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Regulatory and metabolic aspects of the phosphate starvation response of *Corynebacterium glutamicum*

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Content

Abbreviations

ATC	Anhdyrotetracycline
ATCC	American Type Culture Collection
ATP/ADP	Adenosine triphosphate/Adenosine diphosphate
BHI(S)	Brain Heart Infusion (+ Sorbitol)
Cm^R	Chloramphenicol resistance
DTT	Dithiothreitol
EMSA	Electrophoretic gel mobility shift assay
et al.	*et alii*
GC-Tof-MS	Gas chromatography time-of-flight mass spectrometry
IPTG	Isopropyl-thio-β-D-galactopyranoside
Kan^R	Kanamycin resistance
LB	Lysis Broth
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
OD_{600}	Optical density at 600 nm
P	Phosphorus
PAGE	Polyacrylamide gel electrophoresis
P_i	Inorganic phosphate
^{31}P-NMR	Phosphorus nuclear magnetic resonance
RBS	Ribosome binding site
SDS	Sodiumdodecylsulfate
TBE	Tris base - Boric acid - EDTA
TCA	Trichloric acid
TCS	Two-component regulatory system
TE	Tris base – EDTA
TEMED	*N*,*N*,*N*,*N*,-Tetramethylethylenediamine
TNI	Tris base – NaCl - Imidazol
v/v	Volume per volume
w/v	Weight per volume

Further abbreviations not included in this section are according to international standards, as for example listed in the author guidelines of the *FEBS Journal.*

1 Abstract

Phosphorus is an essential element for all living organisms, as it is a prerequisite for cellular metabolism. *Corynebacterium glutamicum*, an industrial amino acid producer, is a Gram-positive soil bacterium that has developed regulatory mechanisms to overcome phosphate (P_i) limitation for growth in its habitats. When P_i is limited, *C. glutamicum* induces P_i-starvation-inducible (*psi*) genes. The encoded proteins allow the utilization of residual P_i and of alternative phosphorus sources. Induction of these *psi* genes is partially dependent on a two-component signal transduction system composed of the sensor kinase PhoS and the response regulator PhoR.

In this work, evidence was obtained that besides PhoSR a second two-component system consisting of the sensor kinase SenX3 and the response regulator RegX3 is involved in the P_i starvation response. Deletion of the chromosomal *regX3* gene was only possible in the presence of a plasmid-borne copy, indicating that RegX3 is essential in *C. glutamicum*. Global gene expression studies with a strain allowing conditional repression of *regX3* in concert with DNA-binding studies indicated that phosphorylated RegX3 activates several genes of the P_i starvation stimulon, i.e. *pstSCAB* (ABC transporter for P_i), *ugpAEBC* (ABC transporter for glycerol 3-phosphate), *phoC* (putative secreted phosphoesterase) and *ushA* (5'-nucleotidase), but also a cluster of four genes two of which are involved in NAD^+ biosynthesis (*ndnR-nadA-nadC-nadS*). Furthermore, whole-cell alkaline phosphatase activity was found to be controlled by RegX3.

In the second part of this thesis, the influence of P_i limitation on the metabolism of *C. glutamicum* analyzed. Metabolite analysis by GC-TOF mass spectrometry of cells cultivated in glucose minimal medium revealed a strongly increased maltose level under P_i limitation. As maltose formation could be linked to glycogen metabolism, the cellular glycogen content was determined. In contrast to cells grown under P_i excess, the glycogen level of P_i-limited cells remained high in the stationary phase. Surprisingly, even acetate-grown cells, which do not form glycogen under P_i excess, do so under P_i limitation and retain it also in stationary phase. Expression of *pgm* and *glgC*, encoding the first two enzymes of glycogen synthesis, phosphoglucomutase and ADP-glucose pyrophosphorylase, was found to be increased 6- and 3-fold under P_i limitation, respectively. Increased glycogen synthesis together with a decreased glycogen degradation might be responsible for the altered glycogen metabolism. Independent from these experimental results, flux balance analysis suggested that an increased carbon flux to glycogen is a solution for *C. glutamicum* to adapt carbon metabolism to limited P_i concentrations.

In summary, this work led to the identification of an additional regulatory system involved in the P_i starvation response and of a link between P_i limitation and glycogen metabolism in *C. glutamicum*.

2 Zusammenfassung

Phosphor ist ein essentieller Nährstoff für alle lebenden Zellen, da er eine Voraussetzung für den zellulären Stoffwechsel darstellt. Das Gram-positive Bodenbakterium *Corynebacterium glutamicum*, das zur industriellen Aminosäure-Produktion eingesetzt wird, hat regulatorische Mechanismen zur Anpassung an Phosphat-Mangel entwickelt. Unter Phosphat-limitierenden Bedingungen wird ein Satz von sogenannten *psi*-Genen (*phosphate starvation-inducible*) induziert, die für Proteine kodieren, welche eine effiziente Nutzung von verbleibendem Phosphat sowie von alternativen Phosphor-Quellen erlauben. Für die Induktion dieser *psi*-Gene ist zum Teil ein Zweikomponentensystem verantwortlich, das aus der Sensorkinase PhoS und dem Antwortregulator PhoR besteht.

In dieser Arbeit konnte gezeigt werden, dass neben PhoSR auch das Zweikomponentensystem SenX3/RegX3 eine Rolle bei der Phosphat-Mangelantwort spielt. Eine Deletion des chromosomalen *regX3*-Gens war nur in Gegenwart einer plasmid-gebundenen *regX3*-Kopie möglich. Dies deutet darauf hin, dass RegX3 in *C. glutamicum* essentiell ist. Globale Genexpressionsanalysen mit einem Stamm, der eine konditionale Repression von *regX3* erlaubte, und anschließende DNA-Bindungsstudien deuten darauf hin, dass der phosphorylierte Antwortregulator RegX3 eine Reihe von Genen des Phosphat-Mangelstimulons aktiviert: *pstSCAB* (ABC-Transporter für Phosphat), *ugpAEBC* (ABC-Transporter für Glycerin-3-Phosphat), *phoC* (putative sekretierte Phosphoesterase) und *ushA* (5'-Nukleotidase). Darüber hinaus gehört das Gencluster *ndnR-nadA-nadC-nadS* zum RegX3-Regulon. Die von *nadA* und *nadB* kodierten Proteine sind an der NAD^+-Biosynthese beteiligt. Enzymassays zeigten außerdem, dass die alkalische Phosphatase-Aktivität intakter Zellen von *C. glutamicum* durch RegX3 reguliert wird.

Im zweiten Teil dieser Arbeit wurde der Einfluss von Phosphat-Mangel auf den Stoffwechsel von *C. glutamicum* untersucht. Eine Metaboliten-Analyse mittels GC-TOF-Massenspektrometrie zeigte einen erhöhten Maltose-Gehalt in Zellen, die auf Glucose-Minimalmedium unter Phosphat-limitierenden Bedingungen kultiviert wurden. Da die Maltose-Bildung im Zusammenhang mit dem Glykogen-Stoffwechsel stehen könnte, wurde der zelluläre Glykogen-Gehalt bestimmt. Im Gegensatz zu Zellen, die unter Phosphat-Überschuss kultiviert worden waren, zeigten Phosphat-limitierte Zellen auch in der stationären Phase einen hohen Glykogen-Gehalt. Überraschenderweise wurde sogar bei Wachstum mit Acetat als Kohlenstoffquelle unter Phosphat-Mangel Glykogen gebildet, nicht aber bei Kultivierung unter Phosphat-Überschuss. Die ersten beiden Gene der Glykogensynthese (*pgm*, Phosphoglucomutase; *glgC*, ADP-Glucosepyrophosphorylase) zeigten einen 6- bzw. 3-fach erhöhten mRNA-Spiegel unter Phosphat-limitierten Bedingungen. Eine erhöhte Glykogensynthese zusammen mit einem verringerten Glykogenabbau ist möglicherweise für den veränderten Glykogen-Stoffwechsel verantwortlich. Unabhängig von diesen Experimenten ergaben Analysen mit einem genom-weiten stöchiometrischen Stoffwechsel-Modell von *C. glutamicum*, dass ein erhöhter Kohlenstoff-Fluss zu Glykogen eine Lösung zur Anpassung des Kohlenstoff-Stoffwechsels an Phosphat-Limitierung darstellt.

3 Introduction

Ohne Phosphor, Kein Gedanke. (Without phosphorus there would be no thoughts.)
By Friedrich Karl Christian Ludwig Büchner

3.1 Phosphorus – an essential element for cellular growth and regulation

Phosphorus (P) is an essential element for all cells as it is a component of e.g. DNA, RNA, and membrane lipids. The common phosphorus source is inorganic phosphate (P_i), which is taken up by bacteria either via secondary transporters or via ATP-driven ABC transporters. Extracellular phosphate esters can be served as an alternative P source. Phosphate esters are hydrolyzed by bacterial phosphatases and the resulting P_i imported into the cells. In addition, some bacteria utilize specific uptake systems for the transport of *sn*-glycerol-3-phosphate as organophosphate. The intracellular P_i is assimilated into cellular metabolites by reactions such as F_1F_0-ATP synthase or glyceraldehyde-3-phosphate dehydrogenase. Moreover, polyphosphate can be formed as a readily available intracellular P_i source.

The key role of phosphorus in metabolism (Fig. 3.1) is indicated e.g. by the fact that 357 of 4,672 Clusters of Orthologus Groups (COGs) (Tatusov *et al.*, 1997) are related to inorganic phosphate and phosphorus-containing molecules (Table 3.1). These 357 COGs fall into 18 functional categories: 70% into the category "Metabolism" (e.g. fructose-1,6-bisphosphatase), 13% into the category "Cellular processes and signaling" (e.g. UDP-N-acetylglucosamine-1-phosphate transferase), and 16% into various other categories. This supports the central role of phosphorus in metabolism and underlines that transport and assimilation of P_i are important tasks for cells.

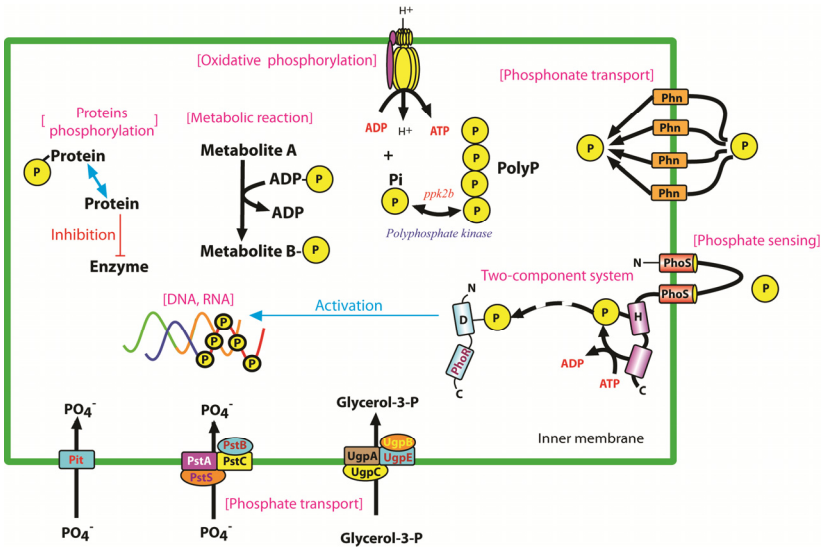

Figure 3.1 Selection of reactions and molecules related to phosphorus in bacterial cells.

Table 3.1 Functional categories of COGs involving phosphorus. 357 COGs among 4,872 COGs from 66 genomes and 192,987 proteins were found by searching with the keyword 'phosph', which is a prefix of 'phosphorylase', 'phosphate', 'phosphatase', 'phosphoribosyl-' and 'phosphotransferase'.

Code	COGs	COGs-phosph	Description of COGs
Information storage and processing			
J	245	4	Translation, ribosomal structure and biogenesis
A	25	1	RNA processing and modification
K	231	2	Transcription
L	238	3	Replication, recombination and repair
B	19	-	Chromatin structure and dynamics
Cellular processes and signaling			
D	72	2	Cell cycle control, cell division, chromosome partitioning
Y	-	-	Nuclear structure
V	46	1	Defence mechanisms
T	152	21	Signal transduction mechanisms
M	188	20	Cell wall/membrane/envelope biogenesis
N	96	1	Cell motility
Z	12	-	Cytoskeleton
W	1	-	Extracellular structures
U	159	-	Intracellular trafficking, secretion, and vesicular transport
O	203	3	Posttranslational modification, protein turnover, chaperones
Metabolism			
C	258	16	Energy production and conversion
G	230	88	Carbohydrate transport and metabolism
E	270	28	Amino acid transport and metabolism
F	95	34	Nucleotide transport and metabolism
H	179	28	Coenzyme transport and metabolism
I	94	26	Lipid transport and metabolism
P	212	28	Inorganic ion transport and metabolism
Q	88	3	Secondary metabolites biosynthesis, transport and catabolism
Poorly characterized			
R	702	48	General function prediction only
S	1346	-	Function unknown

In order to survive in P_i-limited environments, bacteria have developed strategies to cope with this situation. A key element is the Pst system, a high-affinity, low velocity ABC transporter for P_i uptake (Willsky & Malamy, 1980). When P_i is limited, synthesis of the Pst system is highly activated and P_i is mainly taken up by this transporter at the expense of ATP. In many bacteria, alkaline phosphatase (e.g. encoded by the *phoA* gene in *Escherichia coli*) is induced and secreted into the periplasm, where the enzyme hydrolyzes phosphate esters to generate free P_i which can be taken up by the Pst system (Van Dien & Keasling, 1998, Wanner *et al.*, 1988). Some bacteria also utilize non-specific 5'-nucleotidases, acid phosphatases, hexose-6-phosphatase and cyclic phosphodiesterases to provide free P_i in the periplasm (Wanner, 1996).

In bacteria, the expression of genes required to cope with environmental stresses such as P_i limitation are often regulated by two-component regulatory systems (TCS) consisting of a membrane-associated sensor kinase and a response regulator. Upon stimulus recognition, the sensor kinase is autophosphorylated at a histidine residue with ATP and the phosphoryl group is subsequently transferred to an aspartate residue in the N-terminal receiver domain of the cognate response regulator. This results in a conformational change of the response regulator that enables its C-terminal effector domain containing a helix-turn-helix DNA-binding motif to bind to its target promoters and to activate the expression of corresponding genes, such as the Pho regulon in the case of P_i starvation (McCleary & Stock, 1994, Glover *et al.*, 2007, Kocan *et al.*, 2006, Sola-Landa *et al.*, 2005). Mutants lacking the corresponding TCS fail to activate their target genes (Kocan et al., 2006). The Pho regulon that is activated by P_i limitation often includes the *pst* operon encoding the high-affinity ABC transporter for P_i, the *phoA* gene encoding an alkaline phosphatase and the *ugp* operon encoding an *sn*-glycerol-3-phosphate transporter.

On the other hand, P_i-starved cells utilize pyrophosphate (PP_i) and polyphosphate (polyP) to supply intracellular P_i. PP_i is formed during nucleic acid biosynthesis or by metabolic reactions and is hydrolyzed to P_i by pyrophosphatase. PolyP is a linear polymer synthesized by polyphosphate kinase (Ppk) from ATP or nucleoside triphosphates and is degraded by exo- or endo-polyphosphatases (Ppx) to liberate P_i under certain conditions (Stumpf & Foster, 2005, Foster, 2007, Lindner *et al.*, 2009, Zhang *et al.*, 2007).

3.2 Phosphate starvation responses of microorganisms

3.2.1 *Escherichia coli* and the two-component systems PhoBR and CreBC

Escherichia coli possesses two major P_i transporters, the Pst and Pit systems, which are a high-P_i-affinity (K_m: 0.4 μM), low-velocity (V_{max}: 15.9 nmol P_i min^{-1} mg^{-1} of protein) system and a low-P_i-affinity (K_m: 38.2 μM), high-velocity (V_{max}: 55 nmol P_i min^{-1} mg^{-1} of protein) system, respectively (Willsky & Malamy, 1980). When P_i is limited, the Pst system apparently plays a crucial role for P_i import. Also, the *ugpBAECQ* and *glpT* genes encoding an ABC transporter for glycerol-3-phosphate (G3P) and a secondary transporter for G3P, respectively, and the *uhpT* gene encoding a hexose phosphate transporter are induced to take up the organophosphates G3P and hexose-6-phosphate (Winkler, 1996). Phosphonate can be utilized as P source through the phosphonatase pathway (PhnXWRSTUV) and the carbon-phosphorus lyase pathway (PsiD) to generate P_i and phosphorylated compounds (Wanner, 1994). In addition, the porin PhoE is activated under P_i limitation, which forms an anion channel in the outer membrane (Benz *et al.*, 1984).

Under P_i-starved conditions, *E. coli* induces $\underline{P_i}$ starvation inducible genes (called the *psi* genes) encoding for proteins involved in the transport of P_i (the Pst system) and other phosphorus sources (the Ugp system for G3P), in the cleavage of phosphomonoesters, such as alkaline phosphatase (PhoA), 5'-nucleotidase (Nuc), and phosphodiesterase, and proteins involved in cell wall biosynthesis, phosphonate degradation and polyphosphate metabolism (*ppk-ppx*) (Wanner, 1996, Vershinina & Znamenskaia, 2002, Hsieh & Wanner, 2010). The *psi* genes, except for the *ppk-ppx* operon, belong to the Pho regulon, which is defined as the genes that are regulated by the two-component regulatory system PhoB-PhoR, one of 36 TCS in *E. coli*.

The PhoBR TCS, consisting of a sensor kinase encoded by the *phoR* gene and a response regulator encoded by the *phoB* gene, regulates the expression of 31 genes for coping with P_i limitation (Wanner, 1996). Recently, the results of a DNA microarray analysis (Baek & Lee, 2006) suggested that three more genes are PhoB-regulated: *amn* (AMP nucleosidase), *yibD* (encodes a protein involved in metal ion stress) and *ytfK* (a hypothetical protein). The membrane-bound sensor kinase PhoR has autophosphorylation activity and serves as phosphoryl donor for the response regulator PhoB when P_i is limited or dephosphorylates PhoB when P_i is in excess (Ellison & McCleary, 2000, Makino *et al.*, 1989) (Fig. 3.2). The effector domain of PhoB contains a helix-turn-helix motif and binds to the so-called PHO box, a DNA sequence motif consisting of two direct repeats of 7 bp with the well-conserved

sequence CTGTCAT separated by a 4-bp-long segment in the promoter regions of the *pstS*, *phoA*, *phoB*, *ugpB*, *phnC*, *phoH*, and *phoE* genes (Wanner, 1996). PhoB is activated by phosphorylation, resulting in a 150-fold higher DNA binding affinity compared to the unphosphorylated protein. Transcription of the PHO regulon genes is increased through interactions of phosphorylated PhoB with the σ^{70} subunit of RNA polymerase (Makino et al., 1989, Wanner, 1996, Ellison & McCleary, 2000).

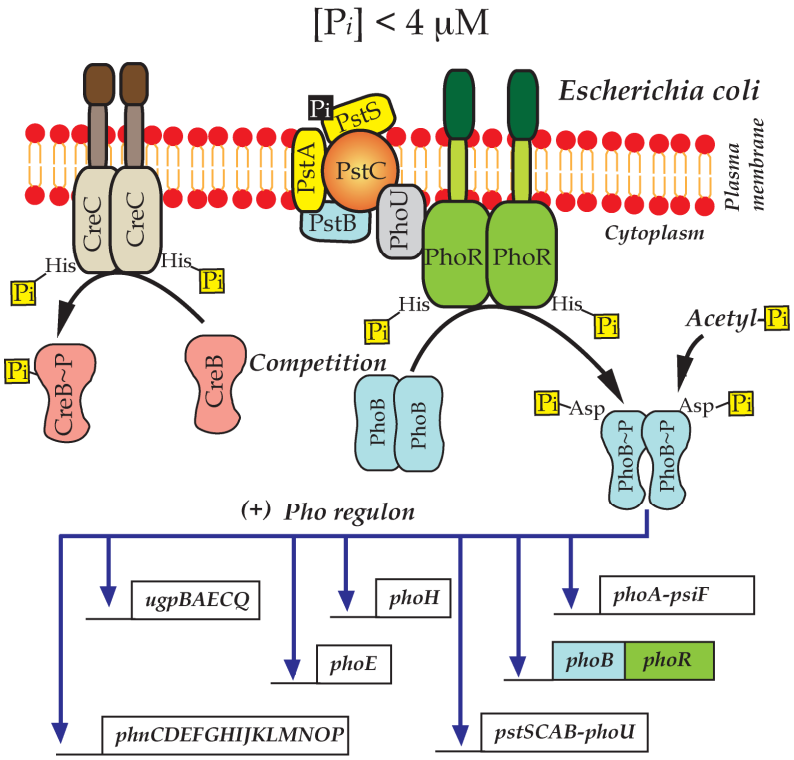

Figure 3.2 Diagram on the regulation of expression of Pho regulon genes in *E. coli*. When the external P_i concentration is less than 4 μM, the sensor kinase PhoR is autophosphorylated and the phosphoryl group is transferred to the response regulator PhoB, causing a conformational change that results in a strongly increased DNA binding affinity of PhoB. Binding of PhoB~P to its target genes is shown by blue arrows. In the absence of PhoR, PhoB competes with CreB for phosphorylation by the sensor kinase CreC. Acetyl phosphate might also be involved in the PhoR-independent phosphorylation of PhoB. The *phoA* gene codes for an alkaline phosphatase, the *phoE* gene for an outer membrane porin channel, the *phoH* gene for an ATP-binding protein of unknown function, the *phoBR* for the TCS, the *pstSCAB* genes for a high affinity P_i ABC transporter, the *phoU* for a chaperone-like PhoB/PhoR inhibitory protein, and the *phnCDEFGHIJKLMNOP* for 14 proteins involved in phosphonate utilization.

In absence of PhoR, PhoB can be phosphorylated in a P_i-independent manner by the sensor kinase CreC (*cre* stands for catabolite repression) or by acetyl phosphate (Wanner, 1996). In the case of CreC, PhoB competes with response regulator CreB for the phosphorylation by a sensor kinase CreC. Acetyl phosphate can activate PhoB (and many other response regulators) via direct chemical phosphorylation (Wanner, 1996). The *ackA-pta*

operon encoding acetate kinase and phosphotransacetylase, i.e. the enzymes that form acetyl phosphate from acetate and ATP or from acetyl-CoA and P_i, respectively, is part of the CreB regulon (Avison *et al.*, 2001). The PhoR-independent phosphorylation of PhoB may also play a role in the presence of PhoR and P_i and allow cross-regulation of the Pho regulon in response to different stimuli.

3.2.2 *Bacillus subtilis* and the regulatory systems PhoPR, ResDE and Spo0A

Bacillus subtilis, a Gram-positive soil bacterium, frequently confronts conditions of nutrient depletion which trigger genetic responses to replenish the depleted nutrients before commitment to sporulation. Phosphorus is of course also an essential element for *B. subtilis*. When the extracellular P_i level decreases to around 0.1 mM, *B. subtilis* utilizes the Pst system encoded by *pstSACB*$_1$*B*$_2$ genes and the organophosphate transporter encoded by the *glpQT* genes, and induces alkaline phosphatases encoded by the *phoA* and *phoB* genes and alkaline phosphodiesterase encoded by the *phoD* gene (Hulett, 1996, Qi & Hulett, 1998, Antelmann *et al.*, 2000). Furthermore, *B. subtilis* represses the *tagAB* and *tagDEF* operons involved in the synthesis of teichoic acid, a major cell wall component, which is a polymer of glycerol or ribitol joined by a phosphate group, and activates genes of the *tuaABCDEFGH* operon which are involved in the synthesis of teichuronic acid, a phosphate-free glucuronic acid containing polysaccharide (Heptinstall *et al.*, 1970, Liu *et al.*, 1998a, Qi & Hulett, 1998). Thus, teichuronic acid replaces teichoic acid in the cell walls of P_i-starved cells and thus saves phosphorus for other purposes.

Like *E. coli*, a two-component regulatory system controls the Pho regulon of *B. subtilis*. Regulation of the P_i starvation response in *B. subtilis* proceeds in a more complicated manner than in *E. coli* and involves three TCS (PhoPR, ResDE and the Spo0A phosphorylation system among 36 sensor kinases and 34 response regulators (Fig. 3.3) (Hulett, 1996, Sun *et al.*, 1996, Fabret *et al.*, 1999, Nakano *et al.*, 2000, Vershinina & Znamenskaia, 2002). The sensor kinase PhoR has autophosphorylation activity and serves as phosphoryl donor for the response regulator PhoP (Abdel-Fattah *et al.*, 2005). Subsequently, PhoP~P activates transcription from the promoters in front of *phoP*, *resA*, *phoA*, *phoB*, *phoD*, *pstS*, and *tuaA* and represses transcription starting from the promoters in front of *resD*, *tagA*, and *tagD* by binding to a DNA sequence motif composed of two direct repeats of TTAACA-like sequences that are separated by approximately 5 bp (Liu *et al.*, 1998b, Antelmann et al., 2000). A *phoR* mutant of *B. subtilis* showed a residual induction of alkaline phosphatase (APase) activity and this APase activity was slightly higher than in a *phoP* mutant (Hulett *et al.*, 1994, Hulett, 1996).

Unlike *E. coli*, acetyl phosphate might not have a significant regulatory role in the control of the Pho regulon due to the fact that *in vitro* phosphorylation studies showed that acetyl phosphate is not able to phosphorylate PhoP (Hulett, 1996).

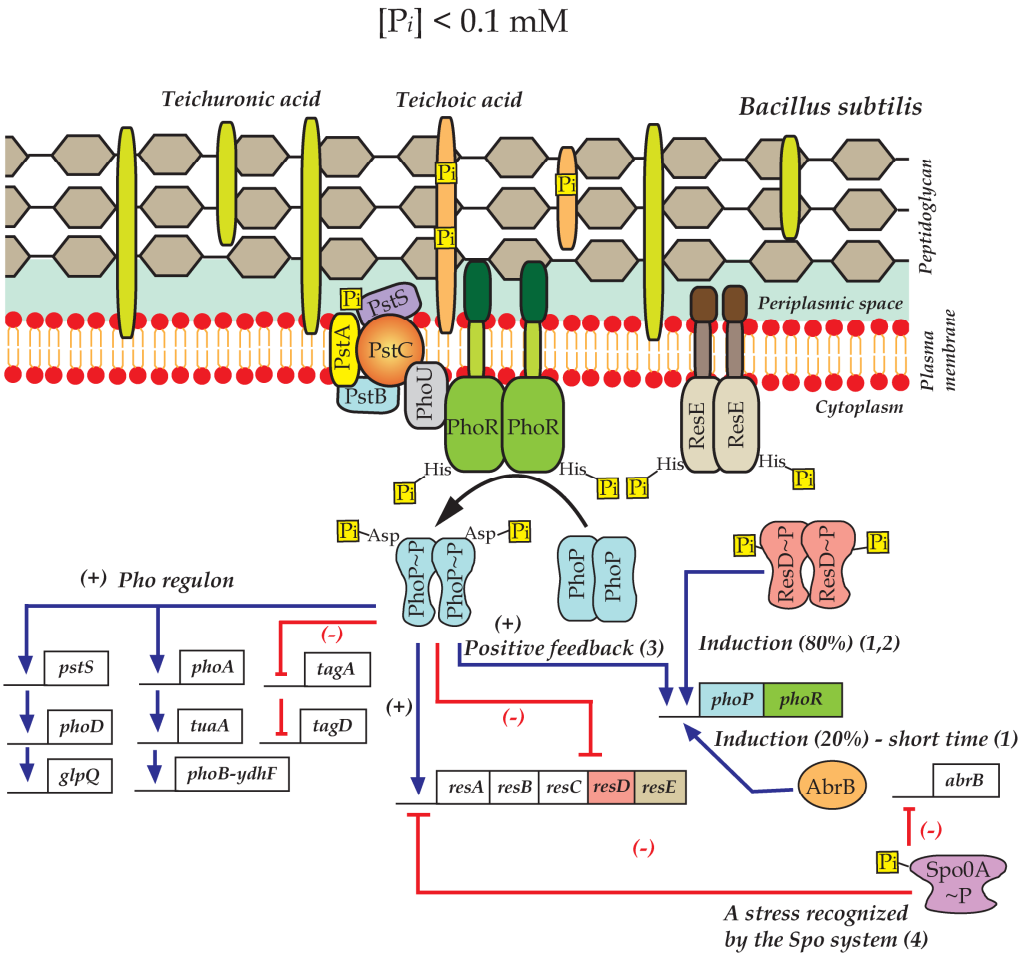

Figure 3.3 Diagram on the regulation of expression of Pho regulon genes in *B. subtilis* by three different two-component regulatory systems. Blue arrows (+) and red lines with blunt ends (-) indicate activation and repression of transcription. When P_i is below 1 mM, phosphorylated ResD and ArbB initiate induction of *phoP* and *phoR* as approximately 80% and 20% of the APase activity, respectively (Hulett, 1996). PhoR phosphorylates PhoP and PhoP~P binds to its target promoters (the *pstSCAB*$_1$*B*$_2$, *phoA*, *phoB*, *phoD*, and *tuaABCDE* genes for the activation; the *tagAB* and *tagDEF* for the repression). PhoP~P activates the *phoPR* genes (positive autoregulation) as well as the *resABCDE* operon (3). The response regulator Spo0A~P represses the expression of *resABCDE* and *abrB* to cease the expression of the Pho regulon (4). Then, Spo0A~P initiates spore formation. The *phoA and phoB* genes encode alkaline phosphatases, the *phoD* gene an alkaline phosphodiesterase, the *glpQ* gene for a glycerophosphodiester phosphodiesterase, the *pstSCAB*$_1$*B*$_2$ genes for a high affinity P_i ABC transporter, the *phoPR* for the TCS, the *resABCED* for proteins involved in cytochrome *c* biosynthesis and the TCS, the *tauABCDEFGH* for proteins in teichuronic acid biosynthesis and the *tagAB* and *tagDEF* for proteins in teichoic acid biosynthesis.

A second two-component system called ResDE, which controls the expression of genes for the synthesis of heme *a* and maturation of cytochrome *c,* is also involved in P_i regulation (Nakano *et al*., 1996, Nakano et al., 2000). Under P_i-limited condition and in the presence of *phoPR*, Δ*resD* and Δ*resE* mutants were found to have 80% and 60% reduced APase activity, respectively (Hulett, 1996, Sun et al., 1996). When P_i is limited, ResD and AbrB activate expression of *phoPR*. The activation by AbrB lasts only for a short period of time (only 20% APase reduction was found in a Δ*abrB* mutant) and is inhibited by the Spo0A system (Jensen *et al*., 1993). The activation of *phoPR* expression by ResD~P leads to synthesis of the PhoPR proteins and then PhoP~P positively regulates the Pho regulon genes including the *phoPR* genes themselve, as well as the *resABCED* genes, thus boosting the P_i starvation response. Expression of genes encoding essential proteins for aerobic and anaerobic respiration are activated upon nutritional limitations (Nakano *et al*., 1996), suggesting that respiratory regulation systems are cross-controlled by the regulatory systems for nutritional stresses, including P_i starvation.

The Spo0A protein is essential for the initialization of sporulation and has been shown to function both as a positive and negative transcriptional regulator (Burbulys *et al*., 1991, Birkey *et al*., 1994). The Pho regulon genes were hyper-induced (500% compared to wild type) in a Δ*spo0A* mutant and were induced for longer periods. The Δ*spo0A-resD,* Δ*spo0A-abrB,* and Δ*spo0A-abrB-resD* double or triple mutants showed 200%, 300%, and 0% of APase activities, respectively (Hulett, 1996, Birkey *et al*., 1998). These data indicated that Spo0A inhibits expression of the Pho regulon by repressing the transcription of the *abrB* and *resDE* genes, thus inhibiting expression of *phoPR*.

In addition to the specific Pho regulon, the large σ^B-dependent general stress was also induced after entry into the stationary phase provoked by P_i starvation (Antelmann et al., 2000). Proteomics and transcriptomics studies of *B. subtilis* showed that the *yjbC*, *jfhM*, and *jxiE* genes might be involved as the Pho regulon genes (Antelmann *et al*., 2000).

Besides *B. subtilis*, its close relative, *B. licheniformis*, an industrial strain for the production of protease and amylase, has been studied with respect to its P_i starvation response (Voigt *et al*., 2004, Hoi *et al*., 2006, Voigt *et al*., 2006). Transcriptome and proteome comparisons showed that the P_i starvation response of *B. licheniformis* is similar to that of *B. subtilis,* as shown e.g. by the induction of the Pst system and of alkaline phosphatase activity. A major difference to *B. subtilis* was that *B. licheniformis* secreted high amounts of phytase, which catalyses the hydrolytic cleavage of phytate (inositol hexakisphosphate) as an important alternative P_i source.

3.2.3 *Mycobacterium* and the two-component systems PhoPR and SenX3-RegX3

Mycobacterium tuberculosis (*Mtb*) is a Gram-positive pathogenic member of the *Corynebacterineae* which causes mortality throughout the world, with two million deaths annually (Cole *et al.*, 1998). Since multi-drug-resistant *Mtb* strains have been found worldwide, more efficient vaccines against *Mtb* are necessary. Thus, it is important to find new virulence factor targets (Pérez *et al.*, 2001). After the complete genome sequencing of *Mtb*, 11 two-component regulatory systems were identified (Cole et al., 1998), one of which was designated PhoPR (encoded by the Rv0757-Rv0758 genes). The response regulator PhoP belongs to the OmpR/PhoB subfamily with an N-terminal regulatory domain and a C-terminal DNA-binding domain.

