cAMP dependent regulation of the TCA cycle by GlxR and oxidative stress response regulation by RosR in *Corynebacterium glutamicum*

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Content

Abbreviations

Amp^R	Ampicillin resistance
ATCC	American Type Culture Collection
BHI(S)	Brain Heart Infusion (+ Sorbitol)
DNase	Desoxyribonuclease
DTT	dithiothreitol
e.g.	for example
et al.	et alii
IPTG	Isopropyl-thiol-ß-D-galactopyranosid
KanR	Kanamycin resistance
LB	Luria Bertani
MOPS	3-(N-morpholino)-propanesulfonic acid
OD600	optical density at 600 nm
ORF	open reading frame
RBS	ribosome binding site
rpm	rounds per minute
qRT-PCR	quantitative real-time PCR
SpecR	Spectinomycin resistance
TBE	Tris base - Boric acid – EDTA
TCA	Trichloric acid
TE	Tris base - EDTA
TNI	Tris base - NaCl – Imidazol
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
WT	wild type
ε	molar extinction coefficient

Abbreviations not included in this list are accordingly to international standards, as for example listed in the author guidelines of the *FEBS Journal*.

1 Summary

DNA-binding transcription factors are key components in the regulation of gene expression in all organisms. They sense changes in the intra- or extracellular environment and trigger appropriate responses by activation or repression of their target genes. In this work, two transcriptional regulators and an adenylate cyclase of the Gram-positive soil bacterium *Corynebacterium glutamicum* have been studied, leading to the following results:

(i) The Crp-like transcriptional regulator GlxR was found to regulate genes of the tricarboxylic acid (TCA) cycle. Expression of the succinate dehydrogenase operon *sdhCAB* and of the P2 transcript of the citrate synthase gene *gltA* is weakly repressed by GlxR by binding to distinct DNA sequence motifs in the promoter regions. On the other hand, evidence was obtained that expression of the fumarase gene *fum* is weakly activated by GlxR. Thus, GlxR appears to be involved in fine-tuning the expression of TCA cycle genes.

(ii) DNA-binding of GlxR is strictly dependent on cAMP and consequently GlxR activity is controlled by the cellular cAMP concentration. Therefore, a mutant lacking the *cyaB* gene encoding the only annotated adenylate cyclase in *C. glutamicum* was characterized. Strain Δ*cyaB* showed growth defects in the presence of acetate or propionate. This phenotype could be reversed by plasmid-borne *cyaB* or by extracellular cAMP. The cAMP level in the *cyaB* mutant was decreased compared to wild type. DNA microarray experiments revealed an altered expression of many of the known or proposed GlxR target genes in the Δ*cyaB* mutant .

(iii) The MarR-type transcriptional regulator RosR (Cg1324) is probably involved in the response to oxidative stress. Direct target genes of RosR were identified by transcriptome comparisons of a Δ*rosR* mutant and the wild type and electrophoretic mobility shift assays (EMSA). Purified RosR was found be a homodimer that binds to a conserved 20-bp inverted repeat (5´-WTTGTTGAYR-YRTCAACWAA-3´). Except for the *narKGHJI* operon encoding a nitrate/nitrite antiporter and dissimilatory nitrate reductase, all other identified target genes were repressed by RosR. They encode four putative monooxygenases, two putative FMN reductases, a protein of the glutathione S-transferase family, a putative polyisoprenoid-binding protein (Cg1322) and RosR itself. *In vitro* DNA-binding of RosR was inhibited by H_2O_2. The oxidation status of cysteine residues was found to be important for redox-dependent DNA-binding, as exchange of Cys64, Cys92 and Cys151 to serine led to a H_2O_2-insensitive RosR variant. According to these data, oxidation or another type of cysteine modification inactivates RosR and causes deactivation or derepression of its target genes. Further support for involvement of RosR in the oxidative stress response was obtained by the significantly increased H_2O_2-sensitivity of a mutant lacking the RosR target gene cg1322.

2 Introduction

Corynebacterium glutamicum was isolated 1956 in a screen for bacteria excreting L-glutamate, which as monosodium glutamate is used as flavour enhancer (Kinoshita et al., 1957). *C. glutamicum* is a predominantly aerobic, non-motile, biotin-auxotrophic soil bacterium. The cells are rodshaped with a typical, somewhat irregular ("coryneform") morphology, and often arranged in V-formations, due to their "snapping" mode of cell division. Taxonomically this species belongs to the class of *Actinobacteria*, which includes Gram-positive eubacteria with high G+C content (Liebl, 2005). *C. glutamicum* serves as a model organism for the *Corynebacterineae*, a suborder of the *Actinomycetales* which amongst others includes the genus *Mycobacterium* including the pathogenic species *Mycobacterium tuberculosis* or *M. leprae* (Stackebrandt et al., 1997).

Soon after its discovery, *C. glutamicum* was used for the industrial production of the amino acids L-glutamate (Kimura, 2005) and L-lysine (Kelle et al., 2005), the latter serving mainly as a feed additive in swine and poultry nutrition. Today, more than 1.5 million tons of L-glutamate and more than 0.85 million tons of L-lysine are produced with *C. glutamicum*. Recent studies indicated that this species may also be attractive for the production of other metabolites such as succinate, lactate or ethanol (Inui et al., 1993a, b; Okino et al., 2009). The current knowledge on this non-pathogenic bacterium has been summarized in two recently published monographs (Burkovski, 2008; Eggeling and Bott, 2005).

The completely sequenced 3.3-Mb genome of *C. glutamicum* (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003) allowed a reliable prediction of 3002 protein-coding genes. *C. glutamicum* possess 127 one-component regulators and 13 two-component regulators, which usually carry a helix-turn-helix (HTH) motif for DNA binding to specific DNA recognition sites (Gough and Chotia, 2002). The largest family of transcriptional regulators is the TetR family with 16 members, followed by the ArsR, GntR, HTH_3 and LysR families (Brune et al., 2005).

For a better understanding of the regulation of gene expression, it is crucial to gain deeper insights into the functionality of these regulatory proteins.

2.1 The TCA cycle and its regulation in *C. glutamicum*

2.1.1 TCA cycle and related pathways

The tricarboxylic acid (TCA) cycle is an amphibolic pathway. Depending on the provided carbon sources, either the complete TCA cycle or at least parts of it are required for growth.

In its catabolic function, acetyl-CoA is completely oxidized to CO_2, leading to generation of ATP by substrate-level phosphorylation and to the formation of reducing equivalents (NADH and reduced menaquinone), which are oxidized by the respiratory chain and drive oxidative phosphorylation (Bott and Niebisch, 2003). In its anabolic function, the TCA cycle provides oxaloacetate and 2-oxoglutarate as the precursors of the aspartate and the glutamate family of amino acids (Cronan and LaPorte, 1996; Guest, 1992). NADPH required for reductive amination of 2-oxoglutarate to L-glutamate is generated in the isocitrate dehydrogenase (Icd) reaction.

C. glutamicum is able to grow on various different substrates, e.g. sugars, ethanol or different organic acids. The quality of these substrates controls whether all enzymes of the TCA are operating or related pathways like the methylcitrate cycle or the glyoxylate cycle are additionally activated. When bacteria grow on carbohydrates, they have to replenish intermediates of the TCA cycle which are used for anabolism. This is accomplished by the anaplerotic reactions of phosphoenolpyruvate (PEP) carboxylase and pyruvate carboxylase, which convert PEP or pyruvate to oxaloacetate. During growth on acetate, ethanol or fatty acids, the central metabolism is entered at the acetyl-CoA level and the glyoxylate bypass is used as anaplerotic reaction to synthesize oxaloacetate from two molecules of acetyl-CoA (Kornberg, 1966). In the glyoxylate bypass isocitrate lyase (AceA) converts isocitrate to glyoxylate and succinate (Fig. 2.1B). In the next step, glyoxylate and acetyl-CoA are condensed to malate by malate synthase (AceB). Malate is then converted by malate quinone oxidoreductase to oxaloacetate for anabolism and gluconeogenesis.

During growth on propionate the methylcitrate cycle is active and converts propionyl-CoA to pyruvate. Methylcitrate synthase converts propionyl-CoA and oxaloacetate to (Z)-2-methylcitrate. This compound is dehydrated by methylcitrate dehydratase to methylaconitate, which then is rehydrated by aconitase to methylisocitrate. This compound is then split by 2-methylisocitrate lyase to succinate and pyruvate (Fig. 2.1C). Succinate is converted in three steps to oxaloacetate, which re-enters the cycle. A comprehensive overview of the enzymes and genes involved in TCA cycle in *C. glutamicum* is described in a recent monograph (Eggeling and Bott, 2005).

2.1.2 Regulation of the TCA cycle

The TCA cycle provides the precursors for the amino acids L-glutamate and L-lysine and intermediates of this cycle such as succinate are also of interest for industrial production (Okino et al., 2009). Therefore, a detailed understanding of the regulation of the TCA cycle is

desirable. Recent studies have shown that this pathway is regulated in a very complex manner, both on the transcriptional and the posttranscriptional level. On the posttranscriptional level, the 2-oxoglutarate dehydrogenase complex (ODHC), which converts 2-oxoglutarate to succinyl-CoA, is regulated by the small inhibitor protein OdhI (Niebisch et al., 2006). Unphosphorylated OdhI is the active form and binds to the E1o subunit of ODHC causing an inhibition of its enzymatic activity. Phosphorylation of OdhI at threonine-14 by serine/threonine protein kinase G (PknG) leads to the inactive form of OdhI, which is no longer able to bind to ODHC. Besides PknG, also the other three serine/threonine protein kinases found in *C. glutamicum*, PknA, PknB and PknL, were found to play some role in OdhI phosphorylation (Schultz et al., 2009). The presence of OdhI was shown to be essential for efficient L-glutamate formation by *C. glutamicum* (Schultz et al., 2007).

On the transcriptional level, several players have been identified that participate in the regulation of TCA cycle gene expression (Fig. 1.1A). AcnR, a TetR-type regulator, is a repressor of the aconitase gene *acn* (Krug et al., 2005). Since the effector molecule of AcnR could be identified yet, the physiological role of *acn* repression by AcnR remains unclear. Besides AcnR, the AraC-type regulator RipA (Wennerhold et al., 2005) and the LuxR-type regulator RamA (Cramer et al., 2006) are involved in the regulation of *acn* expression. In the presence of acetate, the global regulator RamA activates *acn* expression (Emer et al., 2008). Higher aconitase activity is required due to the higher carbon flux in the TCA cycle and the glyoxylate cycle (Wendisch et al., 2000). In conjunction with its antagonist RamB, RamA controls the expression of the glyoxylate bypass genes *aceA* (isocitrate lyase) and *aceB* (malate synthase) (Gerstmeir et al., 2004). RipA is involved in the control of iron homeostasis. It is induced under iron limitation and reduces the expression of *acn* and further genes coding for iron-containing proteins, such as the succinate dehydrogenase structural genes *sdhCAB*, in order to reduce the cellular iron demand (Wennerhold et al., 2005).

The promomter region of *sdhCAB* contains next to the RipA binding motif a sequence (TGTGAGGAAGCTCACC), which might be recognized by the Crp-type regulator GlxR. This regulator will be introduced in the next chapter. Further motifs within the intergenic region between *sdhCAB* and *ramB* belong to global regulators RamA and RamB (Arndt and Eikmanns, 2007; Cramer et al., 2006; Gerstmeir et al., 2004). It could be shown, that *ramB* is negativly autoregulated and activated by RamA (Cramer and Eikmanns, 2007). A little is known about *sdhCAB* regulation so far, but there is indication that the *sdhCAB* operon as well as other TCA cycle genes are subject to a more complex regulation by several regulators to allow adaptation to changing environmental conditions.

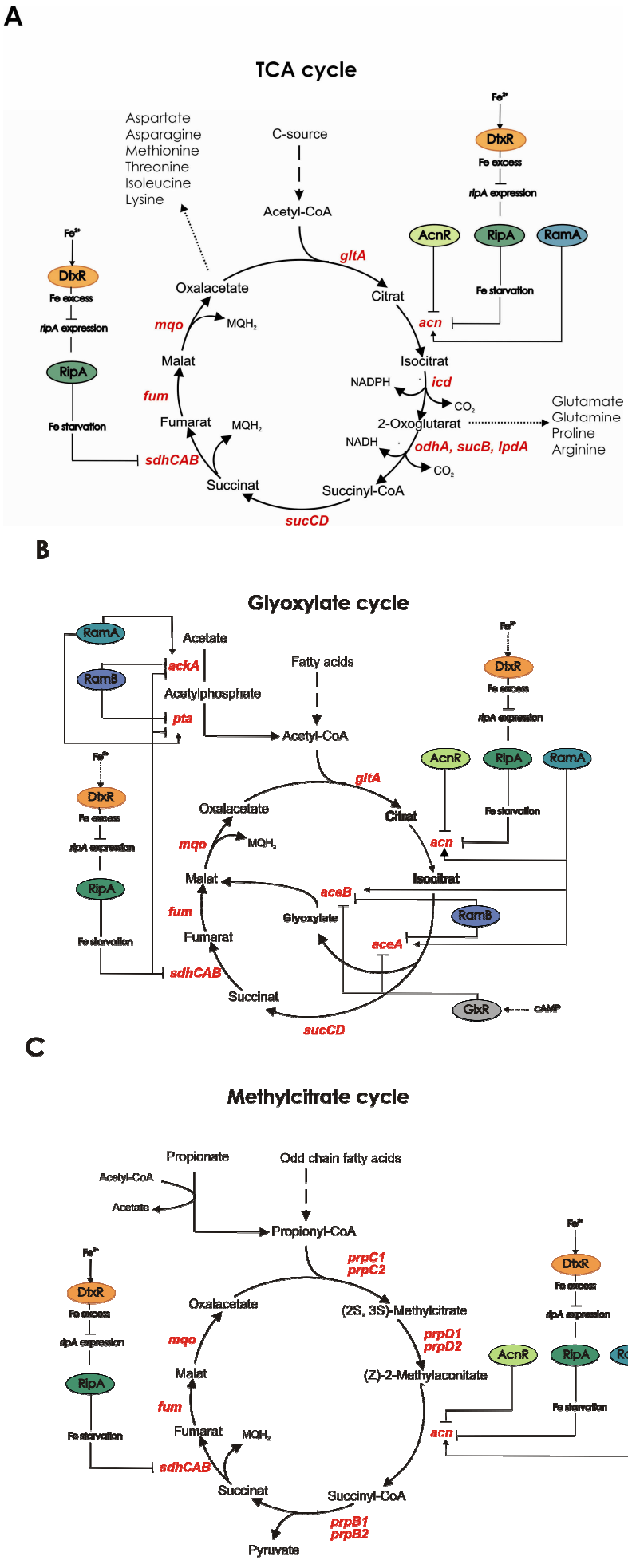
A
TCA cycle
Aspartate
Asparagine
Methionine
Threonine
Isoleucine
Lysine
C-source
Acetyl-CoA
gltA
Citrat
acn
Isocitrat
icd
NADPH
CO2
2-Oxoglutarat
Glutamate
Glutamine
Proline
Arginine
odhA, sucB, lpdA
NADH
CO2
Succinyl-CoA
sucCD
Succinat
sdhCAB
Fumarat
MQH2
fum
Malat
mqo
MQH2
Oxalacetate
Fe2+
DtxR
Fe excess
ripA expression
RipA
Fe starvation
AcnR
RipA
RamA
B
Glyoxylate cycle
RamA
RamB
Acetate
ackA
pta
Acetylphosphate
Fatty acids
Acetyl-CoA
gltA
Citrat
acn
Isocitrat
aceB
Glyoxylate
aceA
Oxalacetate
mqo
MQH2
Malat
fum
Fumarat
sdhCAB
Succinat
sucCD
GlxR
cAMP
C
Methylcitrate cycle
Propionate
Odd chain fatty acids
Acetyl-CoA
Acetate
Propionyl-CoA
prpC1
prpC2
(2S, 3S)-Methylcitrate
prpD1
prpD2
(Z)-2-Methylaconitate
acn
Oxalacetate
mqo
Malat
fum
Fumarat
MQH2
sdhCAB
Succinat
Succinyl-CoA
prpB1
prpB2
Pyruvate

Fig. 2.1. Transcriptional regulation of (A) the TCA cycle, (B) the glyoxylate cycle and (C) the methylcitrate cycle in *C. glutamicum.* The genes encode the following enzymes: *gltA*: citrate synthase, *acn*: aconitase, *icd*: isocitrate dehydrogenase, *odhA*, *sucB, lpdA*: 2-oxoglutarate dehydrogenase, *sucCD*: succinyl-CoA synthetase, *sdhCAB*: succinate dehydrogenase, *fum*: fumarase*, mqo*: malate:menaquinone oxidoreductase, *aceA*: isocitrate lyase, *aceB*: malate synthase, *ackA*: acetate kinase, *pta*: phosphotransacetylase, *prpC1*, *prpC2*: methylcitrate synthase, *prpD1*, *prpD2*: methylcitrate dehydratase, *prpB1*, *prpB2*: 2-methylcitrate lyase. The amino acids derived from oxaloacetate and 2-oxoglutarate and the currently known transcriptional regulators with experimental proven regulation of individual genes are indicated. Lines emanating from transcriptional regulators ending with arrows indicate transcriptional activation and the lines ending with a bar indicate transcriptional repression (Adapted from Bott (2007).

2.2 GlxR, a member of the cAMP receptor protein family

GlxR of *C. glutamicum* is a member of the cAMP receptor protein (CRP) family. Similar to the CRP protein of *Escherichia coli*, GlxR is active when it is complexed with cyclic adenosine monophosphate (cAMP) (Kim et al., 2004). CRP plays an important role in carbon catabolite repression, which is controlled by cAMP in enteric bacteria such as *E. coli* (Epstein et al., 1975). Both in CRP and in GlxR the cAMP-binding domain is located at the N-terminus and the DNA-binding domain with a helix-turn-helix motif at the C-terminus. Amino acid sequence alignments of GlxR and CRP from *E. coli* reveal 29% identity (Fig. 2.2). Interestingly, *glxR* gene from *C. glutamicum* can complement an *E. coli crp* deletion mutant (Kim et al., 2004), indicating that the DNA-binding motifs of the two protein must be quite similar. Within the *Corynebacterineae*, the sequence identities of the GlxR homologs, e. g. Rv3676 of *M. tuberculosis*, to the protein of *C. glutamicum* vary between 76 and 97% (Fig. 2.2).

In the first report on GlxR (Cg0350) the protein was described as a putative regulator of the *dtsR1* gene in *C. glutamicum* and was therefore named Drp (*dtsR1* regulating protein) (Hirano et al., 2001; Kimura, 2002). Gene regulation by GlxR was first demonstrated by Kim et al. (2004), who identified GlxR as a transcriptional regulator in a screen for genes regulating *aceB* (malate synthase) expression and showed direct cAMP-dependent binding of purified GlxR to *aceB* promoter region. Therefore they named the corresponding gene *glxR*, an acronym for glyoxylate bypass regulator. Acetate-grown cells of a *C. glutamicum* strain overexpressing *glxR* showed a decreased activity of the two glyoxylate bypass enzymes isocitrate lyase and malate synthase, indicating a repression of the corresponding genes *aceA* and *aceB* by GlxR. The presence of high cAMP levels in glucose-grown cells supported the hypothesis that GlxR represses *aceA* and *aceB* expression under conditions that do not require the glyoxylate bypass. Besides *aceB*, GlxR was described to repress the genes *gntP* (gluconate permease) and *gntK* (gluconate kinase) of gluconate catabolism (Letek et al., 2006).

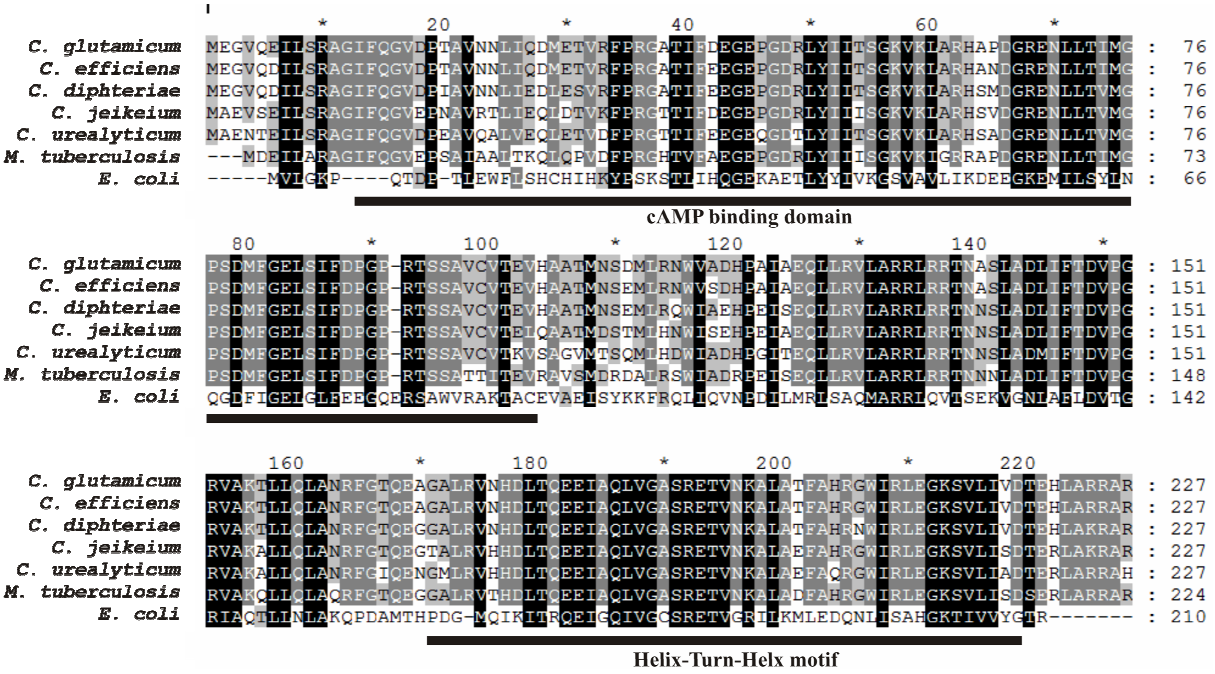

Fig. 2.2. Multiple sequence alignment of GlxR homologs of different *Corynebacterium* species and *M. tuberculosis* and CRP of *E. coli*. The cAMP-binding domain and the DNA binding helix turn helix motif are indicated. The alignment was established using the ClustalX software (Larkin et al., 2007).

The resuscitation promoting factor 2 (*rpf2*) is also negatively controlled by GlxR (Jungwirth et al., 2008). Rpf2 is involved in growth and culturability of *C. glutamicum*. Interestingly, expression of the *rpfA* gene of *M. tuberculosis* is controlled by the transcriptional regulator Rv3676, a GlxR homolog (Rickman et al., 2005).

Further GlxR target genes encode proteins of the glycolytic pathway, the TCA cycle, aromatic compound degradation, aerobic and anaerobic respiration, glutamate uptake, nitrogen assimilation and fatty acid biosynthesis. Consequently, GlxR functions as global regulator in *C. glutamicum* (Han et al., 2008a; Han et al., 2008b; Kohl et al., 2008). To ascertain the influence of GlxR on the expression of its target genes is a crucial task, because most attempts (including our own) to delete the *glxR* gene failed (Kim et al., 2004; Letek et al., 2006; Toyoda et al., 2009). Interestingly, the successfull deletion of *glxR* in strain ATCC13032 was reported in a review article stating that the mutant showed a strong growth defect and altered expression of more than 100 genes compared to wild type (Moon et al., 2007).

2.3 cAMP in *C. glutamicum* and other bacteria

3´-5´-cyclic adenosine monophosphate (cAMP) is a second messenger which is important in many biological processes. It is derived from adenosine triphosphate (ATP) and used for intracellular signal transduction in many different organisms. In humans for example, cAMP is associated with protein kinase function in several biochemical processes, including regulation of gene expression. In this case cAMP activates protein kinase A (PKA) by binding to specific locations on the regulatory subunits (C_2R_2). This causes dissociation of the regulatory and catalytic subunits, thereby activating the latter ones. Active PKA phosphorylates specific serine or threonine residues within its protein targets, leading e.g. to an alteration of the DNA-binding activity of the target protein.

