitoring global gene expression changes in response to P_i availability.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C.glutamicum* wild-type strain ATCC 13032 was used for all experiments. *C. glutamicum* was cultivated in CGXII minimal medium (23) containing 0.03 g of protocatechuic acid per liter and 40 g of glucose per liter as a carbon and energy source. As the sole P source, 13 mM P_i was used under P_i-sufficient conditions and 0.13 mM P_i was used under P_i-limited conditions. For the P_i up-shift experiment, *C. glutamicum* was precultured at 30°C with agitation at 120 rpm in CGIII medium (31). After washing, the cells were cultivated in CGXII medium under P_i-limiting conditions for 24 h, and then cells were inoculated into CGXII medium with various P_i concentrations at a starting optical density at 600 nm (OD_{600}) of 0.6. For the P_i down-shift experiment, cells were first precultured in CGIII medium, then cultured for 24 h in CGXII medium under P_i-sufficient conditions, and finally inoculated into the same medium at a starting OD_{600} of 0.6. Exponentially growing cells from this P_i-sufficient culture were harvested and washed. One aliquot was used for RNA preparation (zero time), and another aliquot was shifted into the CGXII medium with a limiting P_i concentration (0.13 mM) (referred to as P_i down-shift). RNA was prepared 10, 30, 60, 90, 120, and 180 min after the P_i down-shift and was used for global gene expression analysis in a comparison with preshift conditions (zero time). *E.coli* JM109 was used as the host for construction of reporter plasmids and was cultivated in Luria-Bertani medium (39) at 37°C and 170 rpm.

Generation of *C. glutamicum* DNA microarrays. DNA microarrays based on PCR products of *C. glutamicum* were generated for use in global gene expression analyses by using the procedures described previously (24, 34, 48, 50). The genes were amplified in 96-well plates with genomic DNA of *C. glutamicum* ATCC 13032 as the template and gene-specific primers purchased from degussa (Frankfurt, Germany). The identities and quality of the PCR products were checked by gel electrophoresis, and the PCR products were precipitated with isopropanol, resuspended in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), and transferred to 384-well plates as described previously. The PCR products were printed onto poly-L-lysine-coated glass slides by using an arraying robot. The DNA microarrays were rehydrated in a 1× SSC atmosphere, UV cross-linked (650 μJ), and blocked in 230 ml of methyl pyrrolidinone containing 15 ml of 1 M boric acid (titrated to pH 8.0 with sodium hydroxide) and 4.4 g of succinic anhydride. The *C. glutamicum* whole-genome DNA microarray contained 3,673 PCR products covering 2,860 of the 2,994 genes (506 genes in duplicate) described for the genome according to the National Center for Biotechnology Information (NCBI) (accession no. NC003450) and 284 additional putative coding sequences (23 sequences in duplicate). In general, the PCR products were 500 ± 50 bp long and represented regions of the genes which facilitate specific hybridization. Additionally, 100 spots of *C. glutamicum* genomic DNA were used as normalization controls, and 16 spots of λ DNA, 16 spots of *E. coli* DNA, and one spot of the *E. coli aceK* gene were used as negative controls.

Preparation of total RNA. Exponentially growing cells at an OD_{600} of about 5.0 were poured into ice-containing tubes precooled to −70°C and were harvested by centrifugation (5 min, 3,500 × *g*, 4°C) (48). The cell pellet either was directly subjected to RNA isolation or was immediately frozen in liquid nitrogen and stored at −70°C until it was used. For isolation of total RNA, the (frozen) cell pellet was resuspended in 350 μl of RLT buffer of the RNeasy system (Qiagen, Hilden, Germany). After this, 250 mg of 0.1-mm-diameter zirconia-silica beads (Roth, Karlsruhe, Germany) were added, and the cells were disrupted by 15 and 30 s of bead beating with a Silamat S5 (Vivadent, Ellwangen, Germany). After centrifugation (1 min, 13,000 × *g*), the supernatant was used for RNA preparation by using the RNeasy system with DNase I treatment according to manufacturer's instructions. Isolated RNA samples were checked for purity by denaturing formaldehyde agarose gel electrophoresis and spectrophotometrically and were kept at −70°C until they were used (34).

Gene expression analysis with *C. glutamicum* DNA microarrays. 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 (GibcoBRL/Life Technologies, Gaithersburg, Md.) and the fluorescent nucleotide analogues FluoroLink Cy3-dUTP (green) and Cy5-dUTP (red) (Amersham Pharmacia, Little Chalfont, United Kingdom) as described previously (24, 34, 48). The labeled cDNA probes were purified and concentrated by using Microcon YM-30 filter units (Millipore, Bedford, Mass.) (24). Fluorescently labeled cDNA probes containing 1.2 μg of poly(A) (Sigma, Munich, Germany) per ml as a competitor, 30 mM HEPES, and 0.3% sodium dodecyl sulfate in 3× SSC were hybridized to arrays in a humid chamber for 5 to 16 h at 65°C. After hybridization, the arrays were washed in 1× SSC–0.03% sodium dodecyl sulfate and finally in 0.05× SSC (24).

Immediately after stringent washing, the fluorescence intensities at 635 and 532 nm were determined with a GenePix 4000 laser scanner (Axon Inc., Union City, Calif.), and the images were processed as TIFF images. Raw fluorescence data were analyzed quantitatively by using GenePix 3.0 software (Axon Inc.). Data were normalized to the average ratio for *C. glutamicum* genomic DNA. The normalized ratio of the median was taken to reflect the relative RNA abundance for hybridization signals with a green or red fluorescence signal that was at least threefold greater than the median fluorescence background signal. For statistical analysis of the gene expression data (5, 20) *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 (28, 34). Of the genes that showed significantly changed RNA levels ($P < 0.05$), those with at least fourfold-increased or -decreased average RNA levels were considered further and subjected to a hierarchical cluster analysis by the average linkage clustering method (13).

Construction of transcriptional fusions. The promoter regions upstream of the *pstSCAB* and *ugpAEBC* operons were amplified with primers PpstN (′-CG-GGATCC-TGCGGACTGCTGGGAAGATG-3′), PpstC (5′-CCC-AAGCTT-TAAGAATCGGTGATTTTCGTTCC-3′), PugpN (5′-CCC-AAGCTT-TTGGTG CGAAGGATTCCGATTC-3′), and PugpC (5′-CG-GGATCC-TCTGTCCGCC

TTGATCTCTTGG-3′). Amplified fragments were subcloned into the pGEM-T vector (Promega, Madison, Wis.) and then into the corynebacterial promoter-probe pET2 vector (46). The *pstS-cat* and *ugpA-cat* fusion vectors pET2-pst and pET2-ugp were introduced into the *C. glutamicum* wild type by electroporation by using the following conditions: 25 μF, 600 Ω, and 2.5 kV/cm. After electroporation, 1 ml of Luria-Bertani medium was immediately added to each sample. Then the sample was exposed to 46°C for 6 min and incubated at 30°C for 90 min for regeneration. Each promoter activity was measured by determining the chloramphenicol acetyltransferase activity. The cultivation conditions used for *C. glutamicum* ATCC 13032(pET2-pst) and ATCC 13032(pET2-ugp) were the conditions described above for the P_i down-shift experiment. Chloramphenicol acetyltransferase activity was determined as described by Shaw (40), and protein concentrations were determined as described by Gornall et al.(16) with bovine serum albumin as the standard.

