Regulation of the phosphate starvation response in *Corynebacterium glutamicum* by the PhoRS two-component system

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Martina Kočan

aus Bratislava, Slowakei

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Referent: **Prof. Dr. M. Bott**

Korreferent: **Prof. Dr. H. Sahm**

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ABBREVIATIONS

Ap^R	ampicillin resistance
ATCC	American Type Culture Collection
ATP	adenosine triphosphat
BHI	brain heart infusion
bp	base pair
Da	dalton
DMSO	dimethylsulfoxide
dNTP	desoxyribonucleotide triphosphate
dsDNA	double-stranded DNA
g	acceleration of free fall
Km^R	kanamycin resistance
MOPS	3-(N-morpholino)propanesulfonic acid
ORF	open reading frame
ORF	*ORF* encoded protein
P	phosphorus
PCR	polymerase chain reaction
P_i	inorganic phosphate
rpm	rotations per minute
SDS	sodium dodecylsulphate
Tris	tris(hydroxymethyl)-aminomethan
U	unit of enzymatic activity, 1 U = 1 µmol/min
v/v	volume per volume
w/v	weight per volume

I ZUSAMMENFASSUNG

Die Erkennung von Umweltveränderungen und die notwendige Anpassung daran erfolgt in Bakterien in vielen Fällen durch Zweikomponenten-Signaltransduktionssysteme, kurz Zweikomponenten-Systeme genannt. Sie bestehen aus einer meist membrangebundenen Sensorkinase, die in Abhängigkeit von spezifischen Umweltreizen den Phosphorylierungszustand und damit die Aktivität eines Antwortregulators kontrolliert, der in den weitaus meisten Fällen als Transkriptionsregulator fungiert. Im 3.3 Mb-Genom des gram-positiven Bakteriums *Corynebacterium glutamicum* wurden Gene für 13 Zweikomponenten-Systeme identifiziert. Durch gerichtete Mutagenese wurde ein Satz von zwölf Mutanten konstruiert, in denen die Gene für jeweils eine Sensorkinase und den dazugehörigen Antwortregulator deletiert waren. Im einem Fall konnte nur das Gen für die Sensorkinase deletiert werden, nicht aber das für den Antwortregulator.

In dieser Arbeit wurde untersucht, ob eines der Zweikomponentensysteme von *C. glutamicum* an der Anpassung an Phosphatmangel beteiligt ist. Dazu wurden die genannten Mutanten unter Phosphatmangel und unter Phosphatüberschuss kultiviert. Eine der Mutanten wuchs bei Phosphatmangel, nicht aber bei Phosphatüberschuss, deutlich schlechter als der Wildtyp. Der Wachstumsdefekt konnte durch plasmid-gekoppelte Expression der fehlenden Gene komplementiert werden. Diese Resultate wiesen auf eine Funktion des fehlenden Zweikomponentensystems bei der Phosphatmangel-Antwort hin und die entsprechenden Gene wurden daher *phoS* (**Pho**sphat-**S**ensorkinase) und *phoR* (**Pho**sphat-***R****esponse*-Regulator) genannt.

Mit dem Ziel, die Gene zu identifizieren, die durch den Antwortregulator PhoR reguliert werden, wurden DNA-Microarray-Analysen durchgeführt. In einer ersten Serie von Experimenten wurde das Transkriptom der Δ*phoRS*-Mutante vor und nach einem Wechsel von Phosphatüberschuss zu Phosphatmangel verglichen. Dabei zeigte sich, dass mit Ausnahme des *pstSCAB*-Operons die Gene, die im Wildtyp unter Phosphatmangel induziert werden, in der Mutante nicht induziert wurden. Dies erklärt die Wachstumshemmung der Δ*phoRS*-Mutante unter Phosphatmangel. Die *pstSCAB*-Gene, die einen hochaffinen ABC-Tranporter für Phosphat kodieren, wurden in der Δ*phoRS*-Mutante unter Phosphatmangelbedingungen noch partiell induziert, was auf die Existenz eines weiteren, bisher noch unbekannten Regulators der *pst*-Gene hindeutet. In einer zweiten Serie von DNA-Microarray-Experimenten wurde das Transkriptom der Δ*phoRS*-Mutante mit dem des

Wildtyps verglichen. Bei Phosphatüberschuss zeigten in der Mutante 27 Gene eine geringere und 15 Gene eine höhere mRNA-Konzentration als im Wildtyp. Unter Phosphatmangel zeigten alle bekannten Phosphatmangel-Gene (*pstSCAB*, *ugpAEBC*, *glpQ*, *phoH*, *nucH* and *ushA*) in der Mutante einen geringere mRNA-Konzentration als im Wildtyp, was die vorherigen DNA-Microarray-Daten unterstützt. Während im Wildtyp die mRNA-Konzentration des *pitA*-Gens, das für einen niedrig-affinen sekundären Phosphat-Transporter kodiert, nach einem Wechsel zu Phosphatmangel sank, war sie in der Δ*phoRS*-Mutante nach einem solchen Wechsel unverändert, beim Vergleich Δ*phoRS*-Mutante/Wildtyp in der Mutante erhöht. Durch *Primer-Extension*-Analysen der Gene *pstS*, *ugpA* und *phoR* wurden die DNA-Microarray-Daten bestätigt und die Transkriptionsstartpunkte bestimmt. Die genannten Resultate deuten darauf hin, daß PhoR als Aktivator vieler Phosphatmangel-Gene fungiert, aber auch als Repressor des *pitA*-Gens.

Um die Phosphorylierungsreaktionen zu zeigen, die charakteristisch für Zweikomponenten-Systeme sind, wurden die Sensorkinase PhoS und der Antwortregulator PhoR mit einem carboxyterminalen Hexahistidin-*Tag* modifiziert, in *Escherichia coli* überproduziert und anschließend durch Affinitätschromatographie gereinigt. Die Solubilisierung und Reinigung des integralen Membranproteins PhoS erfolgte mit dem Detergenz N,N-Dimethyldodecylamin-N-oxid (LDAO). Mit den isolierten Proteinen konnte die ATP-abhängige Autokinase-Aktivität von solubilisiertem PhoS und der Phosphoryltransfer von phosphoryliertem PhoS auf PhoR nachgewiesen werden.

Die Bindung des Antwort-Regulators PhoR an die Promotorregionen der Operons *pstSCAB*, *ugpAEBC* und *phoRS* sowie des *pitA*-Gens wurde durch Gelretardationsexperimente analysiert. Mit dem vollständigen PhoR-Protein konnte unabhängig von den gewählten Bedingungen keine Bindung gezeigt werden. Mit einem PhoR-Derivat, bei dem die aminoterminalen 125 Aminosäuren durch einen Hexahistidin-*Tag* ersetzt wurden, konnte dagegen eine Bindung an die vier oben genannten Promotorregionen nachgewiesen werden, nicht jedoch an die Promotorregionen von *clpP1P2* und *clpC*, die als Negativkontrollen dienten. Zusätzlich wurde die PhoR-Bindestelle in der *pstS*-Promotorregion durch DNase-I-*Footprint*-Experimente analysiert. Dabei wurde der Bereich 170 bis 205 bp stromaufwärts des *pstS*-Transkriptionsstarts als geschützt identifiziert. Diese Resultate unterstützen eine direkte Regulation von Phosphatmangel-Genen sowie des *pitA*-Gens durch das PhoS-PhoR-Zweikomponentensystem.

II ABSTRACT

In bacteria, the recognition of environmental changes and the necessary adaptations to these changes is in many cases accomplished by two-component signal transduction systems. They are composed of a usually membrane-bound sensor kinase and a response regulator. In response to specific environmental signals, the sensor kinase controls the phosphorylation state of the response regulator and thus its activity. With few exceptions, response regulators function as transcriptional regulators. Within the 3.3 Mb-genome of the gram-positive bacterium *Corynebacterium glutamicum* genes for 13 two-component systems were identified. By directed mutagenesis, a set of 12 strains was constructed each lacking the genes for one sensor kinase and its response regulator. In one case, only the sensor kinase, but not the response regulator could be deleted.

In this work it was tested, whether one of the two-component systems of *C. glutamicum* is involved in the phosphate starvation response. To this end, the mutants mentioned above were cultivated under phosphate excess and phosphate limitation. One of the mutants grew poorer than the wild type under phosphate limitation, but not under phosphate excess. The growth defect could be abolished by expression of the missing genes from a plasmid. These results indicate a function of the deleted two-component system in the phosphate starvation response and therefore the corresponding genes were named *phoS* (**pho**sphate **s**ensor kinase) and *phoR* (**pho**sphate **r**esponse regulator).

With the aim to identify the target genes of response regulator PhoR, DNA microarray analyses were performed. In a first set of experiments, the transcriptom of the Δ*phoRS* mutant before and after a shift from phosphate excess to phosphate starvation was compared. Genes known to be induced by phosphate starvation in the wild type were not induced in the mutant, explaining the impaired growth of the Δ*phoRS* mutant under phosphate limitation. The *pstSCAB* genes encoding a high-affinity ABC transporter for phosphate were still partially induced in the Δ*phoRS* mutant under phosphate limitation, indicating the existence of an additional, currently unknown regulation of the *pst* operon. In a second set of experiments, the transcriptom of the Δ*phoRS* mutant was compared to that of the wild type. Under phosphate excess, 27 genes showed a lower and 15 genes a higher mRNA level in the mutant compared to the wild type. Under phosphate-limited conditions, all of the known P_i starvation-inducible genes (*pstSCAB*, *ugpAEBC*, *glpQ*, *phoH*, *nucH* and *ushA*) showed lower mRNA levels in the deletion mutant, sustaining the previous DNA microarray data. Interestingly, the mRNA level

of the *pitA* gene encoding a low-affinity P_i transporter decreased after a shift from P_i excess to P_i limitation in the wild type, but not in the Δ*phoRS* mutant. By primer extension analysis of *pstS*, *ugpA* and *phoR*, the DNA microarray data were confirmed and the transcriptional start sites of these genes were determined. In summary, the data indicated that the PhoS-PhoR two-component system is responsible for the activation of many phosphate starvation genes, but also for repression of the *pitA* gene.

In order to demonstrate the phosphorylation reactions characteristic for two-component signal transduction systems, the sensor kinase PhoS and the response regulator PhoR were both modified with a carboxyterminal histidine tag, overproduced in *Escherichia coli* and subsequently purified by affinity chromatography. Solubilization and purification of the integral membrane protein PhoS were performed with the detergent N,N-dimethyldodecylamine-N-oxide (LDAO). Both the autokinase activity of solubilized PhoS and the phosphoryl transfer from phosphorylated PhoS to PhoR were demonstrated with the isolated proteins.

The binding of the response regulator PhoR to the promoters of *pstSCAB*, *ugpAEBC*, *phoRS* and *pitA* was analysed by gel shift analyses. With the entire PhoR protein it was not possible to show binding, irrespective of the conditions applied. However, a derivative of PhoR in which the aminoterminal 125 amino acid residues were deleted and replaced by a histidine tag did bind to the four promoter regions mentioned above, but not to the negative control promoters *clpP1P2* and *clpC*. Additionally, the PhoR binding site within the *pst* promoter was analysed by DNase I footprinting, which indicated a protected region localized 170 to 205 bps upstream of *pstS* transcriptional start site.

III INTRODUCTION

1 Phosphate (P_i) starvation response in bacteria

In terms of cellular content, phosphorus (P) is a major element. P compounds serve as building blocks of innumerable biomolecules: P is an essential component of membrane

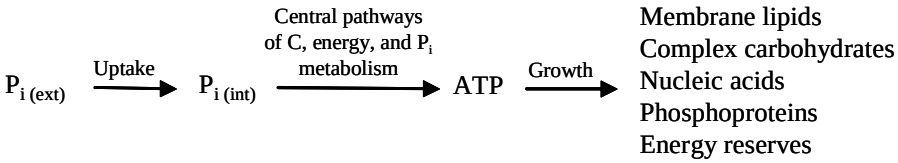
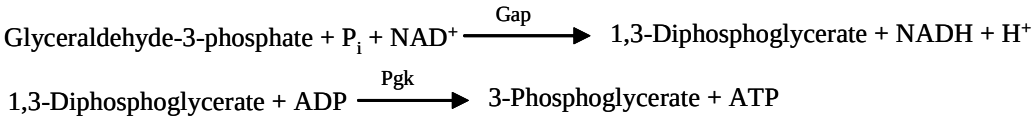


Fig. 1: Pathways of phosphorus assimilation from environmental inorganic phosphate (P_i).

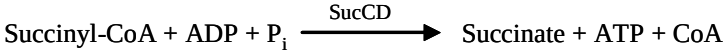
lipids, complex carbohydrates such as lipopolysaccharides and of nucleic acids. Phosphorus is also incorporated into many proteins posttranslationally (Wanner, 1996). P assimilation

I. Oxidative phosphorylation

II. Glycolysis



III. Tricarboxylic acid cycle



IV. Acetate formation

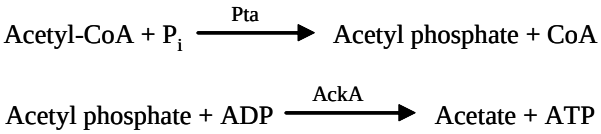


Fig. 2: Some key pathways involving metabolism of intracellular inorganic phosphates.
Notation: AckA, acetate kinase; AtpIBEFHAGDC, F_1F_O-ATP synthase; Gap, glyceraldehyd 3-phosphate dehydrogenase; Pgk, phosphoglycerate kinase; PMF, proton motive force; Pta, phosphotransacetylase; SucCD, succinyl-CoA synthetase.

occurs mainly in the form of phosphate via the reactions of energy and carbon metabolism (Fig. 1, 2). Therefore, a sufficient P supply is essential for optimal energy and carbon metabolism.

1.1 Phosphate starvation response in model bacteria

In bacteria, P is typically 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. These compounds are either 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 (Hulett, 2002; Wanner, 1996).

Bacteria have evolved a signalling system to induce the formation of specific enzymes and transporters that serve to increase the cytoplasmic P_i availability when the supply of P_i becomes limiting. When P_i is in excess, it is transported by *E. coli* into the cell by a low-affinity transporter called PitA. PitA is reported to be expressed constitutively and transports phosphate in a proton motive force-dependent manner (Wanner, 1996). When formed, the PitB transporter encoded by a cryptic homolog of *pitA* is also able to transport P_i into the cell in a manner similar to that used by PitA (Hoffer *et al*., 2001). When the extracellular P_i concentration falls below about 4 μM, *E. coli* stimulates the transcription of at least 30 genes (most of which are organised in operons) that allow the organism to survive this stress situation (Tab. 1), including e.g. genes encoding a high-affinity phosphate uptake system called the Pst system (*pstSCAB*) or genes for a glycerol-3-phosphate uptake system called the Ugp system (*ugpBAEC*), which both belong to the ABC-type transporter family. The induction of all these genes under P_i starvation in *E. coli* is mediated by a two-component regulatory system consisting of the sensor kinase PhoR and the response regulator PhoB. Therefore, the genes form the so-called Pho regulon. Under P_i limitation the sensor kinase PhoR autophosphorylates and then transfers the phosphoryl group to the response regulator PhoB. PhoB~P in turn activates transcription of *phoBR* and other Pho regulon genes. In summary, when P_i is scarce, *E. coli* takes up P_i by the high-affinity transport system Pst, 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 (Wanner, 1996). The *E. coli* Pst system, which transports P_i at the

Tab. 1: Pho regulon genes/operons of *E. coli*.

Gene/operon	Function of the gene products
phoA-psiF	periplasmic alkaline phosphatase and gene encoding a protein of unknown function
phoE	outer membrane porin channel
pstSCAB-phoU	high-affinity ABC-type phosphate uptake system (PstSCAB) and PhoU protein with a possible role in the overall process of P_i assimilation via the Pst system
ugpBAECQ	ABC-type glycerol-3-phosphate uptake system (UgpBAEC) and phosphodiesterase (UgpQ) that hydrolyzes glycerophosphoryl diesters (deacylated phospholipids)
phnCDEFGHIJKLMNOP	14 proteins involved in phosphonate uptake and degradation
phoH	ATP-binding protein of unknown function
phoR	histidine kinase
phoB	response regulator

expense of ATP (Chan and Torriani, 1996; Wanner, 1996) 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 *pst* operon, is not required for P_i transport, and its function is unclear (Steed and Wanner, 1993). The high affinity of the Pst system and its energetization by ATP allow concentrative P_i uptake when extracellular P_i is limited.

Regulation of the P_i starvation response in *B. subtilis* differs substantially from that in *E. coli*. Inorganic phosphate is one of the critical limiting nutrients for biological growth in soil, the natural environment of *B. subtilis* (Ozanne, 1980). Due to the fact that in nature *B. subtilis* may spend much of its existence in a phosphate-deficient state, it has evolved a variety of systems for utilizing this limited nutrient and corresponding phosphate starvation-inducible genes are subject to a very complex regulation (Antelmann *et al*., 2000). When P_i becomes limiting (extracellular P_i concentration lower than 0.1 mM), *B. subtilis* activates 31 genes in 10 operons that constitute the PhoP regulon (Tab. 2), including e.g. genes for two alkaline phosphatases (*phoA*, *phoB*) or genes encoding a high-affinity P_i uptake system (*pstSCAB$_1$B$_2$*)

as well as the *phoPR* and *resABCDE* operons encoding the PhoP/PhoR and ResD/ResE two-component regulatory systems (Hulett, 2002). Thus, upon P_i limitation *B. subtilis* takes up P_i by an ATP-driven high-affinity uptake system and mobilizes phosphate extracellularly by using phosphatases. Moreover, under phosphate starvation conditions the transcription of the teichuronic acid biosynthetic genes *tuaABCDEFGH* is induced and at the same time the transcription of the teichoic acid biosynthetic genes *tagAB* and *tagDEF* is repressed. Teichoic acids, an anionic polymer containing polyglycerol or polyribitol phosphate, are a major cell wall component of *B. subtilis* when grown in phosphate-replete media (Baddiley, 1970; Mauël *et al*., 1989). Approximately 15% of the entire cellular phosphorus content is stored in the form of teichoic acids under these conditions (Archibald *et al*., 1993). During phosphate starvation, however, the cell ceases to produce phosphate-rich teichoic acids and replaces them with a phosphate-free polymer, teichuronic acids. As a result, the cell saves phosphorus for cellular metabolism and DNA.

Tab. 2: Pho regulon genes/operons of *B. subtilis*

Gene/operon	Function of the gene products
phoA	alkaline phosphatase
phoB-ydhF	alkaline phosphatase (PhoB) and unknown lipoprotein
phoD	alkaline phosphodiesterase
pstSCAB$_1$B$_2$	high-affinity ABC-type phosphate transporter
glpQ	glycerophosphoryl diester phosphodiesterase
tuaABCDEFGH	teichuronic acid biosynthesis
tagAB	teichoic acid biosynthesis
tagDEF	teichoic acid biosynthesis
ykoL	protein of unknown function
phoPR	response regulator PhoP and histidine kinase PhoR
resABCDE	cytochrome c biosynthesis (ResA, ResB, ResC), histidine kinase ResE, response regulator ResD

The Pho regulon of *B. subtilis* is controlled by at least three two-component signal transduction systems: PhoP/PhoR, ResD/ResE and the phosphorelay system leading to the phosphorylation of Spo0A (Fig. 3). Two of these systems act as positive regulators, while the

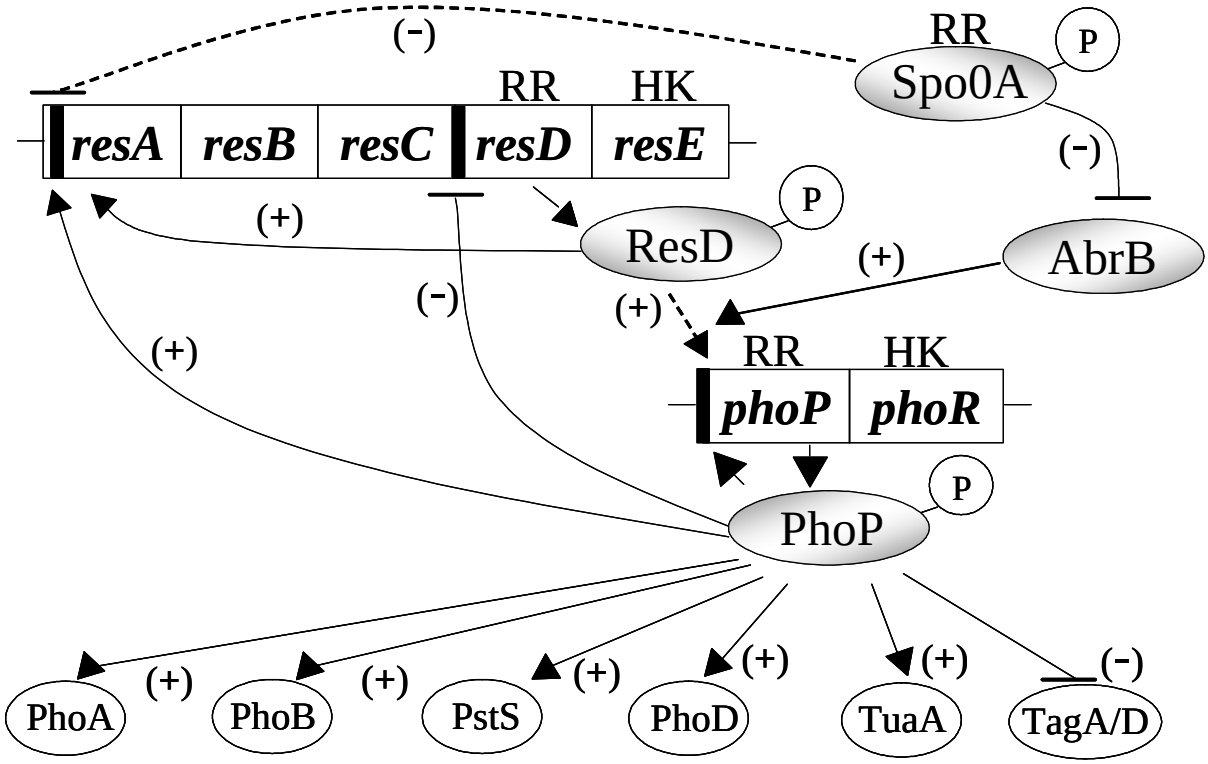


Fig. 3: Model depicting the signal transduction network leading to PhoP induction in *B. subtilis*, a phosphate starvation response. Proteins are indicated by ovals, and genes/operons are symbolized by rectangles. Solid lines indicate that direct interaction has been demonstrated. Dashed lines are utilized for interactions that could either be direct or indirect. Positive regulation is labelled with an arrow and a plus sign (+), while repression is noted by an arrow and a minus sign (-). Two-component system members are labelled either as histidine kinase (HK) or response regulator (RR) (Hulett, 2002).

third is involved in negative regulation of the Pho regulon. Under phosphate-starvation conditions, the histidine protein kinase PhoR phosphorylates the response regulator PhoP. Subsequently PhoP~P activates transcription of *phoPR, resABCDE* and other P_i starvation-inducible genes and represses transcription of e.g. the *tagAB* operon (see Fig. 3). The response regulator ResD along with its cognate histidine kinase ResE functions as regulator of genes involved in aerobic and anaerobic respiration in *B. subtilis.* Moreover, the ResD/ResE two-component system is also required for full-induction of the Pho regulon genes. A positive regulator of the Pho regulon is also the transition-state regulatory protein AbrB. However,

ResDE and AbrB are involved in activation of the Pho regulon genes through separate regulatory pathways. The response regulator Spo0A~P exerts a negative effect on the Pho regulon through its repression of *abrB*, and possibly through repression of *resDE*. Both pathways converge to regulate transcription of the *phoPR* operon (Sun *et al*., 1996).

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 phosphate starvation conditions (Andersen *et al*., 1990) and is a phosphate-binding protein similar to *E. coli* PstS (Chang *et al*., 1994). Subsequently, it was realized that the genome of *M. tuberculosis* harbours three genes encoding phosphate-binding proteins in three operons (*pstBS1C1A2, pstS2, pstS3C2A1*) at one locus (Cole *et al*., 1998; Lefevre *et al*., 1997). By using translational fusions, two P_i starvation-responsive promoters were identified for the *pstBS1C1A2* operon (Torres *et al*., 2001).

1.2 Phosphate starvation response in *Corynebacterium glutamicum*

Corynebacterium glutamicum is a non-pathogenic, non-sporulating aerobic bacterium that has gained interest because of its use in the production of amino acids (Eggeling and Sahm, 1999) and of its emerging role as a model organism for Gram-positive bacteria with high (G+C) content (Nolden *et al*., 2002), which include a number of important pathogens, in particular *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*. *C. glutamicum* was first isolated in 1957 by Japanese scientists, Dr. Udaka and Dr. Kinoshita, from a soil sample (Kinoshita *et al*., 1957; Udaka, 1960). Systematic studies have indicated that *C. glutamicum* can only be isolated from soils contaminated by bird faeces (Woodruff, 1981). While the wild type strain had originally been isolated because of its capability to secrete L-glutamate, which serves as a flavour enhancer in the food industry, meanwhile a number of classically mutagenized and genetically engineered strains have been developed to produce a large number of different amino acids (Eggeling and Sahm, 2001), most notably L-lysine, which is used as a feed additive. Currently, the annual production of L-glutamate and L-lysine amounts to 1 000 000 and 600 000 tons, respectively (Leuchtenberger, 1996; Pfefferle *et al*., 2003).

The phosphate starvation stimulon of *C. glutamicum* was characterized by global gene expression analysis (Ishige *et al*., 2003). 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 the identification of five groups of genes (in total 92) with different expression patterns after the shift. Four of these groups include genes which are not directly involved in phosphorus

metabolism and changed expression presumably due to the exchange of the medium or due to the reduced growth rate observed after the shift. One group, however, comprised 25 genes, most of which are obviously related to phosphorus uptake and metabolism and their mRNA level increased after the shift to P_i limitation. The genes belonging to this group are collectively called *p*hosphate *s*tarvation *i*nducible genes (*psi*) and include e.g. the *pstSCAB* operon, the *ugpAEBC* operon, *glpQ*, *phoH* or *phoRS* (Tab. 3).

Tab. 3: Prominent phosphate starvation inducible genes of *C. glutamicum*. (Ishige *et al*., 2003).

Gene	Function of the gene products	Avg mRNA level after shifting to P_i limitation/avg mRNA level before shifting to P_i limitation					
		10 min	30 min	60 min	90 min	120 min	180 min
pstS		5.0	18.0	23.2	21.5	19.1	13.8
pstC	***pstSCAB* operon**	4.4	19.3	23.6	22.8	20.9	19.1
pstA	high-affinity uptake system for P_i	1.9	5.8	12.5	8.6	7.1	6.9
pstB		2.7	9.0	12.4	14.5	10.3	8.3
ugpA		2.1	15.1	16.8	15.0	11.0	12.5
ugpE	***ugpAEBC* operon**	1.6	9.8	22.3	15.0	11.7	18.1
ugpB	*sn*-glycerol 3-phosphate uptake system	2.2	12.9	22.8	18.7	17.2	14.5
ugpC		1.1	4.2	3.9	4.3	4.5	4.5
glpQ	glycerophosphoryldiester phosphodiesterase	1.5	2.8	4.7	5.0	9.2	7.5
phoH	ATPase related to P_i starv. inducible protein	1.3	1.6	4.6	4.4	5.8	4.7
ushA	UDP-sugar hydrolase/5'-nucleotidase	1.2	7.3	6.5	19.3	10.3	7.2
nucH	nuclease		4.8	19.5	31.7	25.8	16.9
pctB	ABC transporter permease	1.0	2.8	3.0	5.0	2.7	2.3
pctC	ABC transporter, ATP binding protein	1.2	6.3	3.6	3.4	2.9	2.1
Cgl3064	putative secreted phosphoesterase		2.3	5.3	7.3	11.9	6.3
cgtS3	histidine kinase	4.7	1.7	1.0	0.8	0.8	0.9
cgtR3	response regulator	8.1	2.3	1.4	1.1	1.1	1.5
Cgl0596	hypothetical protein		10.8	18.8	17.6	15.0	8.9
Cgl0849	hypothetical protein	1.3	1.3	2.0	2.8	3.0	3.0
Cgl2336	hypothetical protein	1.0	2.8	3.8	4.2	4.6	4.2

According to their predicted functions, the proteins encoded by this group of *psi* genes in *C. glutamicum* catalyze high-affinity uptake of inorganic phosphate and of glycerol 3-phosphate, and hydrolysis of organophosphates and nucleic acids. The *pct* genes are part of the *pctABCD* operon encoding an ABC transporter which might be involved in the uptake of a not yet identified P compound (Ishige *et al*., 2003). The P_i starvation stimulons of *C. glutamicum* and *E. coli* are quite similar, however, that of *E. coli* includes genes for phosphonate uptake and

degradation (Wanner, 1996), whereas *C. glutamicum* lacks homologs of these genes as well as the capability to utilize phosphonates as P sources (Ishige and Wendisch, unpublished data). Compared to the P_i starvation response of *B. subtilis*, that observed in *C. glutamicum* led to different expression changes. The cell wall of *C. glutamicum* lacks teichuronic acids and teichoic acids (Liebl *et al*., 1991) and the genome does not contain homologs of the *B. subtilis tuaABCDEFGH, tagAB* and *tagDEF* genes. Moreover, in *B. subtilis* P_i starvation also results in a σ^B-dependent induction of the genes of the general stress response (Antelmann *et al*., 2000; Pragai *et al*., 2002).

Concerning the kinetics of the P_i starvation response at the gene expression level, the first response of *C. glutamicum* to P_i limitation is increased expression of the *pstSCAB* operon, which is evident already 10 min after a P_i down-shift. The induction of *glpQ* and of the oppositely oriented *ugpAEBC* operon was less pronounced after 10 min but high 30 min after the P_i down-shift. In contrast to *C. glutamicum*, in *E. coli* the corresponding genes form a single operon *ugpBAECQ* (Argast and Boos, 1980; Brzoska *et al*., 1994). Figure 4 presents a

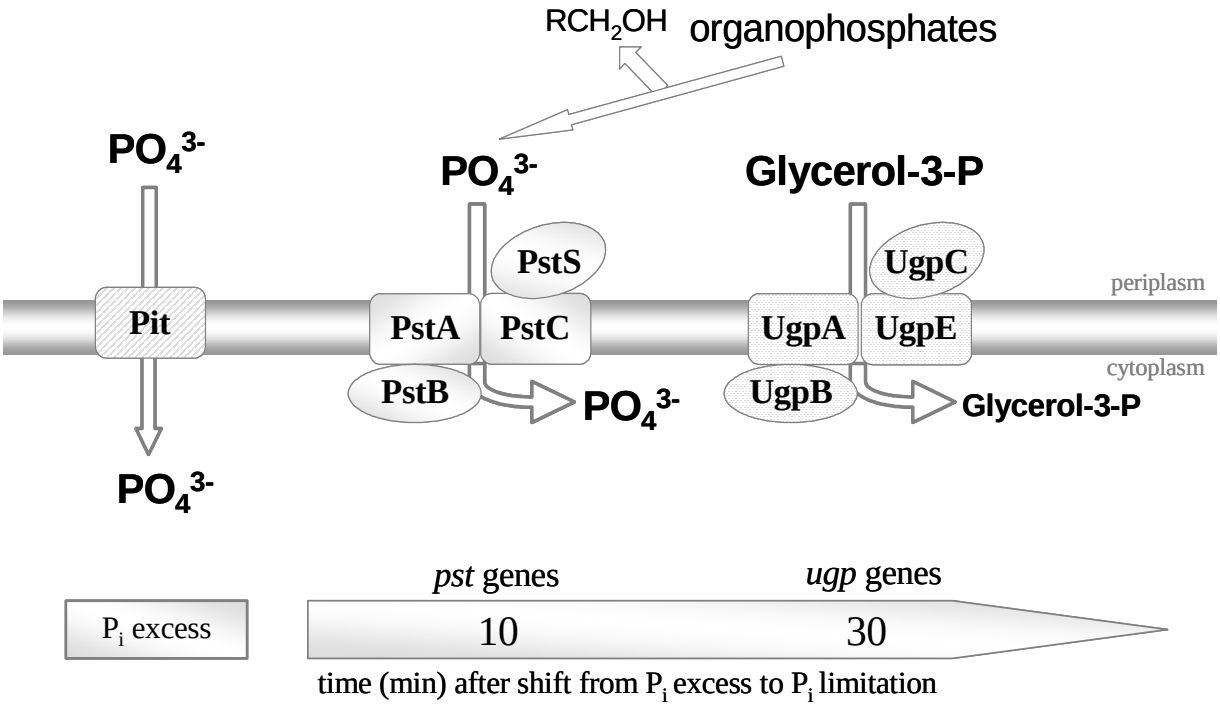


Fig. 4: Basic model of the response of *C. glutamicum* to P_i starvation. Pit - low affinity P_i transporter, PstSCAB - ABC transporter for inorganic phosphate, UgpAEBC – ABC transporter for glycerol-3-phosphate.

basic model of the response of *C. glutamicum* to P_i starvation.

Besides the *pst* genes, also the *cgtR3-cgtS3* genes showed 5- and 8-fold increased mRNA levels already 10 min after the shift from P_i excess to P_i limitation. In contrast to the *pst* genes, however, the *cgtRS3* mRNA levels decreased to their phosphate excess value within 60 min. The *cgtRS3* genes encode a sensor kinase (*cgtS3*) and a response regulator (*cgtR3*) which form a prototypical two-component signal transduction system (Stock *et al*., 2000). In addition, another response regulator gene (*cgtR9*) showed a 7- to 2-fold increased mRNA level 30 to 120 min after the P_i downshift (Ishige *et al*., 2003). The *cgtR9* gene is located next to genes involved in copper metabolism.