In bacteria cAMP is known to be involved in carbon catabolite repression (CCR). It is common that bacteria can metabolize various compounds as carbon source. These carbon sources can either be co-metabolized or certain carbon sources are used preferentially to others. *C. glutamicum* usually metabolizes different carbon sources simultaneously, such as glucose and acetate or glucose and gluconate (Frunzke et al., 2008; Wendisch et al., 2000). However, many other bacteria show a clear preference for certain carbon sources, the most prominent example being the glucose-lactose diauxie found in *E. coli* (Monod, 1942). In this case, glucose is used first and prevents lactose utilization as long as glucose is present. This phenomenon is known as glucose repression or more generally as carbon catabolite repression (CCR) (Magasanik, 1961). In *E. coli* CCR is regulated by the transcription factor CRP (cAMP receptor protein), the second messenger cAMP, the adenylate cyclase CyaB and the EIIA component PtsG of the glucose-specific PTS system. CCR is controlled by the phosphorylation status of $EIIA^{Glc}$. In the presence of glucose, $EIIA^{Glc}$ exists predominantly in the unphosphorylated state because the phosphoryl group, which comes from the donor phosphoenolpyruvate (PEP), is immediately transferred to the sugar (Bettenbrock et al., 2007; Hogema et al., 1998). In this situation the unphosphorylated $EIIA^{Glc}$ inhibits transport mechanisms of alternative substrates. During growth on lactate for example, $EIIA^{Glc}$ remains in its phosphorylated state, thereby activating adenylate cyclase, which converts ATP to cAMP (Feucht and Saier, 1980). Through the elevated intracellular cAMP concentration the transcriptional regulator CRP becomes active and activates expression of many catabolic genes.

Similar to other pathogens, *M. tuberculosis* has developed mechanisms to subvert eukaryotic cAMP signalling. This could either be realized by protein toxins that are adenylate cyclases by themselves or by toxins that modify host adenylate cyclases. Depending on the

strain, the *M. tuberculosis* genome codes for 15 to 17 adenylate cyclase homologues, suggesting an important and complex role of cAMP in *M. tuberculosis* biology (Agarwal and Bishai, 2009).

In *C. glutamicum* little is known about the second messenger cAMP. As described before, the CRP homolog GlxR is dependent on cAMP and the GlxR regulon appears to include more than 100 genes, which is in the range of other bacterial CRPs (Blencke et al., 2003; Liu et al., 2005; Moreno et al., 2001; Yoshida et al., 2001). Unlike in *E.coli,* the cAMP concentration in *C. glutamicum* was reported to be higher in glucose-grown cells than in acetate-grown cells (Kim et al., 2004). Inspection of the genome sequence of *C. glutamicum* indicated that this non-pathogenic species contains only a single adenylate cyclase encoded by *cyaB* (cg0375).

Nucleotide cyclases can be subdivided into six classes depending on their amino acid sequence. Class I nucleotide cyclases are only found in γ-proteobacteria, like the adenylate cyclase of *E. coli* involved in CCR. Class II comprises toxins secreted by pathogens such as *M. tuberculosis* or *Bordetella pertussis* (Agarwal and Bishai, 2009; Dautin et al., 2002). Class III contains the most abundant type of nucleotide cyclases found in metazoa, protozoa, fungi, some archaea, and eubacteria, including CyaB of *C. glutamicum*. Class III nucleotide cylases can be further subdivided into four subclasses (IIIa - IIId). The subdivision is based on the primary sequence of the cyclase homolog domain (CHD), which is quite divergent in class III nucleotide cyclases (Linder and E., 2003) Rather little is known about class IV, V and VI cyclases. Beside the adenylate cyclases, class III contains all known guanylate cyclases. It is known that the adenylate cyclases of class III must form dimers to be active and that the catalytic centre is formed in the dimer interface (Tesmer et al., 1997; Zhang et al., 1997). The selection between adenosine or guanosine triphosphate is related to one lysine and one aspartate residue of each monomer (Tesmer et al., 1997; Tesmer et al., 1999).

The single adenylate cyclase CyaB of *C. glutamicum* consists of an N-terminal membrane-integral domain containing six putative transmembrane helices which is linked via a HAMP domain to a class IIId catalytic domain (CHD). The HAMP domain might function as transmitter domain, as it was found to have a large positive stimulatory effect on the adenylate cyclase activity of Rv3645 of *M. tuberculosis*, which has the same domain composition as CyaB of *C. glutamicum* (Linder et al., 2004).

2.4 The MarR family of transcriptional regulators and regulation of oxidative stress in *C. glutamicum*

2.4.1 The MarR family of transcriptional regulators

The MarR (multiple antibiotic drug resistance regulator) family includes prokaryotic transcriptional regulators which are involved e.g. in the control of virulence factor production, in the response to environmental stresses or in the regulation of the catabolic pathways for aromatic compounds. Proteins of the MarR family often exist as homodimers bound in the DNA-bound and in the free state. The DNA-binding domain is a winged helix-turn-helix (wHTH) motif which was first identified in the eucaryotic transcription factor HNF-3γ (Clark et al., 1993). Eponymous for this subfamily of helix-turn-helix-containing proteins are two "wings" formed by β-strands and loops in the secondary structure of the proteins. "Wing 1" consists of a β-hairpin motif comprising the elements S2-W1-S3 and "wing 2" the elements S3-W2 of the secondary structure elements of the complete wHTH motif : H1-S1-H2-H3-S2-W1-S3-W2 (H: α-helix; S: β-strands; w: loop) (Gajiwala and Burley, 2000). A structural view on the wHTH motif is given in Fig. 2.3, showing the MarR structure from *E. coli*. Only in few cases the structures of some members vary in absence of the second wing or in length of the turn in the wHTH motif (Brennan, 1993; Clark et al., 1993; Zheng et al., 1999). Crystal structures of proteins of the MarR family complexed with their target DNA reveals that helix H3 is essential for DNA recognition (Clark et al., 1993; Jin and Liao, 1999; Jin et al., 1999; Marsden et al., 1998; Zheng et al., 1999). Regulators of the MarR family in most cases repress gene expression by binding to the -10 and/or -35

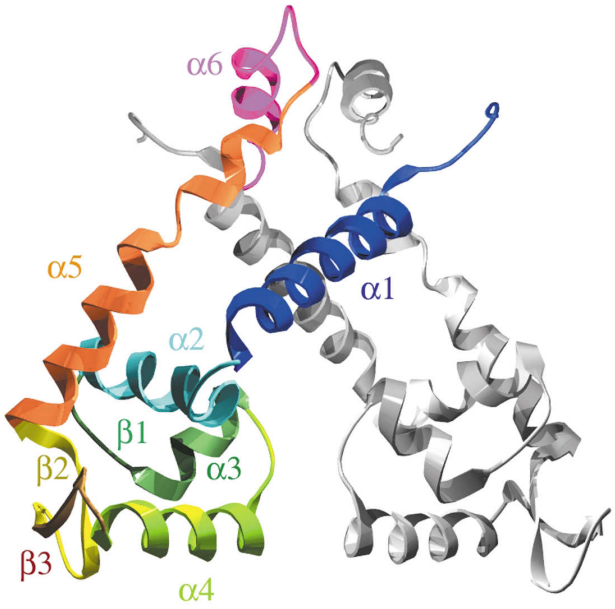

Fig. 2.3. Ribbon representation of the cocrystal structure of the MarR dimer from *E. coli*. The secondary structure elements of one subunit are colored. The wHTH motif (H1-S1-H2-H3-S2-W1-S3-W2) is indicated as follows: H1 (α2)-S1 (β1)-H2 (α3)-H3 (α4, recognition helix)-S2 (β2)-W1-S3 (β3). (Alekshun et al., 2001)

elements of the target promoter, thereby causing a steric inhibition of RNA polymerase binding. Sometimes they also might function as transcriptional activators or have both repressor and activator functions. Furthermore, more than half of the MarR family members are known to be autoregulated (Wilkinson and Grove, 2006).

2.4.2 Regulation of oxidative stress in *C. glutamicum*

Oxidative stress is caused by a formation of harmful superoxide or hydroxl radicals, but the biological defense mechanisms to detoxify the cell prevent the damage of a vast majority of crucial macromolecules such as DNA, lipids and proteins. Bacteria developed various mechanisms to protect themselves against oxidative stress. An example for an enzymatic protection mechanism is the degradation of reactive oxygen species (ROS) like the superoxide anion radical O_2^- to oxygen and hydrogen peroxide. This reaction is catalyzed by superoxide dismutase. In the next step catalase converts H_2O_2 to oxygen and water. Other small proteins like thioredoxin act as cofactors in electron transfer to protect the cell. Another strategy is production of small molecules like glutathione as reducing agent. In *C. glutamicum* mycothiol is present instead of glutathion as reducing agent (Feng et al., 2006).

The genetic response to oxidative stress in *E. coli* is controlled by two transcriptional regulators, SoxRS and OxyR (Pomposiello and Demple, 2001; Zheng and Storz, 2000). Sensing the intracellular redox conditions can be realized via oxidation of the cysteine residues (OxyR) or the oxidation of a $[2Fe2S]^{2+}$ cluster (SoxRS).

Little is known about the regulation of the oxidative stress response in *C. glutamicum* and only some players have been identified yet. In *C. glutamicum*, a homolog of OxyR is present (encoded by cg2109) but not yet characterized, whereas SoxRS homologs are absent. One example of regulating oxidative stress is the interplay between the WhiB-like transcriptional regulators WhcA, WhcE and the sigma factor SigH (Choi et al., 2008; Kim et al., 2005a; Kim et al., 2005b). In this regulation WhcA operates as a repressor while WhcE functions as an activator of gene expression. Overexpression of *whcA* and deletion of *whcE* revealed a higher sensitivity against stress inducing substances, such as diamide (Choi et al., 2008; Kim et al., 2005a; Kim et al., 2005b). WhcA target genes, such as *trxB* (thioredoxin reductase), cg1214 (cysteine sulfinate desulfatase) or cg3405 (quinone reductase) are also regulated in a *sigH* deletion strain, suggesting that WhcA is a transcription factor in the SigH-mediated stress response pathway which includes oxidative stress and heat stress (Kim et al., 2005a). In *M. tuberculosis*, both WhcA and WhcE carry a CXXC motif which seems to play an important role in redox sensing (Alam et al., 2007).

Another transcriptional regulator sensing oxidative stress in *C. glutamicum* was recently described. The protein QorR (encoded by cg1552), a member of the DUF24-family, is a negative regulator of *qor2*, a gene which is induced by the thiol-specific oxidant diamide (Ehira et al., 2009). DNA-binding of QorR is inhibited under oxidizing conditions *in vitro* and the only cysteine residue of QorR, Cys17, was shown to be essential for redox-sensitive regulation of QorR activity. Deletion of *qor2,* encoding a quinone oxidoreductase causes higher sensitivity against diamide. Another indirectly involved player is McbR, the master regulator of sulfur metabolism in *C. glutamicum*. Deletion of *mcbR* resulted in upregulation of several oxidative stress proteins, such as catalase (Krömer et al., 2008; Rey et al., 2005). The reason for oxidative stress in strain Δ*mcbR* is the significant upregulation of sulfite reductase, whose activity results in the formation of superoxide and peroxides (Krömer et al., 2008).

The oxidative stress response plays an important role during the transition from the exponential growth phase to the stationary phase and within the stationary phase. In these situations, the sigma factor SigB is upregulated while the housekeeping sigma factor SigA is downregulated, indicating a replacement of the sigma factor in the RNA polymerase holo enzyme. The genes which are upregulated by SigB in the transition phase include genes encoding anti-oxidative or protection proteins that prepare the cell to successfully confront environmental stresses (Larisch et al., 2007).

2.5 Aims of this work

In this work selected transcriptional regulators in *C. glutamic*um had to be characterized. In the first part, the role of the cAMP-dependent transcriptional regulator GlxR in TCA cycle regulation had to be investigated. A DNA-binding motif similar to the *E. coli* CRP consensus binding site had been identified in the promoter region of the succinate dehydrogenase operon *sdhCAB*, suggesting a regulation by GlxR (Bott, 2007). Besides GlxR, also an adenylate cyclase mutant of *C. glutamicum* should be studied. The second topic was to characterize Cg1324 (named RosR in this work), an uncharacterized member of the MarR family of transcriptional regulators which in the beginning was also assumed to be involved in genetic control of the TCA cycle in *C. glutamicum*. For characterization of RosR, the effect of a *rosR* deletion on global gene expression had to be analyzed with DNA microarrays followed by DNA-binding studies with purified RosR. In this way, the RosR regulon was defined. The work contributes to a better understanding of the transcriptional regulatory network in *C. glutamicum*.

3 Results

The major topic of this doctoral thesis was the characterization of the transcriptional regulators GlxR and RosR and of an adenylate cyclase mutant of *C. glutamicum*. The results are summarized in four articles, one of which has already been published, one of which has been submitted for publication, and two will be submitted in the near future.

The article ”Transcriptional control of the succinate dehydrogenase operon *sdhCAB* of *Corynebacterium glutamicum* by the cAMP-dependent regulator GlxR and the LuxR-type regulator RamA” by M. Bussmann, D, Emer, S. Hasenbein, S. Degraf, B. J. Eikmanns and M. Bott (J. Biotechnol 143: 173-182, 2009) describes the role of the two known global transcriptional regulators GlxR and RamA in the control of the succinate dehydrogenase genes. GlxR was enriched by DNA affinity chromatography with the *sdhCAB* promoter region by S. Hasenbein. Subsequent studies by M. Bussmann confirmed the binding of purified GlxR to the predicted binding motif and demonstrated that the *sdhCAB* operon is weakly repressed by GlxR.

The manuscript “Citrate synthase in *Corynebacterium glutamicum* is encoded by two *gltA* transcripts which are controlled by RamA, RamB, and GlxR” by J. van Ooyen, D. Emer, M. Bussmann, M. Bott, B. J. Eikmanns and L. Eggeling, which has been submitted to the Journal of Biotechnology, describes the transcriptional regulation of citrate synthase gene *gltA*. Citrate synthase (CS) is a major carbon flux control point in the cell and transcribed in two different transcripts, each of which is regulated separately by GlxR, RamA or RamB. Northern blot analyses, EMSAs and CS activity tests by M. Bussmann and J. van Ooyen revealed that the short transcript of *gltA* is under control of GlxR in glucose-grown cells. The complex control of *gltA* expression indicates that CS activity is carefully adapted to the physiological conditions.

An understanding of the physiological role of GlxR requires an understanding of the mechanisms and signals, by which the cAMP concentration in the cell is controlled. In the manuscript “Effects of a *cyaB* deletion on growth, organic acid production and global gene expression in *Corynebacterium glutamicum*” by M. Bussmann, A. Koch-Koerfges and M. Bott, the analysis of a mutant lacking the only annotated adenylate cyclase gene *cyaB* is described. The studies revealed interesting phenotypes of the Δ*cyaB* strain, in particular a strong growth defect in the presence of acetate and propionate and many differently expressed GlxR target genes. Almost all studies described were performed by M. Bussmann. The manuscript will be submitted to the Journal of Biotechnology.

The second transcriptional regulator analyzed in this work belongs to the MarR family and was named RosR for "regulator of oxidative stress response". By transcriptome comparisons of a Δ*rosR* mutant and the wild type and electrophoretic mobility shift assays (EMSAs) with purified RosR, 15 target genes were identified. Except of the *narKGHIJ* operon, all other genes were found to be repressed by the binding of RosR in close neighbourhood of the transcriptional start sites. Noticeable, four of the target genes coded for monooxygenases. DNA-binding by RosR was inhibited by H_2O_2, but the inhibition could be prevented by the exchange of the cysteine residues, which indicates that RosR might respond to oxidative stress. This idea was supported by the increased hydrogen peroxide sensitivity of a mutant lacking cg1322, an important target gene of RosR. The results are described in the manuscript "RosR (Cg1324), a hydrogen peroxide-sensitive MarR-type transcriptional regulator of *Corynebacterium glutamicum"* by M. Bussmann and M. Bott, which will be submitted to the Journal of Biological Chemistry.

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Transcriptional control of the succinate dehydrogenase operon *sdhCAB* of *Corynebacterium glutamicum* by the cAMP-dependent regulator GlxR and the LuxR-type regulator RamA

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ABSTRACT

In experiments performed to identify transcriptional regulators of the tricarboxylic acid cycle of *Corynebacterium glutamicum*, the cAMP-dependent regulator GlxR and the regulators of acetate metabolism RamA and RamB were enriched by DNA affinity chromatography with the promoter region of the *sdhCAB* operon encoding succinate dehydrogenase. The binding of purified GlxR, RamA and RamB was verified by electrophoretic mobility shift assays and the regulatory effects of these proteins on *sdhCAB* gene expression were tested by promoter activity assays and SDH activity measurements. Evidence was obtained that GlxR functions as a repressor and RamA as an activator of *sdhCAB* expression, whereas RamB had no obvious influence under the conditions tested.

1. Introduction

Corynebacterium glutamicum, a Gram-positive soil bacterium, is used for the industrial production of amino acids and a model organism of the suborder *Corynebacterinae* within the order *Actinomycetales*. An overview of the current knowledge on this organism can be found in two recent monographs (Burkovski, 2008; Eggeling and Bott, 2005). The major amino acids produced with *C. glutamicum* are L-glutamate (>1.5 million tons/year) and L-lysine (>0.8 million tons/year), both of which are derived from intermediates of the tricarboxylic acid (TCA) cycle (Eikmanns, 2005). Recent studies have uncovered that this amphibolic pathway is subject to a complex regulation both at the transcriptional and the posttranscriptional level (Bott, 2007).

At the posttranscriptional level, 2-oxoglutarate dehydrogenase complex (ODHC) activity was shown to be controlled by the OdhI protein (Niebisch et al., 2006). In its unphosphorylated form, OdhI binds to the E1o subunit OdhA and inhibits the ODHC enzyme activity. Binding and inhibition can be relieved by phosphorylation of OdhI at threonine-14 by serine/threonine protein kinase PknG. The presence of the OdhI protein is essential for efficient L-glutamate production (Schultz et al., 2007).

At the transcriptional level, several regulators have been identified. Expression of the aconitase gene *acn* was shown to be controlled by three different transcriptional regulators, the TetR-type regulator AcnR (Krug et al., 2005), the AraC-type regulator RipA (Wennerhold et al., 2005) and the LuxR-type regulator RamA (Cramer et al., 2006). Whereas the physiological meaning of *acn* repression by AcnR is not yet clear, the global regulator RamA is responsible for activation of *acn* expression when acetate is used as a carbon source and a higher flux through the TCA cycle is required (Emer et al., 2009). RipA is induced under iron limitation and functions as a repressor of *acn* and several other genes encoding prominent iron proteins. It thus serves to reduce the cellular iron demand and saves iron for essential iron proteins. The *sdhCAB* operon, encoding succinate dehydrogenase (SDH), the second iron-containing enzyme of the TCA cycle of *C. glutamicum*, also belongs to the RipA regulon (Wennerhold et al., 2005).

Interestingly, next to one of the RipA binding sites within the *sdhCAB* promoter region the motif TGTGAGGAAGCTCACC (inverted repeat underlined) was observed which is highly similar to the *Escherichia coli* CRP consensus motif and might be recognized by the CRP homolog GlxR of *C. glutamicum* (Bott, 2007; Han et al.,

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2008). The cAMP-binding transcriptional regulator GlxR was first identified in a screen for genes exerting regulatory effects on *aceB* expression (Kim et al., 2004). Meanwhile, there is clear evidence that GlxR influences transcription of numerous genes in *C. glutamicum* and functions as a global regulator (Han et al., 2007, 2008; Jungwirth et al., 2008; Kohl et al., 2008; Letek et al., 2006). In all cases studied so far, *in vitro* binding of GlxR to its target binding sites was strictly dependent on the presence of cAMP. In contrast to the situation in *E. coli* and other bacteria, the intracellular cAMP level in *C. glutamicum* was reported to be elevated during growth on glucose and low during growth on acetate (Kim et al., 2004).

A problem in elucidating the influence of GlxR on expression of its target genes is caused by the fact that most attempts (including our own) to delete the *glxR* gene failed (Kim et al., 2004; Letek et al., 2006). Therefore, in several studies GlxR was overproduced to determine its effects on the expression of target genes. The construction of a *glxR* deletion mutant was reported in a review article and it was stated that this mutant showed significant growth defects and altered expression of more than 100 genes (Moon et al., 2007). Primary data for this mutant have not been published, however. Very recently, the successful deletion of *glxR* in *C. glutamicum* strain R was reported, however, the mutant grew to slowly to be further analysed (Toyoda et al., 2009). Therefore, it remains unclear whether GlxR is involved in the control of *sdhCAB* expression.

RamA and RamB were shown to be antagonistic transcriptional regulators of the *pta-ack* operon, encoding acetate kinase and phosphotransacetylase as well as of the *aceA* and *aceB* genes, encoding isocitrate lyase and malate synthase (Cramer et al., 2006; Gerstmeir et al., 2004). The gene *mctC*, which encodes an importer of acetate, propionate and pyruvate in *C. glutamicum*, is also regulated by RamA (Jolkver et al., 2009) as well as genes of the ethanol metabolism (Arndt and Eikmanns, 2007; Auchter et al., 2008) and the resuscitation factor *rpf2* (Jungwirth et al., 2008). Typical binding sites of both transcriptional regulators were also found in the intergenic region between the *sdhCAB* operon and the *ramB* gene, which is located upstream of and in the opposite orientation to *sdhCAB* (Arndt and Eikmanns, 2008; Cramer et al., 2006; Gerstmeir et al., 2004). While it has been shown that *ramB* expression is subject to negative autoregulation by RamB and to positive regulation by RamA (Cramer et al., 2007), so far nothing is known about *sdhCAB* expression control by RamA and/or RamB.

In this study, we have identified GlxR, RamA and RamB as binding to the promoter region of the SDH operon *sdhCAB*. Furthermore, we analysed the relevance of the three transcriptional regulators for expression of *sdhCAB*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this work are listed in Table 1. For chloramphenicol acetyltransferase (CAT) and SDH assays, the *C. glutamicum* strains used in this work were precultured in 50 ml of brain heart infusion (BHI, Difco, Detroit, USA) or 2xTY medium (Sambrook et al., 2001) overnight on a rotary shaker at 120 rpm and 30 °C. Washed cells from these cultures were used to inoculate the main cultures with an optical density at 600 nm (OD_{600}) of 1 (glucose medium) or 1.5 (acetate medium). Precultures and main cultures were incubated in 500-ml baffled Erlenmeyer flasks containing 60 ml CGXII minimal medium (Keilhauer et al., 1993) supplemented with 3,4-dihydroxybenzoate (30 mg/l) as iron chelator and glucose, acetate or glucose plus acetate at concentrations indicated in Section 3. If appropriate, kanamycin (25 μg/ml or 50 μg/ml) or spectinomycin (250 μg/ml) was added to the indicated

Table 1
Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	F⁻ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1 gyrA96 relA1 phoA*, host for cloning purposes	Invitrogen
E. coli BL21(DE3)	F⁻ *ompT hsdS*$_B$ (r_B^-, m_B^-) *gal dcm* (DE3); host for overproduction of GlxR, RamA and RamB	Studier and Moffatt (1986)
C. glutamicum ATCC1302	ATCC 13032, wild-type strain	American Type Culture Collection
C. glutamicum RG1	*ramB*-negative mutant of *C. glutamicum* WT	Gerstmeir et al. (2004)
C. glutamicum RG2	*ramA*-negative mutant of *C. glutamicum* WT	Cramer et al. (2006)
Plasmids		
pEKEX3-glxR	Spec^R; *E. coli*/*C. glutamicum* shuttle plasmid carrying *glxR* under the control of a *tac* promoter	This work
pET2	Kan^R; promoter probe plasmid	Vasicova et al., 1998
pET2-sdh1	Kan^R; pET2 derivative with a 495-bp fragment covering the *C. glutamicum sdhCAB* promoter	This work
pET2-sdh3	Kan^R; pET2 derivative with a 100-bp fragment covering the *C. glutamicum sdhCAB* promoter	This work
pET2-sdh4	Kan^R; pET2 derivative with a 67-bp fragment covering the *C. glutamicum sdhCAB* promoter	This work
pET2-sdh8	Kan^R; pET2 derivative with a 349-bp fragment covering the *C. glutamicum sdhCAB* promoter	This work
pET2-sdh9	pET2-sdh8 with deleted GlxR binding site	This work
pET16b	Amp^R; pBR322 *ori*$V_{E.C.}$, P_{T7}, *lacI*; vector for overexpression of genes in *E. coli*, adding an N-terminal decahistidine affinity tag to the synthesized protein	Novagen
pET16b-glxR	Kan^R; pET16b derivative for overproduction of GlxR with an N-terminal decahistidine tag	U. Sorger (Forschungszentrum Jülich) and V.F. Wendisch (University of Münster)
pET28-RamA-6xHis	Kan^R; vector for overexpression of genes in *E. coli*, adding a N-terminal hexahistidyl affinity tag to the synthesized protein	Cramer et al. (2006)
pET29-RamB-6xHis	Kan^R; vector for overexpression of genes in *E. coli*, adding a C-terminal hexahistidyl affinity tag to the synthesized protein	Gerstmeir et al. (2004)
pLysS	Cam^R; pACYC184 derivative carrying the gene for the bifunctional T7 lysozyme	Novagen

Table 2
Oligonucleotides used in this study. Restriction sites are underlined.