The PhoP of *Mtb* has a protein sequence which matches that of the phosphate-dependent PhoR response regulator of *C. glutamicum* with a 66% identity. However, unlike the PhoP protein of other bacteria, which, as the name suggests, represents a P_i-dependent response regulator, PhoP of *Mtb* is not involved in the P_i-dependent regulation but is implicated in virulence due to the fact that growth of a Δ*phoP* mutant was attenuated in human and murine macrophages and mice (Walters *et al.*, 2006, Ryndak *et al.*, 2008). In contrast to most other autoregulated TCS, PhoPR is negatively autoregulated by binding of PhoP~P to the RNA-polymerase binding site and the transcriptional start site of the *phoPR* operon. Global transcription profiling of the *phoP* mutant of the wild-type strain *Mtb* H37Rv revealed that 44 genes of the total 3924 ORFs showed decreased expression in the *phoP* mutant, including genes annotated to encode lipid metabolism enzymes, membrane structural proteins and proteins involved in substrate transport across the cytoplasmic membrane, but not well-known P_i starvation inducible genes (Walters *et al.*, 2006).

The PhoP protein is involved in the virulence of *Mtb* by regulating genes (Fig. 3.4). The PhoP-dependent gene cluster *pks2*, *papA1*, *mmpL8,* and *Rv3822* (corresponding to Rv3825c, Rv3824c, Rv3823c, and Rv3822, respectively), which showed decreased gene expression in the Δ*phoP* mutant, encodes for a polyketide synthase, a polyketide synthase-associated protein, a lipid transporter, and an unknown secreted protein, respectively. Polyketide synthase utilizes C_{16}-C_{20} fatty acid methylmalonyl-CoAs to synthesize phthioceranic and hydroxyphthioceranic acids, which are required for the biosynthesis of the major sulphatide in the sulfolipid-1 (SL-1). Then, SL-1 is transported into the periplasmic space by the MmpL8 membrane protein and SL-1 is further incorporated into the asymmetric outer lipid bilayer that performs important functions in the pathogenicity of *Mtb*.

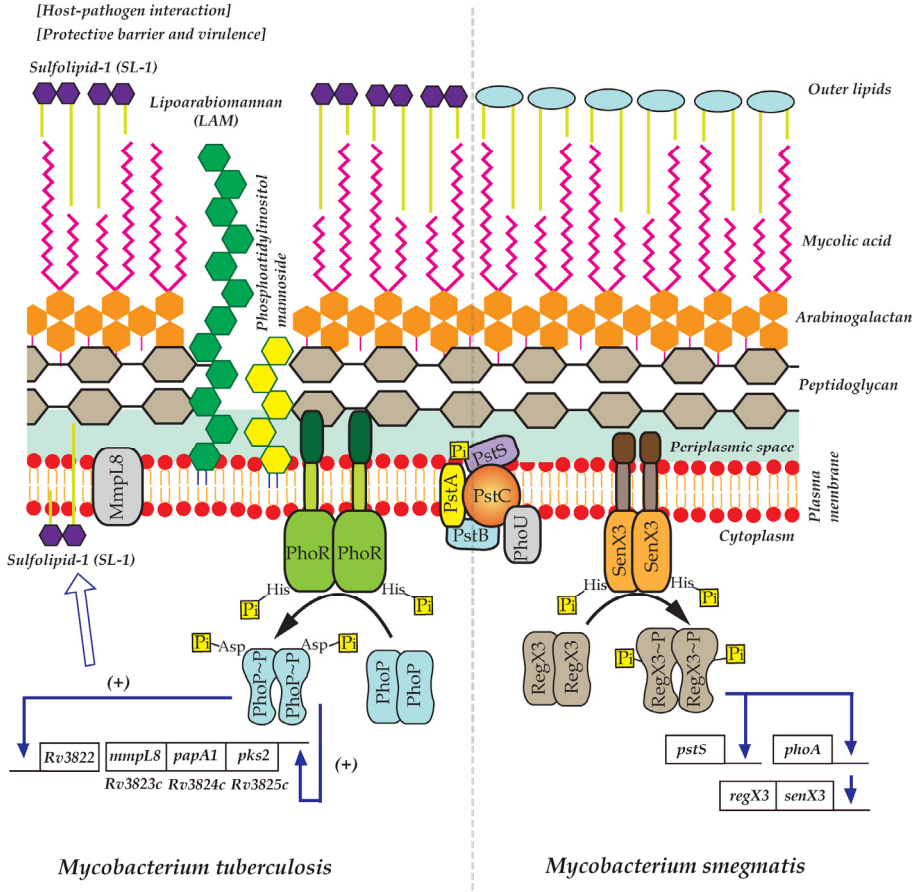

Figure 3.4 Diagram on the regulation of virulence in *M. tuberculosis* by the two-component system PhoPR and on the P_i-dependent regulation by SenX3-RegX3 in *M. smegmatis*. Blue arrows (+) indicate transcriptional activation. As shown in the left half, PhoP is involved in virulence but not in P_i starvation and regulates *pks2*, *papA1*, *mmpL8* and *Rv3822*. The *pks2* gene encodes a polyketide synthase, *papA1* a polyketide synthase-associated protein, *mmpL8* a lipid transporter, and *Rv3822* an unknown secreted protein. These genes are involved in the biosynthesis of sulfolipid-1 (SL-1), which is part of the outer leaflet of the outer membrane that is important for virulence by interacting with the host immune system. Sulfolipid-1 (SL-1) synthesized by Pks2 is transported into the periplasm by the lipid transporter MmpL8 and incorporated in the asymmetric outer lipid bilayer. In addition to PhoPR, SenX3-RegX3 of *Mtb* has also been implicated in virulence, but not in the P_i starvation response. In the right half, the SenX3-RegX3 TCS of *M. smegmatis* is shown to activate the *phoA* gene encoding alkaline phosphatase, the *pst* operon for the high affinity P_i ABC transporter, and the *senX3-regX3* genes themselves. This activation occurs under P_i-limited conditions, indicating the SenX3-RegX3 TCS responds to P_i starvation.

In addition to the PhoPR TCS, the SenX3-RegX3 TCS has also been studied (Himpens *et al.*, 2000, Supply *et al.*, 1997, King-Scott *et al.*, 2007). Global gene expression analysis indicated that 50 genes are controlled by RegX3 (Parish *et al.*, 2003). Some of the down-regulated genes include genes involved in fatty acid degradation, cell wall biosynthesis, lipid biosynthesis, and cell envelope proteins. When Δ*senX3-regX3* mutants were tested by exposure to extreme pH, nutrient limitations, antibiotics, and H_2O_2 to find their particular stimuli, no significant gene expression changes were found (Parish et al., 2003). Thus, the SenX3-RegX3 TCS in *Mtb* might be involved in virulence, like the PhoPR TCS. No *Mtb* TCS has been shown to regulate

gene expression in response to P_i limitation up to now, even though P_i is an essential element taken up by the Pst system and must be acquired for pathogenic infection processes (Braunstein *et al.*, 2000, Lefèvre *et al.*, 1997).

Interestingly, *Mycobacterium smegmatis*, a fast-growing and non-pathogenic mycobacterium, also possesses the SenX3-RegX3 TCS and in this species it was shown to regulate the *phoA* and *pst* gene expression in response to P_i limitation (Glover et al., 2007, Kriakov *et al.*, 2003). A similar function in *Mtb* has yet to be shown.

3.2.4 *Streptomyces* and the two-component system PhoPR

Streptomyces species are soil-dwelling Gram-positive bacteria that produce a large amount of pharmacologically active secondary metabolites including antibiotics (Khosla, 2009, Bentley *et al.*, 2002). High levels of P_i in the medium negatively affect the production of several antibiotics and other secondary metabolites. In these species, the PhoPR TCS (among 33 two-component sensor kinases and 74 two-component regulators in *S. coelicolor*) regulates genes responsible for P_i starvation.

The PhoP protein serves as a DNA-binding regulator and PhoR as a transmembrane sensor protein (Bentley et al., 2002). Phosphorylated PhoP binds to the PHO box (G$_{G/T}$TCAYYYR$_{G/C}$G as a direct repeat unit) in the promoter region of the *pstS* and *phoA* genes to activate synthesis of the Pst system and of an extracellular alkaline phosphatase, respectively (Sola-Landa et al., 2005, Sola-Landa *et al.*, 2008). In addition, the *phoC* and *phoD* genes encoding for an alkaline phosphatase and a phospholipase are regulated by PhoP (Apel *et al.*, 2007). Glycerophosphodiester phosphodiesterase encoded by the *glpQ1* and *glpQ2* genes is also regulated by PhoP to utilize glycerophosphodiesters as a phosphate and carbon source (Santos-Beneit *et al.*, 2008) (Fig. 3.5).

On the other hand, PhoP is also involved in the biosynthesis of secondary metabolites. Large amounts of actinorhodin and undecylprodigiosin were measured in both Δ*phoP* and Δ*phoPR* mutants of *S. lividans* under P_i limitation (Rodríguez-García *et al.*, 2007, Sola-Landa *et al.*, 2003). Overexpression of AfsS encoded by a short ORF located in the 3' region of the regulatory *afsR* gene induced expression of the *actII-ORF4* and *redD* genes (Floriano & Bibb, 1996). The encoded pathway-specific regulators ActII-ORF4 and RedD are involved in the overproduction of actinorhodin and undecylprodigiosin by activating the biosynthesis genes. Thus, the PhoPR system of *Streptomyces sp.* positively regulates the Pho regulon genes and negatively antibiotic synthesis.

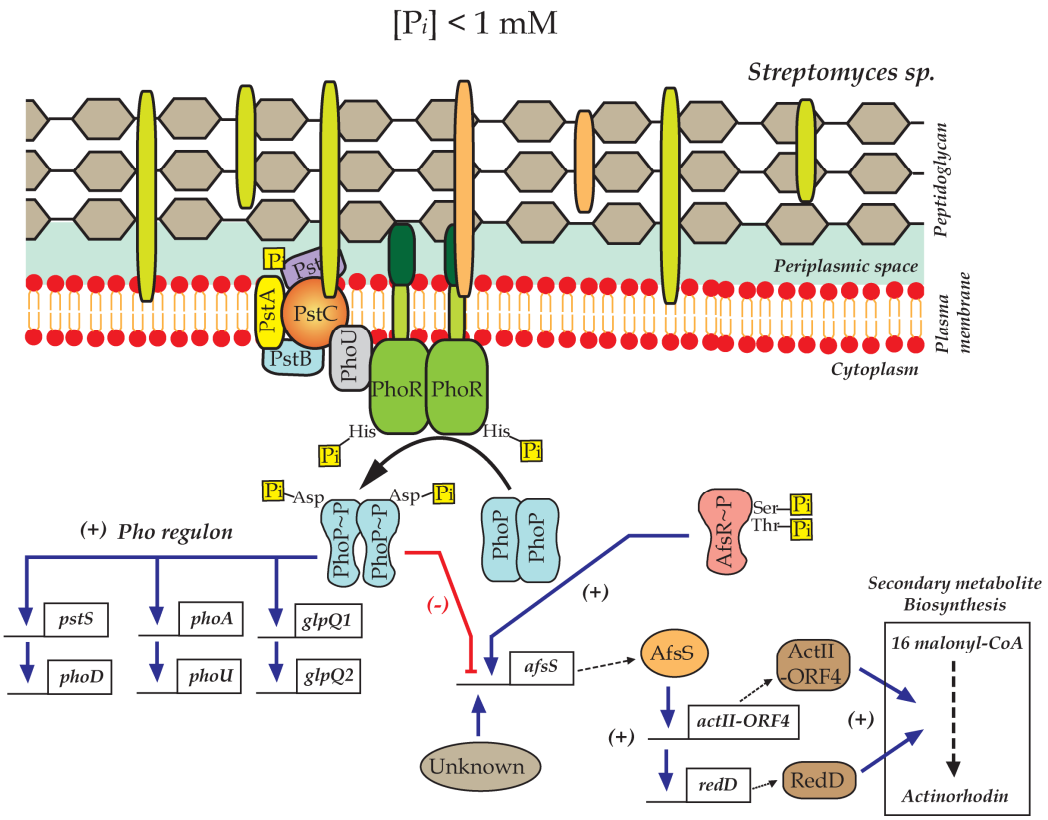

Figure 3.5 Diagram showing regulation processes in *Streptomyces sp.* that are controlled by the two-component system PhoPR. Blue arrows (+) and red lines with a blunt end (-) indicate transcriptinal activation and repression, respectively. Phosphorylated PhoP activates expression from the promoters upstream of *pstS*, *phoA*, *phoD*, *phoU*, *glpQ1* and *glpQ2* genes. The *pstSCAB* genes encode a high affinity P_i ABC transporter, *phoA* and *phoC* for an alkaline phosphatase, *phoD* for a phospholipase, *phoU* gene for a putative regulator of the PHO response, and *glpQ1* and *glpQ2* for glycerophosphodiester phosphodiesterases. On the other hand, PhoP represses the expression of the *afsS* gene which is positively regulated by the regulator AfsR. The AfsS protein activates gene expression of the pathway-specific transcriptional activators ActII-ORF4 and RedD, which activate expression of genes involved in the biosynthesis of actinorhodin.

3.3 Phosphate starvation responses of *Corynebacterium glutamicum*

Corynebacterium glutamicum is a non-pathogenic, soil bacterium that is used industrially to produce L-glutamate and L-lysine (1.5 million tonnes per year and 0.8 million tonnes per year, respectively) (Kalinowski *et al.*, 2003, Wendisch *et al.*, 2006). P is an essential element for *C. glutamicum* as well as for other bacteria. As mentioned in Table 3.1, P metabolism is strongly related to both central metabolism and energy metabolism. P is mainly taken up in the form of P_i, but also as glycerol-3-phosphate. *C. glutamicum* is able to synthesize soluble and granular polyP for diverse functions including as a reservoir of P_i, a substitute for ATP in a kinase reaction, and as an adaption to environmental stress. *C. glutamicum* is able to accumulate up to 600 mM cytosolic P_i in the form of polyP, which is synthesized from ATP or GTP by class II polyphosphate kinase, mainly encoded by the *ppk2B* gene (Lindner *et al.*,

2007). PolyP can be degraded either by polyphosphate/ATP-dependent glucokinase, encoded by the *ppgK* gene (Lindner *et al.*, 2010), or by exopolyphosphatases, encoded by the *ppx1* and *ppx2* genes (Lindner et al., 2009).

A recent global gene expression analysis revealed that 25 genes involved in P metabolism were up-regulated under P_i limitation (0.13 mM P_i), which form the so-called phosphate starvation inducible genes (the *psi* genes) (Ishige *et al.*, 2003). These *psi* genes include the *pstSCAB* operon encoding a high-affinity P_i ABC transporter, the *ugpAEBC* operon encoding glycerol-3-phosphate uptake, *glpQ1* encoding a glyerophosphoryldiester phosphodiesterase, *ushA* encoding an enzyme secreted with UDP-sugar hydrolase and 5'-nucleotide (Rittmann *et al.*, 2005), *nucH* encoding a putative secreted nuclease, *phoC* genes encoding a cell wall-associated alkaline phosphatase, *phoH1* encoding an ATPase of unknown function, and *pctABCD* encoding an ABC transporter of an unknown phosphorus-containing molecule. In addition, the *phoRS* genes encoding the two-component system PhoRS were highly up-regulated when *C. glutamicum* faced P_i starvation.

3.3.1 The two-component regulatory system PhoSR

C. glutamicum possesses 13 TCS that respond to environmental stimuli (Kocan et al., 2006, Kalinowski et al., 2003). The PhoRS TCS consists of a membrane-bound sensor kinase encoded by the *phoS* gene and a response regulator encoded by the *phoR* gene. In a Δ*phoRS* mutant, the increased expression of the *psi* genes was mostly abolished under P_i limitation. These *psi* genes are called the Pho regulon genes of *C. glutamicum (Kocan et al., 2006)* (Fig. 3.6). These Pho regulon genes, including the *pstSCAB* operon, *ugpAEBC* operon, *glpQ1*, *ushA*, *nucH*, *phoH1*, *phoC* and *phoRS* genes, are activated by PhoR~P as indicated by DNA microarray results and electrophoretic mobility shift assays using phosphorylated PhoR and DNA fragments covering the corresponding target promoter regions (Schaaf & Bott, 2007). In addition, PhoR might repress the expression of the *pitA* gene encoding a low-affinity P_i uptake transporter as the *pitA* mRNA level was 4-fold decreased in the wild type after a shift from P_i excess to P_i limitation but unchanged in the Δ*phoRS* mutant. Additional mutational analysis of the DNA-binding sites indicated that the putative PhoR binding site is a degenerated 8-bp direct repeat separated by 3 bp (CCTGTGAANNCCTGTGAA). The *phoRS* genes are subject to positive autoregulation.

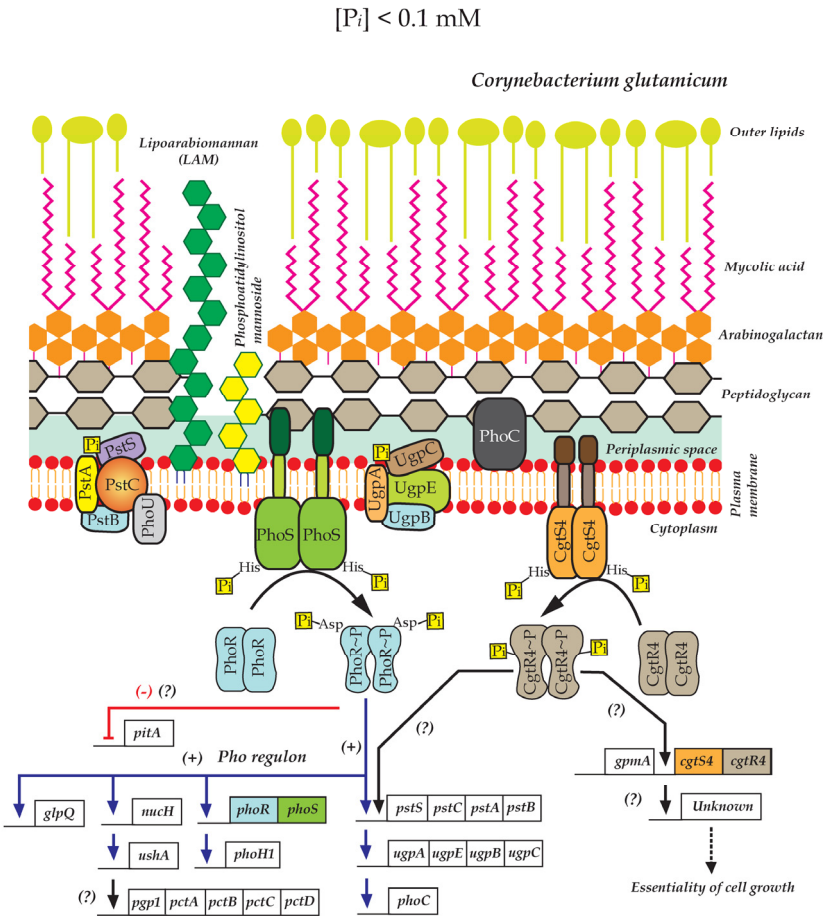

Figure 3.6 Diagram showing P_i-dependent regulatory processes in *C. glutamicum* that are controlled by two-component system PhoRS. Blue arrows (+) and red arrows with blunt ends (-) indicate transcriptional activation and repression, respectively. Phosphorylated PhoR activates expression of the *pstSCAB*, *phoC*, *ugpAEBC*, *glpQ*, *nucH*, *ushA*, *pctA*, and *phoRS* genes and might repress the *pitA* gene by binding to their promoters. The *pstSCAB* genes code for a high affinity P_i ABC transporter, *phoC* for a putative alkaline phosphatase, *ugpAEBC* for a glycerol-3-phosphate uptake, *glpQ1* for a glycerophosphodiester phosphodiesterase, *ushA* for a secreted enzyme with UDP-sugar hydrolase and 5'-nucleotide, *nucH* for a putative secreted nuclease, *pctABCD* for an ABC-type phosphate/phosphonate transport system and *pitA* for a low affinity secondary P_i transporter. The SenX3-RegX3 TCS might also be involved in the phosphate starvation response, a topic that has been studied in this PhD thesis.

3.3.2 The two-component regulatory system SenX3-RegX3

A time-resolved DNA microarray analysis showed that the changes in gene expression of the Δ*phoRS* mutant after shifting from P_i excess to P_i limitation were significantly different in the wild type. None of the Pho regulon genes were induced within an hour, except for the *pstSCAB* operon (Kocan et al., 2006). This result suggested the involvement of an additional regulator of the *pstSCAB* operon for P_i starvation responses. Independently, DNA affinity chromatography using the *pstS* promoter led to the identification of proteins bound to this DNA region. Among these PhoR was found, as expected, but also RegX3, which is the response regulator of the two-component system SenX3-RegX3 (previously named CgtS4-

CgtR4 (Kocan et al. 2006) and is encoded by the *regX3* gene. Evidence has been provided that the *regX3* gene is essential for cell growth (Wessel, 2003). The failure to delete the *regX3* gene could be overcome by conditional repression of *regX3* using the Tet repressor system. A mutant strain allowing conditionally repression of *regX3* (the *C. glutamicum* HMW1 mutant) was used in this PhD thesis to search for target genes of RegX3 by analysing the global gene expression with DNA microarrays.

3.4 Development of microbial metabolomics

Omics technologies have been developed to globally analyse (and when possible quantify) mRNAs, proteins, metabolites and metabolite fluxes in biological systems (Fiehn *et al.*, 2000, Kell, 2004, Weckwerth, 2003). Metabolomics deals with the measurement of the metabolome, which is the full set of metabolites inside and outside of cells (endo- and exo-metabolome), composed of hundreds to thousands of different compounds (Fig. 3.7). In order to measure a metabolome, comprehensive chromatographic technologies coupled with high resolution mass spectrometry have been developed and applied, for example gas chromatography-mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), Fourier transformation ion cyclotron resonance-mass spectrometry (FT-ICR-MS), capillary electrophoresis-mass spectrometry (CE-MS), and liquid chromatography-NMR (LC-NMR) (Mashego *et al.*, 2006, Oldiges *et al.*, 2007, Villas-Bôas *et al.*, 2004). Sophisticated bioinformatic software along with web-based or stand-alone databases are required to convert raw data into meaningful biological data (Kopka *et al.*, 2004, Neuweger *et al.*, 2008, van der Werf *et al.*, 2006, Xia *et al.*, 2009). Each analytical platform has advantages and disadvantages for metabolite profiling and there is no single analytical technology which covers an entire metabolome as yet (Büscher *et al.*, 2009, Werf *et al.*, 2007). Thus, the optimal strategy from each analytical field must be applied to metabolome samples in order to gain a comprehensive description of the metabolome.

Since there has been a massive increase of studies in the field of metabolomics, standard reporting requirements have been issued by the Metabolomics Standards Initiative (MSI) for the dissemination of results and the integration of data with other omics data (Fiehn *et al.*, 2007, van der Werf *et al.*, 2007). In order to obtain high quality data, sample preparation procedures have to be optimized before the samples are analyzed. Particular attention has been paid to the preparation of microbial metabolome samples (Hiller *et al.*, 2007, Wittmann *et al.*, 2004, Villas-Bôas *et al.*, 2005, Bolten *et al.*, 2007). This chapter focuses on sample preparation and metabolome analysis of microorganisms.

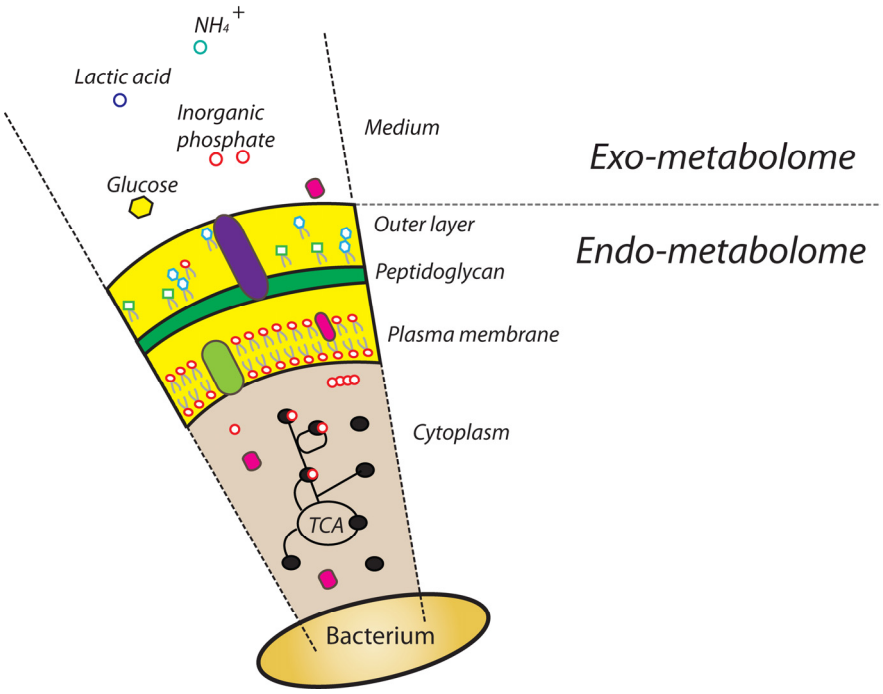

Figure 3.7 Diagram representing the metabolome of a bacterium. The exo-metabolome represents the set of metabolites secreted into the medium as a result of cellular activities or components of the medium. Compared to the exo-metabolome, the endo-metabolome is commonly regarded as 'metabolome' and represents the set of metabolites within the cell.

3.4.1 Sample preparation

The ideal sample preparation method for microorganisms is a one-step method involving (i) the inactivation of all enzyme activities, (ii) the separation of cells from the medium, and (iii) the extraction of metabolites from the cells within seconds (Villas-Bôas et al., 2004). Each task can be achieved separately, by adding a quenching solution such as liquid nitrogen or cold methanol for the inactivation of enzyme activities, by using fast centrifugation and washing with buffers for the separation, and by using methanol or ethanol based methods for metabolite extraction. However, the loss of certain intracellular metabolites into the medium is usually inevitable during the quenching step. Due to the diversity of the physiochemical properties of metabolites, 100% metabolite extraction and recovery are not achievable using just one solvent.

So far, a quenching method using cold methanol, a separation method using centrifugation, extraction methods using pure methanol and a mixture of methanol and water, and a concentration method using freeze-drying have been successfully applied to the intracellular metabolite profiling of *E. coli* and yeast (Hiller et al., 2007, Villas-Bôas et al., 2005, Bolten et al., 2007, Ewald *et al.*, 2009, Villas-Bôas & Bruheim, 2007, Werf et al., 2007).

However, these steps are not suitable for all other microbes such as Gram-positive species like *C. glutamicum* and filamentous fungi, because the particular cell wall structure of *C. glutamicum* causes a metabolite leak during quenching with cold methanol (Wittmann *et al.*, 2004) and filamentous fungi are highly viscous and heterogeneous during the separation and extraction steps (Smedsgaard & Nielsen, 2004, Villas-Bôas et al., 2005). Therefore, strain-specific sample preparation techniques are required for analysing metabolomes of interest.

Several sample preparation methods have been developed for *C. glutamicum* metabolome analysis (Plassmeier *et al.*, 2007, Börner *et al.*, 2007, Strelkov *et al.*, 2004). Loss of intracellular metabolites was calibrated by measuring the quenching solutions and by adding a U-^{13}C-labelled cell extract for isotope dilution mass spectrometry (Mashego *et al.*, 2004, Wu *et al.*, 2004). In this PhD thesis, a silicon oil-layered perchloric acid extraction method (Marienhagen & Eggeling, 2008) was performed for analysing the metabolome of *C. glutamicum*, where the quenching, separation and extraction steps take place within 30 seconds and an additional neutralization step via the addition of potassium hydroxide is required. Further optimized sample preparation methods for comprehensive metabolome analysis including hydrophobic, hydrophilic, and amphiphilic metabolites are necessary.

3.4.2 Metabolome analysis

After metabolite extraction from the cell samples, appropriate analytical methods have to be employed based on the types of metabolites which need to be detected for the purposes of the experiment (Oldiges et al., 2007, Villas-Bôas et al., 2004). GC-MS covers all different classes of thermo-stable metabolites after the derivatization of target metabolites (mainly non-volatile) (Roessner *et al.*, 2000, Schauer *et al.*, 2005, Kopka, 2006). LC-MS does not need additional derivatization and enables the detection of glycolytic intermediates, nucleotides, and isoprenoids (Buchholz *et al.*, 2001, Bartek *et al.*, 2008), and CE-MS is employed for the detection of charged molecules such as carboxylic acids, phosphorylated saccharides, and nucleotides (Soga *et al.*, 2003, Monton & Soga, 2007). Moreover, metabolite analysis in a single cell of yeast has been developed using matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) (Amantonico *et al.*, 2008), and over one million possible masses and extremely complex mixtures can be analysed using FT-ICR-MS (Brown & Kornberg, 2004, Rodgers *et al.*, 2000).

Using both the GC-MS and LC-MS platforms, six different analytical methods were applied to the metabolome of *E. coli* with 694 different metabolites expected from *in silico* analysis and 176 unique metabolites were analysed in cell extracts (Werf et al., 2007). In

addition, six methods using GC-MS, LC-MS, CE-MS enabled the detection of 75 standards from among 91 standard metabolites covering glycolysis, the pentose phosphate pathway, the TCA cycle, redox metabolism, amino acids, and nucleotides (Büscher *et al.*, 2009). The LC-MS/MS method has been developed to measure accurate concentrations of target metabolites in *E. coli*. 103 metabolites in exponentially growing *E. coli* cells were quantified by the intensive LC-MS/MS analytical method and the calculated total metabolite concentration was approximately 300 mM, with glutamate, glutathione, fructose-1,6-bisphosphate, and ATP making up 70% (Bennett *et al.*, 2009, Bennett *et al.*, 2008). In *Sacchromyces cerevisiae*, 11 metabolites in glycolysis and the TCA cycle were quantified using LC-MS and uniformly ^{13}C-labelled cell extracts as internal standards (Wittmann *et al.*, 2005, Wu et al., 2004, Wu *et al.*, 2006). Also, several amino acids in *S. cerevisiae* were measured by HPLC-UV in order to characterize the dynamics of intracellular amino acid pools during batch cultivation (Hans *et al.*, 2001, Bolten & Wittmann, 2008).

A comprehensive analysis of the metabolome of *C. glutamicum* was specifically undertaken using GC-MS and 121 metabolites, including amino acids, organic acids, phosphocarbohydrates, carbohydrates, and fatty acids, among 300 compounds, were identified using two different extraction methods for hydrophobic and hydrophilic metabolites (Börner et al., 2007, Strelkov et al., 2004). A semi-quantitative metabolite profiling using GC-MS was also performed to investigate the central metabolism and the methylcitrate cycle of propionate-grown *C. glutamicum* (Plassmeier *et al.*, 2007). Twenty-seven metabolites in glycolysis, the TCA cycle, the pentose phosphate pathway, and nucleotides in *C. glutamicum* were also quantified using LC-MS/MS in order to understand the *in vivo* cellular activities of this metabolic network (Blombach *et al.*, 2008, Bartek et al., 2008). A combination of metabolome and transcriptome analyses showed that the AmtR-dependent nitrogen control is related to L-lysine biosynthesis through the regulation of the *dapD* gene (Buchinger *et al.*, 2008). In this PhD thesis, GC-MS with either electron ionization or chemical ionization was applied to *C. glutamicum* for the semi-quantitative metabolome analysis of the P_i starvation response.

3.5 Aims of this work

The aim of this PhD thesis was to investigate the regulatory and metabolic aspects of the P_i starvation response of *C. glutamicum*. The first topic addressed in this work concerned the regulatory function of the two-component system SenX3-RegX3. The *C. glutamicum* RegX3 protein shares high sequence identity (67% and 76% respectively) to the PhoP response

regulator of *S. coelicolor* and the RegX3 regulator of *M. smegmatis* and therefore was assumed to be a good candidate for a transcriptional regulator controlling the expression of the *pstSCAB* genes encoding a high-affinity P_i ABC transporter and of alkaline phosphatase genes in response to P_i starvation. For this purpose transcriptome analyses with DNA microarrays of strains allowing conditional repression of *regX3* and DNA-binding studies with purified RegX3 protein should be used to define the target genes of this response regulator. Combined with the previous data on PhoRS TCS, this investigation should lead to a better understanding of the regulatory network involved in the P_i starvation response of the industrial amino acid producer *C. glutamicum*.

The second part of this work was a metabolome analysis in order to investigate how the metabolome of *C. glutamicum* changes in response to P_i starvation. For this purpose, GC-MS and ^{31}P-NMR should be used. Semi-quantitative comparisons of metabolites in cells could hint at metabolic regulations under the control of P_i starvation responses. Furthermore, an independent *in silico* simulation by flux balance analysis could also provide hints to characterize the P_i starvation responses using a large-scale metabolic network.

4 Results

The major aim of this PhD thesis was to unravel the regulatory and metabolic aspects of the P_i starvation response of *C. glutamicum*. The two-component system SenX3-RegX3 of *C. glutamicum* was analysed as a favourite regulatory system for being involved in the P_i starvation response, and semi-quantitative comparisons of the intracellular metabolites of *C. glutamicum* under P_i excess and P_i starvation were performed to study in the influence of P_i on the metabolome. The results from these studies have been summarized in two manuscripts.