RESULTS

Phosphate as a sole P source for growth of *C. glutamicum*. Initially, the growth characteristics of *C. glutamicum* with inorganic phosphate as the sole P source were determined. When *C. glutamicum* was grown in CGXII minimal medium with 40 g of glucose per liter, which contained 13 mM potassium phosphate as the sole P source, the cultures reached a final OD_{600} of 60. When the cells were washed and transferred to CGXII-glucose medium lacking a P source, they still grew and reached a final OD_{600} of 12 (data not shown), which indicated that there were internal P sources, such as polyphosphate (26). *C. glutamicum* cells precultured under P_i-limiting conditions showed almost no growth in the absence of a P source and proportional increases in the cell yield as well as the growth rate with increasing P_i concentrations when they were cultivated in CGXII-glucose medium (Fig. 1). The P_i concentration that supported growth of *C. glutamicum* with a half-maximal growth rate (Monod constant) was calculated to be 0.1 mM. Similar results were obtained when *C. glutamicum* was cultured with sodium phosphate instead of potassium phosphate as the sole P source (data not shown).

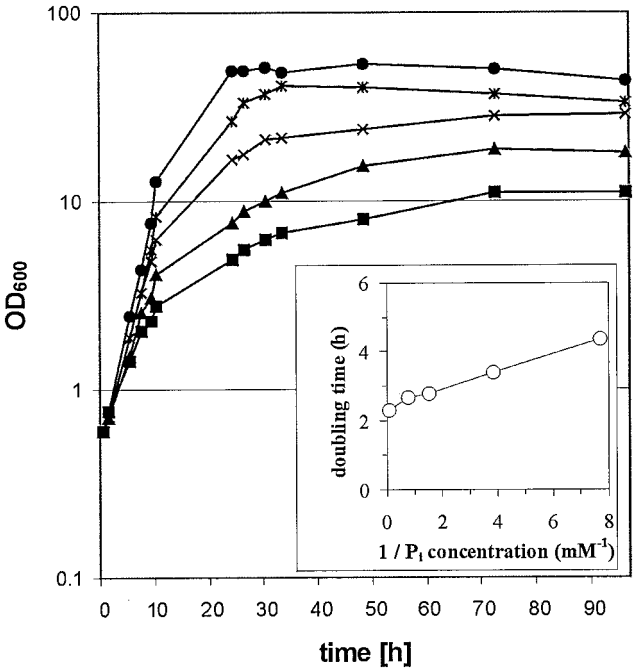


FIG. 1. Growth of *C. glutamicum* ATCC 13032 in CGXII minimum medium with 40 g of glucose per liter and different concentrations of P_i. Cells were precultured in CGXII medium with 0.13 mM potassium phosphate for 24 h before inoculation into CGXII medium containing 0.13 mM (■), 0.26 mM (▲), 0.65 mM (×), 1.3 mM (*), or 13 mM (●) potassium P_i. (Inset) Doubling times plotted against reciprocal P_i concentrations.

Comparison of global gene expression during growth under P_i-limiting and P_i-sufficient conditions. In order to identify genes that were differentially expressed in response to different P_i concentrations in the growth medium, the global gene expression patterns of *C. glutamicum* that was either maintained under P_i-limiting conditions or shifted from P_i-limiting to P_i-sufficient conditions were analyzed by using DNA microarrays. Cells were precultured in CGXII-glucose medium under P_i-limiting conditions (0.13 mM P_i) for 24 h and then used to inoculate fresh medium containing either a sufficient (13 mM) or limiting (0.13 mM) P_i concentration. After 7.5 h, exponentially growing cells (OD_{600}, 4.3 and 2.1, respectively) were harvested, and RNA was prepared. Four independent DNA microarray experiments were carried out, two with potassium phosphate and two with sodium phosphate as the P source. Genes that showed reliable hybridization signals in at least three of four experiments and that were differentially expressed (RNA ratios higher than 4 or lower than 0.25) are listed in Table 1. Under P_i-limiting conditions, 14 genes showed decreased expression (RNA ratios less than 0.25) compared to the expression under P_i-sufficient conditions, and 14 genes showed increased expression (RNA ratios greater than 4) (Table 1). The former group included 12 ribosomal protein-encoding genes, the translation initiation factor gene (open reading frame [ORF] 2648, Cgl1378), and a gene whose function is unknown (ORF 2614). This finding reflected the slower growth under P_i-limiting conditions, as growth rates of 0.30 and 0.16 h^{-1} were observed under P_i-sufficient and P_i-limiting conditions, respectively. It was not clear whether expression of ORF 2614, which codes for a hypothetical protein containing 89 amino acids and does not seem to be part of an operon, was reduced as a consequence of slower growth. The group of genes which exhibited increased expression under P_i-limiting conditions included nine genes which are known to be induced by P_i starvation in other bacteria, one gene (ORF 2851, *nucH*) encoding a putative extracellular nuclease (a member of the endonuclease-exonuclease-phosphatase family, Pfam domain PF03372), and four genes without assigned functions (ORFs 1086, 1598, 1760, and 3082). The known phosphate starvation-inducible genes include *phoH*, *glpQ*, *ugpAEBC* (expression of *ugpC* increased only 2.5-fold), and *pstSCAB* (Table 1). An alkaline phosphatase gene (ORF 1400, Cgl0849) also showed increased expression, albeit to a lesser degree (3.4-fold).

Kinetics of gene expression changes after a shift from P_i-sufficient to P_i-limiting conditions. In contrast to the set of experiments described above, in which gene expression was analyzed in cells grown for 7.5 h under P_i-limiting or P_i-saturating conditions, the experiments described below were performed to detect both the short-term and the long-term responses of *C. glutamicum* after a shift from P_i-excess conditions to P_i-limiting conditions (i.e., the kinetics of the response). Cells growing exponentially under P_i-sufficient conditions (13 mM) were harvested. RNA was prepared from one aliquot, and another aliquot was used to inoculate parallel cultures

TABLE 1. Gene expression changes during growth of *C. glutamicum* under P_i-limiting and P_i-sufficient conditions