Oligonucleotide	Sequence (5′ → 3′)	Relevant characteristics
sdhC1	GATGGCCATGGTCAGCTTCATTGCCCAG	IRD800-labelled
sdhC2	GACGCTCACGCAGCGCCTCCGTCG	IRD800-labelled
PEsdhC-for	ACCTACGGTGCAACTGTTGG	
PEsdhC-rev	CATTAGGCCAGTGATGGCCATG	
Biotin primer	BIO-TEG-AGGAGTCGTCGATGTGGAGACC	3′-Biotinylation
sdhC-fw	AACTATAGCGTGATCACCATCACC	
sdhC-rv	GAGGAGTCGTCGATGTGGAGACCGCCATGGTCAGCTTCATTGCCC	
sdhC-A-rev	GGCACCTCCAGTGTCGTTG	
sdhC-B-for	GAATGTTGAAAAGGCCTCAC	
sdhC-C-for	GATCGGTATCCTGTGAGGAAGC	
sdhC-prom-for	AATAGGATCCCACCTACGGTGCAACTGTTGGG	BamHI
sdhC-prom-rev	AATAGAGCTCGGCACCTCCAGTGTCGTTGTTAC	SacI
glxR-pEKEX-3-for	AATACCCGGGAAGGAATAGTATAGGTGGAAGGTGTACAGGAGATCC	XmaI
glxR-pEKEX-3-rev	AATAGAATTCTTATCGAGCGCGACGTGCCAAATGC	EcoRI
pET2-sdhCAB-mut-for	GTTCGGATCGGTATCCTTGGTTTTAGAATG	
pET2-sdhCAB-mut-rev	CATTCTAAAACCAAGGATACCGATCCGAAC	
sdh1 prom hin 1	ACGCGTCGACGCGCAACTATAGCGTGATCA	SalI
sdh prom hin 2	ACGCGTCGACTGTGTCACCTGCGCAAAGTTG	SalI
sdh3 hin	ACGCGTCGACTTATGTCTCTAAACAGCCAG	SalI
sdh hin 4	ACGCGTCGACGGAGCGCCCCGTGACTGGTT	SalI
sdh rück	CGCGGATCCGGCACCTCCAGTGTCGTTGT	BamHI

final concentrations. *E. coli* DH5α was used as host for all cloning purposes, *E. coli* BL21(DE3)/pLysS for overproduction of GlxR and *E. coli* BL21(DE3) for overproduction of RamA and RamB. The *E. coli* strains were cultivated in LB medium or 2xTY medium (Sambrook et al., 2001) at 37 °C or at 30 °C for GlxR overproduction. If appropriate, ampicillin was added (100 μg/ml).

2.2. General DNA techniques and sequence analyses

Standard methods such as PCR, restriction or ligation were carried out according to established protocols (Sambrook et al., 2001). *E. coli* was transformed as described (Inoue et al., 1990). DNA sequencing was performed with a 3100-Avant genetic analyser (Applied Biosystems, Darmstadt, Germany). Sequencing reactions were carried out with the ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Alternatively, sequencing was performed at Eurofins MWG Operon (Ebersberg, Germany). All oligonucleotides used in this study are listed in Table 2.

2.3. Primer extension analysis

To determine the transcriptional start site (TSS) of *sdhCAB*, primer extension analysis was performed as described previously (Engels et al., 2004) using the IRD800-labelled oligonucleotides sdhC1 and sdhC2 (Table 2). Total RNA was isolated as described (Lange et al., 2003) from *C. glutamicum* wild-type cells grown either in glucose minimal medium or in acetate minimal medium up to the exponential growth phase (OD_{600} = 5–6). The length of the primer extension products was determined by running the four lanes of a DNA sequencing reaction set-up using the same IRD8700-labelled oligonucleotide as for reverse transcription alongside the primer extension products. As template for the sequencing reaction a PCR product obtained with the oligonucleotides PEsdhC-for (starting 349 bp upstream of the *sdhC* start codon, Table 2) and PEsdhC-rev (starting 126 bp downstream of the sdhC start codon, Table 2) and chromosomal DNA of *C. glutamicum* wild-type were used.

2.4. Construction of strains and plasmids

For overexpression of *glxR* in *C. glutamicum*, the *glxR* gene was amplified from chromosomal DNA of *C. glutamicum* wild-type using the oligonucleotides glxR-pEKEx3-for and glxR-pEKEx3-rev (Table 2). The resulting PCR product was cut with XmaI and EcoRI and cloned into the vector pEKEx3 under the control of an inducible *tac* promoter. The resulting plasmid pEKEx3-glxR was transferred by electroporation into the desired *C. glutamicum* strains. Expression plasmid pET16b-glxR encodes a GlxR protein that contains 21 additional amino acids (MGHHHHHHHHHHSSGHIEGRH) at the amino terminus including a factor Xa cleavage site (SGHIEGR). Vector pET28-RamA-6xHis was used for the overproduction of a RamA protein with an N-terminal hexahistidine tag (Cramer et al., 2006), pET29-RamB-6xHis for overproduction of a RamB protein with a C-terminal hexahistidine tag (Gerstmeir et al., 2004). For construction of pET2-based plasmids used for the determination of *sdhCAB* promoter activities, DNA fragments covering the *sdhC* promoter region from −479 to +16 (fragment sdh1), from −84 to +16 (fragment sdh3), from −51 to +16 (fragment sdh4), and from −334 to +16 (fragment sdh8) with respect to the TSS were amplified via PCR. The fragments are shown in Fig. 2A and the primers used are listed in Table 2. The PCR products were digested with SalI/SacI and BamHI and cloned into the promoter probe vector pET2 (Vasicova et al., 1998), resulting in plasmids pET2-sdh8, pET2-sdh1, pET2-sdh3 and pET2-sdh4. Plasmid pET2-sdh9, carrying the *sdhC* promoter region from −334 to +16 with the GlxR binding site deleted was constructed using the QuickChange XL Site Directed Mutagenesis Kit (Stratagene) using pET2-sdh8 as template and the oligonucleotides pET2-sdhCAB-mut-for and pET2-sdhCAB-mut-rev (Table 2). All recombinant plasmids were sequenced in order to exclude unwanted mutations.

2.5. Chloramphenicol acetyltransferase (CAT) assay

For analysing the expression of the *sdhCAB* operon, the desired *C. glutamicum* strains were transformed with plasmids pET2-sdh1, pET2-sdh3 or pET2-sdh4, pET2-sdh8 and pET2-sdh9, which contain different *sdhCAB* promoter fragments in front of a promoter-less CAT gene. The promoter activity test was performed as described previously by measuring the formation of 5-thio-2-nitrobenzoate photometrically at 412 nm and 37 °C (Engels and Wendisch, 2007; Gerstmeir et al., 2003). 1 Unit is defined as the turnover of 2 μmol acetyl-CoA per min.

2.6. Overproduction and purification of GlxR, RamA and RamB

For overproduction of GlxR, *E. coli* BL21(DE3)/pLysS was transformed with expression plasmid pET16b-glxR and cultivated in

LB medium at 37 °C to an OD_{600} of 0.5. Then expression of the target gene was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the culture was incubated for another 3 h at room temperature. After the cells were harvested, purification of GlxR by Ni^{2+}-NTA affinity chromatography was performed essentially as described previously for RipA (Wennerhold et al., 2005). His-tagged RamA and RamB were purified as described before (Cramer et al., 2006) (Gerstmeir et al., 2004). For desalting, the RamA protein was dialysed overnight against 30% (w/v) glycerol in water with Visking dialysis tubes (Serva) with a pore size of 25 Å. For desalting RamB, PD10 columns (GE Healthcare) were used with bandshift buffer containing 10 mM Tris–HCl pH 7.6, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 10% (w/v) glycerol.

2.7. Electrophoretic mobility shift assay (EMSA)

The binding of GlxR to putative target promoters was tested as described previously for RipA (Wennerhold et al., 2005). Purified GlxR protein was incubated with DNA fragments sdh5, sdh6 and sdh7 (final concentration 8–20 nM) in a total volume of 20 µl. The binding buffer contained 20 mM Tris–HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 2.5% (v/v) glycerol, 0.5 mM EDTA and, when desired, 0.2 mM cAMP. The promoter region of a non-target gene of GlxR (*cg3154*) was used as negative control. The reaction mixtures were incubated at room temperature for 20 min and then loaded onto a 15% (w/v) native polyacrylamide gel. Electrophoresis was performed at room temperature and 170 V using 1 × TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) as electrophoresis buffer. The gels were subsequently stained with SybrGreen I according to the instructions of the supplier (Sigma–Aldrich, Taufkirchen, Germany).

To test for binding of RamA or RamB, the *sdhCAB* promoter fragments sdh1, sdh2, sdh3 and sdh4 (see Fig. 2A) were used for EMSA experiments. The respective DNA fragments were generated by PCR and purified by NucleoSpin Extract Kit II (Macherey Nagel). In the binding assays, 50–100 ng of the PCR products were incubated with various amounts of RamA or RamB (0, 0.25, 0.5, and 1 µg) under conditions described previously (Cramer et al., 2006). After incubation for 20 min at RT, the mixtures were separated on a 2% agarose gel in TAE buffer (40 mM Tris–HCl, 20 mM acetate, 1 mM EDTA, pH 7.5) at 70 V and stained with ethidium bromide. As a control, 1 µg BSA was incubated with the fragments.

2.8. SDH activity test

2.8.1. Preparation of membrane fractions for SDH assays

In order to determine the SDH activity, the membrane fraction of the cells was prepared by one of the following methods. (i) Cells from a 400-ml culture grown in CGXII minimal medium with 4% (w/v) glucose to the exponential growth phase (OD_{600} = 5) were harvested by centrifugation (10,000 × *g*, 10 min, 4 °C), washed once with ice-cold 50 mM potassium phosphate buffer pH 7.5 and resuspended in 7 ml of the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 mM diisopropylfluorophosphate. Lysozyme (10 mg/ml) was added and the cell suspension then incubated at 37 °C for 30 min. After disruption using a French pressure cell (207 MPa), intact cells and cell debris were removed by centrifugation (5000 × *g*, 20 min, 4 °C) and the supernatant was subjected to ultracentrifugation (150,000 × *g*, 60 min, 4 °C). The membrane pellet was resuspended in 50 mM potassium phosphate buffer pH 7.5 and directly used for the assay. (ii) *C. glutamicum* cells were grown in 50 ml CGXII medium with 1% (w/v) glucose, 1% (w/v) potassium acetate or with both substrates (0.5% each) to the exponential growth phase (OD_{600} = 5) or to early stationary phase (OD_{600} = 9–20, dependent on the strain), washed twice with 200 mM Tris–HCl pH 7.3 (buffer 1) and subsequently resuspended in 1 ml of this buffer. The cell suspension was mixed in 2 ml screw-cap vials with 250 mg glass beads (0.1 mm diameter, BioSpec Products, Inc., Bartlesville, USA), and the cells were disrupted with a RiboLyser (4 × 35 s at setting 6.5 with intermittent cooling on ice for 5 min; Hybaid, Heidelberg, Germany). Subsequently, glass beads and cellular debris were removed by centrifugation (2650 × *g*, 20 min, 4 °C) and the supernatant was subjected to two ultracentrifugation steps (75,000 × *g*, 30 min, 4 °C) with intermittent washing with buffer 2, containing 50 mM HEPES pH 7.3, 10 mM potassium acetate, 10 mM $CaCl_2$, 5 mM $MgCl_2$ as described (Molenaar et al., 1998). After the second ultracentrifugation step, the membrane pellet was resuspended in 0.5 ml buffer 2.

2.8.2. SDH assay

SDH activity was measured using a previously described method (Spencer and Guest, 1973). The reaction mixture contained 100 mM potassium phosphate buffer pH 7.5, 3 mM KCN, 0.23 mM 2,6-dichlorophenol indophenol (DCPIP), 2.18 mM *N*-methylphenazonium methosulfate (phenazonium metasulfate, PMS), and membrane fraction (0.2–2 µg membrane protein) in a final volume of 1 ml. The reaction was initiated by the addition of 10 mM succinate and the reduction of DCPIP was monitored at 600 nm. The rate of succinate oxidation was calculated using a molar extinction coefficient at 600 nm of 21 mM^{-1} cm^{-1}. One unit of activity is defined as 1 µmol succinate oxidized per min. Protein concentrations were determined with the BCA protein assay kit (Thermo Science, Rockford, USA) using bovine serum albumin (BSA) as standard.

2.9. DNA affinity chromatography and protein identification by MALDI-TOF mass spectrometry

Enrichment of DNA-binding proteins interacting with the upstream region of *sdhCAB* was performed as described previously (Engels et al., 2005). A 600-bp fragment covering the region from −475 to +125 (fragment sdh7; see Fig. 2A) with respect to the TSS of *sdhC* was first amplified using the oligonucleotides sdhC-fw and sdhC-rv (see Table 2). After purification, the fragment was reamplified by PCR with the primer sdhC-fw and biotin primer, resulting in biotin-labelling of the fragment. The fragment was then coupled to Streptavidin-coated magnetic beads (IBA, Göttingen, Germany). Proteins binding unspecifically were washed off with a buffer containing 0.1 M NaCl and specifically bound proteins were subsequently eluted with a buffer containing 2 M NaCl. The proteins present in the high-salt eluate were concentrated by trichloroacetic acid precipitation and the resolubilized proteins were separated on 15% SDS-polyacrylamide gels which were stained with silver. Dominant protein bands in the gel were subjected to peptide mass fingerprinting. After tryptic *in gel* digestion, the masses of the tryptic peptides were determined by MALDI-TOF mass spectrometry and used for the identification of the proteins as described previously (Schaffer et al., 2001).

3. Results and discussion

3.1. Determination of the sdhC transcriptional start site (TSS) by primer extension

To determine the TSS of the *sdhCAB* operon, primer extension experiments were performed using the primers sdhC1 and sdhC2 and total RNA isolated from glucose- and acetate-grown *C. glutamicum* wild-type cells. As shown in Fig. 1, a "C" residue located 16 bp upstream of the translational start site of *sdhC* was identified as TSS ("+1") with primer sdhC1. The intensity of the signal was significantly stronger when RNA isolated from acetate-grown cells was used compared to RNA from glucose-grown cells. This indicates that

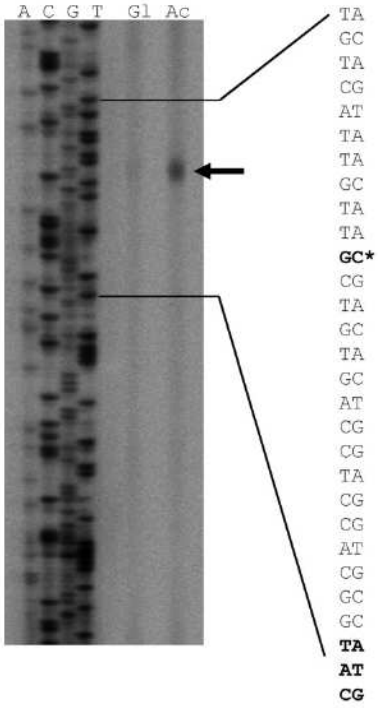

Fig. 1. Determination of the transcriptional start site of *sdhC* using primer sdhC1 and total RNA isolated from *C. glutamicum* wild-type cells grown on glucose (lane Gl) or acetate (lane Ac). The "C" residue representing the identified transcription start site and the ATG start codon of *sdhC* are indicated in bold.

sdhCAB expression is activated when acetate is used as a carbon source. The experiments performed with primer sdhC2 confirmed the results obtained with primer sdhC1 (data not shown). The *sdhC* TSS identified here differs by one nucleotide from the one reported previously using the RACE method (Brune et al., 2006).

3.2. Enrichment of GlxR, RamA and RamB by DNA affinity chromatography with the sdhC–ramB intergenic region

In order to identify transcriptional regulators of the SDH operon *sdhCAB* in *C. glutamicum*, DNA affinity chromatography experiments were performed as described in Section 2 with the 600-bp biotinylated promoter fragment sdh7 (Fig. 2A). Several proteins present in extracts of both acetate- and glucose-grown cells were enriched strongly by the sdh7 promoter fragment, indicating a specific interaction (data not shown). The respective protein bands from SDS-polyacrylamide gels were subjected to tryptic in-gel digestion and the resulting peptides analysed by MALDI-TOF mass spectrometry. The peptide mass fingerprints led to the identification of the proteins GlxR (Cg0350; 5–15 identified peptides, sequence coverage 40–63%), RamA (Cg2831; 16 identified peptides, sequence coverage 53%), RamB (Cg0444; 18–21 identified peptides, sequence coverage 45–48%) and DtxR (Cg2103; 8 identified peptides, sequence coverage 42%).

The band containing GlxR was enriched much stronger from glucose-grown cells than from acetate-grown cells. RamA was isolated from acetate-grown cells and DtxR from glucose-grown cells, whereas RamB was enriched from both extracts. Although the GlxR regulon of *C. glutamicum* has recently been studied in some detail (Han et al., 2008; Kohl et al., 2008), a regulatory effect of GlxR on *sdhCAB* or *ramB* expression has not been described so far. In contrast, DtxR, the global regulator of iron homeostasis in *C. glutamicum*, was previously shown to bind to the *sdhC–ramB* intergenic region (Brune et al., 2006; Wennerhold and Bott, 2006). DtxR has been proposed to function as an activator of *sdhCAB* expression under iron excess and in fact, the *sdhCAB* mRNA levels were found to be decreased in Δ*dtxR* mutants (Brune et al., 2006; Wennerhold and Bott, 2006). However, this reduction is also due to repression of *sdhCAB* by RipA, which is induced in Δ*dtxR* mutants (Wennerhold et al., 2005). Therefore, activation of *sdhCAB* by DtxR has not yet been demonstrated unequivocally. The other proteins, RamA and RamB, were previously also shown to bind to the *sdhC–ramB* intergenic region and to control the transcription of *ramB* (see Section 1 and (Cramer et al., 2007)). However, the function of RamA and RamB in expression control of the *sdhCAB* operon has not been investigated so far.

3.3. cAMP-dependent binding of purified GlxR to the sdhC–ramB intergenic region

In order to confirm that GlxR binds specifically to the *sdhCAB* promoter, the GlxR protein modified by an N-terminal decahistidine tag was overproduced using *E. coli* BL21(DE3) containing plasmids pLysS and pET16b-glxR and purified by Ni^{2+}-chelate affinity chromatography (Fig. 3A). The purified regulator was then used to perform EMSA experiments. As shown in Fig. 3B, GlxR bound to the *sdhC* promoter fragment sdh7 in a strictly cAMP-dependent manner, whereas a control fragment covering the promoter region of *cg3145* was not bound. In order to exclude effects of the histidine tag on binding affinity, the tag was cleaved off with factor Xa and the resulting GlxR protein was found to have the same affinity to the *sdhC* promoter as the histidine-tagged protein (data not shown). The *sdhC* promoter region contains a putative GlxR binding sequence (TGTGAGGAAGCTCACC) centered 198 bp upstream of the TSS of *sdhC* (Fig. 2A). EMSAs with the 232 bp fragment sdh5 containing this motif and with the 196 bp fragment sdh6 lacking this motif showed that it was essential for GlxR binding (Fig. 3C).

3.4. GlxR acts as repressor of the sdhCAB operon

To test whether GlxR has a regulatory effect on the expression of the *sdhCAB* operon, promoter activity and SDH activity tests were carried out. As our own attempts and that of others to construct a *glxR* deletion mutant failed (see Section 1), a GlxR-overproducing strain was used to test the effects of elevated GlxR levels. For this purpose, strain *C. glutamicum* (pEKEX3-glxR) carrying the *glxR* gene under the control of an IPTG-inducible *tac* promoter, was transformed with pET2-sdh8 carrying the DNA region from −334 to +16 with respect to the *sdhC* TSS in front of the promoter-less *cat* reporter gene. Expression of *cat* was measured as CAT activity. In an experiment schematically depicted in Fig. 3D, cells of an induced culture (i.e., *glxR* overexpressed by addition of IPTG) were compared to cells of a non-induced culture in CGXII minimal medium with 4% (w/v) glucose. Cells from the induced culture showed a 30% lower CAT activity (0.132 μmol min^{-1} (mg protein)$^{-1}$) than cells from the non-induced culture (0.168 μmol min^{-1} (mg protein)$^{-1}$). A similar result was obtained when strain ATCC 13032 carrying pET2-sdh8 and pEKEX3-glxR was compared with strain ATCC 13032 carrying pET2-sdh8 and pEKEx3. When cultured in the presence of 0.5 mM IPTG, the strain overexpressing *glxR* showed a CAT activity of 0.098 μmol min^{-1} (mg protein)$^{-1}$, whereas the control strain not overexpressing *glxR* had a CAT activity of 0.141 μmol min^{-1} (mg protein)$^{-1}$. When acetate instead of glucose was used as carbon source, *glxR* overexpression resulted in a 15% lower CAT activity compared to the reference strains (0.275 *vs.* 0.310 μmol min^{-1} (mg protein)$^{-1}$).