The first part of this PhD thesis is a manuscript entitled 'SenX3-RegX3 (CgtS4-CgtR4), an essential two-component regulatory system of *Corynebacterium glutamicum* involved in the phosphate starvation response'. This paper summarizes the regulatory role of the RegX3 response regulator of *C. glutamicum* for the P_i starvation response using DNA microarray and DNA-binding analyses. Besides the PhoRS TCS, the two-component system SenX3-RegX3, among 13 two-component regulatory systems in *C. glutamicum*, also activates the expression of genes involved in the P_i starvation response. In addition, SenX3-RegX3 regulates genes involved in NAD^+ biosynthesis.

The main difficulty in this study was the essentiality of the *regX3* gene of *C. glutamicum*. To perform DNA microarray analysis, the TetR repressor system was employed to conditionally repress the *regX3* gene, as gene deletion was not possible. Successful gene silencing of the *regX3* gene in the *C. glutamicum* HMW1 (see the manuscript for details) mutant and its DNA microarray analysis are described in this manuscript. 17 genes showed a significant change (at least 2-fold) in mRNA levels in the *C. glutamicum* HMW1 mutant in the absence of anhydrotetracycline (ATC, analogue of tetracycline), compared to the same mutant in the presence of ATC. Repression of *regX3* by TetR occurs in the absence of ATC. This allowed the identification of putative target genes of the RegX3 regulator, several of which are known to be involved in the P_i starvation response. When analysed by EMSA, purified RegX3 was found to bind to the promoter regions of *pstS*, *phoC, ugpA*, and *ushA*, whose mRNA levels were reduced at reduced cellular RegX3 levels and thus are presumably activated by RegX3. In addition, RegX3 bound to the promoter region of a cluster of four genes, at least two of which are involved in NAD^+ biosynthesis (*ndnR-nadA-nadC-nadS*). Expression of these genes was also reduced when *regX3* expression was repressed. Furthermore, evidence was obtained that RegX3~P induces genes with alkaline phosphate activity under P_i starvation conditions in *C. glutamicum*.

The second manuscript, entitled 'A link between phosphate starvation responses and glycogen metabolism in *C. glutamicum* revealed by metabolomics', describes the influence of P_i limitation on the metabolism of *C. glutamicum* using a metabolomics approach. A ^{13}C-stable isotope-assisted metabolite identification method using GC-MS was developed to accurately identify the metabolites. Isotope-assisted metabolome analysis revealed that intracellular P_i, lactate, L-alanine, L-proline, and TCA intermediates were lowered in P_i-limited cells. In addition, the level of intracellular maltose in phosphate-limited cells was increased 28-fold. This significant change in maltose in P_i-starved cells stimulated the analysis of intracellular glycogen in *C. glutamicum* under P_i excess and P_i limitation, since the elevated maltose pool might be related to an altered glycogen metabolism.

Under P_i excess a glycogen pool in *C. glutamicum* wild type was formed and then degraded again within the exponential phase, whereas P_i-starved cells formed a glycogen pool in the exponential phase, but also retained it in the stationary phase. Interestingly, even acetate-grown cells, which do not form glycogen under P_i excess, did so under P_i limitation conditions. Chloramphenicol acetyltransferase assays supported the findings of an increased flux of glucose 6-phosphate into the glycogen pathway and reduced fluxes into glycolysis and the pentose phosphate pathway. Independently, the *in silico* simulation predicted an increased rate of glycogen synthesis at low P_i uptake and high glucose uptake rates. The same behaviour was also observed when acetate was used as the carbon source instead of glucose. These predictions are in agreement with the experimental results. In conclusion, our study showed that altered glycogen metabolism resulting in an increased and more stable glycogen pool was another consequence of P_i limitation.

4.1 SenX3-RegX3 (CgtS4-CgtR4), an essential two-component regulatory system *of C. glutamicum* involved in the phosphate starvation response

SenX3-RegX3 (CgtS4-CgtR4), an essential two-component regulatory system of *Corynebacterium glutamicum* involved in the phosphate starvation response

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Running title: SenX3-RegX3 two-component system of *C. glutamicum*

Keywords: *Corynebacterium glutamicum*, two-component signal transduction, phosphate starvation response, CgtS4-CgtR4, SenX3-RegX3

In previous studies the phosphate (P_i) starvation stimulon of *Corynebacterium glutamicum*, a Gram-positive soil bacterium, has been characterized and the two-component signal transduction system PhoS-PhoR was found to be involved in the activation of P_i starvation-inducible genes. Here we provide evidence that besides PhoSR a second two-component system composed of the sensor kinase SenX3 (CgtS4) and the response regulator RegX3 (CgtR4) is involved in the P_i starvation response. Deletion of the chromosomal *regX3* gene was only possible in the presence of a plasmid-borne copy, indicating that RegX3 is an essential response regulator in *C. glutamicum*. Global gene expression studies with a strain allowing conditional repression of *regX3* in concert with DNA-binding studies indicated that phosphorylated RegX3 activates several genes of the P_i starvation stimulon, i.e. *pstSCAB* (ABC transporter for P_i), *ugpAEBC* (ABC transporter for glycerol 3-phosphate), *phoC* (putative secreted phosphoesterase) and *ushA* (5'-nucleotidase), but also a cluster of four genes two of which are involved in NAD^+ biosynthesis (*ndnR-nadA-nadC-nadS*). Furthermore, whole-cell alkaline phosphatase activity was found to be controlled by RegX3. In summary, evidence for the involvement of the SenX3-RegX3 two-component system of *C. glutamicum* in the regulation of the P_i starvation response and in NAD^+ biosynthesis is presented.

Phosphorus (P) is an essential nutrient for all cells, as it is a constituent of DNA, RNA, phospholipids, ATP, NAD(P)H and numerous metabolites. Inorganic phosphate (P_i) is the most common phosphorus source for bacteria. P_i is taken up by secondary or primary transporters and assimilated into ATP. Polyphosphate can serve as a P_i storage compound. When P_i becomes limiting, most bacteria activate a set of genes encoding transporters and enzymes that allow the efficient capture of residual P_i or make P_i available by cleavage of e.g. organic phosphate esters. This is termed the phosphate starvation response. Characteristic features of this response are (i) increased synthesis of a high-affinity ABC transporter for P_i uptake, the Pst system, (ii) increased synthesis of secreted enzymes with phosphoesterase activity, such as alkaline phosphatase (AP), and (iii) control by two-component regulatory systems (TCS).

The ATP-dependent Pst transport system is able to take up P_i even at low extracellular concentrations. It is encoded by at least four genes, such as *pstSCAB* in *Escherichia coli* (10), $pstSCAB_1B_2$ in *Bacillus subtilis* (1, 28), *pstSCAB* in

Mycobacterium smegmatis (9) or *pstSCAB* in *Streptomyces lividans* (5, 7). They encode a secreted P_i-binding protein (PstS), two integral membrane proteins (PstA and PstC) and an ATP-binding protein (PstB). Secreted phosphoesterases hydrolyse phosphate esters to release P_i which can subsequently be taken up by the Pst system. The most prominent example is APase, which is encoded e.g. by the *phoA* gene in *E. coli* (22, 37), *M. smegmatis* (9, 20) and *S. lividans* (33), *phoA* or *phoB* in *B. subtilis* (1, 12). Responsible for the increased expression of these genes under P_i limitation are TCS, such as those encoded by the *phoBR* genes in *E. coli* (29, 39), *phoPR* in *B. subtilis* (12, 35), *senX3-regX3* in *M. smegmatis* (9) or *phoPR* in *S. lividans* (8, 33). It is assumed that upon sensing P_i limitation the sensor kinases of these TCS are autophosphorylated on a conserved histidine residue and the phosphoryl group is transferred to a conserved aspartate residue in the receiver domain of the cognate response regulator. This triggers a conformational change of the response regulator and enables the carboxyterminal DNA-binding domain to bind to the promoter of the target genes and to activate their expression.

However, bacteria have also developed strain-specific P_i-starvation responses and regulations. In a mutant of *E. coli* lacking the sensor kinase PhoP, the response regulator PhoB competes with the response regulator CreB for phosphorylation by the senor kinase CreC of the catabolite repression two-component CreBC system (2). CreB regulates a number of catabolic genes, e.g. the *ackA-pta* operon encoding acetate kinase and phosphotransacetylase or *talA* encoding transalolase. Acetylphosphate was also suggested to be involved in the modulation of the PhoP-PhoB activity (38, 40). These results indicate a possible cross-regulation of carbon metabolism and P_i assimilation in *E. coli*. In *B. subtilis*, which can sporulate in order to survive extreme environmental conditions, several additional regulators besides the PhoPR TCS are involved in the Pi starvation response, i.e. AbrB, ResDE and Spo0A (11). The two-component system ResDE and the transition-state regulator ArbB mediate the initiation of P_i-dependent regulation by the two-component system PhoPR and the Spo0A system represses the Pho response by regulating ResDE and ArbB pathways (16). In *S. lividans*, high accumulation of actinorhodin in a Δ*phoP* mutant has been reported (33), which indicated that the biosynthesis of secondary metabolites is strongly repressed by PhoPR two-component system.

The P_i-starvation response of *Corynebacterium glutamicum*, a soil bacterium used for production of more than two million tons of amino acids per year (17), was initially studied by transcriptomics using whole-genome DNA microarrays (14). Comparison of the mRNA profiles before and at different times after a shift from P_i excess to P_i limitation revealed a group of *psi* genes that cope with P_i limitation. This group includes the *pstSCAB* operon, the *ugpAEBC* operon encoding an ABC transporter for uptake of glycerol 3-phosphate, *glpQ1* encoding a glycerophosphoryl diester phosphodiesterase, *ushA* encoding a secreted enzyme with UDP-sugar hydrolase and 5'-nucleotidase activity (30), *nucH* encoding a putative secreted nuclease which possibly plays a role in liberating P_i from extracellular nucleic acids, *phoH1* encoding an ATPase of unknown function, the *pctABCD* operon encoding an ABC transporter which might be involved in the uptake of a yet unknown phosphorus-containing compound, *phoC* encoding a putative cell wall-associated phosphatase, and the *phoRS* genes encoding a TCS (14).

Subsequent studies with a Δ*phoRS* mutant indicated that the TCS composed of the sensor kinase PhoS and the response regulator PhoR is involved in the response of *C. glutamicum* to P_i starvation (19). *In vitro* studies showed that PhoR is phosphorylated by PhoS and that phosphorylation increased the DNA-binding affinity of PhoR. PhoR~P bound to many of the P_i starvation-inducible genes, but with different affinities (31). A preliminary 19-basepair (bp) binding motif composed of two loosely conserved 8-bp direct repeats separated by three basepairs was suggested (31).

In the Δ*phoRS* mutant of *C. glutamicum* the *pstSCAB* genes still showed significantly increased expression after a shift from P_i excess to P_i starvation, although not as strong as in the wild type (19). This suggested that an additional P_i-responsive regulatory system is present in *C. glutamicum* and involved in the regulation of the *pstSCAB* genes. Studies in phylogenetically related bacteria such as *M. smegmatis* suggested that the TCS annotated as CgtS4 (cg0483) and CgtR4 (cg0484) could be involved in P_i-dependent regulation. Therefore, the role of these proteins, which were renamed to SenX3 (*senX3*) and RegX3 (*regX3*) due to their similarity to the corresponding protein of mycobacteria, was analysed in this work.

MATERIALS AND MEHTODS

Bacterial strains and standard growth conditions. Bacterial strains and plasmids used or constructed in this work are listed in Table 1. *Escherichia coli* DH5α

(Invitrogen) was used as host during the construction of recombinant plasmids and was grown aerobically at 37°C on a rotary shaker at120 rpm in LB medium. *E. coli* BL21(DE3) or *E. coli* C43(DE3) was used for overproduction of the proteins SenX3 and RegX3 and grown aerobically at 30°C on a rotary shaker at 120 rpm in LB medium. *C. glutamicum* ATCC 13032 (wild type) and its derivatives were cultivated at 30°C on at rotary shaker at 120 rpm in brain heart infusion medium (Difco) or in CGXII minimal medium (18) containing 30 mg/l 3,4-dihydroxybenzoate as iron chelator and 222 mM glucose as carbon source. When appropriate, the media contained chloramphenicol (25 μg/ml for *E. coli* and 7.5 μg/ml for *C. glutamicum*) or kanamycin (50 μg/ml for *E. coli* and 25 μg/ml for *C. glutamicum*).

Construction of plasmids and mutants. The oligonucleotides used as PCR primers in this study are listed in Table S1. Plasmids were constructed by standard molecular genetic methods and confirmed by DNA sequence analysis. For the deletion of the chromosomal *senX3-regX3* genes, plasmid pK19*mobsacB*Δ*cgtSR4* was used (19). For expression of *senX3 and regX3* in *C. glutamicum*, plasmids pXMJ19-senX3-regX3, pXMJ19-regX3, pXMJ19-regX3-D52N, and pXMJ19-regX3$_{His}$ were constructed, which are based on the *E. coli*/*C. glutamicum* shuttle vector pXMJ19 (15). In the case of pXMJ19-senX3-regX3, a PCR reaction was performed with oligonucleotides senX3-regX3-PstI-fw and senX3-regX3-XbaI-rv, by which a 1988 bp fragment was obtained which included the *senX3* and *regX3* coding regions including 31 bp upstream of the proposed *senX3* start codon. In order to remove the major part of the *senX3* gene from plasmid pXMJ19-senX3-regX3, it was digested with HindIII and NruI, resulting in a 1.08 kb and a 7.48 kb fragment. After Klenow fill-in, the 7.48 kb fragment was religated to form pXMJ19-regX3. In order to create pXMJ19-regX3-D52N containing an aspartate to asparagine exchange at position 52 of RegX3, pXMJ19-regX3 was mutagenized with the Quick-change Site Directed Mutagenesis Kit (Stratagene, La Jolla, USA) using oligonucleotides regX3-D52N-fw and regX3-D52N-rv, see Table S1). These oligonucleotides introduced another silent mutation, by which a DraI restriction site was introduced into pXMJ19-regX3-D52N, allowing an easy distinction from its parent plasmid by restriction analysis. In order to test the *in vivo* functionality of RegX3 with a C-terminal histidine tag, a 1.97 kb XbaI-SmaI fragment from plasmid pET24b-RegX3$_{His}$ was isolated and cloned into pXMJ19 cut with the same enzymes.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	F^- *thi-1 endA1 hsdR17*($r^- m^+$) *supE44 ΔlacU169* (ϕ80*lacZΔM15*) *recA1 gyrA96 relA1*; host for cloning	Invitrogen
E. coli BL21(DE3)	F^- *ompT hsdSB*($r_B^- m_B^-$) *gal dcm (*λcIts857 *ind1* Sam7 *nin5 lacUV5*-T7 gene 1); host for overproduction of plasmid-encoded recombinant proteins	(34)
E. coli C43(DE3)	Derivative of *E. coli* BL21(DE3); more suitable for the expression of membrane proteins	(24)
C. glutamicum ATCC 13032	Biotin-auxotrophic wild type strain	(17)
C. glutamicum MW1	Derivative of ATCC 13032 with an in-frame deletion of the *senX3-regX3* gene after the transformation of pXMJ19-senX3-regX3	This work
C. glutamicum MW2	Derivative of ATCC 13032 with an in-frame deletion of the *senX3-regX3* gene after the transformation of pXMJ19-regX3	This work
C. glutamicum MW3	Derivative of ATCC 13032 with an in-frame deletion of the *senX3-regX3* gene after the transformation of pXMJ19-regX3(D52N)	This work
C. glutamicum HMW1	Derivative of ATCC 13032 with an in-frame deletion of the *senX3-regX3* gene after the transformation of pXMJ19-ASK-IBC3C-regX3	This work
Plasmids		
pET24b	Kan^r; P_{T7} *lacI oriV* from pBR322; *E. coli* expression vector for overproduction of proteins with an C-terminal hexahistidine tag	Novagen
pET24b-SenX3$_{His}$	Kan^r; pET24b derivative for overproduction of SenX3 with an C-terminal hexahistidine tag (SenX3$_{His}$)	This work
pET24b-RegX3$_{His}$	Kan^r; pET24b derivative for overproduction of RegX3 with an C-terminal hexahistidine tag (RegX3$_{His}$)	This work
pET24b-Strep	Kan^r; pET24b with Strep-tag II sequence (WSHPQFEK)	(23)
pET24b- RegX3$_{Strep}$	Kan^r; pET24b-Strep derivative for overproduction of RegX3with an C-terminal hexahistidine tag (RegX3$_{strep}$)	This work
pK19mobsacBΔcgtSR4	Kan^r; pK19mobsacB derivative containing a crossover PCR product covering the up- and downstream regions of the *senX3-regX3* genes	(19)
pXMJ19	Cm^r; *E. coli – C. glutamicum* shuttle vector for gene expression under P_{tac} ($oriV_{E.c.}$, $oriV_{C.g}$, $lacI^q$)	(15)
pASK-IBA3C	Cm^r; *E. coli* vector for gene expression under P_{tetA} and TetR repressor ($oriV_{E.c.}$)	IBA GmbH (Göttingen)
pASK-IBA3C-regX3	Cm^r; pASK-IBC3C derivative for expression of the *regX3* gene under control of P_{tetA} and TetR repressor	This work
pXMJ19-ASK-IBA3C-regX3	Cm^r; plasmid composed of pXMJ19 and pASK-IBC3C-regX3 ($oriV_{E.c.}$ $oriV_{C.g}$) allowing regulated expression of *regX3* in *C. glutamicum*	This work
pXMJ19-senX3-regX3	Cm^r; pXMJ19 derivative for expression of the *senX3-regX3* genes in *C. glutamicum*	This work
pXMJ19-regX3	Cm^r; pXMJ19 derivative for expression of the *regX3* gene in *C. glutamicum*	This work
pXMJ19-regX3(D52N)	Cm^r; pXMJ19 derivative for the synthesis of a RegX3 protein with a D52N exchange	This work
pXMJ19-regX3$_{His}$	Cm^r; pXMJ19 derivative for the synthesis of a RegX3 protein with a C-terminal His tag	This work

Due to the essentially of the *regX3* gene, the search by DNA microarray analysis for genes whose expression is influenced by RegX3 required a strain in which the *regX3* gene could be conditionally repressed. For this purpose, the *regX3* gene was amplified by PCR using oligonucleotides regX3-BsaI-fw and regX3-BsaI-rv and inserted into the expression vector pASK-IBA3C (IBA GmbH, Göttingen, Germany) using two Bsa1 restriction sites. In the resulting plasmid pASK-IBA3C-regX3, expression of *regX3* is controlled by the *tetA* promoter of transposon Tn*10*, which can be repressed by the TetR repressor in the absence of tetracyclin or anhydrotetracyclin (ATC). TetR is also encoded on the pASK-IBA3C vector. In order to allow replication in *C. glutamicum*, plasmid pASK-IBA3C-regX3 was linearized with SpeI and ligated with the shuttle vector pXMJ19 also cut with SpeI. The resulting plasmid pXMJ19-ASK-IBA3C-regX3 was transferred into a *C. glutamicum* strain in which the suicide plasmid pK19mobsacB-ΔcgtSR4 has been integrated via a single homologous recombination into the chromosome. In the presence of ATC, a *sacB*-based selection was made for a second homologous recombination event by which the vector part of pK19*mobsacB*-ΔcgtSR4 is excised from the chromosome, leading either to a deletion of the chromosomal *senX3-regX3* genes or to the restoration of the wild type situation.

For overproduction of SenX3 and RegX3 in *E. coli* and purification, several expression plasmids were constructed, which encode a SenX3 protein with a C-terminal hexahistidine tag (pET24b-SenX3$_{His}$, LEHHHHHH), a RegX3 protein with a C-terminal hexahistidine tag (pET24b-RegX3$_{His}$, VEHHHHHH) and a RegX3 protein with a C-terminal StrepTag-II (pET24b-RegX3$_{Strep}$, VEWSHPQFEK).

Global gene expression analysis. *C. glutamicum* strain HMW1 was precultivated in BHI medium with 200 ng/mL of ATC and subsequently for 24 h in CGXII medium in the presence or in the absence of ATC (200 ng/mL). These cells were then used to inoculate the main cultures (CGXII medium with or without ATC) to an initial OD_{600} of 0.8. Exponentially growing cells from each culture were harvested at an OD_{600} of 2.5, washed and used for RNA preparation. The preparation of RNA and the synthesis of fluorescently labeled cDNA were carried out as described previously (21). Custom-made DNA microarrays printed with 70-mer oligonucleotides for *C. glutamicum* ATCC 13032 were obtained from Operon (Cologne, Germany) and are based on the genome sequence entry with accession no. NC_006958 (17). Hybridization and stringent washing of the microarrays were performed according to the instructions of the supplier.

Hybridization was carried out for 16 to 18 h at 42°C by using a microarray user interface hybridization system (BioMicro Systems, Salt Lake City, UT). After being washed, the microarrays were dried by centrifugation (5 min at 1,600 x *g*) and the levels of fluorescence at 532 nm (for Cy3-dUTP) and 635 nm (for Cy5-dUTP) were determined with 10-µm resolution by using a GenePix 6000 laser scanner (Axon Instruments, Sunnyvale, CA). Quantitative image analysis was carried out using GenePix image analysis software (GenePix Pro 6.0; Axon Instruments), and the results were saved as GenePix results files. For data normalization, GenePix results files were processed using the Bioconductor/R packages limma and marray (http://www.bioconductor.org). Processed and normalized data, as well as experimental details (minimum information about a microarray experiment) (3), were stored in the in-house microarray database for further analysis (27). The transcriptome comparison of strain HMW1 grown in the presence or absence of ATC was performed three times starting from independent cultures. The criteria for identifying genes with significantly changed mRNA levels were as follows: (i) flags ≤ 0 (GenePix Pro 6.0), (ii) signal/noise ≥ 5 for Cy5 or Cy3, (iii) ratio of median ≥2 (ATC^-/ATC^+), and (iv) statistically significant change (*p*-value <0.05 in a Student t-test).

Overproduction and purification of SenX3 and RegX3. For overproduction of $SenX3_{His}$, $RegX3_{His}$, and $RegX3_{Strep}$, *E. coli* BL21(DE3) or *E. coli* C43(DE3) transformed with pET24b-$SenX3_{His}$, pET24b-$RegX3_{His}$, or pET24b-$RegX3_{Strep}$ was grown in LB medium at 30°C on a rotary shaker at 120 rpm until the cultures had reached an OD_{600} of 0.5. Then, target gene expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the cultures were incubated for another 3 h at room temperature. Subsequently, cells were harvested, washed once in TNI5 buffer (20 mM Tris/HCl, pH 7.9, 300 mM NaCl, and 5 mM imidazole; the number in TNI5 indicates the milimolar imidazole concentration) or in TE buffer for purification of $RegX3_{Strep}$ (10 mM Tris/HCl, pH 7.5, 1 mM EDTA), and resuspended in the same buffer. After addition of a protease inhibitor cocktail (Complete, Mini, EDTA-free; Roche Diagnostics, Mannheim, Germany), cells were disrupted using a French press. Intact cells and cell debris were removed by centrifugation (30 min at 5,000 × *g*; 4°C) and the supernatant was subjected to ultracentrifugation (1 h at 150,000 × *g*; 4°C). For the solubilization of $SenX3_{His}$, the membran fraction obtained after ultracentrifugation was resuspended in 1 ml TNI5 buffer and the protein concentration was determined

using the Bradford assay. A 10% (w/w) solution of the detergent dodecyl-β-D-maltoside (DDM; 2 g DDM/ g of protein) was slowly added to the membrane suspension until a concentration of 2 g dodecyl-β-D-maltoside per g protein was obtained. The suspension was stirred on ice for 1 h and then subjected to ultracentrifugation (20 min at 150,000 × *g*; 4°C).

$SenX3_{His}$ and $RegX3_{His}$ present in the supernatant of the ultracentrifugation were purified by nickel chelate affinity chromatography using nickel-charged His-Bind resin (Merck, Darmstadt, Germany) equilibrated with TNI5. After washing the column with TNI50, specifically bound proteins were eluted with TNI100 or TNI200 for $RegX3_{His}$ or $SenX3_{His}$, respectively. Protein-containing fractions were pooled and the elution buffer was exchanged against BS buffer containing 50 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, and 10% (v/v) glycerol. $RegX3_{Strep}$ present in the supernatant of the ultracentrifugation step was applied to a StrepTactin-Sepharose column with a bed volume of 2 ml (IBA, Göttingen, Germany) equilibrated with buffer W (100 mM Tris/HCl, pH 8.0, 1 mM EDTA). The column was washed with 10 mM of buffer W and specifically bound proteins were eluted with buffer E (buffer W with 2.5 mM D-desthiobiotin). Fractions containing $RegX3_{Strep}$ were pooled. Overproduction and purification of the proteins was followed by SDS-PAGE and staining of the proteins with Coomassie brilliant blue.

***In vitro* phosphorylation assay.** For the determination of the autophosphorylation activity, 1.07 μM $SenX3_{His}$ was incubated with 0.17 μM [γ-^{32}P]-ATP (10 mCi/mL) and 40 μM nonradioactive ATP. The assay mixture was incubated at room temperature and at different time points, aliquots were removed, mixed with an equal volume of 2x SDS loading buffer (124 mM Tris, pH 6.8, 20% glycerol, 4.6% SDS, 1.4 M β-mercaptoethanol, 0.01% bromohenol blue), and placed on ice. Subsequently, without prior heating, the samples were subjected to SDS-PAGE using a 12% separating gel. After being dried, the gel was analyzed with a BAS-1800 phosphor imager (Fujifilm). For the determination of phosphorylation of RegX3 by SenX3, a twofold molar excess of purified $RegX3_{Strep}$ was added to the $SenX3_{His}$ and ATP containing assay mixture after 30 min incubation and the sample was incubated for another 31 min. Aliquots were removed every 10 minutes.

Electrophoretic mobility shift assay (EMSA). EMSAs were performed essentially as described previously (41). DNA fragments used in the EMSAs were synthesized by

PCR, purified with the PCR purification kit (Qiagen, Hilden, Germany) and eluted in EB buffer (10 mM Tris/HCl, pH 8.5). The purified DNA fragments were mixed with $RegX3_{His}$ either in the unphosphorylated state or after phosphorylation with $SenX3_{His}$. The reaction mixtures included 100 ng target DNA and a 0- to 90-fold molar excess of $RegX3_{His}$ protein in a total volume of 20 µL of BS buffer. For phosphorylation of RegX3, it was incubated for 60 min with SenX3 (half the concentration of RegX3) and 5 mM ATP at room temperature before adding the DNA fragments. For protein-DNA interaction, the mixtures were incubated for 30 min at room and then separated on a 15% native polyacrylamide gel. Electrophoresis was performed at room temperature and 170 V using 1x TBE (89 mM Tris, 9 mM boric acid, and 2 mM EDTA) as electrophoresis buffer. The gels were subsequently stained with Sybr®Green I or GelRed™ according to the instructions of the supplier (Sigma-Adrich) and photographed.

Northern blot analysis. Total RNA of *C. glutamicum* wild type was prepared from cultures growing exponentially in CGXII glucose medium with either 13 mM or 0.13 mM P_i. 5 µg RNA was separated by formaldehyde agarose gel electrophoresis and blotted onto a nylon membrane (Hybond-N+, Amersham Bioscience). The RNA was fixed to the membrane by UV crosslinking. For synthesis of the probe, the oligonucelotides (senX3-NR-fw/-rv and regX3-NR-fw/-rv) were used for the synthesis of a digoxigenine (DIG)-labeled DNA probe. The nylon membrane with the crosslinked RNA was hybridized with the DIG-labeled probe at 50°C, and after washing with 1x SSC buffer (20x SSC, 3 M NaCl, 300 mM sodium citrate) at a temperature of 50°C, detection was performed using anti-digoxigenin-AP and CDP-*Star* (Roche). Chemiluminescence was detected by an LAS-3000 imaging system (Fuji).

Primer extension analysis. For non-radioactive primer extension analysis of the *senX3*, *regX3*, and *phoC* genes, total RNA was isolated from exponentially growing *C. glutamicum* wild type cultivated in CGXII glucose medium containing 0.13 mM P_i. Primer extension with 10-13 µg of total RNA was performed using IRD800-labeled oligonucleotides (senX3-PE-rv-IRD800, regX3-PE-rv-IRD800, phoC-PE-rv-IRD800) (MWG Biotech, Ebersberg, Germany) as described previously (6). The template for DNA sequence analysis used to localize the 3′-end of the primer extension product was amplified in a standard PCR reaction using unlabeled oligonucleotides (see Table S1 in the supplementary materials).

Alkaline phosphatase assay. For the determination of alkaline phosphatase (AP) activity, the method described by Glover *et al.* (9) was used with minor modifications. Cells were precultivated in BHI medium supplemented with 2% (w/v) glucose for 16 h, washed once with 0.9% (w/v) NaCl and transferred to CGXII medium containing only 1/100 of the regular P_i concentration (0.13 mM P_i instead of 13 mM P_i) in order to deplete internal polyphosphates. After a second precultivation in P_i-limited CGXII medium, cells were grown in CGXII medium containing either 13 mM or 0.13 mM P_i to an OD_{600} of 1. Samples (corresponding to 1 ml culture of OD_{600} = 10) of *C. glutamicum* cultures were taken after 8 h, 12 h and 24 h after start of the cultivation. After centrifugation, cells were washed with 1 mL TT buffer (1 M Tris/HCl, pH 8.0, 0.2% Tween 40) and resuspended in 100 µl of this buffer. After addition of 900 µL of substrate solution (10 mM *p*-nitrophenyl phosphate, 10 mM $MgCl_2$), the tubes containing 2.5 mg cell dry weight/ml were incubated at 37°C for exactly 30 min in the dark. After centrifugation, the absorbance of the supernatant was immediately measured at 405 nm and the specific alkaline phosphatase activity was calculated using an extinction coefficient of 18.3 $mM^{-1}cm^{-1}$ for *p*-nitrophenolate. In control experiments it was shown that AP activity increased linearly over time up to 180 min and was linearly dependent on cell dry weight up to at least 6 mg/ml.

RESULTS

***In silico* analysis of the *C. glutamicum senX3-regX3* (*cgtSR4*) genes and the encoded proteins.** The genome sequence of the *C. glutamicum* type strain ATCC 13032 (13, 17) revealed the presence of 13 two-component signal transduction systems. In this study, we aimed at a functional analysis of the two-component system encoded by *cgtS4* (cg0483) and *cgtR4* (cg0484) (19). As these genes and the encoded proteins are highly similar to the *senX3* and *regX3* genes and the encoded proteins from mycobacteria, we decided to use the latter names also for *C. glutamicum*.

The *senX3-regX3* genes are located downstream of *gpmA* encoding phosphoglycerate mutase, a glycolytic enzyme catalysing the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. This organisation is conserved in corynebacteria and mycobacteria. The coding region of *senX3* according to the annotation by (17) starts with a GTG start codon 10 bp downstream of the stop codon of *gpmA* and specifies a protein of 413 amino acid residues. The *senX3* annotation proposed by (13) is different and starts 91 bp

downstream of the *pgmA* start codon with ATG. The derived protein consists of 386 amino acid residues. Although sequence alignments with SenX3 homologs from other bacteria favor the start codon located 10 bp downstream of *pgmA*, experimental determination of the aminoterminal sequence of native SenX3 is required to determine the start codon unequivocally. The ATG start codon of *regX3* overlaps with the TGA stop codon of *senX3*. 693 bp downstream of the stop codon of *regX3* the following open reading frame (cg0485) starts, which encodes a protein of unknown function.

The sensor kinase SenX3 (calculated mass 45,165 Da) belongs to the class I histidine protein kinases. The C-terminal kinase core contains the sequence motifs characteristic for histidine kinases, i.e. the H-, N-, D-, F- and G-box. The phosphorylatable histidine residue is located at position 166 (based on the 413 amino acid residue protein). The N-terminal portion of SenX3 (amino acid residues 1 - 150) is presumably involved in signal recognition. Hydropathy analysis using TopPred-II (4) revealed that the N-terminus (amino acid residues 1-21) is very hydrophobic and predicted to form a transmembrane segment. A second region of lower hydrophobicity stretches from amino acid residues 43 – 63 and has characteristics of an amhiphilic helix. It is important to point out that the predicted aminoterminal transmembran helix lacks in the alternative annotation for SenX3. The response regulator RegX3 (232 amino acid resiudes, predicted mass 25.999 Da) belongs to the PhoB/OmpR subfamily and is composed of an aminoterminal receiver domain and a carboxyterminal DNA-binding domain. The phosphorylated aspartate residue is located at position 52.

Evidence for essentiality of *regX3* in *C. glutamicum*. In order to analyse the function of the SenX3-RegX3 system in *C. glutamicum*, attempts were made to construct a marker-free deletion mutant lacking both *senX3* and *regX3* using the suicide vector pK19mobsacB (32) and an established protocol (26). After the first homologous recombination of the plasmid pK19mobsacBΔcgtSR4 into the chromosome and selection for the second recombination event, 40 clones were tested by PCR and found to represent wild type. This indicated that *senX3* and/or *regX3* are essential for *C. glutamicum*. To support this assumption, the second recombination event was performed with the strain obtained after the first homologous recombination (13032::pK19mobsacBΔcgtSR4) carrying either an expression plasmid for *senX3* and *regX3* (pXMJ19-senX3-regX3), or an expression plasmid for *regX3* only (pXMJ19-regX3) or, as negative control, the vector only (pXMJ19). In the presence of pXMJ19-senX3-regX3, ten kanamycin-sensitive and sucrose-tolerant clones obtained after the

second recombination event were analyzed by PCR, five of which contained a chromosomal *senX3-regX3* deletion. This was also confirmed by Southern blot analysis using SphI digested chromosomal DNA (data not shown). In the presence of pXMJ19-regX3, six out of nine clones showed the *senX3-regX3* deletion, whereas in the presence of pXMJ19 nine out of nine clones tested showed the wild type situation. According to these results, *regX3*, but not *senX3* is essential for *C. glutamicum*.