2 Aim of the work

The aim of this PhD thesis was to search for regulators involved in the phosphate starvation response in *C. glutamicum.* The project was part of the BMBF-Kompetenznetzwerk-Cluster "Use of DNA chip technology for analysis of global regulatory networks in *Corynebacterium glutamicum*". As outlined above, two-component signal transduction systems play a central role in the P_i starvation response of many bacteria studied in this respect, e.g. *E. coli* or *B. subtilis*. Therefore, it appeared possible that this is true also in *C. glutamicum*. In the 3.3 Mb genome sequence of this species (Kalinowski *et al*., 2003), genes encoding 13 two-component systems were identified, all of which are presumably active in transcriptional regulation of gene expression (Schaffer and Bott, in preparation). However, with one exception, the function of the different two-component systems could not be deduced from sequence comparisons or other in silico analyses. In order to test an involvement in the P_i starvation response, a set of *C. glutamicum* mutants each lacking one two-component systems was tested for growth under P_i limitation. This screening approach led to the identification and subsequent characterization of the PhoRS two-component system, which presumably is the central regulator of the P_i starvation response in *C. glutamicum*.

IV MATERIALS AND METHODS

1 Bacterial strains

The bacterial strains used in this work are listed in Table 4. All the strains were kept as glycerol stock cultures at both -20 °C and -70 °C for long-term storage or on agar plates at 4 °C for short-term storage.

Tab. 4: Bacterial strains used in this work.

Strain	Relevant phenotype	Source/Reference
Escherichia coli		
DH5α	F^- *thi-1 endA1 hsdR17* (r^-, m^-) *supE44* Δ*lacU*169 (Φ80*lacZ*Δ*M15*) *recA1 gyrA96 relA1*; host for cloning procedures	Bethesda Research Laboratories
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^-, m_B^-) *gal dcm* (λcIts857 *ind1* Sam7 *nin5 lacUV5*-T7 gene 1); host for overproduction of recombinant proteins from pET expression plasmids; contains a chromosomally integrated T7 RNA polymerase gene under the control of the *lac*UV5 promoter	Studier & Moffatt (1986)
C43(DE3)	BL21(DE3) derivative, carrying not identified mutation(s); host for overproduction of membrane proteins	Miroux & Walker (1996)
Corynebacterium glutamicum		
ATCC13032	wild type	Abe *et al*. (1967)
13032Δ*cgtRS3* later named 13032Δ*phoRS*	ATCC13032 derivative, deletion of *cgtRS3*	S. Schaffer
13032Δ*citAB*	ATCC13032 derivative, deletion of *citAB*	S. Schaffer

13032Δ*mtrAB*	ATCC13032 derivative, deletion of *mtrAB*	Möker et al. (2004)
13032Δ*cgtRS1*	ATCC13032 derivative, deletion of *cgtRS1*	S. Schaffer
13032Δ*cgtRS2*	ATCC13032 derivative, deletion of *cgtRS2*	S. Schaffer
13032Δ*cgtRS4* pXMJ19CgtR4	ATCC13032 derivative, deletion of *cgtRS4* enabled by the presence of plasmid pXMJ19CgtR4	Wessel (2003)
13032Δ*cgtRS5*	ATCC13032 derivative, deletion of *cgtRS5*	S. Schaffer
13032Δ*cgtRS6*	ATCC13032 derivative, deletion of *cgtRS6*	S. Schaffer
13032Δ*cgtRS7*	ATCC13032 derivative, deletion of *cgtRS7*	S. Schaffer
13032Δ*cgtRS8*	ATCC13032 derivative, deletion of *cgtRS8*	S. Schaffer
13032Δ*cgtRS9*	ATCC13032 derivative, deletion of *cgtRS9*	S. Schaffer
13032Δ*cgtRS10*	ATCC13032 derivative, deletion of *cgtRS10*	S. Schaffer
13032Δ*cgtRS11*	ATCC13032 derivative, deletion of *cgtRS11*	S. Schaffer

2 Plasmids

In Table 5 the plasmids used in this work are listed. Further information on individual plasmids is provided in the text.

Tab. 5: Plasmids used in this work.

Plasmid	Relevant characteristics	Source/Reference
pEKEx2	Km^R, *C. glutamicum/E. coli* shuttle vector for regulated gene expression (P_{tac}, $lacI^Q$, pBL1 $oriV_{C.g.}$, pUC18 $oriV_{E.c.}$)	Eikmanns *et al.* (1991)
pEKEx2-*phoRS*	pEKEx2 derivative carrying *phoRS* with a C-terminal StrepTag-II	This work
pET24b	Km^R, expression vector with a T7*lac* promoter and the possibility to construct fusion proteins with a C-terminal His_6-tag	Novagen, Madison, USA

pET24b-*phoR*	Km^R, pET24b derivative for the overproduction of *C. glutamicum* PhoR with a C-terminal His_6-tag ($PhoR_{His}$)	This work
pET24b-*phoS*	Km^R, pET24b derivative for the overproduction of *C. glutamicum* PhoS with a C-terminal His_6-tag ($PhoS_{His}$)	This work
pET16b	Ap^R, expression vector with a T7*lac* promoter and the possibility to construct fusion proteins with an N-terminal His_{10}-tag cleavable by the factor Xa protease	Novagen, Madison, USA
pET16b-*phoRΔ1-125*	Ap^R, pET16b derivative for the overproduction of *C. glutamicum* PhoRΔ1-125 with an N-terminal His_{10}-tag (PhoRΔ1-125)	This work
pET16b-*phoRΔ1-139*	Ap^R, pET16b derivative for the overproduction of *C. glutamicum* PhoRΔ1-139 with an N-terminal His_{10}-tag (PhoRΔ1-139)	This work
pK19*mobsacB*	Km^R, mobilisable suicide vector for allelic exchange in *C. glutamicum* (pK18 $oriV_{E.c.}$, *sacB*, *lacZ*α)	Schäfer *et al.* (1994)
pK19*mobsacB*Δ*cgtRS3*	Km^R, pK19*mobsacB* derivative carrying *cgtRS3* deletion construct	S. Schaffer
pK19*mobsacB*Δ*citAB*	Km^R, pK19*mobsacB* derivative carrying *citAB* deletion construct	S. Schaffer
pK19*mobsacB*Δ*mtrAB*	Km^R, pK19*mobsacB* derivative carrying *mtrAB* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS1*	Km^R, pK19*mobsacB* derivative carrying *cgtRS1* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS2*	Km^R, pK19*mobsacB* derivative carrying *cgtRS2* deletion construct	S. Schaffer

pK19*mobsacB*Δ*cgtRS4*	Km^R, pK19*mobsacB* derivative carrying *cgtRS4* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS5*	Km^R, pK19*mobsacB* derivative carrying *cgtRS5* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS6*	Km^R, pK19*mobsacB* derivative carrying *cgtRS6* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS7*	Km^R, pK19*mobsacB* derivative carrying *cgtRS7* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS8*	Km^R, pK19*mobsacB* derivative carrying *cgtRS8* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS9*	Km^R, pK19*mobsacB* derivative carrying *cgtRS9* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS10*	Km^R, pK19*mobsacB* derivative carrying *cgtRS10* deletion construct	S. Schaffer
pK19*mobsacB*Δ*cgtRS11*	Km^R, pK19*mobsacB* derivative carrying *cgtRS11* deletion construct	S. Schaffer
pXMJ19	Cm^R, *C. glutamicum/E. coli* shuttle vector for regulated gene expression (P_{tac}, $lacI^Q$, pBL1 $oriV_{C.g.}$, pK18 $oriV_{E.c.}$)	Jacoby *et al.* (1999)
pXMJ19CgtR4	pXMJ19 derivative carrying *C. glutamicum cgtR4*	Wessel (2003)

2.1 Construction of the complementation plasmid pEKEx2-*phoRS*

In order to complement the growth phenotype of the *C. glutamicum* deletion mutant Δ*phoRS*, plasmid pEKEx2-*phoRS* (Fig. 5) was constructed which encodes the unmodified response regulator PhoR and the sensor kinase PhoS modified with a carboxyterminal StrepTag-II (WSHPQFEK). Expression of the corresponding genes (coding regions plus 40 bp DNA upstream of the *phoR* start codon) is controlled by the *tac* promoter. The corresponding sequence of the *phoRS* genes was amplified from chromosomal DNA of *C. glutamicum* ATCC13032 using primers phoRS-SbfI-fw and phoRS-KpnI-rv. The polymerase chain reaction (PCR) was performed with the Expand High Fidelity PCR System composed of

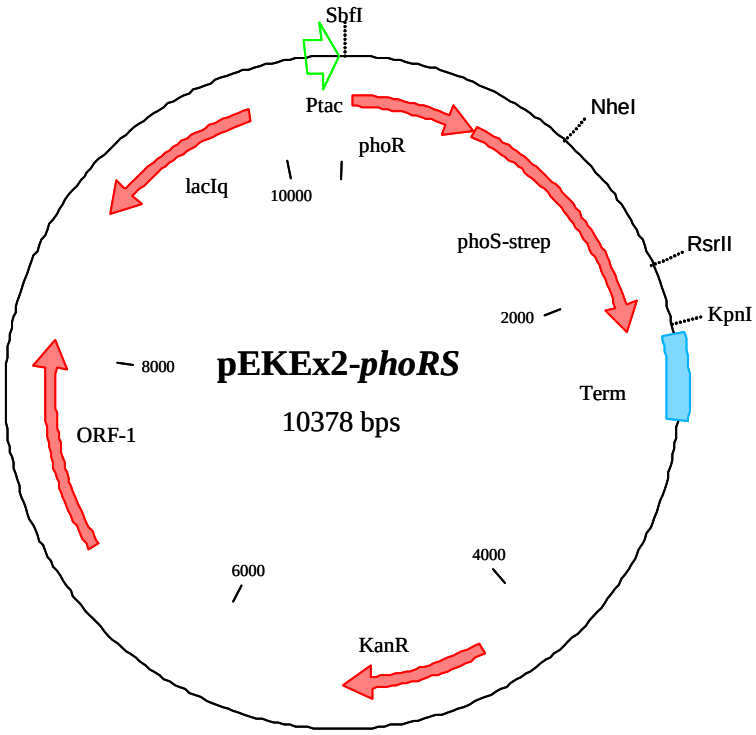


Fig. 5: Schematic illustration of plasmid pEKEx2-*phoRS*.

a unique enzyme mix containing a thermostable *Taq*-DNA polymerase and a thermostable proofreading *Pwo*-DNA polymerase. The PCR product of 2263 bp encoding the two-component system PhoRS was purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). After digestion of the product with *Sbf*I and *Kpn*I, the DNA fragment was gel purified using the QIAEX kit (Qiagen) and cloned into the expression vector pEKEx2 cut with the same enzymes. The ligation reaction was performed with the Rapid DNA ligation kit (Roche Diagnostics). The ligation mixture was used to transform *E.coli* DH5α. The transformants were selected on LB plates with kanamycin. Ten plasmids isolated from recombinant clones were analysed by restriction analysis with *Sbf*I and *Kpn*I, two of which showed the expected restriction pattern (2.2 kb and 8.1 kb) and were further analysed by DNA sequence analysis. In one of the plasmids (pEKEx2-*phoRS*-5), several mutations were identified within the phoRS coding region, whereas the second one (pEKEx2-*phoRS*-3) contained only a single point mutation, i.e. an A instead of T 872 bp downstream of the *phoS* start codon, which leads to a Leu → Gln exchange at position 291 of the PhoS sequence. In order to eliminate this mutation, a 750 bp NheI-RsrII fragment of pEKEx2-*phoRS*-3 was replaced by the corresponding fragment of plasmid pET24b-phoS (see below). DNA sequence

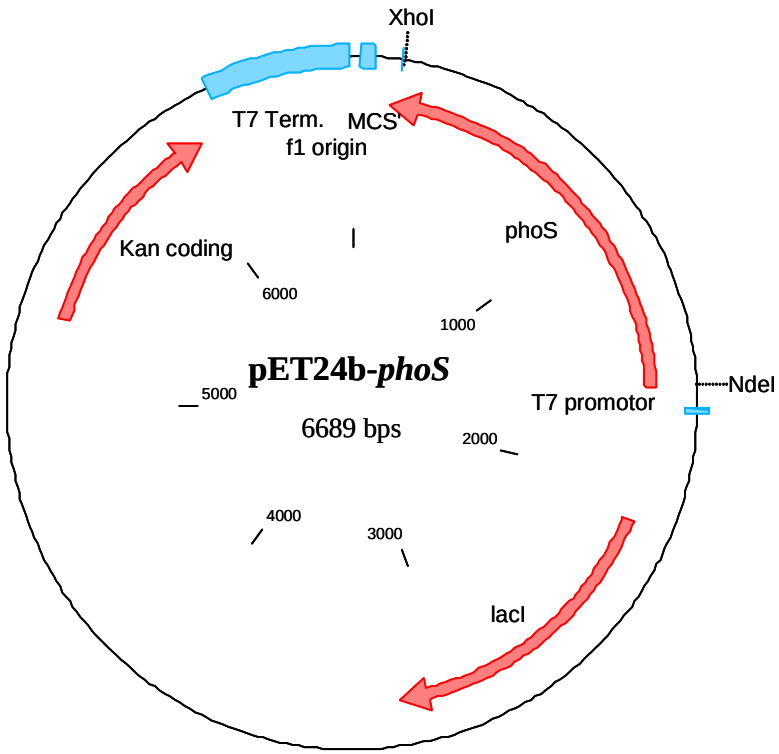


Fig. 6: Schematic illustration of plasmid pET24b-*phoS*.

analysis confirmed that the resulting plasmid pEKEx2-*phoRS* contained no mutations in the *phoRS* coding sequence and this plasmid was used then for the complementation studies.

2.2 Construction of expression plasmids

For overproduction and purification of the *C. glutamicum* sensor kinase PhoS from *E. coli*, a T7 expression system was used and PhoS was modified by a carboxyterminal His-tag. The expression plasmid pET24b-*phoS* (Fig. 6) was constructed as follows. The coding region of the *C. glutamicum phoS* gene was amplified by PCR from chromosomal DNA of *C. glutamicum* ATCC13032 using primers that introduced an *Nde*I restriction site at the initiation codon (phoS-*Nde*I-fw) and an *Xho*I restriction site in front of the stop codon (phoS-*Xho*I-rv). PCR was performed with the Expand high fidelity PCR system (Roche Diagnostics). After digestion of the PCR product with *Nde*I and *Xho*I, the DNA fragment was purified from an agarose gel using the QIAEX kit (Qiagen) and cloned into the expression vector pET24b (Novagen) cut with the same enzymes. The resulting plasmid pET24b-*phoR* was sequenced with the T7 promoter primer and the T7 terminator primer. The cloning strategy resulted in a protein designated $PhoS_{His}$ (493 amino acids, 53.4 kDa) that contained eight additional amino

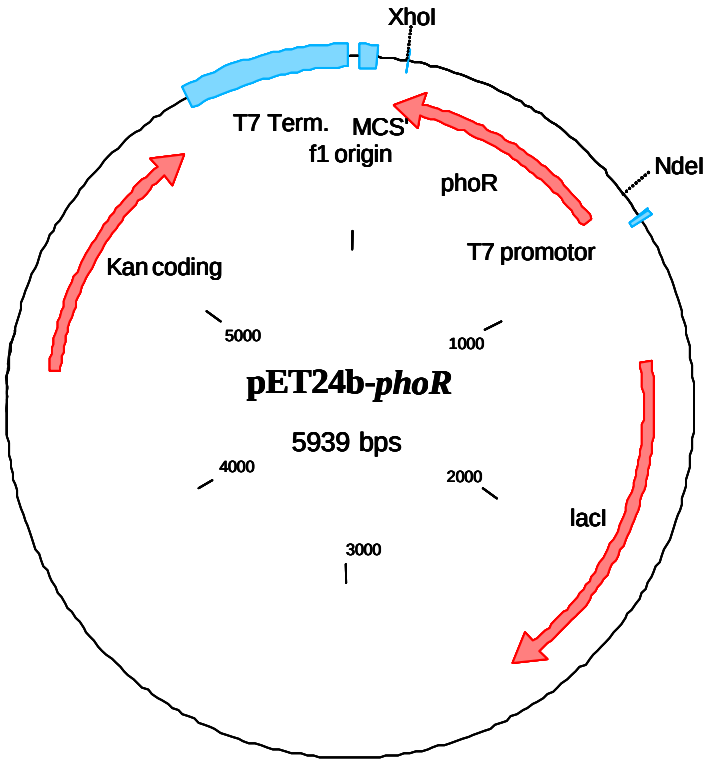


Fig. 7: Schematic illustration n of plasmid pET24b-*phoR*.

acid residues (LEHHHHHH) at the carboxyterminus. Synthesis of $PhoS_{His}$ is controlled by a T7*lac* promoter (Studier *et al.*, 1990).

To overproduce and purify the *C. glutamicum* response regulator PhoR from *E. coli*, the expression plasmid pET24b-*phoR* (Fig. 7) was constructed using the same procedures as described above for $PhoS_{His}$. Primers phoR-*Nde*I-fw and phoR-*Xho*I-rv introduced an *Nde*I restriction site at the initiation codon and an *Xho*I restriction site in front of the stop codon, respectively. The cloning strategy resulted in protein $PhoR_{His}$ (243 amino acids, 27.4 kDa) that contained eight additional amino acid residues (LEHHHHHH) at the carboxyterminus.

For the purification of the DNA-binding domain of PhoR, the aminoterminal border of this domain was estimated with the help of a sequence alignment of the PhoR orthologs from other *Corynebacterium* and *Mycobacterium* species (Fig. 15). It shows two highly conserved parts corresponding the receiver domain (N-terminal) and the DNA-binding domain (C-terminal) which are connected by a non-conserved region which presumably represents a linker region. For further studies, the DNA-binding domain was purified with the linker region and without the linker region. The first derivative was named PhoRΔ1-125, where amino acids 1 to 125 of PhoR are missing. The second derivative was named PhoRΔ1-139,

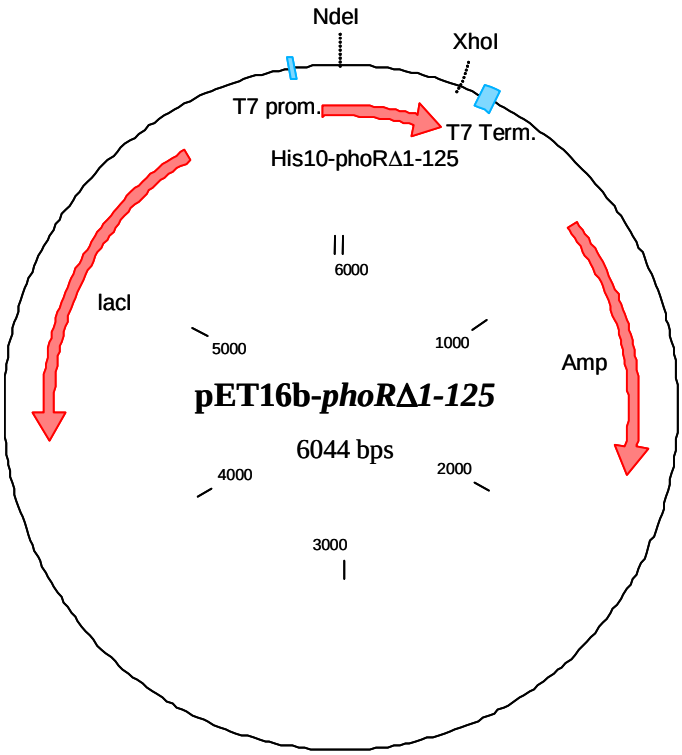


Fig. 8: Schematic illustration of plasmid pET16b-*phoRΔ1-125*.

where amino acids 1 to 139 of PhoR are missing. For the construction of plasmids encoding PhoRΔ1-125 and PhoRΔ1-139, a T7 expression system was used and both proteins were modified by an aminoterminal His-tag rather than by a carboxyterminal His-tag. To this end, the expression plasmids pET16b-*phoRΔ1-125* (Fig. 8) and pET16b-*phoRΔ1-139* were constructed using the same procedures as described above. The corresponding sequences of the binding domain were amplified by PCR (Expand high fidelity PCR system; Roche Diagnistics). The primers PhoR-bin1-NdeI-fw (for PhoRΔ1-125) and PhoR-bin2-NdeI-fw (for PhoRΔ1-139) introduced an *NdeI* restriction site at the newly introduced start codon and primer PhoR-bin1-XhoI-rv introduced an *XhoI* restriction site after the stop codon. The resulting 0.4-kb fragments were gel purified and subsequently cloned into the expression vector pET16b cut with the same enzymes. The cloning strategy led to PhoR derivates missing amino acids 1-125 or 1-139 to of wild-type PhoR. The resulting proteins PhoRΔ1-125 (132 amino acids, 15.0 kDa) and PhoRΔ1-139 (118 amino acids, 13.6 kDa) consisted of 111 or 97 amino acids of wild-type PhoR fused an aminoterminal region including ten histidine residues followed by factor Xa cleavage site (IEGR). Synthesis of PhoRΔ1-125 and PhoRΔ1-139 was controlled identically as described above for $PhoS_{His}$.

3 Oligonucleotides

The oligonucleotides used as primers for DNA sequencing, PCR, probe generation and primer extension are listed in Table 6. They were purchased from MWG Biotech, Ebersberg, Germany.

Tab. 6: Sequence of the oligonucleotides used in this work. In some cases oligonucleotides were designed to introduce recognition sites for restriction endonucleases (recognition sites underlined, restriction endonucleases indicated in parentheses) or a sequence for the StrepTag-II (printed in bold).

Name	Sequence (5'-3')	Use
phoRS-SbfI-fw	CC GCG GCC TGC AGG TAA TCA TTC CGC TAA TGG ACA CCA A (*Sbf*I)	Construction of pEKEx2-*phoRS*
phoRS-KpnI-rv	TAA ATA GGT ACC TTA **CTT CTC GAA CTG CGG GTG GCT CCA** AGA AAC CGC CGG CAA GGT GAT (*Kpn*I)	Construction of pEKEx2-*phoRS*
pSE380-F	IRD800-CGG TTC TGG CAA ATA TTC TG	Sequencing primer
phoRS-seq1	IRD800-GC AAC GTC GTG GAA TCC TAC	Sequencing primer
phoRS-seq2	IRD800-GAT CGA CGT GTC CCG CAG TG	Sequencing primer
pstS_promreg_fw	AC ATG AAT ACT TCC GAT CGT ATT AAA AGC	PCR primer for the amplification of the *pstS* promoter region
pstS_promreg_rv	C GGA CTG CTG GGA AGA TGC ACC TT	PCR primer for the amplification of the *pstS* promoter region
ugpA_promreg_fw	T TGA GCC CTA GGG GAG TGG TCG G	PCR primer for the amplification of the *ugpA* promoter region

ugpA_promreg_rv	A CCC AAT GAA GGT TGA TGT GGG CGA	PCR primer for the amplification of the *ugpA* promoter region
phoR_promreg_fw	ATA ATC CCA ATT TCA ACT CTT TCA GTA CA	PCR primer for the amplification of the *phoR* promoter region
phoR_promreg_rv	AT TCC TGG CAT CAT GAC ATC GAG GAT	PCR primer for the amplification of the *phoR* promoter region
pitA_promreg_fw	ATG AAG TTA AAA ACA TTT TCC GTG AAG C	PCR primer for the amplification of the *pitA* promoter region
pitA_promreg_rv	C AAT GGA TGT GGC CAT CGC ATT GC	PCR primer for the amplification of the *pitA* promoter region
PclpC-cat-1	TAT ATC TAG AAT GGG TCT CCT AGT TGA TTA ATC AG	PCR primer for the amplification of the *clpC* promoter region
SC-clpC-rv2	TAT ATC TAG ACT CGA ACA TGT CTC TCC CCT TA	PCR primer for the amplification of the *clpC* promoter region
clpP-activator-fw	TTC GGT GAA GAA GAA GTA GCT GAG AC	PCR primer for the amplification of the *clpP1P2* promoter region
clpP-BS2-rv	TGC GCT CAC TCA GGA GGC GAT CAA AG	PCR primer for the amplification of the *clpP1P2* promoter region
pstS_prext1	IRD800-GCC TGC AGT AAC TGC GCC CAC AAG G	Primer extension analysis
pstS_prext2	IRD800-GGA GTC GGA GCA AGC TAC AAG AGC GA	Primer extension analysis

ugpA_prext2	IRD800-GC AAG CCA CCA TTC TGT CCG CCT TGA T	Primer extension analysis
ugpA_prext3	IRD800-AG TGC GGC GGC AAG CCA CCA TT	Primer extension analysis
phoR_prext1b	IRD800-GTC AGA CTG GTT GTC CAT TTT TAT C	Primer extension analysis
pitA_prext1	IRD800-TG ACG GTA AAT GCC CAG GAT AGA GTT CG	Primer extension analysis
pitA_prext2	IRD800-AAG CGT AGA AAC CAA ATG TCG ATT TTG GAT	Primer extension analysis
phoS-NdeI-fw	C GGC CCG CAT ATG GAA AAT CCT TAT GTT GCT GCG C (*Nde*I)	Construction of pET24b-*phoS*
phoS-XhoI-rv	TAA ATA CTC GAG AGA AAC CGC CGG CAA GGT GAT C (*Xho*I)	Construction of pET24b-*phoS*
phoR-NdeI-fw	C GGG CCC CAT ATG GAC AAC CAG TCT GAC GGA CA (*Nde*I)	Construction of pET24b-*phoR*
phoR-XhoI-rv	T ATA TAT CTC GAG GCT ACG TGG GGT GCG CAG AAC (*Xho*I)	Construction of pET24b-*phoR*
PhoR-bin1-NdeI-fw	C GGG CCC CAT ATG CGC GGT GGA GCA GTT GAA G (*Nde*I)	Construction of pET16b-*phoRΔ1-125*
PhoR-bin1,2-XhoI-rv	ATA TAT CTC GAG TTA GCT ACG TGG GGT GCG CAG AAC (*Xho*I)	Construction of pET16b-*phoRΔ1-125;* pET16b-*phoRΔ1-139*
PhoR-bin2-NdeI-fw	C GGG CCC CAT ATG TAC GCA GAC CTC ACC CTC AA (*Nde*I)	Construction of pET16b-*phoRΔ1-139*
pET-promoter-IRD	IRD800-CGA AAT TAA TAC GAC TCA CTA TAG G	Sequencing primer
pET-terminator-IRD	IRD800-TAT GCT AGT TAT TGC TCA GCG GTG	Sequencing primer
pstS_footpr_fw	T CCA GAA TTA CAA GAG ATT TAC GTA GTT GG	DNase I footprint analysis

pstS_footpr_rv	GT AAC TGC GCC CAC AAG GGC GAT GG	DNase I footprint analysis
pstS_footpr_fw_IRD800	IRD800-T CCA GAA TTA CAA GAG ATT TAC GTA GTT GG	DNase I footprint analysis
pstS_footpr_rv_IRD800	IRD800-GT AAC TGC GCC CAC AAG GGC GAT GG	DNase I footprint analysis

4 Media and growth of bacteria

The *E. coli* strains were routinely grown at 37 °C in Luria-Bertani (LB) medium (Sambrook *et al*., 1989) or on LB agar plates (LB containing 1.5% (w/v) Bacto agar (Becton Dickinson)). For overproduction of heterologous proteins with *E. coli* BL21(DE3) or C42(DE3), the temperature was changed from 37 °C to room temperature after IPTG addition at an OD_{600} of 0.6-0.8. For the preparation of competent cells for long-term storage (in detail described later) the cells were grown at 18 °C in SOB medium (Miller and Nickoloff, 1995).

The *C. glutamicum* strains were grown either in rich media (LB or Brain-Heart-Infusion (BHI, from Difco) with 2 % (w/v) glucose) or in CGXII minimal medium with 4 % (w/v) glucose (Keilhauer *et al*., 1993) and different concentrations of phosphate. After electroporation the cells were regenerated in BHIS medium (BHI medium with 0.5 M sorbitol) and subsequently plated on BHIS plates (BHI agar (Difco) with 1.5 % (w/v) Bacto agar).

The 5 ml-liquid cultures were grown in glass tubes at 180 rpm and cultures with a volume of 20 ml or more were grown in Erlenmeyer flasks at 120 - 130 rpm. For selective growth, the media were supplemented with ampicillin (100 µg/ml), chloramphenicol (10 µg/ml for *E. coli* and *C. glutamicum*) or kanamycin (50 µg/ml for *E. coli*, 25 µg/ml for *C. glutamicum*).

Composition of cultivation media (final concentrations):

LB medium: 10 g/l tryptone (Difco), 5 g/l yeast extract (Difco), 5 g/l NaCl

SOC medium: 20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl after sterilization with autoclave addition of 1 ml per 100 ml each the 2

M glucose solution and 2 M magnesium solution (1 M $MgCl_2$ x 6 H_2O and 1 M $MgSO_4$ x 7 H_2O), both sterile filtrated

SOB medium: 20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 0.2 g/l KCl, after sterilization with autoclave addition of 1.3 g/l $MgCl_2$ $2H_2O$ (sterile filtrated)

CGIII medium: 10 g/l tryptone (Difco), 10 g/l yeast extract (Difco), 2.5 g/l NaCl, pH 7.4, after sterilization with autoclave addition of 2 % (w/v) glucose (autoclaved separately)

CGXII medium: 20 g/l $(NH_4)_2SO_4$, 5 g/l urea, 1 g/l KH_2PO_4, 1 g/l K_2HPO_4, (for P_i-limited conditions: 0.01 or 0.005 g/l KH_2PO_4 and 0.01 or 0.005 g/l K_2HPO_4), 0.25 g/l $MgSO_4 \cdot 7\ H_2O$, 10 mg/l $CaCl_2$, 42 g/l MOPS, 0.2 mg/l biotin, 1 ml/l trace element solution, pH 7 adjustment, after sterilization with autoclave addition of 1 ml/l protocatechuic acid (30 g/l dissolved in diluted NaOH and sterile filtrated) and of 4 % (w/v) glucose (autoclaved separately)

Trace element solution: 10 g/l $FeSO_4 \cdot 7\ H_2O$, 10 g/l $MnSO_4 \cdot H_2O$, 1 g/l $ZnSO_4 \cdot 7\ H_2O$, 0.2 g/l $CuSO_4 \cdot 5\ H_2O$, 20 mg/l $NiCl_2 \cdot 6\ H_2O$, to dissolve HCl was added (final pH approximately 1)

BHIS medium: 37 g/l BHI and 91 g/l sorbitol (autoclaved separately)

4.1 Growth measurement

Growth of bacteria in liquid culture was monitored by measuring the optical density at 600 nm (OD_{600}) in a LKB Ultrospec Plus photometer (Pharmacia). To guarantee the linear relationship between cell density and optical density, the cultures were diluted with growth medium so that the OD_{600} was not higher than 0.4.

4.2 Growth at different phosphate concentrations

For analysing the influence of different phosphate concentrations on the growth of *C. glutamicum* wild type, the 12 two-component deletion mutants and the complementation strain Δ*phoRS*/pEKEx2-*phoRS*, the strains were cultivated in CGXII minimal medium. As

sole P source, 13 mM P_i was used under P_i-sufficient conditions and 0.065 or 0.13 mM P_i under P_i-limited conditions. The following cultivation scheme was routinely applied in these experiments. First, all strains were incubated 9 hours at 30 °C in 5 ml LB medium and then 0.5 ml was inoculated into 50 ml CGIII medium. After overnight growth, cells were harvested by centrifugation (4 °C, 10 min, 5200 *g*), washed with CGXII medium without phosphate and carbon sources, and used to inoculate 60 ml CGXII medium with a reduced phosphate concentration ($[P_i]$ = 0.065 or 0.13 mM) to an OD_{600} of 0.6. After 24 hours at 30 °C and 120 rpm, cells were harvested and washed again. Finally, these cells were used to inoculate two 500-ml Erlenmeyer flasks with 60 ml CGXII medium containing either the regular phosphate concentration ($[P_i]$ = 13 mM) or a limited phosphate concentration ($[P_i]$ = 0.065 or 0.13 mM). The starting OD_{600} was 0.6 and the Erlenmeyer flasks contained two baffles to improve oxygen supply.

For the experiment aimed at the qualitative analysis of alkaline phosphatase activity, the wild type and the two-component deletion mutants were incubated 48 hours at 30 °C on CGXII plates with excess phosphate (13 mM). Subsequently, the strains were streaked onto CGXII plates containing either 13 mM, 1.3 mM or 0.13 mM phosphate and incubated 48 hours at 30 °C. For each phosphate concentration, plates with and without 40 µg per ml of X-phosphate (5-bromo-4-chloro-3-indolyl phosphate) were used.