In order to further support the repressing effect of GlxR on *sdhCAB* expression, the GlxR binding motif on the pET2-sdh8

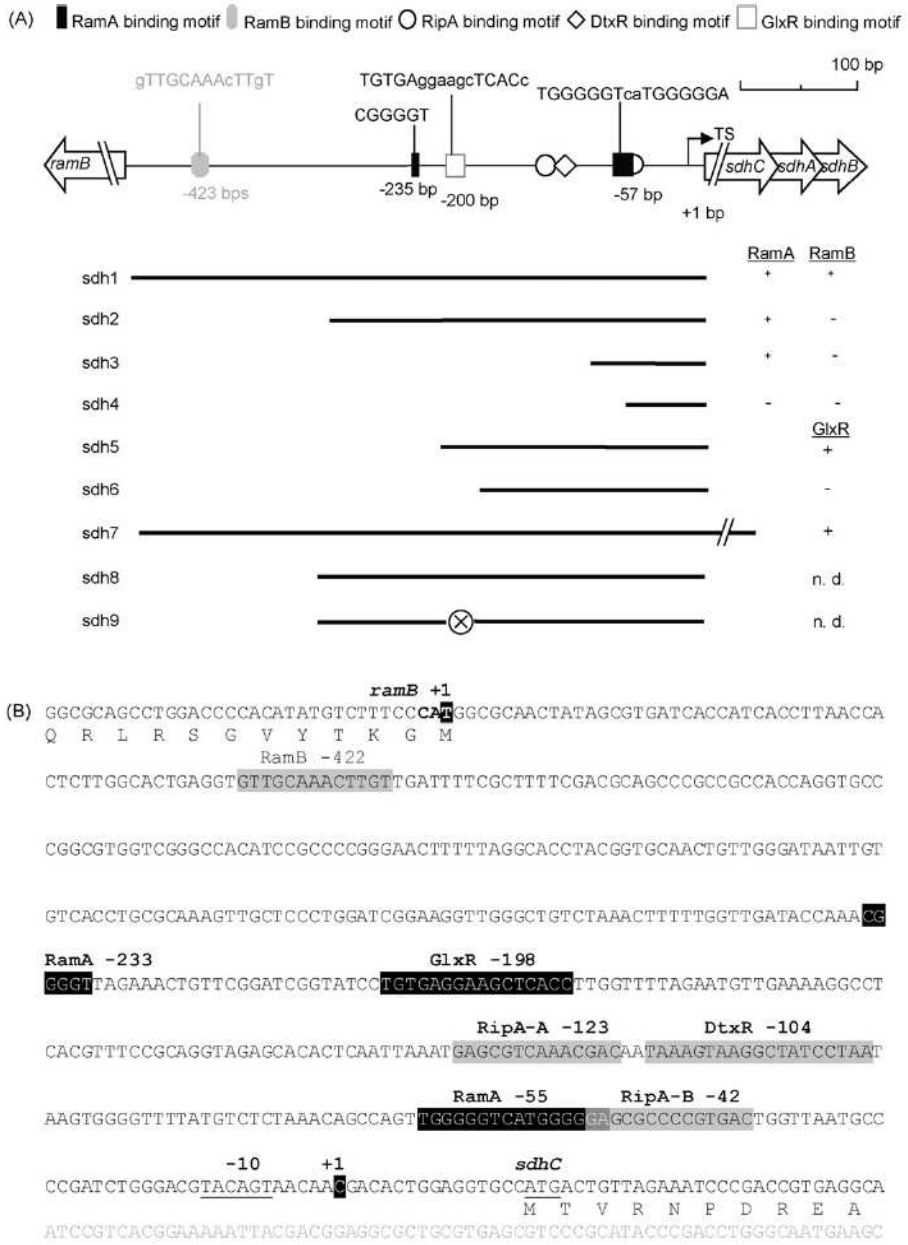

Fig. 2. Scheme (A) and nucleotide sequence (B) of the promoter region of the SDH operon *sdhCAB* with known or presumed binding sites for the transcriptional regulators RamA, RamB, GlxR, DtxR and RipA. Divergently transcribed is *ramB*. The TSS (TS) of the *sdhCAB* operon is located 16 bp upstream of the *sdhC* start codon. The numbers beneath the indicated binding sites of RamA, RamB and GlxR in (A) indicate the position of the center of the sites relative to the TS. Nucleotides identical to the RamA and RamB consensus binding sites are marked as capitals. The promoter fragments sdh1 (−479 to +16 with respect to TS), sdh2 (−307 to +16), sdh3 (−84 to +16), sdh4 −51 to +16), sdh5 (−216 to +16), sdh6 (−181 to +16), sdh7 (−475 to +125) sdh8 (−334 to +16) and sdh9 (−334 to +16) were used in the EMSAs with purified RamA, RamB and GlxR protein and/or for reporter gene assays. Binding of the respective fragments to RamA, RamB or GlxR is indicated on the right side, where "+"indicates binding and "–" indicates no binding.

reporter plasmid was deleted using site directed mutagenesis. The resulting plasmid pET2-sdh9 was transferred into *C. glutamicum* with pEKEX3-glxR and CAT activities were determined after growth in the presence and absence of 0.5 mM IPTG. This time, cells of the IPTG-induced culture showed the same CAT activity (0.23 μmol min^{-1} (mg protein)$^{-1}$) as the uninduced reference culture (0.25 μmol min^{-1} (mg protein)$^{-1}$). These results support the assumption that the reduced CAT activity observed with the native *sdhCAB* promoter is due to negative regulation by GlxR.

In a further series of experiments, SDH activities of *C. glutamicum* (pEKEx3) and *C. glutamicum* (pEKEx3-glxR) were compared. In the *glxR* overexpressing strain, SDH activity

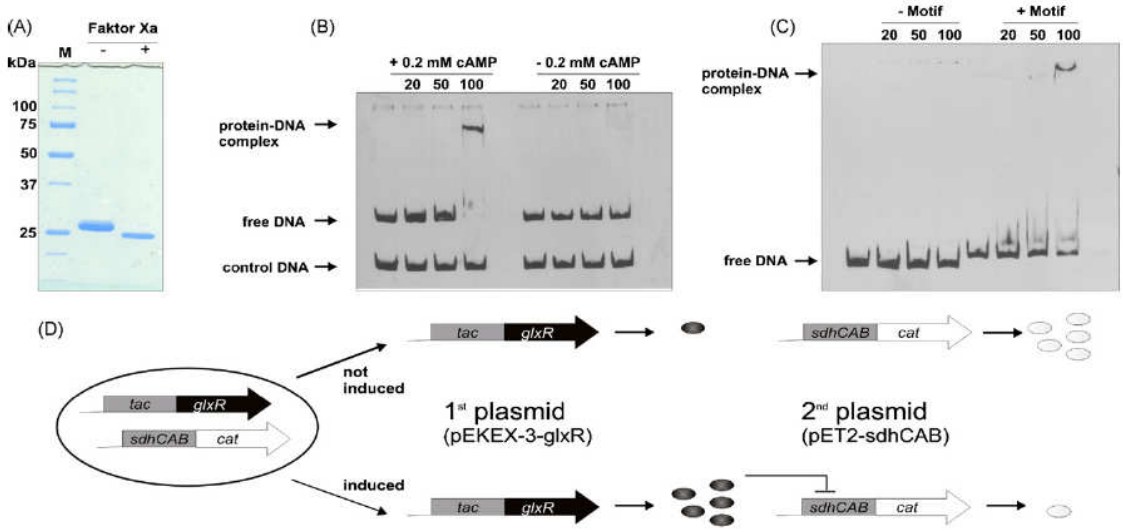

Fig. 3. cAMP-dependent binding of purified GlxR to the *sdhCAB* promoter. (A) Coomassie-stained SDS-polyacrylamide gel showing purified GlxR before (lane "–") and after cleavage (lane "+") of the N-terminal histidine tag by factor Xa. (B) EMSAs with GlxR and the *sdhCAB* promoter region (fragment sdh7 in Fig. 2A). DNA fragments (100 ng) covering the promoter region of the *sdhCAB* operon or of a gene used as negative control (cg3154) were incubated for 20 min at room temperature with a 0–100-fold molar excess of purified GlxR either in the absence of cAMP or in the presence of 0.2 mM cAMP. Samples were then separated by native PAGE (15%) and stained with SybrGreen. (C) Influence of the proposed GlxR binding sequence in the *sdhCAB* promoter region on binding of GlxR. For this experiment, a 232-bp DNA fragment (sdh5 in Fig. 2A) with the putative GlxR binding site and a 196-bp fragment (sdh6 in Fig. 2A) lacking this site were used for EMSAs with GlxR in the presence of 0.2 mM cAMP. (D) Scheme of the two plasmid system that was used to determine the influence of GlxR on *sdhCAB* promoter activity. Plasmid pEKEX3-glxR contains the *glxR* gene under the control of an inducible *tac* promoter, plasmid pET2-sdh8 carries the reporter gene *cat* under control of the *sdhCAB* promoter. Cells were cultivated in CGXII minimal medium containing 4% (w/v) glucose to an OD_{600} of 5 and then 0.5 mM IPTG was added to induce *glxR* overexpression and water to the reference culture.

(1.69 μmol min^{-1} (mg protein)$^{-1}$) was 30% lower than in the reference strain (2.44 μmol min^{-1} (mg protein)$^{-1}$), in agreement with the results of the promoter activity assays. Taken together, the results indicate that GlxR does not only function as a repressor of the glyoxylate genes *aceA* and *aceB* and the gluconate catabolism genes *gntK* and *gntP* (Kim et al., 2004; Letek et al., 2006), but also as a repressor of the TCA cycle genes *sdhCAB*. However, as the effect of GlxR overproduction on *sdhCAB* expression was not very strong, at least under the conditions used here, the primary function of GlxR might be in fine-tuning expression.

The GlxR binding site in the *sdhC* promoter is located in a relatively long distance (198 bp) to the mapped TSS (see Fig. 2A). In *E. coli* and other bacteria, the majority of the repressor binding sites have been found to be located in the region between −30 to +10 with respect to the TSS (Collado-Vides et al., 1991, 2009). However, Kohl et al. (2008) recently described several GlxR binding sites located in a distance of more than 200 bp upstream of the translational start site of the respective gene presumably controlled by GlxR. In fact, 6 out of 20 GlxR binding sites in front of genes with known TSSs were found to be located more than 40 bp upstream of the TSS (Kohl et al., 2008). Thus, a long distance between the GlxR binding site and the transcription start site obviously is not uncommon in *C. glutamicum*. Explanations for this feature with respect to the *sdhCAB* operon might be the existence of a second, not yet identified TSS of *sdhCAB*, or a scenario in which GlxR binding interferes with the function of a transcriptional activator of *sdhCAB* expression, such as RamA (see below).

3.5. Binding of purified RamA and RamB to the sdhC–ramB intergenic region

To investigate whether also RamA and RamB specifically interact with the *sdhCAB* promoter region, the binding of purified hexahistidyl-tagged RamA and RamB proteins were tested by EMSA experiments using the DNA fragments sdh1, sdh2, sdh3 and sdh4 shown in Fig. 2A. The results of the experiments with RamA are depicted in Fig. 4A, those with RamB in Fig. 4B. The EMSAs shown in Fig. 4A show retardation of fragment sdh1, sdh2 and sdh3, but not of fragment sdh4. In case of fragments sdh1 and sdh2, two distinct RamA/DNA complexes were observed, suggesting that two RamA binding sites exist in the region between the *sdhCAB* operon and the *ramB* gene. In contrast, with fragment sdh3 only one and with fragment sdh4 no RamA/DNA complex was observed. In accordance with these results, two motifs with similarity to the RamA consensus sequence (A/C/TG_{4-6}T/C or A/GC_{4-6}A/G/T; Cramer et al., 2006) were identified in the *sdhC* promoter region, the proximal one still being present on fragment sdh3, but not on sdh4 (Fig. 2A). The *sdhC*-proximal binding site represents a tandem, characterized by two single motifs interrupted by two spacer nucleotides, whereas the distal one represents a single CGGGGT stretch (Fig. 2). According to our results, both binding sites are functional with respect to interaction with RamA. Whereas binding of purified RamA to the *sdhC*-distal motif and its functionality in control of *ramB* expression has been shown before (Cramer et al., 2007), binding of RamA to the tandem motif was not demonstrated yet. The EMSAs with purified RamB showed a retardation only with fragment sdh1, but not with fragments sdh2, sdh3 and sdh4 (Fig. 4B). This confirms the functionality of the RamB binding site centered 422 bp upstream of the *sdhCAB* transcriptional start and 58 bp upstream of *ramB* translational start, which was previously shown to be involved in the autoregulation of *ramB* expression (Cramer et al., 2007).

3.6. RamA acts as an activator of the sdhCAB operon

The binding studies revealed an interaction of RamA and RamB with the *sdhCAB* promoter region (Fig. 4). To test for an effect of RamA and/or RamB on *sdhCAB* transcription, promoter fusion experiments were performed with the *ramB* deletion mutant

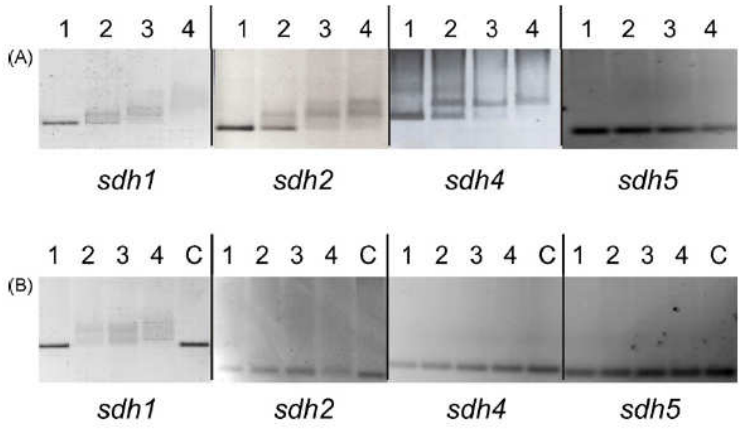

Fig. 4. Binding of purified RamA (A) and RamB (B) to the *ramB-sdhCAB* intergenic region. The fragments sdh1, sdh2, sdh3 and sdh4 used for the EMSAs (50–100 ng) are shown in Fig. 2. Lanes 1–4 contain 0, 0.25, 0.5, or 1 μg RamA or RamB protein, respectively; lane C shows a control with 1 μg bovine serum albumin instead of RamB.

C. glutamicum RG1, the *ramA* deletion mutant *C. glutamicum* RG2 and the parental wild-type strain of *C. glutamicum*. For this purpose, plasmids pET2-sdh1, pET2-sdh3 and pET2-sdh4 were transformed into the wild-type strain and into both mutants and after growth in minimal medium containing 1% (w/v) glucose, 1% (w/v) potassium acetate or glucose plus acetate (0.5% each), the specific CAT activities (i.e., the *sdhCAB* promoter activities) were determined.

As shown in Table 3, about two-fold higher CAT activities were detected when *C. glutamicum* (pET2-sdh1) was grown on acetate instead of glucose. In accordance, previous DNA microarray experiments revealed about two-fold higher mRNA levels of *sdhC*, *sdhA* and *sdhB* in acetate-grown cells compared to glucose-grown cells of *C. glutamicum* (Gerstmeir et al., 2004; Hayashi et al., 2002; Muffler et al., 2002). Four-fold lower specific CAT activities in the RamA-deficient strain *C. glutamicum* RG2 (pET2-sdh1) than in *C. glutamicum* wild-type (pET2-sdh1) indicates that RamA is involved in the activation of the *sdhCAB* operon both on glucose and on glucose plus acetate. In contrast, the RamB-deficient strain *C. glutamicum* RG1 (pET2-sdh1) showed about the same CAT activities as *C. glutamicum* wild-type (pET2-sdh1) on either substrate (Table 3). The binding of RamB therefore obviously has no strong effect on *sdhCAB* expression, at least under the conditions tested. An indirect effect of RamB as shown previously for expression control of the aconitase gene (Emer et al., 2009) thus can also be excluded.

Table 3
Specific CAT activities of *C. glutamicum* wild-type and its Δ*ramA* and Δ*ramB* mutants carrying different *sdhCAB* promoter fragments in the promoter probe vector pET2. Cells were grown in CGXII minimal medium with 1% (w/v) glucose or 1% (w/v) potassium acetate or both substrates (0.5% each) and harvested in the exponential growth phase (OD_{600} = 5).

C. glutamicum strain	Specific CAT activity (U/mg protein)[a]		
	Glucose	Glucose + acetate	Acetate
Wild-type (pET2_sdh1)	0.21 ± 0.10	0.34 ± 0.10	0.54 ± 0.08
RG2 (pET2_sdh1) [Δ*ramA*]	0.05 ± 0.02	0.09 ± 0.05	n.g.[b]
RG1 (pET2_sdh1) [Δ*ramB*]	0.25 ± 0.10	0.39 ± 0.11	0.61 ± 0.20
Wild-type (pET2_sdh3)	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01
RG2 (pET2_sdh3) [Δ*ramA*]	<0.01 ± 0.01	<0.01 ± 0.00	n.g.[b]
RG1 (pET2_sdh3) [Δ*ramB*]	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Wild-type (pET2_sdh4)	<0.01 ± 0.00	<0.01 ± 0.00	<0.01 ± 0.00
RG2 (pET2_sdh4) [Δ*ramA*]	<0.01 ± 0.00	<0.01 ± 0.00	n.g.[b]
RG1 (pET2_sdh4) [Δ*ramB*]	<0.01 ± 0.00	<0.01 ± 0.00	<0.01 ± 0.00

[a] The values are means ± standard deviation of 3–5 independent cultivations and two determinations per experiment.
[b] As previously reported, the RamA-deficient strain *C. glutamicum* RG2 shows no growth (n.g.) with acetate as sole carbon source (Cramer et al., 2006).

The promoter activities of *C. glutamicum* (pET2-sdh3) and also those of *C. glutamicum* RG1 (pET2-sdh3) were found to be 10–25-fold lower when compared to those of *C. glutamicum* (pET2-sdh1) or *C. glutamicum* RG1 (pET2-sdh1), respectively. These results might indicate the importance of the *sdhC*-distal RamA binding motif for *sdhCAB* activation. However, the lower promoter activities might also be due to the lack of the DtxR binding site on fragment sdh3 (see Fig. 2A). The RamA-negative *C. glutamicum* RG2 (pET2-sdh3) showed even two-fold lower CAT activities when compared to the respective wild-type and RamB-negative RG1 derivatives, indicating an activating effect of RamA on *sdhCAB* expression mediated by binding to the tandem motif. In contrast, no carbon source-dependent activation was observed with all *C. glutamicum* strains carrying plasmids pET2-sdh3 and no activity at all was detected with the three strains carrying pET2-sdh4 (Table 3).

The results of the promoter fusion experiments suggest (i) that both the proximal and the distal RamA binding sites have significance for *sdhCAB* expression, (ii) that RamA activates *sdhCAB* expression about four-fold and (iii) that RamB apparently does not have a function in *sdhCAB* expression control under the conditions tested. Since the *sdhC–ramB* intergenic region contains, aside from the RamA and RamB binding sites, also binding sites for GlxR, RipA and for DtxR (see Fig. 2A), it remains unclear whether the lower specific CAT activities (and thus, the lower *sdhCAB* promoter activities) observed with fragments sdh3 and sdh4 are only due to the lack of the RamA binding site(s). A missing activation of *sdhCAB* expression by DtxR could also contribute to this result.

To further test the influence of RamA and RamB on expression of the *sdhCAB* operon, SDH enzyme activities were determined in membrane fractions of cells grown in minimal medium with 1% (w/v) glucose or 1% (w/v) potassium acetate or a mixture of both (0.5% each). Cells harvested in the exponential and early stationary growth phase were analysed. The results given in Table 4 show about six-fold lower SDH activities in the *ramA* mutant *C. glutamicum* RG2 when compared to the wild-type strain in the exponential phase, roughly corresponding to the results obtained by the promoter fusion experiments. Also in accordance with the observed promoter activities, no strong differences in SDH activities were observed when the wild-type was compared to the RamB-negative strain RG1 (Table 4). In the stationary phase, SDH activities of all strains were higher than in the exponential phase (Table 4). Slightly higher SDH activities in wild-type cells grown on acetate and 4–5-fold lower SDH activities in the RamA-deficient RG2 strain than in

Table 4
Specific SDH activities in membrane preparations of *C. glutamicum* wild-type and in *ramA*- and *ramB*-negative strains *C. glutamicum* RG2 and RG1, respectively. Cells were grown in CGXII minimal medium containing 1% (w/v) glucose or 1% (w/v) acetate or both substrates (0.5%) and harvested in the exponential or stationary growth phase.

C. glutamicum strain	Growth phase	Specific SDH activity (U/mg protein)[a]		
		Glucose	Glucose + acetate	Acetate
Wild-type	Exponential phase	0.61 ± 0.24	0.66 ± 0.23	0.75 ± 0.30
RG2 [Δ*ramA*]		0.09 ± 0.03	0.10 ± 0.02	n.g.[b]
RG1 [Δ*ramB*]		0.44 ± 0.16	0.64 ± 0.13	0.92 ± 0.25
Wild-type	Stationary phase	0.82 ± 0.12	1.14 ± 0.04	1.33 ± 0.3
RG2 [Δ*ramA*]		0.23 ± 0.03	0.23 ± 0.10	n.g.[b]
RG1 [Δ*ramB*]		1.02 ± 0.06	1.59 ± 0.11	1.72 ± 0.07

[a] The values are means ± standard deviation of six independent cultivations. The specific SDH activities in this series of experiments were lower than those observed with *C. glutamicum* (pEKEx3) and *C. glutamicum* (pEKEx3-glxR) (see Section 3.4). This might be due to different methods used to prepare the membrane fraction (see Section 2.8).
[b] n.g., no growth.

the wild-type correspond to the results obtained with cells harvested in the exponential growth phase (Table 4).

Taken together, the results on SDH activities in *C. glutamicum* corroborate (i) that, independent of the presence of acetate and also independent of the growth phase, RamA functions as an activator of *sdhCAB* expression and (ii) that RamB exerts no influence on *sdhCAB* expression under the conditions tested. In contrast to RamA, RamB obviously has relevance only for expression control of the *ramB* gene. Furthermore, our results suggest, that *sdhCAB* expression in stationary phase cells of *C. glutamicum* is slightly higher than in exponentially-growing cells. This latter conclusion is in accordance with recent data on transcriptional regulation patterns of TCA cycle genes in glucose-grown *C. glutamicum* R cells (Han et al., 2008).

The results in Tables 3 and 4 show that RamA activates *sdhCAB* expression and thus probably is of high relevance for optimal SDH activity in *C. glutamicum*. We recently also found that RamA activates the expression of the aconitase gene *acn* when the cells grow on acetate (Emer et al., 2009). However, a weak activation of *acn* by RamA was also observed during growth on glucose. Toyoda et al. (2009) just recently found that RamA in *C. glutamicum* also activates the expression of *gapA*, encoding glyceraldehyde-3-phosphate dehydrogenase, when the cells grow on glucose. Thus, RamA positively controls the expression of genes for at least three key enzymes in the central pathways of glucose metabolism in *C. glutamicum*. In accordance, we observed restricted growth of the RamA-deficient mutant *C. glutamicum* RG2 on media containing either glucose or glucose plus acetate (Cramer et al., 2006). In the first culture, growth impairment concerned the final OD_{600}, however, with successive cultivation in CGXII minimal glucose medium growth became more and more restricted (in growth rate and final OD_{600}) and finally stopped after 10–14 generations (2–3 cultivations; data not shown). This phenotype of *C. glutamicum* RG2 corroborates the relevance of RamA.

DNA affinity chromatography experiments revealed the binding of GlxR, RamA and RamB to the *sdhCAB* promoter region. The subsequent studies indicated that only GlxR and RamA are involved in *sdhCAB* expression control. Jungwirth et al. (2008) found the promoter region of the resuscitation promoting factor 2 (Rpf2) to bind also GlxR, RamA and RamB and all three transcriptional regulators were shown to be involved in regulation of *rpf2* expression. Toyoda et al. (2009) also showed binding of RamA and of GlxR to the *gapA* promoter region, although it remained unclear whether GlxR in fact regulates *gapA* expression. However, all these observations suggest that the RamA and GlxR regulons at least partially overlap and that both regulators have common targets. However, the interplay of the two regulators and also a possible interplay with RamB remains to be elucidated.

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Citrate synthase in *Corynebacterium glutamicum* is encoded by two *gltA* transcripts which are controlled by RamA, RamB, and GlxR

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ABSTRACT

Citrate synthase (CS) is located at a major flux control point in metabolism and is required for both the tricarboxylic acid and the glyoxylic acid cycle activity. Here we show that the CS gene *gltA* of *Corynebacterium glutamicum* is monocistronic but that two transcripts are formed with their transcript initiation sites located 121 bp and 357 bp upstream of the translational start codon, respectively. Northern blot analyses revealed that during growth on acetate the short transcript prevails, whereas during growth on glucose the long transcript is dominant. Further Northern blots, reporter gene fusions, and CS activity measurements in mutants deleted of the transcriptional regulators RamA and RamB or with the global regulator GlxR overexpressed, revealed a complex scenario on the involvement of these regulators in *gltA* transcription. This was confirmed by the direct interaction of isolated RamA, RamB and GlxR proteins with specific *gltA* promoter regions. The combined analyses point to an elaborated control of *gltA* transcript formation which is possibly required as a dedicated mechanism to balance the total CS activity according to the physiological requirement .