In further experiments, pXMJ19 derivatives encoding modified RegX3 proteins were tested. In the presence of pXMJ19-regX3$_{His}$, seven out of 12 clones tested after the second recombination event showed the *senX3-regX3* deletion, indicating that a C-terminal His tag (VEHHHHHH) does not interfere with the essential function of RegX3. In the presence of pXMJ19-regX3-D52N, two out of four clones carried the *senX3-regX3* deletion, indicating that phosphorylation of RegX3 is not required for its essential function in *C. glutamicum*.

Construction of a *C. glutamicum* strain for conditional *regX3* expression. In order to get hints on the target genes of the essential response regulator RegX3, a Δ*senX3-regX3* mutant was constructed in the presence of plasmid pXMJ19-ASK-IBA3C-regX3. This plasmid carries the *regX3* gene under the control of the *Escherichia coli tetA* promoter and also the *tetR* gene encoding the TetR repressor of the *tetA* promoter. Repression of the *tetA* promoter by TetR can be relieved by addition of anhydrotetracycline (ATC). The chromosomal deletion was performed in the presence of ATC and 2 out of 16 clones tested carried a *senX3-regX3* deletion. In Fig. 1A, a PCR analysis of one of these clones named HMW1 (Δ*senX3-regX3*/pXMJ19-ASK-IBA3C-regX3) is shown. As shown in Fig. 1B, when colonies from a BHI agar plate containing ATC were streaked on BHI plates with or without ATC, single colonies were formed only in the presence of ATC, but not in its absence, confirming the essentiality of *regX3* and the functionality of the conditional expression system.

In liquid CGXII medium containing 4% (w/v) glucose as carbon source, the growth behaviour of strain HMW1 was different than on BHI plates. When CGXII medium was inoculated with cells from BHI medium with ATC, the cultures showed similar growth rates of 0.16 h^{-1} with and without ATC. When cells of the ATC-containing culture were used to inoculate fresh CGXII medium with ATC, almost the same growth rate of 0.15 ± 0.002 h^{-1} as in the preculture was observed and a final OD_{600} of about 60 was reached, similar to the wild type. The reduced growth rate of strain HMW1 compared to the wild

type (0.4 h^{-1}) must be caused either the lack of *senX3* and/or a different expression level of *regX3*. When cells of the preculture without ATC were used to inoculate fresh CGXII medium without ATC, a strongly decreased growth rate of 0.06 ± 0.01 h^{-1} was observed and the final OD_{600} reached was only 8.2 ± 0.52. Thus, in liquid media the inhibitory effect of a reduced RegX3 level on growth required a longer precultivation in the absence of ATC to be detected.

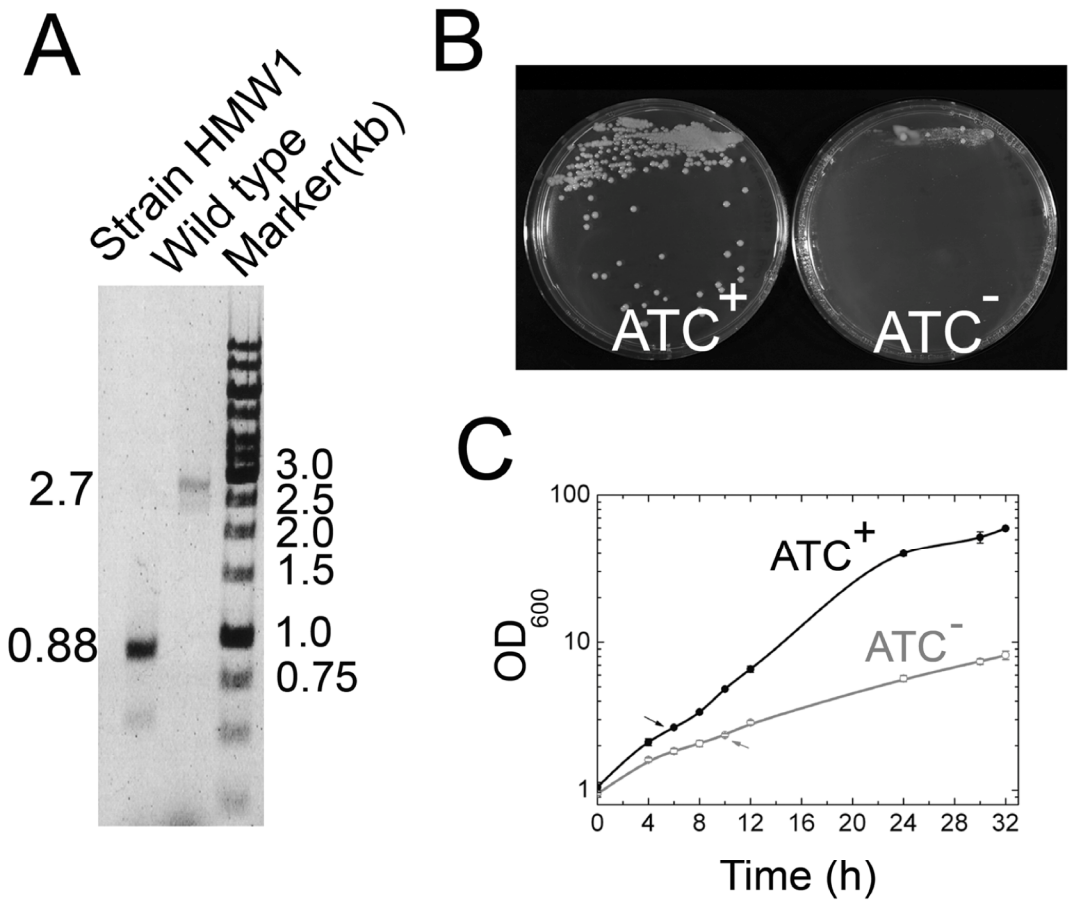

FIG. 1. Genomic and growth analysis of the *C. glutamicum* mutant strain HMW1 allowing conditional repression of *regX3*. Strain HMW1 contains a deletion of the chromosomal *senX3-regX3* genes and plasmid pXMJ19-ASK-IBA3C-regX3. Expression of *regX3* by this plasmid is controlled by the *tetA* promoter of transposon Tn*10* and is repressed by TetR in the absence of anhydrotetracycline. (A) Confirmation of the *senX3-regX3* deletion in *C. glutamicum* HMW1 by PCR using the oligonucleotides ΔsenX3-regX3-seq-fw and ΔsenX3-regX3-seq-rv. As expected, a 2.7 kb DNA fragment was amplified from chromosomal DNA of the wild type and a 0.88 kb fragment from chromosomal DNA of strain HMW1. (B) Growth of *C. glutamicum* HMW1 on BHI agar plate in the presence (ATC^+) or absence (ATC^-) of anhydrotetracycline. As inoculum colonies from a BHI agar plate with ATC were used. (C) Growth of *C. glutamicum* HMW1 in CGXII glucose minimal medium supplemented or not supplemented with 200 ng/ml ATC. The cells were precultivated first in BHI medium without ATC and subsequently in CGXII medium without ATC. The arrows indicate the time points at which samples for transcriptome analysis were taken.

Transcriptome analyses using strain HMW1. In order to hints on the target genes of RegX3, DNA microarray-based transcriptome comparisons were performed using strain HMW1 cultivated in the absence and in the presence of ATC**.** RNA was isolated from exponentially growing cells at an OD_{600} of 2.5. As shown in Table 2, four genes showed a more than two-fold increased mRNA level in the absence of ATC, i.e. *aceA* (cg2560, isocitrate lyase), *metE* (cg1290, cobalamin-independent methionine synthase), cg1977 (putative secreted protein), and cg1190 (hypthetical protein). 13 genes showed a more than two-fold decreased mRNA in the absence of ATC. The mRNA level of *regX3* was most strongly reduced with a ratio of 0.01 (i.e. a 100-fold lower *regX3* mRNA level was observed in the cells grown in the absence of ATC compard to cells grown in the presence of ATC).

TABLE 2. Genes showing ≥ 2-fold changed mRNA levels in *C. glutamicum* strain HMW1 after growth without (ATC^-) or with (ATC^+)

C. glutamicum locus tag no.	Annotation	Gene	mRNA ratio (ATC^-/ATC^+)[a]	*p*-value[b]
cg2560	Isocitrate lyase	*aceA*	2.84	0.001
cg1290	5-Methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	*metE*	2.42	0.000
cg1977	Putative secreted protein		2.18	0.003
cg1190	Hypothetical protein cg1190		2.05	0.010
cg1218	A transcriptional repressor/a member of Nudix	*ndnR*	0.46	0.014
cg1216	Quinolinate synthetase	*nadA*	0.35	0.002
cg1215	Nicotinate-nucleotide pyrophosphorylase	*nadC*	0.30	0.008
cg1214	Cysteine sulfinate desulfinase/cysteine desulfurase or related enzyme	*nadS*	0.34	0.002
cg3393	Putative secreted phosphoesterase	*phoC*	0.40	0.006
cg1568	Glycerol-3-phosphate ABC transport system, permease component	*ugpA*	0.37	0.005
cg1569	Glycerol-3-phosphate ABC transport system, permease component	*ugpE*	0.36	0.014
cg1570	Glycerol-3-phosphate ABC transport system, secreted compontent	*ugpB*	0.38	0.019
cg2843	Phosphate ABC transport system, permease component	*pstB*	0.44	0.008
cg2844	Phosphate ABC transport system, permease component	*pstA*	0.36	0.011
cg2845	Phosphate ABC transport system, permease component	*pstC*	0.22	0.013
cg2846	Phosphate ABC transport system, secreted component	*pstS*	0.23	0.007
cg0484	Two component response regulator RegX3 (CgtR4)	*regX3*	0.10	0.003

[a] The mRNA ratios are averages from at least three experiments.
[b] $P < 0.05$ as determined by a *t*-test.

Eight of the 13 genes are members of the phosphate starvation stimulon, i.e. the *pstSCAB* genes encoding an ABC transporter for P_i uptake, the *ugpABCD* genes encoding an ABC transporter for glycerol 3-phosphate uptake, and *phoC* encoding a putative alkaline phosphatase. The residual four genes are clustered and include *nadS* (cg1214) encoding a cysteine sulfinate desulfinase/cysteine desulfurase or related enzyme, *nadC* (cg1215) encoding nicotinate-nucleotide pyrophosphorylase, *nadA* (cg1216) encoding quinolinate synthetase, and *ndnR* (cg1218) encoding a transcriptional repressor of the NAD de novo biosynthesis genes. These genes, i.e. *ndnR-nadA-nadC-nadS*, are involved in de novo NAD biosynthesis.

Autophosphorylation of SenX3 and phosphoryl group transfer to the response regulator RegX3. The DNA microarray analyses had indicated that several P_i starvation-inducible genes might be targets of RegX3. To test whether RegX3 binds to the promoter regions of these genes and how phosphorylation effects the DNA-binding properties, RegX3 with a carboxyterminal StrepTag-II ($RegX3_{Strep}$) and its sensor kinase SenX3 with a carboxyterminal hexahistidine tag ($SenX3_{His}$) were overproduced in *Escherichia coli* and purified by StrepTactin and Ni^{2+}-NTA affinity chromatography, respectively (Fig. 2A). In the case of SenX3, the protein was localized in the membrane fraction and solubilized by dodecylmaltoside before purification. As shown in Fig. 2B, the isolated SenX3 protein showed autophosphorylation activity when incubated with [γ-^{32}P]-ATP. The maximal phosphorylation level was observed 10 min after of the reaction. When purified RegX3 was added to the reaction mixture, it became phosphorylated within one minute to almost the maximal level (Fig. 2B), which then remained constant for 30 min. Thus, the phosphorylation reactions typical for two-component signal transduction systems could be demonstrated for the SenX3-RegX3 system of *C. glutamicum*.

Primer extension and Northern blot analysis. In order to determine the transcriptional start sites of *phoC*, *senX3* and *regX3*, primer extension analyses were performed using the oligonucleotides phoC-PE-rv-IRD800, senX3-PE-rv-IRD800 and regX3-PE-rv-IRD800 and total RNA of wild type cells grown in CGXII glucose minimal medium under P_i limitation (0.13 mM). As shown in Fig. 3A, the putative transcriptional start site of *phoC* was found to be a G residue located 150 bp upstream of the ATG start codon proposed in the annotation by Kalinowski *et al.* (17) which predicts a protein of 1461 amino acid residues. In the case of *senX3*, a possible

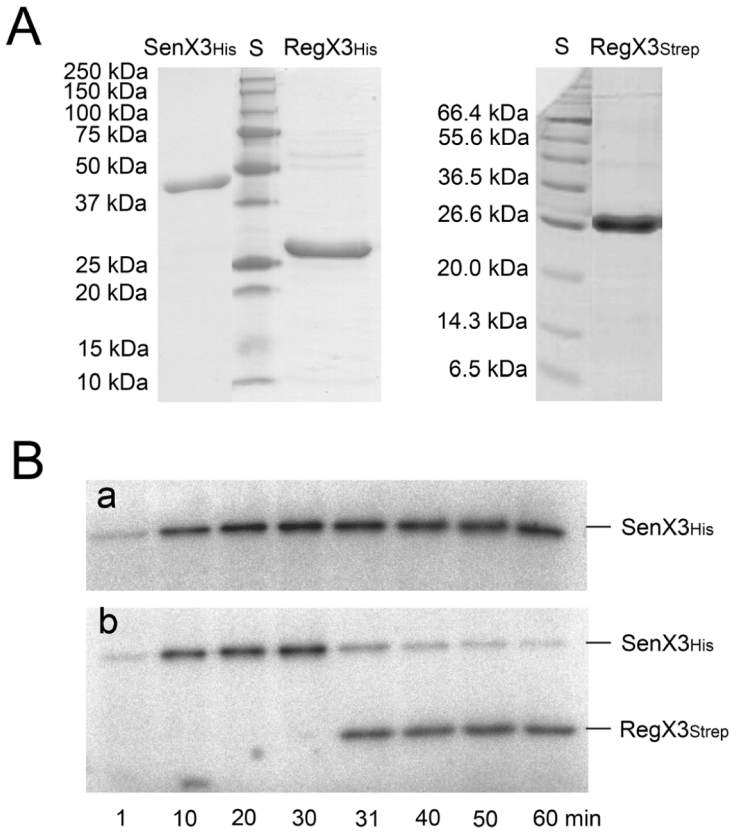

FIG. 2. Purification of SenX3 and RegX3 and phosphorylation studies. (A) Coomassie-stained SDS-polyacrylamide gel showing purified $SenX3_{His}$, $RegX3_{His}$ and $RegX3_{Strep}$. S, protein standard (Bio-Rad). (B) Autophosphorylation of SenX3 and phosphoryl group transfer from SenX3 to RegX3. 1.6 µM $SenX3_{His}$ in TKMD buffer (50 mM Tris-HCl pH 7.5, 200 mM KCl, 5 mM $MgCl_2$, and 5 mM dithiothreitol) was incubated at room temperature with 0.17 µM [γ-^{32}P]-ATP (~3,000 Ci/mmol; 10 mCi/ml) and 40 µM nonradioactive ATP in a total volume of 60 µl. Two parallel assays (a and b) were processed. After 1, 10, 20 and 30 min, 5 µl samples were removed, mixed immediately with 5 µl 2 × SDS loading buffer and stored on ice. Immediately after removal of the 30-min sample, 20 µl TKMD buffer was added to assay and 20 µl of a $RegX3_{Strep}$ solution in TKMD buffer was added to assay b (final RegX3 concentration 1.98 µM). The resulting concentrations of SenX3 and ATP were 1.07 µM and 26.78 µM, respectively. 7.5 µl samples were removed after 31 min, 40 min, 50 min and 60 min, mixed with 7.5 µl 2 x SDS loading buffer and stored on ice. 8 µl of the samples taken from 1 to 30 min and 12 µl of the samples taken from 31 to 60 min were separated electrophoretically on a 12% SDS polyacrylamide gel (without prior heating). Subsequently, the gels were dried and analysed with a BAS-1800 phosphor imager (Fujifilm).

transcriptional start site was located at an A residues located 29 bp upstream of the predicted GTG start codon (Fig. 3B). However, this start site is located within the coding region of the phosphoglucomutase gene and is not preceded by a typical -10 region. Although it appeared likely that *senX3* and *regX3* are cotranscribed, we also performed primer extension with an oligonucleotide annealing within the 5'-end of the *regX3* gene. Surprisingly, a primer exentension product was formed starting at a C residue located 30 bp upstream of the ATG start codon of *regX3* and thus within the

senX3 coding region (Fig. 3C). Although the prediced -10 region (TACGAC or ACGACT) did not fit very well with consensus sequence (TANAAT) for the house-keeping sigma factor (25), the primer extension experiment indicated that *regX3* could possess an own promoter.

To test independently whether *senX3* and *regX3* are transcribed separately, Northern blot analyses were performed with RNA hybridization probes for *senX3* and for *regX3*. Total RNA of wild- type cells grown either under P_i excess (13 mM) or P_i limitation (0.13 mM) was used. As shown in Fig. 4, with both probes a hybridizing band of about 2.6 kb was detected, which could represent an mRNA species covering both *senX3* and *regX3*. With the *senX3* probe, an additional hybridizing fragment of 1.5 kb was identified, which was not observed with the *regX3* probe. On the hand, a weak band of about 1.0 kb was observed with *regX3* probe, but not with the *senX3* probe. These results support the existence of both a common and individual senX3 and regX3 mRNA species.

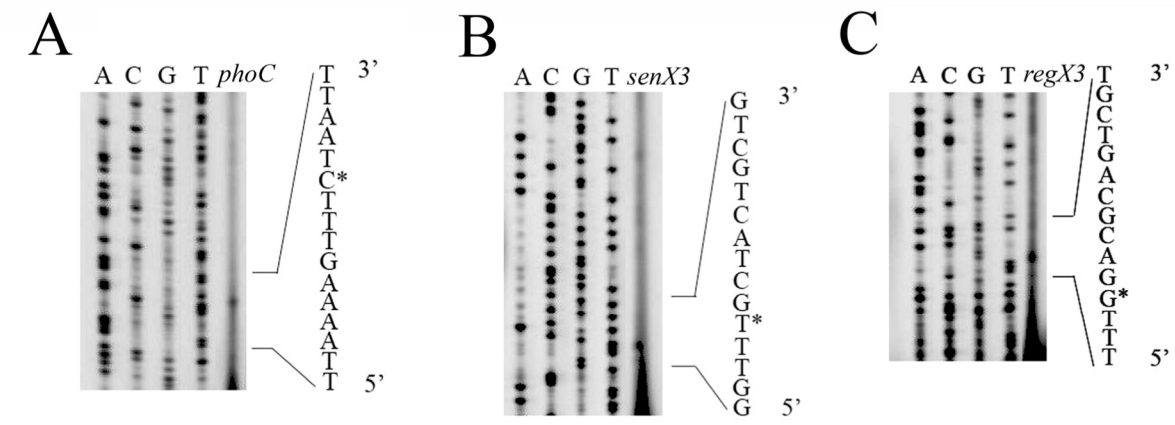

FIG. 3. Identification of putative transcription start site of the *phoC*, *senX3* and *regX3* genes by primer extension. 10 µg of total RNA isolated from *C. glutamicum* wild type grown on CGXII glucose medium under P_i limitation (0.13 mM) was used as template for primer extension analysis of *phoC* (A), *senX3* (B) and *regX3* (C). The presumed transcriptional start site is indicated by an asterisk. The corresponding sequencing reactions (lane A, C, G and T) were generated using the same IRD-800-labeled oligonucleotide (phoC-PE-rv-IRD800, senX3-PE-rv-IRD800, and regX3-PE-rv-IRD800) as in the primer extension reactions and PCR products covering the corresponding promoter region as the template DNA.

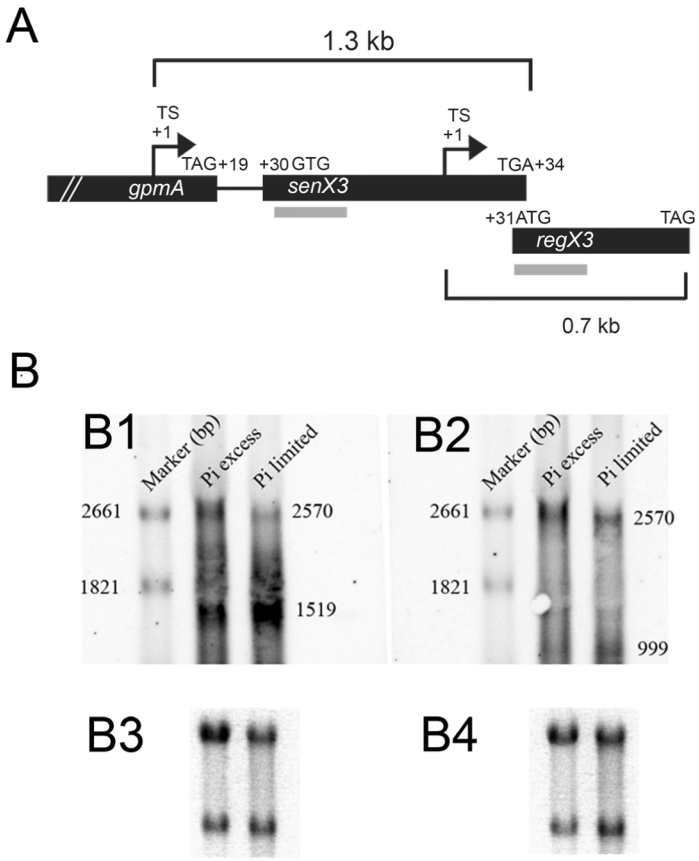

FIG. 4. Northern blot analysis of the *senX3-regX3* genes. A, Scheme of the senX3-regX3 region. The possible transcription start sites of *senX3* and *regX3* are indicated and the position of the probes used for Northern hybridization. B, 5 µg total RNA isolated from *C. glutamicum* wild type grown either P_i excess (13 mM) or P_i limitation (0.13 mM) were separated on a denaturing agarose gel and hybridized with DIG-labelled RNA probes against *senX3* (571 bp, panel B1) and *regX3* (561 bp, panel B2). In panels B3 and B4, the application of comparable amounts of RNA in the gels used for Northern hybridization was controlled by staining the RNA with GelRed (Biotium Inc., CA, USA).

Binding of RegX3 to putative target promoters. Binding of RegX3 to the promoter regions of those genes or operons was tested, which showed a decreased mRNA level in the transcriptome comparison reported above and are might be activated by RegX3. Purified $RegX3_{His}$ was used either unphosphorylated or after phosphorylation for 1 h with SenX3 and ATP. As shown in Fig. 5, binding of unphosphorylated RegX3 was observed with the promoter regions of *pstS*, *ugpA*, *phoC*, *ushA*, and cg1418. The *ushA* gene (cg0397) is part of the P_i-starvation stimulon (14) and encodes a 5'-nucleotidase (30). Its mRNA ratio in the DNA microarray experiment with strain HMW1 was 0.55 (p-value 0.05) and therefore not included in Table 2. No binding of unphosphorylated RegX3 was observed with a DNA fragment covering the 3'-end of *pgmA* and the 5'-end

of *senX3*, which harbours the putative *senX3* promoter. Phosphorylation of RegX3 increased its apparent binding affinity to the promoter regions of *pstS*, *ugpA*, *phoC* and *ushA* about two-fold, whereas still no binding to the putative *senX3* promoter region occurred.

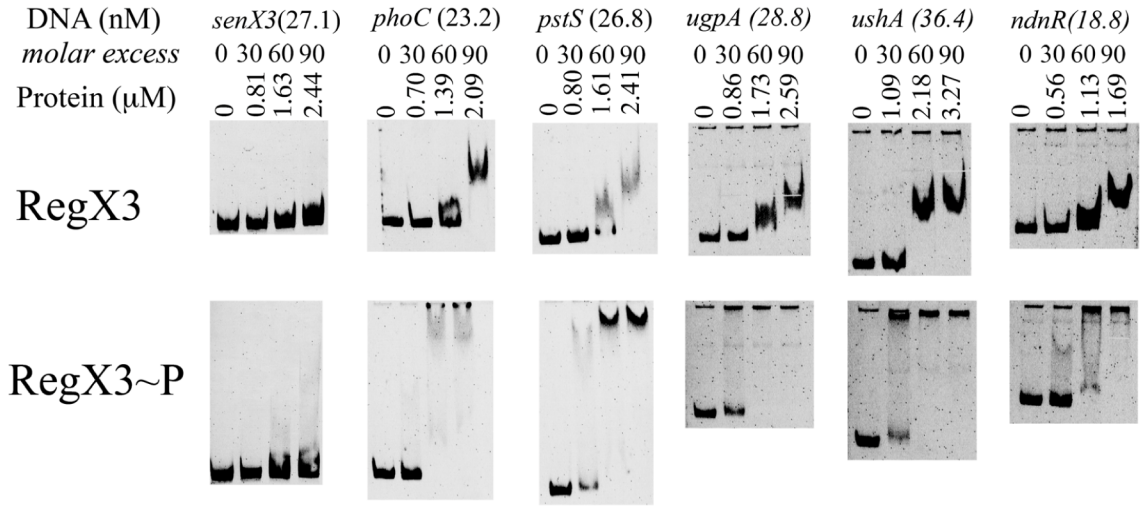

FIG. 5. Binding of RegX3 and RegX3~P (His-tagged versions) to selected promoter regions. DNA fragments (100 ng) covering the promoter regions of *senX3*, *phoC*, *pstS*, *ushA*, and *ndnR* were incubated for 20 min at room temperature with increasing concentrations of either unphosphorylated RegX3 or RegX3 that had been at least partially phosphorylated by incubation for 60 min with SenX3 and ATP. Subsequently, the reaction mixtures (20 μL) were separated by electrophoresis on a native 15% polyacrylamide gel and the gels were stained with SybrGreen. The promoter regions of *pstS* and *phoC* genes used in this experiment are denoted as fragments F1 in Fig. 6.

To define the RegX3 DNA-binding site more precisely, EMSAs were performed with subfragments of the *pstS* and *phoC* promoter regions and phosphorylated RegX3. As shown in Fig. 6, two regions were identified in the *pstS* promoter that are involved in RegX3 binding, extending from -39 to -79 bp and from -105 to -140 bp with respect to the *pstS* transcription start site (19). In the case of the *phoC*, the RegX3 binding site extends from -29 to -49 bp with respect to the transcriptional start site. Despite the delimitation of the RegX3 binding sites in the *pstS* and *phoC* promoters, various attempts to define a consensus binding site for RegX3 present in all of the four proposed target genes in *C. glutamicum* were not successful.

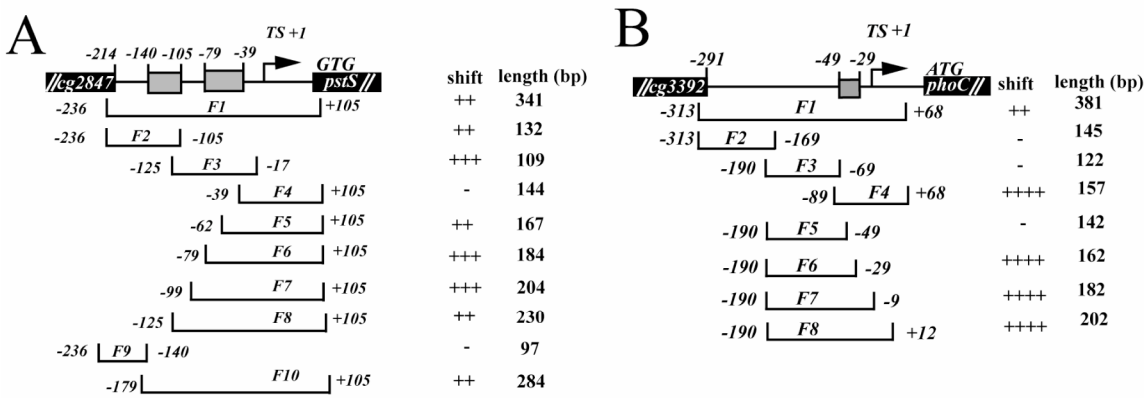

FIG. 6. Search for the RegX3 binding sites in the promoter regions of *pstS* (A) and *phoC* (B) using EMSAs with different DNA fragments and phosphorylated RegX3. The numbers indicate the position of the fragments relative to the transcriptional start site (+1). Binding affinity of the fragments were divided into six categories: ++++, 50% of the fragment shifted with a <30-fold molar excess of RegX3~P; +++, 50% of the fragment shifted with a 30-fold molar excess of RegX3~P; ++, 50% of the fragment shifted with a >30- to 60 fold molar excess of RegX3~P; +, 50% of the fragment shifted with a >60-fold molar excess of RegX3~P; and -, fragment not shifted with RegX3~P. Oligonucelotides used for amplification of the DNA fragments by PCR are listed in Table S1, EMSAs are shown in Fig. S1.

Role of RegX3 for alkaline phosphatase activity. Alkaline phosphatase (AP) activity is a convenient reporter for P_i limitation. We tested strains differing by the absence or presence of *senX3* and *regX3* or containing a mutated version of *regX3* (*regX3*-D52N) for AP activity using intact cells. As shown in Table 3, the AP activity of the wild type cultivated under P_i limitation was 6-fold higher (after 8 h) than that of cells cultivated under P_i excess. This confirms that *C. glutamicum*, as many other bacteria, responds to P_i limitation by the increased synthesis of secreted enzymes with alkaline phosphatase activity that release P_i from organic phosphoesters which can subsequently be taken up by the Pst transport system and used for growth. In contrast to the wild type, strain MW1 showed high AP activity both under P_i excess and P_i limitation. It was 12-fold and 2-fold higher than that of the wild type under P_i excess and P_i limitation, respectively. Strain MW1 lacks the chromosomal *senX3-regX3* genes, but expresses the genes from plasmid pXMJ19-senX3-regX3, even in the absence of IPTG. An increased level of RegX3 might be responsible for the increased AP activity. Strain MW2 showed even 50-fold higher AP activity than the wild type under P_i excess. As strain MW2 differs from strain MW1 by the lack of the *senX3* gene, the result may be caused by the absence of a SenX3 phosphatase activity, which dephosphorylates RegX3. Under P_i limitation, the AP activity of strain MW2 was 5-fold lower than under P_i excess, but still

comparable to that of strain MW1. A surprising result was obtained with strain MW3, which differs from MW2 only by a D52N mutation in the RegX3 protein, which is expected to prevent phosphorylation of RegX3. Strain MW2 should very low AP activity both under P_i limitation and P_i excess, suggesting that phosphorylated RegX3 is required to express the responsible gene(s). This poses the question how RegX3 is phosphorylated in strain MW2 lacking the cognate SenX3 histidine kinase. In summary, the results described above strongly indicate that the SenX3-RegX3 system is involved in the control of AP activity in *C. glutamicum*.

TABLE 3. Specific whole-cell alkaline phosphatase activity of different strains of *C. glutamicum* grown in CGXII glucose minimal medium either under P_i excess or P_i limitation. AP activity was measured after 8 h, 12 h and 24 h. The experiment was performed twice with comparable results.

Time (h)	Whole-cell alkaline phosphatase activity (nmol min^{-1} (mg cell dry weight)$^{-1}$)							
	C. glutamicum wild type		*C. glutamicum* MW1		*C. glutamicum* MW2		*C. glutamicum* MW3	
	13 mM P_i	0.13 mM P_i	13 mM P_i	0.13 mM P_i	13 mM P_i	0.13 mM P_i	13 mM P_i	0.13 mM P_i
8	0.163 ±0.003	0.960 ±0.029	1.985 ±0.066	1.886 ± 0.065	8.180 ± 1.112	1.520 ± 0.087	0.038 ± 0.045	0.050 ± 0.011
12	0.121 ±0.002	0.931 ±0.117	1.581 ± 0.061	1.523 ± 0.081	3.234 ± 0.378	0.849 ± 0.080	0.108 ± 0.001	0.039 ± 0.006
24	0.023 ±0.009	0.958 ±0.319	0.825 ± 0.106	1.017 ± 0.159	0.789 ± 0.920	0.397 ± 0.003	0.009 ± 0.002	0.024 ± 0.001

DISCUSSION

Previous studies showed that in *C. glutamicum* another regulatory system besides the two-component system PhoSR is involved in the activation of the *pstSCAB* genes under P_i limitation (14, 19, 31). *In silico* analysis showed that the *senX3-regX3* genes (previously annotated as *cgtS4-cgtR4*) of *C. glutamicum* encode a two-component regulatory system with high similarity to the SenX3-RegX3 system of mycobacteria, which in *Mycobacterium smegmatis* was shown to be involved in regulating P_i-dependent gene expression (9). The response regulator RegX3 of *M. smegmatis* was found to be essential, but the reasons for essentiality are not yet clear (9). The results obtained in this study indicated that also *C. glutamicum* the *regX3* gene is an essential gene, as its chromosomal deletion was only possible in the presence of a plasmid-borne *regX3* copy. To investigate the essential function of RegX3 and its target genes in *C.*

glutamicum, a genome-wide DNA microarray analysis was performed by comparing the gene expression profiles of the strain HMW1 either in the absence or presence of ATC. Genes showing decreased mRNA levels in the presence of decreased RegX3 levels (Table 2) were considered as putative target genes which expression is activated by RegX3. Using electrophorectic mobility shift assays with purified protein, we could show binding of RegX3 to the promoter regions of the P_i starvation-inducible genes *pstSCAB*, *ugpAEBC*, *phoC* and *ushA*. Although the RegX3 binding sites within the promoters of *pstS* and *phoC* could be delimited to comparably small regions, the attempts to find a consensus binding site present in all identified target promoters by bioinformatic tools failed.