ORF	NCBI no.	Annotation	Gene	Avg mRNA level under P_i-limiting conditions/ avg mRNA level under P_i-sufficient conditions[a]
442	Cgl0065	Phosphate starvation-inducible protein	*phoH*	4.6
976	Cgl0485	Ribosomal protein L10		0.1
988	Cgl0493	Ribosomal protein S12		0.2
989	Cgl0494	Ribosomal protein S7		0.1
992	Cgl0514	Ribosomal protein S3		0.1
993	Cgl0515	Ribosomal protein L16/L10E		0.2
994	Cgl0516	Ribosomal protein L29		0.2
999	Cgl0521	Ribosomal protein L14		0.2
1000	Cgl0522	Ribosomal protein L24		0.2
1020	Cgl0539	Ribosomal protein L18		0.2
1022	Cgl0540	Ribosomal protein S5		0.1
1050	Cgl0566	Ribosomal protein L17		0.2
1086	Cgl0596	Hypothetical protein		9.6
1421	Cgl0869	Ribosomal protein L28		0.2
1598	Cgl1021	Hypothetical membrane protein		4.1
1760	Cgl1170	Hypothetical protein		10.1
2614	(855659–855946)	Questionable ORF		0.2
2637	Cgl1387	Glycerophosphoryl diester phosphodiesterase	*glpQ*	6.4
2639	Cgl1385	Glycerol-3-phosphate ABC-type transporter, periplasmic component	*ugpB*	13.6
2640	Cgl1384	Glycerol-3-phosphate ABC-type transporter, permease component	*ugpE*	10.7
2641	Cgl1383	Glycerol-3-phosphate ABC-type transporter, permease component	*ugpA*	12.6
2648	Cgl1378	Translation initiation factor IF-3		0.2
2851	Cgl2592	Predicted extracellular nuclease	*nucH*	9.4
2871	Cgl2575	Phosphate ABC-type transporter, periplasmic component	*pstS*	17.8
2873	Cgl2574	Phosphate ABC-type transporter, permease component	*pstC*	12.1
2875	Cgl2573	Phosphate ABC-type transporter, permease component	*pstA*	7.5
2876	Cgl2572	Phosphate ABC-type transporter, ATPase component	*pstB*	9.3
3082	Cgl2336	Hypothetical protein		4.2

[a] The relative mRNA levels under P_i-limiting and P_i-sufficient conditions are averages from four experiments. Only ORFs with P values of <0.05 as determined by a Student's t test and with relative mRNA levels equal to or greater than 4 or equal to or less than 0.25 are shown.

with medium containing a limiting P_i concentration (0.13 mM). From these cultures RNA was prepared 10, 30, 60, 90, 120, and 180 min after transfer. By using DNA microarrays the global gene expression at each of these times was compared to that of the culture before the transfer (zero time). Table 2 summarizes the observed expression differences and lists genes that were reliably detected and showed at least at one time point expression that was significantly altered by at least a factor of 4. Subsequently, hierarchical cluster analysis of these genes revealed five groups or subclusters of genes which showed similar expression profiles under the conditions used (Fig. 2).

Subcluster 1 included 25 genes which showed increased expression at a relatively early stage of adaptation and exhibited high expression levels until 180 min after the onset of P_i limitation. Except for ORF 1760, a gene encoding a hypothetical protein similar to *yceI* in *E. coli*, this subcluster contained all of the genes that showed increased expression in the experiments described above (Table 1): *pstSCAB*, *glpQ*, *ugpAEBC*, *nucH*, and the hypothetical genes ORFs 1086, 3081, and 3082 (Table 2). In addition, subcluster 1 comprised genes presumably involved in P metabolism, including the phosphate starvation-inducible gene *phoH* (represented by two PCR products), the Cgl0328 gene putatively encoding a 5′-nucleotidase or related esterase, the genes encoding an ABC transporter of unknown function (ORFs 2553 and 2554), and a gene encoding a phosphoesterase (ORF 3442). Furthermore, a number of genes not obviously related to P metabolism showed increased RNA levels after the shift to P_i limitation; these genes included the genes encoding methylcitrate lyase (*prpB1*), methylcitrate synthase (*prpC1*), ferrochelatase (ORF 2849), two hypothetical proteins (ORFs 3081 and 3362), a permease of unknown function (ORF 2261), and glucose-1-dehydrogenase (ORF 2262). It is not yet clear whether these proteins play a major role, direct or indirect, in adaptation of *C. glutamicum* to P_i starvation. With regard to the kinetics of expression changes, the genes of subcluster 1 can be ordered. Expression of the high-affinity P_i uptake system genes *pstSCAB* increased first after the shift to phosphate starvation conditions (Fig. 3A); this was followed by increases in expression of the *sn*-glycerol 3-phosphate uptake genes *ugpAEBC* and *glpQ* (Fig. 3B) and of the genes encoding extracellular nuclease (ORF 2851, *nucH*), 5′-nucleotidase or the related esterase/5′-nucleotidase (Cgl0328, ORF 766), and the hypothetical genes ORFs 1086, 3081, and 3082 (Fig. 3C). All other genes in subcluster 1 exhibited smaller and slower increases in expression (Fig. 3D).

Subcluster 2 (Fig. 2) included nine genes whose expression was transiently increased after the P_i down-shift, but the RNA levels slowly reached the same levels as before the shift (Fig. 3E and Table 2). Five of these genes are part of a chromosomal locus encoding a copper-exporting ATPase, an associated protein, a two-component regulatory system, a hypothetical protein, a multicopper oxidase, and a thiol-disulfide interchange protein (ORFs 376 to 383). Additionally, genes encoding a response regulator (ORF 2831), a nonheme ferritin (ORF 3472), a putative cation efflux protein, and two hypothetical proteins (ORFs 399 and 2779) belonged to subcluster 2.

TABLE 2. Changes in gene expression at different times during the P_i starvation response of *C. glutamicum*