1. Introduction

Corynebacterium glutamicum is a Gram-positive bacterium that was isolated from soil in a screen for bacteria that excrete L-glutamate (Kinoshita *et al.,* 1957). It has gained considerable interest as a model organism for the suborder *Corynebacterineae*, which also includes the family *Mycobacteriaceae*, due to the elementary cell wall architecture *C. glutamicum* possesses and its ease of handling. In addition, it has naturally a long standing interest because of its use for the large-scale production of L-glutamate and L-lysine and further amino acids, exceeding together an annual production of more than 2 million tons with an estimated steady increase of about 5% per year. Monographs treating this model bacterium have been published recently (Burkovski 2008; Eggeling and Bott 2005). A key reaction in central metabolism is catalysed by the citrate synthase (CS) enabling the cellular

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provision of reducing equivalents and building blocks via citric acid cycle activity or glyoxylic acid cycle activity (Arndt and Eikmanns, 2008). There are two major types of CS enzymes characterized by their regulatory and structural properties. In Gram-negative bacteria, the type II CS occurs which is strongly and specifically inhibited by NADH. This CS forms a hexameric molecule not found in other organisms (Weitzmann and Jones 1968). In contrast, the dimeric type I CS does not show allosteric properties. This latter type of enzyme is found in Gram-positive bacteria, archaea and eukaryotes. The CS of *C. glutamicum* appears to be a homotetramer (Radmacher *et al*., 2007) albeit in *C. glutamicum* ssp. *flavum* the enzyme has been reported to consist of two subunits (Shiio *et al.,* 1977). However, it is clear that the enzyme is neither regulated in its activity by NADH nor 2-oxoglutarate as is typical for the type II CS (Eikmanns *et al*., 1994, Shiio *et al*., 1977). Interestingly, it was observed that during growth on acetate the total CS activity is about 1.5-fold increased as compared to growth on glucose (Eikmanns *et al*., 1994, Eikmanns, 2005). This agrees with the determined higher flux via CS on acetate-grown cells (Wendisch *et al.,* 2000), and would suggest a flux control via the total CS amount. Opposed to *Bacillus subtilis* or *B. methanolicus* (Jin and Sonenshein, 1994a, 1994b; Brautaset *et al*., 2003), for instance, *C. glutamicum* has only one citrate synthase encoded by *gltA*, which is monocistronic (Eikmanns *et al.,* 1994). Its inactivation led to the absence of CS activity and citrate auxotrophy. Under special conditions, however, when the repressor PrpR is mutated, the methylcitrate synthase PrpC2 also present in *C. glutamicum* can take over CS activity (Radmacher *et al*., 2007). In contrast to other genes of the glyoxylate cycle and the tricarboxylic acid cycle such as the aconitase and succinate dehydrogenase genes *acn* and *sdhCAB*, respectively (Emer *et al*., 2009, Bussmann *et al*., 2009), there is only little information on transcriptional control of *gltA* expression in *C. glutamicum.* Comparative transcriptome analyses indicated an about two-fold up-regulation of *gltA* expression in acetate-grown cells as compared to glucose-grown cells (Gerstmeir *et al.,* 2003, Muffler *et al.,* 2002, Hayashi *et al.,* 2002). Recently, the regulator GlxR was found to bind upstream of *gltA* (Han *et al.,* 2008b). This global cAMP-dependent regulator was originally identified as a regulator involved in *aceB* expression control encoding malate synthase (Kim *et al.,* 2004), and has recently been shown to interact with promoter regions of 50 further genes, including regulatory genes which sense carbon source availability (Kohl *et al*., 2008). In addition, we observed upstream of *gltA* motifs resembling the binding motif for RamB (Gerstmeir *et al*., 2004), and a close inspection suggested that also RamA-binding sequences might be present (Cramer *et al.,* 2006). RamA and RamB were originally identified as regulators involved in expression control of genes of acetate metabolism, such as the *pta-ack* operon, encoding phosphotransacetylase and acetate kinase, respectively, and of *aceA* and *aceB*, encoding the two enzymes specific to the glyoxylate cycle (Gerstmeir *et al.,* 2004, Cramer *et al.,* 2006). However, it recently became clear that both RamA and RamB have also broader significance in controlling the central metabolism and were found to control the expression of an increasing number of genes, like *adh*, *ald*, *acn*, *sdhCAB, gapA*, *cspB*, *mctC* and *rpf2* (Arndt and Eikmanns 2007, Auchter *et al.,* 2009, Emer *et al.,* 2009, Hansmeier *et al.,* 2006, Jolkver *et al.,* 2009, Jungwirth *et al.,* 2008, Toyoda *et al.,* 2008). Based on the significance of CS activity for central metabolism and the indications of a subtle control of its synthesis at the transcriptional level, we here report on our discoveries on *gltA* transcription in *C. glutamicum*.

2. Materials and Methods

2.1 Plasmids, bacteria, and culture conditions

Plasmids, oligonucleotides and bacterial strains used in this study and their relevant characteristics and sources are given in Tab. 1. As a minimal medium for *C. glutamicum*, the MOPS-buffered CGXII was used (Bott and Eggeling, 2005) containing either 2% (w/v) potassium acetate or 4% (w/v) glucose and *C. glutamicum* was grown at 30 °C as 50 ml cultures in 500 ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Growth was followed by measuring the optical density at 600 nm (OD). If appropriate, kanamycin or spectomycin were added to a final concentration of 25 or 250 µg/ml, respectively. *E. coli* BL21(DE3)/pLysS was used for overproduction of GlxR and *E. coli* BL21(DE3) for overproduction of RamA and RamB.

2.2 Construction of plasmids

The promoter probe vector pET2 (Vasikova *et al.,* 1998) was used for construction of transcriptional fusions of *gltA* promoter fragments with the promoterless *cat* gene, encoding the chloramphenicol acetyl transferase (CAT). The fragments gltA1, gltA3 and gltAP2 were generated by PCR. For overexpression of *glxR* in *C. glutamicum*, the *glxR* gene was amplified from chromosomal DNA of *C. glutamicum*. The resulting PCR product was treated with *Xma*I and *Eco*RI and cloned into pEKEx3. The latter plasmid was derived from pEKEx2 which was cleaved with *Xho*I/*Stu*I, blunted and used to insert the spectinomycin resistance gene derived from pEC-S18mob2 as a 1176 bp *Bgl*II fragment. For construction of the expression plasmid pET16b-glxR containing an N-terminal decahistidine tag, the *glxR* coding region was amplified by PCR. The resulting PCR product was digested with *Xma*I and *Eco*RI and cloned into pET16b. The GlxR protein encoded by this plasmid contains 21 additional amino acids (MGHHHHHHHHHHSSGHIEGRH) at the amino terminus, including the factor Xa cleavage site SGHIEGR. All constructs made were verified by sequencing.

Table 1
Plasmids, oligonucleotides and bacterial strains used in this work.

Plasmid, oligonucleotide, or strain	**Characteristics / sequence** [a]	**Source, reference, or primer use**
Plasmids		
pET2	Promoter test vector, promoterless *cat* gene, Km^R	Vasicova *et al.,* 1998
pET2_gltA1	pET2 with 718 bps promoter fragment	This work
pET2_gltA3	pET2 with 263 bps promoter fragment	This work
pET16b	N-terminal 10x histidyl fusion vector, Amp^R	Novagen
pET16b-glxR	Kan^R; pET16b derivative for overproduction of GlxR with an N-terminal decahistidine tag	This work
pEKEx2	Kan^R; expression vector for use in *E. coli* and *C. glutamicum*	Eikmanns *et al*., 1994
pEKEx3	P*tac*, $Spec^R$, derived from pEKEx2	This work
pEKEx3-glxR	pEKEx3 with *glxR*	This work
pLysS	Cam^R ; pACYC184 derivate carrying the gene for the bifunctional T7 lysozyme	Novagen
pK19*mobsacB*	Vector enabling allelic exchange in *C. glutamicum*	Schäfer *et al.,* 1994
pK19*mobsacB*ΔP1	Vector enabling deletion of PgltA1 in WT	This work
pK19*mobsacB*ΔP12	Vector enabling deletion of PgltA12 in WT	This work
Oligonucleotides		

gltA1 prom hin 1	acgcgtcgacggaagtcggtcatgtcttcg	fragment *gltA1*
gltA prom hin 2	acgcgtcgacgtggcgaattgcatgggga	fragment *gltA2*
gltA prom hin 3	acgcgtcgaccagcacatcggcacaattgatc	fragment *gltA3*
gltA rück	cgcggatccgactacttccgtaatccggaa	fragments *gltA1, gltA2 and gltA3*
gltASonde+	atgtttgaaagggatatcgtggctac	Northern probe *gltA*
gltASonde-	gaacggttgccttatcaagctgtgc	Northern probe *gltA*
glxR-pEKEx3-for	aatacccgggaaggaatagtataggtggaaggtgtacaggagatcc	for pEKEx3-glxR
glxR-pEKEx3-rev	aatagaattcttatcgagcgcgacgtgccaaatgc	for pEKEx3-glxR
ΔPgltA-160_5'f	ctgacccaacaactataaccctgaagc	for pK19*mobsacB*ΔP1
ΔPgltA-160_5'r	agccaattcccccacaatcacgttgg	for pK19*mobsacB*ΔP1
ΔPgltA-160_3'f	tccgaacaaatatgtttgaaaggg	for pK19*mobsacB*ΔP1
ΔPgltA-160_3'r	tgtgcctcatcgagtgggttcagc	for pK19*mobsacB*ΔP1
ΔPgltA_B 5' in	cccactcgagaaacatgcatagcgttttcaatagttcggtgtcg	for pK19*mobsacB*ΔP12
ΔPgltA_B 5' out	ccccccgggggggcctagggaaaggatgatctcgta	for pK19*mobsacB*ΔP12
ΔgltA 3' in	tatgcatgtttctcgagtgggccgaacaaatatgtttgaaaggat	for pK19*mobsacB*ΔP12
ΔgltA 3' out	tgctctagagcatgaactgggacttgaagtcctc	for pK19*mobsacB*ΔP12t
gltA 3' p1	ccgtattgttcgcaatcggtcg	RLM-Race analysis
gltA 3' p2	gcagctcggtgcagcaggcaac	RLM-Race analysis
gltA 5' p2	gaactcgccaccggggtagtgc	RLM-Race analysis
gltA 5' p1	tctcagacagcatcttgcccagg	RLM-Race analysis
gltA UTR RT	cgactcggtggagccagtgctc	RLM-Race analysis
gltA-fw	ctagcaccctagattcttcacgc	fragment *gltA4*
gltA-1-rev	cagtagccacggtcacgcc	fragment *gltA4*
gltA-2-for	ggcgtgaccgtggctactg	fragment *gltA5*
gltA-rv	gaggagtcgtcgatgtggagacccaggacaacaccgttgttaccc	fragment *gltA5*
Strains		
E. coli DH5α	*F⁻ thi-1 endA1 hsdR17(r-m+) supE44 ΔlacU169 (Φ80lacZΔM15) recA1 gyrA96 relA1*	Invitrogen
E. coli BL21(DE3)	F⁻ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* DE3); host for overproduction of GlxR, RamA and RamB	Studier *et al.*1986
E. coli BL21(DE3)/pET28-RamAx6His	F⁻ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* DE3); contains plasmid pET28-RamAx6His (Kan^R); host for overproduction of RamA	Cramer *et al.,* 2006
E. coli BL21(DE3)/pET29-RamBx6His	F⁻ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* DE3); contains plasmid pET29-RamBx6His (Kan^R); host for overproduction of RamB	Gerstmeir *et al.,* 2004
C. glutamicum	ATCC13032, wild type (WT)	American Type Culture Collection
WTΔramB	*ramB* deletion mutant of WT, alias RG1	Gerstmeir *et al.,* 2004
WTΔramA	*ramA* deletion mutant of WT, alias RG2	Cramer *et al.,* 2006
WTΔP1gltA	WT deleted of 160 bp PgltA1-fragment	This work
WTΔP12gltA	WT deleted of 540 bp PgltA1-fragment	This work

[a] sequences are shown in their 5` to 3` direction, and restriction sites are underlined

2.3 Enzyme assays

To determine chloramphenicol acetyl transferase (CAT) activities, *C. glutamicum* was grown in minimal medium to the exponential growth phase to an OD of about 5, washed twice with 20 ml of 100 mM Tris-HCl buffer pH 7.8 and resuspended in 1 ml of the same buffer. The cell suspension was filled into 2 ml screw-cap vials together with 250 mg of glass beads, 0.1 mm diameter (BioSpec Products, Bartlesville, USA) and the cells were disrupted with a RiboLyser (Hybaid, Heidelberg, Germany). After disruption, glass beads and cellular debris were removed by centrifugation (20 min, 4°C, 5 000 rpm) and the supernatant was used for the assays. The specific CAT activities were determined as described by Gerstmeir *et al.* (2004), except that 0.4 mM acetyl-CoA (instead of 0.1 mM) was used. Citrate synthase activity was determined as described (Radmacher and Eggeling, 2007).

2.4 Preparation of His-tagged RamA, RamB and GlxR fusion proteins

Plasmids pET28-RamAx6His and pET29-RamBx6His were used for the isolation of hexahistidyl (His)-tagged RamA and His-tagged RamB fusion proteins (Gerstmeir *et al.,* 2004; Cramer *et al.,* 2006). The proteins were isolated from recombinant *E. coli* BL21(DE3) cells using nitrilotriacetic acid affinity chromatography. RamA was desalted by dialysis overnight against 30% (w/v) glycerol in water using Visking dialysis tubes (Serva, Heidelberg, Germany) with a pore size of 25 Å. RamB protein was transferred into bandshift buffer containing 10 mM Tris-HCl pH 7.6, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 10% (w/v) glycerol by use of PD10 colums (GE-Healthcare). The RamB solution was finally made to contain 30% glycerol and both proteins were stored at -20°C until use. For overproduction of GlxR, recombinant *E. coli* BL21(DE3)/pLysS was used and cultivated in LB medium at 37°C to an OD of 0.5. Expression of the target gene was induced by addition of 1 mM IPTG followed by a further 3 h incubation at room temperature (RT). Cells were harvested and GlxR isolated essentially as described previously for RipA (Wennerhold *et al.,* 2005).

2.5 Promoter binding assays with RamA, RamB and GlxR

The binding of RamA or RamB protein to promoter fragments was tested in electrophoretic mobility shift assays. Fragments were generated by PCR using chromosomal DNA from *C. glutamicum* WT as a template using the primers listed in Tab. 1, and purified using the NucleoSpin Extract Kit II (Macherey and Nagel, Düren, Germany). In the binding assays, 50 to 100 ng of the PCR products were incubated with increasing amounts of RamA or RamB (0, 0.25, 0.5 and 1 µg) under the conditions described in Cramer *et al.* (2006). After incubation for 20 min at RT, the mixtures were separated on a 2% agarose gel in TAE-buffer (40 mM Tris-HCl, 20 mM acetate, 1 mM EDTA, pH 7.5) at 70 V and stained with ethidium bromide. As a control, 1 µg BSA was incubated with each fragment. The binding of GlxR to promoter fragments was tested as described previously for RipA (Wennerhold *et al.,* 2005). Purified GlxR protein was incubated with 100 ng DNA fragments (final concentration 8 nM) in a total volume of 20 µl. Purified GlxR protein was incubated with DNA fragments in a final concentration 20 nM and a total volume of 20 µl. The binding buffer contained 20 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 2.5% (v/v) glycerol, 0.5 mM EDTA and, when desired, 0.2 mM cAMP. The reaction mixtures were incubated at RT for 20 min and then loaded onto a 15% native polyacrylamide gel. Electrophoresis was performed at RT and 170 V using 1x TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) as electrophoresis buffer. The gels were subsequently stained with SybrGreen I according to the instructions of the supplier (Sigma-Aldrich, Taufkirchen, Germany) and photographed.

2.6 Northern blot analysis

20 ml portions of exponentially growing cultures were chilled on ice and cells harvested immediately by centrifugation (5 min, 3000 g, 4°C) (Polen and Wendisch 2004). Cells were disrupted by bead beating and RNA isolated using the RNeasy-kit (Qiagen, Hilden, Germany). 5 µg RNA were separated by formaldehyde–agarose gel electrophoresis. RNA was transferred onto nylon membranes (Hybond N+; Amersham) by downward capillary blotting and immobilized by UV cross-linking. For probe construction, the DIG DNA Labelling and Detection kit (Roche, Basel, Switzerland) was used to generate DIG-dUTP labelled PCR products. The nylon membranes with fixed total RNA were hybridized with the digoxigenin (DIG)-labelled probes using standard stringency conditions (55°C, 50% formamide). DIG detection was performed according to the manufacturer's instructions. The chemiluminescent mRNA bands were recorded using the Fujifilm LAS-3000 Mini CCD camera and image analyzing system together with the software AIDA 4.15 (raytest Inc., Willmington, USA). Equal loading was normalized based on quantification of the fluorescence of ribosomal RNAs in ethidium bromide-stained gels.

2.7 RLM-Race

10 µg RNA was ligated by incubating with 40 U T4 RNA containing 10 U RNAse Inhibitor (Promega, Madison, USA) in 1x T4 RNA ligase buffer and a total volume of 25 µl. The reaction was incubated for 1 h at 37°C followed by heating at 65°C for 10 min. After adjustment of the total volume to 400 µl, proteins were removed by phenol-chloroform extraction. The RNA was resuspended in 10 µl dimethylpyrocarbonate treated water and the total sample used for cDNA synthesis, applying the Superscript II RT-kit (Invitrogen, Carlsbad, USA) with gene-specific primer gltA-UTR-RT. cDNA was amplified in a first PCR using the primer pair gltA-3'-p1 / gltA-5'-p1 with the product serving as template for the second (nested) PCR using primers gltA-3'-p2 / gltA-5'-p2. Products were cloned in *Sma*I linearized pUC18 and sequenced. P1gltA was identified in 5 out of 10 clones, whereas in the other five clones different shorter inserts were found, probably due to RNAse activity. P2gltA was identified in 7 out of 16 clones, whereas in the others also only various shorter inserts were present.

3. Results

3.1 Citrate synthase activities

RamA and RamB are regulators of acetate metabolism in *C. glutamicum* (Arndt and Eikmanns 2008, Bott 2007). Following the observation that upstream of *gltA* binding sites for RamB have been recognized (Gerstmeir *et al.,* 2004), we determined CS activities in *ramA* and *ramB* deletion mutants. For this purpose, the strains were grown on salt medium CGXII with either glucose or acetate as carbon source. Growth of WTΔramA with acetate was not possible due to complete lack of isocitrate lyase and malate synthase activity in this mutant (Cramer *et al.,* 2006). With glucose as a substrate, the growth rate of WTΔramA was reduced from 0.40 to about 0.20 h^{-1},

Table 2

Citrate synthase (CS) activity in *C. glutamicum* WT and the *ramA* and *ramB* deletion mutants.

	Specific CS activity (µmol min^{-1} (mg protein)$^{-1}$	
C. glutamicum strain	glucose	acetate
WT	0.99 ± 0.02	1.26 ± 0.03
WTΔramB	1.88 ± 0.06	1.58 ± 0.02
WTΔramA	0.12 ± 0.02	n.g.

n.g., no growth

whereas with WTΔramB the growth rates on either substrate were hardly influenced (data not shown). Cells were harvested in the exponential growth phase and their CS activities determined (Tab. 2). In *C.*

glutamicum WT the CS activity was significantly higher during growth on acetate as compared to growth on glucose. Interestingly, CS activity was increased on both substrates in WTΔramB, and in contrast, reduced on glucose in WTΔramA. These results suggested that RamB acts as repressor and RamA as an activator of *gltA*, as it was observed for several genes of the central metabolism (Arndt and Eikmanns, 2008).

3.2 CS is encoded by two transcripts

A scheme on the *gltA* locus is given in Figure 1. Downstream of *gltA* the *fkpB* gene encoding peptidyl-prolyl cis-trans isomerase is located, whereas upstream, and separated by 726 bps, the phosphoserine transaminase gene *serC* is present (Peters-Wendisch *et al.,* 2005). We performed a Northern analysis to assess the transcriptional organisation of *gltA*. For this purpose, RNA was extracted from exponential-phase cells of the WT grown on CGXII-glucose, and analysed by Northern hybridisation with a 500-bp *gltA*-specific RNA probe covering the central part of the gene. As a result two transcripts of about 1800 and 1500 bps were detected (Fig. 1). This unexpected observation prompted us to delete the previously identified promoter P1*gltA* (Eikmanns *et al.,* 1994), as a 160 bp chromosomal fragment to result in strain WTΔP1*gltA* (Fig. 1). In addition strain WTΔP12*gltA* was made, where a larger fragment of 540 bps was deleted. Whereas strain WTΔP1*gltA* still exhibited 15% of the activity of the WT, in extracts of strain WTΔP12*gltA* no CS activity was detectable. When RNA of WTΔP1gltA was probed in the Northern analysis, the shorter transcript was absent , whereas the long transcript was still formed (Fig. 1). This is naturally shorter than that in the WT due to the chromosomal deletion. The faint band in all hybridizations present is due to unspecific interaction with 16S rRNA which is the abundant species in RNA preparations. Since in principle the large 1800 bp transcript could encode *gltA*

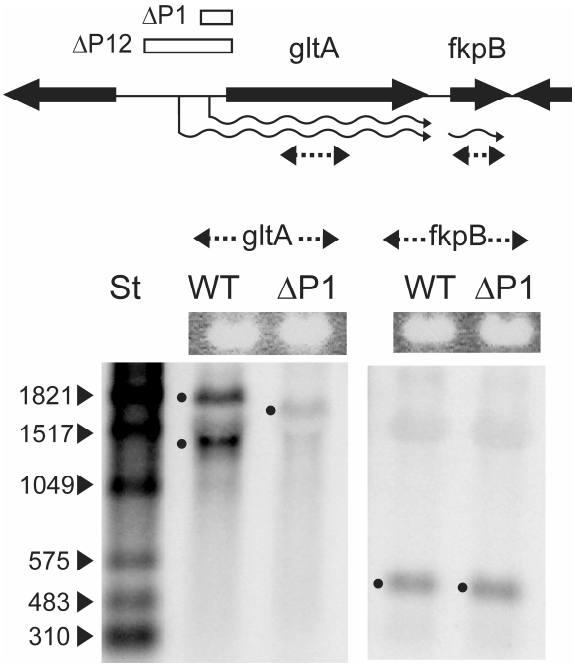

Fig. 1. Organization of the *gltA* region and its transcriptional analysis. On top is shown the chromosomal organisation of *gltA* and *fkpB* together with the localisation of the two probes used for Northern blot analyses which are indicated as double headed arrows with broken lines. The open bars marked ΔP1 and ΔP12 correspond to the chromosomal regions deleted in WTΔP1*gltA* and WTΔP12*gltA*, respectively. The wavy lines indicate the transcripts obtained in the Northern analysis shown in the lower part of the figure. Probed was RNA of the wild type (WT) and of the WTΔP1*gltA* deletion mutant (ΔP1) with either the *gltA* or *fkpB* probe. The 16S rRNA as visible in the ethidium-bromid stained gel which serves standardisation is shown on top of the blot. Hybridising probes were recorded by chemoluminescence with relevant signals marked by a dot. On the left standards (St) are given, marked with arrow heads and with their sizes in bp as indicated.

plus *fkpB* (Han *et al.,* 2008a), an additional Northern blot analysis was made using a probe specific to *fkpB* (Fig. 1). This confirmed that *gltA* is encoded by two transcripts of different length and that *fkpB* is transcribed separately as a monocistronic unit.

3.3 Determination of the 5' and 3' UTR of the two transcripts

To determine the 5´- and 3´-ends of the *gltA* transcripts, RNA of the WT was circularized with T4-RNA ligase and cDNA synthesized using the *gltA*-specific primer *gltA*UTRRT (RLM-RACE). Subsequent PCR amplification of the 5´- and 3´-junction and sequencing identified one transcriptional start site located 121 bp upstream from the first nucleotide of the translation initiation codon. This matches exactly the transcription initiation site determined in a primer extension analysis (Eikmanns *et al.,* 1994), and this is identical to P1gltA. As an additional novel transcription initiation site P2gltA located 357 bp upstream of the translation initiation codon was identified. The 3´-untranslated region ends 81 bps after the stop codon. It exhibits a high potential to form a stable stem-loop structure with a low $\Delta G^{0'}$ value of -34.8 kJ mol^{-1}. From this analysis it follows that the two different *gltA* promoters yield transcripts with a length of 1754 and 1517 bps, respectively, both with the same 3´-end.