Besides the P_i starvation-inducible genes, the gene cluster *ndnR-nadA-nadC-nadS* was identified as a possible target of RegX3. The essential *nadA* and *nadC* genes are annotated as encoding quinolinate synthase and a quinolinate phosphorylribosyltransferase, two enzymes involved in NAD^+ biosynthesis (36). The function of the NdnR protein is not yet clear, although it has been annotated as ADP-ribose pyrophosphatase and shown as a transcriptional repressor of the gene cluster *ndnR-nadA-nadC-nadS*. It contains a catalytic domain of the Nudix hydrolase family, where Nudix stands for nucleoside diphosphate linked to some other moiety x. The protein encoded by *nadS* belongs to the class-V pyridoxal-phosphate-dependent aminotransferase family (NifS/IscS subfamily), but similar to NdnR, its exact function is not yet known. However, the essential *nadA*, *nadC*, or *nadS* gene deletion mutants showed the nicotinate auxotrophy (36), which might explain the essentiality of the regX3 gene.

Phosphorylation by its cognate sensor kinase SenX3 increased the DNA-binding affinity of RegX3, indicating that the phosphorylated form is responsible for the activation of expression of *pstSCAB*, *ugpAEBC*, *phoC*, *ushA* and *ndnR-nadA-nadC-nadS*. Phosphorylation of RegX3 was required to induce AP activity in a strain lacking SenX3. Therefore, additional phosphoryl donors are expected to exist for RegX3 besides SenX3. These could be either other sensor kinases, such as PhoS, or low-molecular weight donors such as acetyl phosphate. Although *phoC* is a candidate for an AP regulated by RegX3, preliminary studies suggest that another protein is responsible for the AP activity detected. Deletion of the chromosomal *regX3* gene was possible in the presence of a plasmid-encoded RegX3 derivative which presumably cannot be

phosphorylated due to an D52N exchange. Therefore, the essential function of RegX3 apparently does not depend on its phosphorylation.

In summary, our studies provided evidence for the essentiality of the response regulator RegX3 in *C. glutamicum* and for the involvement of RegX3 in the P_i-starvation response and NAD^+ biosynthesis. Future studies aim at a clarification of the essentiality of RegX3 and of the stimuli controlling the activity of the sensor kinase SenX3.

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Supplemental materials (SenX3-RegX3 two-component system of *C. glutamicum*)

TABLE S1. Oligonucleotides used in this work. Restriction sites are underlined.

Name	Sequence (5′ → 3′)	Restriction Enzyme	Remark
Oligonucleotides for the construction of plasmids			
regX3-BsaI-fw	NNNNNNGGTCTCNAATGACGAGAATCCTGATCGT	BsaI	pASK-IBA3C-regX3
regX3-BsaI-rv	NNNNNNGGTCTCNGCGCTCAGCTCGAATTTGTAGCCCA	BsaI	pASK-IBA3C-regX3
regX3-PstI-fw	GGCGGCTGCAGCACCTATTCGTACGACTGCGTC	PstI	pXMJ19-regX3
regX3-XbaI-rv	TGGTTCTAGACTACAGCTCGAATTTGTAGCC		pXMJ19-regX3
senX3-regX3-PstI-fw	GGCGGCTGCAGCAAACCAGGGTAATAAGTAGC	PstI	pXMJ19-senX3-regX3
senX3-regX3-XbaI-rv	TGGTTCTAGACTACAGCTCGAATTTGTAGCC	XbaI	pXMJ19-senX3-regX3
regX3-D52N-fw[1]	GACATCGTCCTT*TTAA+ACCTCATGCTCCCA	DraI	pXMJ19-regX3(D52N)
regX3-D52N-rv	TGGGAGCATGAGGTTTAAAAGGACGATGTC	DraI	pXMJ19-regX3(D52N)
senX3-His-NdeI-fw	GGCGGCTGCATATGAGCACTCTTCTTGCTTTCGTAT	NdeI	pET24b-SenX3$_{His}$
senX3-His-XhoI-rv	GGCGCTCGAGTGATTTTTCCTTTCGGCGCCCAG	XhoI	pET24b-SenX3$_{His}$
regX3-His-NdeI-fw	GGCGGCTGCATATGACGAGAATCCTGATCGTTGAA	NdeI	pET24b-RegX3$_{His}$
regX3-His-XhoI-rv	GGCGCTCGAGCTACAGCTCGAATTTGTAG	XhoI	pET24b-RegX3$_{His}$
regX3-Strep-NdeI-fw	GGCGGCTGCATATGACGAGAATCCTGATCGTTGAA	NdeI	pET24b-RegX3$_{Strep}$
regX3-Strep-XhoI-rv	GGCGCTCGAGCTACAGCTCGAATTTGTAG	XhoI	pET24b-RegX3$_{Strep}$

Name	Sequence (5′ → 3′)	Restriction Enzyme	Remark
Oligonucleotides for EMSAs			
pstS-f1-fw	GCCACGTAGTTTACGAAAAGTA		
pstS-f1-rv	GGAGCGCTTAAGAGTGAGGTT		
phoC-f1-fw	TGCGCTGGTTCCATCTGCTT		
phoC-f1-rv	GGCAGGATGAGGTTGAGAAA		
ugpA-f1-fw	GCTCGCTTAACACCCCATAA		
ugpA -f1-rv	ACGGGAAAGCCTCTTCATTA		
ushA-f1-fw	GCTCGCTTAACACCCCATAA		

ushA -f1-rv	ACGGGAAAGCCTCTTCATTA
senX3-f1-fw	GTTAAGCACCTTGACGGCAT
senX3-f1-rv	GCGACGCATCCGATCTTT
cg1218-f1-fw	ATACGGCCATCTGGATTTCA
cg1218-f1-rv	CGGTTGGCCAGTTGTATTTC
pstS-f2-fw	GCCACGTAGTTTACGAAAAGTA
pstS-f2-rv	ACTCGTAACATTTGGAGACTC
pstS-f3-fw	GAGTCTCCAAATGTTACGAGT
pstS-f3-rv	CCTTGAAATGACTAACTCAGGCT
pstS-f4-fw	AGCCTGAGTTAGTCATTTCAAGG
pstS-f4-rv	GGAGCGCTTAAGAGTGAGGTT
phoC-f2-fw	TGCGCTGGTTCCATCTGCTT
phoC-f2-rv	CATCTAGAGGGGGTGTCTTTTG
phoC-f3-fw	CAAAAGACACCCCCTCTAGATG
phoC-f3-rv	GTTTACTTTAGCGAGAGTAGC
phoC-f4-fw	GCTACTCTCGCTAAAGTAAAC
phoC-f4-rv	GGCAGGATGAGGTTGAGAAA
pstS-f5-fw	GTTCATAAGTTGTTAACCAAATT
pstS-f5-rv	GGAGCGCTTAAGAGTGAGGTT
pstS-f6-fw	CCGAACGATAGCCCAGAGTT
pstS-f6-rv	GGAGCGCTTAAGAGTGAGGTT
pstS-f7-fw	GTGGGTAGTGGCAGAATTTG
pstS-f7-rv	GGAGCGCTTAAGAGTGAGGTT
pstS-f8-fw	GAGTCTCCAAATGTTACGAGT
pstS-f8-rv	GGAGCGCTTAAGAGTGAGGTT
pstS-f9-fw	GCCACGTAGTTTACGAAAAGTA
pstS-f9-rv	GGCAATTTTCGACCCCTGCG
pstS-f10-fw	AGAATCGGTGATTTTCGTTCC
pstS-f10-rv	GGAGCGCTTAAGAGTGAGGTT
phoC-f5-fw	CAAAAGACACCCCCTCTAGATG

phoC-f5-rv	GGGATCAAGGTGAATTAGGT
phoC-f6-fw	CAAAAGACACCCCCTCTAGATG
phoC-f6-rv	ATCTGTCATTGCTTTTCGGT
phoC-f7-fw	CAAAAGACACCCCCTCTAGATG
phoC-f7-rv	TTCATGTGAACTATAGGAGA
phoC-f8-fw	CAAAAGACACCCCCTCTAGATG
phoC-f8-rv	ATTAAAAGTTTCTAATTAAA

Name	Sequence (5′ → 3′)	Restriction Enzyme	Remark
Oligonucleotides for Primer extension and Northern blot analysis			
senX3-PE-fw	GTTAAGCACCTTGACGGCAT		
senX3-PE-rv	ATGAGGACCACGCCCAATACG		
senX3-PE-rv-IRD800	ATGAGGACCACGCCCAATACG		IRD800 modified
regX3-PE-fw	CGTGGTCATGATCCGAGTAA		
regX3-PE-rv	GATTCCTCATCTTCAACGATCA		
regX3-PE-rv-IRD800	GATTCCTCATCTTCAACGATCA		IRD800 modified
phoC-PE-fw	TGCGCTGGTTCCATCTGCTT		
phoC-PE-rv	TGCGCTGGTTCCATCTGCTT		
phoC-PE-rv-IRD800	TGCGCTGGTTCCATCTGCTT		IRD800 modified
senX3-NR-fw	ATGCGTCGCCACAAGTCCG		
senX3-NR-rv	TGGAAAGGGAGATCAGTTCG		
regX3-NR-fw	ATGACGAGAATCCTGATCGT		
regX3-NR-rv	AATTCGGTCGATGAGCTGTC		

[1] In the oligonucleotide regX3-D52N-fw a silent mutation (*) was introduced in the 50th codon of *regX3* (CTC → CTT) in order to create a DraI restriction site and a point mutation (+) was introduced in the 52nd codon (GAC → AAC) causing an D52N exchange at the amino acid sequence level.

TABLE S2. Concentration of RegX3~P (μM) used for EMSA with DNA fragments of the promoter region of the *pstS* gene

Name of DNA fragment	Concentration of RegX3~P (μM) used			
Molar excess	0	30	60	90
F2	0	1.73	3.45	5.18
F3	0	2.09	4.18	6.28
F4	0	1.58	3.17	4.75
F5	0	1.37	2.73	4.10
F6	0	1.24	2.48	3.72
F7	0	1.12	2.24	3.35
F8	0	0.99	1.98	2.97
F9	0	2.35	4.70	7.05
F10	0	0.67	1.34	2.01

TABLE S3. Concentration of RegX3~P (μM) used for EMSA with DNA fragments of the promoter region of the *phoC* gene

Name of DNA fragment	Concentration of RegX3~P (μM) used			
Molar excess	0	30	60	90
F2	0	1.57	3.14	4.72
F3	0	1.87	3.74	5.61
F4	0	1.45	2.90	4.36
F5	0	0.60	1.20	1.80
F6	0	1.57	3.14	4.72
F7	0	1.87	3.74	5.61
F8	0	1.45	2.90	4.36

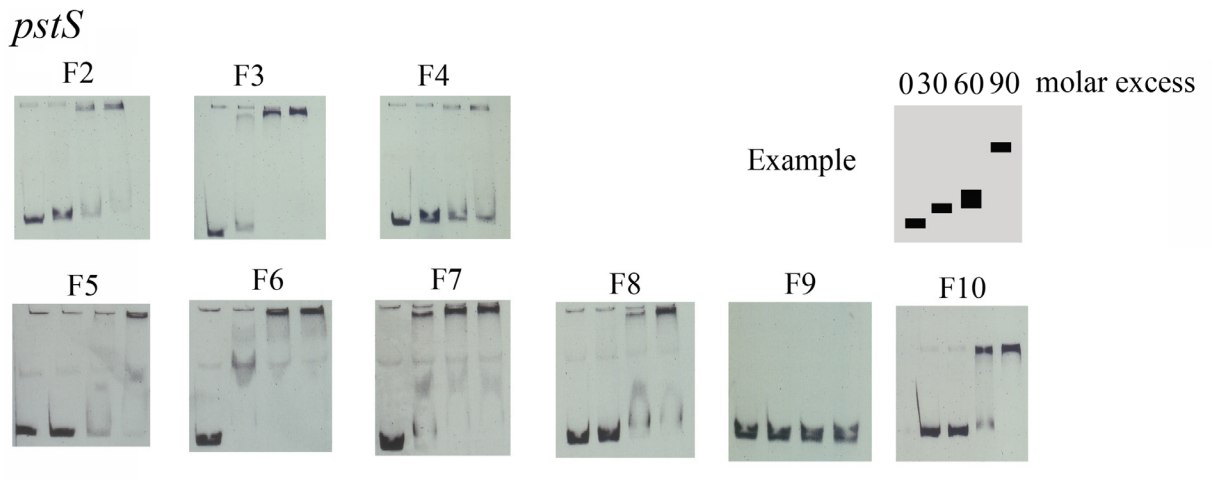

FIG. S1. Binding of RegX3~P and RegX3 to DNA fragments of the promoter region of the *pstS* gene for the identification of binding site. Concentrations of RegX3~P(μM) used for EMSA are listed in the Table S2. EMSA with fragment F1 of the *pstS* gene is shown in Fig. 3 of the manuscript.

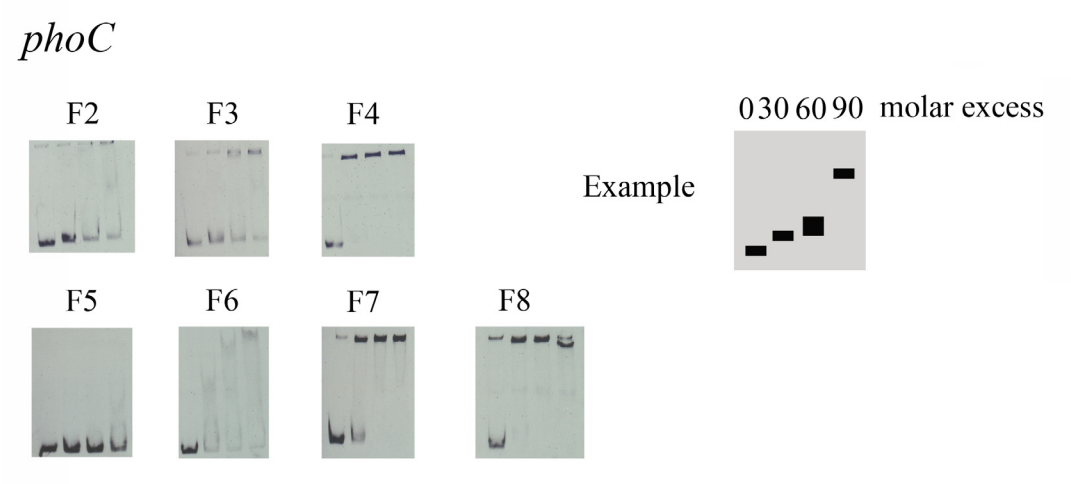

FIG. S2**.** Binding of RegX3~P and RegX3 to DNA fragments of the promoter region of the *phoC* gene for the identification of binding site. Concentrations of RegX3~P(μM) used for EMSA are listed in the Table S3. EMSA with fragment F1 of the *phoC* gene is shown in Fig. 3 of the manuscript.

4.2 A link between phosphate starvation and glycogen metabolism in *C. glutamicum* revealed by metabolomics

A link between phosphate starvation and glycogen metabolism in *Corynebacterium glutamicum* revealed by metabolomics

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Running title: Glycogen storage under P_i limitation in *C. glutamicum*

In this study we analyzed the influence of phosphate (P_i) limitation on the metabolism of *Corynebacterium glutamicum*. Metabolite analysis by GC-TOF mass spectrometry of cells cultivated in glucose minimal medium revealed a strongly increased maltose level under P_i limitation. As maltose formation could be linked to glycogen metabolism, the cellular glycogen content was determined. In contrast to cells grown under P_i excess, the glycogen level of P_i-limited cells remained high in the stationary phase. Surprisingly, even acetate-grown cells, which do not form glycogen under P_i excess, do so under P_i limitation and retain it also in stationary phase. Expression of *pgm* and *glgC*, encoding the first two enzymes of glycogen synthesis, phosphoglucomutase and ADP-glucose pyrophosphorylase, was found to be increased 6- and 3-fold under P_i limitation, respectively. Increased glycogen synthesis together with a decreased glycogen degradation might be responsible for the altered glycogen metabolism. Independent from these experimental results, flux balance analysis suggested that an increased carbon flux to glycogen is a solution for *C. glutamicum* to adapt carbon metabolism to limited P_i concentrations.

Phosphorus is an essential nutrient for all cells and required e.g. for the biosynthesis of nucleotides, NAD(P)H, DNA, and RNA, but also for the regulation of protein activity by phosphorylation of histidine, aspartate, serine, threonine, or tyrosine residues. A common phosphorus source is inorganic phosphate (P_i) and cells have developed mechanisms for the acquisition, assimilation and storage of P_i. When P_i becomes limiting, many bacteria induce the synthesis of proteins that enable them to capture the residual P_i resources more efficiently and to make alternative phosphorus sources accessible. The corresponding genes are collectively named P_i starvation-inducible genes or *psi* genes. The P_i-starvation response, in particular its regulation, has been most carefully studied in *Escherichia coli* (45) and *Bacillus subtilis* (14).

We recently started to characterize the P_i starvation response in *Corynebacterium glutamicum*, a Gram-positive soil bacterium used industrially for the production of more than two millions tons of amino acids per year, mainly L-glutamate and L-lysine (12). An overview on the biology, genetics, physiology, and application of *C. glutamicum* can be found in two recent monographs (3, 6). Phosphorus constitutes 1.5 % - 2.1 % of the cell dry weight of *C. glutamicum* (24), part of which can be present as polyphosphate (22, 29). Several of the enzymes involved in polyphosphate metabolism have been characterized recently, such as a class II polyphosphate kinase (28), the exopolyphosphatases Ppx1 and Ppx2 (26), a polyphosphate/ATP-dependent

glucokinase (25), or a polyphosphate/ATP-dependent NAD^+ kinase (27). The P_i starvation stimulon of *C. glutamicum* was determined using whole-genome DNA microarrays (15). Comparison of the mRNA profiles before and at different times after a shift from P_i excess to P_i starvation led to the identification of a group of genes that are presumably required to cope with limited P_i supply. This group includes the *pstSCAB* operon encoding an ABC transporter for high-affinity P_i uptake, the *ugpAEBC* operon encoding an ABC transporter for uptake of glycerol 3-phosphate, *glpQ1* encoding a glycerophosphoryl diester phosphodiesterase, *ushA* encoding a secreted enzyme with UDP-sugar hydrolase and 5'-nucleotidase activity (33), *nucH* encoding a putative secreted nuclease which possibly plays a role in liberating P_i from extracellular nucleic acids, *phoC* (NCgl2959/*cg3393*) which may encode a cell wall-associated phosphatase (46), *phoH1* encoding an ATPase of unknown function, and the *pctABCD* operon encoding an ABC transport system which might be involved in the uptake of a yet unknown phosphorus-containing compound (15). *C. glutamicum* lacks homologs of genes for phosphonate degradation, as well as the capability to utilize phosphonates as P sources (15).

In most bacteria analyzed in this respect, the P_i starvation response is controlled by two-component signal transduction systems, e.g., the PhoBR system in *E. coli* (13) or the PhoPR system in *B. subtilis* (14). Our previous studies revealed that in *C. glutamicum* a two-component system composed of the sensor kinase PhoS and the response regulator PhoR is involved in the activation of phosphate starvation-inducible genes (21). Studies with purified proteins showed that phosphorylation by PhoS increased the DNA-binding affinity of PhoR, which bound to many of the P_i starvation-inducible genes, but with different affinities (34).

The study reported here was initiated by the question how the metabolism of *C. glutamicum* responds to P_i limitation. Our results reveal a link between P_i limitation and glycogen metabolism, which was also used for metabolic simulations based on a genome-wide metabolic model.

MATERIALS AND METHODS

Strains and cultivation. The strains and plasmids used in this study are listed in Table 1. *C. glutamicum* wild type ATCC 13032, its *glgC* disruption mutant (36), and its Δ*sugR* mutant (9) were precultivated aerobically at 30°C in baffled 500-ml shake flasks on a rotary shaker at 120 rpm using CGIII complex medium (10 g peptone, 10 g yeast extract, and 25 g NaCl per liter) supplemented with 222 mM glucose. After washing the cells with 0.9 % (w/v) NaCl, they were

transferred to the defined CGXII minimal medium (18) supplemented with protocatechuic acid (30 mg/liter) as an iron chelator and either 222 mM glucose or 300 mM potassium acetate as carbon source. For the analysis of response of *C. glutamicum* to P_i limitation at the metabolite level, the cells were precultivated twice in CGXII glucose medium with 0.13 mM P_i and after washing they were inoculated into CGXII glucose medium and different P_i conditions (13 mM, 0.65 mM, 0.26 mM, and 0.13 mM). P_i was added as KH_2PO_4 and K_2HPO_4. Samples for metabolite analysis were taken after 8, 12, and 24 h cultivation. For *in vivo* ^{13}C-labeling of the metabolites, cells were grown in CGXII medium containing 222 mM uniformly ^{13}C-labeled glucose (Cambridge Isotope Laboratory, Andover, MA) as sole carbon source under the conditions described above, including two precultivations with ^{13}C-labeled glucose.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	F- *thi-1 endA1 hsdR17*(r^- m^-) *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *recA1 gyrA96 relA1*	Invitrogen
C. glutamicum ATCC 13032	Biotin-auxotrophic wild type strain	(19)
C. glutamicum Δ*sugR*	ATCC 13032 derivative with an in-frame deletion of *sugR*	(9)
C. glutamicum IMC	ATCC 13032 derivative with a disruption of *glgC*	(36)
Plasmids		
pET2	Kan^r; promoter-probe vector for *C. glutamicum*	(44)
pET2-*pgm*	Kan^r; pET2 with a 423-bp fragment covering the *pgm* promoter (-413 to +10 with respect to the proposed translational start site)	This study
pET2-*glgC*	Kan^r; pET2 with a 406-bp fragment covering the *glgC* promoter (-402 to +4 with respect to the proposed translational start site)	This study

Extraction of metabolites and sample preparation for metabolite profiling. 1-ml samples of triplicate cultures with known OD_{600} were added into 2 ml-Eppendorf tubes containing 500 mg of silicon oil (δ = 1.05 g/cm^3) and 300 µl 20% (v/v) perchloric acid ($HClO_4$, δ = 1.18 g/cm^3). The tubes were centrifuged immediately at 13,000 rpm for 30 s in order to separate the cells from the culture supernatant and to inactivate metabolism by the acid treatment. After careful removal of the supernatant, the samples were mixed and neutralized with 185 µl of 6 N potassium hydroxide. The pH was controlled by the indicator bromthymol blue, which is green at pH 7. Aliquots of the polar phase, corresponding to 5.0 mg cell dry weight [an OD_{600} of 1 corresponds to 0.25 mg cell dry weight per ml; (16)] were lyophilized for at least 2 days. Subsequently, the dried cell extracts were treated for 90 min at 35°C with 50 µl methoxyamine hydrochloride in pyridine (20 mg/ml) and subsequently trimethylsilated with 80 µl N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) for 4 hr at 35°C to derivatize the metabolites for GC-TOF mass spectrometry (MS) analysis.

Metabolite pattern analysis. Metabolite pattern analysis was used as an initial approach to determine whether the metabolite patterns of cells differ depending on P_i availability. In a first series of experiments, GC-TOF MS data sets were obtained from cells cultured for 24 h in CGXII glucose medium with different P_i concentrations (13 mM, 0.65 mM, 0.25 mM, and 0.13 mM of P_i). In a second series, GC-TOF MS data sets were obained from three time points (8 h, 12 h and 24 h) of cultures grown in CGXII glucose medium under either P_i excess (13 mM) or P_i starvation (0.13 mM). To perform metabolite pattern analysis, for each sample a dataset containing 2517 mass fragments (whose chemical identities were unknown) differing either in mass or retention time and identified in all analyzed samples by MarkerLynx software, was exported to SIMCA-P+ software (Umetrics AB, Umeå, Sweden). These datasets were analysed by partial least-squares discriminant analysis (PLS-DA) (47).

GC-(EI/CI)-TOF MS. The derivatized metabolite samples prepared as described above were analyzed by GC-TOF MS using both the electron ionization (EI+) mode and chemical ionization (CI+) mode. The GC-TOF MS system was composed of an Agilent gas chromatograph 6890N (Agilent, Santa Clara, USA) equipped with a Gerstel MultiPurpose Sampler MPS2 (Gerstel, Mülheim, Germany) and a GCT Premier™ benchtop orthogonal acceleration time-of-flight mass spectrometer (Waters, Milford, USA). The system was operated using the MassLynx software (version 4.1, Waters, Milford, USA). GC was performed using a 30 m × 0.25 µm DB-5MS column (J&W Scientific, Folsom, USA) with a constant flow rate of 1 ml/min of helium as the

carrier gas in the column. After washing the needle of the injector syringe with hexane and methanol, 1 µl of sample was injected with a split ratio of 2:1 at 280°C to a glass liner (4 mm inner diameter) filled with glass wool. For GC separation, oven temperature stayed initially for 2 min at 85°C, then increased to 320°C by 15°C/min, where it was hold for 5 min. Transfer of the samples from the GC to the mass spectrometer was performed at 150°C.

Evaporated chemicals were ionized by either the EI or CI method. For the electron ionization (EI+), an ionization energy was tuned at 70 eV according to the operation manual using heptacosafluorotributylamine ($(C_4F_9)_3N$) as internal reference. For positive chemical ionization (CI+), isobutene (0.7 bar, 10^{-5} mbar in the source) as a reagent gas generated ionized $[MH]^+$, $[M + C_4H_9]^+$, and $[M + C_3H_3]^+$ quasi-molecules at an electron energy of 50 eV. Tuning for CI+ mode was performed according to the operation manual using 2,4,6-tri(trifluoromethyl)-1,3,5-triazine as internal reference. Mass ions were detected with scan time set to 0.9 s and the inter-scan set to 0.1 s in the centroid mode using lock mass at 218.9856 m/z. Mass fragment patterns were analyzed to identify metabolites using NIST MS search with several public libraries and a home-made library.

Metabolite identification by GC-(EI/CI)-TOF MS based on the use of naturally and uniformly ^{13}C-labelled metabolites. GC-MS has been widely used to analyze volatile chemicals and derivatized chemicals because of high sensitivities and the availability of standard mass fragment libraries. In general, the identification process in GC-MS depends on the matching score of mass fragment patterns between analytes and standards with the same retention time or the index number only when mass fragment libraries of standards are available. However, a number of peaks with low matching scores (below 700) show ambiguous metabolite identification or remain unknown because of the incompleteness of biological metabolite databases and the possibility of undesired reactions by using highly reactive derivatization chemicals. Therefore, in this study an additional procedure was used to identify 'real' metabolites among possible chemicals from derivatized cell extracts. The method is based on the comparison of uniformly ^{13}C-labeled metabolites with naturally labeled metabolites, which were measured separately. By combining all information obtained from the extracts of the cultures grown with either unlabeled or ^{13}C-labelled glucose using the EI/CI ionization methods, metabolites were identified by several criteria as outlined in supplementary Fig. S1. In the case of mass fragments that still remained unknown after this protocol, identification was attempted based on the exact mass measurement using a Matlab script, which searches possible chemical compositions online

in publicly available chemical databases (Pubchem and KEGG compound DB). The details of this procedure are described in the supplemental data.

Analysis of phosphorus-containing metabolites using ^{31}P *in vivo* NMR spectroscopy. ^{31}P NMR analysis was performed essentially as described (28). Cultures were harvested in the mid-exponential growth phase (8 h) and in the stationary phase (24 h). 1 g of wet cell pellet was suspended in 4 ml absolute ethanol, mixed for 1 min, and centrifuged for 10 min at 4,400 x *g* and 4°C. The supernatant was discarded, and the pellet was resuspended in a mixture of 1.1 ml fresh bi-distilled water, 0.3 ml 1 M EDTA (pH 8.2) and 0.6 ml D_2O. 700 µl of 2 ml cell suspension were transferred into a 5-mm NMR tube and analyzed. During preparation, all samples were kept on ice or were kept frozen until further use.

The ^{31}P-NMR spectra were measured at 5°C on a Varian Inova 400 MHz spectrometer. An amount of D_2O sufficient to obtain a stable lock signal was added to each sample prior to measurement. The following parameters were used: frequency, 161,985 MHz; excitation pulse width, 9.25 µs; pulse repetition delay, 1 s; and spectral width, 18.35 kHz. Routine spectra were acquired with 4,096 scans. Chemical shifts were referenced to 85% orthophosphoric acid (0 ppm). Standards of P_i and polyphosphate ("P68" with polymerization from 10 to 40; BK Giulini Chemie, Ladenburg, Germany) were prepared having final concentrations of 10 mM, in terms of P_i (29). Signals were integrated with the MestRe Nova (Mestrelab research, Santiago, Spain) to quantify total intracellular P_i and phosphorus containing metabolites, e.g. phosphomonoesters such as sugar phosphates, NDP-glucose or polyphosphate.

Measurement of intracellular glycogen contents. The glycogen contents of *C. glutamicum* were determined by the enzymatic method as described previously (31, 35). A culture volume corresponding to 12.5 mg cell dry weight [an OD_{600} of 1 corresponds to 0.25 mg/ml cell dry weight; (16)] was centrifuged and the cells were washed twice with TN buffer (50 mM Tris-HCl pH 6.3, 50 mM NaCl). After centrifugation, the cell pellet was resuspended in 1 ml of 40 mM potassium acetate buffer pH 4.2 and transferred to 2 ml safe-lock Eppendorf tubes filled with 250 mg zirconia/silica beads (0.1 mm diameter). After inactivation of cell-bound glycosidic activity by incubation at 99 °C for 5 min, the cells were disrupted by 2 x 30 s bead beating at 4,500 rpm using a Silamat S5 (Ivoclar Vivadent, Ellwangen, Germany). The cell debris and glass beads were separated from the supernatant by centrifugation (13,000 x *g*, 20 min) and the supernatant was collected and stored at -20°C until use. Each sample was divided into two 100 µl aliquots (labelled sample A and sample B). 2 µl amyloglucosidase (10 mg/ml, Roche Diagnostics,

Mannheim, Germany) was added to sample A to degrade glycogen to free glucose, whereas sample B served as reference. Both samples were incubated for 2 hr at 57 °C with shaking at 850 rpm. Subsequently, the glucose concentration in the two samples was determined using a coupled enzymatic assay with hexokinase and glucose 6-phosphate dehydrogenase (Roche Diagnostics, Mannheim, Germany) by measuring the NADH formed at 340 nm. Finally, the glycogen content was calculated in mg per g of cell dry weight (CDW) after subtracting the glucose concentration of the reference sample B from that of the test sample A.

Construction of transcriptional fusions and chloroamphenicol acetyltransferase (CAT) assays. For CAT assays, DNA fragments covering the promoter regions of the *pgm* and *glgC* genes were amplified and cloned into the corynebacterial promoter-probe vector pET2 (44), resulting in plasmids of pET2-*pgm* and pET2-*glgC*, respectively (Table 3). The cloned fragments were controlled by DNA sequence analysis. The plasmids were introduced into wild type *C. glutamicum* and the transformed strains were cultivated as outlined above. The CAT assays were performed as described previously (9).

Constraint-based analysis using a genome-scale model of *C. glutamicum*. Flux balance analysis (FBA) was performed using a slightly modified version of the genome-scale model of *C. glutamicum* ATCC 13032 (20) consisting of 446 metabolic reactions and 411 metabolites. The following additional reactions for glycogen formation and degradation were considered as described in (36):

GlgC: $G1P + ATP \rightarrow ADP\text{-}GLC + PP_i$

GlgA: $ADP\text{-}GLC \rightarrow GLGN + ADP$

GlgP, MalP: $GLGN + P_i \rightarrow G1P$

For this network steady-state flux distributions were calculated by FBA using linear programming-based optimization of growth as cellular objective function. In order to obtain feasible phenotypic spaces related to the experimentally observed P_i starvation response of *C. glutamicum*, the flux cone was constrained to different combinations of uptakes rates for P_i and glucose or acetate as carbon source (32). In addition, only byproducts that were experimentally observed during the experiments were allowed to be formed. For these fluxes upper bounds were defined that were estimated from the quantitative experimental data generated by HPLC analysis. This refers to lactate, acetate, fumarate and malate for glucose-grown cells, whose formation rates were estimated to be 0.10, 0.02, 0.01 and 0.01 mmol g_{CDW}^{-1} h^{-1}, respectively. All

simulations were performed under MATLAB (Mathworks, R2008b) using the COBRA toolbox with LP-solver GLPK (1).