For analysing the response to a shift from P_i excess to P_i limitation (P_i down-shift) by DNA chip analysis, *C. glutamicum* wild type and the deletion mutant Δ*phoRS* were incubated 9 hours at 30 °C in 5 ml LB and then 0.5 ml were inoculated into 50 ml CGIII medium. After 24 hours at 30 °C and 120 rpm, cells were harvested by centrifugation (4 °C, 10 min, 5200 *g*) washed with CGXII medium without phosphate and carbon sources, and used to inoculate 60 ml CGXII medium with the regular phosphate concentration (13 mM) to an OD_{600} of 0.6. After 24 hours at 30 °C and 120 rpm, cells were harvested and washed again. Subsequently, cells were cultivated in 60 ml CGXII medium with 13 mM phosphate starting with an OD_{600} of 0.6. Cells were grown to mid-log phase (OD_{600} = 4 - 5). An aliquot (20 ml) of the culture was used for RNA preparation (0 min), the rest of the culture was centrifuged (4 °C, 5 min, 3500 *g*), washed and the cells resuspended in 60 ml CGXII medium with 0.065 mM phosphate. After incubation at 30 °C and 120 rpm for 10, 30 or 60 minutes after the P_i down-shift, cells were harvested and used for RNA isolation. For each time point (including the time 0 min) the cells were grown in separate flask and from one flask (60 ml culture) 3 x 20 ml of

cells was poured into ice-loaded tubes (per each 50 ml falcon tube with 20 ml culture 9 g of ice was used) pre-cooled to -20 °C. After centrifugation (5 min, 3500 *g*, 4 °C), the cell pellet was either directly subjected to RNA isolation or immediately frozen in liquid nitrogen and stored at -70 °C until use.

5 Molecular biological techniques involving DNA

5.1 Isolation of DNA

The isolation of plasmids from *E. coli* cultures was carried out using the QIAprep Spin Miniprep kit (Qiagen), which is based on the alkaline lysis method (Birnboim and Doly, 1979). Plasmids from *C. glutamicum* were isolated according to the modified protocol of the alkaline lysis method (Schwarzer and Pühler, 1991). The plasmid DNA was resuspended or eluted in EB Buffer (Qiagen, 10 mM Tris/HCl, pH 8.5). Chromosomal DNA from *C. glutamicum* was isolated as described by Eikmanns *et al*. (1994). For DNA precipitation with ethanol, 0.1 volume 3 M sodium acetate (pH 5.2) and 3 volumes ethanol were added to the DNA solution. The precipitated DNA was then centrifuged 30 min at 15 000 *g* and 4 °C, washed with ice-cold 70 % (v/v) ethanol, air-dried and resuspended in EB Buffer. The DNA concentration was calculated by measuring the extinction (E) at 260 nm. The value E_{260} = 1 corresponds to 50 µg/ml dsDNA (Sambrook *et al.*, 1989). The ratio E_{260}/E_{280}, which reflects protein impurities of the DNA solution, should lie between 1.8 and 2.

5.2 Recombinant DNA techniques

Restriction of DNA was carried out as suggested by the suppliers Roche Diagnostics or New England Biolabs. The DNA cleavage with restriction endonucleases was accomplished in 20 µl (analytic scale) or 50 µl (preparative scale) reaction mixtures with 10-30 U of restriction enzyme in the appropriate buffer. The mixture was incubated at least 1 h at 37 °C (unless the enzyme required a different incubation temperature).

DNA fragments were separated by electrophoresis in 0.8 to 2 % agarose gels with TAE buffer (40 mM Tris-HCl pH 7.5, 40 mM acetic acid, 1 mM EDTA) and subsequently purified from agarose gels either with the QIAEX II Gel Extraction Kit (Qiagen) or with the QIAquick Gel Extraction Kit (Qiagen).

The ligation of DNA fragments was performed with the Rapid DNA Ligation kit (Roche Diagnostics) according to the instructions of the supplier. The ligation reactions were incubated for approx. 1 h at room temperature.

5.3 Preparation of competent cells and transformation

Fresh chemically competent *E. coli* cells were prepared from a culture grown in 250 ml SOB medium (1 L Erlenmeyer flask) at 18-23 °C. This culture was inoculated with several colonies from a fresh LB plate. After reaching an OD_{600} of 0.6, the culture was stored on ice for 10 min and then centrifuged for 10 min at 2500 *g*. The pellet was resuspended in 80 ml ice-cold TB buffer (10 mM Pipes buffer, 15 mM $CaCl_2 \cdot 2H_2O$, 0.25 M KCl, adjusted to pH 6.8 with KOH, 55 mM $MnCl_2 \cdot 2H_2O$, sterile filtrated) and incubated on ice for 10 min. The culture was centrifuged again for 10 min at 2500 *g* and the pellet was resuspended gently in 20 ml TB buffer with DMSO added to a final concentration of 7 % (v/v). Subsequently, the cells were gently mixed by swirling and incubated on ice for 10 min. Aliquots of 0.2 ml were transferred into cooled Eppendorf cups, frozen in liquid nitrogen and stored at -70 °C. For transformation, the competent *E. coli* cells were thawed on ice and 50 µl were incubated on ice with 10 µl of the ligation mixture or with 1 µl plasmid DNA for 0.5 h. Then the cells were heat-shocked for 90 sec at 42 °C, mixed with 0.5 ml SOC medium and incubated for 1 h at 37 °C. Aliquots of the cell suspension were then spread on agar plates containing the appropriate antibiotics.

The transformation of competent *C. glutamicum* cells by electroporation was carried out as described by van der Rest *et al.* (1999). For transformation with replicative plasmids, the competent cells were prepared as follows: First the cells were grown in 20 ml LB medium (containing 2 % glucose) overnight at 30 °C. Then 100 ml of Epo-medium (LB medium with 4 g/l isonicotinic acid hydrazide (isoniazid), 25 g/l glycine, 1 % (v/v) Tween 80, sterile filtrated) was inoculated with the preculture to an OD_{60} of 0.3. The cells were incubated for 28 h at 18 °C and afterwards kept on ice for 30 min with gentle shaking every 5 to 10 min. After centrifugation for 10 min at 5200 *g* and 4 °C, the cells were resuspended in 40 ml sterile, ice-cold 10 % (v/v) glycerol. This washing procedure was repeated three more times. Finally, the cells were resuspended in 2 ml sterile, ice-cold 10 % (v/v) glycerol and aliquots of 100 µl were frozen in liquid nitrogen and stored at -70 °C. For electroporation, 100 µl of these competent cells were thawed on ice and mixed with 1-2 µl plasmid DNA (100-250 ng DNA for replicative plasmids, 1-2 µg DNA for suicide plasmids). Then the cells were transformed into an ice-cold sterile electroporation cuvet (electrode distance 0.2 cm) and subjected to an

electrical impulse. The parameters were adjusted as follows: voltage, 2.5 kV; capacity, 25 µF; resistance, 600 Ω. The obtained time constant should be 10-12 msec. Immediately after the pulse 0.5 ml BHIS medium was added to the sample and mixed carefully. After transfer to a sterile 2-ml Eppendorf cup the cells were subjected to a heat shock at 46 °C for 6 min. Afterwards the cells were gently shaken 1 h at 30 °C for regeneration and spread on selective BHIS agar plates.

5.4 Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify DNA fragments *in vitro* (Mullis und Faloona, 1987) and to control chromosomal deletions. All oligonucleotides that were used as primers are listed in Tab. 6. For analytical PCR *Taq*-DNA-Polymerase (Qiagen or Sigma, Deisenhofen) was used. The amplification of DNA fragments that were subsequently cloned into plasmids was carried out with the Expand high fidelity PCR system (Roche Diagnostics), which uses a combination of *Taq*-DNA polymerase and the proofreading *Pwo*-DNA polymerase (Barnes, 1994).

PCR was performed in a Primus 25 thermocycler (MWG Biotech). The standard reaction mixture (100 µl) consisted of 0.5 µM of each primer, 1 µg genomic DNA (template), 200 µM dATP, dGTP, dCTP and dTTP and 2.5 U *Taq*-DNA polymerase. An initial denaturation step for 5 min at 95 °C was followed by 25 to 35 cycles of denaturation (30 s at 95 °C,), primer annealing (30 s at annealing temperature T_A) and primer elongation (t_E s at 72 °C). The annealing temperature T_A was in general 4 °C lower than the lowest predicted melting temperature T_M of the primers. The calculation of T_M was based on the nucleotide composition of the primers: T_M (°C) = 4·(G + C) + 2·(A + T). The elongation time t_E was determined according to the size of the amplified DNA fragment. Routinely, an elongation time of 60 s was applied for the amplification of 1000 bp. When using the Expand High Fidelity kit the reaction conditions suggested by the supplier were also considered.

In order to control the *C. glutamicum* deletion mutants, one colony was resuspended in 100 µl water, heated 10 min at 95 °C, mixed and cooled on ice. Subsequently, 5 µl of this suspension was used as template in a 20 µl PCR assay. 10 µl of the PCR mixture was then analysed by agarose gel electrophoresis. Amplified DNA fragments from PCR reactions were purified using the QIAquick spin PCR purification kit (Qiagen). Alternatively, purification was achieved through agarose gel electrophoresis followed by gel extraction. DNA fragments

were purified from agarose gels either with the QIAEX II Gel Extraction kit (Qiagen) or the QIAquick Gel Extraction kit (Qiagen).

5.5 DNA sequence analysis

DNA was sequenced according to the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The sequence reaction was performed with DYEnamic Direct Cycle Sequencing Kit (Amersham Pharmacia Biotech, Freiburg, Germany) using the 5`-end fluorescently IRD800 labelled oligonucleotides (Tab. 6). Afterwards, the fluorescently labelled sequencing products could be detected with LiCor DNA sequencer 4200 from MWG Biotech. For the sequencing, the following DNA-primer premix was prepared:

DNA-primer premix: x µl DNA (130-160 ng per kb DNA template, max 16 µl DNA template)
1 µl labelled oligonucleotide (2 pmol)
(17 - x) µl distilled water

The DNA-primer premix was divided into 4 PCR-reaction tubes each 4 µl of DNA-primer premix. Afterwards into each PCR-reaction tube 1 µl one of ddATP-, ddCTP-, ddGTP- or ddTTP- was added. The samples were processed according to following scheme in termocycler:

The reaction conditions (example):

DNA denaturation	3 min	95 °C	
DNA denaturation	30 sec	95 °C	30 x
primer annealing	30 sec	57 °C	
primer elongation	1.5 min	70 °C	

The annealing temperature T_A was in general 4 °C lower than the lowest predicted melting temperature T_M of the primers (see 5.4). After the sequencing reaction was stopped by addition of 2 ml formamide loading dye (Sequencing Kit) and the samples were stored at -20 °C in dark. Shortly before the loading samples on sequencing gel, these were heated 5-10 min at 70 °C (denaturation) and put on ice. Subsequently, 1-1.5 µl was loaded onto a denaturing 4.6 % (w/v) Long Ranger (Biozym, Hamburg, Germany) sequencing gel and separated in a

Long Read IR DNA sequencer 4200 (Licor Inc., Lincoln, USA). The sequencing gel with separation length 66 cm was prepared from following solutions:

4.6 ml	"Long Ranger"–acrylamide-bisacrylamide solution (50 %, Biozym)
21 g	urea (Sigma, Deisenhofen)
5 ml	10 x TBE (890 mM Tris base, 890 mM boric acid, 20 mM EDTA, final pH 8.3)
32 ml	bidestilled water
500 µl	DMSO
50 µl	TEMED
350 µl	10 % (w/v) ammonium peroxodisulphate (APS)

The electrophoresis conditions were as follows:

voltage	2200 V
current	37 mA
power	50 W
temperature	45 °C

Alternatively, sequence analysis was performed by AGOWA GmbH. Computer-assisted DNA and protein sequence analyses, e.g. the search for restriction sites or open reading frames, was performed by using the software Clone Manager for Windows (Version 5.02, Scientific & Educational Software). Comparison of DNA and protein sequences or data bank searches to identify sequence homologues were performed with the programs BLASTX, BLASTN and BLASTP (Altschul *et al*., 1997) at the website of the National Center for Biotechnology Information (NCBI, Washington, USA) and with the bioinformatics suite ERGO (Integrated Genomics, Inc., Chicago, U.S.A.). Multiple sequence alignments were done with the program ClustalW 1.8 (Thompson *et al*., 1994).

6 Molecular biological techniques involving RNA

Since RNases are very stable and active enzymes, all the lab material and solutions used for RNA purification and analysis had to be treated prior to use in order to eliminate possible RNase contaminations. For this purpose, they were autoclaved twice. Materials and solutions for RNA isolation obtained from commercial suppliers were RNase-free already.

6.1 Isolation of total RNA

RNA was isolated with the RNeasy kit (Qiagen). 20 ml of a *C. glutamicum* culture grown to mid-log phase (OD_{600} = 4 - 5) was poured into ice-loaded tubes (50 ml falcon tube 9 g ice per 20 ml culture) pre-cooled to -20 °C and centrifuged (5 min, 3500 *g*, 4 °C). The cell pellet was either directly subjected to RNA isolation or immediately frozen in liquid nitrogen and stored at -70 °C until use. For isolation of total RNA, the (frozen) cell pellet was resuspended in 350 µl of RLT buffer of the RNeasy system (Qiagen). Afterwards, 250 mg of 0.1 mm zirconia/silica beads (Roth, Karlsruhe, Germany) were added and cells were disrupted by bead-beating for 15 sec and 30 sec using a Silamat S5 (Vivadent, Ellwangen, Germany). After centrifugation (1 min, 13 000 *g*), the supernatant was used for RNA preparation 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. Formaldehyde agarose gel in total volume of 45 ml consists of 0.8 – 2 % agarose, 31.5 ml RNase free water, 5 ml 10 x formaldehyde gel buffer (0.2 M MOPS, 0.05 M natrium acetate, 0.01M EDTA, pH 7.0 adjusted with NaOH, sterilization with autoclave) and 8.5 ml formaldehyde (added after temperature of gel-mix below 50 °C). The samples were run in 1 x formaldehyde gel buffer. (Polen *et al*., 2003). The concentration of RNA was determined by measuring the extinction at 260 nm: E_{260} = 1 corresponds to 40 µg RNA/ml. RNA samples were kept at -70 °C until use.

6.2 DNA chip technology

The *C. glutamicum* whole-genome DNA microarrays used in this work were prepared in the group of Dr. V. Wendisch. They contain 3673 PCR products (size approx. 500 bp) covering 2860 of the 2994 genes (506 in duplicate) described for the genome according to NCBI NC003450 and 284 further putative coding sequences (23 in duplicate). Additionally, 100 spots of *C. glutamicum* genomic DNA are present as normalization controls and 16 spots of phage λ DNA, 16 spots of *E. coli* genomic DNA and 1 spot of the *E. coli aceK* gene as negative controls (Polen and Wendisch, 2004). These DNA samples were spotted by a robotic system onto defined positions of poly-L-lysine-coated glass microscope slides. In order to saturate free ε-amino groups of poly-L-lysine and to immobilize and denature the DNA probes, the DNA chips were treated chemically and thermally before hybridisation with fluorescently labelled cDNA. In a first step, the DNA on the slides was rehydrated in a humid chamber over a 1 x SSC solution and subsequently dried very fast on a heating plate at 100 °C

(2-3 sec). Then the DNA was linked covalently to poly-L-lysine by exposure to UV light (650 µJ, Stratalinker, Stratagene, La Jolla, USA). The free e-amino groups were subsequently blocked with 180 mM succinic anhydride in order to eliminate unspecific binding of the fluorescently labelled cDNA probes (Shalon et al., 1996). For denaturation of the double-stranded DNA, the DNA chips were incubated for 1.5 min in water of 95 °C and the single-stranded DNA was fixed by incubation the chips in cold ethanol. After drying of the chips by centrifugation (5 min, 50 *g*) they were stored until use in an exsiccator.

Identical amounts (20-25 µg) of total RNA from the two cultures, whose transcriptomes should be compared, were used for random hexamer-primed synthesis of fluorescently labelled cDNA by reverse transcription with Superscript II (GibcoBRL/ Life Technologies, Gaithersburg, USA) using the fluorescent nucleotide analogues FluoroLink Cy3-dUTP ($\lambda_{absorption\ max}$ 550 nm, $\lambda_{fluorescence\ max}$ 570 nm, green, Amersham Pharmacia Biotech, Freiburg) or Cy5-dUTP ($\lambda_{absorption\ max}$ 649 nm, $\lambda_{fluorescence\ max}$ 670 nm, red, Amersham Pharmacia Biotech, Freiburg) (Wendisch *et al.*, 2001). In total volume of 15 µl was 20-25 µg of each RNA annealed to 500 ng of random hexanucleotide primers (Amersham Pharmacia Biotech, Freiburg). The RNA-primer mix was incubated at 65 °C for 10 min and transferred to ice for 2 min. The reaction mixtures (30 µl) consist of 3 µl 1mM Cy3-dUTP (culture 1) or 3 µl 1 mM Cy5-dUTP (the culture 2), 6 µl 1.Strang buffer (final conc. 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 0.6 µl nucleotides mix dNTPs (final conc. 500 µM dATP, dGTP, dCTP, 200 µM dTTP, Invitrogen) and 2 µl Superscript II reverse transcriptase (final conc. 400 U) (Life Technologies, Inc., Rockville, MD). Subsequently were these mixtures incubated for 10 min at room temperature and then transferred to a 42 °C water bath for 110 min for cDNA synthesis. Reactions were stopped by adding 1o µl 0.1 N NaOH and incubating for 10 min at 70 °C to hydrolyse RNA. Reaction mixtures were neutralized with 10 µl 0.1 N HCl. To remove not inbuilt nucleotides, the mixtures (50 µl) were diluted by adding 500 µl H_2O and the labelled cDNA probes were purified and concentrated using Microcon YM-30 filter units (Millipore, Schwalbach) (Khodursky *et al.*, 2003). The Cy3-labelled and Cy5-labelled cDNAs were mixed together, again filled up to 500 µl with H_2O and concentrated to volume of 14.5 µl. Afterwards, 1.2 µg/µl polyA (Sigma, Deisenhofen) or 1.2 µg/µl poly-(dI-dC) was added as competitor in order reduce unspecific background fluorescense. To the probe was added 3 µl 20 x SSC with 0.48 µl HEPES (1 M, pH 7) in order to assure stringent hybridisation conditions and 0.45 µl 10% (v/v) SDS was added to reduce the surface tension (Zimmer *et al.*, 2000).After DNA denaturation for 2 min at 100 °C and

cooling for 5-10 min at room temperature, the probe was added to the DNA chip below a special cover slip (LifterSlip, Erie Scientific, New Hampshire, USA). In order to avoid drying of sample solution during hybridisation at 65 °C, two lines of droplets of 3 x SSC solution were applied at each edge of DNA chip plate. The chip was then incubated in hybridisation chamber (Die Tech Inc., York Haven, USA) for 5-16 h at 65 °C in water bath (Zimmer *et al*., 2000). To remove the sample solution and unspecific bound fluorescently labelled DNA after hybridization, the chips were washed at room temperature for 5 min in 1 x SSC with 0.03% (w/v) SDS and afterwards in 0.05 x SSC (Zimmer *et al*., 2000). Immediately after stringent washing the chip was dried by centrifugation (5 min, 50 *g*) and the fluorescence intensities of Cy3 at 570 nm (excitation with monochromatic light of 532 nm) and of Cy5 at 670 nm (excitation with monochromatic light of 635 nm) were measured using a GenePix 4000 laser scanner (Axon Inc., Union City, USA) and processed as TIFF-images. Each spot of the measurement corresponded to an area of 10 x 10 µm on the chip. Raw fluorescence data were analysed quantitatively using GenePix Pro 3.0 Software (Axon Inc., USA). A normalization factor was calculated which sets the average fluorescence ratio of the 100 spots with *C. glutamicum* genomic DNA to one (e.g. if the measured ratio of Cy3/Cy5 fluorescence of these 100 spots was 1.2, the normalization factor would be 1.2). The ratio determined for all other spots was divided by this factor and thus normalized to *C. glutamicum* genomic DNA. The normalised ratio of median was taken to reflect the mRNA ratio of the two samples for those spots whose green or red fluorescence signal was at least threefold above the median fluorescence background. For statistical analysis of the gene expression data (Hommais *et al*., 2001), p-values for the independent replicate experiments were calculated based on the Student's *t*-test (heterscedastic *t*-test provided by the Microsoft Excel software) by using log-transformed fluorescence ratios for individual genes on the one hand and for genomic DNA on the other hand (Polen *et al*., 2003). Of the genes showing significantly changed RNA levels (p-values < 0.05) those genes with at least two-fold increased or decreased average RNA ratios were considered further.

6.3 Primer extension analyses

Non-radioactive primer extension analyses were performed using IRD800-labelled oligonucleotides (Tab. 6). 20 µg of total RNA were combined with 2 pmol labelled oligonucleotide and 2 µl of 5 x annealing buffer (50 mM Tris-HCl pH 7.9, 1.25 M KCl) in a total volume of 10 µl. The reaction was heated to 65 °C for 5 min and then slowly (0.5 °C/2 min) cooled to 42 °C in a thermocycler. In order to perform reverse transcription, 23 µl water

were combined with 10 µl 5 x reverse transcription buffer (250 mM Tris-HCl pH 8.3, 125 mM KCl, 15 mM $MgCl_2$), 5 µl 100 mM DTT, 1 µl deoxyribonucleotides (25 mM dATP, dCTP, dGTP and dTTP), 0.5 µl actinomycin D (5 mg/ml in ethanol) and 0.5 µl SuperScript II RNase H^- Reverse Transcriptase (200 units/µl; Life Technologies, Bethesda, U.S.A.) in that order and the entire mix was added to the RNA immediately after the annealing step. After incubation for one hour at 42 °C, the reaction was stopped by adding 120 µl RNase A reaction mix (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 100 µg/ml sonicated salmon sperm DNA, 200 µg/ml RNase A, added freshly), incubated for one hour at 37 °C and the DNA precipitated overnight after adding 17 µl 3 M sodium acetate (pH 5.2) and 380 µl ice-cold ethanol. Subsequently, the samples were centrifuged (20 min at 15 000 *g*, 4 °C). After washing the pellet with 500 µl 70% (v/v) ethanol and air-drying, the DNA was dissolved in 2 µl of water and 2 µl formamide loading dye (Epicentre Technologies, Madison, USA). 1 µl was then loaded onto a denaturing 4.3 % (w/v) Long Ranger (Biozym, Hamburg, Germany) sequencing gel and separated in a Long Read IR DNA sequencer 4200 (Licor Inc., Lincoln, USA). The sequencing gel with separation length 42 cm was prepared from following solutions:

2.85 ml	"Long Ranger"–acrylamide-bisacrylamide solution (50 %, Biozym)
13 g	urea (Sigma, Deisenhofen)
3.1 ml	10 x TBE (890 mM Tris base, 890 mM boric acid, 20 mM EDTA, final pH 8.3)
19.9 ml	bidistilled water
310 µl	DMSO
31 µl	TEMED
217 µl	10 % (w/v) ammonium peroxodisulphate (APS)

The electrophoresis conditions were as follows:

voltage	1500 V
current	31.5 mA
power	35 W
temperature	50 °C

The length of the primer extension products was determined by running the four lanes of a sequencing reaction set up using the same oligonucleotide as for reverse transcription

alongside the primer extension products. Except for the phoR gene, the transcriptional start of all genes analysed by primer extension was determined using two different oligonucleotides (pstS_prext1 and pstS _prext2 for *pstS*, ugpA_prext2 and ugpA _prext3 for *ugpA* and phoR_prext1b for *phoR*, see Tab. 6). The corresponding sequencing reactions were generated by using the same IRD-800 labelled oligonucleotide as in the primer extension reactions as well as PCR products, which cover the region of the respective transcriptional start site, as template DNA. Oligonucleotides used for preparation of the template PCR products were phoR_promreg_fw and phoR_promreg_rv for *phoR*, pstS_promreg_fw and pstS_promreg_rv for *pstS*, ugpA_promreg_fw and ugpA_promreg_rv for *ugpA* (for sequences see Table 6). The PCR product of *phoR* promoter region (P_{phoR}) starts 108 bp upstream of ATG start codon and ends 208 bp downstream from 1st nucleotide of the start codon. The *pstS* PCR product starts 1171 bp upstream of GTG start codon of *pstS* and ends 207 bp downstream from 1st nucleotide of the start codon. The *ugpA* PCR product starts 199 bp upstream of GTG start codon of *ugpA* and ends 208 bp downstream from 1st nucleotide of the start codon.

7 Biochemical methods

7.1 Protein purification

7.1.1 Purification of $PhoS_{His}$

The purification of the integral membrane protein $PhoS_{His}$ was also carried out *via* Ni^{2+} chelate affinity chromatography. A 2-l culture of *E. coli* C43(DE3) containing the expression plasmid pET24b-*phoS* was first grown in LB medium with kanamycin (50 µg/ml) at 37 °C and 130 rpm until the OD_{600} reached a value between 0.3 and 0.5. Then, IPTG was added to a final concentration of 1 mM and incubation was continued for another 4 h at room temperature. Subsequently, the cells were harvested by centrifugation, washed once in 50 ml of buffer $TNI5^+$ and stored at -20 °C. To purify $PhoS_{His}$, the cells were resuspended in the $TNI5^+$ buffer (4 ml/g cell wet weight) containing two protease inhibitors, diisopropyl fluorophosphate (1 mM) and phenylmethylsulfonylfluorid (1 mM), and 0.2 mg DNase I/ml. Then the cells were lysed by three passages through a French pressure cell at 207 Mpa. Intact cells and cell debris were removed by centrifugation (30 min at 27 000 *g*, 4 ^{o}C). The resulting cell-free extract was subjected to ultracentrifugation for 90 min at 120 000 *g* and 4 ^{o}C thereby separating the membrane fraction from the soluble cell fraction. The pellet containing the membrane fraction was resuspended in approx. 4 ml $TNI5^+$ buffer and ultracentrifuged again.

After this washing step, the membrane pellet was resuspended in a small volume of $TNI5^+$ buffer supplemented with 10% glycerol. After determination of the protein concentration, an aliquot containing 50 mg of protein was used for further purification and the rest was stored -20 °C. All further steps were performed at 4 °C. The membrane fraction (50 mg protein) was mixed with the detergent lauryldimethylamine oxide (LDAO, Calbiochem-Novabiochem Corporation) at a final concentration of 2% (v/v) and gently stirred for 45 min in an ice bath. After ultracentrifugation for 20 min at 150 000 *g*, the supernatant containing the LDAO-solubilized proteins was run by gravity through a Ni^{2+}-NTA-agarose Superflow column (3 ml bed volume, Qiagen) equilibrated with buffer $TNI5^+$ plus 0.1% (v/v) LDAO. Subsequently, the column was washed with three volumes of buffer $TNI20^+$ plus 0.1% (v/v) LDAO followed by three volumes of buffer $TNI50^+$ plus 0.1% (v/v) LDAO. Then, the $PhoS_{His}$ protein was eluted with buffer $TNI100^+$ plus 0.1% (v/v) LDAO. Purification was monitored by SDS-PAGE. A buffer exchange was performed by gel filtration (PD-10 columns; Pharmacia) and the purified protein was stored in TKMD buffer plus 0.1% (v/v) LDAO at 4 °C. Due to the fact that the LDAO detergent in the buffer disturbed the routine methods for the determination of protein concentrations (see below), the protein of these samples was precipitated before concentration determination. The protein sample and the standard were first diluted with water to final volume of 1 ml. After addition of 0.1 ml 0.15% (w/v) sodium deoxycholate the mixture was allowed to stand for 5 min at room temperature. For precipitation, 0.1 ml 70 % (w/v) TCA (trichloroacetic acid) was added and samples were centrifuged for 5 min at 9 000 *g*. The supernatant was carefully removed and the pellet was resuspended in 50 µl 0.1 N NaOH. Finally, protein concentration was determined using BCA kit (see below).

7.1.2 Purification of $PhoR_{His}$ and its C-terminal domain

E. coli BL21(DE3) containing the expression plasmid pET24b-*phoR* was grown in LB medium with kanamycin (50 µg/ml) and *E. coli* BL21(DE3) containing either plasmid pET16b-*phoRΔ1-125* or pET16b-*phoRΔ1-139* was grown in the same medium with ampicillin (100 µg/ml). 2 l cultures in 5-l Erlenmeyer flasks (without baffles) were incubated at 37 °C and 130 rpm until the OD_{600} reached a value between 0.3 and 0.5. Then, synthesis of the PhoR proteins was induced by the addition of 1 mM IPTG (isopropylthio-β-D-galactoside) and the cultures were incubated for another 3-5 h at room temperature. Cells were harvested by centrifugation, washed once in 50 ml of buffer $TNI5^+$ (buffer $TNI5^+$ consists of 20 mM Tris-HCl, pH 7.9, 300 mM NaCl, 5% glycerol (w/v) and 5 mM imidazole (the number indicates the millimolar imidazole concentration)) and stored at -20 °C. To monitor the

production of the recombinant protein, 1-ml culture samples were taken prior and after induction by IPTG. These samples were centrifuged (5 min 15 000 *g*) and the cells resuspended in OD_{600} x 100 µl 1 x SDS loading buffer for the analysis by SDS-PAGE (see below). For the disruption of the cells from the 2-l culture, they were resuspended in the $TNI5^+$ buffer (4 ml/g cell wet weight). After addition of two protease inhibitors, diisopropyl fluorophosphate (1 mM) and phenylmethylsulfonylfluorid (1 mM), and of 0.2 mg DNase I/ml, the cells were disrupted by three passages through a French pressure cell (207 Mpa, SLM AMINCO® Spectronic Instruments, Rochester, U.S.A.). Intact cells and cell debris were removed by centrifugation (30 min at 27 000 *g*, 4 °C). The cell-free extract was then ultracentrifuged (60 min at 150 000 *g*, 4 °C). Aliquots of all fractions were removed for analysis by SDS-PAGE. After filtration through a 0.2 µm filter, the soluble cell fraction (supernatant of the ultracentrifugation step) was loaded onto a Ni^{2+}-nitrilotriacetic acid (NTA)-agarose column containing 2 ml bed volume of His-bind resin (Novagen) for $PhoR_{His}$ or 3 ml bed volume of Ni-NTA resin (Qiagen) for PhoRΔ1-125 and PhoRΔ1-139 equilibrated with buffer $TNI5^+$. Subsequently, the column was washed with 20 ml of buffer $TNI5^+$ followed by 6 ml of buffer $TNI50^+$. Then, the $PhoR_{His}$ protein was eluted with 8 ml of buffer $TNI100^+$. In the case of PhoRΔ1-125 and PhoRΔ1-139 the column was washed with 6 ml of buffer $TNI100^+$ and then the protein was eluted with 8 ml of buffer $TNI200^+$. A buffer exchange was performed by gel filtration with PD-10 columns (Pharmacia) and the purified proteins were stored in TKMD buffer at 4 °C (Meyer *et al.*, 1997). With increasing age the DNA-binding efficiency of the PhoRΔ1-125 decreased. Therefore the protein was kept for maximally 3 day at 4°C when using it for DNA-binding studies.

7.2 Protein concentration determination

Protein concentrations were routinely determined with the Bradford protein assay (BioRad Laboratories) using bovine serum albumin as standard. A semi-quantitative assay to roughly determine the protein content of fractions after column chromatography was performed by mixing 50 µl of the individual fractions with 250 µl of Bradford reagent (BioRad Laboratories) and inspection of the colour. Alternatively to the Bradford assay, the BCA (*bic*inchonic *a*cid) protein assay (BCA-Test, Smith *et al.*, 1985) was applied as described by the manufacturer (Pierce).

7.3 SDS polyacrylamide gel electrophoresis

SDS-PAGE was carried out essentially as described by Laemmli (1970) using the MiniProtean-II equipment from BioRad. For native PAGE, the same solutions were used except that SDS was omitted from the gel and electrophoresis buffers and from the sample loading buffer. In general 12 % or 15 % separating gels and 4 % or 6 % collecting gels were used. The samples were mixed with 2× SDS loading buffer (117 mM Tris/HCl, pH 6.8, 3.3 % (w/v) SDS, 2 % (v/v) β-mercaptoethanol, 10 % (w/v) glycerol, 0.01 % (w/v) bromphenolblue; composition according to an Amersham Pharmacia Biotech protocol) and denatured at 95 °C for 5-10 min. As protein standards the "Perfect Protein Marker" (Novagen) or the "Broad Range Protein Marker" (New England Biolabs) were used. Electrophoresis was performed at a constant voltage of 200 V for approx. 45 min at room temperature. Coomassie staining was routinely used to visualize the proteins after gel electrophoresis. The gel was washed twice in demineralized water and then stained in GelCode Blue Stain Reagent (Pierce).

8 *In vitro* phosphorylation studies

8.1 *In vitro* phosphorylation of $PhoS_{His}$ and phosphotransfer to $PhoR_{His}$

In vitro phosphorylation of $PhoS_{His}$ was performed with the purified protein at a final concentration of 2.9 μM. First the protein was incubated at room temperature in TKMD buffer (buffer TKMD consists of 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol (DTT)) and with final concentration 0.73 μM γ-[^{32}P]-ATP (3000 Ci/mmol, 10 mCi/ml, Amersham Bioscience, Freiburg). After 1 and 30 min 5 μl aliquots were removed, mixed with an equal volume of 2 x SDS loading buffer and kept on ice until all samples had been processed. Subsequently, without prior heating, the samples were subject to SDS-PAGE (15% separating gel) for 45 min at 200 V. The short run time allowed to keep the radioactive front (containing γ-[^{32}P]-ATP) within the gel. The gel with the radioactive front cut off was then transferred to a Whatman 3MM paper, covered with Saran wrap and dried for approximately 45 min on a vacuum gel dryer (60 °C). Finally, the gel was analyzed with a PhosphoImager (Fuji BAS 1800) and with the software program Aida 2.11 (Raytest Straubenhardt).