3.4 Transcript intensities on acetate, on glucose and in mutants

RamA and RamB were originally identified as playing prominent roles during growth on acetate (Gerstmeir *et al.,* 2004; Cramer *et al.,* 2006). The obvious influence of both regulators on CS activity (see above) prompted us to compare the *gltA* transcript intensities during growth on acetate and glucose. For this purpose, cells of the WT were pregrown on acetate and glucose, inoculated into new medium with the same substrates and RNA isolated during exponential growth after 2 and 4 hrs of incubation, where the optical densities were about 2 and 5, respectively. The result for the Northern blot analysis, as shown in Fig. 2A, demonstrates that the short transcript is predominant during growth on acetate. This is the case at both time points of the analysis, and even at later time points over the entire cultivation time the short transcript is dominant (data not shown). During growth on glucose at the early time point the long transcript prevails. However, on this substrate with cultivation time a shift in the transcript intensities occurs which is already apparent at the second sampling point. This is probably due to the known formation of acetate during growth on glucose which occurs already from the beginning of the cultivation and is accumulating to concentrations exceeding 30 mM (Han et al., 2008a). This result let us to suppose that a separate carbon source-dependent regulation of the two *gltA*

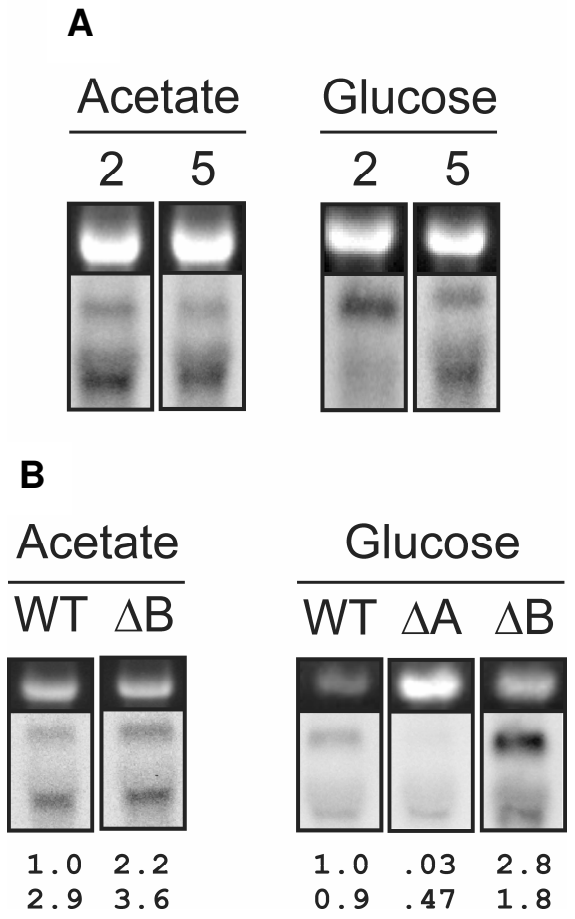

Fig. 2. Formation of the two *gltA* transcripts during growth on acetate or glucose and in deletion mutants. Shown are the result of 4 different analyses, with the ethidium bromid stained gels in the upper rows which show the 16S rRNA before blotting and the chemiluminescence recordings of the hybridised probe underneath. In **A** is shown transcript formation in the wild type during exponential growth on acetate or glucose, respectively, at OD 2 and 5. In **B** is shown transcript formation in WT and its Δ*ramA* and Δ*ramB* deletion mutants. Sampling was at an optical density of about 8, and transcripts were additionally quantified by densitometry. The ratios give an approximate indication on the amount of the respective transcript present in relation to the total amount of the 16S rRNA and thelong transcript set to 1. Blots generated from independent cultivations gave comparable results.

promoters exists. We next assayed whether the amounts of transcripts differ in the absence of either RamA or RamB. For this purpose RNA of WTΔramA, and WTΔramB was prepared and analysed as before. There is a clear increase of both transcripts in *C. glutamicum* WTΔramB, and a clear decrease in WTΔramA (Fig. 2B). These results indicate that RamA and RamB exert a strong counteracting effect on both transcripts. In summary, the data demonstrate that in the wild type of *C. glutamicum* both *gltA* transcripts are under strong transcriptional control, but the particular mRNA formation nevertheless results overall in only a slightly changed CS activity (Tab. 2).

3.5 CAT activities in ramA and ramB mutants

To provide further support for carbon source-dependent regulation of the transcripts and the involvement of RamA and RamB, we fused fragment gltA1 covering both promoter regions and fragment gltA3 covering the proximal promoter region only with *cat* as reporter gene (Fig. 3). The resulting plasmids pET2_gltA1 and pET2_gltA3 were used to transform *C. glutamicum* WT, WTΔramA and WTΔramB and the recombinants were grown on CGXII medium with glucose or acetate as substrate to determine the resulting CAT activities (Tab. 3). The data obtained with the long insert in pET2_gltA1 in the three strains are in excellent agreement with the CS activity due to chromosomal *gltA* expression (see Tab. 2). Also the reduced activity in the wild type background due to pET2_gltA3 is in accord with the promoter organisation since in this case only promoter P1gltA drives transcription. Importantly, relevant features of regulation due to *ramA* deletion are retained with the small fragment. In the absence of RamA the CAT activity due to pET2_gltA3 is about 50% of the WT (0.10 as compared to 0.19 µmol min^{-1} $mg(protein)^{-1}$), which agrees with the densitometric estimation of the transcripts in the Northern blot, where in absence of RamA the amount of the small transcript is reduced to approx. 50% (Fig. 2). Furthermore, the Northern blot revealed that in WTΔramA the long transcript was hardly detectable, and this is reflected in the only minor increase in CAT activity when

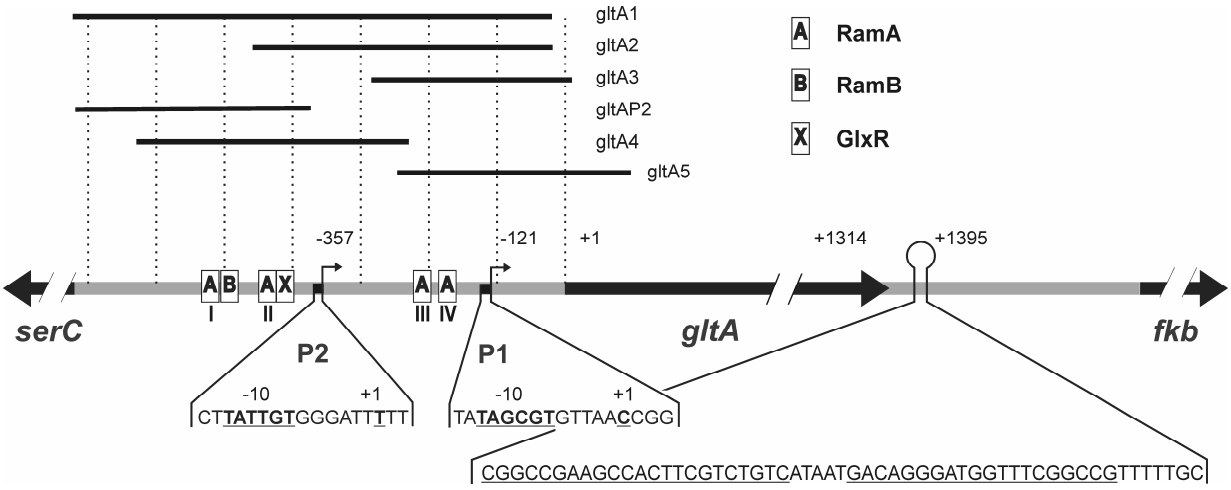

Fig. 3. Overview on *gltA* analysis and transcript formation. Given are the promoter regions of the *gltA* transcripts together with RamA, RamB and GlxR binding sites. In addition the fragments used in the electrophoretic mobility shift assays (EMSAs) are indicated, as well as those used for the *cat* fusions. The binding sites are choosen according to the EMSAs and based on the sequence identities to the known binding motifs. The RamA binding sites are numbered I - IV and the specific localization of the binding sites with respect to the translation initiation site +1, is as follows: RamA I, -528 to -522; RamB, -511 to -499; RamA II, -443 to -438; GlxR, -417 to -403; RamA III, -218 to -211; RamA IV, -184 to -178. Shown is furthermore the common terminator of both transcripts

comparing in the WTΔramA background pET2_gltA3 with pET2_gltA1 (Tab. 3). Regarding RamB, it is evident that the derepression due to the absence of RamB is also bound to the small fragment, since a rather high CAT activity is obtained when *ramB* is deleted. CAT activity is slightly increased with pET2_gltA1 on both substrates.

3.6 In vitro binding of RamA and RamB

To obtain information on *in vitro* interactions of RamA and RamB with *gltA* promoter regions, electrophoretic mobility shift assays (EMSAs) were performed. RamA and RamB proteins were isolated and incubated with the DNA fragments gltA1, gltAP2 gltA2, or gltA3 (Fig. 4A). Using RamA, all fragments shifted, including fragment gltA3 and gltAP2, both spanning regions specific to either P1gltA or P2gltA. Indeed, four unambiguous RamA binding motifs with similarity to the consensus sequence A/C/TG$_{4-6}$T/C or A/GC$_{4-6}$ A/G/T (Cramer *et al.,* 2006) are present on the fragments analysed. Two are in front of P1gltA, centered at position -181 and -214, respectively, with respect to the *gltA* translational start, and two are present upstream of P2gltA, centered at position -440 and - 525 (Fig. 3A). Coupled with the data from the Northern blot and the CAT assays, the gel shift experiments suggest that RamA directly interacts with both promoter regions to activate formation of the two transcripts. In the EMSAs with RamB protein, a retardation was observed with fragment gltA1 and gltAP2, whereas with fragment gltA2 or gltA3 no clear shift was obtained (Fig. 4B). This result was unexpected since the *in vivo* data generated, i.e. the Northern blots and the CAT activities, demonstrated that the transcript formed by P1gltA activity also responds to the presence of RamB (see discussion).

Table 3

Specific chloramphenicol acetyltransferase (CAT) activities of *C. glutamicum* strains carrying different *gltA* promoter fragments in plasmid pET2 that were grown in minimal medium with either 1% glucose or 1% acetate and harvested in the exponential growth phase at an OD of about 5.

	Specific CAT activity (µmol min^{-1} (mg protein)$^{-1}$ [a]	
C. glutamicum strain	Glucose	Acetate
WT pET2_gltA1	0.48 ± 0.13	0.65 ± 0.11
WTΔramB pET2_gltA1	1.28 ± 0.33	0.87 ± 0.20
WTΔramA pET2_gltA1	0.14 ± 0.06	n.g.[b]
WT pET2_gltA3	0.19± 0.08	0.30 ± 0.12
WTΔramB pET2_gltA3	1.06 ±0.29	0.63 ± 0.22
WTΔramA pET2_gltA3	0.10 ±0.05	n.g.[b]

[a] The values are means ± standard deviation of three to five independent cultivations and two determinations per experiment.
[b] n.g, no growth.

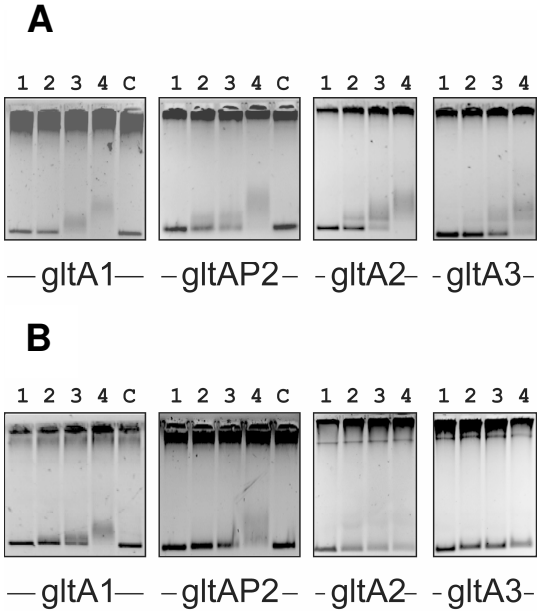

Fig. 4. *In vitro* interaction of RamA (**A**) and RamB (**B**) proteins with fragments gltA1, gltAP2, gltA2 and gltA3 (see Fig. 34). The respective protein used was 0 µg for lanes 1, 0.25 µg for lanes 2, 0.5 µg for lanes 3, and 1 µg for lanes 4 (molar excess of about 40, 60 and 120). Controls made with 1 µg bovine serum albumin are given in lanes C.

3.7 Consequences of glxR overexpression and in vitro binding of GlxR

Besides RamA and RamB, also GlxR is a probable candidate involved in *gltA* control (Han *et al.,* 2008a). Since *glxR* is essential, we assessed its relevance for *gltA* transcription by overexpression of *glxR* in *C. glutamicum* WT pEKEx3-glxR and compared the CS activity to WT pEKEx3 as control. During growth on glucose the specific activity was reduced by 41%, and on acetate by 25%, suggesting repression of *gltA* by GlxR. Furthermore, a direct analysis of GlxR interaction with *gltA* promoter regions was assayed. GlxR was overexpressed, isolated, and incubated with two DNA fragments spanning either the

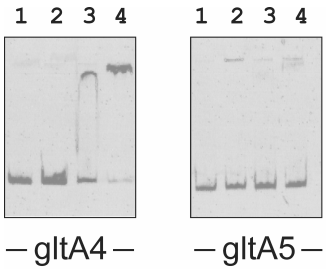

Fig. 5. GlxR involvement in *gltA* expression. The electrophoretic mobility shift obtained with DNA fragments gltA4 and gltA5 is shown. The GlxR protein used was 0 µg for lane 1, 0.5 µg for lane 2, 1 µg for lane 3, and 2 µg for lane 4 (molar excess of 50, 100 and 200).

P2gltA (fragment gltA4) or the P1gltA region (fragment gltA5) (Fig. 5). A shift of fragment gltA4 was obtained, which was strictly dependent on the presence of 0.2 mM cAMP, a known effector of GlxR (Kim *et al.,* 2004). No binding occurred with fragment gltA5 even in presence of cAMP (data not shown).

4. Discussion

In *Escherichia coli, gltA* expression is under control of ArcA (Park *et al.,* 1994). Under aerobic conditions, deletion of *arcA* results in a two-fold increased expression, and this control is independent of the global carbon metabolism regulators Crp and FruR. There is an early report that two transcripts might encode *gltA* in *E. coli* (Wilde and Guest, 1986). However, further details on transcriptional regulation of this key enzyme in *E. coli* are not available. *B. subtilis* has two citrate synthase genes, *citA* and *citZ* (Jin and Sonenshein, 1994a), with *citZ* organized in an operon together with the genes for isocitrate dehydrogenase and malate dehydrogenase. Whereas a *citA* null mutant does not show a growth defect, a *citZ* mutant results in a partial glutamate auxotrophy, and it is the latter gene which is repressed 2-fold by glucose (Jin and Sonenshein, 1994b). Genome wide DNA array analyses with *C. glutamicum* grown on glucose or acetate have shown that a carbon-source dependent expression of *gltA* exists (Muffler *et al.,* 2002; Gerstmeir *et al.,* 2003). We here found an elaborate and pronounced *gltA* expression control in *C. glutamicum*. The *gltA* gene is monocistronic and essential for growth of *C. glutamicum* on mineral salts medium (Eikmanns *et al.,* 1994). There are two transcripts and the Northern blot experiments show that the short transcript prevails during growth on acetate, whereas the long one is dominant during growth on glucose, at least in the initial stages of glucose utilisation. Expression assays done by Northern blot and with transcriptional fusions suggest that both the P1 and P2 promoters have higher activity in the presence of RamA and lower activity in the presence of RamB. A direct effect of RamA at both promoters is suggested by the EMSA experiments, and by the locations of candidate RamA binding sites (Fig. 3). However, despite the presence of candidate RamB binding sites in both the P1 and P2 promoter regions, the EMSA experiments are consistent with a direct effect for RamB only at P2. This lack of congruence between the in vivo expression assays and the in vitro gel shift assays might be explained if another effector was needed for full binding of RamB in vitro, and this effector was missing from the gel shift assays. The function of RamA and RamB is well studied for the acetate- and ethanol-utlisation genes *pta-ack, aceA, aceB, ald* and *adhA* (Cramer *et al.,* 2006; Gerstmeir *et*

al., 2004; Auchter *et al.,* 2009; Arndt and Eikmanns, 2007), as well as for the resuscitation factor gene *rpf2,* or the transporter gene *mctC* (Jungwirth *et al.,* 2008; Jolkver *et al.,* 2009). In these cases it is demonstrated too, that RamA serves as an activator, whereas RamB acts as a repressor. The three proteins RamA, RamB, and GlxR were originally isolated as regulators involved in *aceA* and *aceB* contol (Cramer *et al*., 2006; Gerstmeir *et al*., 2004; Kim *et al*., 2004). These genes encode isocitrate lyase and malate synthase of the glyoxylate cycle, and the enzymes have high activity on acetate whereas on glucose there is almost no activity present. However, in addition to these two enzymes also citrate synthase, aconitase, succinate dehydrogenase, fumarase and malate:quinone oxidoreductase are required for a functional glyoxylic acid cycle, with these genes being also required for the citric acid cycle. Albeit not as strong increased in activity as isocitrate lyase and malate synthase, also the enzymes shared by the glyoxylate and tricarboxylic acid cycle are noticeable increased during growth on acetate (Eggeling and Bott 2005). These elevated activities during growth on acetate and the higher metabolite fluxes through these reactions on this substrate asks for a common control of these enzymes. Indeed, RamA excerts a positive influence not only on *gltA* transcript formation, but also on that of *acn* (aconitase) (Emer *et al*., 2009), and *sdhCAB* (succinate dehydrogenase) (Bussmann *et al*., 2009). Similarly, also RamB additionally controls *acn*, albeit in an indirect manner. In addition to RamA and RamB, GlxR is involved in control of *gltA* transcription, preferentially repressing the long transcript, and also *sdhCAB* is repressed by GlxR (Bussmann *et al*., 2009). Thus, the global regulators RamA, RamB, and GlxR, exhibit a connectivity of regulatory interactions on the control of these key genes in central metabolism, as suggested for further examples of carbon metabolism in *C. glutamicum* from *in silico* studies (Kohl *et al*., 2008).The *gltA* gene is unique among the glyoxylate- and tricarboxylic acid cycle genes as it is transcribed with 2 messages which are separately controlled. A reason at hand is that the encoded CS is located at the entry point of both cycles. As one control element we have as yet detected GlxR which exerts a slightly stronger effect on repression of the long transcript. However, it is possible that still further unknown regulatory features might exist to enable specific control for formation of both transcripts, like different mRNA stability, for instance. It is most striking that in the wild type the strong and in part counteracting control by the regulators involved results in an only slightly altered CS activity (Shiio *et al*., 1977; Eikmanns *et a*l., 1994). It is at best about 1.3-fold altered when comparing cells grown on acetate with those grown on glucose (Tab. 2), but this largely agrees with the sum of the transcript ratio on acetate plus glucose, which is 1.5 (Fig. 2). One reason could be that the elaborated transcriptional control serves to provide a sensitive and fast mechanism to adapt the total transcript level and thus CS activity to the physiological requirements. Indeed there are indications that the *in vivo* flux via CS activity in *C. glutamicum* operates close to the maximum activity provided by the enzyme. Mutants showing about 1/3 of CS activity exhibit reduced growth (Radmacher *et al.,* 2007). Therefore, it is a tempting idea that possession of a non-allosterically regulated CS type I, as is present in *C. glutamicum*, might be compensated by a flux control at this key position in central metabolism exerted by the total enzyme amount.

Acknowledgements

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Effects of a *cyaB* deletion on growth. organic acid production and global gene expression in *Corynebacterium glutamicum*

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ABSTRACT

The *cyaB* gene of *Corynebacterium glutamicum* ATCC 13032 encodes a class III adenlyate cyclase and is the only annotated adenylate cyclase gene in this species. A Δ*cyaB* deletion mutant showed similar growth behaviour as the wild type in minimal media containing glucose. gluconate or ethanol as carbon source. In contrast. a strong growth defect was observed in media containing acetate or propionate as carbon sources. When acetate was present in addition of glucose. gluconate or ethanol. growth of the Δ*cyaB* mutant was also inhibited. The growth defects of the mutant could be reversed by plasmid-borne *cyaB* or by addition of extracellular cAMP. The cAMP level was found to be decreased in the Δ*cyaB* mutant compared to the wild type. Transcriptome comparisons of the Δ*cyaB* mutant with the wild type using cells grown with glucose or a glucose-acetate mixture revealed that many target genes of the cAMP-binding transcriptional regulator GlxR were differentially expressed.

1. Introduction

Corynebacterium glutamicum. a Gram-positive soil bacterium. is used for the industrial production of amino acids. the most important ones being L-glutamate and L-lysine. and serves as a model organism of the suborder *Corynebacterinae* within the order *Actinomycetales*. Major aspects of the biology and the industrial use of this species and close relatives have been summarized recently (Burkovski, 2008; Eggeling and Bott, 2005).

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In the genome sequence of *Corynebacterium glutamicum* ATCC13032 (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003) only one gene encoding an adenylate cylase has been annotated (*cyaB*. cg0375). whereas 16 adenylate cyclase genes have been annoted in the genome of the relative *Mycobacterium tuberculosis* (Shenoy et al., 2004). Nucleotide cyclases are enzymes that convert nucleotide triphosphates NTP´s into the respective second messenger 3´-5´-cyclic nucleotide monophosphate (cNMP). Based on their amino acid sequences. the metal-dependent nucleotide cyclases can be divided into six classes . Class I nucleotide cyclases are exclusive to γ–proteobacteria. One example is the nucleotide cyclase of *Escherichia coli* which is involved in catabolite repression

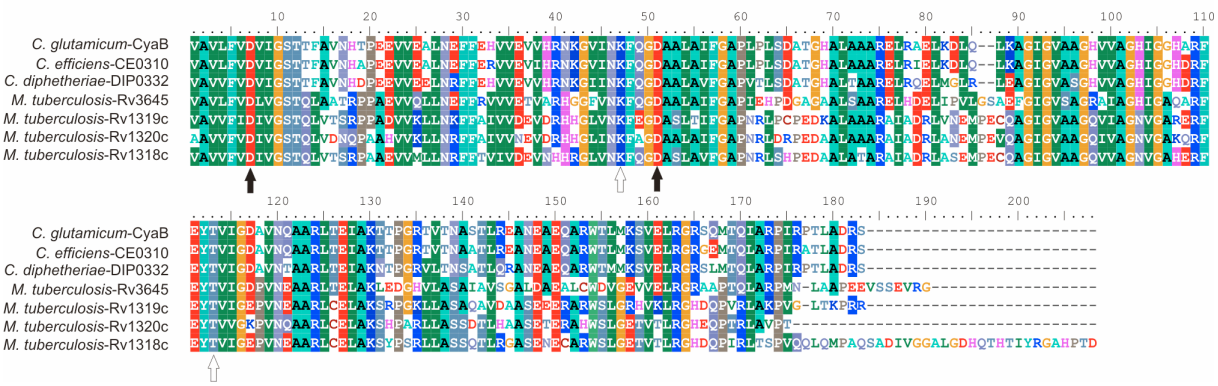

Fig. 1. Multiple sequence alignment of the cyclase domains of *C. glutamicum* adenylate cyclase CyaB and its homologs in *C. diphtheriae*. *C. efficiens* and *M. tuberculosis*. Black arrows indicate metal binding residues. open arrows indicate substrate specifying residues.

(Feucht and Saier, 1980; Harwood et al., 1976). Class II nucleotide cyclases are toxins secreted for example by *Bordetella pertussis* (Dautin et al., 2002). For class IV. V and VI nucleotide cyclases not much is known or described. Class III nucleotide cyclases. which are the most abundant type of cAMP-forming enzymes. are found in metazoa. protozoa. fungi. green algae. eubacteria. and archaea. They have to form dimers to be active and the catalytic centre is formed at the dimer interface (Tesmer et al., 1997; Zhang et al., 1997). Class III nucleotide cyclases include besides the adenylate cyclases also all known guanylate cyclases. The adenylate and guanylate cyclase structures contain a central four stranded anti-parallel β-sheet and α-helices on either site (Tesmer et al., 1997). Crystal structures and mutagenesis studies of a chimeric mammalian enzyme helped to understand the catalytic mechanism and substrate binding (Tesmer et al., 1997; Tesmer et al., 1999). These studies showed that one lysine and one aspartate residue of each monomer are essential for substrate binding and crucial for selection between ATP or GTP. Two aspartate residues (D396 and D440) are involved in binding the metal cofactor (Mg^{2+} or Mn^{2+}). Class III nucleotide cylases can be further subdivided into four subclasses (IIIa - IIId). The subdivision is based on the primary sequence of the cyclase homolog domain (CHD). which is quite divergent in class III nucleotide cyclases (Linder and E., 2003).

One of the 16 nucleotide cyclases that are present in the pathogenic *M. tuberculosis*. the one encoded by Rv3645 shows 43% sequence identity to *C. glutamicum* CyaB and consists of an N-terminal membrane-integral domain containing six putative transmembrane helices which is linked to a class IIId catalytic homolog domain (CHD). CyaB of *C. glutamicum* also possesses six putative transmembrane helices in the N-terminal part that are linked via a HAMP domain to the CHD. In Rv3645 the HAMP domain shows a large positive stimulatory effect on the activity of the catalytic domain (Linder et al., 2004). Besides Rv3645. three other cyclases (Rv1318c. Rv1319c. Rv1320c) of the same domain composition show a high sequence identity to CyaB (36 - 39%). especially in the CHD (Fig. 1). In all *Corynebacterium* species of known genome sequence CyaB is well conserved and always the only annotated nucleotide cyclase. The CyaB homologs of *C. diphtheriae* and *C. efficiens* for example show a sequence identity of 72 and 90 %. respectively. to *C. glutamicum* CyaB (Fig. 1).