ORF	NCBI no.	Annotation	Gene	Avg mRNA level after shift of P_i-limiting conditions/avg mRNA level before shift to P_i-limiting conditions[b]					
				10 min	30 min	60 min	90 min	120 min	180 min
195	Cgl2814	Sulfate adenylate transferase subunit 1		2.2[c]	0.3[c]	0.2[c]		0.4[c]	
312	Cgl2917	Putative integral membrane transport protein, similar to shikimate transport protein *shiA*		**10.0**			0.4	0.6	0.5[c]
348	Cgl2946	Hypothetical membrane protein		1.2[c]	0.7	0.3[c]	0.2[c]	0.2[c]	0.1[c]
376		Similar to heavy-metal-transporting ATPases		2.2[c]	5.7[c]	3.4	2.5	1.3	1.0
378	Cgl2965	Two-component system, response regulator		1.5	7.1[c]	6.0[c]	4.3[c]	2.1[c]	1.3
380	Cgl2966	Hypothetical protein			8.3[c]	6.4[c]	2.7[c]	1.3	1.0
381	Cgl2967	Putative multicopper oxidase	*cumA*	1.8[c]	9.3[c]	6.1[c]	3.1[c]	1.3	0.8
383	Cgl2968	Hypothetical protein		1.8	5.7[c]	3.4		1.0	0.6[c]
399	Cgl2979	Hypothetical protein		3.9[c]	4.8[c]	1.7	1.1	1.1	0.9
403	Cgl2982	Single-stranded DNA-binding protein	*ssb*		0.6[c]			0.2[c]	0.2[c]
441	Cgl0065	Phosphate starvation-inducible protein	*phoH*	1.1	1.8	3.3[c]	4.4[c]	4.7[c]	4.5[c]
442	Cgl0065	Phosphate starvation-inducible protein	*phoH*	1.4	1.4	5.9[c]	4.3[c]	6.8[c]	4.8[c]
766	Cgl0328	5′-Nucleotidase or related esterase		1.2	7.3[c]	6.5[c]	**19.3**[c]	**10.3**[c]	7.2[c]
854	Cgl0388	Hypothetical membrane protein		0.2[c]	0.1[c]	0.2[c]	0.3[c]	1.9	1.3
856	Cgl0390	ABC-type transporter, permease component, similar to heme uptake systems		0.4[c]	0.2[c]	0.3	0.5	1.6	
857	Cgl0391	ABC-type transporter, ATPase component, similar to heme uptake systems		0.3[c]	0.2[c]	0.3	0.5	1.5	0.9
976	Cgl0485	Ribosomal protein L10		0.8	0.8	0.2[c]	0.1[c]	0.1[c]	0.0[c]
978	Cgl0486	Ribosomal protein L7/L12		0.7	0.8	0.2[c]	0.3[c]	0.3	0.2[c]
988	Cgl0493	Ribosomal protein S12		1.5[c]	0.8	0.3[c]	0.3[c]	0.2[c]	0.2[c]
992	Cgl0514	Ribosomal protein S3		0.9	0.7	0.2[c]	0.1[c]	0.1[c]	0.1[c]
993	Cgl0515	Ribosomal protein L16/L10E			0.7			0.1[c]	0.1[c]
994	Cgl0516	Ribosomal protein L29		0.9	0.7	0.2[c]			0.1[c]
999	Cgl0521	Ribosomal protein L14					0.3[c]		0.2[c]
1000	Cgl0522	Ribosomal protein L24		1.2	0.8	0.2[c]	0.2[c]	0.1[c]	0.1[c]
1020	Cgl0539	Ribosomal protein L18		1.0	0.7	0.2[c]	0.2	0.2	0.1[c]
1044	Cgl0560	Translation initiation factor IF-1		1.1	0.4	0.3[c]	0.4	0.2[c]	0.4
1047	Cgl0562	Ribosomal protein S11		1.1	0.7	0.2[c]		0.2[c]	0.2[c]
1048	Cgl0563	Ribosomal protein S4		1.0	0.8	0.3[c]		0.2[c]	0.2[c]
1050	Cgl0566	Ribosomal protein L17		1.0	0.7	0.3[c]		0.2[c]	0.2[c]
1086	Cgl0596	Hypothetical protein			**10.8**[c]	**18.8**[c]	**17.6**[c]	**15.0**[c]	8.9[c]
1168	Cgl0665	Similar to vibriobactin utilization protein *viuB*			0.1[c]	0.3[c]		1.3	0.8
1169	Cgl0666	ABC-type transporter, ATPase component, similar to iron uptake systems			0.2[c]	0.2[c]		1.1	0.7
1170	Cgl0667	ABC-type transporter, permease component, similar to iron uptake systems			0.1[c]	0.2[c]		1.0	
1173	Cgl0669	ABC-type transporter, periplasmic component, similar to iron uptake systems		0.2[c]	0.2[c]	0.2[c]	0.3[c]	1.0	0.7
1205	Cgl0695	Methylisocitrate lyase	*prpB1*			3.3[c]	3.5[c]	4.7[c]	3.9[c]
1206	Cgl0696	Methylcitrate synthase	*prpC1*	0.6	1.2	3.7[c]		4.2[c]	2.9[c]
1346	Cgl0807	Siderophore-interacting protein			0.1[c]	0.2[c]		1.1	
1347	Cgl0808	ABC-type cobalamin/Fe^{3+}-siderophore transport		0.1[c]	0.1[c]	0.1[c]	0.2[c]	1.3	0.8
1349	Cgl0810	ABC-type cobalamin/Fe^{3+}-siderophore transport		0.1[c]	0.1[c]	0.2[c]	0.2[c]	1.0	0.5
1351	Cgl0812	ABC-type cobalamin/Fe^{3+}-siderophore transport		0.2[c]	0.2[c]		0.3[c]		
1352	Cgl0813	ABC-type cobalamin/Fe^{3+}-siderophore transport			0.1[c]				
1420		Similar to ribosomal protein L33 of *E. coli* (putative sequencing error)		1.1	0.6	0.2[c]		0.4	0.2[c]
1421	Cgl0869	Ribosomal protein L28		1.0	0.8	0.2[c]	0.2[c]	0.2[c]	0.2[c]
1558	Cgl0982	AraC-type DNA-binding domain-containing protein		0.2[c]	0.2[c]	0.2[c]	0.4[c]	2.6	1.1
1598	Cgl1021	Hypothetical membrane protein		0.8	1.6[c]	4.5[c]	7.8[c]	7.8[c]	6.9[c]
1645	Cgl1067	Cysteine sulfinate desulfinase		1.1[c]	1.2	0.5[c]	0.3[c]	0.2[c]	0.3[c]
1646	Cgl1068	Nicotinate-nucleotide pyrophosphorylase		1.0	1.0	0.3[c]	0.1[c]	0.1[c]	0.1[c]
1647	Cgl1069	Quinolinate synthase		0.8	1.1	0.3[c]	0.1[c]	0.1[c]	0.1[c]
1655	Cgl1077	2-Polyprenyl-6-methoxyphenol hydroxylase		4.6[c]	1.6	0.9[c]	1.0	1.0	0.9
1722	Cgl1139	Methionine synthase II			0.7	0.2[c]		0.2[c]	0.6[c]
1855	Cgl1248	Siderophore-interacting protein		0.4[c]	0.3[c]			4.3	
1875	Cgl2996	*myo*-Inositol-1-phosphate synthase		0.8[c]	1.3[c]	0.8	0.4[c]	0.2[c]	0.5[c]
2146	Cgl2035	ABC-type transport systems, periplasmic protein, similar to iron(III) dicitrate uptake systems		0.3[c]	0.2[c]	0.3[c]		1.6	1.0
2168		Questionable ORF		1.6[c]	1.1	0.3[c]		0.2[c]	0.2[c]
2261	Cgl2133	Predicted Co/Zn/CD cation transporter		0.8	1.2	2.0[c]	2.7[c]	3.5[c]	4.8[c]
2262	Cgl2134	Dehydrogenase		0.6[c]	1.2	2.3[c]	2.5[c]	3.3[c]	4.4[c]
2553	Cgl1460	ABC-type transporter, ATPase component		1.0	2.8	3.0[c]	5.0[c]	2.7[c]	2.3