The phosphotransfer from $PhoS_{His}$ to $PhoR_{His}$ was monitored with the use of radioactively labelled γ-[^{32}P]-ATP. $PhoS_{His}$ (2.7 μM final concentration) was incubated at

room temperature in TKMD buffer in the presence of γ-[^{32}P]-ATP (0.67 µM final concentration, 3000 Ci/mmol, 10 mCi/ml, Amersham Bioscience, Freiburg), as described above for the phosphorylation of $PhoS_{His}$. After 31 min purified $PhoR_{His}$ (8.2 µM final concentration) was added to the mixture of $PhoS_{His}$ and γ-[^{32}P]-ATP. In distinct time intervals, 5 µl aliquots were removed, mixed with 5 µl 2 x SDS loading buffer and stored on ice. The probes were then subject to SDS-PAGE (15% separating gel) at 200 V for 45 min. The gel was dried and analyzed with a PhosphoImager (Fuji BAS 1800). Additionally, the gel was also stained with Coomassie blue (GelCode Blue Stain Reagent (Pierce)).

9 DNA-protein interaction studies

9.1 Gel retardation assays

For the gel retardation assays with $PhoR_{His}$, PhoRΔ1-125 and PhoRΔ1-139 the promoter regions upstream of the operons *pstSCAB* (1383 bp), *ugpAEBC* (407 bp), *phoRS* (317 bp) and *pitA* (565 bps) were amplified by PCR from chromosomal DNA of *C. glutamicum* ATCC13032 using the primer pairs pstS_promreg_fw/pstS_promreg_rv, ugpA_promreg_fw/ ugpA_promreg_rv, phoR_promreg_fw/phoR_promreg_rv, and pitA_promreg_fw/ pitA_promreg_rv, respectively. The promoter regions that were used as a negative control were *clpC* (300 bps) and *clpP1P2* (548 bps) and they were amplified by PCR also from chromosomal DNA of *C. glutamicum* ATCC 13032 using primer pair PclpC-cat-1/SC-clpC-rv2 for *clpC* promoter region and clpP-activator-fw/clpP-BS2-rv for *clpp1p2* promoter region. Subsequently, the different PCR products were purified from agarose gel and then radioactively labelled using the protocol suggested by enzyme and buffers supplier USB Corporation, Cleveland, USA.

5`-end labeling protocol:

x µl (1pmol)	DNA - purified PCR product
5 µl	T4 PNK buffer (10 x) (T4 PNK buffer: 0.5 M Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 100 mM 2-mercaptoethanol, USB Corporation, Cleveland, USA)
0.6 µl (2 pmol)	[γ-^{32}P]-ATP (10 mCi/ml, Amersham Pharmacia Biotech, Freiburg)
(20 - x) µl	distilled water
3 µl	T4 polynucleotide kinase (PNK) diluted 1:10 (to 3U/µl)with T4 PNK

dilution buffer (50 mM Tris-HCl, pH 8)

The mixture was incubated 30 min at 37 °C. Unconsumed [γ-^{32}P]-ATP was removed using the QIAquick-spin PCR purification kit (Qiagen) and DNA was eluted with 40 µl distilled H_2O. This 40 µl solution of labelled DNA was calculated for 20 protein-DNA binding reactions. The protein-DNA binding reaction were prepared in 5 x BS buffer (1 x BS buffer consists of 50 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA and 10 % glycerol). They contained in a total volume of 20 µl (calculated for 10 reactions) 2 µg poly-(dI-dC) as competitor DNA, 1.6 µl of the labelled DNA fragment (2 nM final concentration) and 4 µl of the different protein dilutions (0 - 19.5 µM final concentrations for $PhoR_{His}$ and 0 - 2.3 µM final concentrations for PhoRΔ1-125 and PhoRΔ1-139). The proteins were diluted in 1 x BS buffer. In the case of $PhoR_{His}$ also the phosphorylated protein was prepared and than added to reaction mixture with DNA. The $PhoR_{His}$ was phosphorylated either with $PhoS_{His}$ plus ATP or using acetylphosphate. The $PhoR_{His}$ phosphorylation mixture with $PhoS_{His}$ plus ATP was prepared in total volume of 20 µl and consists of 5 µl different $PhoR_{His}$ dilutions (0 - 19.5 µM final concentrations), 9 µl $PhoS_{His}$ (final conc. 1.1 µM), 4 µl 1 x BS buffer and 2 µl 50 mM ATP. In the case of phosphorylation with acetylphosphate, the protein was phosphorylated in 20 µl reaction mix consisting of 5 µl of the different $PhoR_{His}$ dilutions (0 - 19.5 µM final concentrations) 10 µl 100mM lithium potassium acetyl phosphate (Sigma, Deisenhofen) (pH 7.5), 4 µl 1 x BS buffer and 1 µl H_2O. The phosphorylation protein mixtures were then incubated for 1 hour at 37 °C before adding into protein-DNA binding reaction mix.

The DNA-protein mixtures were incubated for 30 min at room temperature, then 2 µl of a 0.1 % xylene cyanol solution was added as tracking dye and 10 µl was separated in a 10 % native polyacrylamide gel (acrylamide:bisacrylamide 75:1 (w/w)) with 0.5 x TBE (1 x TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, final pH 8.3) as running buffer. Electrophoresis was carried out at constant voltage of 180 V while the cooling the entire electrophoresis chamber (MiniProtean II, BioRad) in a polystyrene box filled with ice. The gel was then transferred to a Whatman 3MM paper, covered with Saran wrap and dried for approx. 45 min on a vacuum gel dryer (60 °C). Finally, the gel was analysed with a PhosphoImager (Fuji BAS 1800) or with an X-ray film (Fuji).

9.2 DNase I footprint assays

Labelled DNA fragments were obtained by amplification with 5'-end IRD800 labelled oligonucleotides (MWG Biotech, Ebersberg, Germany). The *pstS* regulatory region was amplified with the oligonucleotide pairs pstS_footpr_fw/pstS_footpr_rv_IRD800 (labelled non-template strand) and pstS_footpr_fw_IRD800/pstS_footpr_rv (labelled template strand). These labelled PCR products were purified using Microcon YM-30 filter units (Millipore, Bedford, USA). The PCR mixture (100 µl) was applied on Microcon YM-30 and centrifuged 5 min 14 000 *g*. Subsequently, samples were washed with 500 µl H_2O and centrifuged at 14 000 *g* until the volume was approx. 8 µl. Finally, the Microcon YM-30 was inverted and DNA solution was transferred into eppendorf tube by centrifugation (2 min, 15 000 *g*). The DNA purity was controlled by agarose gel electrophoresis.

The binding reactions were prepared in two types of binding buffers: 1) in 1 x BS buffer (the same which was used for the gel retardation assay), in this case the salt solution (100 mM $MgCl_2$, 50 mM $CaCl_2$) was added after incubation of protein with DNA or 2) in "footprint" 5 x binding buffer (100 mM Tris-HCl pH 7.5, 5 mM EDTA, 50% (v/v) glycerol, 5 mM DTT, 0.05% (v/v) Triton X-100 and 500 mM NaCl), in this case the salt solution was added before incubation of protein with DNA. In a total volume of 200 µl 1.5-3 nM of labelled fragments were combined with 0.5 µl poly-[d(IC)] (1 µg/µl) and different amounts of purified PhoRΔ1-125 (0 – 2.3 µM, final concentrations). Then the binding reactions were incubated for 30 minutes at RT. Subsequently, 10 µl salt solution (added only to binding reaction with 1 x BS buffer) and afterwards 5 µl 0.125 µg/ml DNase I in DNase I buffer (10 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 50 mM KCl and 1 mM DTT) was added and digestion allowed to proceed for exactly two minutes. The reaction was terminated by the addition of 700 µl ice-cold stop solution (645 µl 96 % Ethanol (v/v), 5 µl salmon sperm DNA (4 µg/µl), 50 µl saturated ammonium acetate solution) and the DNA was precipitated overnight at -20 °C. After centrifugation (20 min at 15 000 *g*, 4 ^{o}C), the DNA pellet was washed with 500 µl 70 % (v/v) ethanol, air-dried and dissolved in 2.5 µl water and 2.5 µl formamide loading dye (Epicentre Technologies Corp., Madison, U.S.A.). 1.2 µl was then loaded onto a denaturing 4.6% (w/v) Long Ranger (Biozym, Hamburg, Germany) sequencing gel (separation length 66 cm) and separated in a Long Read IR DNA sequencer (Licor Inc., Lincoln, U.S.A.) (for details see 5.5). The length of the reaction products was determined by running the four lanes of a sequencing reaction set up using the same oligonucleotide as for reverse transcription alongside the primer extension products.

V RESULTS

1 Screening for two-component system involved in the P_i starvation response

When inorganic phosphate (P_i), the usually preferred phosphorus source, becomes limiting, many bacteria (e.g. *Escherichia coli* or *Bacillus subtilis*) stimulate the transcription of genes which allow them to cope with P_i starvation. These genes encode e.g. a high affinity ABC transport system for P_i (Pst system) (Willsky and Malamy, 1976; Willsky and Malamy, 1980), an ABC uptake system for glyceraldehyde-3-phosphate (Ugp system) (Argast and Boos, 1980; Brzoska *et al*., 1994), alkaline phosphatases (Coleman, 1992; Kim and Wyckoff, 1991), which cleave phosphate from organic phosphate esters, or a glycerophosphoryldiester phosphodiesterase (White, 2000). The induction of these genes in *E. coli* is mediated by a two-component regulatory system consisting of the sensor kinase PhoR and the response regulator PhoB. The genes regulated by PhoB form the so-called Pho regulon (Wanner, 1993). Regulation of the phosphate starvation genes by two-component regulatory systems was not only found in *E. coli*, but also in several other bacteria such as *Bacillus* (Hulett *et al*., 1994), *Pseudomonas* (Anba *et al*., 1990), *Agrobacterium* (Charles and Nester, 1993; Mantis and Winans, 1993) or *Synechococcus* (Aiba and Mizuno, 1994; Nagaya *et al*., 1994). The first aim of this work was to investigate, if also in *Corynebacterium glutamicum* a two-component signal transduction system is involved in the induction of phosphate starvation response.

1.1 Growth of *C. glutamicum* wild type and two-component deletion mutants under P_i-limited conditions

C. glutamicum contains 13 two-component systems (TCS), all of which are presumably active in transcriptional regulation of gene expression. However, with one exception, their specific function could not be proposed with the help of bioinformatics. In previous studies it was shown that 12 of the *C. glutamicum* TCS are not essential since mutants could be constructed lacking the corresponding genes (strains Δ*cgtSR1*, Δ*cgtSR2*, Δ*cgtSR3*, Δ*cgtSR5*, Δ*cgtSR6*,Δ*cgtSR7*, Δ*cgtSR8*, Δ*cgtSR9*, Δ*cgtSR10*, Δ*cgtSR11*,Δ*citAB*, and Δ*mtrAB*) according to the method described by Niebisch and Bott (2001). In the case of the CgtSR4 TCS, deletion of the *cgtSR4* genes was only possible in the presence of a plasmid-encoded copy of *cgtR4*

(strain *Cg*Δ*cgtRS4*/pXMJ19CgtR4), indicating that CgtR4 is essential for *C. glutamicum* (Wessel, 2003).

All 13 mutants were tested for their ability to grow in glucose minimal medium with different P_i concentrations. The aim of the experiment was to investigate if one or more two-component deletion mutants are impaired in their ability to grow under phosphate-limited conditions. If such mutants can be found, the corresponding two-component systems are good candidates for being involved in the P_i starvation response. *C. glutamicum* ATCC13032 (wild type) and the 13 deletion mutants were pre-cultured in CGXII glucose medium under P_i-limiting conditions (0.13 mM P_i) for 24 hours and then used to inoculate fresh CGXII medium containing either sufficient (13 mM) or limiting P_i concentrations (0.13 mM) and medium

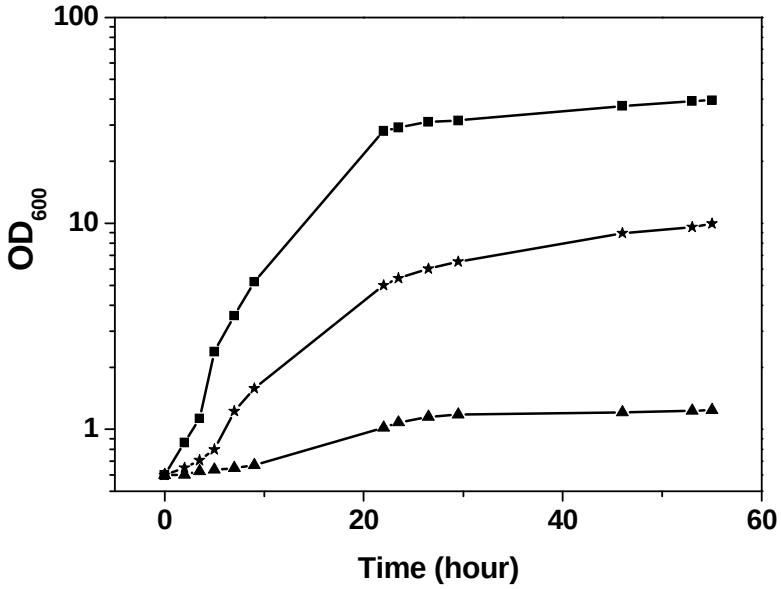


Fig. 9: Growth of *C. glutamicum* wild type in CGXII minimal medium containing 4% (w/v) glucose and either 13 mM P_i (■), 0.13 mM P_i (∗) or no added P_i (▲). The cultures (60 ml in 500 ml-Erlenmyer flask with two baffles) were incubated at 30 °C and 120 rpm. Cells were pre-cultured in CGXII medium with 0.13 mM potassium phosphate for 24 hours before inoculation of the same medium.

without added phosphate. Fig. 9 presents the growth of the wild type. As expected, growth was significantly impaired at decreased phosphate concentrations. Without added phosphate, there was not even one doubling of the cells. The precultivation with 0.13 mM phosphate for 24 hours was necessary in order to reduce the content of P storage compounds (polyphosphate

(reference)) present in *C. glutamicum.* The results shown in Fig. 9 confirm the data from Ishige *et al*. (2003).

In medium without added phosphate, there was only minimal growth of the wild type and the deletion mutants, resulting in less than one doubling. However, there was one interesting observation: the "growth" of all deletion mutants was slightly impaired in comparison with the wild type. Fig. 10 shows as an example the growth of the wild type and of the two deletion mutants Δ*cgtSR7* and Δ*cgtSR8*. This indicates that all mutants have a yet unknown disadvantage compared to the wild type under these conditions.

Under phosphate excess conditions (13 mM P_i), all mutants grew almost like the wild type except for strains Δ*mtrAB* and Δ*cgtSR4*/pXMJ19CgtR4, which grew somewhat slower (Fig. 11C and 11E). The growth impairment of the Δ*mtrAB* mutant was already obvious in the precultivation and the starting OD_{600} of the main culture therefore was lower than for the other strains. It was recently shown by Möker *et al*. (2004) that the *mtrAB* mutant initially grows like the wild type in CGXII medium with 13 mM phosphate, however, due to an acidification of the medium the mutant stops growing much earlier than the wild type, leading to a decreased final density. Concerning strain Δ*cgtSR4*/pXMJ19CgtR4, the growth difference

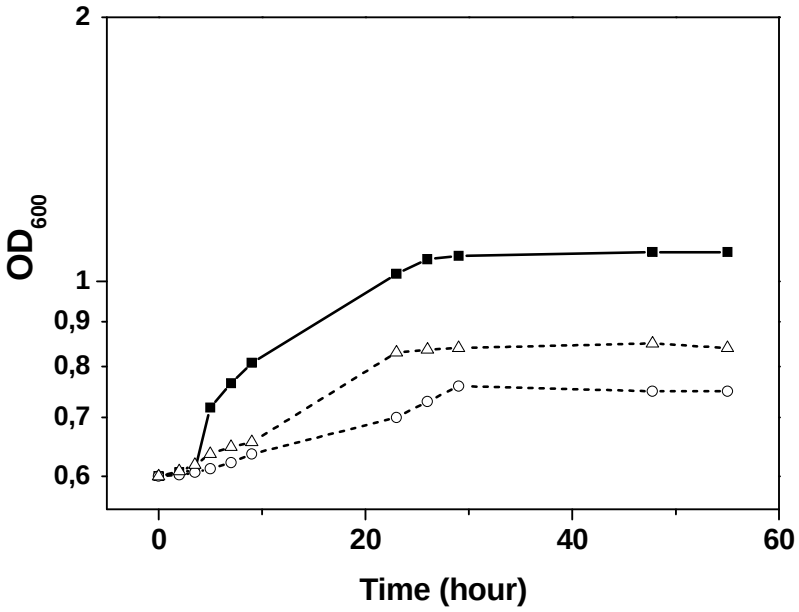
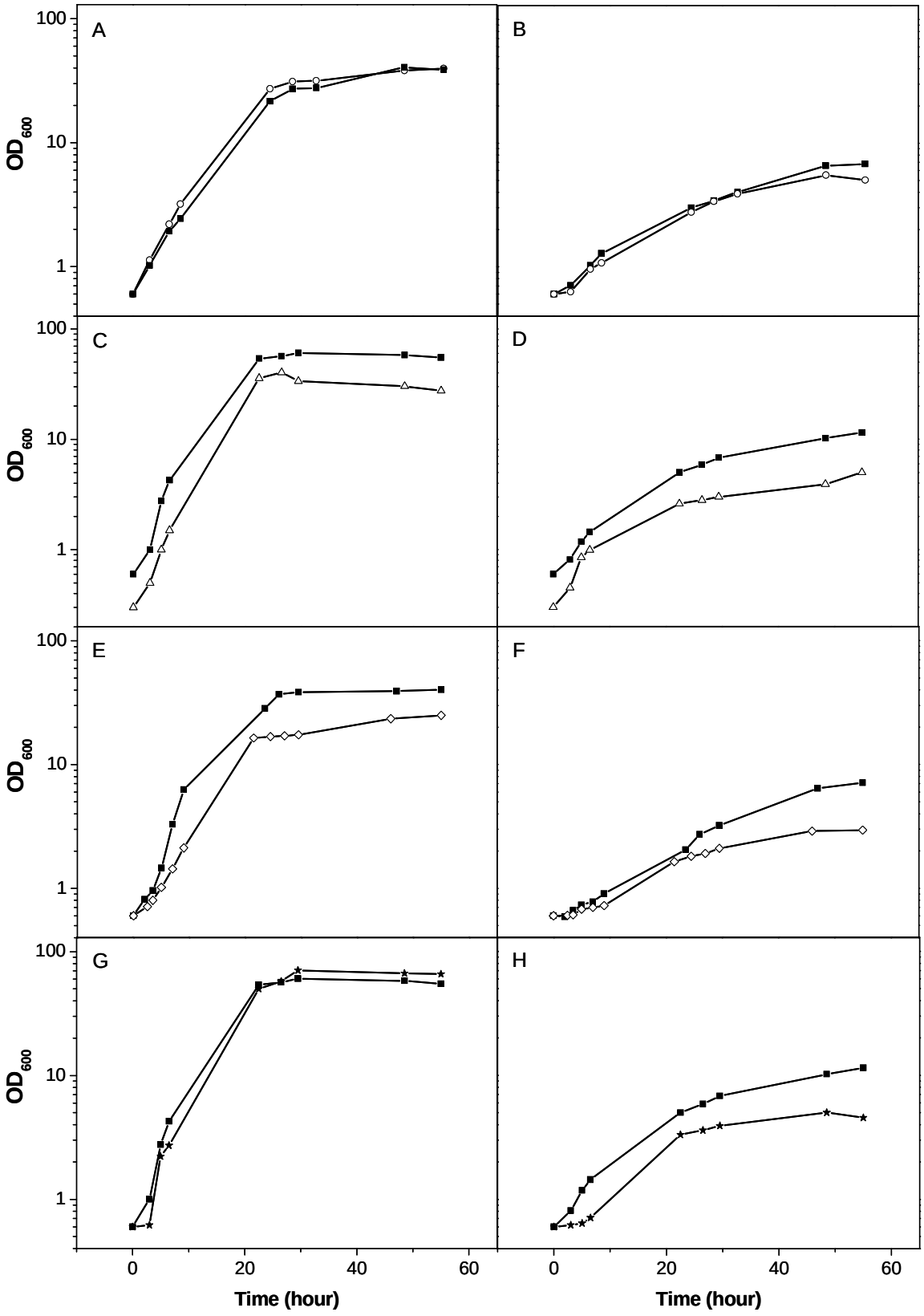


Fig. 10: Growth of *C. glutamicum* wild type (■) and two-component deletion mutantΔ*cgtSR7* (Δ) and Δ*cgtSR8* (○) in CGXII minimal medium with 4% (w/v) glucose and without added P_i at 30 °C and 120 rpm. Cells were pre-cultured in CGXII medium with 0.13 mM potassium phosphate for 24 hours before inoculation of the same medium.

to the wild type was also observed previously by Wessel (2003), in CGXII medium with regular phosphate concentration (13 mM) wild type reached the final OD_{600} of 40.3 however the strain Δ*cgtSR4*/pXMJ19CgtR4 only 25.

The most important results were obtained from the growth behaviour at the limited phosphate concentration (0.13 mM). According to the growth curves, the mutants could be divided into three groups. The first group consists of deletion mutants Δ*cgtSR1*, Δ*cgtSR2*, Δ*cgtSR5*, Δ*cgtSR6*, Δ*cgtSR7*, Δ*cgtSR8*, Δ*cgtSR9*, Δ*cgtSR10*, Δ*cgtSR11* and Δ*citAB* that grew almost like the wild type. Fig. 11B shows as an example the growth of deletion mutant Δ*cgtSR1* belonging to this group. The second group includes mutants Δ*mtrAB* and Δ*cgtSR4*/pXMJ19CgtR4, whose growth was impaired under phosphate starvation compared to the wild type (Figs. 11D and 11F). However, since these mutants showed a growth defect already under phosphate excess, this impairment might not be specific for P_i limitation. Finally, the last group consists of only one deletion mutant, i.e. Δ*cgtSR3*. Whereas this mutant grew like the wild type under phosphate abundance (Fig. 11G), growth under phosphate limitation was severely impaired (Fig. 11H). This result was confirmed in three independent experiments and indicates that the CgtSR3 TCS plays an important role in the regulation of the phosphate starvation response in *C. glutamicum*. Therefore the corresponding genes *cgtS3* and *cgtR3* were renamed ***phoS*** for phosphate sensor kinase and ***phoR*** for phosphate response regulator. Correspondingly, mutant Δ*cgtSR3* was renamed Δ*phoRS*.

Fig. 11 (next page): Growth of *C. glutamicum* wild type (■) and different two-component deletion mutants (Δ*cgtSR1* (o), Δ*mtrAB* (Δ), Δ*cgtSR4*/pXMJ19CgtR4 (◊) and Δ*cgtSR3* (∗)) in CGXII minimal medium with 4% (w/v) glucose and either 13 mM phosphate (panels A, C, E, G) or 0.13 mM phosphate (panels B, D, F, H) at 30 ^{o}C and 120 rpm. Cells were pre-cultured in CGXII medium with 0.13 mM potassium phosphate for 24 hours before inoculation of the same medium. The growth curves for the wild type represent separate cultivations for each panel.


A
B
C
D
E
F
G
H
OD600
100
10
1
0
20
40
60
Time (hour)

1.2 Complementation of mutant 13032Δ*phoRS* with plasmid pEKEx2-*phoRS*

In order to confirm that the deletion of the *phoRS* genes is responsible for the growth impairment of the Δ*phoRS* mutant under P_i limitation, a complementation study was performed. To this end, plasmid pEKEx2-*phoRS* was constructed, which contains the *phoRS* genes (coding regions plus 40 bp DNA upstream of the *phoR* start codon) under the control of the IPTG-inducible *tac* promoter. The natural *phoR* promoter (see chapter 3 of the Results section) was not included. *C. glutamicum* wild type and Δ*phoRS* mutant, both carrying the vector pEKEx2, and the complementation strain Δ*phoRS*/pEKEx2-*phoRS* were pre-cultured in CGXII glucose medium with 25 µg/ml kanamycin under P_i-limiting conditions (0.065 mM) for 24 hours and then used to inoculate fresh medium with 25 µg/ml kanamycin containing either sufficient (13 mM) or limiting P_i-concentrations (0.065 mM).

While the deletion mutant *C. glutamicum* Δ*phoRS*/pEKEx2 showed the expected growth impairment, the complementation strain grew like the wild type (Fig. 12). This result was

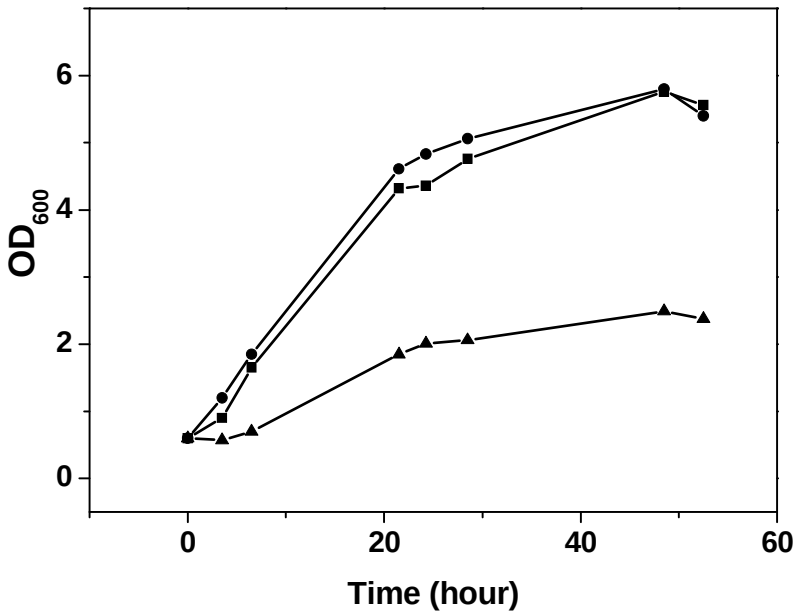


Fig. 12: Growth of the *C. glutamicum* strains 13032/pEKEx2 (■), 13032Δ*phoRS*/pEKEx2 (▲) and the complementation strain 13032Δ*phoRS*/pEKEx2-*phoRS* (●) in CGXII minimum medium with 4% (w/v) glucose, 25 µg/ml kanamycin, 0.065 mM P_i and without IPTG. Cells were pre-cultured in CGXII medium with 0.065 mM potassium phosphate for 24 hours before inoculation of the same medium.

obtained in three independent experiments and confirmed that the growth defect of strain Δ*phoRS* under P_i limitation is due to the deletion of the *phoRS* genes. According to our study the presence of IPTG was not required to obtained complementation, indicating that there is a basal expression of the *phoRS* genes from the pEKEx2-*phoRS* plasmid which is sufficient to obtain complementation.

1.3 Characterization of the *phoRS* genes and its products

In Fig. 13, the genomic organisation of the *phoRS* genes is shown. The genes located up- and downstream are presumably not involved in the P_i starvation response. The *phoR* gene has a length 708 bps and encodes a protein composed of 235 amino acids with a predicted mass of 26 350 Da. Analysis of the protein sequence with PFAM (Bateman *et al.*, 2004)

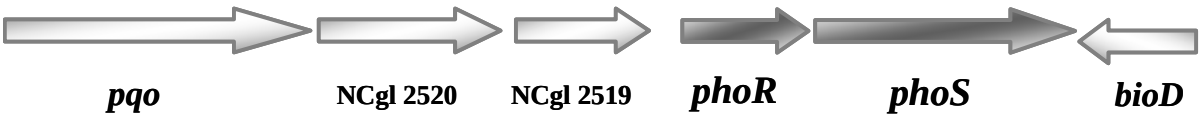


Fig. 13: Genomic organization of the two-component system *phoRS* in *C. glutamicum* ATCC 13032. Annotations: *pqo*, pyruvate quinine oxidoreductase; NCgl 2520, putative amino acid processing enzyme; NCgl 2519, bacterial regulatory protein (MerR family); *phoR*, phosphate response regulator; *phoS*, phosphate sensor kinase; *bioD*, dethiobiotin synthetase.

reveals that it contains two conserved domains (Fig. 14), i.e. an N-terminal response regulator receiver domain (PF00072) extending from residues 9 – 128 and a C-terminal transcriptional regulatory domain (PF00486) extending from residues 156 – 230, which is almost always found associated with the response regulator receiver domain. The aspartate residue which presumably is phosphorylated was located at position 59. The PhoR protein is highly conserved in other members of *Corynebacterianeae* and the orthologs from *C. efficiens*, *C. diphtheriae*, *M. tuberculosis*, *M. bovis* and *M. marinum* have 91%, 81%, 62%, 62% and 65% sequence identity to PhoR of *C. glutamicum*, respectively. Fig. 15 shows an amino acid sequence alignment of these proteins.

Eight bps downstream from the *phoR* gene the putative start codon of the *phoS* gene is localized. The *phoS* gene has a length of 1458 bps and encodes a protein of 485 amino acids with a predicted mass of 52 365 Da. As many other histidine kinases, PhoS contains two putative transmembrane helices extending from residues 44 - 64 and from 184 – 204. PFAM

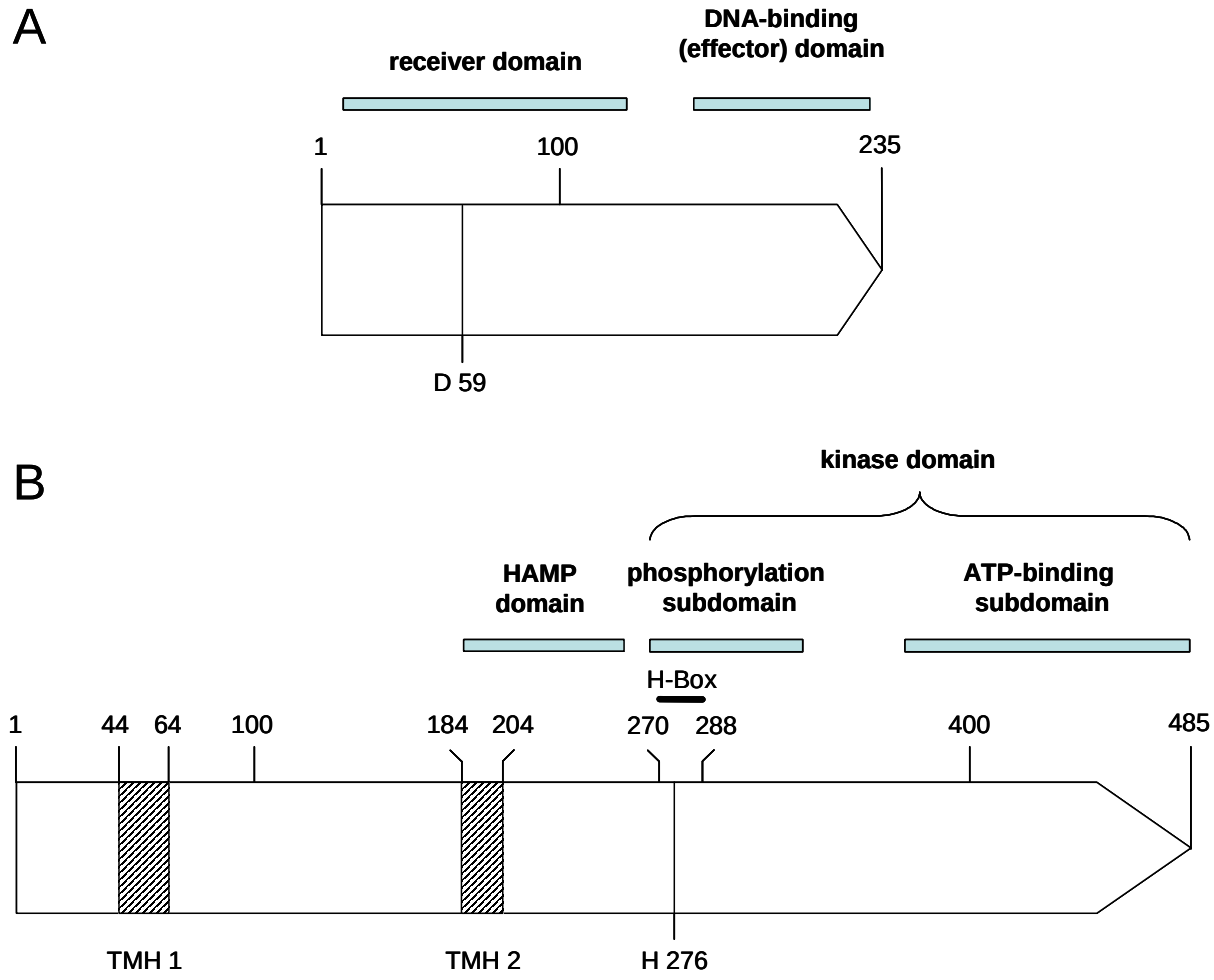


Fig. 14: Predicted domain structure of the response regulator PhoR (A) and the sensor kinase PhoS (B) from *C. glutamicum*. The response regulator consists of the N-terminal receiver domain with the aspartate residue, which is predicted to be phosphorylated, and the C-terminal DNA-binding (effector) domain (A). The sensor kinase PhoS consists of the two putative transmembrane helices (TMH 1 and TMH 2), the HAMP domain (*H*istidine kinases, *A*denylyl cyclases, *M*ethyl binding protein, *P*hosphatases) and C-terminal kinase domain, which includes ATP-binding subdomain and phosphorylation subdomain with histidine residue, which is predicted to be phosphorylated.

analysis of PhoS reveals three domains, i.e. a HAMP domain (PF00672) extending from residues 185-255, a histidine kinase A phosphoacceptor domain (PF00512) extending from residues 266 – 330 and the DNA gyrase B- and Hsp90-like ATPase domain (PF02518) extending from residues 373 to 484 (Fig. 14). The histidine residue which presumably is phosphorylated was located at position 276. Like the PhoR protein, the PhoS protein is highly conserved within the Corynebacterianeae. The orthologs from *C. efficiens*, *C. diphtheriae*, *M.*

tuberculosis, M. bovis and *M. marinum* possess 73%, 48%, 41%, 41% and 41% sequence identity, respectively. Fig. 16 shows an amino acid sequence alignment of these proteins.