The product of the CyaB-catalysed reaction. cAMP. functions as a second messenger. In *C. glutamicum*. the transcriptional regulator GlxR is known to bind cAMP. GlxR. which was first identified as a regulator of the glyoxylate bypass genes (Kim et al., 2004). functions as a global regulator with numerous of target genes (Bussmann, 2009; Han et al., 2008a; Han et al., 2008b; Jungwirth et al.,

2008; Kohl et al., 2008; Kohl and Tauch, 2009). The fact that most attempts (including our own) to delete the *glxR* gene failed (Kim et al., 2004; Letek et al., 2006; Toyoda et al., 2009). triggered the question. whether the only annotated adenlyate cyclase gene *cyaB* can be deleted. The results reported here and in a recent publication (Cha et al., 2009) show that *cyaB* is not essential in *C. glutamicum* and that a *cyaB* mutant has interesting phenotypes. which in the future should help to clarify the roles of CyaB and GlxR in the regulation of metabolism.

2. Materials and Methods

2.1. Bacteria. plasmids. oligonucleotides and culture conditions

Strains and plasmids used in this work are listed in Table 1. For growth experiments. precultures and main cultures were cultivated at 30°C and 120 rpm in 500-ml baffled Erlenmeyer flasks containing 60 ml CGXII minimal medium (Keilhauer et al., 1993) supplemented with

Table 1
Bacterial strains. plasmids and oligonucleotides used in this study.

Strain. plasmid or oligonucleotide	Characteristics or sequence	Source or reference
Strains		
E. coli DH5α	F⁻ *thi-1 endA1 hsdR17*(r-m+) *supE44* Δ*lacU169*Φ80*lacZ*Δ*M15*) *recA1 gyrA96 relA1*	Invitrogen
C. glutamicum ATCC 13032	ATCC 13032. wild-type strain (WT)	American Type Culture Collection
C. glutamicum Δ*cyaB*	WT with an in frame deletion of the *cyaB* gene (cg0375)	This work
C. glutamicum Δ*cyaB*-pAN6-cyaB	Δ*cyaB* carrying pAN6-cyaB expression plasmid	This work
Plasmids		
pAN6-cyaB	Kan^R; pEKEx2 derivative carrying the *cyaB* gene under the control of an IPTG-inducible *tac* promoter	This work
pK19mobsacB-ΔcyaB	Kan^R; pK19mobsacB derivative containing a overlap extension PCR product covering the up- and downstream regions of *cyaB*	This work
Oligonucleotides		
pAN6-cyaB-Pst-for	5´-AAACTGCAGGTGCTCGGCACGAATGTGTTTG-3´	Primers for pAN6-cyaB cloning; *Pst*I and *Nhe*I restriction site are underlined
pAN6-cyaB-Nhe-rev	5´-AAAGCTAGCTTAGGACCTATCCGCCAACGTC-3´	
Aden-1-hind-for	5´-ATTAAAGCTTCGGGGTGGCTGCCTCCCATG-3´	Primers for pK19mobsacB-Δ*cyaB* cloning; *Hin*dIII and *Xba*I restriction sites are underlined
Aden-2-rev	5´-CGTTTAGGTTTAGTGGCTGGGCAAACAGCATTACTGCGAGCGCACC-3´	
Aden-3-for	5´-CCCAGCCACTAAACCTCCCCGGCGGCCTATTCGGCCGACGTTG-3´	
Aden-4-xba-rev	5´-ATTATCTAGACAATCACGCCGCGTACATCGC-3´	
test-del-cyaB-for	5´-CAATTGCTGCGGGACGATGTG-3´	Primers for confirmation of the *cyaB* deletion
test-del-cyaB-rev	5´-GATTCACTGCCTAAAGGTGCG-3´	

3.4-dihydroxybenzoate (30 mg/l) as iron chelator. As carbon sources, glucose, gluconate, propionate, acetate, ethanol or mixtures of acetate and glucose, acetate and gluconate or acetate and ethanol were used at concentrations indicated in the Results and Discussion section. If appropriate. kanamycin (25 or 50 µg/ml) was added to the indicated final concentrations. *E. coli* DH5α was used as host for all cloning purposes and grown at 37°C in LB medium (Sambrook et al., 1989).

2.2. Construction of cyaB in frame deletion mutant

In-frame *cyaB* deletion mutants of *C. glutamicum* ATCC 13032 were constructed as described previously (Niebisch and Bott, 2001). For this purpose. the *cyaB* upstream and downstream regions (~500 bp) were amplified with the Expand High Fidelity kit (Roche Diagnostics. Mannheim. Germany) using the oligonucleotide pairs Aden-1-hind-for. Aden-2-rev. Aden-3-for and Aden-4-xba-rev. respectively (Table 1). The resulting PCR products were subsequently fused by overlap-extension PCR using oligonucleotides Aden-1-hind-for and Aden-4-xba-rev. After digestion with HindIII and XbaI. the ~1kb DNA fragment was cloned into pK19*mobsacB* (Schäfer et al., 1994) cut with the same restriction enzymes to yield pK19*mobsacB*-Δ*cyaB*. Unwanted mutations were excluded by DNA sequencing of the PCR-derived part of the plasmid. Subsequently. plasmid pK19*mobsacB*-Δ*cyaB* was transferred by electroporation (van der Rest et al., 1999) into competent cells of *C. glutamicum* strain ATCC 13032 and the transformation mixture was plated on BHI agar (Difco, Detroit, USA) containing 25 µg kanamycin/ml. After selection for the first and second recombination events. genomic DNA of kanamycin-sensitive and sucrose-resistant clones was analyzed by PCR with the primers test-del-cyaB-for and test-del-cyaB-rev (Table 1) in order to distinguish between wild-type and Δ*cyaB* clones. In the case of the wild-type situation. a PCR product of 1.5 kb was obtained. in the case of a successful *cyaB* deletion a 1-kb PCR product. Furthermore. the PCR fragment obtained from a Δ*cyaB* mutant was controlled by sequencing.

2.3. Quantitative determination of carbon sources and organic acids

Glucose. gluconate. acetate. ethanol and organic acid concentrations in supernatants were determined by a newly developed method based on ion exchange chromatography using an Agilent 1100 HPLC system (Agilent Technologies. Waldbronn. Germany) equipped with a cation exchange column (organic acid refil column. 300 x 8 mm. CS-Chromatographie Service GmbH. Langerwehe. Germany). Substances were eluted with a flow rate of 0.4 ml min^{-1} for 42 min at 40°C with 100 mM H_2SO_4. Eluted organic acids or carbon sources were detected by a diode array detector (DAD G1315B) at a wavelength of 215 nm or by a refraction index detector (Agilent 1200 Series) (optical unit temperature 35°C). Quantification was performed using calibration curves obtained with external standards. An example of an elution chromatogram of separated external standards in one single run is shown in Fig. S1.

2.4. Determination of cAMP concentrations

For measurement of the intracellular cAMP concentration. cells were prepared as described previously (Siegel et al., 1977). The cAMP concentration was measured with the Biotrak cAMP enzyme immunoassay system (GE Helthcare; RPN 2251) and related to *C. glutamicum* cell dry weight based on the correlation that an OD_{600} of 1 corresponds to 0.25 g dry weight l^{-1} (Kabus et al., 2007).

2.5. Global gene expression analysis

Preparation of RNA and synthesis of fluorescently labelled cDNA were carried out as described (Möker et al., 2004). Custom-made DNA microarrays for *C. glutamicum* ATCC 13032 printed with 70mer oligonucleotides were obtained from Operon (Cologne. Germany) and are based on the genome sequence entry NC_006958 (Kalinowski et al., 2003). Hybridization and stringent washing of the microarrays were performed according to the instructions of the supplier. Hybridization was carried out for 16–18 h at 42°C using a MAUI hybridization system (BioMicro Systems. Salt Lake City. USA). After washing the microarrays were dried by centrifugation (5 min. 1600 *g*) and fluorescence was determined at 532 nm (Cy3-dUTP) and 635 nm (Cy5-dUTP) with 10 mm resolution using an Axon GenePix 6000 laser scanner (Axon Instruments. Sunnyvale. USA). Quantitative image analysis was carried out using GenePix image analysis software and results were saved as GPR-file (GenePix Pro 6.0. Axon Instruments). For data normalization. GPR-files were processed using the BioConductor/R-packages limma and marray (http://www.bioconductor.org). Processed and normalized data as well as experimental details (MIAME. (Brazma et al., 2001)) were stored in the in-house microarray database for further analysis (Polen and Wendisch, 2004). Using the DNA microarray technology. the genome-wide mRNA concentrations of *C. glutamicum* wild type were compared with those of the mutant strain Δ*cyaB*. The strains were cultivated in CGXII minimal medium with either 200 mM glucose. or glucose plus acetate (100 mM each). RNA used for the synthesis of labelled cDNA was prepared from cells in the exponential growth phase. For each comparison. three independent DNA microarray experiments were performed. each starting from an independent culture. To filter for differentially expressed genes and reliable signal detection in each of the six comparisons. the following quality filter was applied: (i) flags _ 0 (GenePix Pro 6.0). (ii) signal/noise ≥ 3 for Cy5 (F635Median/B635Median. GenePix Pro 6.0) or Cy3 (F532Median/B532Median. GenePix Pro 6.0). (iii) ≥ twofold change in the comparison Δ*cyaB* mutant versus wild type in minimal medium. and (iv) significant change ($P < 0.05$) in Student's *t*-test (Excel. Microsoft).

Table 2
Growth characteristics of *C. glutamicum ΔcyaB* in CGXII minimal medium.

Carbon source(s)	Growth rate (h^{-1})		Substrate consumption rate (nmol min^{-1} (mg DW^{-1}))	
	WT	Δ*cyaB*	WT	Δ*cyaB*
Glucose	0.39 ± 0.0	0.40 ± 0.01	94 ± 2	86 ± 2
Gluconate	0.33 ± 0.01	0.33 ± 0.01	73 ± 2	77 ± 3
Acetate	0.25 ± 0.01	0.09 ± 0.01	168 ± 3	126 ± 8
Ethanol	0.22 ± 0.02	0.22 ± 0.01	159 ± 5	174 ± 7
Glucose/Acetate	0.36 ± 0.04	0.18 ± 0.01 – 0.2 ± 0.01	41 ± 4 / 112 ± 7	26 ± 1 - 38 ± 3 / 79 ± 5
Gluconate/Acetate	0.23 ± 0.01	0.17 ± 0.01	28 ±3 / 77 ± 3	23 ± 2/75 ± 3
Acetate/Ethanol	0.25 ± 0.01	0.06 ± 0.01	94 ± 2/111 ± 3	-/-

For growth of *ΔcyaB* on Glucose/Acetate. growth rate and glucose consumption were calculated for the first 10 h and after 10 h.

3. Results

3.1. Deletion of the adenylate cyclase gene cyaB of C. glutamicum and growth of the resulting mutant strain on different carbon sources.

In order to analyse the function of the only annotated adenylate cyclase of *C. glutamicum*. the corresponding *cyaB* gene was deleted in-frame in the *C. glutamicum* type strain ATCC 13032 as described in Materials and Methods. Of 9 kanamycin-sensitive and sucrose-resistant clones analysed by PCR after the second recombination event. 4 showed the desired cyaB deletion and 5 represented the wild-type situation. Thus. no difficulties were encountered in obtaining the deletion mutant.

For comparative growth experiments. *C. glutamicum* Δ*cyaB* and the wild type strain were cultivated in CGXII minimal medium containing different carbon sources. No significant differences in growth and carbon source consumption were observed with glucose (200 mM). gluconate (100 mM) or ethanol (1% + 7 mM glucose) (Figs. 2A. 2B and 2E) and a similar result was obtained with pyruvate (200 mM) (data not shown). A minor difference during growth on glucose was observed for acetate secretion after 18 h. which was lower in the Δ*cyaB* mutant (15 mM) than the wild type (30 mM) (data not shown). No acetate or similar acetate concentrations were secreted during growth on the other carbon sources mentioned above.

When cultivated with acetate (243 mM) as carbon source. the *cyaB* deletion mutant showed a strong growth defect with a growth rate of 0.09 h^{-1} in comparison to 0.25 h^{-1} of the wild type (Fig. 2D). Morever, the mutant reached only a much lower final optical density (OD_{600} of about 5) than the wild type (OD_{600} of about 20). A growth defect of the Δ*cyaB* mutant was also observed with propionate (200 mM) as carbon source (Fig. 2C). The cultures of the mutant reached an OD_{600} of about 4. that of the wild type of about 20. The growth behaviour of the wild type on propionate showed a long lag phase which was also effected by the substrate switch from glucose in the preculture to propionate in the main culture. This effect was also described previously (Claes et al., 2002).

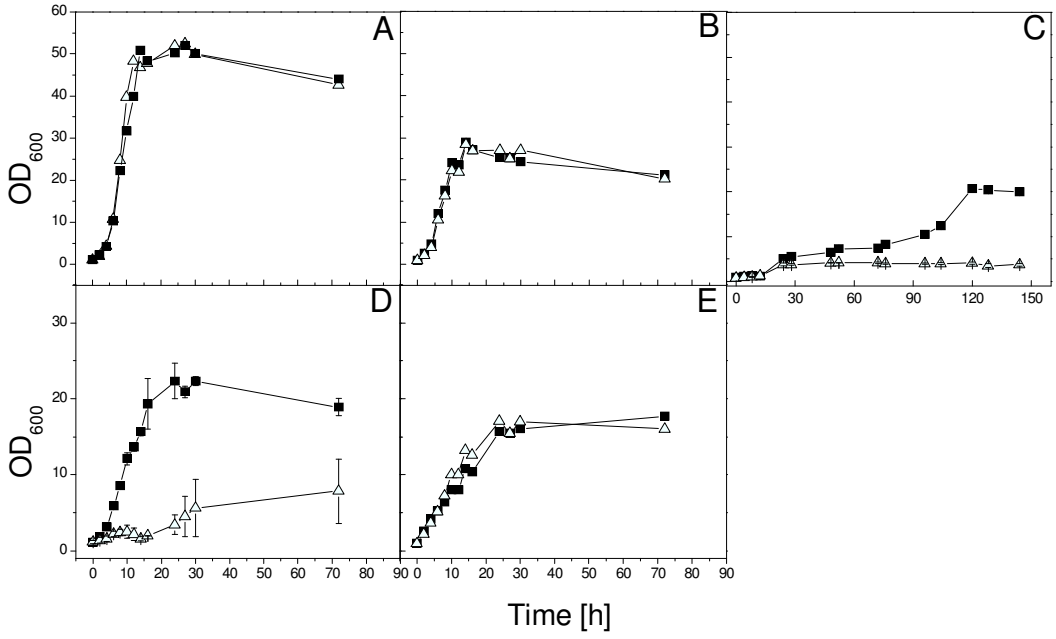

Fig. 2. Growth of *C. glutamicum* wild type (black squares) and its Δ*cyaB* mutant (open triangles) in CGXII minimal medium containing the following carbon sources: (A) 200 mM glucose; (B) 200 mM gluconate; (C) 200 mM sodium propionate; (D) 243 mM sodium acetate; (E) 217 mM Ethanol + 7 mM Glucose.

3.2. Growth properties of the ΔcyaB mutant on carbon source mixtures containing acetate

Due to the inhibitory effect of acetate on growth of the Δ*cyaB* mutant. we also tested growth on acetate-glucose. acetate-gluconate and acetate-ethanol mixtures. It has been shown previously that *C. glutamicum* coutilizes acetate and glucose (Wendisch et al., 2000) and also gluconate and glucose (Frunzke et al., 2008) . In contrast. during growth on ethanol-glucose mixtures. ethanol is consumed after glucose. representing one of the few examples of clear catabolite repression in *C. glutamicum* (Arndt et al., 2008). As shown in Fig. 3. the presence of acetate had a negative impact on growth and carbon source consumption of the Δ*cyaB* mutant. When cultivated with a mixture of glucose and acetate (100 mM each). the Δ*cyaB* strain showed a much lower growth rate (0.13 h^{-1}) than the wild type (0.36 h^{-1}) (Fig. 3A). Therefore. also the kinetics of acetate and glucose consumption were slower in the Δ*cyaB* strain (Figs. 3D and 3G). Acetate consumption rates of 79 ± 5 nmol mg^{-1} min^{-1} and 112 ± 7 nmol mg^{-1} min^{-1} were calculated for the Δ*cyaB* mutant and the wild type. respectively. Glucose consumption by the Δ*cyaB* strain was very slow in the first 12 hours with an uptake rate of 26 ± 1 nmol mg^{-1} min^{-1}. After 12 hours the consumption rate increased to 38 ± 3 nmol mg^{-1} min^{-1} but was still lower than the wild type rate of 41 nmol mg^{-1} min^{-1}. The final optical density measured after 48 h was also significantly lower for the mutant (OD_{600} of 34) than for the wild type (OD_{600} of 40).

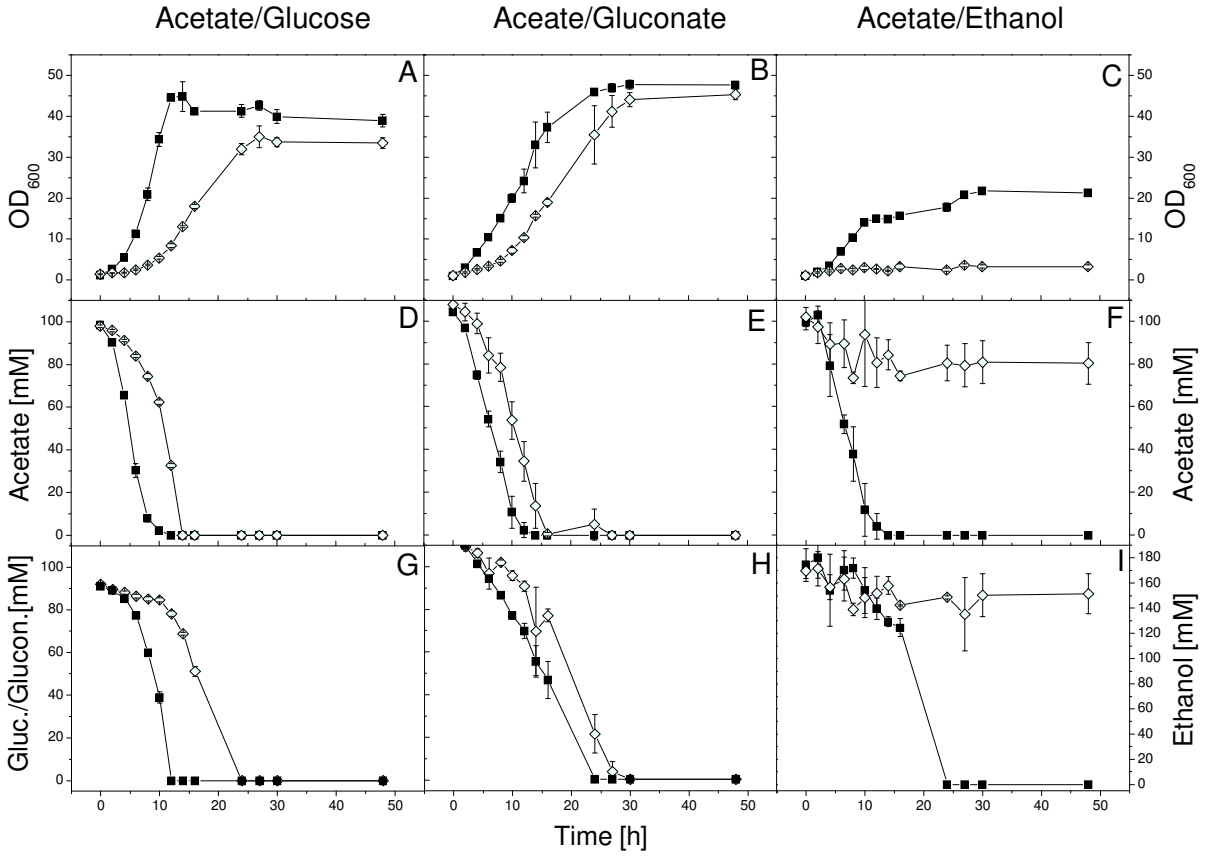

Fig. 3. Growth and substrate consumption of *C. glutamicum* wild type (black squares) and its Δ*cyaB* mutant (open diamonds) in minimal medium containing acetate and a second or third carbon source. Panels A – C show growth (OD_{600}) on 100 mM acetate and 100 glucose (A). 100 mM acetate and 100 mM gluconate (B). and 100 mM acetate and 217 mM ethanol (7 mM glucose). Panels D – F shows acetate consumption for growth experiments of A – C. Panel G shows glucose consumption for growth experiment A. panel H shows gluconate consumption for growth experiment B. and panel I shows ethanol consumption for growth experiment C.

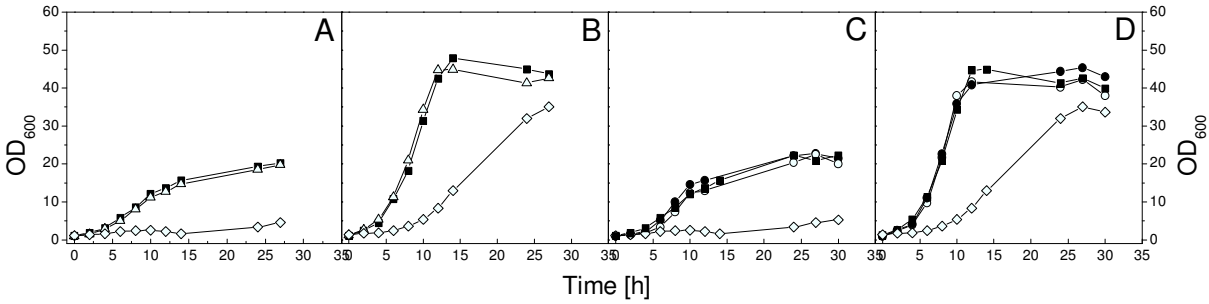

Fig. 4. Complementation of Δ*cyaB* mutant with plasmid encoded *cyab* (A. B) and 10 mM extracellular cAMP (C. D). Growth of *C. glutamicum* wild type (black squares). Δ*cyaB* (open diamonds) and Δ*cyaB*-pAN6-cyaB (open triangles) in CGXII minimal medium containing 200 mM sodium acetate (A) or 100 mM glucose and 100 mM sodium acetate (B). Growth of *C. glutamicum* wild type (black circles) and Δ*cyaB* (open circles) in CGXII minimal medium with 10 mM cAMP (no cAMP: WT = black squares. Δ*cyaB* = open diamonds) containing 200 mM sodium acetate (C) or 100 mM glucose and 100 mM sodium acetate (D). (cAMP used in this study: Adenosine 3′. 5′-cyclic monophosphate. free acid. Boehringer Mannheim)

When cultivated in acetate-gluconate medium (100 mM each) the Δ*cyaB* mutant showed again a reduced growth rate (0.17 h^{-1}) compared to the wild type (0.23 h^{-1}) (Fig. 3B) and also the substrate consumption rates are somewhat lower for the mutant (75 ± 3 nmol acetate mg^{-1} min^{-1} and 23 ± 2 nmol gluconate mg^{-1} min^{-1}) than for the wild type (77 ± 3 nmol acetate mg^{-1} min^{-1} and 28 nmol ± 3 gluconate mg^{-1} min^{-1}) (Figs. 3E and 3H). The final optical density was similar for wild type and mutant.

The strongest differences in growth behaviour between wild type and Δ*cyaB* mutant was observed in media containing acetate (100 mM). ethanol (247 mM) and a minor concentration of glucose (7 mM) as carbon sources. In this medium the mutant did not grow at all. whereas the wild type reached a final OD_{600} of of 22 (Fig. 3C). The wild type showed a diauxic growth behaviour. which correlated with the observation that in the first growth phase primarily acetate was used and in the second growth phase ethanol.