RESULTS

Influence of different P_i concentrations on the metabolite pattern of *C. glutamicum* cells. In order to determine the consequences of P_i limitation at the metabolite level, metabolome analysis was performed by GC-TOF mass spectrometry (GC-TOF MS) and the resulting data were analyzed by PLS-DA as a tool for multivariate data analysis (23). For this purpose, *Corynebacterium glutamicum* wild type was cultivated at four different initial P_i concentrations (13 mM, 0.65 mM, 0.26 mM, and 0.13 mM) in CGXII glucose minimal medium. The highest concentration is the one regularly used in this medium. Growth of the cells was comparable to that reported previously (15) and is shown in Fig. 1A. The cells were collected after 24 h of cultivation, intracellular metabolites were extracted and analyzed by GC-TOF MS, and PLS-DA was performed with the data from 2517 mass fragments that were identified. The resulting score plot for metabolite pattern analysis is shown in Fig. 1B. It is obvious that the samples obtained from cells grown with 13 mM, 0.65 mM and 0.13 mM P_i formed three distinct groups, whereas the samples obtained from cells grown with 0.26 mM P_i formed two subgroups, one being located close to the 0.65 mM group and the other next to the 0.13 mM group. This analysis was based on mass fragment patterns only rather than on a set of identified metabolites, and it clearly indicated that different P_i concentrations in the medium affect the metabolite composition within the cells. For the following experiments, 13 mM P_i was used for P_i excess conditions and 0.13 mM for P_i-limiting conditions.

In a second series of experiments, *C. glutamicum* was grown in glucose medium either with 13 mM P_i or with 0.13 mM P_i and samples for GC-TOF MS were taken after 8 h, 12 h and 24 h of cultivation. The experiment was performed in triplicate starting with independent cultures and resulted in 18 metabolite data sets that again were analyzed by PLS-DA. As shown in Fig. 1C, except for two data sets (P_i excess culture after 8 h and P_i-limed culture after 12 h) which overlapped, all other data sets formed distinct groups on the score plot. For further analysis, cells cultivated for 24 h were used.

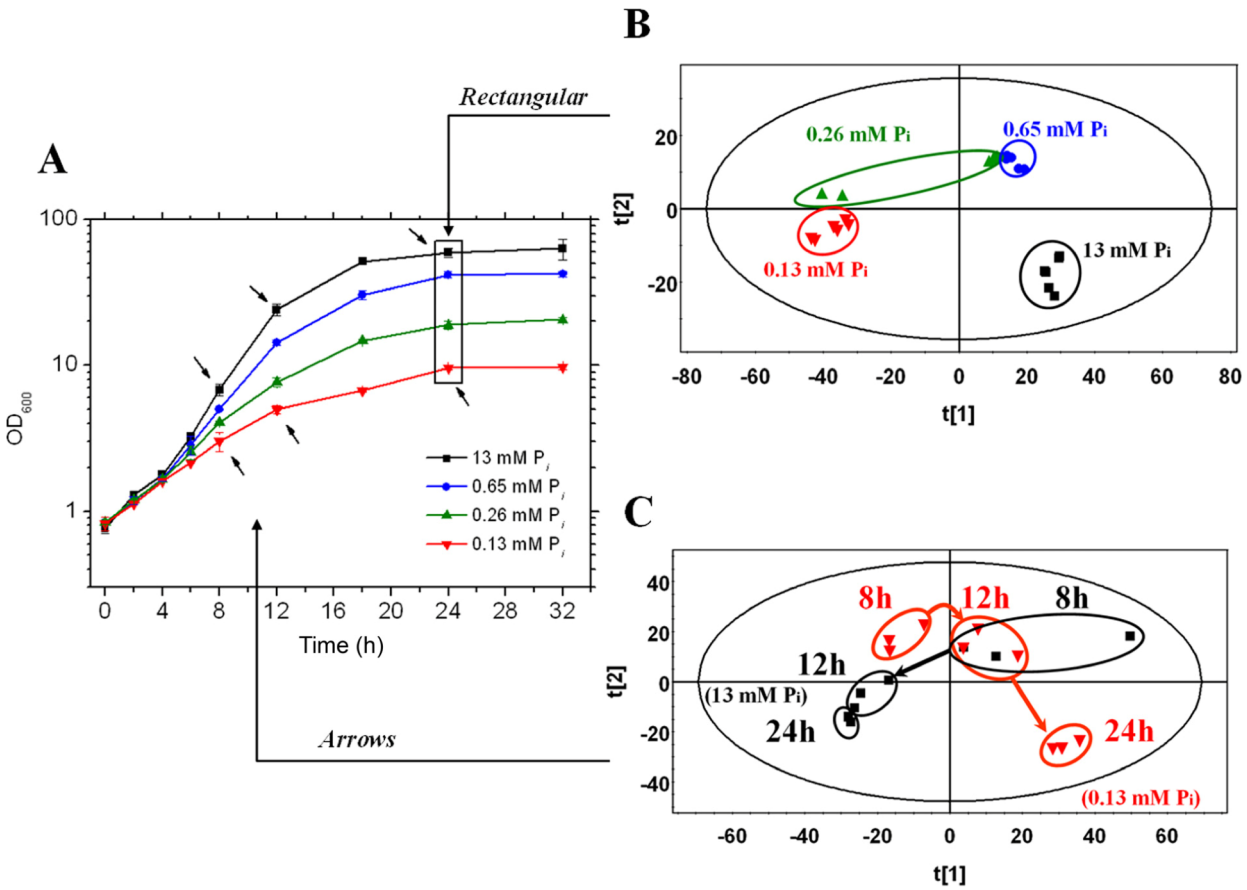

FIG. 1. Influence of growth with different P_i concentrations on the metabolome of *C. glutamicum*. (A) Growth of *C. glutamicum* ATCC 13032 in CGXII minimum medium with 222 mM of glucose and different concentration of inorganic P_i. Cells were precultured twice in CGXII glucose medium with 0.13 mM potassium P_i and then transferred to CGXII medium containing 0.13 mM (red inverted triangle), 0.26 mM (green triangle), 0.65 mM (blue circle), or 13 mM (black rectangular) inorganic P_i. The experiment was performed in triplicate and mean values and standard deviations are shown. After 24 h, samples of all cultures were taken and used for metabolite analysis by GC-TOF MS. The 2517 mass fragments detected in all samples were used for PLS-DA, representing one symbol of the score plots. (B) PLS-DA score plot of the metabolome samples taken after 24 h of growth with different P_i concentrations (black squares, 13 mM P_i; blue dots, 0.65 mM P_i; green triangles, 0.26 mM P_i; red inverted triangle, 0.13 mM P_i). The x-axis, t[1], and the y-axis, t[2], represent vectors for the most significant components of the matrix x of mass ion abundances. The plot shows a directionality of the metabolite pattern from P_i excess to limitation. (C) PLS-DA score plot of the samples taken after 8, 12 and 24 h from cultures grown in CGXII glucose medium with either 13 mM P_i (black square) or 0.13 mM P_i (red inverted triangle).

Semi-quantitative comparison of metabolites in cells cultivated under P_i excess or P_i starvation. In order to get more detailed insights into the changes at the metabolite level that occur under P_i limitation, a semi-quantitative comparison was performed in which the metabolomes of cells grown in triplicate for 24 h under P_i excess (13 mM) or P_i starvation (0.13 mM) were analysed by GC-TOF MS using both electron ionization and chemical ionization. For this purpose, one culture was grown with naturally labelled glucose, whereas the other one was grown with $^{13}C_6$-glucose. The availability of uniformly ^{13}C-labelled metabolites was useful for the identification of the mass fragments detected by GC MS (see supplementary data).

Table 2 lists metabolites that could be unequivocally identified in the samples. The pool of cytoplasmic P_i was calculated to be three-fold lower in the P_i-limited cells, which is in contrast to the 100-fold lower P_i concentration in the medium at the start of the cultivation. It reflects the capability of the cells to maintain a comparably high cytoplasmic P_i concentration also when the external P_i is limiting, due to the activation of the P_i starvation response. The lower level of lactic acid in P_i-limited cells corresponds to the fact that P_i-limited cultures did not produce L-lactate, whereas the P_i excess cultures did (Fig. 2A). Lactate excretion during growth on glucose is usually caused by oxygen limitation and due to the much lower glucose consumption rate of the P_i-limited cells, no oxygen limitation occurred. The detection of glycolic acid in *C. glutamicum* extracts was unexpected, as this compound has not been described yet as a metabolite in this organism and no pathway is known leading to glycolate in *C. glutamicum*. L-Alanine was detected in two forms, as a twofold trimethylsilyl-modified form and a threefold trimethylsilyl-modified form. Both forms showed a reduced level in the P_i-limited cells. As L-alanine is derived from pyruvate, this result might be a consequence of a reduced glycolytic flux and a reduced pyruvate pool during P_i limitation. Also three intermediates of the tricarboxylic acid (TCA) cycle showed reduced levels in P_i-limited cells, such as succinate, malate and fumarate. L-proline, which is formed from L-glutamate, showed a strongly reduced pool under P_i limitation, which could be due to a reduced TCA cycle flux and reduced levels of NADPH or ATP.

The metabolites that showed the most strongly increased levels in P_i-limited cells were glucose (115-fold) and maltose (28-fold). Whereas the ratio measured for glucose might be caused by contamination through the high concentration of external glucose that was still present in the medium of the P_i-limited cultures after 24 h, the increased pool of maltose cannot be explained in this way. As outlined below, it is related to glycogen metabolism.

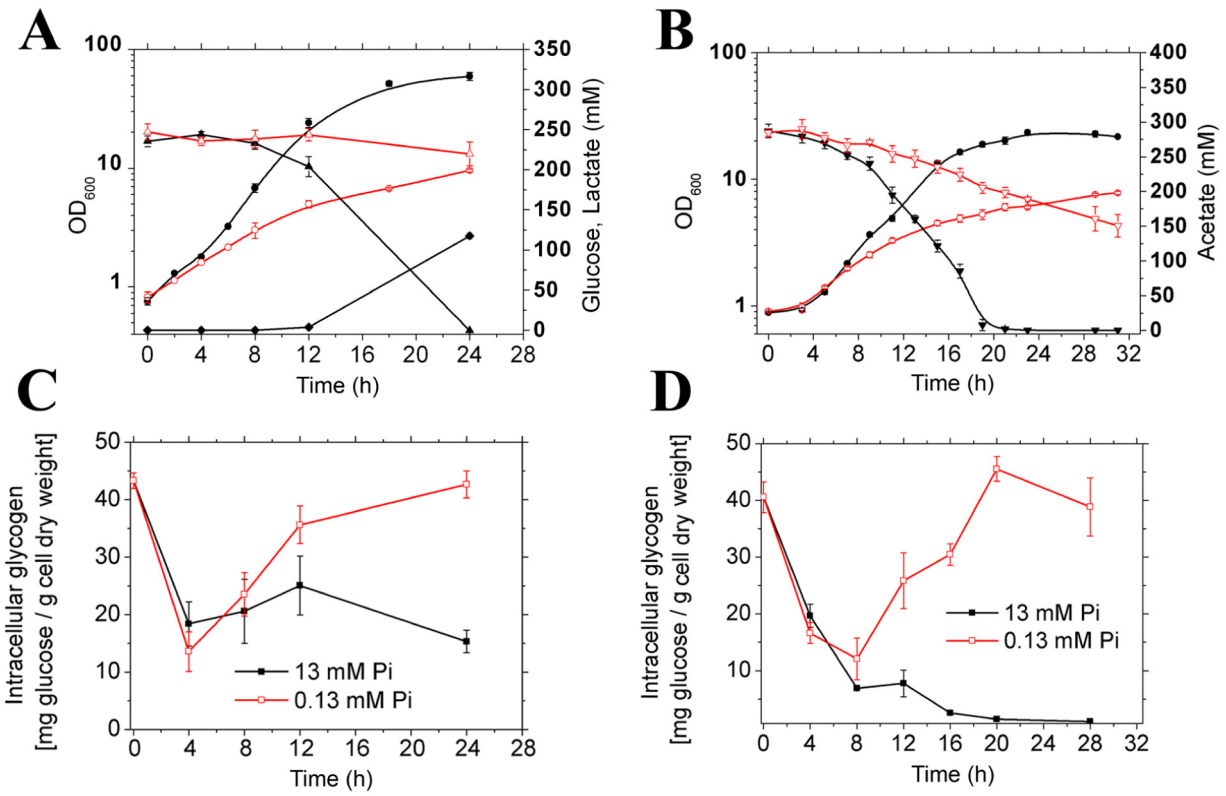

FIG. 2. In panels A-D, growth (OD_{600}, circles), carbon source consumption (triangles) and the cellular glycogen pools (squares) are shown for *C. glutamicum* cultivated in CGXII minimal medium with 222 mM glucose (panels A and C) or with 300 mM potassium acetate (panels B and D) either under P_i excess (13 mM, black symbols) or P_i limitation (0.13 mM, red symbols). The inoculum was precultivated twice in the same medium under P_i limitation. In panel A lactate formation is shown in addition (rhombic symbols), which did not occur during growth on acetate (panel B). Mean values and standard deviations of triplicate cultures are shown.

TABLE 2. Relative ratio of identified metabolites (integrated area of chromatographic peak under P_i limiting conditions vs. under P_i excess conditions) during growth of *C. glutamicum* under P_i limitation and P_i excess

Name	**Derivati -zations**	**Monoiso- topic mass (Da)**	**RT[1]**	**RI[2]**	**Area[3] (P_i limitation/P_i excess)**	**Area ratio (P_i limit./ P_i excess)**	**p-value[4]**
Lactic acid	2TMS[5]	234.1108	5.48	1033	7337 ±295 / 384667 ±48336	0.02	0.005
Glycolic acid	2TMS	220.0951	5.67	1041	15300 ± 2307 / 5483 ±361	2.79	0.017
L-Alanine	2TMS	223.1267	5.97	1053	9963 ±1767 / 36233 ±13799	0.27	0.100
	3TMS	305.1663	8.76	1279	5900 ±335 / 96467 ±20766	0.06	0.017
Phosphate	3TMS	314.0959	7.85	1236	53233 ±28407 / 170000 ±17578	0.31	0.003
L-Proline	2TMS	259.1424	8.15	1250	5400 ±1542 / 63433 ±7250	0.09	0.003
Succinic acid	2TMS	262.1057	8.32	1258	49300 ±4078 / 81567 ±15815	0.60	0.048
Fumaric acid	2TMS	260.0900	8.68	1275	3907 ±457 / 7900± 123	0.49	0.006
Malic acid	2TMS	350.1401	9.96	1441	3880 ±98 / 17833 ±981	0.22	0.001
Glucose[6]	5TMS, 1MeOx[7]	569.2876	13.44	1852	433667 ±12423 / 3757 ±45	115.44	0.0003
Maltose[8]	8TMS, 1MeOx	947.459	16.38	2659	3553 ±983 / 127 ±27	28.05	0.027
Trehalose[9]	8TMS	918.4324	18.49	2661	433333 ±26312 / 476000 ±17349	0.91	0.158

1 RT, retention time (min) during gas chromatography
2 RI, retention index calculated using alkane standards (C10 to C40)
3 Mean values and standard deviation from triplicate experiments
4 p-value determined by a Student's t-test
5 TMS, trimethylsilyl
6 Glucose levels are relatively uncertain due to possible contamination by glucose of the medium during the extraction process; Pi-limited cells retain high glucose concentrations in the medium after 24 h of cultivation
7 MeOX, methoxyamine
8 Maltose was detected only by the EI mode due to higher molecular mass and was extracted with cold methanol.
9 Trehalose data were included despite a p-value >0.05 as it can serve as a precursor for maltose by the action of trehalose synthase (TreS).

Phosphorus-containing metabolite profiling of *C. glutamicum* using ^{31}P-NMR spectroscopy. To complement GC-MS analysis, *in vivo* ^{31}P-NMR was applied to measure different intracellular phosphorus-containing metabolites in *C. glutamicum* cells cultivated either under P_i excess or limitation. The results are summarized in Fig. 3. The cytoplasmic P_i concentration after 24 h of growth was calculated to be 17.5 ± 0.72 mM under P_i excess and 1.8 ± 0.01 mM under P_i limitation. This difference (9.7-fold) is larger than the one determined by GC-MS (3-fold), but reflects the native situation accurately. The concentration of phosphomonoesters was also found to be much lower in P_i-starved cells (4.0 ± 0.16 mM after 24 h) compared to P_i-excess cells (27.1 ± 1.4 mM after 24 h). Similarly, the concentration of NDP-glucose was about 6-fold lower in P_i-starved cells (0.16 ± 0.13 mM after 24 h) compared to P_i-excess cells (1.07 ± 0.28 mM after 24 h). Polyphosphate was only detected in stationary-phase cells grown under P_i excess, but not in cells grown under P_i limitation, as expected.

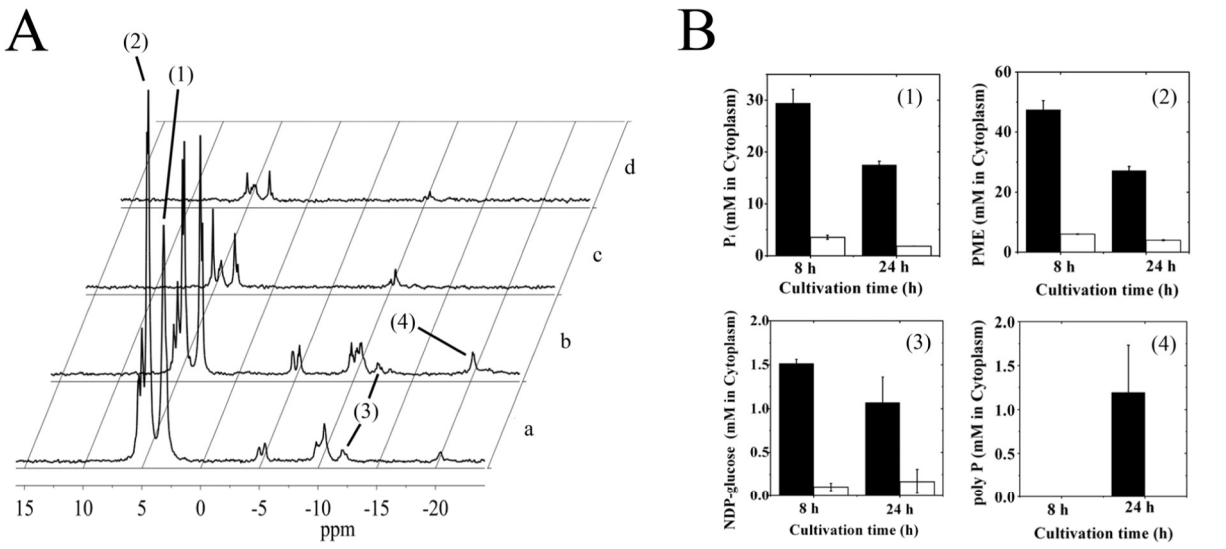

FIG. 3. *In vivo* ^{31}P-NMR spectrum in panel A and measurements to determine the cytoplasmic concentrations of intracellular inorganic P_i, phosphate monoesters (PME), NDP-glucose and polyphosphate (polyP) in cells cultivated for 8 h (a) and 24 h (b) in CGXII glucose minimal medium with 13 mM P_i (black bars) or for 8 h (c) and 24 h (d) in CGXII glucose minimal medium with 0.13 mM P_i (white bars) in panel B. Signals representing intracellular inorganic P_i (1), phosphate monoesters (2), NDP-glucose (3) and polyphosphate (4) are marked in the ^{31}P-NMR spectra, which were recorded using a Varian Inova 400 MHz spectrometer operating at a ^{31}P frequency of 161.985 MHz as described in Materials and Methods. The experiment was performed twice with comparable results.

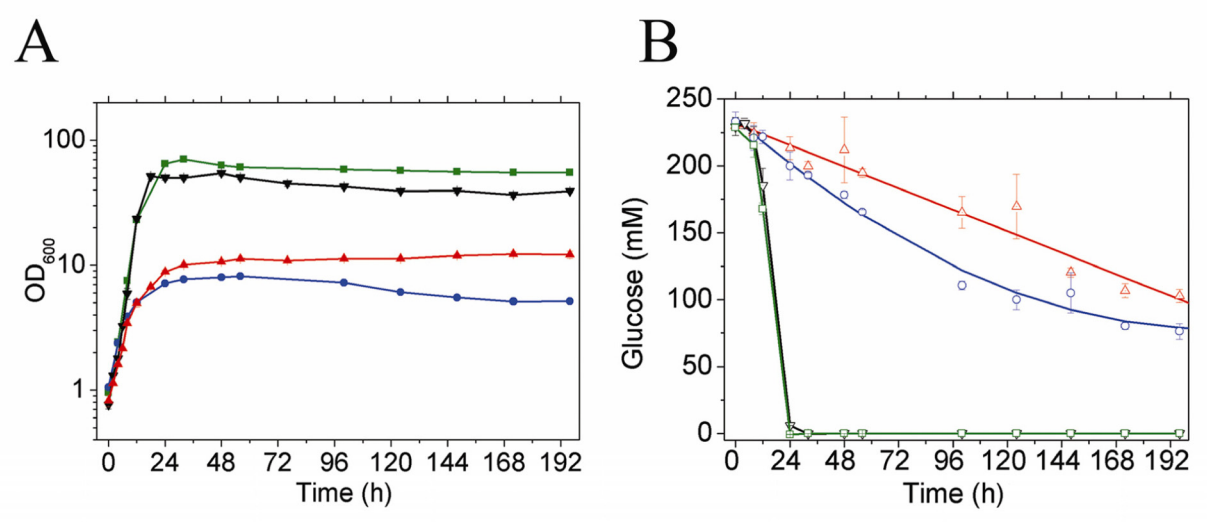

FIG. 4. Growth (A) and glucose consumption (B) of *C. glutamicum* wild type (black and red curves) and the Δ*sugR* mutant (green and blue curves) in CGXII minimal medium with 4% (w/v) glucose and either 13 mM P_i (black and green curves) or 0.13 mM P_i (red and blue curves). Mean values and standard deviations of triplicate cultures are shown.

Influence of the transcriptional regulator SugR on the glucose uptake rate under P_i limitation. The DeoR-type transcriptional regulator SugR represses genes of the phosphoenolpyruvate phosphotransferase system (PTS), genes of several glycolytic enzymes and a variety of further metabolic genes (8, 9, 11, 40-42). The repressing function of SugR has been reported to be relieved by several sugar phosphates, i.e. fructose 6-phosphate, fructose 1-phosphate, glucose 6-phosphate or fructose 1,6-bisphosphate (9, 11, 42). The levels of these metabolites are high when cells grow on sugars, such as glucose or fructose, and low when the cells grow on gluconeogenic carbon sources, such as acetate. As the level of P_i monoesters in glucose-grown cells was found to be much lower in P_i-starved cells, the question arose whether the low glucose uptake rates observed for cells growing under P_i limitation [46 nmol min^{-1} (mg of protein)$^{-1}$ compared to 192 nmol min^{-1} (mg of protein)$^{-1}$ under P_i excess] might be related to the activity of SugR.

To test this possibility we analysed growth and glucose consumption of a Δ*sugR* mutant of *C. glutamicum* (9) under P_i excess and P_i starvation. As shown in Fig. 4, the Δ*sugR* mutant grew similar to the wild type under P_i limitation, but to a lower optical density. Under P_i excess, on the other hand, the Δ*sugR* mutant reached a higher optical density than the wild type. Under P_i excess,

the glucose consumption rate of the Δ*sugR* mutant was comparable to that of the wild type, whereas it was 2.5-fold higher (114 nmol min^{-1} (mg of protein)$^{-1}$) under P_i limitation (period from 8 h to 24 h after start of the cultivation was used for calculation). This supports the assumption that the low glucose consumption rate under P_i limitation is at least partially due to repression of the PTS genes for glucose uptake (*ptsG*, *ptsI*, *ptsH*) and of glycolytic genes by SugR, due to low levels of its effector metabolites. The major reason for the low glucose consumption rate under P_i limitation might, however, be a low rate of phosphoenolpyruvate (PEP) formation.

Influence of P_i limitation on the glycogen pool of *C. glutamicum*. *C. glutamicum* cells growing on glucose, fructose, or sucrose accumulate glycogen up to 90 mg per g of cell dry weight (CDW) in the early exponential growth phase and degrade the polymer when the sugar becomes limiting. In contrast, only marginal amounts of glycogen are formed in cells growing on the gluconeogenic substrates acetate or lactate (36). Recent studies revealed a close connection between maltose and glycogen metabolism (38). The finding of a strongly increased maltose pool in P_i-limited cells prompted us to measure also the glycogen content of cells. As shown in Fig. 2, there was a significant discrepancy under P_i-limiting and P_i-excess conditions. Comparable to previously published data (36) cells grown on glucose under P_i excess accumulated glycogen up to 30 mg glucose per g CDW in the early exponential growth phase and then started to degrade it before reaching the stationary phase. In contrast, P_i-limited cells accumulated glycogen up to 24 h to a level of about 45 mg glucose per g CDW. Even more surprising was the observation that also cells grown with acetate as sole carbon source formed glycogen under P_i limitation up to levels comparable to that of glucose-grown cells (Fig. 2C and D). In contrast and in agreement with previous results (36), no glycogen was formed by acetate-grown cells under P_i excess (Fig. 2D). The high glycogen level that was measured at time zero in all cultures resulted from the precultivation of the inoculum under P_i-limiting conditions. Based on these results, P_i limitation causes glycogen accumulation in *C. glutamicum*.

The results described above raised the question whether also other types of growth limitations have an influence on glycogen accumulation. Therefore, the glycogen content was measured in *C. glutamicum* cells cultivated either under nitrogen excess and nitrogen limitation or under iron excess and iron limitation. Both types of stresses have been studied in the past and key players involved in the adaptation to these stresses have been identified [for reviews see (4) and (10)]. As shown in Fig. S4, these limitations did not cause an accumulation of glycogen in the stationary

phase during growth on glucose or acetate. This indicates that glycogen accumulation is one of the specific responses of the cell to P_i limitation.

Influence of P_i limitation on the expression of *pgm* and *glgC*. Glycogen synthesis in *C. glutamicum* involves four enzymes, i.e. phosphoglucomutase (*pgm*) catalyzing the conversion of glucose 6-phosphate to glucose 1-phosphate, ADP-glucose pyrophosphorylase (*glgC*), which converts glucose 1-phosphate and ATP to ADP-glucose and pyrophosphate, glycogen synthase (*glgA*) converting [α-1,4-glucan]$_n$ and ADP-glucose to [α-1,4-glucan]$_{n+1}$ and ADP, and branching enzyme (*glgB*), which introduces α-1,6-glycosidic bonds into linear α-1,4-glucans. To test the influence of P_i limitation on the expression of *pgm* and *glgC*, the corresponding promoter regions were cloned into the promoter probe vector pET2 containing a promoterless chloramphenicol acetyltransferase reporter gene and the resulting plasmids pET2-*pgm* and pET2-*glgC* were transferred into *C. glutamicum* wild type. As shown in Table 3, expression of the phosphoglucomutase gene *pgm* was 6-fold higher in cells grown for 24 h under P_i limitation compared to cells grown for 24 h under P_i excess. In the case of the ADP-glucose pyrophosphorylase gene *glgC* the expression level was 3-fold higher under P_i limitation compared to P_i excess. These results indicate that genes of the glycogen synthesis pathway are activated or derepressed under P_i limitation and increased levels of the two enzyme activities could at least partially be responsible for the increased flux of glucose 6-phosphate into the glycogen pathway and reduced fluxes into glycolysis and the pentose phosphate pathway.

TABLE 3. Influence of the P_i concentration on the expression of the *pgm* and *glgC* genes in *C. glutamicum*.

Growth condition	Chloramphenicol acetyltransferase activity [1] (nmol min^{-1} (mg protein)$^{-1}$)	
	C. glutamicum pET2-*pgm*	*C. glutamicum* pET2-*glgC*
P_i excess (13 mM)	31 ± 5	195 ± 37
P_i limitation (0.13 mM)	189 ± 37	583 ± 6

[1]The indicated strains were cultivated for 24 h in CGXII medium with 222 mM glucose under either P_i excess (13 mM P_i) or P_i limitation (0.13 mM). Chloramphenicol acetyltransferase activities were determined in cell-free extracts. Means and standard deviations derived from three independent cultivations are given.

***In silico* simulation of the phosphate starvation response using a genome-scale model of *C. glutamicum*.** In order to study the influence of P_i limitation on metabolism *in silico* by flux balance analysis (FBA), a genome-scale model of *C. glutamicum* (20) was expanded by including two reactions required for glycogen synthesis (ADP-glucose pyrophosphorylase and glycogen synthase) and one reaction responsible for glycogen degradation, which represents both glycogen phosphorylase and maltodextrin phosphorylase. The resulting optimal phenotypes referring to growth under variation of glucose and P_i uptake are shown in Figure 5A. As expected, under the precondition of a sufficient glucose uptake rate (>4 mmol (g CDW)$^{-1}$ h^{-1}), the growth rate is linearly dependent on the P_i uptake rate. However, there is a discrepancy between the simulated maximal growth rate and the experimentally determined growth rate under P_i-limiting conditions. the latter being located at a point where no steady state flux solution of the network exists. To reach the experimentally observed growth rate of about 0.16 h^{-1} under P_i limitation in the simulation, glucose and phosphate uptake rates have to be significantly higher (>2 mmol (g CDW)$^{-1}$ h^{-1} and >0.1 mmol g (CDW)$^{-1}$ h^{-1}, respectively). A similar observation was made for the results obtained for the acetate-grown cells. Here discrepancies were found for both, phosphate-limiting and phosphate-excess conditions (Fig. 5B). As expected, simulations showed that increasing the glucose or acetate uptake rate at low phosphate uptake rates did not lead to higher cellular growth.

Since a strong influence of P_i limitation on the glycogen pool was observed (see above), the influence of varying glucose and phosphate uptake rates on glycogen formation was tested *in silico*. As shown in Fig. 5C, an increased rate of glycogen synthesis was predicted at low P_i uptake and high glucose uptake rates. The same behaviour was also observed when acetate instead of glucose was used as carbon source (Fig. 5D). These predictions are in agreement with the experimental results.

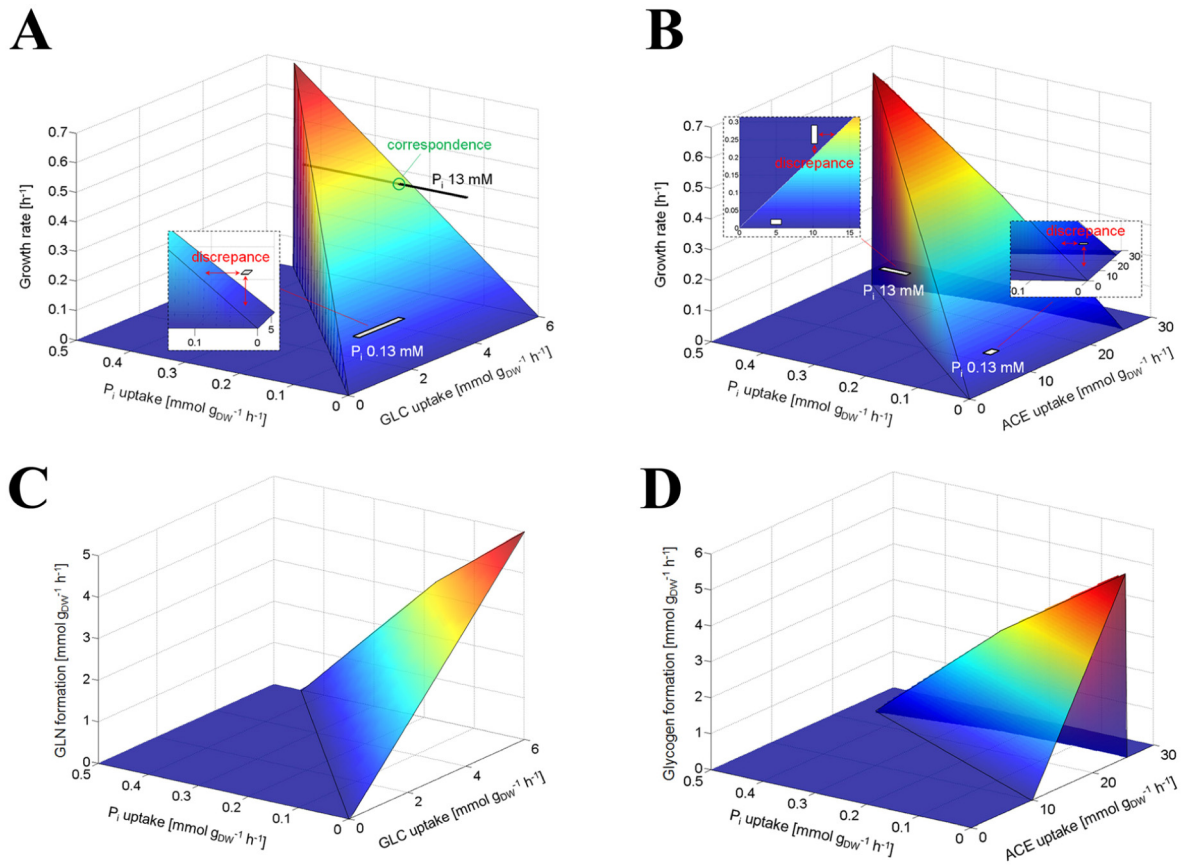

FIG. 5. In panels A-D, the simulated phenotypes under growth optimization of the genome-scale metabolic network model are shown. *In silico* solutions of growth rates and glycogen (GLN) formation under variation of P_i uptake in combination with either glucose (GLC) uptake (panels A and C) or acetate (ACE) uptake (panels B and D) form three-dimensional surfaces in each case. For comparison measured growth rates for P_i-limited and -excess cultures including experimental errors are mapped as light-gray rectangles.