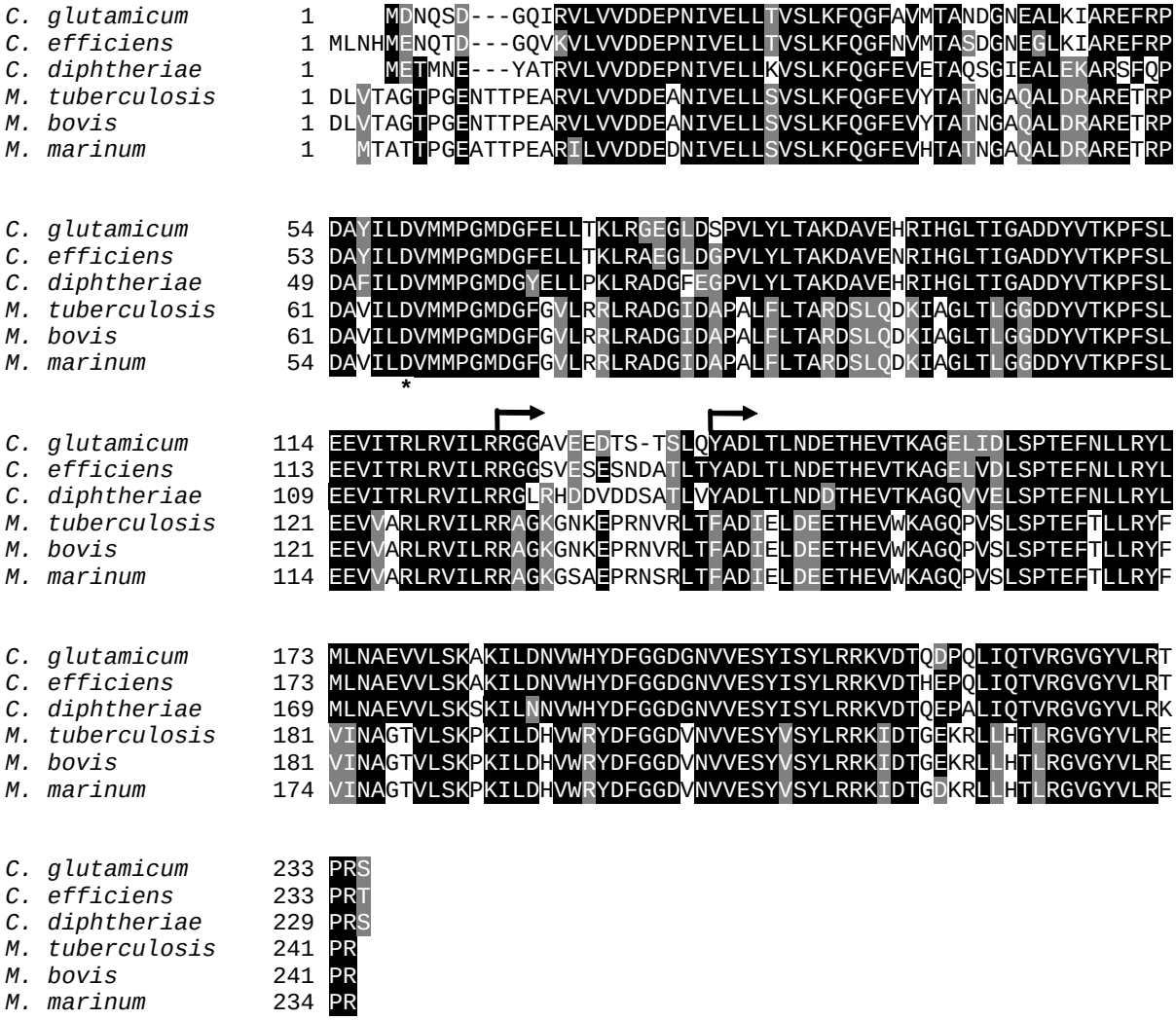


Fig. 15: Sequence alignment of the PhoR protein from *C. glutamicum* with orthologous proteins from *C. efficiens, C. diphtheriae, M. tuberculosis, M. bovis* and *M. marinum.* The amino acids that are identical in at least three sequences are in black boxes, conservatively exchanged amino acids in grey. The highly conserved aspartate residue, which is predicted to be phosphorylated, is marked by an asterisk. Arrows indicate the N-termini of the output domains PhoRΔ1-125 and PhoRΔ1-139. Sequences were extracted from the bioinformatics software ERGO (Integrated Genomics, Inc., Chicago, U.S.A.) and aligned with the help of Jalview and the Boxshade program (http://www.ch.embnet.org/ software/BOX_form.html).

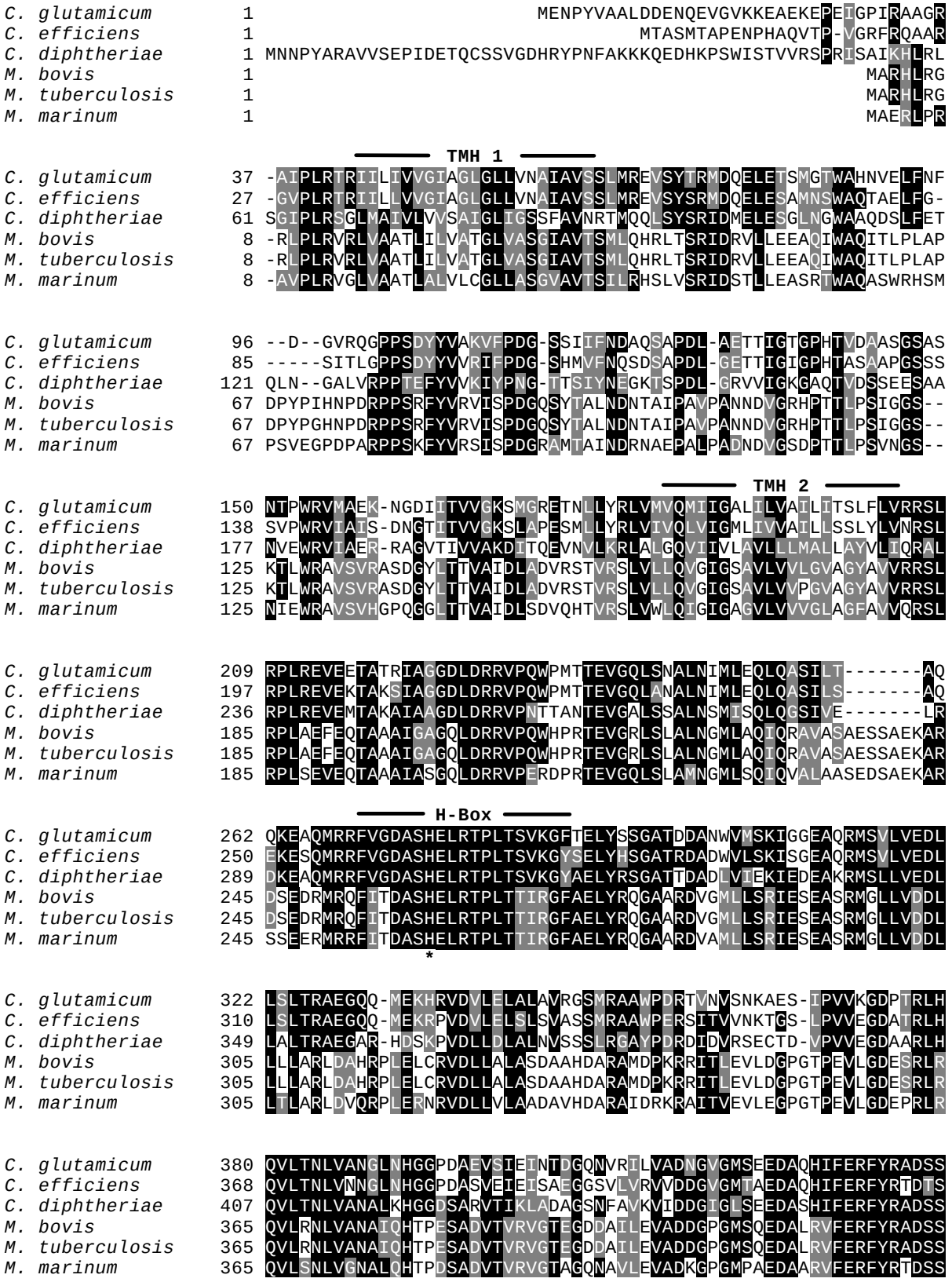

C. glutamicum
C. efficiens
C. diphtheriae
M. bovis
M. tuberculosis
M. marinum
TMH 1
TMH 2
H-Box
*

Fig. 16: Sequence alignment of the PhoS from *C. glutamicum* with homologous proteins from *C. efficiens, C. diphtheriae, M. bovis, M. tuberculosis, M. marinum.* The aminoacids that are identical in at least three sequences are in black boxes, conservative replaced amino acids in grey. The highly conserved aspartate residue, which is predicted to be phosphorylated, is marked by an asterisk.
Sequences were extracted from bioinformatics software ERGO (Integrated Genomics, Inc., Chicago, U.S.A.) and aligned with the help of Jalview and Boxshade program (http://www.ch.embnet.org/ software/BOX_form.html).

1.4 Qualitative analysis of alkaline phosphatase activity in *C. glutamicum* wild type and two-component deletion mutants

In the following experiments, wild type and mutants were tested for the production of alkaline phosphatase. The induced synthesis of alkaline phosphatase in response to phosphate limitation is a classical example of enzyme induction in bacteria. Moreover, alkaline phosphatase is a traditional reporter because its activity is easy to detect. X-phosphate (5-bromo-4-chloro-3-indolyl phosphate), which is used as substrate in the experiments, is a colourless soluble compound, but upon hydrolysis of the phosphate group, the indolyl moiety is released and forms a blue product upon oxidation (Fig. 17).

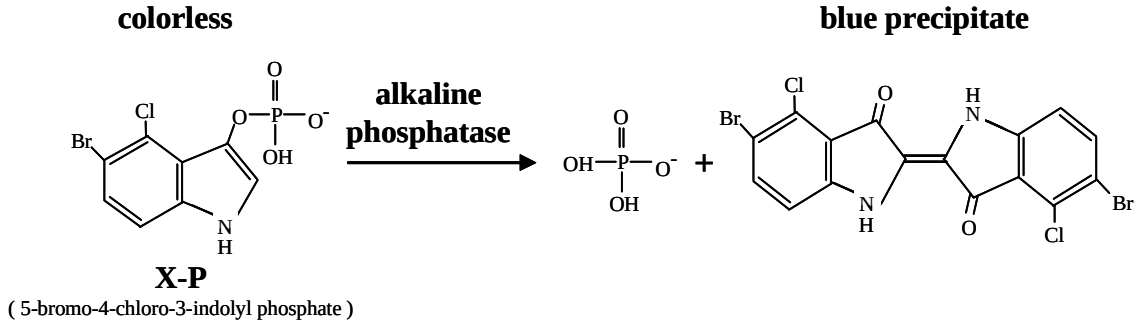


Fig. 17: Following the alkaline phosphatase reaction. The X-phosphate (5-bromo-4-chloro-3-indolyl phosphate) substrate produces a colored product on cleavage by alkaline phosphatase.

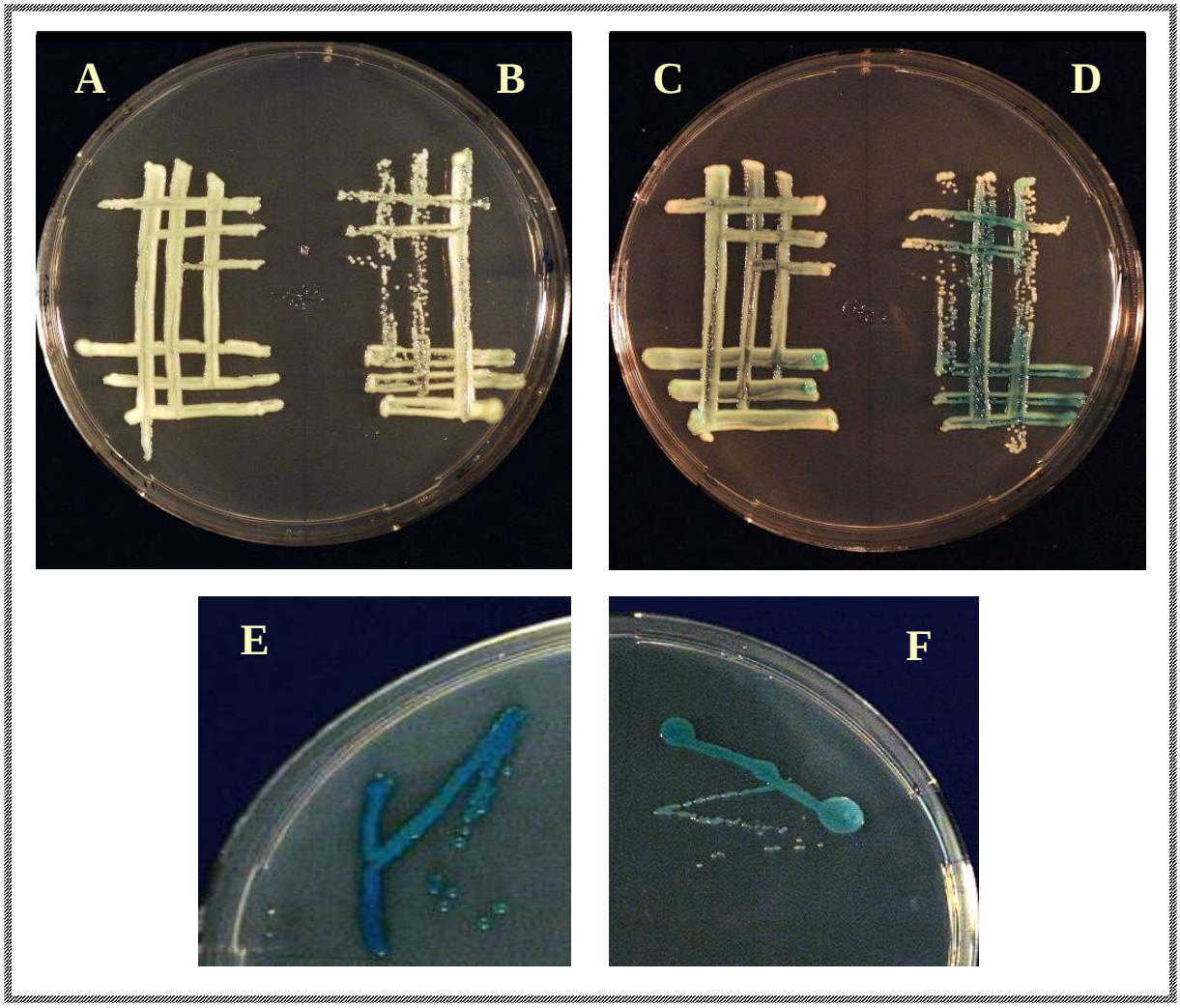


Fig. 18: Growth of *C. glutamicum* wild type (A, C, E) and strain Δ*cgtSR4*/pXMJ19CgtR4 (B, D, F) on CGXII plates with 4% (w/v) glucose, 40 µg/ml X-phosphate and different concentrations of phosphate: 13 mM (A, B), 1.3 mM (C, D) and 0.13 mM (E, F). The plates were incubated for 48 hours at 30°C.

Growth of the wild type and strain *Cg*Δ*cgtSR4*/pXMJ19CgtR4 on CGXII agar plates with three different concentrations of phosphate and with X-phosphate is shown in Fig. 18. Under conditions of phosphate excess, all strains did not change colour on the plates with X-phosphate. However, on plates with 1.3 mM phosphate, all mutants and the wild type became lightly blue. Finally, under phosphate-limited conditions (0.13 mM phosphate), the wild type and all mutants changed their colour into blue. The strain 13032Δ*cgtSR4*/pXMJ19CgtR4 became blue earlier on X-P plates with 1.3 mM phosphate than the other mutants and the wild type (Fig. 18D).

The results shown in Fig. 18 confirm that *C. glutamicum* wild type possesses an alkaline phosphatase whose synthesis is induced under P_i-limiting conditions. However, since this induction was also observed in the Δ*phoRS* deletion mutant, the relevant alkaline phosphatase gene/s is/are apparently not controlled by the PhoRS two-component system. The observation that the induction of alkaline phosphatase in strain 13032Δ*cgtSR4*/pXMJ19CgtR4 occurs earlier or is stronger than in the wild type points to a role of the CgtSR4 system in the transcriptional regulation of this protein.

2 Global analysis of the P_i starvation response in *C. glutamicum* wild type and the deletion mutant Δ*phoRS* with DNA microarrays

With the aim to identify the target genes of the response regulator PhoR, DNA microarray analyses were performed. In the first set of experiments the transcriptome of the *C. glutamicum* mutant Δ*phoRS* before and after a shift from phosphate excess to phosphate limitation was compared. The main task of these experiments was to determine whether the Δ*phoRS* mutant is still able to express the *psi* (*p*hosphate *s*tarvation *i*nducible) genes known to be induced after a shift to phosphate starvation in the wild type (Fig. 19A) (Ishige *et al*., 2003). In the second set of experiments, the transcriptome of the wild type was compared to that of the Δ*phoRS* deletion mutant after cultivation under phosphate-excess and after a shift to phosphate-limited conditions.

2.1 Transcriptome changes in strain Δ*phoRS* after a shift from P_i excess to P_i limitation

In a first set of DNA microarray experiments the gene expression changes in strain Δ*phoRS* after a shift from P_i excess to P_i starvation were investigated. Cells growing exponentially under P_i-sufficient conditions (13 mM) were harvested. RNA was prepared from one aliquot whereas the other aliquot was used to inoculate three parallel cultures with medium containing a limiting P_i concentration (0.065 mM). From these cultures RNA was prepared 10, 30 and 60 min after the transfer. The mRNA levels of the cells after the shift were compared to the mRNA levels of the cells before the shift. This experiment was performed in triplicate starting with independent cultures. In Table 7 those genes are listed that showed a significantly altered mRNA level at least at one time point and in two of three

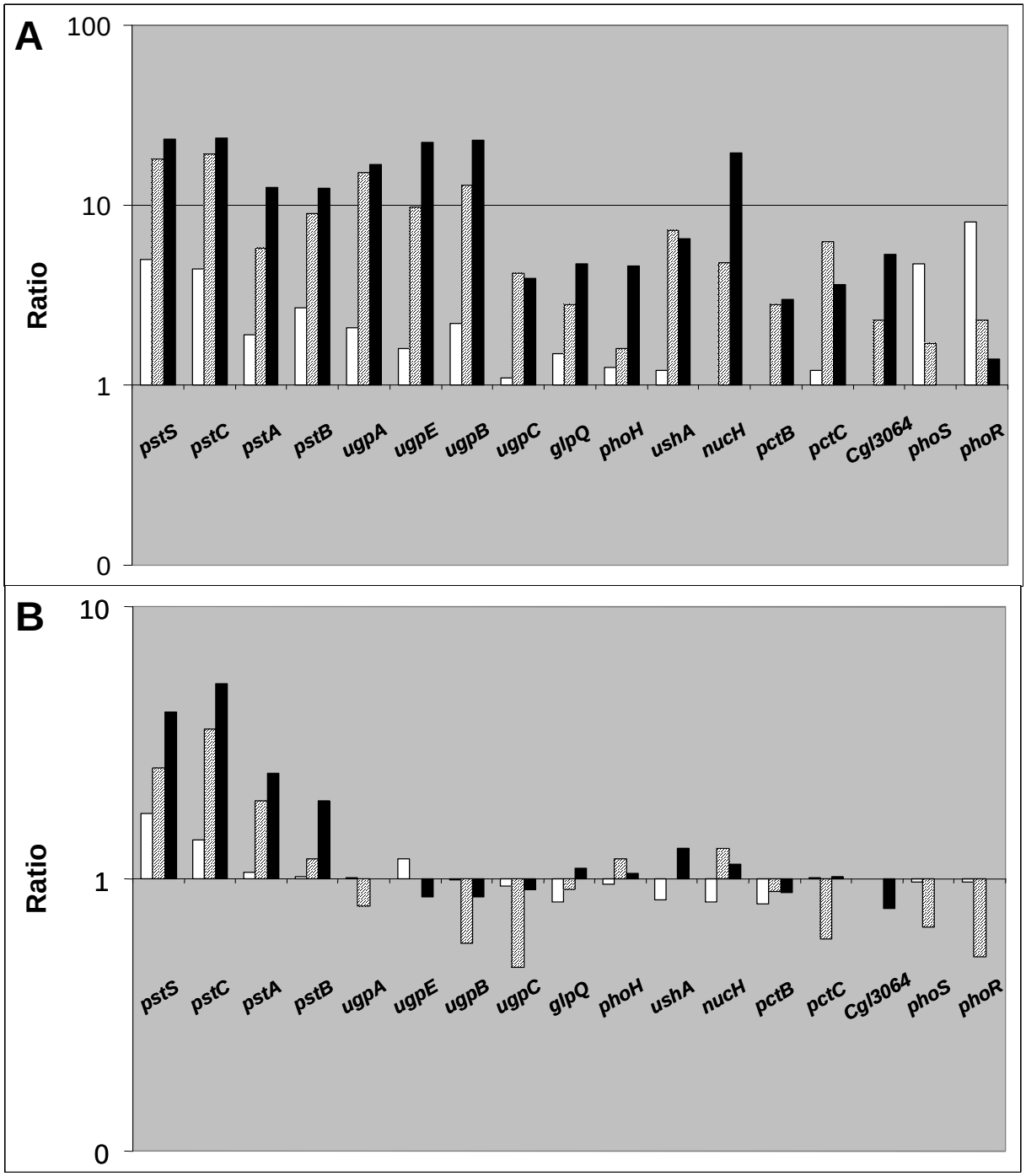


Fig. 19: Relative mRNA level of *psi* (*p*hosphate *s*tarvation *i*nducible) genes in *C. glutamicum* wild type (A) and Δ*phoRS* (B). The ratio represents the mRNA level 10 min (□), 30 min (▨) and 60 min (■) after the onset of P_i starvation versus the mRNA level immediately before the onset of P_i starvation. The experiment was performed three times for each time point and average mRNA ratios were calculated. The criteria used for selection of RNA ratios were a signal to noise ratio of ≥3 for either Cy3- or Cy5-fluorescence. Notation: see Table 3. The graph A was designed from data published by Ishige *et al*. (2003).

independent experiments. In total, 34 genes showed an increased and 47 genes a decreased mRNA level after the shift. Except for the *pst* genes, none of the prominent *psi* genes, e.g. the *ugpAEBC* operon, *glpQ* or *ushA*, were induced either 10, 30 or 60 min after the shift (Fig. 19B). This is in marked contrast to the situation in the wild type (Ishige *et al*., 2003) and supports a central role of the PhoRS TCS in the phosphate starvation response of *C. glutamicum*. In the case of the *pstSCAB* operon, a 2- to 5-fold increased mRNA level was observed 30 min and even more pronounced 60 min after the shift. Since this induction level is lower than in the wild type, the data might indicate that the *pst* operon is controlled both by the PhoRS system and another, yet unknown regulator.

According to DNA microarray data of Ishige *et al*. (2003) concerning *C. glutamicum* wild type, 92 genes showed altered mRNA levels after a shift from P_i excess to P_i limitation. Hierarchial cluster analysis of these genes revealed five groups with characteristic expression profiles. Four of these groups included genes most of which are not directly involved in phosphorous (P) metabolism and changed expression presumably due to the exchange of the medium or due to the reduced growth rate observed after the shift. As a consequence of P_i starvation, expression of some ribosomal protein genes decreased, genes involved in iron metabolism showed transiently reduced expression and genes involved in copper metabolism and in protocatechuate degradation showed transiently increased expression in *C. glutamicum* wild type (Ishige *et al*., 2003). In the strain Δ*phoRS* the expression of *psi* genes did not change after the shift to P_i limitation, however many of the genes belonging to the other four groups showed similarly altered mRNA levels as in the wild type. This supports the assumption that the *phoRS* deletion specifically affects the expression of the *psi* genes.

2.2 Transcriptome comparison of the Δ*phoRS* mutant and the wild type before and after a shift from P_i excess to P_i limitation

The Δ*phoRS* deletion mutant and the wild type cells were grown in regular CGXII medium under P_i-sufficient conditions (13 mM). In the exponential growth phase (OD_{600} ca. 4-5) cells were harvested and total RNA was prepared. Subsequently, the mRNA levels of the two strains were compared with DNA microarrays. The experiment was performed three times starting with independent cultures each time. Genes with at least 2-fold altered mRNA levels that showed reliable hybridization signals in at least two of the experiments are listed in Table 8.

Under phosphate excess, 15 genes showed a lower mRNA level and 27 genes an increased mRNA level in the wild type compared to the mutant. Concerning the genes that were induced in wild type, no obvious functional connection could be deduced (Tab. 9). Except the *phoR* and *phoS* genes, the highest induction showed the *porB* gene encoding an anion-specific porin, which is involved in the transport of hydrophilic compounds across the outer hydrophobic barrier of mycolic acids (Costa-Riu *et al*., 2003). Besides *porB*, the genes of the *gluABCD* operon coding for a high affinity glutamate transport system had higher mRNA levels in the wild type. Functional expression of the *gluABCD* genes in *Escherichia coli* enable this strain to growth on glutamate as the only carbon source (Burkovski and Krämer, 1995; Kronemeyer *et al*., 1995). However it is important to mention that all of the genes with an increased mRNA level in the wild type under P_i excess also showed increased mRNA levels under P_i limitation (except for putative glycosyltransferase, Cg0438) (Tab. 8), showing that the difference is independent of the P_i concentration. Moreover, it is surprising that more than 10 of the genes with an increased mRNA level in the wild type are organised at one locus of the genome starting at NCgl0674 and ending at NCgl0697. The possibility exists that the increased mRNA level of these genes results from a duplication of the corresponding genome region, which could be tested by a comparison of the genomic DNA using DNA microarrays. The group of genes with a lower mRNA level in the wild type under P_i excess involves six *psi* genes, i.e. *phoH1*, *nucH* and the *ugp* operon. This suggests the possibility that the PhoR not only activates these genes under P_i limitation, but also represses them under P_i excess. However this hypothesis remains to be studied.

Completing the transcriptome comparison of wild type and Δ*phoRS* mutant, the mRNA levels of the two strains were compared after a shift from P_i excess to P_i limitation. Cells of the mutant and the wild type cultivated in CGXII medium with 13 mM phosphate were harvested in the exponential growth phase (OD_{600} = 4-5) and used to inoculate CGXII medium with 0.065 mM P_i. 10, 30 and 60 min after the shift cells were harvested and used for RNA preparation and subsequent microarray analysis. In total, 80 genes showed at least two-fold altered mRNA levels after the shift, 55 of which had an increased and 25 a decreased mRNA level in the wild type. Table 8 summarizes the observed expression differences and lists genes that were reliably detected and showed at least at one time point a significantly altered mRNA level. 60 min after the shift, the prominent P_i starvation inducible genes *pstSCAB, ugpAEBC, glpQ, phoH, nucH* and *ushA* showed higher mRNA levels in the wild type compared to the deletion mutant (Fig. 20), confirming the assumption that their induction is dependent on PhoRS. The only exception were the *pctB and pctC* genes, which had similar

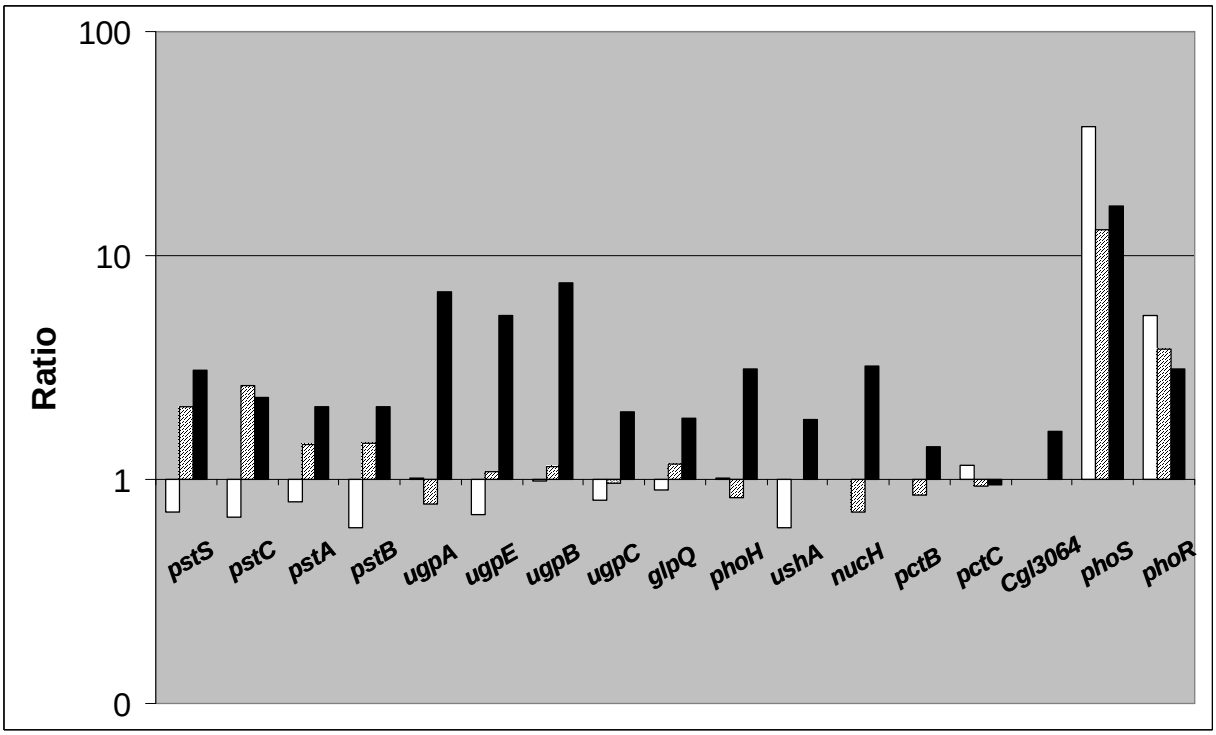


Fig. 20: Relative mRNA level of *psi* (*p*hosphate *s*tarvation *i*nducible) genes in *C. glutamicum* wild type versus strain Δ*phoRS*. The ratio represents the mRNA level 10 min (□), 30 min (▨) and 60 min (■) after the onset of P_i starvation in wild type versus the mRNA level at the same time points after the onset of P_i starvation in strain Δ*phoRS*. The experiment was performed three times for each time point and average RNA ratios were calculated. The criteria used for selection of RNA ratios were a signal to noise ratio of ≥3 for either Cy3- or Cy5-fluorescence. Notation: see Table 3.

mRNA levels in wild type and mutant. Interestingly, the transcriptome comparison revealed that 60 min after the shift to P_i limitation the mRNA level of the *pitA* gene encoding a low-affinity P_i transporter was two-fold lower in the wild type compared to the Δ*phoRS* mutant (Tab. 8). This observation might indicate that *pitA* is repressed by PhoR under P_i limitation.