Continued on following page

TABLE 2—*Continued*

ORF	NCBI no.	Annotation	Gene	Avg mRNA level after shift of P_i-limiting conditions/avg mRNA level before shift to P_i-limiting conditions[b]					
				10 min	30 min	60 min	90 min	120 min	180 min
2554	Cgl1459	ABC-type transporter, permease component		1.2	6.3	3.6[c]	3.4[c]	2.9[c]	2.1
2614		Questionable ORF		1.2[c]	0.7	0.3[c]	0.2[c]	0.2[c]	0.1[c]
2624	Cgl1399	Arginine repressor	*argR*	5.0[c]	0.7	0.7[c]	0.8	0.8	0.8
2625	Cgl1398	Ornithine carbamoyltransferase	*argF*	5.5[c]	0.6	0.6[c]	0.6[c]	0.7	0.7
2637	Cgl1387	Glycerophosphoryl diester phosphodiesterase	*glpQ*	1.5	2.8[c]	4.7[c]	5.0[c]	9.2[c]	7.5[c]
2638	Cgl1386	Glycerol-3-phosphate ABC-type transporter, ATPase component	*ugpC*	1.1	4.2[c]	3.9[c]	4.3[c]	4.5[c]	4.5[c]
2639	Cgl1385	Glycerol-3-phosphate ABC-type transporter, periplasmic component	*ugpB*	2.2	**12.9**[c]	**22.8**[c]	**18.7**[c]	**17.2**[c]	**14.5**[c]
2640	Cgl1384	Glycerol-3-phosphate ABC-type transporter, permease component	ugpE	1.6[c]	9.8[c]	**22.3**[c]	**15.0**[c]	**11.7**[c]	**18.1**[c]
2641	Cgl1383	Glycerol-3-phosphate ABC-type transporter, permease component	*ugpA*	2.1	**15.1**[c]	**16.8**[c]	**15.0**[c]	**11.0**[c]	**12.5**[c]
2647	Cgl1379	Ribosomal protein L35		1.6[c]	1.0	0.4[c]	0.3[c]	0.2[c]	0.2[c]
2648	Cgl1378	Translation initiation factor IF3		2.0[c]	1.1	0.3[c]	0.2[c]	0.1[c]	0.2[c]
2779	Cgl1281	Co/Zn/Cd efflux system component		3.8[c]	4.7[c]	1.9[c]	1.0	0.6[c]	0.7[c]
2831	Cgl2607	Two-component system, response regulator		8.1[c]	2.3	1.4	1.1	1.1	1.5[c]
2832	Cgl2606	Two-component system, sensory transduction		4.7[c]	1.7	1.0	0.8	0.8	0.9
2849	Cgl2594	Predicted deacetylase			4.7	2.0[c]	2.1	2.1[c]	
2851	Cgl2592	Predicted extracellular nuclease	*nucH*		4.8[c]	**19.5**[c]	**31.7**[c]	**25.8**[c]	**16.9**[c]
2871	Cgl2575	Phosphate ABC-type transporter, periplasmic component	*pstS*	5.0[c]	**18.0**[c]	**23.2**[c]	**21.5**[c]	**19.1**[c]	**13.8**[c]
2873	Cgl2574	Phosphate ABC-type transporter, permease component	*pstC*	4.4[c]	**19.3**[c]	**23.6**[c]	**22.8**[c]	**20.9**[c]	**19.1**[c]
2875	Cgl2573	Phosphate ABC-type transporter, permease component	*pstA*	1.9	5.8[c]	**12.5**[c]	8.6	7.1[c]	6.9[c]
2876	Cgl2572	Phosphate ABC-type transporter, ATPase component	*pstB*	2.7[c]	9.0[c]	**12.4**[c]	**14.5**	**10.3**[c]	8.3[c]
2895		Questionable ORF		1.3	0.7	0.3[c]	0.2[c]	0.2[c]	0.1[c]
3081	Cgl2336	Hypothetical protein		1.0	2.8[c]	3.8[c]	4.2[c]	4.6[c]	4.2[c]
3082	Cgl2336	Hypothetical protein			4.3[c]	5.3	5.0[c]		5.3[c]
3086		Questionable ORF		0.5[c]	0.2	0.4[c]	0.3[c]	0.5[c]	
3326	Cgl1067	Cysteine sulfinate desulfinase			0.6			0.2[c]	
3347	Cgl1714	Hypothetical protein		0.2[c]	0.2	0.3[c]		4.2	2.5
3362		Questionable ORF				1.8[c]	2.7	4.7[c]	
3442	Cgl3064	Hypothetical membrane protein, similar to phosphoesterases			2.3	5.3	7.3	**11.9**[c]	6.3
3458	Cgl3075	ABC-type transport systems, periplasmic component, similar to iron uptake systems		0.1[c]	0.1[c]	0.1[c]	0.1[c]	0.5	0.3
3472	Cgl2526	Ferritin-like protein		**10.3**[c]	**10.3**[c]	5.8[c]	5.5[c]	1.8	1.6
64185	Cgl2389	Acyl-CoA:acetate CoA transferase beta subunit		7.3[c]	1.5			0.4	
64187	Cgl2393	Predicted hydrolase/acyltransferase		7.7[c]	1.7				0.8
66073	Cgl2397	Protocatechuate 3,4-dioxygenase beta subunit		5.2[c]	1.8	1.3	1.1	1.1	1.0
66513	Cgl2392	Acetyl-CoA acetyltransferase		4.4[c]	1.8	0.6	0.5	0.7	
69134	Cgl2642	Phosphotransferase system IIC component		1.3	0.5	0.4[c]	0.2[c]	0.3[c]	0.2

[a] The relative mRNA levels 10, 30, 60, 90, 120, and 180 min after the shift to P_i-limiting conditions compared to the levels under preshift conditions are averages from three experiments. Only ORFs whose mRNA levels were equal to or greater than 4 or equal to or less than 0.25 at at least one time are shown.
[b] Boldface type indicates relative mRNA levels that were equal to or greater than 10.
[c] $P < 0.05$ as determined by a *t* test.

Subcluster 3 contained nine genes whose expression rapidly increased after the P_i down-shift, but as soon as 30 min after the P_i down-shift preinduction RNA levels were reached (Fig. 3F). Five of these genes (ORFs 1655, 64185, 64187, 66073, and 66513) encode enzymes or subunits of enzymes predicted to be involved in the degradation of protocatechuate, which is present in CGXII medium to facilitate iron uptake, and similar compounds to yield acetyl coenzyme A (acetyl-CoA) and succinyl-CoA. Additionally, the genes encoding a putative transporter (ORF 312), the arginine repressor and ornithine carbamoyltransferase (ORFs 2624 and 2625), and a sensor kinase (ORF 2832) showed short-term expression increases after the onset of P_i limitation. Interestingly, expression of the gene encoding the corresponding response regulator (ORF 2831) transiently increased in a similar manner (Table 2 and Fig. 3F).