DISCUSSION

Previous studies on the response of bacteria to P_i-limiting conditions mainly focused on gene expression, regulators and enzymes involved in the P_i starvation response. In the work presented here, the influence of P_i limitation on metabolite levels was analysed by GC-MS using *C. glutamicum* as model organism. An important result was the detection of strongly elevated maltose levels under P_i limitation, which raises the question how this disaccharide is formed in cells growing on glucose. The only pathway that has been described in literature for *C. glutamicum* is the conversion of trehalose to maltose by trehalose synthase (TreS). TreS was shown to be the only enzyme present in *C. glutamicum* capable of converting trehalose to maltose and *vice versa* and evidence was presented that TreS is mainly responsible for trehalose degradation (48). Trehalose, which serves as a stress protection compound and as a prerequisite for mycolate production, is synthesized either from UDP-glucose and glucose 6-phosphate via the OtsA-OtsB pathway or from malto-oligosaccharides or α-1,4-glucans via the TreY-TreZ pathway (43, 48). The cytoplasmic trehalose level as determined by GC-MS was much higher compared to the maltose level (factor 10^3 to 10^4) and was only slightly decreased under P_i-limitation compared to P_i-excess.

Besides TreS, one alternative enzyme candidate could also play a role in maltose formation. The protein encoded by cg1012 shows sequence similarity to the *E. coli* maltodextrin glucosidase MalZ. MalZ removes glucose residues from the reducing end of maltodextrins which are composed of more than two glucose residues (i.e. maltotriose, maltotetratose etc.) and forms maltose as an endproduct (5). The presence of this enzyme activity in *C. glutamicum* is not yet clear (38), but would offer an alternative explanation for the high internal glucose level of P_i-limited cells (besides the possibility that it is due to contamination from residual extracellular glucose).

Recent studies have indicated that *C. glutamicum* catabolizes maltose in the same way as *E. coli* (2) by MalQ (cg2523), a maltodextrin glucanotransferase (also called amylomaltase) which forms from any maltodextrin, including maltose, larger maltodextrins and glucose. Glucose can then be phosphorylated either by an ATP-dependent glucokinase (30) or by a polyphosphate/NTP-dependent glucokinase (25) to glucose 6-phosphate and catabolized, whereas the maltodextrins are degraded by maltodextrin phosphorylase (MalP) to glucose 1-phosphate, which is converted to glucose 6-phosphate by phosphoglucomutase (38). In *Mycobacterium tuberculosis* and *M. smegmatis* maltose formed from trehalose by TreS is incorporated into

glycogen by the consecutive action of the maltose kinase Pep1 and the maltosyltransferase GlgE (7, 17). Genes encoding homologs of Pep1 (*cg2530*) and GlgE (*cg1382*) were also identified in *C. glutamicum*, but have not yet been characterized (G. M. Seibold, unpublished data).

In a previous study, the presence of maltose in cells of *C. glutamicum* cultivated on glucose under P_i excess was reported (39). Thus, maltose might be a regular metabolite in *C. glutamicum* not only during growth on maltose. The question, whether the increased maltose pool observed under P_i-limitation is caused by an increased synthesis or by a decreased degradation or both cannot be answered at the moment.

Triggered by the close connection of maltose and glycogen metabolism we found that P_i starvation also had a strong influence on the cellular glycogen pool. Whereas under P_i excess glycogen is formed in the early exponential phase and then degraded again, P_i-starved cells form a glycogen pool in the exponential phase, but retain it also in the stationary phase, irrespective of whether glucose or acetate were used as carbon source. The high glycogen pool under P_i starvation could be due to increased synthesis, as suggested by the increased expression of *pgm* and *glgC*. The regulators responsible for this increased expression are not yet known. The global transcriptional regulator RamA was recently shown to function as an activator of *glgC* (37), but current knowledge suggests that RamA responds to a metabolite involved in acetate catabolism rather than to P_i. Besides an increased glycogen synthesis rate, also a decreased glycogen degradation rate could be responsible for the altered glycogen pool under P_i limitation. Glycogen degradation in *C. glutamicum* involves glycogen phosphorylase (GlgP), which phosphorolytically cleaves α-1,4-glycosidic bonds at the non-reducing ends of glycogen and forms glucose 1-phosphate and phosphorylase-limited dextrins (pl-dextrins). The debranching enzyme (GlgX) converts the pl-dextrins to linear maltodextrins ($2 < n < 20$; n, number of glucose molecules), which are then further degraded by MalP to glucose 1-phosphate (38). As GlgP and MalP both require P_i, their activity might be limited at reduced cytoplasmic P_i concentrations.

The *in silico* simulation data based on a stoichiometric genome-scale metabolic model (20) that was modified to include glycogen synthesis and degradation reactions predicted increased glycogen formation in exponentially growing cells under P_i limitation with glucose or acetate as carbon sources. However, the model was not able to correctly predict the experimentally determined growth rates under P_i starvation. Reasons for the observed discrepancies could be a lack of qualitative information and/or quantitative accuracy of the model, which mainly includes all biomass-related reactions and rough estimations of stoichiometric coefficients. Furthermore,

the model does not incorporate any kind of P_i-dependent regulation of central metabolism and storage pool metabolism (glycogen, polyphosphate). An inclusion of regulation would necessitate the formulation of mechanistic models, which is currently impossible due to the lack of quantitative knowledge of regulatory and metabolic processes. Therefore the results from our stoichiometric analysis should be regarded as a first step in simulating the complex metabolism of carbohydrate storage pools like glycogen.

Central carbon metabolism and energy metabolism are inevitably connected to the availability of P_i, as key enzymatic reactions require P_i as substrate, such as glyceraldehyde 3-phosphate dehydrogenase and F_1F_0-ATP synthase. In addition, many reactions require ATP or ADP as substrates, such as phosphofructokinase, 3-phosphoglycerate kinase and pyruvate kinase. As the cytoplasmic concentrations of P_i, ADP and ATP are lower under P_i limiting conditions, a reduced enzyme activity and consequently a reduced glycolytic flux can be envisaged, resulting in a reduced PEP synthesis rate and thus a reduced glucose consumption rate. In this study, another consequence of P_i limitation was found, namely an altered glycogen metabolism resulting in an increased and more stable glycogen pool. To our knowledge, such a link has not yet been described. Further studies are required to elucidate the molecular details of this connection.

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Supplemental materials (Glycogen storage under P_i limitation in *C. glutamicum*)

Metabolite extraction using cold 50% (v/v) methanol

A culture sample of 1 ml was rapidly transferred into a 50 ml Falcon tube containing 5 g of ice precooled to -25°C and centrifuged for 2 min at 5311 x g and -20°C using a Sigma 4K15 centrifuge with four swing-out rotors (No.11150) (Sigma Labzentrifugen GmbH, Osterode am Harz, Germany) and also precooled to -25°C. Ice and supernatant were immediately discarded, the cell pellet resuspended in 1 ml cold (0°C) 2.6% NaCl precooled and then centrifuged again for 2 min at 5000 rpm and -20°C. The supernatant was discarded, 2 ml of 50% (v/v) methanol precooled to -30°C was added to the cell pellet and the tube vigorously mixed for 1 min. Then, samples were frozen in liquid nitrogen, thawed at room temperature, in a water bath and mixed for 1 min. The freeze-thaw cycle was repeated twice in order to achieve leakage of the cells and release of the intracellular metabolites. After the third thawing step, the cell extract was centrifuged for 10 min at 5311 x g and -4°C. 200 µL of the supernatant (cell-free extract) was transferred into a 0.5 ml Eppendorf tube and vaccum-dried overnight using a Vacufuge Centrifuge 5310 (Eppndorf AG, Hamburg, Germany) with fixed angle rotor in water-based mode. The extract was finally stored in a vacuum desiccator until it was used for GC-MS analysis.

Metabolite identification by GC-(EI/CI)-TOF-MS

The process used for the identification of "real" metabolites in the GC-MS spectra is schematically summarized in Fig. S1. It included the measurement of samples which differed only by the fact that the cultures were grown with either naturally labelled glucose or uniformly ^{13}C-C_6-labelled glucose. These samples were analysed both by GC-EI-MS and GC-CI-MS. The m/z shift of mass fragments owing to ^{13}C isotope incorporation allowed us to determine the number of carbon atoms in the metabolites. The application of the identification procedure shown in Fig. S1 resulted in three "cases".

In case 1, "real" metabolites were identified as follows: Mass fragment patterns obtained by GC-EI-MS of the naturally labelled metabolites were used to search the NIST mass library for possible hits. Hits with a score above 700 were considered further. The unprotonated intact masses measured by GC-CI-MS of the naturally labelled sample were compared with the intact masses (including derivatization) calculated for the hits obtained by GC-EI-MS. "Real" hits should have the same mass in both cases. In addition, the number

of carbon atoms obtained by subtraction of the unprotonated m/z value obtained by GC-CI-MS of the ^{13}C-labelled sample from the unprotonated m/z value obtained by GC-CI-MS of the naturally labelled sample has to match the number of carbon atoms prediced for the hit from the NIST mass library. As an example the identification of a peak with a retention time of 8.76 min is described. The m/z pattern (188 m/z, 73 m/z, 100 m/z, 189 m/z, 147 m/z, 262 m/z, 190 m/z, 59 m/z, 114 m/z, 263 m/z) obtained by GC-EI-MS yielded a high-score hit of 900 after searching the NIST library with NIST MS Search Program, i.e. L-alanine. The m/z value (unprotonated) at 8.76 min elution time obtained by GC-CI-MS of the ^{12}C sample (305.1625) matched the hit predicted from the GC-EI-MS analysis. In agreement, the number of three carbon atoms determined as described above matched with the predicted compound, L-alanine ($C_3H_7O_2N$).

In case 2, putative metabolites identified usually in small chromatographic peaks, but also in some large peaks (hexamethydisiloxan and its derivatives), were excluded as artifacts based on one of the following reasons: (i) The mass fragment pattern search in the NIST mass library led to top 10 hits with a score below 700. (ii) No mass shift was observed when comparing the ^{12}C- and ^{13}C-labelled samples, both in GC-EI-MS and GC-CI-MS. (iii) The unprotonated m/z value for the intact mass obtained by GC-CI-MS of the ^{12}C sample did not match with the intact mass predicted for the hits obtained by GC-EI-MS and NIST library search. A particular exception represents phosphoric acid, which represents a "real" metabolite, although no mass shift can be observed in the comparison of the ^{12}C- and ^{13}C-labelled samples.

Case 3 includes metabolites that could not be identified by searching the NIST library but by a procedure based on the measurement of the exact protonated mass of the intact compound (accuracy within 5 mDa) by GC-CI-MS. The mass obtained form the ^{12}C sample was first analysed using the elemental composition tool provided by the MarkerLynx software (Waters) to obtain possible chemical formulas. The resulting hits were then processed by the Matlab script "Metabolite Composition Analyzer" (Fig. S2), which eliminates the masses caused by the chemical derivatizations and analyzes the remaining hits for the H/C ratio, the N, O, P and S ratio, and the number of C atoms obtained by the comparison of the ^{12}C and ^{13}C-labelled samples. The hits that "survived" these checks were used to search automatically in public databases (PubChem and KEGG compound DB) for metabolites with the same chemical composition. This procedure was successful for candidates whose derivatized m/z was below 400 (Fig. S3). Metabolite candidates with m/z above 400 still await identification.

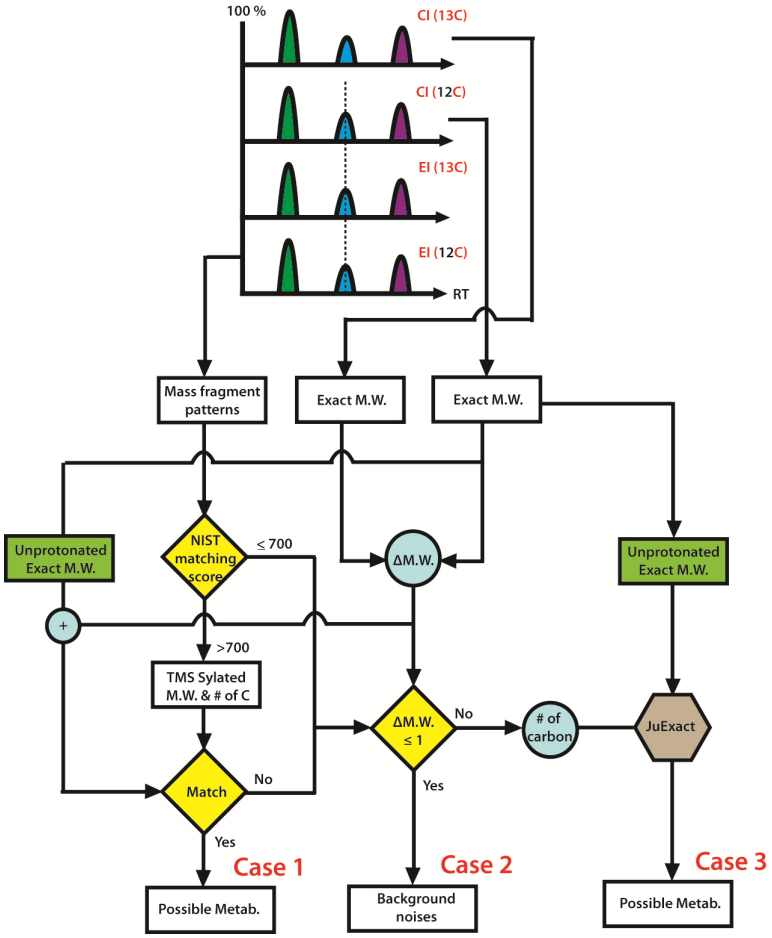

Fig. S1. Process to identify metabolites using GC-EI/CI-TOF mass spectrometry of ^{12}C-and ^{13}C-labelled cell extracts of *C. glutamicum*. For this purpose, cells were grown with either naturally labelled glucose (^{12}C sample) or $^{13}C_6$-labelled glucose (^{13}C sample). The extracted metabolites were analysed both by GC-EI-MS and GC-CI-MS. For details see text.

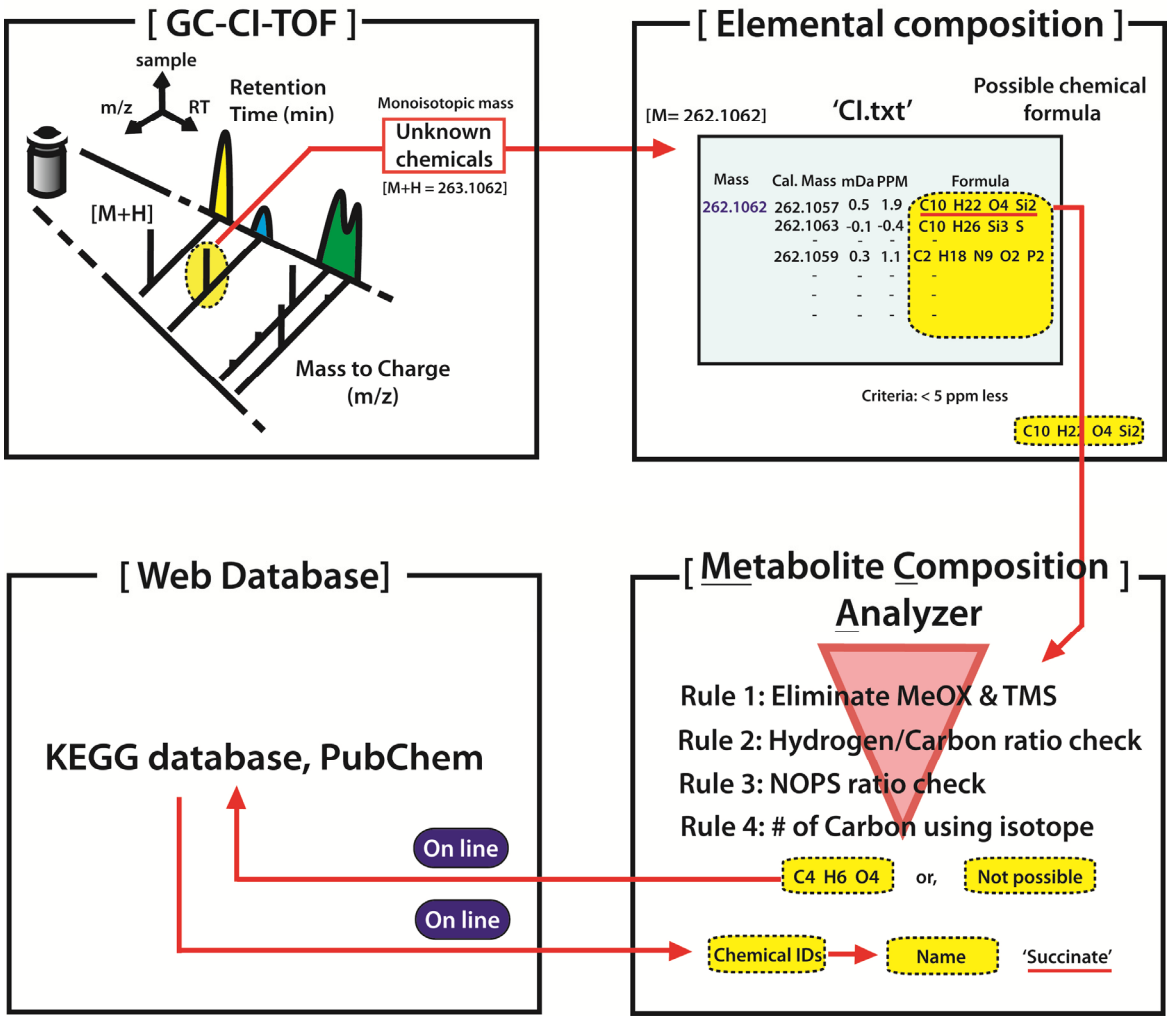

Fig. S2. A workflow to search metabolite candidates for unknown peaks and mass fragments using a Matlab script, called 'Metabolite Composition Analyzer' connected online to public chemical databases. In order to suggest metabolite candidates from unknown peaks, possible elemental compositions were calculated using the Elemental composition 4.0 tool provided by MassLynx software and the results were imported into a Metabolite Composition Analyzer tool that eliminates impossible candidates by applying a number of rules. The remaining candidates were used to search automatically in public databases (PubChem and KEGG compound DB) for metabolites with the same chemical composition. The results were shown as either text file or xml file.

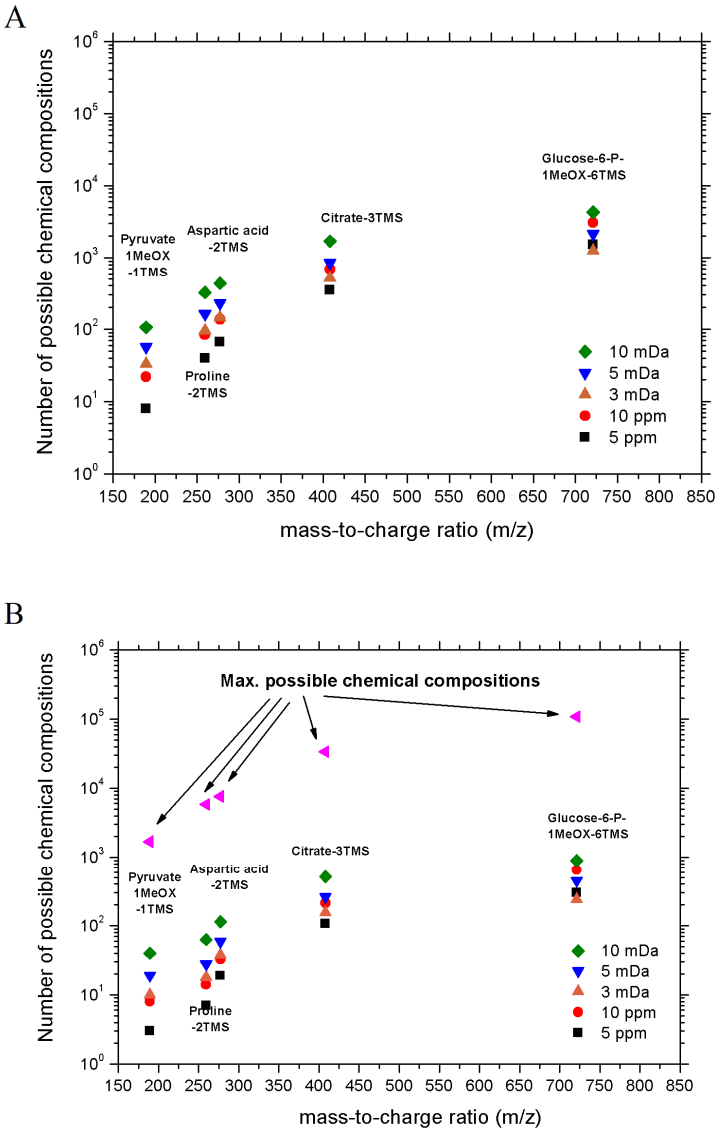

Fig. S3. Number of possible chemical compositions based on exact m/z measurement using only mathematical combination (A) or additionally chemical rules such as LEWIS, SENIOR, isotopic pattern, hydrogen/carbon ratios, and elemental ratios (B). Five different derivatized metabolites were used for calculation of the possible chemical compositions with different measurement errors (5 ppm, 10 ppm, or 3 mDa, 5 mDa, and 10 mDa), i. e. pyruvate-1MeOX-1TMS ($C_7H_{15}O_3NSi$, 189.0821 Da), proline-2TMS ($C_{11}H_{25}O_2NSi_2$, 259.1424 Da), aspartic acid-2TMS ($C_{13}H_{31}O_4NSi_3$, 349.1561 Da), citrate-3TMS ($C_{15}H_{32}O_7Si_3$, 408.1456 Da), and glucose 6-phosphate-1MeOX-6TMS ($C_{25}H_{64}O_9NPSi_6$, 721.2934 Da). In panel A, the mathematically possible combinations of the following chemical elements were calculated and displayed (n = number) that would result in the experimentally determined mass: C (n = 1–29), H (n = 1-101), O (n = 0-10), N (n = 0-5), S (n = 0-5), P (n = 0-5), Si (n = 1-11). In panel B, possible chemical rules were applied in addition, including isotopic patterns (details given in the manual of MarkerLynx, Waters), to determine the maximally possible chemical compositions. For derivatized compounds with a mass >400 Da, the number of possible compositions is above 1000 for both variants. In these cases, additional rules besides the ones used in the Metabolite Composition Analyzer tool are required to eliminate false positives.

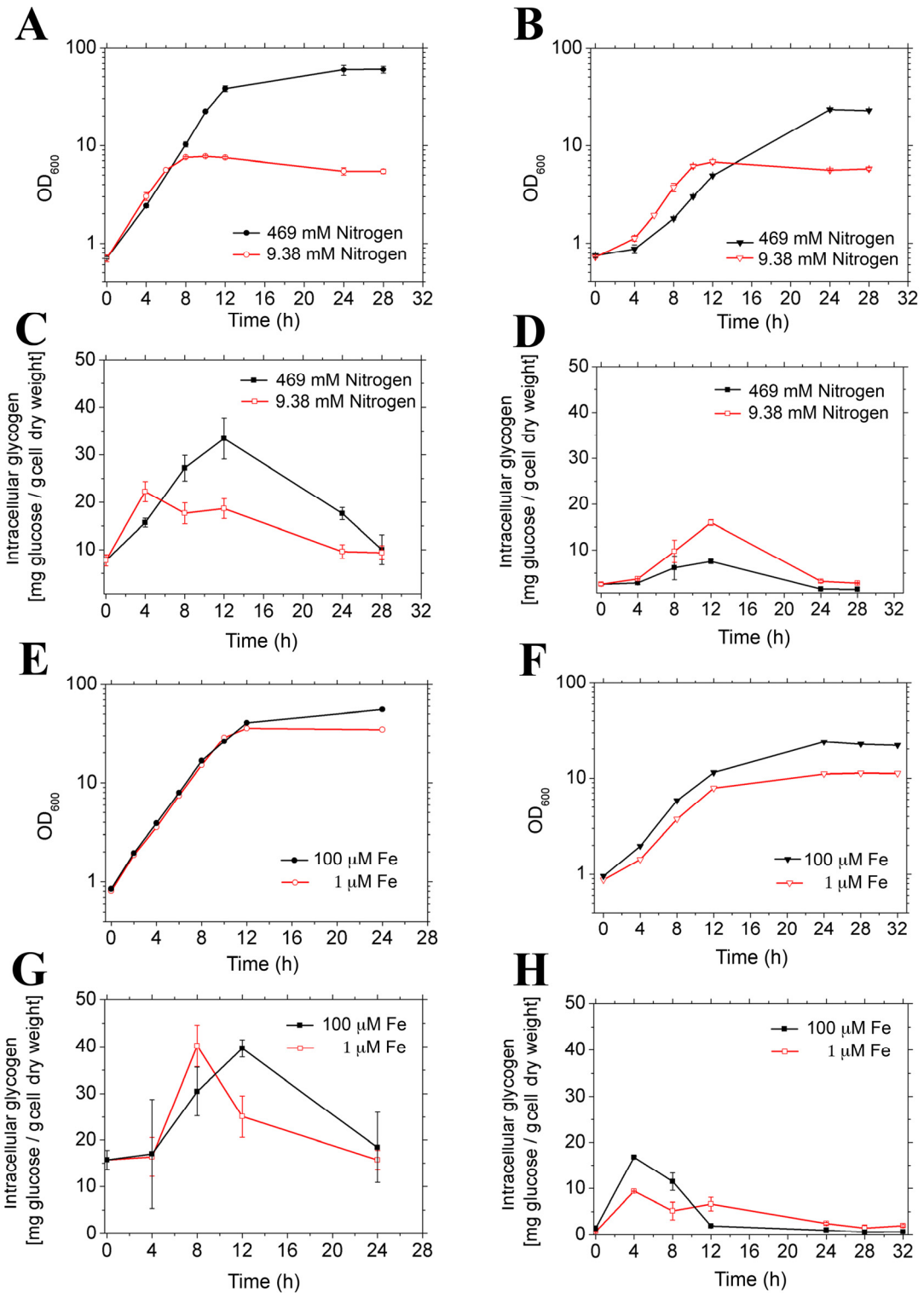

FIG. S4. Growth of and glycogen levels in *C. glutamicum* cultivated in CGXII minimal medium with either 222 mM glucose (left panels) or 300 mM potassium acetate (right panels). In panels A to D, the cells were grown under nitrogen excess and nitrogen limitation, wherease in panels E to H they were cultivated under iron excess and iron limitation. For nitrogen limitation, the concentrations of ammonium sulfate and urea were reduced to 1/50 of the original CGXII medium (20 g/l ammonium sulfate, 5 g/l urea). For iron excess and limitation, the medium contained either 100 µM $FeSO_4$ or 1 µM $FeSO_4$. The inoculum was precultivated twice under nitrogen or iron limitation.

5 Discussion

5.1 Genetic regulation of P_i starvation responses in *C. glutamicum*

The key response to P_i starvation by bacteria is to obtain P from all possible sources by expressing transporter machinery and secreting proteins in order to provide sufficient P for cellular activities. In order to efficiently scavenge P_i, bacteria have evolved complex regulatory systems that activate the expression of genes for target proteins such as high-affinity P_i and glycerol 3-phosphate ABC transporters (Pst and Ugp transport systems, respectively), and secreted alkaline phosphatases in response to P_i availability. In particular, the Pst system and alkaline phosphatases are induced in most bacterial species studied so far with respect to P_i limitation. These P_i starvation inducible genes are usually controlled by a two-component regulatory system, often designated as 'PhoPR' system.

Additionally, bacteria have developed strain-specific regulation systems to respond to P_i availability. *E. coli* utilizes the two-component system CreBC for the cross-regulation of the Pho regulon by carbon regulatory systems (Wanner, 1996). The Spo0A system of *B. subtilis* takes over the control of P_i regulation from the PhoPR and ResDE systems for sporulation (Hulett, 2002). In the case of *M. tuberculosis*, the name 'PhoPR' is misused since its function is related to the virulence of *Mtb* (Ryndak *et al.*, 2008), but *M. smegmatis* utilize the two-component system SenX3-RegX3 for its P_i starvation response (Glover *et al.*, 2007). The PhoPR system in *S. lividans* additionally controls antibiotic biosynthesis genes (Sola-Landa *et al.*, 2003).

In previous studies by our group and in this PhD thesis, the regulatory systems involved in the P_i starvation responses of the Gram-positive soil bacterium *C. glutamicum* have been uncovered using DNA microarray analysis and electrophoretic gel mobility shift assays. Under P_i limited conditions, *C. glutamicum* induces the *psi* genes which are regulated by the two-component system PhoRS (Ishige et al., 2003, Kocan et al., 2006, Schaaf & Bott, 2007) and the two-component system SenX3-RegX3. Like the other bacteria mentioned above, *C. glutamicum* could have secondary functions of the two-component systems. The fact that *regX3* could not be deleted suggests that RegX3 plays an essential role in cell growth. This essentiality of the *regX3* gene could lead to another regulatory function of the SenX3-RegX3 regulatory system. Essential two-component systems have also been described in other bacteria, such as *B. subtilis* (Dubrac *et al.*, 2008), *M. smegmatis* (Glover *et al.*, 2007), or *Staphylococcus aureus* (Martin *et al.*, 1999).

The following three sections are focused on (i) Pho regulation by PhoRS and SenX3-RegX3, (ii) the essentiality of the *regX3* gene, and (iii) the proposed model of phosphate starvation in *C. glutamicum*.

5.1.1 Regulation of P_i-dependent genes by PhoRS and SenX3-RegX3

Many two-component regulatory systems involve OmpR/PhoB-like response regulators. These proteins are characterized by a conserved N-terminal regulatory domain including an aspartate residue to which a phosphoryl group can be transferred from the cognate sensor kinase, and a C-terminal effector domain comprising a helix-turn-helix DNA-binding motif which allows binding to the promoter region of target genes. The sensor kinases of TCS usually contain two transmembrane helices (TMH), often a HAMP domain (Histidine kinase, Adenylyl cyclases, Methyl binding protein, and Phosphatase) and always the conserved kinase domain composed of the phosphorylation subdomain and the ATP-binding subdomain.

The domain composition of the *C. glutamicum* sensor kinases and response regulators has been summarized in the supplementary material of Kocan *et al.* (2006). The histidine residues at position 276 in PhoS (485 amino acids) and at position 166 in SenX3 (413 amino acids) can be autophosphorylated, which was confirmed by *in vitro* phosphorylation assays with radioactive [γ-^{32}P] ATP (Wessel, 2003, Kocan, 2005, Schaaf & Bott, 2007). The mechanism of stimulus recognition remains unclear both for PhoS and SenX3. The response regulators PhoR (235 amino acids) and RegX3 (232 amino acids) are phosphorylated at asparate residues at position 59 in PhoR and position 52 in RegX3, which leads to a conformational change of proteins leading to an improved DNA-binding affinity to the promoter regions of the target genes.

Twenty-five P_i inducible genes of *C. glutamicum* were identified using a time-resolved DNA microarray analysis after a shift from P_i excess to P_i limitation conditions (Ishige et al., 2003), encoding P uptake systems, secreted proteins and the PhoRS TCS. Some *psi* genes are regulated by two transcriptional regulators, PhoR and RegX3, which was shown by both DNA microarray and EMSA analyses. Genetic regulation of phosphate uptake systems and secreted proteins is discussed in the following parts along with P_i-independent regulations in polyP biosynthesis.

Activation of the high-affinity P_i-uptake system

As expected, when bacteria face P_i limitation conditions, uptake systems are induced to import P sources into the cells. The major P_i uptake systems consist of the high-affinity Pst ABC transporter and a low-affinity secondary transporter (PitA). The Pst ABC transporter encoded by the *pstSCAB* operon is composed of a periplasmic P_i-binding protein (PstS), two integral membrane proteins (PstC and PstA), and a cytoplasmic ATP-binding protein (PstB). The Pit transporter is encoded by the *pitA* gene and is a single-component secondary transporter, which may be responsible for P_i symport with a divalent metal ion.

In bacteria, the Pst system is usually regulated by a two-component regulatory system. In *C. glutamicum*, transcription of the Pst system is regulated by both RegX3 and PhoR. In response to P_i starvation, the amount of PhoR~P could be increased by positive feedback regulation and PhoR~P boosts gene expression of the Pst system. However, RegX3 seems to constitutively regulate the *pstSCAB* operon due to the fact that the mRNA levels of *pstSCAB* were decreased by 4-fold in *C. glutamicum* HMW1 mutant grown under P_i excess conditions and in the absence of ATC, compared to the mutant in the presence of ATC (see the manuscript for details). A basal level of expression of the Pst system controlled by RegX3 could enable *C. glutamicum* to quickly respond to P_i limitation. A binding site of RegX3 (position -79 to -39 with respect to the transcriptional start site) was located near the promoter region, where RegX3 may be able to interact with the σ^{70} sigma factor of RNA polymerase to enhance transcription of the *pstSCAB* genes. Interestingly, the global regulator GlxR, which regulates about 150 target genes in *C. glutamicum*, also binds to the promoter regions of the *pst* and *ugp* operons, which was confirmed by EMSA in the presence of its effecter molecule, cAMP (Sorger-Herrmann, 2006, Kohl & Tauch, 2009, Panhorst *et al.*, 2010, Brinkrolf *et al.*, 2007), and a binding site of GlxR (-133 to -118 with respect to the transcriptional start site) was predicted to exist in the promoter region of *pstS* (Kohl *et al.*, 2008). These data provide evidence for cross-regulation of the Pho regulon by carbon regulatory systems.