TABLE 7. Changes in gene expression at different times during the P_i starvation response of *C. glutamicum* Δ*phoRS*

CG	NCBI	ORF	Annotation	Gene	Avg mRNA level after shifting to P_i-limiting conditions/avg mRNA level before shifting to P_i-limiting conditions[a]		
					10 min	30 min	60 min
Cg0160	NCgl0123	518	hypothetical protein		0.43[b]	0.34	0.65
Cg0229	NCgl0181	573	glutamine 2-oxoglutarate aminotransferase large SU	*gltB*	1.28	1.95	2.59[b]
Cg0230	NCgl0182	575	glutamine 2-oxoglutarate aminotransferase	*gltD*	6.41[b]	5.83[b]	6.24[b]
Cg0310	NCgl0251	671	catalase	*katA*	3.49[b]	3.24[b]	1.55[b]
Cg0464	NCgl0375	851	copper-transporting ATPase	*ctpA*	10.32[b]	21.51	5.16[b]
Cg0467	NCgl0378	855	cobalamin/Fe3+-siderophores transport system, secreted component		0.42[b]	0.40	0.63[b]
Cg0468	NCgl0379	856	cobalamin/Fe3+-siderophores transport systems, permease component		0.33	-	0.83[b]
Cg0469	NCgl0380	857	cobalamin/Fe3+-siderophores transport system, ATPase component		0.41[b]	0.50	0.69
Cg0470	NCgl0381	3543	conserved secreted protein		0.16[b]	0.22[b]	0.58
Cg0500	NCgl0405	894	bacterial regulatory protein, LysR family		2.07[b]	2.74	2.21[b]
Cg0569	NCgl0465	971	cation-transporting ATPase		8.60[b]	4.77	2.65[b]
Cg0589	NCgl0482	3549	ABC transporter, nucleotide binding/ATPase protein		0.06[b]	0.10[b]	0.44
Cg0590	NCgl0483	3550	cobalamin/Fe3+-siderophores transport system, permease component		0.22[b]	0.12	0.51
Cg0591	NCgl0484	3551	cobalamin/Fe3+-siderophores transport system, permease component		0.15[b]	0.21	0.26
	NCgl0628	1160	methylaconitase	*prpD2*	0.33[b]	0.47[b]	0.55[b]
Cg0760	NCgl0629	1161	probable methylisocitric acid lyase	*prpB2*	0.37[b]	0.53[b]	0.49[b]
Cg0762	NCgl0630	1162	methylcitrate synthase	*prpC2*	0.22[b]	0.39	0.40
Cg0767	NCgl0635	1168	siderophore-interacting protein		0.16[b]	0.18	0.62
Cg0768	NCgl0636	1169	ABC-type cobalamin/Fe3+-siderophores transport system, ATPase component		0.25[b]	0.09	0.36
Cg0769	NCgl0637	1170	ABC-type cobalamin/Fe3+-siderophores transport system, permease component		0.16[b]	0.09	0.23
Cg0771	NCgl0639	1173	DTXR/iron regulated lipoprotein precursor, secrete	*irp1*	0.14[b]	0.10	0.29
Cg0812	NCgl0678	1223	acetyl/propionyl-CoA carboxylase beta chain	*dtsR1*	0.26[b]	0.24[b]	0.48[b]

Cg0921	NCgl0773	1346	siderophore-interacting protein		0.13[b]	0.12[b]	0.51[b]
Cg0922	NCgl0774	1347	ABC-type cobalamin/Fe3+-siderophores transport system, secreted component		0.11[b]	0.12[b]	0.27[b]
Cg0924	NCgl0776	1349	ABC-type cobalamin/Fe3+-siderophores transport sys		0.07[b]	0.10[b]	0.18[b]
Cg0926	NCgl0777	1350	ABC-type cobalamin/Fe3+-siderophores transport system, permease component		1.24	0.17	-
Cg0927	NCgl0778	1351	ABC-type cobalamin/Fe3+-siderophores transport system, permease component		0.24[b]	0.28[b]	0.38[b]
Cg0928	NCgl0779	1352	ABC-type cobalamin/Fe3+-siderophores transport system, ATPase component		0.65[b]	0.12	0.82
Cg0957	NCgl0802	1386	fatty acid synthase	*fas-IB*	0.46	0.16	0.27[b]
Cg0957	NCgl0802	1388	fatty acid synthase	*fas-IB*	0.57	0.18	0.38[b]
Cg1120	NCgl0943	1558	AraC-type DNA-binding domain-containing protein		0.21[b]	0.20	0.42[b]
Cg1121	NCgl0944	1559	permease of the major facilitator superfamily		0.49[b]	0.92	1.26
Cg1343	NCgl1141	1785	probable respiratory nitrate reductase oxidoreduct	*narH*	4.40[b]	1.91	1.08[b]
Cg1344	NCgl1142	1786	nitrate reductase 2, alpha subunit	*narG*	2.26[b]	1.87	1.13
Cg1405	NCgl1200	1855	siderophore-interacting protein		0.34[b]	0.17	0.46
Cg1412	NCgl1205	1861	ribose/xylose/arabinose/galactoside ABC-type transport system, permease component		0.48[b]	0.79	0.91
Cg1413	NCgl1206	1862	secreted sugar-binding protein		0.40[b]	0.78	0.54
Cg1418	NCgl1209	1865	ABC-type cobalamin/Fe3+-siderophores transport system secreted component		0.22[b]	0.25	0.50[b]
Cg1419	NCgl1210	1866	putative Na+-dependent transporter		0.32[b]	0.22	0.47
Cg1419	NCgl1210	3557	putative Na+-dependent transporter		0.39[b]	0.35	0.48
Cg1447	NCgl1232	2779	Co/Zn/Cd efflux system transmembrane protein		7.16	6.08	7.18[b]
Cg1695	NCgl1444	2376	putative plasmid maintenance system antidote protein		3.17[b]	2.18	2.05[b]
Cg1737	NCgl1482	2425	aconitase	*acn*	1.87	3.69[b]	2.25[b]
Cg1785	NCgl1521	2480	high-affinity ammonia permease	*amt*	1.77	2.07[b]	4.46
Cg1930	NCgl1646	3347	putative secreted hydrolase		0.14[b]	0.21[b]	0.51[b]
Cg2118	NCgl1859	2019	transcriptional regulator of sugar metabolism, DeoR family		4.08[b]	0.93	0.69[b]
Cg2120	NCgl1861	2021	sugar specific pts sytem, fructose/mannitol-specific transport protein	*ptsF*	3.64[b]	0.66	0.41[b]
Cg2181	NCgl1915	2093	ABC-type peptide transport system, secreted component		0.68[b]	0.53[b]	0.31[b]
Cg2183	NCgl1917	2095	ABC-type peptide transport system, permease component		0.71	0.58	0.44[b]

Cg2234	NCgl1959	2146	ABC-type cobalamin/Fe3+-siderophores transport system, secreted component	.	0.26[b]	0.24	0.55[b]
Cg2283	NCgl2001	2205	conserved hypothetical protein		0.33[b]	0.36	0.62
Cg2422	NCgl2127	3245	lipoate-protein ligase B	*lipB*	1.99[b]	2.17	1.46[b]
Cg2423	NCgl2128	3243	lipoic acid synthase	*lipA*	1.65[b]	2.12[b]	1.26[b]
Cg2726	NCgl2393	3022	putative membrane protein		15.60[b]	3.46	4.46[b]
Cg2743	NCgl2409	3511	fatty acid synthase	*fas-IA*	0.42[b]	0.39	0.46[b]
Cg2777	NCgl2434	3480	putative membrane protein		0.47[b]	0.37	0.55[b]
Cg2782	NCgl2439	3472	ferritin-like protein	*ftn*	18.82[b]	17.89	12.14[b]
Cg2843	NCgl2483	2876	ABC-type phosphate transport system, ATPase component	*pstB*	1.02[b]	1.19	1.94[b]
Cg2844	NCgl2484	2875	ABC-type phosphate transport system, permease component	*pstA*	1.06[b]	1.93	2.43[b]
Cg2845	NCgl2485	2873	ABC-type phosphate transport system, permease component	*pstC*	1.39[b]	3.55	5.21[b]
Cg2846	NCgl2486	2871	ABC-type phosphate transport system, secreted component	*pstS*	1.74[b]	2.55	4.10[b]
Cg2925	NCgl2553	69134	enzyme II sucrose protein		3.83[b]	0.93[b]	0.56[b]
Cg3116	NCgl2717	197	phosphoadenosine-phosphosulfate reductase	*cysH*	2.53[b]	-	0.95
Cg3118	NCgl2718	198	sulfite reductase (Hemoprotein)	*cysI*	3.17[b]	1.41	0.79
Cg3132	NCgl2731	212	putative membrane protein		2.53[b]	1.00	1.13[b]
Cg3195	NCgl2787	281	flavin-containing monooxygenase (FMO)		0.40[b]	0.53	0.88
Cg3227	NCgl2817	313	putative L-lactate dehydrogenase	*lldA*	18.31[b]	11.82	1.32
Cg3281	NCgl2859	375	probable cation-transporting ATPase transmembrane protein		3.77[b]	2.03	1.47[b]
Cg3282	NCgl2860	376	cation transport ATPase		14.95[b]	4.27	4.54
Cg3286	NCgl2864	380	putative secreted protein		3.54[b]	2.44	2.67[b]
Cg3287	NCgl2865	381	secreted multicopper oxidase		4.13[b]	2.81	1.57
		382	hypothetical protein		2.92[b]	2.56	2.31
Cg3303	NCgl2877	399	Transcriptional regulator PadR-like family		7.37[b]	4.16	4.18[b]
Cg3327	NCgl2897	1881	starvation-iduced DNA protecting protein	*dps*	3.76[b]	3.51	2.44[b]
Cg3335	NCgl2904	1888	malic enzyme	*mez*	0.46[b]	0.45	0.38[b]
Cg3386	NCgl2952	3430	maleylacetate reductase	*tcbF*	0.41[b]	0.37	0.49
Cg3387	NCgl2953	3431	permease of the major facilitator superfamily		0.32[b]	0.32	0.50
Cg3390	NCgl2956	3438	sugar phosphate isomerase/epimerase		0.38[b]	0.58	1.15[b]
Cg3391	NCgl2957	3439	myo-inositol 2-dehydrogenase	*idhA1*	0.41[b]	0.43	0.38

Cg3404	NCgl2970	3458	ABC-type cobalamin/Fe3+-siderophores transport system, secreted component	0.06[b]	0.07[b]	0.18
		190	hypothetical protein	0.23[b]	0.27	0.52
		2754	hypothetical protein	-	0.80	2.35[b]

[a] The relative mRNA levels 10, 30 and 60 min after the shift to P_i limiting conditions compared to the levels under preshift conditions are averages from at least two of three experiments. Only ORFs whose mRNA levels were equal to or greater than 2 or equal to or less than 0.5 at least one time are shown.

[b] $P<0.05$ as determined by a t test

TABLE 8. Changes in gene expression at different times during the P_i excess, as well as during P_i starvation response of *C. glutamicum* wild type versus *C. glutamicum* Δ*phoRS*

CG	NCBI	ORF	Annotation	Gene	Avg mRNA level after shifting to P_i-limiting conditions WT/avg mRNA level after shifting to P_i-limiting conditions Δ*phoRS*			
					0 min	10 min	30 min	60 min
Cg0085	NCgl0064	442	ATPase related to phosphate starvation-inducible protein	*phoH1*	0.44	1.02[b]	0.83	3.11[b]
Cg0230	NCgl0182	575	glutamine 2-oxoglutarate aminotransferase	*gltD*	0.39	1.22[b]	0.86	0.09[b]
Cg0411	NCgl0334	787	putative membrane protein		0.62	0.30	0.28[b]	0.32[b]
Cg0418	NCgl0341	798	putative aminotransferase		2.11[b]	2.22[b]	2.21[b]	2.77
Cg0438	NCgl0353	822	putative glycosyltransferase		2.01	0.69[b]	0.81	0.75[b]
Cg0464	NCgl0375	851	copper-transporting ATPase	*ctpA*	0.70	0.28[b]	0.24[b]	0.30[b]
Cg0522	NCgl0425	920	cytochrome c biogenesis protein transmembrane prot	*ccsA*	0.63	2.30[b]	2.98[b]	2.08
Cg0523	NCgl0426	921	membrane protein required for cytochrome c biosynthesis		0.87	2.19[b]	2.99[b]	1.94[b]
Cg0524	NCgl0427	922	cytochrome c assembly membrane protein	*ccsB*	0.96	2.55[b]	3.02[b]	2.75[b]
Cg0545	NCgl0445	944	putative low-affinity phosphate transport protein	*pitA*	1.31[b]	1.09	1.11[b]	0.45
Cg0706	NCgl0584	1106	conserved hypotetical membrane protein		0.29	0.38[b]	0.36[b]	0.23[b]
Cg0753	NCgl0623	1152	secreted protein		-	3.29[b]	1.50[b]	1.07[b]
	NCgl0628	1160	methylaconitase	*prpD2*	0.47	1.02	0.78[b]	3.92[b]
Cg0760	NCgl0629	1161	probable methylisocitric acid lyase	*prpB2*	0.48	1.08	0.83[b]	2.20[b]
Cg0762	NCgl0630	1162	methylcitrate synthase	*prpC2*	0.85	0.82	0.81[b]	2.46[b]
Cg0802	NCgl0670	1211	biotin carboxylase and biotin carboxyl	*accBC*	1.81	2.98[b]	1.62[b]	2.59[b]
Cg0808	NCgl0674	1217	lipopolysaccharide biosynthesis acyltransferase	*wbpC*	2.63	2.92[b]	2.24[b]	2.18[b]
Cg0809	NCgl0675	1219	nucleotide-binding protein		-	3.53[b]	2.44	1.74
Cg0810	NCgl0676	1220	conserved hypothetical protein		2.54	2.94[b]	2.13[b]	2.44[b]
Cg0814	NCgl0679	1224	bifunctional biotin ligase/biotin operon repressor	*birA*	2.50	2.19[b]	2.34[b]	2.31[b]
Cg0820	NCgl0684	1230	phosphoribosylaminoimidazole carboxylase catalytic	*purE*	2.99	2.84[b]	2.12[b]	3.20[b]
Cg0821	NCgl0685	1231	conserved hypothetical protein		3.31	2.26[b]	2.38[b]	2.97[b]

Cg0825	NCgl0689	1235	short chain dehydrogenase, N-terminal fragment		2.31	2.64[b]	2.51[b]	2.59[b]
Cg0825	NCgl0689	1236	short chain dehydrogenase, N-terminal fragment		3.84	2.71[b]	3.05[b]	2.53[b]
Cg0831	NCgl0694	1242	sugar ABC transporter, permease protein		2.26	2.74[b]	2.76[b]	3.26[b]
Cg0832	NCgl0695	1243	ABC transporter, membrane spanning protein		2.35	2.45[b]	2.38[b]	3.10[b]
Cg0833	NCgl0696	1244	conserved hypothetical protein		2.57	2.38[b]	2.79[b]	2.71[b]
Cg0834	NCgl0697	1245	bacterial extracellular solute-binding protein		2.33	3.72[b]	3.36[b]	2.12[b]
Cg0839	NCgl0701	1250	hypothetical protein		1.93	2.39[b]	1.80[b]	2.23[b]
Cg1109	NCgl0933	1546	anion-specific porin precursor	*porB*	5.55	3.70[b]	4.20[b]	4.17[b]
Cg1167	NCgl0985	1605	putative secreted protein		2.03	3.49[b]	2.90[b]	2.78[b]
Cg1179	NCgl0994	1616	sensory BOX/GGDEF family protein		0.33	0.24[b]	0.25	0.27[b]
Cg1181	NCgl0995	1617	glycosyltransferase, probably involved in cell wall biogenesis		0.36	0.34[b]	0.31	0.29[b]
Cg1182	NCgl0996	1619	putative membrane protein		0.27	0.33[b]	0.25[b]	0.26[b]
Cg1215	NCgl1023	1646	putative nicotinate-nucleotide pyrophosphorylase	*nadC*	1.04[b]	0.72	0.87[b]	0.31[b]
Cg1216	NCgl1024	1647	quinolinate synthetase	*nadA*	0.59	0.79[b]	0.80[b]	0.32[b]
Cg1218	NCgl1025	1648	ADP-ribose pyrophosphatase		0,77	0,54[b]	0,66[b]	0.22[b]
Cg1325	NCgl1125	1762	putative stress-responsive transcriptional regulator		0.58	0.35[b]	0.43[b]	0.52[b]
Cg1487	NCgl1262	2737	3-isopropylmalate dehydratase large subunit	*leuC*	0.84	2.45[b]	1.62[b]	1.73
Cg1488	NCgl1263	2736	3-isopropylmalate dehydratase small subunit	*leuD*	0.89	2.97[b]	2.10[b]	1.86[b]
Cg1568	NCgl1329	2641	sn-glycerol-3-phosphate transport system permease protein	*ugpA*	0.29	1.01	0.78	6.87[b]
Cg1569	NCgl1330	2640	sn-glycerol-3-phosphate transport system permease protein	*ugpE*	0.58	0.70	1.08[b]	5.40[b]
Cg1570	NCgl1331	2639	secreted sn-glycerol-3-phosphate-binding protein	*ugpB*	0.24	0.99[b]	1.15[b]	7.52[b]
Cg1571	NCgl1332	2638	ABC-type sugar transport systems, ATPase component	*ugpC*	0.85	0.81[b]	0.96	2.01[b]
Cg1785	NCgl1521	2480	high-affinity ammonia permease	*amt*	1.00	0.92[b]	0.56[b]	0.26[b]
Cg2136	NCgl1875	2040	glutamate uptake system ATP-binding protein	*gluA*	2.52	1.53	1.97[b]	2.21[b]
Cg2137	NCgl1876	2041	glutamate secreted binding protein	*gluB*	2.41	1.50[b]	3.34[b]	3.60[b]
Cg2138	NCgl1877	2042	glutamate permease	*gluC*	2.78	1.97[b]	2.87[b]	3.47[b]
Cg2139	NCgl1878	2043	glutamate permease	*gluD*	1.99	1.17	2.14	3.95
Cg2380	NCgl2088	2306	putative membrane protein		2.17	2.28[b]	2.62[b]	2.06[b]
Cg2600	NCgl2284	1644	transposase	*tnp1d*	1.70	4.50[b]	3.43[b]	3.47[b]
Cg2600	NCgl2284	3044	transposase	*tnp1d*	2.76	3.15[b]	3.01[b]	3.07[b]

Cg2600	NCgl2284	3021	transposase	*tnp1d*	2.45	2.72[b]	2.74[b]	3.01[b]
Cg2657	NCgl2336	2947	putative membrane protein - fragment		0.81	0.63[b]	0.48[b]	0.33[b]
Cg2725	NCgl2392	1091	transposase	*tnp1b*	2.61	3.01[b]	3.96	3.82[b]
Cg2725	NCgl2392	3021	transposase	*tnp1b*	2.45	2.72[b]	2.74[b]	3.01[b]
Cg2725	NCgl2392	1644	transposase	*tnp1b*	1.70	4.50[b]	3.43[b]	3.47[b]
Cg2725	NCgl2392	3044	transposase	*tnp1b*	2.76	3.15[b]	3.01[b]	3.07[b]
Cg2726	NCgl2393	3022	putative membrane protein		1.32	0.28[b]	0.28[b]	0.26[b]
Cg2747	NCgl2411	3507	secreted peptidase, M23/M37 family	*mepA*	0.45	0.36[b]	0.43	0.30[b]
Cg2782	NCgl2439	3472	ferritin-like protein	*ftn*	0.69	1.17	0.50	0.17[b]
Cg2799	NCgl2452	2918	putative secreted protein	*pknE*	1.14	2.07[b]	2.23[b]	1.92[b]
Cg2808	NCgl2461	2907	transposase	*tnp13a*	0.88	2.67[b]	2.56[b]	1.78
Cg2842	NCgl2482	2877	putative phosphate uptake regulator	*phoU*	0.74	-	0.84[b]	2.18[b]
Cg2843	NCgl2483	2876	ABC-type phosphate transport system, ATPase component	*pstB*	-	0.61[b]	1.46[b]	2.12[b]
Cg2844	NCgl2484	2875	ABC-type phosphate transport system, permease component	*pstA*	0.52	0.80[b]	1.44	2.12
Cg2845	NCgl2485	2873	ABC-type phosphate transport system, permease component	*pstC*	0.54	0.68[b]	2.63[b]	2.32[b]
Cg2846	NCgl2486	2871	ABC-type phosphate transport system, secreted component	*pstS*	0.58	0.72[b]	2.13[b]	3.08[b]
Cg2868	NCgl2503	2851	predicted extracellular nuclease	*nuc*	0.45	-	0.72	3.19[b]
Cg2887	NCgl2517	2832	probable two component sensor kinase	*cgtS3*	3.28	5.40[b]	3.82[b]	3.11[b]
Cg2888	NCgl2518	2831	putative two component response regulator	*cgtR3*	8.66[b]	37.44[b]	13.00[b]	16.74[b]
Cg2914	NCgl2542	1234	transposase	*tnp5b*	2.01[b]	2.70[b]	1.58[b]	1.93[b]
Cg3082	NCgl2684	155	bacterial regulatory proteins, ArsR family		-	0.32[b]	-	-
Cg3285	NCgl2863	378	putative two component response regulator	*cgtR9*	0.28	0.37[b]	0.69[b]	0.92[b]
Cg3286	NCgl2864	380	putative secreted protein		0.28	0.32[b]	0.70	0.69[b]
Cg3287	NCgl2865	381	secreted multicopper oxidase		-	0.30[b]	0.61	0.73
		382	hypothetical protein		-	0.35[b]	0.77	0.74
Cg3322	NCgl2893	1873	putative secreted membrane-fusion protein		0.24	0.34[b]	0.35[b]	0.27[b]
Cg3327	NCgl2897	1881	starvation-iduced DNA protecting protein	*dps*	1.01[b]	0.49[b]	0.35[b]	0.27[b]
Cg3343	NCgl2912	1898	putative secreted membrane protein		0.53	2.90[b]	1.23[b]	0.96[b]
Cg3399	NCgl2965	3452	permease of the major facilitator superfamily		0.62	0.44[b]	0.67	1.24

[a] The relative mRNA levels under pre-shift conditions as well as mRNA levels 10, 30 and 60 min after the shift to P_i limiting conditions in the *C. glutamicum* wild type compared to the mRNA levels under the same conditions in *C. glutamicum* Δ*phoRS* are averages from at least two of three experiments. Only ORFs whose mRNA levels were altered at least two-fold at one time point are shown.

[b] $P<0.05$ as determined by a t test.

TABLE 9. Changes in gene expression during the P_i excess of *C. glutamicum* wild type versus *C. glutamicum* Δ*phoRS*

CG	NCBI	ORF	Annotation	Gene	Avg mRNA level under P_i-excess conditions WT/avg mRNA level under P_i excess conditions Δ*phoRS*
					P_i excess
Cg2888	NCgl2518	2831	putative two component response regulator	*cgtR3*	8.66[b]
Cg2887	NCgl2517	2832	probable two component sensor kinase	*cgtS3*	3.28
Cg1109	NCgl0933	1546	anion-specific porin precursor	*porB*	5.55
Cg0825	NCgl0689	1236	short chain dehydrogenase, N-terminal fragment		3.84
Cg0825	NCgl0689	1235	short chain dehydrogenase, N-terminal fragment		2.31
Cg0821	NCgl0685	1231	conserved hypothetical protein		3.31
Cg0820	NCgl0684	1230	phosphoribosylaminoimidazole carboxylase catalytic	*purE*	2.99
Cg2136	NCgl1875	2040	glutamate uptake system ATP-binding protein	*gluA*	2.52
Cg2137	NCgl1876	2041	glutamate secreted binding protein	*gluB*	2.41
Cg2138	NCgl1877	2042	glutamate permease	*gluC*	2.78
Cg2139	NCgl1878	2043	glutamate permease	*gluD*	1.99
Cg2600	NCgl2284	3044	transposase	*tnp1d*	2.76
Cg2600	NCgl2284	3021	transposase	*tnp1d*	2.45
Cg2725	NCgl2392	3044	transposase	*tnp1b*	2.76
Cg2725	NCgl2392	1091	transposase	*tnp1b*	2.61
Cg2725	NCgl2392	3021	transposase	*tnp1b*	2.45
Cg0808	NCgl0674	1217	lipopolysaccharide biosynthesis acyltransferase	*wbpC*	2.63
Cg0831	NCgl0694	1242	sugar ABC transporter, permease protein		2.26
Cg0832	NCgl0695	1243	ABC transporter, membrane spanning protein		2.35
Cg0833	NCgl0696	1244	conserved hypothetical protein		2.57
Cg0834	NCgl0697	1245	bacterial extracellular solute-binding protein		2.33
Cg0810	NCgl0676	1220	conserved hypothetical protein		2.54
Cg0814	NCgl0679	1224	bifunctional biotin ligase/biotin operon repressor	*birA*	2.50

Cg2380	NCgl2088	2306	putative membrane protein		2.17
Cg0418	NCgl0341	798	putative aminotransferase		2.11[b]
Cg1167	NCgl0985	1605	putative secreted protein		2.03
Cg0438	NCgl0353	822	putative glycosyltransferase		2.01
Cg2914	NCgl2542	1234	transposase	*tnp5b*	2.01[b]
Cg0760	NCgl0629	1161	probable methylisocitric acid lyase	*prpB2*	0.48
Cg0657	NCgl0628	1160	methylaconitase	*prpD2*	0.47
Cg2747	NCgl2411	3507	secreted peptidase, M23/M37 family		0.45
Cg2868	NCgl2503	2851	predicted extracellular nuclease	*nuc*	0.45
Cg0085	NCgl0064	442	ATPase related to phosphate starvation-inducible protein	*phoH1*	0.44
Cg0230	NCgl0182	575	glutamine 2-oxoglutarate aminotransferase	*gltD*	0.39
Cg1181	NCgl0995	1617	glycosyltransferase, probably involved in cell wall biogenesis		0.36
Cg1179	NCgl0994	1616	sensory BOX/GGDEF family protein		0.33
Cg0706	NCgl0584	1106	conserved hypotetical membrane protein		0.29
Cg1568	NCgl1329	2641	sn-glycerol-3-phosphate transport system permease protein	*ugpA*	0.29
Cg1569	NCgl1330	2640	sn-glycerol-3-phosphate transport system permease protein	*ugpE*	0.58
Cg1570	NCgl1331	2639	secreted sn-glycerol-3-phosphate-binding protein	*ugpB*	0.24
Cg1571	NCgl1332	2638	ABC-type sugar transport systems, ATPase component	*ugpC*	0.85
Cg3285	NCgl2863	378	putative two component response regulator	*cgtR9*	0.28
Cg3286	NCgl2864	380	putative secreted protein		0.28
Cg1182	NCgl0996	1619	putative membrane protein		0.27
Cg3322	NCgl2893	1873	putative secreted membrane-fusion protein		0.24

[a] The relative mRNA levels under P_i excess conditions in the *C. glutamicum* wild type compared to the mRNA levels under the same conditions in *C. glutamicum* Δ*phoRS* are averages from at least two of three experiments. Only ORFs whose mRNA levels were altered at least two-fold are shown.

[b] $P<0.05$ as determined by a t test.

3 Primer extension analyses of the *pstS*, *ugpA* and *phoR* promoters

The primer extension analysis was performed in order to identify the transcriptional start sites of the phosphate starvation inducible genes *pstS*, *ugpA* and *phoR*. Since the intensity of the primer extension products is proportional to the mRNA concentration, this method also allows a qualitative comparison of the mRNA levels in different samples, if the reverse transcriptase reactions contain equal amounts of template RNA. Thus, the primer extension analyses also served to verify the DNA microarray data. The conditions used for growth of *C. glutamicum* wild type and the Δ*phoRS* mutant in these experiments were the same as in the DNA chip experiments.

In the case of *pstS*, which is the first gene of *pstSCAB* operon, the extension products obtained with either of two different primers indicated that transcription starts 80 bp upstream of the GTG start codon with CGT. Primer extension products were observed both in the wild type and the Δ*phoRS* mutant after P_i starvation for 60 min, whereas no signal was observed in both strains after growth with phosphate excess. The signal intensity was much higher in the wild type compared to the Δ*phoRS* mutant (Fig. 21A). These results confirmed the data obtained from DNA microarray analysis where the *pst* genes were only partially induced in the Δ*phoRS* mutant compared to the wild type. We thus obtained further evidence that induction of *pstSCAB* by P_i starvation is dependent on PhoRS and a second unknown regulator. In the case of *ugpA*, which is the first gene of *ugpAEBC* operon, the primer extension experiments with two different primers indicated that transcription starts 1 bp upstream of the GTG start codon with CGT. Our expectations based on DNA chip results were in this case also fulfilled since the *ugpA* primer extension product was only observed in wild type cells grown under phosphate limitation (Fig. 21B). This result confirmed that PhoRS is required for P_i starvation-triggered induction of *ugpAEBC*. Concerning the *phoR* gene, primer extension analysis with one primer indicated that transcription starts 27 bp upstream of the ATG start codon with AAG. The primer phoR_prext1b used in this experiment anneals within the coding region of the *phoR* gene that is still present in the deletion mutant Δ*phoRS*. Due to this fact it was possible to determine the mRNA level of *phoR* also in the deletion mutant Δ*phoRS*. Figure 21C shows that the *phoR* primer extension product was observed after 10 min of P_i limitation in the wild type, but not in the Δ*phoRS* mutant. This result confirmed the DNA microarray data, where the mRNA levels of *phoR* and

phoS were 8- and 5-fold increased 10 min after a shift from P_i excess to P_i limitation, but returned to pre-shift levels within 60 min (Ishige *et al.*, 2003). In addition, they indicate that *phoRS* induction is dependent on the PhoRS two-component system, i.e. the *phoRS* genes are positively autoregulated. However, this conclusion is based on the assumption that the drastically shortened *phoRS* mRNA in the mutant has the same half-life as the wild-type *phoRS* mRNA. Primer extension analysis was also performed to identify the transcriptional start site of the low-affinity phosphate transporter gene *pitA.* Despite the fact that the experiment was performed several times with up to 40 µg template RNA, it was not possible to obtain a reliable result. A putative start site was found 55 bp upstream of the *pitA* start codon.

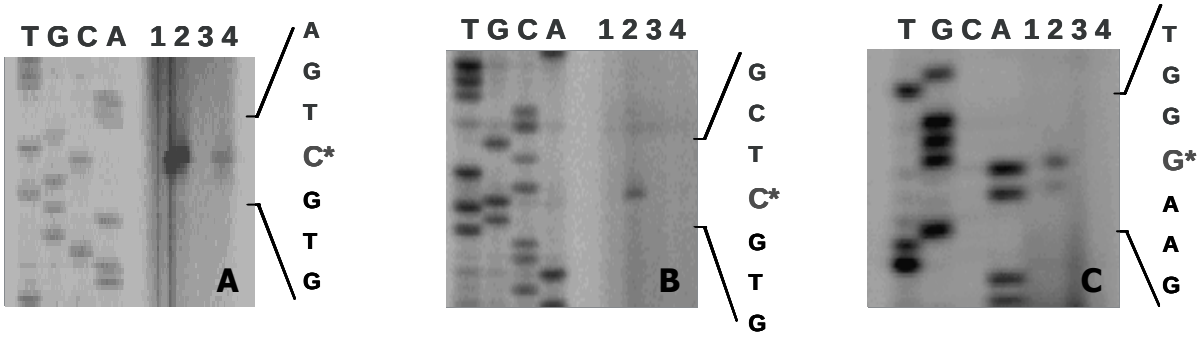


Fig. 21: Comparison of mRNA levels and determination of the transcriptional start sites of the *C. glutamicum pstS, ugpA* and *phoR* genes by primer extension analysis. The reverse transcriptase reactions were performed with the oligonucleotides pstS_prext2 (*pstS*; panel A), ugpA_prext2 (*ugpA*; panel B) and phoR_prext1b (*phoR*; panel C), respectively, and 20 µg of total RNA isolated from the following strains: wild type grown under phosphate excess (lane 1), wild type 60 min (panels A and B) or 10 min (panel C) after a shift from 13 mM P_i to 0.065 mM P_i (lane 2), Δ*phoRS* mutant grown under P_i excess (lane 3), and Δ*phoRS* mutant 60 min (panels A and B) or 10 min (panel C) 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 labelled oligonucleotide as in the primer extension reactions as well as PCR products, which cover the region of the respective transcriptional start site, as template DNA.

4 *In vitro* characterisation of the two-component system PhoRS

The analysis of *C. glutamicum* deletion mutants had revealed that the two-component system PhoS/PhoR plays an important role in the regulation of the phosphate starvation response. The RNA studies described above indicated that the PhoRS system is required for induction of the *psi* genes (e.g. *pstSCAB*, *ugpAEBC*, *phoRS* etc.) and eventually also for repression of the *pitA* gene under P_i starvation. In order to determine whether these effects are direct, the binding of the response regulator PhoR to the promoter region of these genes/operons had to be tested *in vitro*. According to the signal transduction mechanism determined for other sensor kinase/response regulator systems, it was assumed that PhoR has to be phosphorylated by PhoS before it binds to its target promoters. In order to demonstrate the autophosphorylation of PhoS, the phosphoryl transfer to PhoR and the binding of PhoR to DNA *in vitro*, tagged derivatives of PhoS and PhoR were constructed, overproduced in *E. coli* and purified.

4.1 Overproduction and purification of $PhoS_{His}$

For the purification of the sensor kinase PhoS, an expression plasmid for overexpression of *phoS* in *E. coli* was constructed (pET24-*phoS*) that encodes a PhoS protein with eight additional amino acid residues at its C-terminus (LEHHHHHH), including a hexahistidine tag. Cultures of *E. coli* C43(DE3)/pET24-*phoS* were grown at 37 °C to an OD_{600} of ~0.6 before adding IPTG and then the growth was continued for another 4 h at room temperature. After IPTG induction, the plasmid pET24b-*phoS* directed the formation of a protein of the expected size (53.4 kDa), as could be demonstrated by SDS-PAGE analysis of the cells (Fig. 22, lane 2). According to its hydropathy profile, PhoS contains two transmembrane helices extending from residues 44 to 64 and from 184 to 204 and therefore should be an integral membrane protein. SDS-PAGE analysis of the fractions obtained after ultracentrifugation of the cell-free extract revealed $PhoS_{His}$ to be present in the pellet, showing that $PhoS_{His}$ was indeed located in the membrane fraction (Fig. 22, lane 4). In order to purify $PhoS_{His}$, it was solubilized from the membrane with the detergent lauryldimethylamineoxide (LDAO) at a concentration of 2% (v/v), separated from the insoluble protein fraction by ultracentrifugation (Fig. 22, lane 5), and isolated by Ni^{2+} chelate affinity chromatography (Fig. 22, lane 6). The eluted protein was essentially pure as estimated by Comassie-stained SDS polyacrylamide

gels (Fig. 22, lane 6). Approximately 45 % of PhoS could be solubilized from the membrane and the final yield of $PhoS_{His}$ was approx. 0.2 mg per g cell paste (wet weight).