Subcluster 4 (Fig. 2) comprised 29 genes that showed decreased RNA levels 60 min after the onset of P_i limitation and continued to exhibit low levels at later times. Most of these genes code for proteins involved in translation or DNA replication; 15 genes code for ribosomal proteins (ORFs 976, 978, 988, 992 to 994, 999, 1000, 1020, 1047, 1048, 1050, 1420, 1421, and 2647), two genes code for translation initiation factors (ORFs 1044 and 2648), and one gene codes for the single-stranded DNA-binding protein (ORF 403). Reduced expres-

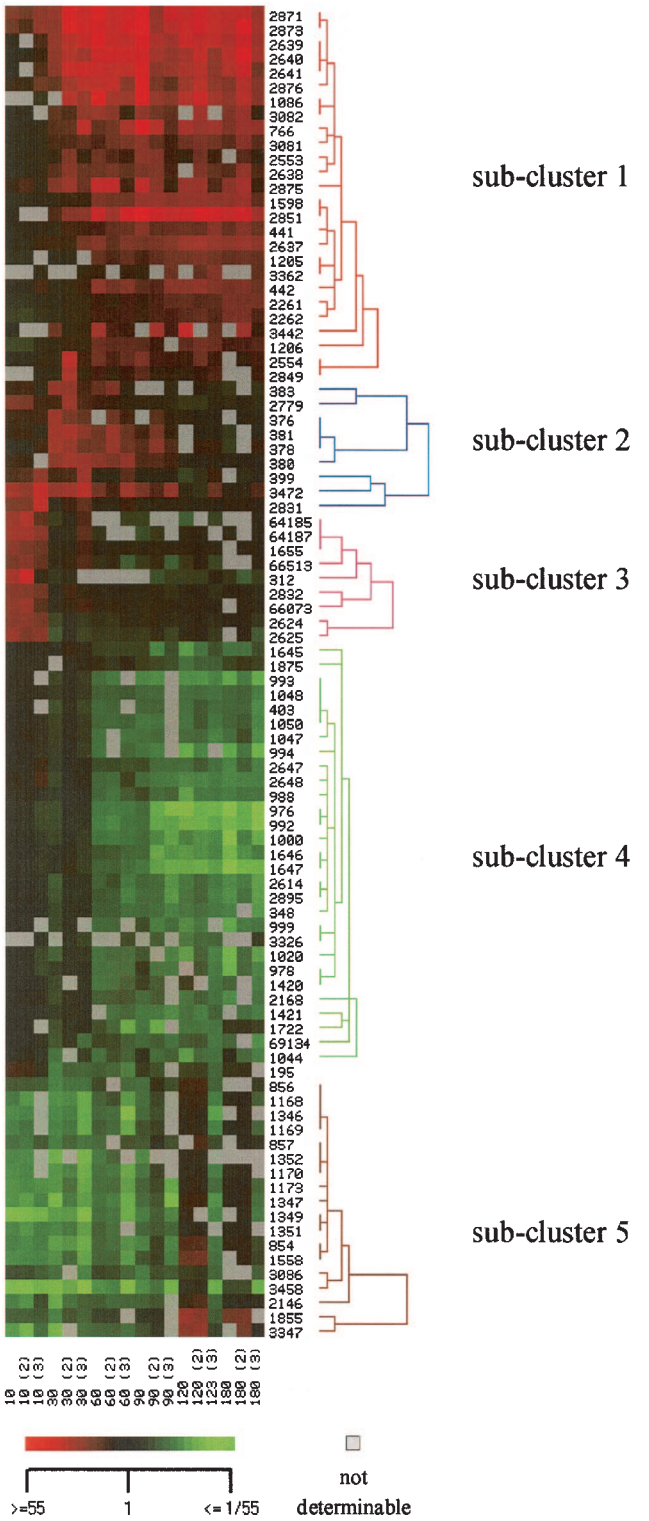


FIG. 2. Hierarchical cluster analysis of gene expression changes during the response of *C. glutamicum* ATCC 13032 to P_i starvation. Gene expression data from 12 microarray experiments (columns) and 92 genes (lines) are represented. The microarray experiments included comparing gene expression of *C. glutamicum* ATCC 13032 before and 10, 30, 60, 90, 120, and 180 min after a shift from P_i-sufficient conditions to P_i-limiting conditions. Subclusters 1 to 5 are indicated by red, blue, pink, green, and brown, respectively. The scale bar indicates the color coding of the relative RNA levels.

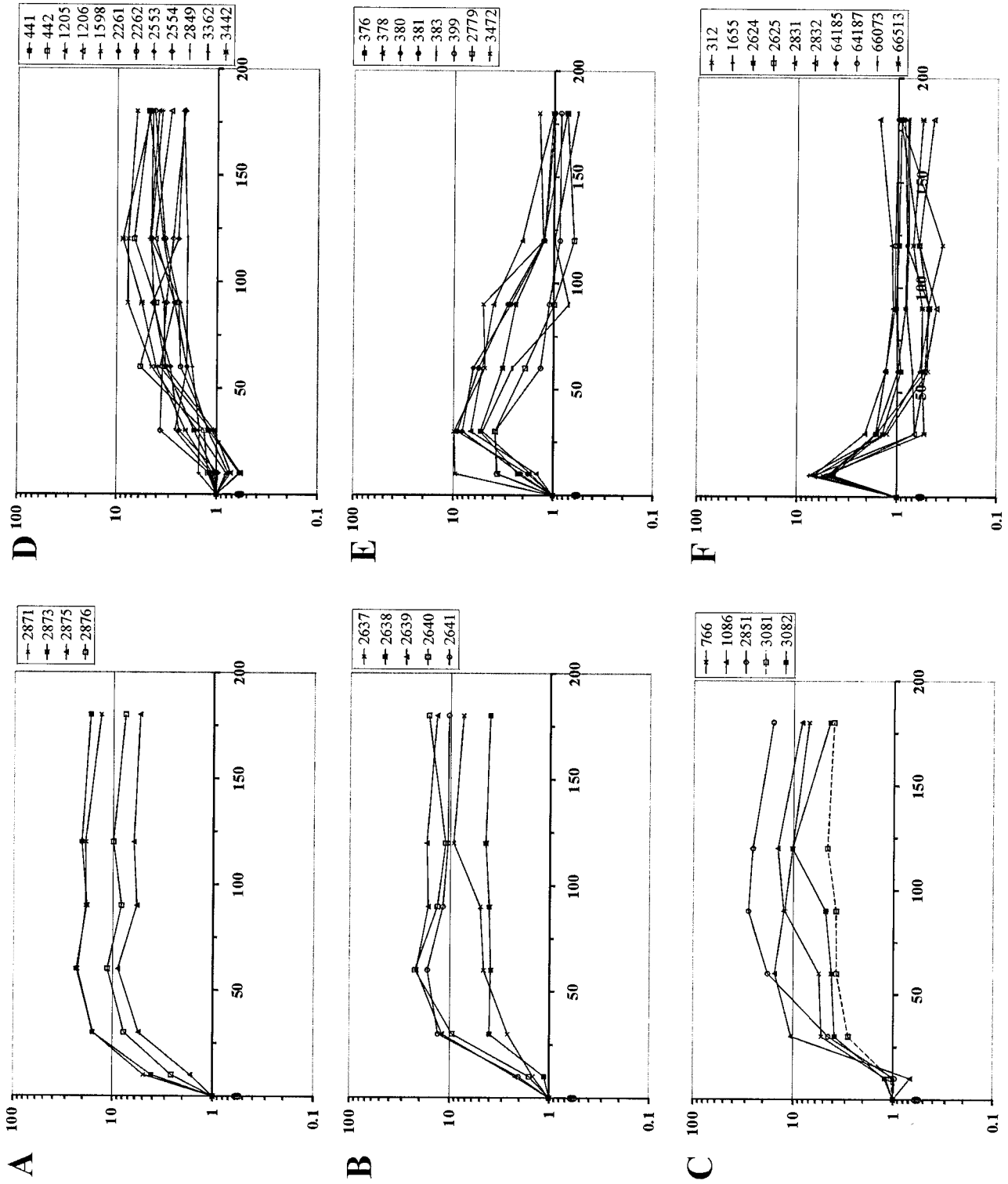
sion of these genes, as well as four hypothetical genes (ORFs 348, 2168, 2614, and 2895) and seven other genes (ORFs 1645 to 1647, 1722, 1875, 3326, and 69134), was correlated with reduced growth after the shift to P_i-limiting conditions.