The Pit system, a low-affinity P_i transporter in *E. coli*, is believed to be constitutively expressed and presumably is responsible for P_i uptake when P_i is in excess. The *pitA* gene is usually not part of the Pho regulons. However, the comprehensive function of the Pit system is not yet understood (Wanner, 1996). In *C. glutamicum*, gene expression of *pitA* after the shift to P_i limitation was not decreased in the Δ*phoRS* mutant, but its mRNA level decreased by 4-fold in the wild type after the shift to P_i limitation. The binding site of PhoR in the *pitA* promoter region was predicted to be at position -6 to -24 with respect to the transcriptional

start site. Thus, PhoR may interfere with the transcription of the *pitA* gene by blocking the promoter region (Kocan, 2005, Schaaf & Bott, 2007). These results suggest a possible repression of the *pitA* gene by the PhoRS system when P_i is limited. No evidence was found for the regulation of *pitA* expression by RegX3.

Glycerol-3-phosphate is an alternative P source for bacteria and is taken up by the Ugp ABC transport system, which is a periplasmic binding protein-dependent transport system. *E. coli* utilizes the Ugp system encoded by the *ugpBAECQ* operon, which is activated by the PhoBR TCS, and the Ugp system is inhibited by high intracellular P_i concentration. Also, *C. glutamicum* possesses the Ugp system encoded by the *ugpAEBC* operon, which is regulated by both the RegX3 and PhoR regulators. Like the Pst system, RegX3 constitutively activates the Ugp system and it is additionally activated by PhoR when P_i is limited.

Genes regulated by the PhoBR TCS (*phnCDEFHIJKLMNOP* and *phnXWRSTUV*) are involved in phosphonate uptake and degradation in *E. coli*. In *C. glutamicum*, the expression of *pctB* and *pctC*, which are part of the *pctABCD* operon encoding an ABC-type transport system for a yet unknown P-containing compound, were induced after a shift to P_i limitation and might be regulated by PhoR. However, based on DNA microarray analysis, no evidence was found to support the regulation of *pctABCD* by RegX3.

Activation of genes encoding alkaline phosphatase and secreted proteins

Another general P_i starvation response of bacteria is the induction of alkaline phosphatase (APase). Bacterial APase is secreted into the periplasmic space in order to hydrolyze phosphate esters to generate free inorganic phosphate to be taken up by the Pst system. In *C. glutamicum*, the *phoB*, *phoC*, and *phoD* genes have been annotated to encoded proteins with APase or phosphodiesterase activity. Only the *phoC* gene is induced under P_i limitation and its expression is regulated by both RegX3 and PhoR. Neither *C. glutamicum* strains with a single deletion (Δ*phoC*) nor with a triple deletion (Δ*phoB*, Δ*phoC*, and Δ*phoD*) showed a reduction in APase activity when P_i was limited. This result strongly indicates that other genes/proteins, which have not yet been identified, are responsible for the measured APase activity. Moreover, a glycerophosphodiester phosphodiesterase encoded by the *glpQ* gene was also induced during P_i starvation and regulated by PhoR, but not RegX3.

Nucleotides, DNA and nucleotide sugars present in natural habitats can also serve as alternative P sources. Bacteria secret enzymes to break them down, rather than transport them directly into the cell. In *E. coli*, UDP-sugar hydrolase encoded by the *ushA* gene serves as an periplasmic enzyme with phosphatase activity, but no transcriptional control has been

reported (Wanner, 1996). Like UshA from *E. coli*, the UshA protein of *C. glutamicum* has 5'-nucleotidase and UDP-sugar hydrolase activity (Rittmann *et al*., 2005); however, the *ushA* gene is transcriptionally regulated by both RegX3 and PhoR. In addition, the *nucH* gene encoding an extracellular nuclease is regulated by PhoR, but not RegX3.

P_i-independent regulation of the polyP pathway

Bacteria can synthesize inorganic polyphosphate (polyP), which is a chain of phosphate residues linked by phosphoanhydride bonds. PolyP performs various functions depending on the cells and the environment (Brown & Kornberg, 2004, Kuroda *et al.*, 2001). PolyP in *E. coli* can be synthesized by polyphosphate kinase (Ppk) from ATP and is increased in response to amino acid starvation and multiple stresses in the stationary phase. In *E. coli*, (p)ppGpp is involved in polyP accumulation by inhibiting the activity of polyphosphatase, and polyP induces RecA- and RpoS-mediated responses (Tsutsumi *et al.*, 2000, Kuroda *et al.*, 1997). Unlike *E. coli*, mycobacteria require polyP to induce the *rel* gene involved in both (p)ppGpp biosynthesis and hydrolysis. Also, a signalling pathway involving the two-component system MprAB, Poly P and σ^E was suggested (Manganelli, 2007).

In *C. glutamicum*, polyP is synthesized by Ppk encoded by the *ppk2B* gene and degraded by either polyphosphate/ATP-dependent glucokinase (Ppgk) or exopolyphosphatase encoded by the *ppx1* and *ppx2* genes (Lindner et al., 2009, Lindner et al., 2010, Lindner et al., 2007). ^{31}P-NMR studies showed that *C. glutamicum* utilized internal polyP for supplying P_i under P_i-limiting conditions (Pallerla *et al.*, 2005). However, the genes obviously involved in polyP degradation were not induced under P_i limitation. In addition, no evidence was obtained that these genes are regulated by the PhoRS and SenX3-RegX3 systems. The two-component system CgtSR2, which is a homologue of the MprA-MprB TCS of *M. tuberculosis*, has not been characterized, and the role of (p)ppGpp in polyP metabolism have not yet been studied in *C. glutamicum*.

5.1.2 The essentiality of the *regX3* gene

Although rare, a number of essential two-component systems have been reported, such as MtrB/MtrA of *Mycobacterium tuberculosis (Fol et al., 2006)* and WalK/WalR of *Bacillus subtilis* (Dubrac *et al*., 2008). Deletion of the *walKR* genes in *B. subtilis* was not successful. The WalR response regulator directly activates genes encoding a hydrolase (YocH), an endopeptidase (LytE) and a cell wall-associated protein (YdjM) (all involved in cell wall metabolism) and represses genes encoding teichoic acid biosynthesis enzymes (TagABDEF), a peptidoglycan

deacetylase (YjeA), a modulator of autolysin activity (YoeB) and a fatty acid desaturase (Des) (Okajima *et al.*, 2008, Fukushima *et al.*, 2008, Howell *et al.*, 2006). In a growing cell, lipid II and WalK sense cell wall precursors, and the activated WalK phosphorylates WalR to continue the synthesis of autolysin for cell wall remodelling, cell elongation and cell division. In a non-growing cell, autolysin biosynthesis could be lowered by de-repressing the YoeB and YjeA modulators and not activating cell division proteins (FtsA and FtsZ). If the WalK/WaklR system was altered, the plasticity of peptidoglycan during cell division could be affected and this would disrupt the cell membrane, causing cell death.

Among the 13 TCS of *C. glutamicum*, the one encoded by *senX3-regX3* (originally designated *cgtS4-cgtR4*) could not be deleted although numerous attempts were made (Wessel, 2003). In this PhD thesis, the *C. glutamicum* HMW1 strain for conditional repression of the *regX3* gene was constructed using the TetR repressor system. TetR binds to the operator *tetO* in the *tetA* promoter region in the absence of tetracycline to repress the transcription of the gene of interest. Using DNA microarray analysis, global gene expression of *C. glutamicum* HMW1 in the presence and in the absence of anhydrotetracyline (ATC) in the medium was compared. 17 genes showed significantly at least 2-fold changed mRNA levels (see the manuscript for details). Interestingly, the mRNA levels of a predicted operon consisting of *ndnR* (*cg1218)*, *nadA*, *nadC*, and *nadS (cg1214)* were decreased by 2- to 3-fold in the *C. glutamicum* HMW1 mutant in the absence of ATC. As purified RegX3 was found to bind the *ndnR* promoter region, with a higher affinity in the phosphorylated state (see the manuscript for details), the data suggest that RegX3 regulates this operon, which may play a vital function in cell growth.

In *C. glutamicum* the *ndnR* (*cg1218*) gene encodes a putative ADP-ribose pyrophosphatase (ADPRase) and a transcriptional repressor of the NAD^+ *de novo* biosynthesis genes *nadA* and *nadC*, which encoded quinolinate synthase (NadA) and quinolinate phosphoryl ribosyl transferase (NadC), and the *nadS* gene encoding a protein with cystein desulfurase activity for facilitating Fe-S cluster assembly of NadA (Teramoto *et al.*, 2010). Biosynthesis of pyridine and NAD^+ is linked to the biosynthesis of purine nucleotides through the pyridine nucleotide cycle (PNC) (Fig. 5.1). Detailed studies of the PNC in *C. glutamicum* have not been performed yet but possible metabolic pathways in PNC are shown in Figure 5.1 based on the genome annotation.

One hypothesis for the essentiality of the *regX3* gene in *C. glutamicum* is that RegX3 constitutively activates transcription of the essential *nadA* and *nadC* genes in the *ndnR-nadA-nadC-nadS* operon. In the absence of RegX3, the reduced expression of *nadA* and *nadC* might

cause nicotinate auxotrophy, similar to *nadA* and *nadC* deletion mutants (Teramoto *et al.*, 2010). Therefore, we hypothesized that RegX3 might be involved in the biosynthesis of NAD^+ by positively regulating the *ndnR-nadA-nadC-nadS* genes.

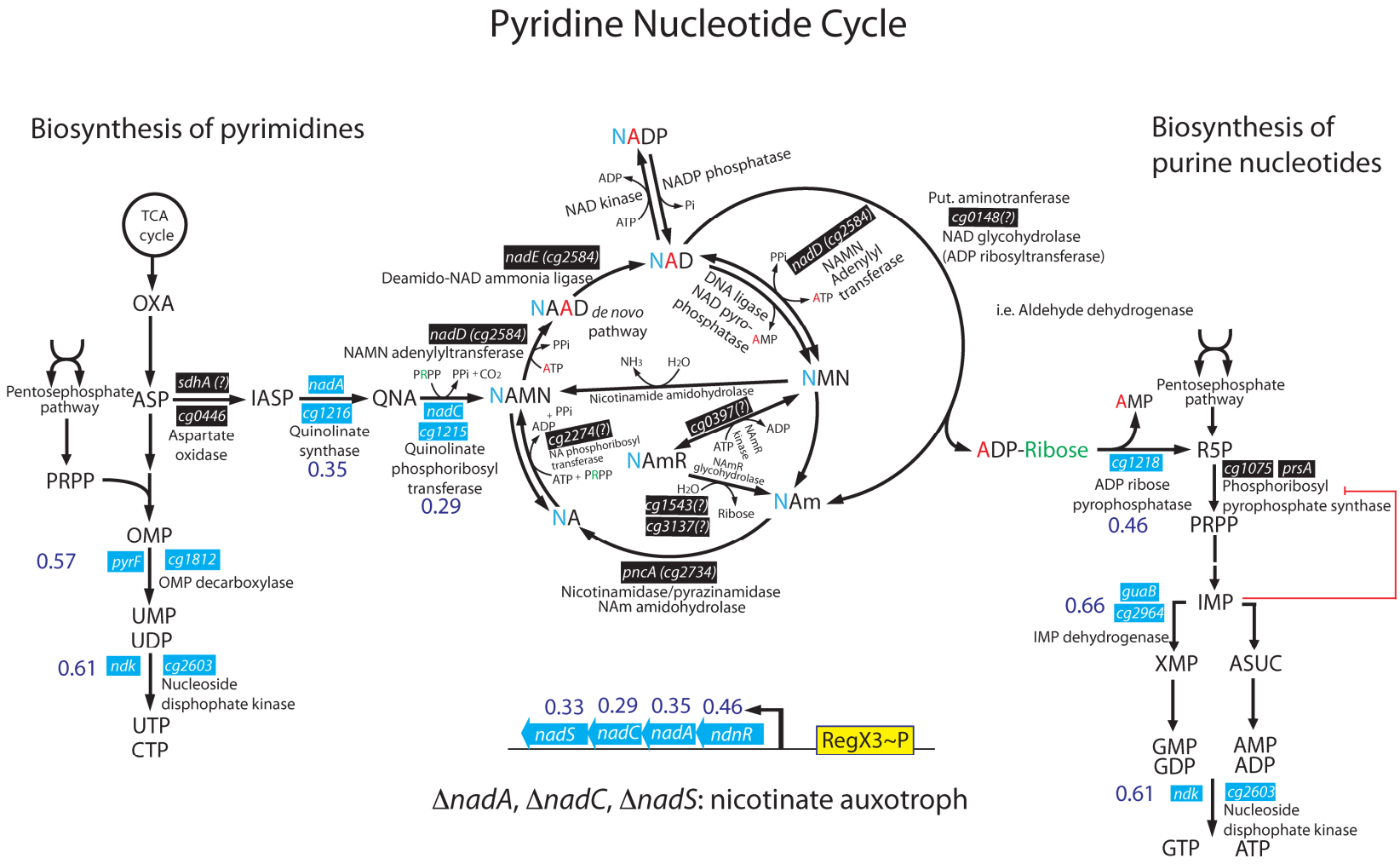

Figure 5.1 Possible pathways of pyridine nucleotide cycle mediating the biosynthesis of NAD^+ in *C. glutamicum*. Blue boxes represent significantly decreased gene expressions in *C. glutamicum* HMW1 in the absence of ATC, compared to the mutant in the presence of ATC. The number represents the ratio of mRNA (ATC^-/ATC^+ of *C. glutamicum* HMW1 mutant; see the detail in the manuscirpt). Names of metabolite were described in the reference (Penfound & J.W., 1996).

5.1.3 Regulatory model of the phosphate starvation response of *C. glutamicum*

In the P_i starvation response of *C. glutamicum*, the two-component systems PhoRS and SenX3-RegX3 stimulate the expression of P_i starvation inducible genes to supply P when P_i is limited (Fig. 5.2). The roles of PhoRS and SenX3-RegX3 are described in the text in section 5.1.1. An additional feature of the SenX3-RegX3 TCS is related to the essentiality of RegX3 described in the text in section 5.1.2. However, the reason for essentiality remains unclear.

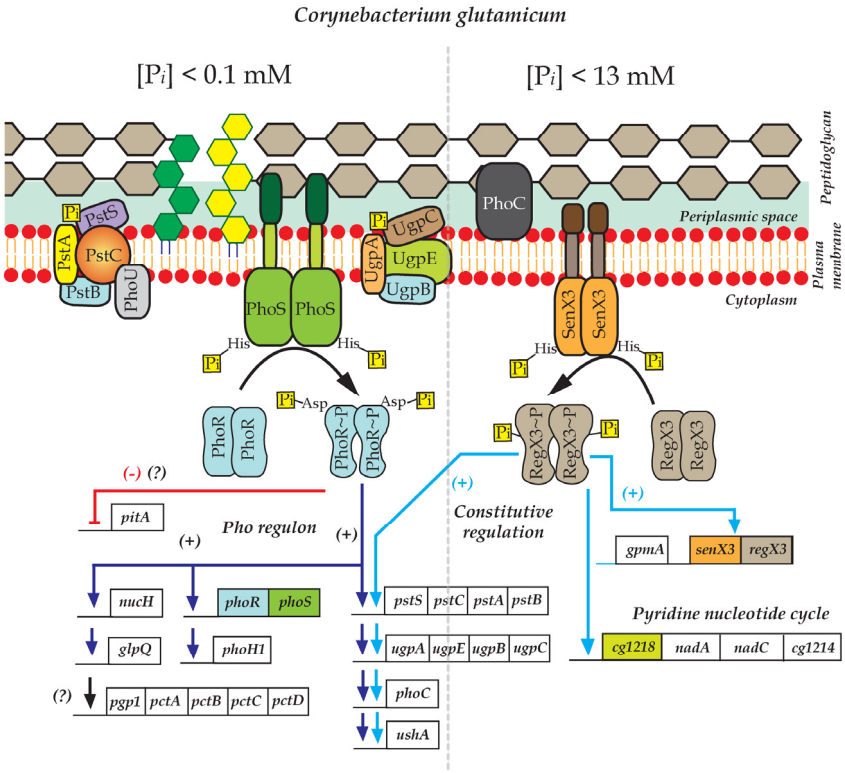

Figure 5.2 Postulated model of P_i starvation responses in *C. glutamicum* regulated by the two-component systems PhoRS and SenX3-RegX3. Blue arrows (+) and red lines with blunt ends (-) indicate transcriptional activation and repression, respectively. This model is based primarily on DNA microarray analyses and EMSA with wild type and mutant strains. RegX3~P presumably activates the target genes shown by the light blue arrows, even under P_i excess. When P_i is limited, PhoR~P is formed and also activates the same target genes as well as other *psi* genes, shown by dark blue arrows. In addition, PhoR~P may repress the *pitA* gene (see in the text 5.1.1). The RegX3 response regulator could regulate the essential genes responsible for the NAD^+ biosynthesis.

5.2 Cross-regulation of carbon metabolism and the P_i starvation response

Over 70% of 357 COGs involving proteins catalyzing reactions involving P-containing molecules were linked to cellular metabolism (Table 3.1). Numerous reactions require P_i, PP_i, or ATP as a cofactor (24%, 7% and 19% of the *in silico* reactions of *E. coli*, respectively) (Reed *et al.*, 2003). Therefore, phosphate-containing molecules are crucial for cellular metabolism,

including carbon metabolism and energy metabolism. This could be one of the main reasons for bacteria to develop regulatory systems and transporters to secure minimum levels of P. Bacteria utilize two-component regulatory systems to sense and respond to P_i limiting conditions, for example, PhoBR of *E. coli* and PhoRS of *C. glutamicum*. The respective Pho regulons include genes for P-transporters, alkaline phosphatases, and other secreted enzymes (see text in section 5.1.1). However, they do not include genes involved in polyP metabolism. This leads to the question of whether or not there any P_i-dependent or -independent regulation mechanisms of carbon and P metabolism in bacteria. Only a few studies have tried to answer this question.

E. coli induces the Pho regulon by PhoB via a P_i-dependent PhoR control and two P_i-independent controls via the sensor kinase CreC and the Pta-AckA pathway (Wanner & Wilmes-Riesenberg, 1992, Wanner, 1996). One possible role of acetylphosphate, which is a substrate and product of both, phosphotransacetylase and acetate kinase, is the phosphorylation of PhoB. This phosphorylation by acetylphosphate suggested a cross-regulation between the two-component regulatory systems and the cell metabolism of P_i and carbon. Another role of acetylphosphate is the regulatory coupling of P_i and ATP. In this regulatory coupling mode, the ratio of ATP-to-acetylphosphate in cells may be detected, leading to induction of the Pho regulon. In *S. lividans*, the *pst* gene regulated by PhoP encodes the high-affinity P_i binding protein PstS which accumulates under high fructose concentrations but not under high glucose concentrations (Díaz *et al.*, 2005). This fact suggests that additional P_i uptake via the Pst system is required because the activation of carbohydrate metabolism driven by an excess availability of carbon from certain sources causes a dramatic reduction of intracellular P_i. However, the complete regulatory links between carbon and phosphate metabolism have not yet been revealed in *E. coli* and *S. lividans*.

In *C. glutamicum*, several binding sites for PhoR and RegX3 are present in the *pstS* promoter region, but also binding sites for two other transcriptional regulators, RamB and GlxR, based on DNA-affinity chromatography and EMSAs (Sorger-Herrmann, 2006, Panhorst et al., 2010) (Fig. 5.3). GlxR is a global regulator, which regulates e.g. genes involved in the glyoxylate bypass, gluconate metabolism, glycolysis, and gluconeogensis. It was shown to bind to the *pstS* promoter region, acting as a transcriptional activator when acetate was used as the carbon source. In addition, the global transcriptional regulator RamB, which regulates e.g. acetate metabolism, was also postulated to activate transcription of the *pst* operon under P_i limitation and with glucose as the sole carbon source. On the other hand,

RamB was believed to repress it under P_i limitation and with acetate as the sole carbon source. Thus, RamB could regulate the *pstS* promoter, depending on the carbon source and the P_i availability. These interactions between the *pstS* promotor and GlxR and RamB were suggested as the regulatory link between carbon and phosphorus metabolism. However, further studies are required to really understand the cross-regulation of carbon and phosphate metabolism in *C. glutamicum*.

5.2.1 Glycogen metabolism of *C. glutamicum* under P_i limitation

In this PhD thesis, a metabolomics approach was used for the semi-quantitative analysis of intracellular metabolites in *C. glutamicum* in order to analyse the consequences of P_i limitation at the metabolite level. Such an approach has not been reported so far in bacteria. Metabolome analysis of *C. glutamicum* cells grown under conditions of either P_i excess or P_i limitation was performed using GC-MS and U-^{13}C-labelled glucose as sole carbon source. Lipid profiles of whole cells including fatty acids transmethylated from phospholipids and mycolic acids were not altered by P_i limitation. Interestingly, among several significant changes in metabolite pools, the level of intracellular maltose was highly increased (28-fold). This result triggered the analysis of intracellular glycogen, since maltose metabolism is connected to glycogen metabolism. From the intracellular glycogen analysis we concluded that glycogen accumulation and retainment in *C. glutamicum* were only observed under P_i limitation conditions but not under nitrogen or iron limitation conditions. Thus, P_i starvation apparently leads to a redirection of carbon metabolism towards glycogen biosynthesis and the limitation of degradation of glycogen.

A larger accumulation of glycogen in *C. glutamicum* under P_i limitation could be possible by the redirection of carbon fluxes from glycolysis or the pentose phosphate pathway into the glycogen biosynthesis pathway by elevated expression of the *pgi* and *glgC* genes (encoding phosphoglucoisomerase and ADP-glucose pyrophosphorylase, respectively). During the glycogen accumulation process, P_i could be gained by the conversion of glucose-1-phosphate and ATP to ADP-glucose and pyrophosphate (PP_i). On the other hand, limitation of the availability of intracellular P_i could alter the amount of degradation of the accumulated glycogen catalysed by GlgP (glycogen phosphorylase) to form glucose-1-phosphate. Or, the conversions of free glucose and maltodextrin as products of glycogen degradation could be limited to form glucose-1-phosphate by Glk (glucokinase) and MalP (maltodextrin phosphorylase) due to the limitation of ATP and P_i availability, respectively.

Glycogen accumulation in response to P_i starvation reveals another link between phosphate and carbon metabolism (Fig. 5.3).

In general, glycogen is known as a major carbon storage compound in bacteria. In addition, it is involved in environmental survival, colonization, and virulence (Chang *et al.*, 2004, Sambou *et al.*, 2008). In *C. glutamicum*, glycogen biosynthesis is connected with osmotically regulated trehalose biosynthesis (Tzvetkov *et al.*, 2003, Seibold & Eikmanns, 2007). In addition, the transcriptional regulator RamA was shown to activate expression of the *glgC* and *glgA* genes, whereas RamB was shown to repress expression of the *glgA* gene (Seibold *et al.*, 2010). The regulation of *glgC* and *glgA* genes by RamB or RamA under P_i limitation has not been investigated so far. Further studies are required to understand the roles of RamA and RamB in the cross-regulation of carbon and phosphate metabolism. A recent study showed that in *E. coli* the *glgC* gene encoding an allosteric ADP-glucose pyrophosphorylase (activated by fructose-1,6-bisphosphate and inhibited by AMP) is positively regulated by the Mg^{2+}-dependent PhoP-PhoQ regulatory system at low Mg^{2+} concentrations, since Mg^{2+}-bound ATP is necessary for GlgC activity (Montero *et al.*, 2009).

Interestingly, the *in silico* simulation using flux balance analysis showed that the predicted growth rate under sufficient P_i conditions was considerably similar to what we observed in the experiment with glucose or acetate. However, there was a clear discrepancy between the predicted growth rate under P_i limitation and the corresponding experimental values. These discrepancies were not corrected by computationally changing glucose or acetate uptake rates. This discrepancy may have occurred due to a lack of quantitative accuracy of the genome-scale model or of the information on P_i-dependent regulation. Nevertheless, at low P_i uptake rates and high glucose uptake rates an increased rate of glycogen biosynthesis was predicted. This behaviour was also shown experimentally when acetate instead of glucose was used as the sole carbon source.

The current *in silico* genome-scale model lacks P_i-dependent regulations and condition-dependent biomass formation, which could lead to inaccurate conclusions from simulation analyses of the P_i starvation response. Therefore, to gain a complete understanding of the P_i starvation response using the *in silico* model, genetic regulations and quantitative knowledge must be incorporated into this model, in addition to the results of the stoichiometric reaction network.

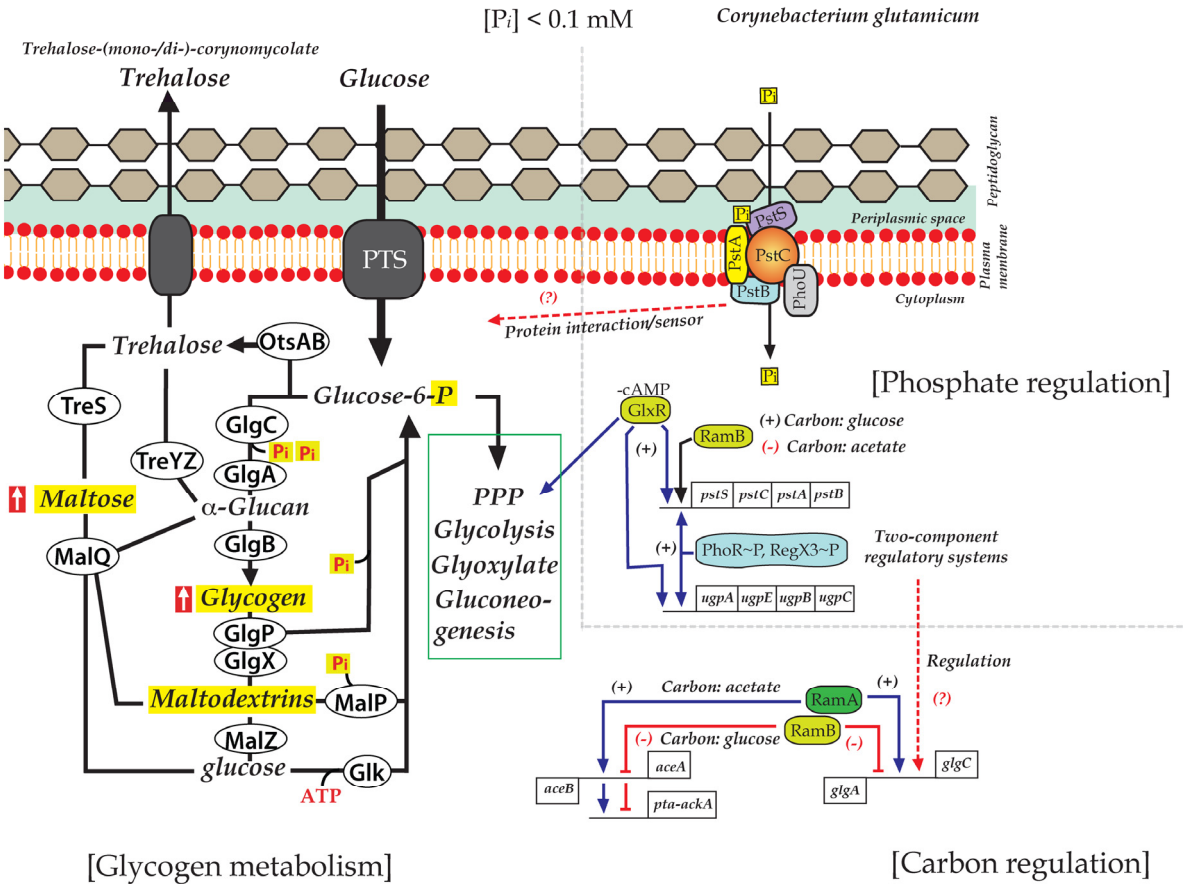

Figure 5.3 Glycogen metabolism of *C. glutamicum* and postulated model of the cross-regulation of carbon and phosphate metabolism (see in the text 5.2). Blue arrows (+) and red lines with blunt ends (-) indicate transcriptional activation and repression, respectively. The global regulators GlxR and RamB regulate the expression of the *pstSCAB* operon, which is also regulated by the two-component systems PhoRS and SenX3-RegX3. Increased maltose and glycogen levels were found in P_i-starved cells, representing a link between carbon and phosphorus metabolism. The transcriptional regulator RamB was shown to repress expression of *glgA* and *glgC*. Thus, lack of RamB repression could be responsible for the observed increased expression of *glgA* and *glgC* under P_i limitation.

5.2.2 Glucose uptake under P_i limitation in *C. glutamicum*

The lowered levels of phosphomonoesters in glucose-grown cells under P_i limitation measured by ^{31}P-NMR predicted a low glucose uptake rate under this condition. Indeed, the glucose uptake rate of P_i-starved *C. glutamicum* cells was found to be decreased by 4-fold. A low rate of PEP formation from the limited availability of P_i could be partially responsible for the low glucose uptake of P_i-starved cells, since the rate of PEP formation via glycolysis is tightly connected to glucose uptake via the PTS system. The redirection of glucose 6-phosphate into glycogen biosynthesis of course reduces PEP formation and thus glucose uptake.

In addition, the carbon source-dependent transcriptional regulators might be involved in phosphate control. The DeoR-type transcriptional regulator SugR represses genes of the

PTS system when the levels of fructose 6-phosphate or fructose 1-phosphate are low, which is the case under P_i limitation. The fact that a 2.5-fold higher glucose uptake rate was observed under P_i limitation for the Δ*sugR* mutant compared to the wild type supported the fact that SugR is at least partially responsible for the reduced glucose uptake. Thus, the transcriptional regulator SugR could be also involved in controlling the cross-regulation of carbon and phosphate metabolism.

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7 Appendix

7.1 Influence of the phosphate limitation on L-lysine producer, *C. glutamicum* DM1945.

C. glutamicum is a gram-positive soil bacterium with importance in industrial biotechnology. Currently more than two million tons of amino acids are produced with *C. glutamicum*, in particular L-glutamate used for flavour enhancer and L-lysine used for feed additive. The influence of phosphate limitation on L-lysine production of *C. glutamicum* DM1945 that is one of industrial lysine producer was tested (Fig. S7.1). As a result, there exist optimum conditions for high lysine production and high cell density near 0.65 mM phosphate concentration in the CGXII medium.

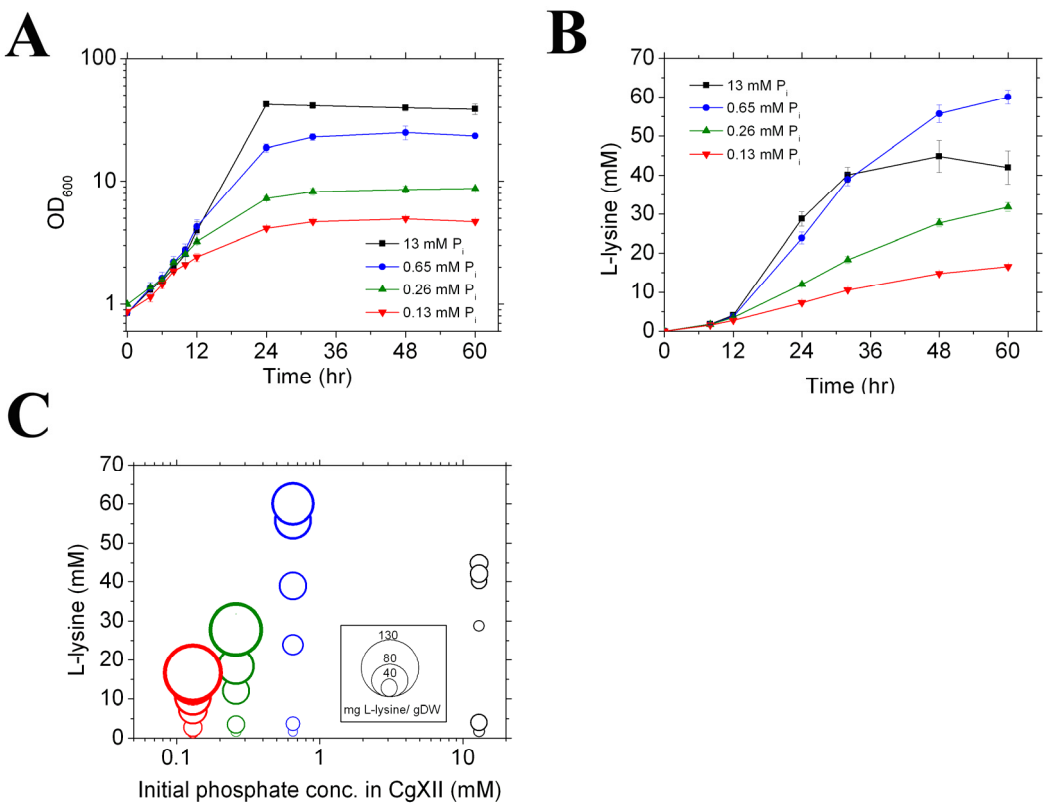

Fig. S7.1 The influence of phosphate limitation on L-lysine production of *C. glutamicum* DM1945. (A) Growth and (B) L-lysine production of *C. glutamicum* DM1945 in CGXII minimum medium with 222 mM of glucose and different concentrations of inorganic phosphate. Cells were precultured twice in CGXII glucose medium with 0.13 mM potassium phosphate and then transferred to CGXII medium containing 0.13 mM (red inverted triangle), 0.26 mM (green triangle), 0.65 mM (blue circle), or 13 mM (black rectangular) inorganic phosphate. (C) Bubble plot for the correlation of L-lysine production to different concentrations of inorganic phosphate in the CGXII medium (Size of bubble, mM L-lysine/g of cell dry weight).

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Declaration

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

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