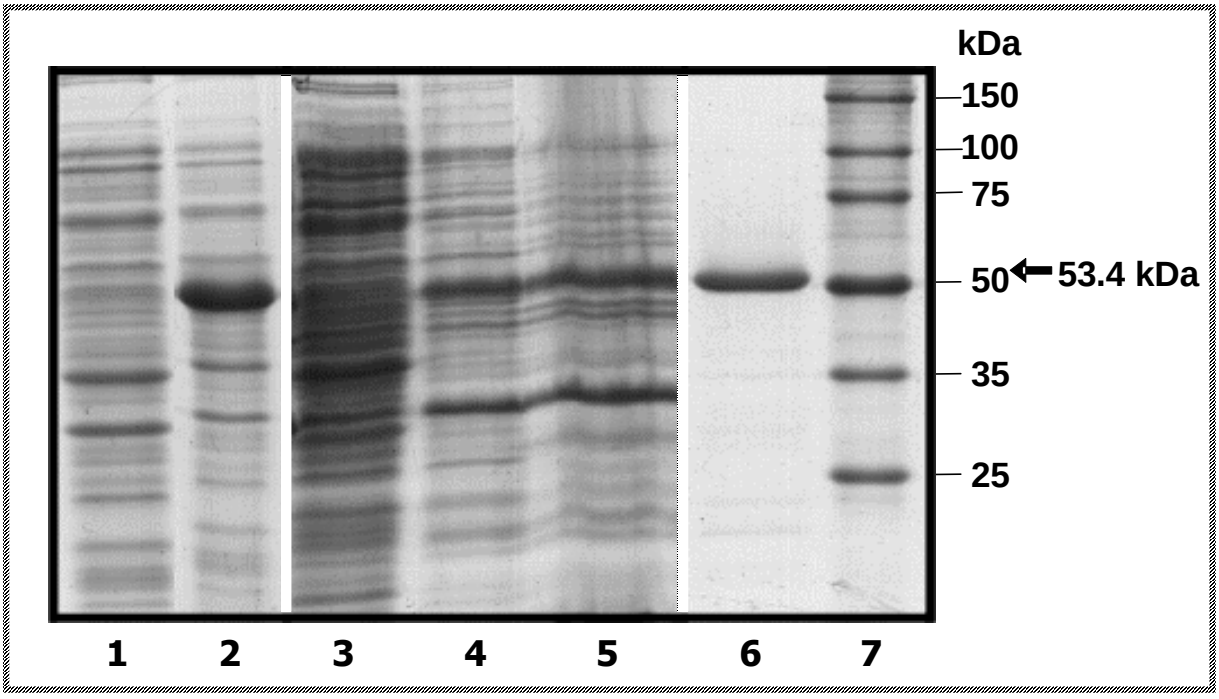


Fig. 22: Overproduction and isolation of $PhoS_{His}$. Coomassie-stained SDS polyacrylamide gel containing the following samples: whole-cell lysate of *E. coli* C43(DE3)/pET24b-*phoS* prior to (lane 1) and 4 h after (lane 2) IPTG induction, soluble cell fraction (lane 3), membrane fraction (lane 4), LDAO-soluble proteins (lane 5), purified $PhoS_{His}$ protein (lane 6) after affinity chromatography on Ni^{2+}-NTA agarose, and protein standard (lane 7).

4.2 Overproduction and purification of $PhoR_{His}$ and its C-terminal domain

In order to purify the response regulator PhoR, a PhoR derivative with a C-terminal $(His)_6$ tag was cloned into plasmid pET24b. This plasmid (pET24-*phoR*) was transferred into the *E. coli* strain BL21(DE3). After induction with IPTG, a dominant protein band was observed after SDS-PAGE of whole-cell extracts (Fig. 23, lane 2). Its apparent size of about 27 kDa corresponds well with the predicted molecular mass of $PhoR_{His}$ (27.4 kDa). In the first overproduction culture, the cells were incubated at 37°C after IPTG addition, which resulted in the formation of inclusion bodies (data not shown). Therefore, in the following cultivations cells were incubated at room temperature after IPTG induction rather than at 37 °C. After this

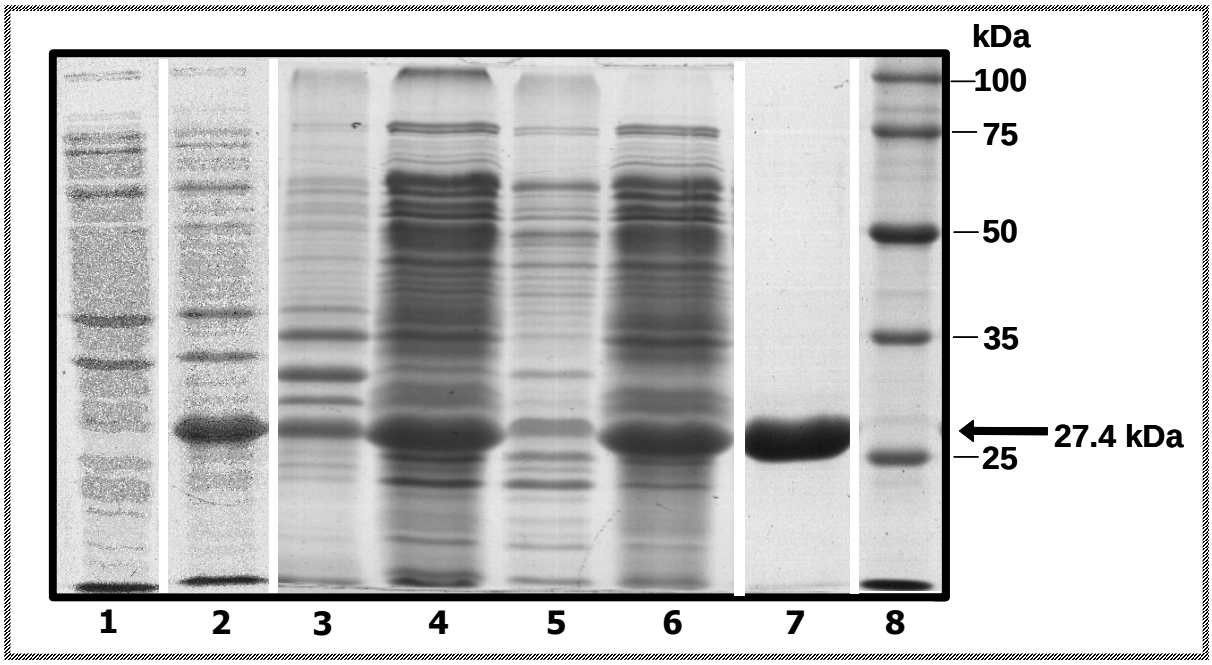


Fig. 23: Overproduction and isolation of $PhoR_{His}$. Coomassie-stained SDS polyacrylamide gel containing the following samples: whole-cell lysate of *E. coli* BL21(DE3)/pET24b-*phoR* prior to (lane 1) and 5 h after (lane 2) IPTG induction, low-speed centrifugation pellet (lane 3), cell free extract (lane 4), membrane fraction (lane 5), soluble cell fraction (lane 6), purified $PhoR_{His}$ protein (lane 7) after affinity chromatography on Ni^{2+}-NTA agarose, and protein standard (lane 8).

change, a major portion of the overproduced protein was found in the supernatant after low-speed centrifugation of cell-free extract (Fig. 23, lane 4). After ultracentrifugation, the band corresponding to PhoR protein was present in supernatant (Fig. 23, lane 6) indicating that PhoR has been obtained in soluble form. Finally, purification of PhoR was achieved by Ni^{2+} chelate affinity chromatography. The eluted protein was essentially pure as estimated by Comassie-stained SDS polyacrylamide gels (Fig. 23, lane 7). The yield of $PhoR_{His}$ was approximately 5 mg per g cell paste (wet weight).

As indicated below, the attempts to show binding of $PhoR_{His}$ to its presumed target promoters failed. Since there was evidence in the literature that the isolated C-terminal binding domain of some response regulators is constitutively active (Molle *et al*., 2003), we decided to isolate also the separated DNA binding domain of PhoR and to analyse its binding properties *in vitro*. Based on an amino acid sequence alignment, two variants of the PhoR binding domain were constructed, one including the linker region that connects the receiver

domain and the DNA-binding domain (PhoRΔ1-125) and one without the linker region (PhoRΔ1-139). The corresponding coding regions were cloned into the expression plasmid pET16b, resulting in the expression plasmids pET16b-*phoRΔ1-125* and pET16b-*phoRΔ1-139*. Both PhoR derivatives contain an N-terminal $(His)_{10}$-tag. These plasmids were transferred into *E. coli* BL21(DE3). The resulting strains were grown at 37 °C before adding IPTG and

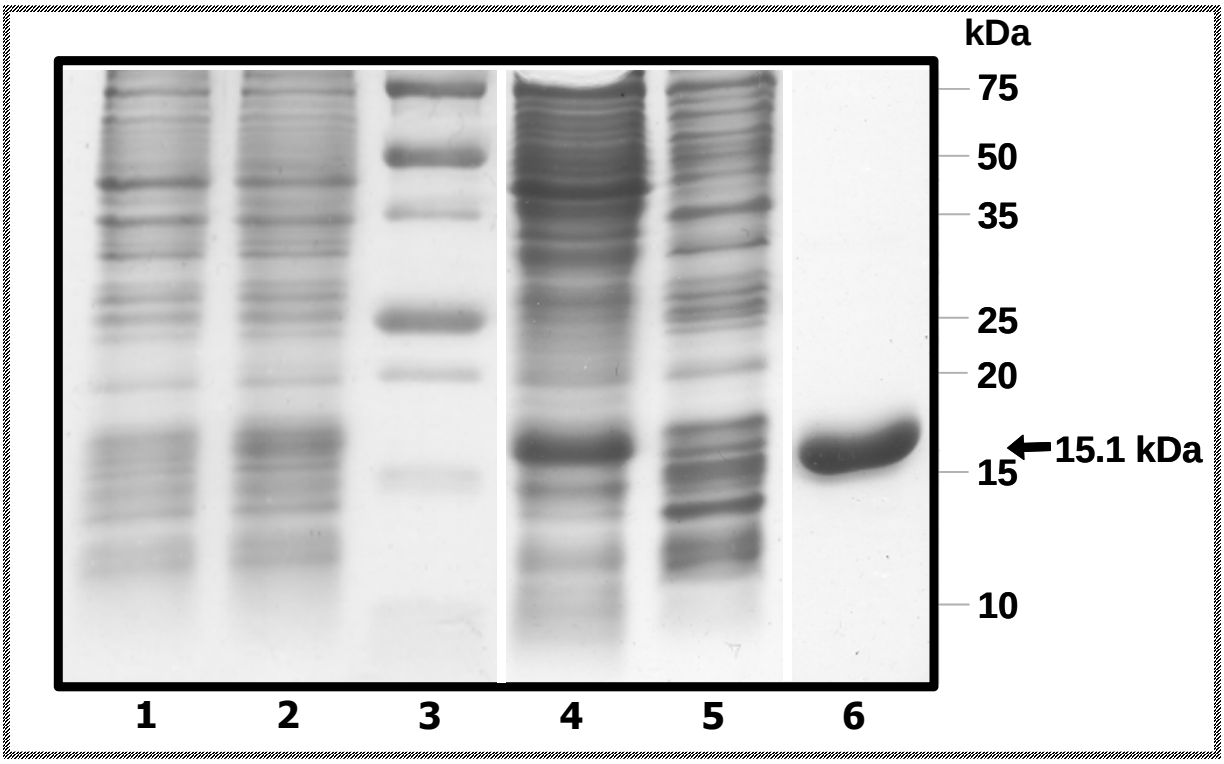


Fig. 24: Overproduction and isolation of PhoRΔ1-125. Coomassie-stained SDS polyacrylamide gel containing the following samples: whole-cell lysate of *E. coli* BL21(DE3)/pET16b-*phoRΔ1-125* prior to (lane 1) and 4 h after (lane 2) IPTG induction, protein standard (lane 3), soluble cell fraction (lane 4), membrane fraction (lane 5), purified PhoRΔ1-125 protein (lane 6) after affinity chromatography on Ni^{2+}-NTA agarose.

then growth was continued at room temperature. After induction with IPTG, protein bands of the expected size (15.1 kDa and 13.6 kDa) were observed after SDS-PAGE of whole-cell extracts (Fig. 24, lane 2 and Fig. 25, lane 2). Fractionation of the cells revealed that the proteins were present in the supernatant obtained after ultracentrifugation of the cell-free extract, indicating that PhoRΔ1-125 and PhoRΔ1-139 have been obtained in a soluble form (Fig. 24, lane 4 and Fig. 25, lane 3). Purification of both proteins was achieved by Ni^{2+} chelate

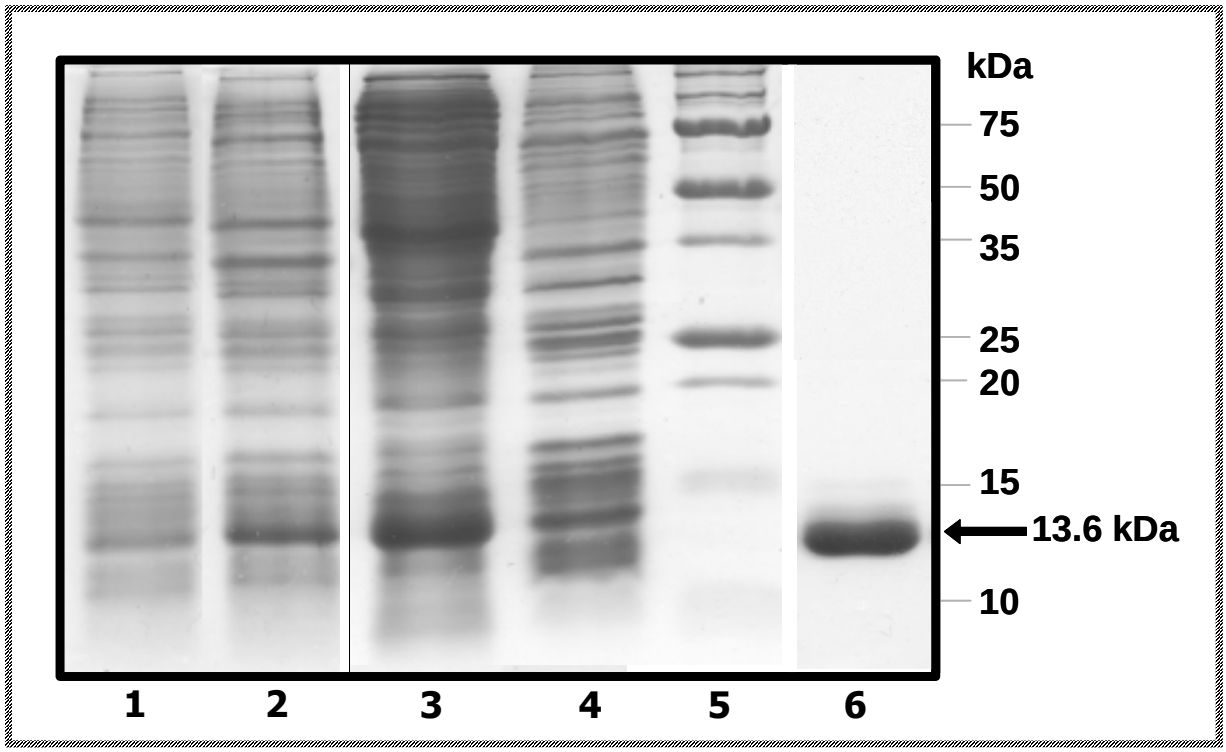


Fig. 25: Overproduction and isolation of PhoRΔ1-139. Coomassie-stained SDS polyacrylamide gel containing the following samples: whole-cell lysate of *E. coli* BL21(DE3)/pET16b-*phoRΔ1-139* prior to (lane 1) and 4 h after (lane 2) IPTG induction, soluble cell fraction (lane 3), membrane fraction (lane 4), protein standard (lane 5), purified PhoRΔ1-139 protein (lane 6) after affinity chromatography on Ni^{2+}-NTA agarose.

affinity chromatography. The eluted proteins were essentially pure as estimated by Coomassie-stained polyacrylamide gels (Fig. 24, lane 6 and Fig. 25, lane 6).

4.3 Phosphorylation studies with $PhoS_{His}$ and $PhoR_{His}$

With the purified $PhoS_{His}$ and $PhoR_{His}$ proteins, the reactions characteristic for two-component signal transduction systems were tested, i.e. the autokinase activity of PhoS and the phosphoryltransfer from phosphorylated PhoS to PhoR.

In order to test the autokinase activity of PhoS, the purified His-tagged protein was incubated with γ-[^{32}P]-ATP (final conc. 0.7 μM) and subsequently analyzed by SDS-PAGE and autoradiography. As shown in Fig. 26, $PhoS_{His}$ (final conc. 2.7 μM) was capable of autophosphorylation (Fig. 26A, lanes for 1 and 30 min). When $PhoR_{His}$ (final conc. 8.2 μM) was added to the reaction mixture containing phosphorylated $PhoS_{His}$ (indicated by the arrow

in Fig. 26A), a rapid phosphotransfer from $PhoS_{His}$~P to $PhoR_{His}$ occurred (Fig. 26A, lanes for 32 and 60 min). $PhoR_{His}$ alone could not be phosphorylated by γ-[^{32}P]-ATP (data not shown). This result indicates that the *C. glutamicum* $PhoS_{His}$ protein produced in *E. coli* functions as an autokinase and as a kinase of $PhoR_{His}$ *in vitro*. Surprisingly, addition of $PhoR_{His}$ to the reaction mixture containing phosphorylated $PhoS_{His}$ resulted in the appearance of two labelled bands after SDS-PAGE, one having the size expected for $PhoR_{His}$, i.e. ~27 kDa, and the other one having a size of ~50 kDa. The latter most likely represents a PhoR dimer and its presence in an SDS gel presumably was possible because the samples were not heat-denatured before application to the gel. After Coomassie-staining of the gel, only the protein bands of 53 kDa and of 27 kDa corresponding to the monomeric PhoS and PhoR proteins could be detected (Fig. 26B). This means that both the phosphorylated dimeric PhoR and the phosphorylated monomeric PhoR represent only a very small fraction of the total PhoR protein present on the gel.

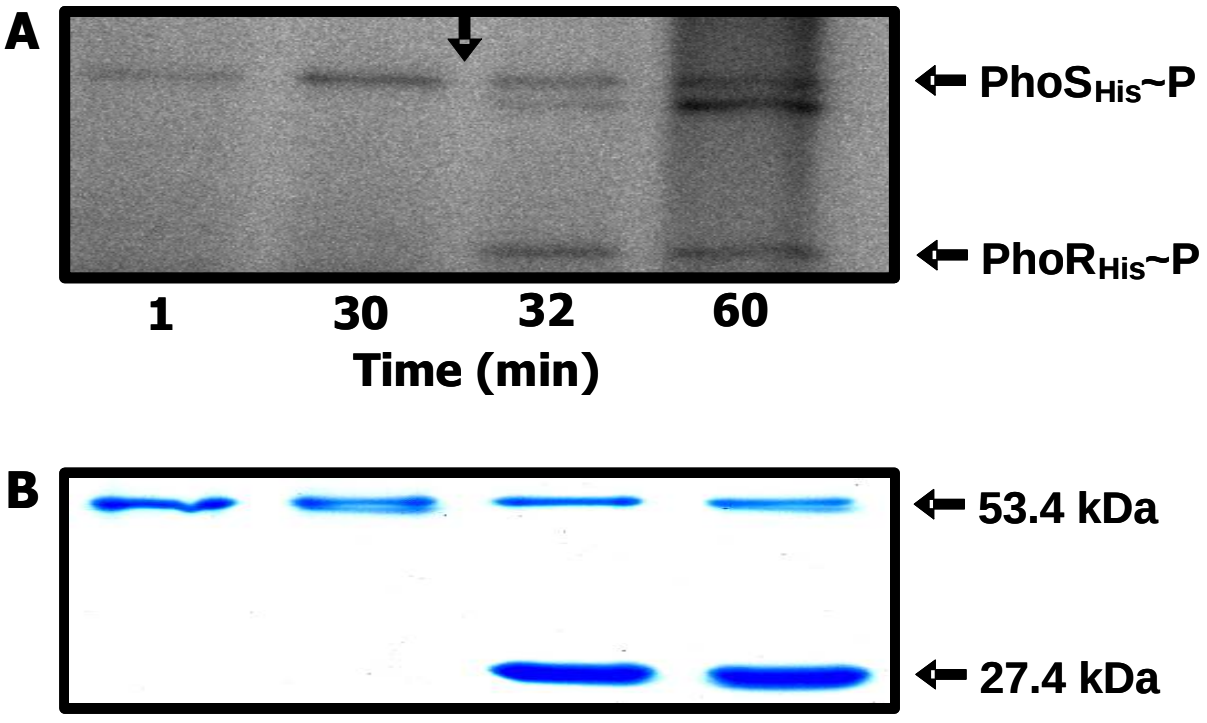


Fig. 26: Autophosphorylation of $PhoS_{His}$ and phosphotransfer from $PhoS_{His}$ to $PhoR_{His}$. $PhoS_{His}$ was phosphorylated for 30 min with γ-[^{32}P]-ATP (0.67 µM), and then a 3-fold excess of $PhoR_{His}$ was added. At the time points indicated, 5 µl aliquots corresponding to 0.27 µg PhoS were removed, mixed with 5 µl 2x SDS loading buffer, and subject to gel electrophoresis. Autoradiography was performed with a PhosphoImager (A) afterwards the gel was stained in Coomassie blue (B).

4.4 DNA-binding studies with $PhoR_{His}$ and its C-terminal domain

4.4.1 Gel retardation assays

The DNA microarray and primer extension studies had indicated that the response regulator PhoR acts as positive regulator of the *psi* genes (e.g. *pstSCAB*, *ugpAEBC*, *phoRS* etc.) and possibly also as a negative regulator of the *pitA* gene. In order to show if these genes are direct target genes of PhoR, gel retardation assays were performed with the purified PhoR derivatives and DNA fragments covering the promoter regions of the operons *pstSCAB*, *ugpAEBC* and *phoRS* and of the monocistronic *pitA* gene. As negative controls, DNA regions covering the promoter regions of the *clpP1P2* operon and of the *clpC* gene (Engels *et al.*, 2004) were used. The DNA probes were generated by PCR and radioactively labelled using γ-[^{32}P]-ATP and T4 polynucleotide kinase.

In a first series of experiments, binding of the $PhoR_{His}$ protein to its target promoters was tested. In order to determine the effect of phosphorylation on the DNA-binding capability of PhoR, the protein was used either as isolated or after 1 h incubation at 37 °C with $PhoS_{His}$ and ATP or after incubation with acetyl phosphate, which might also lead to phosphorylated PhoR. Serial dilutions of the PhoR protein (either untreated or treated as described above) were incubated with a constant amount of the [^{32}P]-labelled DNA fragment for 20 min at room temperature and then the mixtures were separated on a native polyacrylamide gel, which subsequently was dried and analysed with a Phosphoimager. However, with none of the promoter regions tested a shift occurred in the presence of $PhoR_{His}$, either untreated or treated. As an example, the experiments with the *ugpA*, *pstS* and *phoR* promoter regions are shown in Fig. 27. Despite the fact that all PCR products used for the gel shift assays were purified as a single band from agarose gels before labelling, in all cases two or three labelled fragment of different sizes were observed after autoradiography (Fig. 27). There is no clear explanation for this phenomenon, however one possible reason could be that the DNA isolated from the agarose gel contains both double-stranded and single-stranded form, both of which are phosphorylated.

The result that no binding of the $PhoR_{His}$ protein to any of the proposed target promoters could be shown can be explained either by the assumption that regulation of the analysed promoters by PhoR occurs indirectly via another regulator or by the assumption that the experimental conditions were insufficient for binding. For example, if only the phosphorylated PhoR is capable of binding, the concentration of phosphorylated PhoR might

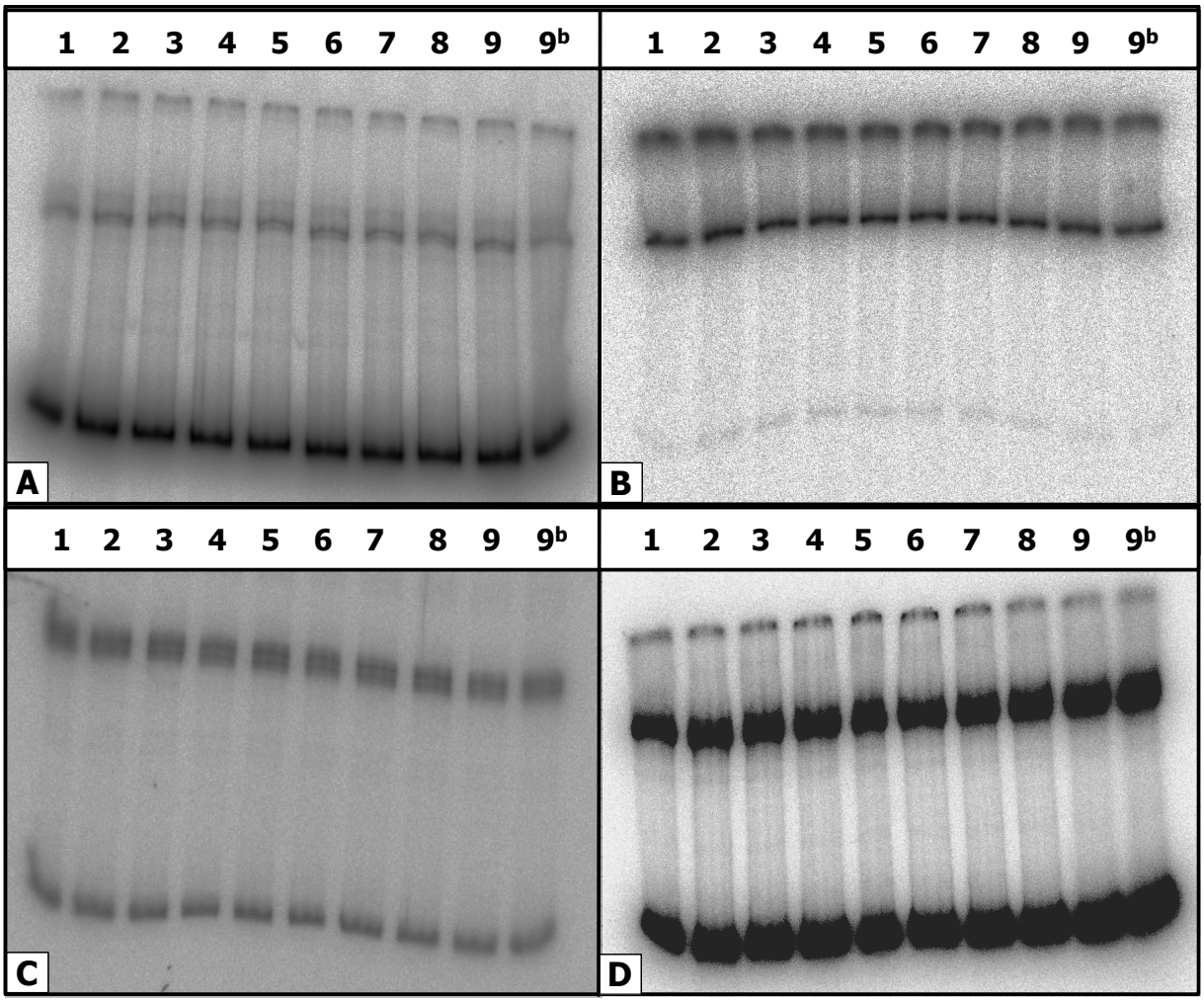


Fig. 27: Gel shift assays with the promoter regions of *ugpA* (A, D, 407 bp), *pstS* (B, 1383 bp) or *phoR* (C, 317 bp) and the response regulator $PhoR_{His}$ preincubated for 1 h at 37 °C with 1.1 µM $PhoS_{His}$ plus 5 mM ATP (A-C) or with 50 mM acetyl phosphate (D). The concentration of $PhoR_{His}$ protein in the reaction mixtures increased from left to right. For figures A-C: lane 1: 0 µM, lane 2: 0.02 µM, lane 3: 0.04 µM, lane 4: 0.08 µM, lane 5: 0.16 µM, lane 6: 0.31 µM, lane 7: 0.63 µM, lane 8: 1.25 µM, lane 9: 2.5 µM. For figure D: lane 1: 0 µM, lane 2: 0.2 µM, lane 3: 0.39 µM, lane 4: 0.78 µM, lane 5: 1.56 µM, lane 6: 3.12 µM, lane 7: 6.25 µM, lane 8: 12.5 µM, lane 9: 19.5 µM final concentration. The binding reactions contained the $PhoR_{His}$ concentrations indicated above, approximately 2 nM labelled DNA fragment (DNA was labelled with [γ-^{32}P]-ATP, as described in Materials and Methods) and the DNA competitor poly-(dI-dC) (2 µg in 20 µl reaction mixture) in a buffer composed of 50 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 10 % glycerol (final concentrations). In the lanes labelled with ^{b}, the reaction mixtures contained an excess of unlabelled DNA fragment (35 nM) instead of poly-(dI-dC).

have been too low to detect binding. Alternatively, the C-terminal hexahistidine tag of the PhoR protein used might interfere with DNA binding. In order to test the latter two possibilities, the gel retardation assays were repeated with the PhoR derivatives PhoRΔ1-125 and PhoRΔ1-139. These derivatives lack the N-terminal receiver domain and might possess constitutive DNA-binding activity, as shown for other response regulators (Molle *et al.*, 2003). Moreover, their His tag is located at N-terminal end rather than on the C-terminal end. The two constructs differ by the presence (PhoRΔ1-125) or absence (PhoRΔ1-139) of the linker region connecting the receiver domain and the DNA-binding domain of PhoR.

In a first experiment, binding of PhoRΔ1-139 and PhoRΔ1-125 to the *ugpA* promoter was tested. With the shorter derivative, only very weak binding was observed and therefore this protein was not used in the further experiments. However, the PhoRΔ1-125 protein did bind to the *ugpA* promoter fragment, leading to its retardation in the gel electrophoresis. As shown in Fig. 28, with increasing amounts of PhoRΔ1-125 in the binding reactions, the free labelled DNA fragment gradually disappeared and a new labelled band appeared corresponding to a DNA-protein complex. A retarded band was seen already at the lowest PhoRΔ1-125 concentration used, i.e. 20 nM. Besides the *ugpA* promoter fragment, PhoRΔ1-125 also shifted the promoter fragments of *pstS* (Fig. 28B), *phoR* (Fig. 28C) and *pitA* (Fig. 28E). In the case of the *pstS* promoter fragment, three labelled species were observed already in the absence of protein. Addition of increasing concentrations of PhoRΔ1-125 led to a shift of two of the labelled fragments, whereas the third one located immediately at the upper end of the gel was not retarded. In this experiment, the first clearly visible retardation was observed at a PhoRΔ1-125 concentration of 290 nM. In the case of the *phoR* promoter, two labelled bands are visible in the absence of protein. Only the faster migrating one was shifted by PhoRΔ1-125, with retardation becoming clearly visible at a concentration of 290 nM. In the case of the *pitA* promoter, the DNA fragment was also clearly retarded at a PhoRΔ1-125 concentration of 290 nM. Whereas the complex formed by PhoRΔ1-125 and the *ugpA* promoter fragment appeared as a distinct band in the gel, the complexes formed with the promoter regions of *pstS*, *phoR* and *pitA* were less well defined.

The specificity of the bandshifts described above was confirmed by the observation that addition of an approximately 20-fold excess of unlabelled *ugpA* probe (data not shown), *pstS* probe (Fig. 28B, lane 6^b), *phoR* probe (Fig. 28C, lane 6^b) and *pitA* probe (Fig. 28E, lane 6^b) to the respective binding reactions led to an inhibition of the retardation of the labelled fragment. Further support for the specificity of the interaction between PhoRΔ1-125 and the promoter

regions of *ugpA, pstS, phoR* and *pitA* was obtained by the fact that fragments covering the promoter regions of *clpP1P2* (Fig. 28D) and *clpC* (data not shown) were not retarded by high concentrations of this protein.

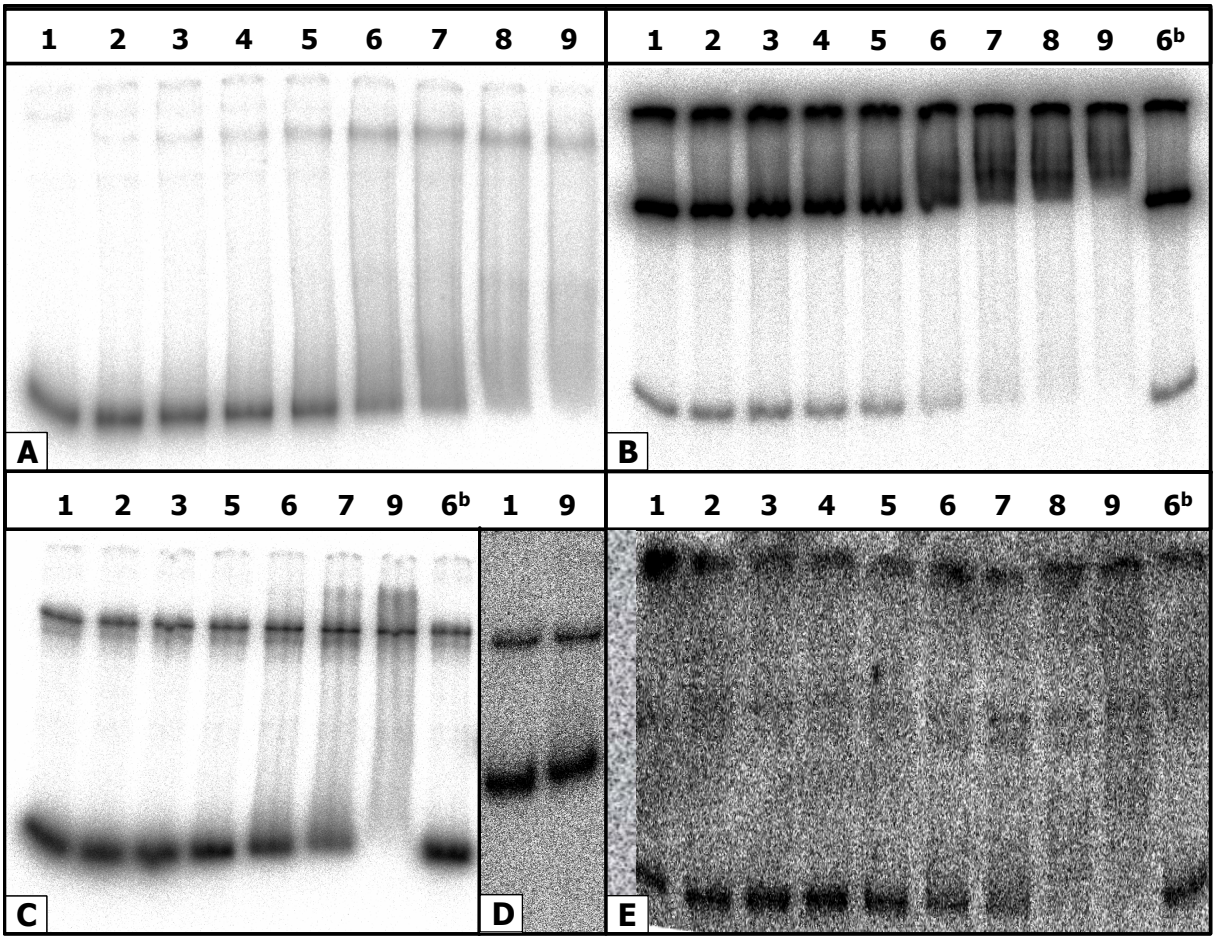


Fig. 28: Gel shift assays with the DNA-binding domain of the response regulator PhoR (PhoRΔ1-125) and the promoter regions of *ugpA* (A, 407 bp), *pstS* (B, 1383 bp), *phoR* (C, 317 bp), *clpP1P2* (D, 548 bp) and *pitA* (E, 565 bp). The concentrations of the PhoRΔ1-125 protein present in the binding reactions were as follows: lane 1: 0 µM, lane 2: 0.02 µM, lane 3: 0.04 µM, lane 4: 0.07 µM, lane 5: 0.14 µM, lane 6: 0.29 µM, lane 7: 0.58 µM, lane 8: 1.15 µM, lane 9: 2.3 µM. The binding reactions contained the PhoRΔ1-125 concentrations indicated above, approximately 2 nM labelled DNA and poly-(dI-dC) (2 µg in 20 µl reaction mixture) competitor DNA in a buffer composed of 50 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 10 % glycerol (final concentrations). In the lanes labelled with ^{b}, the reaction mixtures contained an excess of unlabelled DNA fragment (35 nM) instead of poly-(dI-dC).