Subcluster 5 included 18 genes which exhibited transiently reduced expression after the P_i down-shift. The RNA levels were lower 10, 30, 60, and 90 min after the onset of P_i limitation, but after 120 and 180 min the RNA levels were the same as those before the shift. Fifteen of these genes are presumably involved in iron metabolism and putatively encode a heme transport system and associated proteins (ORFs 854 to 857), two ferric siderophore transport systems and an associated protein (ORFs 1168 to 1173, 1346 to 1348, and 1349 to 1352), two ferric dicitrate-binding proteins (ORFs 3458 and 2146), and a siderophore utilization protein (ORF 1855). Besides these putative iron metabolism genes, two genes encoding hypothetical proteins (ORFs 3347 and 3086) and a gene encoding a transcriptional regulator gene (ORF 1558) belong to subcluster 5.

Construction and analysis of *pstS-cat* and *ugpA-cat* transcriptional fusions. To determine whether the increased RNA levels of the *pstSCAB* and *ugpAEBC* operons are due to transcriptional control and in order to confirm the expression changes observed in the DNA microarray experiments, we constructed and analyzed transcriptional fusions of the promoter regions upstream of *pstS* and *ugpA*. The *pstS* and *ugpA* promoter regions were fused to the promoterless chloramphenicol acetyltransferase gene on the promoter-probe vector pET2 (46), and the resulting plasmids were introduced into *C. glutamicum* wild-type strain ATCC 13032. Using the cultivation protocol used for the kinetic analysis of gene expression changes, we determined expression of the plasmid-borne transcriptional fusions *pstS-cat* and *ugpA-cat.* Expression of *pstS-cat* increased immediately after the P_i down-shift and reached maximal levels about 60 min after the shift. The temporal expression profile of *pstS-cat* is in very good agreement with the profile obtained in the DNA microarray experiments (Fig. 4). Similarly, expression of the transcriptional fusion *ugpA-cat* increased after the P_i down-shift. Thus, we suggest that increased expression of the *pstSCAB* and *ugpAEBC* operons in response to reduced P_i availability is due primarily to transcriptional regulation. It is noteworthy that both expression of the *ugpA-cat* fusion and the RNA levels of the *ugpAEBC* operon genes showed a delayed increase when they were compared to the profile obtained for the *pstSCAB* operon.

DISCUSSION

We identified the P_i starvation stimulon of *C. glutamicum*, and we determined the kinetics of the response to P_i starvation. A number of genes involved in phosphorus metabolism showed increased expression upon P_i starvation. The predicted functions of the proteins encoded by this group of genes include high-affinity uptake of inorganic phosphate, uptake of organophosphates, such as glycerol 3-phosphate, and hydrolysis of organophosphates and nucleic acids. Thus, the P_i starvation stimulons of *C. glutamicum* and *E. coli* are similar. However, 21 of 38 genes known to belong to the P_i starvation stimulons of *E. coli* and *Salmonella enterica* serovar Typhimurium are involved in phosphonate metabolism (47),


A
2871
2873
2875
2876
B
2637
2638
2639
2640
2641
C
766
1086
2851
3081
3082
D
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442
1205
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2779
3472
F
312
1655
2624
2625
2831
2832
64185
64187
66073
66513
100
10
1
0.1
50
100
150
200

FIG. 3. Kinetics of changes in gene expression during the response of *C. glutamicum* ATCC 13032 to P_i starvation. Changes in gene expression 10, 30, 60, 90, 120, and 180 min after a shift from P_i-sufficient to P_i-limiting conditions compared to preshift conditions taken from Table 2 are shown for selected ORFs (see text for details). (A to D) Kinetics of changes in gene expression for subcluster 1 genes (Fig. 2): genes encoding the putative high-affinity phosphate transporter *pstSCAB* (ORFs 2871 to 2876) (A), the glycerophosphoryl diester phosphodiesterase and the *sn*-glycerol 3-phosphate transport system (*glpQ* and *ugpAEBC*, ORFs 2637 to 2641) (B), and the putative extracellular nuclease (ORF 2851, *nucH*), 5′-nucleotidase or a related esterase (Cgl0328, ORF 766), and the hypothetical proteins (ORFs 1086, 3081, and 3082) (C) and all other subcluster 1 genes (D). (E and F) Kinetics of changes in gene expression for the subcluster 2 genes (Fig. 2) except ORF 2831 (E) and for subcluster 3 genes and ORF 2831 (F).

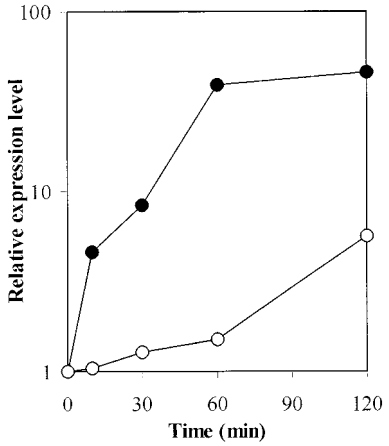

FIG. 4. Expression of the *pstS-cat* and *ugpA-cat* transcriptional fusions in *C. glutamicum* ATCC 13032(pET2-pst) and ATCC 13032 (pET2-ugp) during the response to P_i starvation. The activities obtained from two or more independent cultivations varied less than 20% and were normalized to the activity before the shift to P_i starvation conditions. Symbols: ●, *pstS-cat* fusion; ○, *ugpA-cat* fusion.

whereas *C. glutamicum* lacks homologs of genes for phosphonate degradation (NCBI accession no. NC003450), as well as the capability to utilize phosphonates as P sources (unpublished results). On the other hand, a *C. glutamicum* gene encoding a putative 5′-nucleotidase or related esterase showed increased expression upon P_i starvation, but its *E. coli* homolog, *ushA*, is not a known member of the P_i starvation stimulon (47). The role of the *C. glutamicum* 5′-nucleotidase or related esterase (Cgl0328) in adaptation to low-P_i conditions remains to be studied.