4.4.2 DNase I footprinting assays

In order to determine the PhoR binding site in front of the *pstS* operon, DNase I footprinting assays were performed with the PhoR binding domain PhoRΔ1-125. A 558 bp DNA fragment encompassing the *pstS* regulatory region (from -430 to +128 with respect to the *pstS* transcription start site) was obtained by PCR amplification the olignucleotides pstS_footpr_rv and pstS_footpr_fw_IRD800, the latter of which contained an IRD800 label at the 5'-end. With the intention to optimize the conditions for binding of PhoRΔ1-125 to the *pst*S promoter region, two different buffers were used and compared. In the first case, the binding reaction was prepared in the buffer that was also used for the gel retardation assays (50 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA and 10 % glycerol). In order to reach the optimal salt concentration for DNase I (which partially degrades the DNA) but at the same time not to disturb protein-DNA complex, the concentration of salts was increased by addition of the 10 µl salt solution (100 mM $MgCl_2$, 50 mM $CaCl_2$), into a total volume of 210 µl meaning that final concentrations were 14.8 mM $MgCl_2$ and 2.4 mM $CaCl_2$ after incubation of the protein with DNA and before the addition of DNase I. The second type of buffer in which the binding reactions were prepared was called "footprint" buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5% (v/v) glycerol, 0.5 mM DTT, 0.005% (v/v) Triton X-100 and 50 mM NaCl), since it had been used previously for DNase I footprinting with another protein and its target promoters (Engels *et al*., 2004). In this case, 10 µl of the salt solution (100 mM $MgCl_2$, 50 mM $CaCl_2$) was added into the reaction mixture (total volume 200 µl) meaning that the final concentrations were 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ before incubation of protein with DNA.

As can be seen in Fig. 29, the use of the band-shift buffer resulted in a better resolution of the DNase I footprint than the "footprint" buffer. However, with both buffers a protection from DNase I digestion of the *pstS* promoter region by PhoRΔ1-125 was observed. The protected region stretched from approximately -170 to -205 relative to the *pstS* transcriptional start site (Fig. 30).

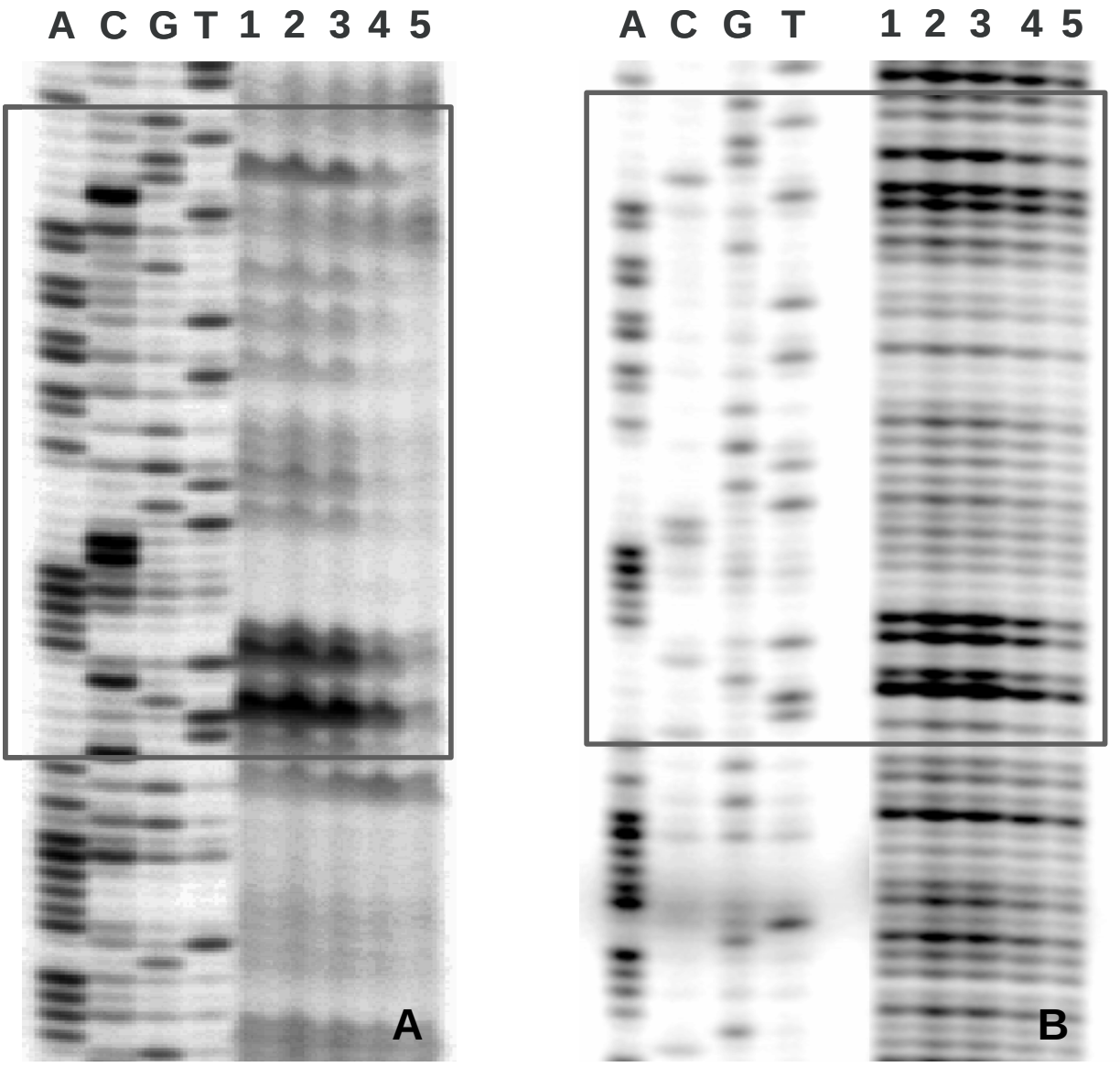


Fig. 29: DNase I footprints with PhoRΔ1-125 and the *pstS* promoter region using "footprint" binding buffer (A) or band-shift buffer (the same which was used for the gel retardation assay) (B). The concentration of PhoRΔ1-125 protein present in the reaction mixtures was as follows: lane 1: 0 μM, lane 2: 0.04 μM, lane 3: 0.14 μM, lane 4: 0.58 μM, lane 5: 2.3 μM. The binding reactions consisted of PhoR Δ1-125 protein, 2 nM labelled DNA sample and 0.5 μg poly-(dI-dC) competitor DNA. Protected regions are indicated by frames. The corresponding sequencing reactions were set up using the same IRD-800-labelled oligonucleotide as for generating the labelled footprinting probes and PCR products identical in sequence to the footprinting probes, as template DNA.

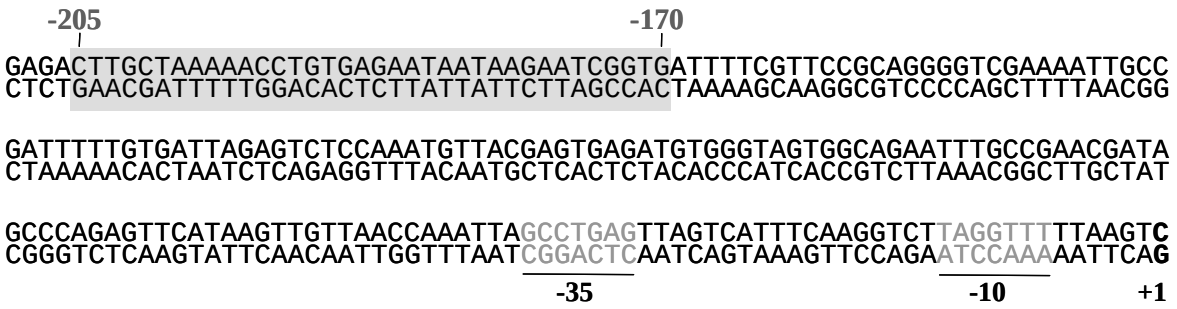


Fig. 30: DNA sequence of the *pstS* promoter region. Indicated are the transcriptional start site (+1) as determined by primer extension, the -10 and -35 regions (underlined) and the PhoR binding site as determined by DNase I footprints (grey box).

VI DISCUSSION

1 Evidence for the involvement of the PhoR/PhoS two-component system in the phosphate starvation response of *C. glutamicum*

In many bacteria whose phosphate starvation response has been studied in some detail, e.g. *Escherichia coli* or *Bacillus subtilis*, two-component signal transduction systems play a central regulatory role in this process. In the work presented here, strong evidence was obtained that this is also the case in *Corynebacterium glutamicum.* Phenotypic, genetic and biochemical data indicate that the two-component system PhoRS plays a key role in the adaptation of *C. glutamicum* to phosphate starvation. Screening of 13 *C. glutamicum* deletion mutants each lacking one specific two-component system revealed that one of them, 13032Δ*phoRS*, is specifically impaired in its ability to grow under P_i limitation. A complementation experiment confirmed 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 by DNA microarray studies aimed at the elucidation of the P_i starvation stimulon of *C. glutamicum* (Ishige *et al*., 2003). In these studies, the mRNA levels of *phoR* and *phoS* were strongly increased 10 min after a shift from P_i excess to P_i limitation but reached pre-shift levels within 60 min. Of the other 24 two-component genes present in *C. glutamicum*, only *cgtR9* showed an altered expression after the shift. Its mRNA level was strongly increased 30 min after the shift and remained increased up to 120 min after the shift (Ishige *et al*., 2003). The rapid and transient induction of the *phoRS* genes suggest their involvement in the P_i starvation response.

Transcriptome comparisons with DNA microarrays of the Δ*phoRS* mutant before and after a shift from P_i excess to P_i limitation revealed that except for *pstSCAB* none of the other *psi* genes were induced within 60 min after the shift, i.e. *ugpAEBC*, *glpQ*, *phoH*, *ushA* and *nucH*. This was in marked contrast to the wild type, in which these genes are strongly induced within 60 min after the shift (Ishige *et al.*, 2003). This difference was confirmed by direct comparisons of the transcriptome of the wild type and the Δ*phoRS* mutant, which showed an increased mRNA level of the *psi* genes in the wild type. The results of the microarrays regarding the expression pattern of the *pst*, *ugp* and *phoRS* genes was confirmed

independently by primer extension analysis. The induction of the *pstSCAB* genes in the Δ*phoRS* mutant suggests the presence of an additional regulator, which is not yet known. A situation where more than one regulator is involved in the phosphate starvation response is not unusual and known for example in *B. subtilis*, where at least three two-component systems interact during phosphate starvation regulation (Birkey *et al.*, 1998). In the case of the genes *pctB* and *pctC* the absence of reliable data preclude a statement on their regulation by the PhoS/PhoR system.

Hierarchical cluster analysis of the genes showing altered expression in the *C. glutamicum* wild type after a shift from P_i excess to P_i-limiting conditions led to the identification of five subclusters, one containing the *psi* genes and four containing predominantly genes which are not directly involved in phosphorus metabolism (Ishige *et al*., 2003). Subcluster 2 includes genes connected with copper metabolism (transiently increased expression after the shift) and subcluster 5 genes connected with iron metabolism (transiently reduced expression after the shift). These genes showed the same expression pattern in the Δ*phoRS* mutant after the shift from P_i excess to P_i limitation, confirming that their expression changes are independent from PhoRS. Subcluster 3 contains, besides others, several genes presumably involved in the degradation of protocatechuate, which is present in the CGXII medium to facilitate iron uptake. These genes have increased mRNA levels after the shift, because the fresh medium contains protocatechuate, which can serve as a carbon source. In a few of the microarray experiments with the Δ*phoRS* mutant these genes were also induced after the shift (orf1655 4x; orf64185 1x, orf64187 1x, orf66073 2x, orf66513 2x), again indicating that the altered expression of these genes is independent of PhoRS. Subcluster 4 contains predominantly genes coding for ribosomal proteins, whose expression begins to decrease approx. 60 min after the shift in the wild type, due to the reduced growth rate. In the Δ*phoRS* mutant, these genes were unchanged, presumably because the changes were analysed only up to 60 min after the shift and not later, as in the wild type. In summary, these results clearly show that specifically the expression of the *psi* genes is impaired in the Δ*phoRS* mutant, whereas the altered expression of the many other genes is independent of PhoRS.

Regarding the expression of the *phoRS* genes, the primer extension studies suggest a positive autoregulation, since a *phoRS* transcript was only detected in the wild type 10 min after a shift to P_i limitation, but not in the Δ*phoRS* mutant. However, the negative result in the mutant might also be due to a rapid degradation of the extremely short *phoRS* mRNA formed in the Δ*phoRS* mutant. Additional studies will be required to unequivocally confirm a positive

autoregulation of the *phoRS* genes.

Concerning the expression of the *pitA* gene encoding a low affinity phosphate transporter, the current data suggest that the PhoRS two-component system represses *pitA* under P_i-limited conditions. The DNA microarray analyses showed a 2-fold decreased mRNA level of *pitA* in the wild type compared to the Δ*phoRS* mutant 60 min after the shift to P_i limitation. Since the *pitA* mRNA level was unaltered in the Δ*phoRS* mutant after a shift from P_i excess to P_i limitation, a decrease in the wild type had to be assumed. Indeed, inspection of the microarray results obtained for the wild type by Ishige *et al*. (2003) revealed that the *pitA* mRNA level was 2- to 8-fold decreased 60 min after the shift and later. The reason why *pitA* is absent in the tables given by Ishige *et al*. is that its average mRNA ratio was below the cut-offs used (≥4 or ≤0.25). In summary, the *pitA* gene appears to be repressed in *C. glutamicum* wild type under P_i-limited conditions in a PhoSR-dependent manner. This is in contrast to *E. coli*, where *pitA* appears to be constitutively expressed (Harris *et al*., 2001). It is not yet clear, whether repression of *pitA* in *C. glutamicum* has an advantage except that precursors and energy required for the synthesis of PitA are saved. The indications that PhoR can act not only as an activator, but also as a repressor resembles the situation in *B. subtilis*, where the response regulator PhoP is required for activation of several genes (see Introduction) but also for repression of the *tagAB* and *tagCDE* operons (Liu *et al*., 1998).

Many bacteria secrete alkaline phosphatases under P_i limitation, in order to obtain P_i from organophosphates which subsequently is taken up by the Pst system. In order to test for alkaline phosphatase activity and regulation in *C. glutamicum*, growth of the wild type and of the two-component deletion mutants on CGXII glucose plates with different P_i concentrations and X-phosphate as indicator was analysed. Indeed, the colonies became blue under P_i limiting conditions, confirming the presence of an inducible alkaline phosphatase activity. However, this activity was also present in the Δ*phoRS* mutant, indicating that at least one alkaline phosphatase exists whose induction under P_i limitation is independent of the PhoS/PhoR system. In a recent survey of *C. glutamicum* genes presumably involved in P metabolism, two alkaline phosphatase genes, *phoB* (Cg2700, NCgl2371) and *phoD* (Cg2485, NCgl2185), and one putative phosphoesterase gene (Cg3393, NCgl2959) were identified (Wendisch and Bott, 2004). Only the latter one was found to be induced under P_i starvation in the wild type (Ishige *et al.*, 2003). In the screening experiment it was observed that the alkaline phosphatase activity in strain 13032Δ*cgtSR4*/pXMJ19CgtR4 was induced earlier or

stronger than in the wild type and the other two-component deletion mutants. This indicates a positive role of the CgtS4-CgtR4 two-component system in the induction. Previous studies on this system also suggested an involvement of CgtR4 in the regulation of some *psi* genes (Wessel, 2003). Remarkably, the proteins with the highest sequence identity to CgtS4 and CgtR4 in *Streptomyces lividans*, PhoP and PhoR, were shown to be involved in the phosphate starvation response (Sola-Landa *et al*., 2003). *S. lividans* deletion mutants lacking *phoP* or *phoR-phoP* were unable to grow in minimal medium at a low P_i concentration (10 µM). The PhoP-PhoR system was shown to be required for expression of the alkaline phosphatase gene *phoA* and for high-affinity phosphate uptake (Sola-Landa *et al*., 2003). Moreover, the biosynthesis of two secondary metabolites, actinorhodin and undecylprodigiosin, was significantly increased in both solid and liquid medium in the Δ*phoP* or Δ*phoR-phoP* deletion mutants.

In *Mycobacterium smegmatis* the alkaline phosphatase gene *phoA* was recently described and shown to be up-regulated under P_i starvation, but constitutively expressed in strains with mutations in genes of the *pst* operon. Unlike *E. coli* PhoA, the *M. smegmatis phoA* gene product is a lipoprotein that is exported to the cell surface but remains associated with the cell membrane (Kriakov *et al*., 2003). Recent studies have suggested a lack of inducible phosphatase activity in *M. bovis* BCG upon phosphate starvation (Braibant and Content, 2001), which is in agreement with the fact that no orthologue of the *M. smegmatis phoA* gene is present in the genome of *M. bovis* BCG.

2 Functional analysis of PhoR and PhoS *in vitro*

With the aim to demonstrate the phosphorylation reactions typical for two-component signal transduction systems, the PhoS and PhoR proteins of *C. glutamicum* were overproduced in *E. coli* and purified by means of a C-terminal histidine tag (Figs. 22 and 23). The solubilization of the membrane-integral PhoS protein was achieved using LDAO as detergent. Both the autokinase activity of $PhoS_{His}$ and the phosphoryltransfer from phosphorylated $PhoS_{His}$ to $PhoR_{His}$ could be demonstrated (Fig. 26). The fact that the solubilized PhoS protein shows autokinase activity is not self-evident, since several other membrane-bound sensor kinases were shown to be inactive in this state and active only after reconstitution into proteoliposomes, e.g. the histidine kinases DcuS and KdpD of *E. coli*. DcuS is part of DcuS/DcuR TCS that controls the expression of genes in response to extracellular C_4-dicarboxylates such as fumarate or succinate (Janausch *et al*., 2004; Abo-Amer *et al*.,2004). KdpD is part of the KdpD/KdpE TCS that regulates expression of the

kdpFABC genes for high-affinity K^+ uptake in response to K^+ availability or medium osmolarity (Jung *et al.*, 1997; Heermann *et al.*, 2003). The reason for the different behaviour of solubilized histidine kinases is not yet clear.

In the phosphotransfer experiment with γ-[^{32}P]-ATP (Fig. 26), two labelled bands with apparent sizes of 27 kDa and 50 kDa appeared on the autoradiograph besides PhoS immediately after the addition of $PhoR_{His}$ to phosphorylated $PhoS_{His}$. These bands presumably represent the monomeric and dimeric forms of $PhoR_{His}$ (predicted mass 27.4 kDa). After staining of the gel used for autoradiography with Coomassie blue, only the monomeric forms of $PhoS_{His}$ and $PhoR_{His}$ were visible, showing that only minor amounts of the proteins became labelled. This might be due to the very low concentration of γ-[^{32}P]-ATP used in these experiments. The question whether the dimer of PhoR was formed as a consequence of the phosphorylation or was present already before cannot be answered from these studies. Gel filtration with unphosphorylated PhoR could be used to determine the native mass of the protein. Moreover, phosphorylation-dependent changes in the quarternary structure of PhoR could be demonstrated by non-denaturing PAGE, as it has been done for the response regulator Spo0A of *B. subtilis* (Muchová *et al.*, 2004). There is also evidence for other response regulators that they form dimers upon phosphorylation, e.g. the *E. coli* response regulator PhoB which is involved in adaptive response to phosphate starvation (McCleary, 1996).

3 Interaction of PhoR with promoter sites

After the identification of the transcriptional start sites of the phosphate starvation inducible genes *phoR*, *pstS* and *ugpA*, the interaction between the response regulator PhoR and its presumed target promoters was studied. According to the signal transduction model of many others two-component signal transduction systems, it was assumed that the binding of PhoR to its target sequence is dependent on the phosphorylation status. The gel retardation assay was carried out with unphosphorylated $PhoR_{His}$ as well as with $PhoR_{His}$ that had been preincubated with $PhoS_{His}$ and ATP and therefore should be at least partially phosphorylated. Unfortunately this attempt was not successful and it was not possible to show binding of $PhoR_{His}$ to either the *ugpA*, *pstS* or *phoR* promoter (Fig. 27). A possible reason could be that the amount of the phosphorylated PhoR was not sufficient to show the binding. Therefore, an attempt was made to phosphorylate PhoR by an alternative method. Several, but not all (Hulett *et al.*, 1996) bacterial response regulators (e.g. PhoB of *E. coli*) become

phosphorylated *in vitro* when incubated with acetyl phosphate (Lukat *et al*., 1992; McCleary *et al*., 1996). In the case of our studies with $PhoR_{His}$, preincubation with acetyl phosphate did not result in the binding of the protein to its presumed target promoters (Fig. 27). With the intention to simplify the analysis by bypassing the requirement for PhoR phosphorylation, the isolated DNA-binding domain of PhoR was tested for its ability to bind to target promoters. The isolated DNA-binding domains of some response regulators, e.g. Spo0A of *B. subtillis* or PrrA of *Rhodobacter sphaeroides*, were shown to be constitutively active (Ireton *et al*., 1993; Laguri *et al*., 2003). Moreover, to avoid a possible hindrance of protein-DNA binding by a C-terminal His-tag, the binding domain of PhoR was provided with an N-terminal His-tag. With the PhoRΔ1-125 protein, a gel retardation could be shown with the promoter regions of *pstS, ugpA, phoR* and *pitA*, but not of *clpP1P2* and *clpC*.

By DNase I footprints with the PhoRΔ1-125 protein and the promoter region of *pstSCAB* operon a binding region was localized 170 to 205 bps upstream of the *pstS*

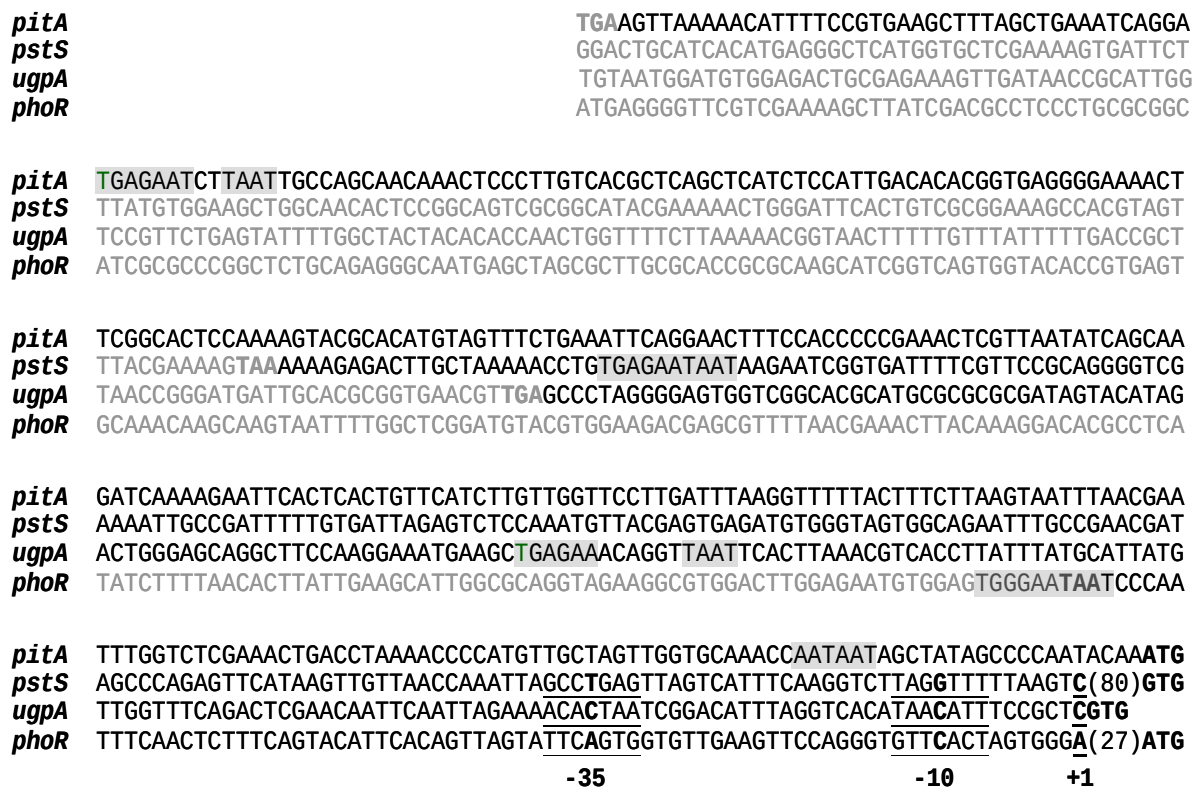


Fig. 31: Alignment of the promoter regions of *pstS, ugpA, phoR* and *pitA* from *C. glutamicum.* Indicated are the translational start sites (bold), the transcriptional start sites (if determined, bold and underlined (+1)) the -10 and -35 regions (underlined), the partial sequence of gene localized upstream of the *pstS, ugpA, phoR* or *pitA* (in grey) and the suggested conserved motifs (grey boxes). Numbers in the parentheses indicate bps between transcriptional and translational start point.

transcriptional start site. However, this experiment was performed only once and therefore needs to be confirmed. Using this method with further target promoters (*ugpAEBC*, *phoRS* and *pitA*), it may be possible to determine a PhoR consensus binding site in the future. In Fig. 31 an alignment of the promoter regions of *pstS*, *ugpA*, *phoR* and *pitA* is shown, which does reveal obvious conserved motifs.

4 Model of the phosphate starvation response in *C. glutamicum*

From the results obtained in this work a model for the regulation of the phosphate starvation response in *C. glutamicum* can be derived (Fig. 32). After a shift from P_i excess to P_i starvation, rapid induction of several *psi* genes is observed which allow the cells to take up the limiting P_i resources more efficiently (Pst and Ugp systems) and to obtain phosphate from organophosphates (GlpQ, UshA, NucH). This rapid induction presumably depends on the PhoS/PhoR two-component system. Moreover, evidence was obtained that the expression of the *pitA* gene for low-affinity P_i uptake is repressed by the PhoS/PhoR system under P_i limitation. There is also an indication that PhoR might repress some of the *psi* genes under P_i excess, a feature which has not been included in the model. Besides the PhoS/PhoR system, another regulator is involved in the P_i starvation response since the *pst* genes were induced after a shift to P_i limitation independent of PhoS/PhoR. It appears possible that this second system also regulates some other *psi* genes and is responsible for a long-term response to P_i starvation. (Fig. 32).

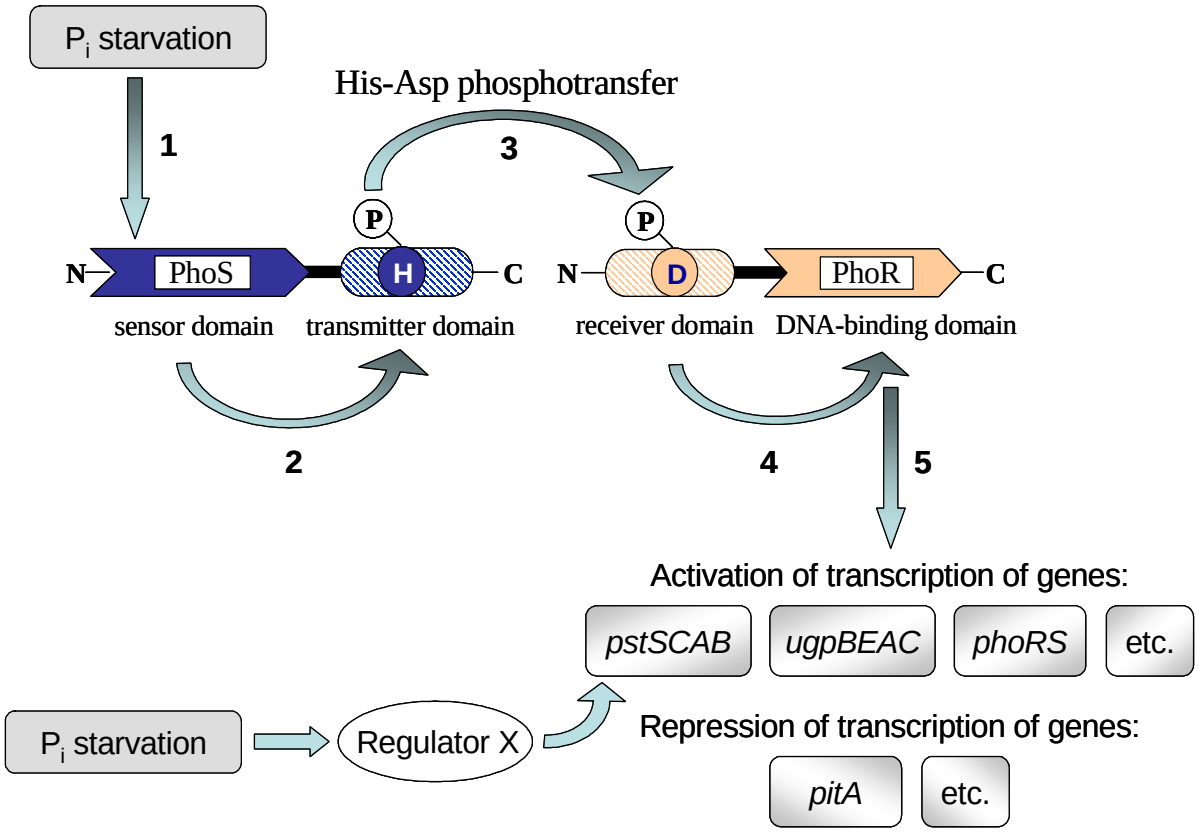


Fig. 32: Model of signal transduction network of PhoR/PhoS two-component system under phosphate limitation.1: A transmembrane histidine kinase PhoS is activated by phosphate starvation perceived by its N-terminal sensor domain.; 2: The activated protein autophosphorylates a histidine residue of the transmitter domain; 3: The phosphoryl group is transferred from the histidine residue of the histidine kinase PhoS to a aspartic acid residue located in the receiver domain of the response regulator PhoR; 4: conformational changes of PhoR; 5: The activated response regulator PhoR stimulates or repress transcription of its target genes. Under P_i limitation is *pst* operon controlled both by the PhoRS system and another, yet unknown regulator X.

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CURRICULUM VITAE

Personal data

Name	Martina Kočan , born Mičková
E-mail	martina_kocan@yahoo.com
Date of birth	29th of September, 1975
Family status	married, no children

Education

1982-1990	Primary school Haanova in Bratislava, Slovakia
1990-1994	High School Jura Hronca in Bratislava, Slovakia
1994-1998	Faculty of Chemical and Food Technology Slovak Technical University in Bratislava, Slovakia BACHELOR OF SCIENCE Discipline: Biochemistry and Biotechnology
1998-2000	Faculty of Chemical and Food Technology Slovak Technical University in Bratislava, Slovakia MASTER OF SCIENCE Discipline: Biochemistry and Biotechnology Study branch: Biochemistry and Microbiology Topic of the Masters thesis: Anti-Microbial Activity of New Cuprous Complexes with Bioactive Ligands
2001-2005	PhD study in the Institut für Biotechnologie 1 (IBT 1), Forschungszentrums Jülich under supervision of Prof. Dr. M. Bott. Topic of the PhD thesis: Regulation of the phosphate starvation response in *Corynebacterium glutamicum* by the PhoRS two-component system

Hereby I declare that this work was performed only by myself and with help of mentioned references.

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