On the gene expression level, the first response of *C. glutamicum* to P_i starvation is increased expression of *pstSCAB* encoding the putative high-affinity P_i uptake system (Fig. 3). As the *pstSCAB* expression kinetics determined by DNA microarray analysis correlated well with the expression kinetics of a plasmid-borne *pstS′-′cat* transcriptional fusion, control of *pstSCAB* expression in response to reduced P_i availability is due primarily to transcriptional regulation. The increases in expression of *pstSCAB* after P_i starvation were the most pronounced both in *C. glutamicum* and in *E. coli*, in which transcripts of the *pstSCAB-phoU* operon were shown to be rapidly processed posttranscriptionally (1). The *E. coli* Pst system, which transports P_i at the expense of ATP (10, 47), is composed of the periplasmic P_i-binding protein PstS, two integral membrane proteins (PstC and PstA), and the ATP-binding protein PstB. The protein encoded by *phoU*, which is part of the operon, is not required for P_i transport, and its function is unclear (41). The high affinity of the Pst system and coupling transport to ATP hydrolysis allow concentrative P_i uptake when extracellular P_i is scarce.

The increases in expression of *ugpQ*, predicted to code for a glycerophosphoryl diester phosphodiesterase, and increases in expression of the oppositely oriented *ugpAEBC* operon, encoding an ABC transporter for glycerol 3-phosphate, were less pronounced but occurred shortly after the increases in expression of *pstSCAB* (Fig. 3). In *E. coli*, the corresponding genes form a single operon, *ugpBAECQ* (6, 9), which is controlled in

response to the P_i availability. In *E. coli* and *S. enterica* serovar Typhimurium, four P_i starvation-inducible uptake systems for organophosphates have been identified: Ugp and GlpT for glycerol 3-phosphate, UhpT for hexose 6-phosphates, and PgtP for phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate (47). While the *C. glutamicum* genome contains homologs of the genes encoding the Ugp system, it lacks homologs of the genes encoding GlpT, UhpT, and PgtP (NCBI accession no. NC003450). However, expression of an operon encoding an ABC transport system which might be involved in the uptake of an as-yet-unidentified P compound increased after P_i starvation. Compared to P_i starvation in *B. subtilis*, P_i starvation in *C. glutamicum* led to different expression changes. The cell wall of *C. glutamicum* lacks teichuronic acid and teichoic acid (29), and the genome does not contain homologs of the *B. subtilis tuaABCDEFGH* operon for teichuronic acid biosynthesis or the teichoic acid biosynthesis operons *tagAB* and *tagDEF* (NCBI accession no. NC003450). In *B. subtilis*, P_i starvation also results in σ^B-dependent increases in expression of genes of the general stress response (4, 35). While no homologs of *B. subtilis yjbC*, *ysnF*, and *yvgO* were identified in the *C. glutamicum* genome, expression of homologs of *B. subtilis yfhM* (Cgl0297), *csbD* (Cgl0229), *yheK* (Cgl1407 and Cgl2853), and *ykzA* (Cgl2385) did not significantly increase during the *C. glutamicum* P_i starvation response.

Several genes obviously involved in P metabolism are not part of the P_i starvation stimulon of *C. glutamicum*. Expression of a gene homologous to *E. coli pitA* encoding a low-affinity P_i transporter (45) and expression of a gene encoding a predicted sodium-dependent phosphate transporter (Cgl2744) were not significantly changed in *C. glutamicum* upon P_i starvation. Whereas *C. glutamicum* accumulates up to 600 mM P units as polyphosphate (26), expression of the *C. glutamicum* homologs of the *E. coli* exopolyphosphatase gene *ppx* (2) (Cgl0408 and Cgl0977), expression of the homolog of the *Pseudomonas aeruginosa* polyphosphate kinase II gene *ppk2* (49) (Cgl0917 and Cgl2714), and expression of the homolog of the *M. tuberculosis* polyphosphate glucokinase gene *ppgK* (21) (Cgl1910) were unchanged. Similarly, in *E. coli* the *ppk-ppx* operon is not part of the PhoB regulon (47), whereas phosphate-dependent regulation of genes involved in polyphosphate synthesis has been found in *Saccharomyces cerevisiae* (33) and *Acinetobacter* species (14, 15).

As a consequence of P_i starvation, expression of at least some ribosomal protein genes decreased. Significantly decreased expression of these genes was first observed 60 min after the shift from P_i-sufficient to P_i-limiting conditions. It is well known that in *E. coli* ribosome synthesis regulation involves growth rate-dependent control and stringent control (17). Apparently, the increases in expression of the genes and operons for uptake of P_i and glycerol 3-phosphate precede the decrease in ribosomal protein gene expression. Thus, the P_i starvation response is initiated before growth ceases and not as a consequence of the end of growth.

The transiently reduced expression of genes involved in iron metabolism and the transiently increased expression of genes involved in copper metabolism and in protocatechuate degradation after the shift to P_i-limiting conditions are not understood. In the shift experiment, changes in the concentrations of the medium components iron, copper, and protocatechuate, which was used as an iron chelator to facilitate iron uptake and optimal growth of *C. glutamicum* (30), might have influenced expression of genes involved in the transport and metabolism of these components. However, it is unlikely that a medium component was limiting before the shift as cells were growing exponentially and had only reached a low cell density (OD_{600}, 5) compared to the density which is supported by the medium (OD_{600}, 60). Furthermore, it is noteworthy that P_i seems to play a direct role in iron transport in *Haemophilus influenzae* (8), as well as in iron-dependent regulation in the related organism *Corynebacterium diphtheriae* (37).

Currently, the regulatory mechanism(s) governing the P_i starvation response of *C. glutamicum* is unknown. In *E. coli* and *B. subtilis*, the two-component regulatory systems PhoBR and PhoPR, respectively (4, 47), are involved in P_i-dependent regulation. In the case of *B. subtilis*, the Spo0 phosphorelay and the ResDE two-component system also play roles in the P_i starvation response (4). During the *C. glutamicum* P_i starvation response genes of two two-component regulatory systems showed transiently increased expression (subcluster 2) (Fig. 2). The genes of one system (ORFs 377 and 378, Cgl2964 and Cgl2965) are adjacent to genes for copper metabolism and showed up to sevenfold increases in expression. The genes of the other system (ORFs 2831 and 2832, Cgl2606 and Cgl2607) showed five- to eightfold-increased expression only 10 min after the shift to P_i starvation conditions. Future studies should reveal the roles of these systems for P_i-dependent control of gene expression.

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