3.3. Complementation studies with C. glutamicum *ΔcyaB*.

The Δ*cyaB* strain was transformed with plasmid pAN6-cyaB, which carries the *cyaB* gene under the control of an IPTG-inducible *tac* promoter. and tested for growth in minimal medium containing either acetate (200 mM) or a glucose-acetate mixture (100 mM each) as carbon sources. As shown in Figs. 4A and 4B. the Δ*cyaB* strain containg pAN6-cyaB grew like the wild type, showing that indeed the *cyaB* deletion was the cause of the observed growth defects. As an alternative to complementation with a *cyaB* expression plasmid we also tested if extracellular cAMP was able to reverse the growth defect of the Δ*cyaB* strain. As shown in Figs. 4C and 4D. the addition of 10 mM cAMP had no effect on growth of the wild type on acetate or glucose-acetate mixtures. but the *cyaB* mutant grew like the wild type in the presence of 10 mM cAMP. This result indicates that the growth phenotype of the Δ*cyaB* is caused by a reduced cAMP level due to the absence of adenylate cylase.

3.4. Influence of the cyaB deletion on the cAMP concentration

The cAMP concentration was determined in triplicate with a commercially available immunoassay using cells harvested in the exponential growth phase (OD_{600} = 5). When cultivated on glucose. the cAMP concentration of the Δ*cyaB* mutant (18 ± 2 pmol (mg protein)$^{-1}$) was only about half that of the wild type (30 ± 12 pmol (mg protein)$^{-1}$). When grown with a glucose-acetate mixture. the cAMP concentration of the wild type was about 3-fold higher than on glucose alone (88 ± 22 pmol (mg

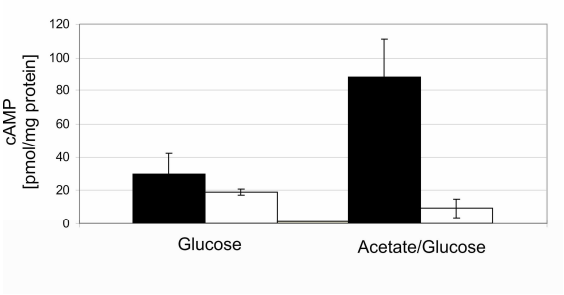

Fig. 5. Intracellular cAMP concentrations in WT (black) and Δ*cyaB* (white). Cells were grown in CGXII minimal medium containing 200 mM Glucose or 100 mM Sodium acetate and 100 mM Glucose. For measurements cells were harvested in the exponential growth phase (OD_{600} = 5).

protein)$^{-1}$) and about 10-fold higher than the concentration measured in the Δ*cyaB* mutant (9 ± 5 pmol (mg protein)$^{-1}$). These results support the function of CyaB as adenylate cyclase.

3.5. Expression profile of C. glutamicum ΔcyaB vs. wild type with GlxR target and strongest regulated genes.

The transcriptome comparisons of glucose-grown cells of the Δ*cyaB* mutant and the wild type revealed significant expression differences (mRNA ratio mutant/wild type either ≥2 or ≤0.5) for 25 genes (Table 3). Of the 25 differently expressed genes on glucose 7 genes are involved in transport mechanisms. As GlxR target genes within this group, *gntP* (gluconate permease) and *pstA* (ABC-type phosphate transport system) could be identified. The *gntP* gene, coding the permease involved in gluconate utilisation had an increased mRNA level in the Δ*cyaB* mutant and the *pstA* gene which is part of the *pstSCAB* operon coding for a high affinity ABC transporter for phosphate uptake had a decreased mRNA level in the Δ*cyaB* mutant. The other genes of the operon also showed at least 3-fold lower mRNA levels in all three experiments, but in these cases the p-value were above the threshold of 0.05. Genes of central metabolism like *prpB2, prpD2, prpC2*, coding for proteins involved in propionate utilization, were mostly upregulated. The only downregulated gene is *pck* which codes for phosphoenolpyruvate carboxy-kinase which catalysis the anaplerosis reaction from oxaloacetate to phosphoenolpyruvate. Except for *prpC2* and *ackA*, all the differently expressed genes of central metabolism are GlxR target genes. Furthermore, 9 genes coding for proteins of unknown function or proteins involved in other cellular processes are differently expressed in the Δ*cyaB* mutant but none is a GlxR target gene (Table 3).

The transcriptome comparisons of cells grown with an acetate-glucose mixture revealed 160 genes with an at least 3-fold changed mRNA ratio (Δ*cyaB*/WT), and thus a much larger number than observed in glucose-grown cells (Table 4). Of overall 160 differently expressed genes, 31 genes are involved in transport mechanisms of *C. glutamicum*. Within this group the *ptsS* gene, coding for the PTS system II for sucrose uptake, showed the highest increased mRNA level with an mRNA ratio of 30 (Δ*cyaB*/WT). Beside *ptsS*, other genes, coding for PTS components (*ptsG. ptsI. ptsF*) also showed also an increased mRNA level in the Δ*cyaB* mutant. The *pstA* gene (ABC-type phosphate transport system) had a decreased mRNA level in the Δ*cyaB* mutant as observed in the comparison of glucose grown cells. Focused on GlxR target genes, only the *pts* genes, *pstA* and *gluC* (glutamate permease) are present as GlxR targets within the group of genes with products involved in transport mechanisms. Regulated genes of central metabolism or respiration in the Δ*cyaB* mutant were mostly GlxR target genes. The strongest effect (mRNA ratio Δ*cyaB*/WT of 80 and 96) was observed for *sucCD*, coding for succinyl-CoA synthetase (Table 4), catalysing the reaction of succinate to succinyl-CoA in the TCA cycle. The two genes coding for proteins involved in propionate utilisation (*prpD1* and *prpC2*) showed an increased mRNA level in the Δ*cyaB* mutant as observed in the comparison of cells grown only on glucose. Also the other genes of the operons coding for proteins involved in propionate utilisation showed at least 3-fold increased

Table 3
Comparison of mRNA levels in glucose-grown cells of *C. glutamicum* wild type and the mutant strain Δ*cyaB* using DNA microarrays.

Gene ID	Gene name	Annotation	mRNA ratio Δ*cyaB*/WT	Predicted or verified GlxR target gene
		Transport		
		Non-PTS components		
cg2610		ABC-type dipeptide/oligopeptide/nickel transport system. secreted component	2.56	
cg2939		ABC-type dipeptide/oligopeptide/nickel transport system	2.68	
cg2938		ABC-type dipeptide/oligopeptide/nickel transport system. permease component	2.15	
cg3216	*gntP*	gluconate permease	2.36	v
cg0545	*pitA*	putative low-affinity phosphate transport protein	1.57	
cg0770		ABC-type cobalamin/Fe^{3+}-siderophores transport system. permease component	0.44	
cg2844	*pstA*	ABC-type phosphate transport system. permease component	0.08	p
		Central metabolism and respiration		
cg3096	*aldA*	aldehyde dehydrogenase	2.83	v
cg2836	*sucD*	succinyl-CoA synthetase alpha subunit	1.91	v
cg2837	*sucC*	succinyl-CoA synthetase subunit beta	1.08	v
cg0759	*prpD2*	methylcitrate dehydratase	2.72	p
cg0760	*prpB2*	methylisocitrate lyase	2.95	p
cg0762	*prpC2*	methylcitrate synthase	2.43	
cg3047	*ackA*	acetate kinase	2.00	
cg3107	*adhA*	Zn-dependent alcohol dehydrogenase	1.79	v
cg3169	*pck*	phosphoenolpyruvate carboxykinase (GTP)	0.53	v
		Further metabolism		
cg3195		flavin-containing monooxygenase (FMO)	3.23	
cg1612		acetyltransferase	3.08	
cg0645	*cytP*	cytochrome p450	2.53	
cg0998		trypsin-like serine protease	2.26	
cg2924		*cysS*. cysteinyl-tRNA synthetase	2.04	
cg1147	*ssuI*	FMN-binding protein required for sulfonate and sulfonate ester utilization	1.83	
cg2354	*tnp2e*	transposase ISCg2e	0.07	
cg0375	*cyaB*	putative adenylate cyclase	0.01	
		Proteins of unknown function		
cg1665		putative secreted protein	0.43	

The mRNA ratios represent mean values of at least two DNA microarray analyses starting from independent cultures. Only ratios with a p-value ≤ 0.05 are shown. The strains were cultivated in CGXII minimal medium with 200 mM glucose. RNA was isolated in the exponential growth phase at an OD_{600} of 5.

mRNA levels in all three experiments, but in these cases the p-values were above the threshold of 0.05. Next to the regulated genes of central metabolism and respiration, 21 genes involved in nitrogen, sulfur or iron metabolism had an altered mRNA level in the Δ*cyaB* mutant. All these 21 genes showed a significant increase in mRNA level, but only the genes of nitrogen metabolism (*amtB*, *gltB*, and *gltD*) are GlxR target genes. In the group of 37 genes of further metabolism, the strongest change in mRNA level in the Δ*cyaB* mutant was observed for GlxR target genes (cg3045, cg0347 and *fabG1*). Overall only 5 out of the 37 genes are GlxR target genes.

The transcriptome comparisons also revealed differently expressed genes coding for transcriptional regulators. Two of the differently expressed regulator genes are

GlxR targets (*whiB1* and *fruR*). The WhiB-like family of proteins is present throughout the actinomycetes, but absent from all other organisms evaluated so far (Molle et al., 2000; Soliveri et al., 2000). In *M. tuberculosis whiB* genes are involved in virolence or multi-drug resistance (Morris et al., 2005; Steyn et al., 2002). FruR is described as a potential activator of the *pts*-genes *ptsI*, *ptsH* and *ptsF* in the presence of fructose in *Corynebacterium glutamicum* strain R (Tanaka et al., 2008). The strongest regulation was observed for *ripA* and *sufR* (Nakunst et al., 2007; Wennerhold et al., 2005), coding regulators of iron and sulfur metabolism which might explain the differently expressed genes of iron and sulfur metabolism. Furthermore, 40 genes coding for proteins of unknown function showed an altered mRNA level in the Δ*cyaB* mutant but none is known as GlxR target. Taken together, the transcriptome comparisons of acetate-glucose grown cells of the Δ*cyaB* mutant and the wild type revealed much more differently expressed genes. Genes of central metabolism with changed mRNA levels in both comparisons were mostly GlxR target genes. Additionally all mRNA levels of experimentally verified GlxR target genes in both transcriptome comparisons (acetate-glucose and glucose-grown cells), are summarised in Table 5.

Table 4
Comparison of the mRNA levels of acetate-glucose-grown cells of *C. glutamicum* wild type compared with the mutant strain *ΔcyaB* using DNA microarrays.

Gene ID	Gene name	Annotation	mRNA ratio Δ*cyaB*/WT	Predicted or verified GlxR target gene
		Transport		
		PTS components		
cg2925	*ptsS*	PTS system enzyme II for sucrose	30.58	p
cg2120	*ptsF*	fructose-specific enzyme II BC component of PTS	7.27	p
cg1537	*ptsG*	glucose-specific enzyme II BC component of PTS	4.16	v
cg2117	*ptsI*	phosphoenolpyruvate:sugar phosphotransferase system enzyme I	3.84	v
		Non-PTS components		
cg2443		permease of the major facilitator superfamily	19.26	
cg2610		ABC-type dipeptide/oligopeptide/nickel transport system. secreted component	14.58	
cg0464	*ctpA*	copper-transporting ATPase	5.53	
cg3282		cation transport ATPase	4.62	
cg0589		ABC transporter. nucleotide binding/ATPase protein	4.15	
cg3367		ABC-type multidrug transport system. ATPase component	4.07	
cg3368		ABC-transporter permease protein	4.01	
cg3295		cation transport ATPase	3.82	
cg3112		predicted permease	3.51	
cg1228		ABC-type cobalt transport system. ATPase component	3.37	
cg1229	*ykoC*	transmembrane component YkoC of energizing module of thiamin-regulated ECF transporter for hydroxymethylpyrimidine	3.28	
cg1661	*arsB3*	arsenite permease	3.27	
cg1231	*chaA*	Ca^{2+}/H^{+} antiporter	3.08	
cg1411		ABC-type sugar (aldose) transport system. ATPase component	3.02	
cg1224	*phnB2*	similarity to alkylphosphonate uptake operon protein PhnB of *Escherichia coli*	3.00	
cg2844	*pstA*	ABC-type phosphate transport system. permease component	0.43	p
cg0545	*pitA*	putative low-affinity phosphate transport protein	0.32	

cg1169		Na^+-dependent transporters of the SNF family	0.32	
cg2524		putative beta (1→2) glucan export composite transmembrane/ATP-binding protein	0.32	
cg2138	*gluC*	glutamate permease	0.30	v
cg3053		permease of the major facilitator superfamily	0.30	
cg3395	*proP*	proline/ectoine carrier	0.29	
cg2557		predicted Na^+-dependent transporter	0.29	
cg1016	*betP*	glycine betaine transporter	0.25	
cg0176		permease (TCDB 9.A.29. putative 4-toluene sulfonate uptake permease (TSUP) family)	0.19	
cg1108	*porC*	porin	0.15	
cg0133		p-aminobenzoyl-glutamate transporter	0.06	
		Central metabolism and respiration		
cg2119	*pfkB*	1-phosphofructokinase protein	6.62	v
cg3107	*adhA*	Zn-dependent alcohol dehydrogenase	26.18	v
cg3096	*aldA*	aldehyde dehydrogenase	8.98	v
cg0762	*prpC2*	methylcitrate synthase	5.51	
cg0796	*prpD1*	propionate catabolic protein PRPD	3.98	p
cg2836	*sucD*	succinyl-CoA synthetase alpha subunit	96.21	v
cg2837	*sucC*	succinyl-CoA synthetase subunit beta	80.41	v
cg3047	*ackA*	acetate kinase	0.45	
cg3169	*pck*	phosphoenolpyruvate carboxykinase (GTP)	0.32	v
cg1343	*narH*	respiratory nitrate reductase	0.31	v
cg1341	*narI*	respiratory nitrate reductase γ chain	0.28	v
cg1342	*narJ*	respiratory nitrate reductase δ subunit	0.31	v
cg1344	*narG*	respiratory nitrate reductase α subunit	0.28	v
cg1345	*narK*	putative nitrate/nitrite transporter	0.24	v
cg1853	*glpD*	glycerol-3-phosphate dehydrogenase	0.29	
cg1725	*mcmB*	methylmalonyl-CoA mutase. large subunit	0.22	
cg1726	*mcmA*	methylmalonyl-CoA mutase. small subunit	0.18	
		Nitrogen metabolism		
cg2261	*amtB*	low affinity ammonium uptake protein	60.33	p
cg0229	*gltB*	glutamine 2-oxoglutarate aminotransferase large subunit	32.03	v
cg0230	*gltD*	glutamine 2-oxoglutarate aminotransferase small subunit	9.61	v
		Sulfur metabolism		
cg1380	*ssuA*	aliphatic sulfonate binding protein	7.82	
cg1147	*ssuI*	FMN-binding protein required for sulfonate and sulfonate ester utilization	7.21	
cg1214		cysteine sulfinate desulfinase/cysteine desulfurase or related enzyme	5.52	
		Iron metabolism		
cg1759		predicted metal-sulfur cluster biosynthetic enzyme	7.73	
cg1760	*sufU*	cysteine desulfhydrase	11.01	
cg1761	*sufS*	cysteine desulfhydrase/selenocysteine lyase	18.20	
cg1762	*sufC*	iron-regulated ABC transporter ATPase subunit	12.99	
cg1763	*sufD*	components of an uncharacterized iron-regulated ABC-type transporter	13.57	
cg1764	*sufB*	component of an uncharacterized iron-regulated ABC-type transporter	12.94	
cg0591		cobalamin/Fe^{3+}-siderophores transport system. permease component	4.97	
cg0768		ABC-type cobalamin/Fe^{3+}-siderophores transport system. ATPase component	4.83	
cg0924		ABC-type cobalamin/Fe^{3+}-siderophores transport system	4.77	

cg0590		cobalamin/Fe^{3+}-siderophores transport system. permease component	4.35	
cg0767		siderophore-interacting protein	4.18	
cg0769		ABC-type cobalamin/Fe^{3+}-siderophores transport system. permease component	3.79	
cg1418		ABC-type cobalamin/Fe^{3+}-siderophores transport system secreted component	3.78	
cg0770		ABC-type cobalamin/Fe^{3+}-siderophores transport system. permease component	3.51	
cg0467		cobalamin/Fe^{3+}-siderophores transport system. secreted component	3.50	
		Further metabolism		
cg0345		metal-dependent hydrolase of the TIM-barrel fold	64.38	p
cg0347		acyl dehydratase	53.54	p
cg0344	*fabG1*	3-oxoacyl-(acyl-carrier protein) reductase	25.97	v
cg3195		flavin-containing monooxygenase (FMO)	24.27	
cg3411		copper chaperone	9.93	
cg2796		MMGE/PRPD family protein (putative 2-methylcitrate dehydratase)	9.34	
cg1476	*thiC*	thiamine biosynthesis protein	7.43	
cg0645	*cytP*	cytochrome p450	6.75	
cg1218		ADP-ribose pyrophosphatase	6.25	
cg1215	*nadC*	nicotinate-nucleotide pyrophosphorylase	5.54	
cg1216	*nadA*	quinolinate synthetase	4.65	
cg1612		acetyltransferase	5.24	
cg1118		pyrimidine reductase. riboflavin biosynthesis	4.86	
cg0404		nitroreductase family	4.67	
cg1580	*argC*	N-acetyl-γ-glutamyl-phosphate reductase	4.42	
cg2591	*dkgA*	2.5-diketo-D-gluconic acid reductase	4.36	
cg0998		trypsin-like serine protease	4.33	
cg1930		putative secreted hydrolase	3.98	
cg2962		uncharacterized enzyme involved in biosynthesis of extracellular polysaccharides	3.95	
cg0291		3.4-dioxygenase beta subunit	3.94	
cg1055	*menG*	ribonuclease activity regulator protein RraA	3.75	
cg2999		putative ferredoxin reductase	3.37	
cg2617	*vanB*	vanillate demethylase	3.36	p
cg2838		predicted dithiol-disulfide isomerase	3.20	
cg1583	*argD*	acetylornithine aminotransferase	3.11	p
cg1134	*pabAB*	p-aminobenzoate synthase component I and II	3.08	
cg2238	*thiS*	sulfur transfer protein involved in thiamine biosynthesis	3.02	
cg2924		*cysS*. cysteinyl-tRNA synthetase	2.17	
cg3275	*fdxA*	ferredoxin	7.26	
cg3346	*leuS*	leucyl-tRNA synthetase	0.32	
cg1332		putative secreted hydrolase	0.31	
cg0538		putative serine protease (ClpP class)	0.31	
cg2928	*nagB*	N-acetylglucosamine-6-phosphate isomerase	0.29	
cg3054	*purT*	5'-phosphoribosylglycinamide transformylase	0.28	
cg2398	*plsC*	1-acyl-sn-glycerol-3-phosphate acetyltransferase	0.24	
cg0723	*crtE*	geranylgeranyl-pyrophosphate sythase	0.23	
cg1628		hydrolase of the α/β superfamily	0.20	
cg0375	*cyaB*	putative adenylate cyclase	0.00	
		DNA/RNA metabolism		
cg3274		site-specific recombinases. DNA invertase Pin homolog-fragment	21.77	
cg0844		type II restriction enzyme. methylase subunit	0.33	
cg2241		putative transcriptional accessory protein. RNA	0.32	

		binding		
cg1457	*dnaQ2*	DNA polymerase III. putative ε subunit	0.28	
		Transcriptional regulators		
cg1120	*ripA*	transcriptional regulator of AraC family	26.77	
cg1765	*sufR*	predicted transcriptional regulator	13.71	
cg0878	*whiB1*	transcription factor	4.75	p
cg2118	*fruR*	transcriptional regulator of sugar metabolism. DeoR family	3.41	v
cg0709	*cgtR7*	two component response regulator	0.27	
cg2500		bacterial regulatory protein. ArsR family	0.28	
		Transposases		
cg1023	*tnp6b*	transposase ISCg6a	9.99	
cg1030	*tnp6c*	transposase ISCg6c	4.08	
cg1022	*tnp6a*	transposase ISCg6a	3.62	
cg0692	*tnp1c*	transposase ISCg1c	3.02	
		Proteins of unknown function		
cg0470		conserved secreted protein	54.47	
cg3156		putative secreted protein	26.51	
cg0366		hypothetical protein cg0366	14.64	
cg3277		uncharacterized ACR. double-stranded beta-helix domain	10.82	
cg1227		hypothetical protein cg1227	8.32	
cg0160		hypothetical protein	7.55	
cg1940		putative secreted protein	6.51	
cg3280		putative secreted protein	6.20	
cg2651		conserved hypothetical protein-fragment	5.91	
cg3268		hypothetical protein	5.14	
cg3372		hypothetical protein	4.86	
cg3283		hypothetical protein predicted by Glimmer	4.75	
cg0466		conserved secreted protein	4.71	
cg0463		hypothetical protein	4.05	
cg1230		hypothetical protein	3.60	
cg1232		hypothetical protein	3.59	
cg1087		hypothetical protein	3.50	
cg0364		membrane protein	3.47	
cg2853		conserved hypothetical protein-fragment	3.47	
cg3269		hypothetical protein	3.45	
cg3270		hypothetical protein predicted by Glimmer	3.44	
cg1202		hypothetical protein	3.32	
cg0372		hypothetical protein	3.31	
cg0105		hypothetical protein	3.37	
cg1091		hypothetical protein	3.23	
cg2025		hypothetical protein predicted by Glimmer	3.21	
cg3337		hypothetical protein	3.19	
cg1660		hypothetical protein	3.17	
cg2564		hypothetical protein	3.14	
cg1050		hypothetical protein	3.07	
cg2803		hypothetical protein	0.33	
cg0411		hypothetical protein	0.33	
cg0620		secreted protein	0.32	
cg2128		putative secreted or membrane protein	0.33	
cg3214		hypothetical protein	0.26	
cg1562		hypothetical protein	0.21	
cg1348		membrane protein containing CBS domain	0.19	
cg0905	*psp2*	putative secreted protein	0.16	
cg1349		membrane protein containing CBS domain	0.11	

The mRNA ratios represent mean values of at least two DNA microarray analyses starting from independent cultures. Only ratios with a p-value ≤ 0.05 are shown. The strains were cultivated in CGXII minimal medium with 100 mM glucose and 100 mM acetate. RNA was isolated in the exponential growth phase at an OD_{600} of 5.

4. Discussion

Nucleotide cyclases or CHDs are well spread over bacterial genomes. Over 200 CHDs of various domain composition have been sequenced (Linder and E., 2003; Shenoy et al., 2004). In many cases more than one class III adenylate cyclase is found in a bacterial genome, as for example, in *M. tuberculosis*. In *C. glutamicum* only one adenylate cyclase (CyaB) is annotated. The domain composition indicates that CyaB might recognize extracellular signals and stimulate cytosolic effectors. However, a signal or ligand which might be recognized could not yet be identified.

First of all, an in frame deletion mutant was constructed to study the biological function of the exclusive adenylate cycle in *C. glutamicum*. Unexpectedly, a low level of cAMP was detected in the constructed deletion mutant *C. glutamicum* Δ*cyaB*. The results showed a decreased cAMP level in *ΔcyaB* cells grown in glucose medium (Fig. 5). In contrast to recent Δ*cyaB* studies published by Cha et al. (2009), we detected a significantly lower level of intracellular cAMP in the wild type and Δ*cyaB* strains.

The published cAMP-level by Kim et al. (2004) for glucose-grown wild type cells was even higher as reported in Cha et al. (2009). These unexpected varying results support the idea of a major difference in sample preparation and question the absolute cAMP concentration in *C. glutamicum* cells. The cAMP level in cells grown in acetate/glucose mixture was significant higher compared to the level of glucose-grown cells contrary to the decreased cAMP level in acetate medium compared to glucose medium reported by (Cha et al., 2009; Kim et al., 2004). The source of remaining cAMP in the Δ*cyaB* mutant has not been identified yet and needs to be further analysed.

Deletion of *cyaB* in *C. glutamicum* revealed a growth defect in minimal medium containing acetate or medium mixtures supplied with acetate (acetetate/glucose; acetate/gluconate; acetate/ethanol) (Figs. 2 and 3). Complementation studies providing plasmid encoded *cyaB* or extracellular cAMP showed that the growth phenotype is specifically caused by the *cyaB* deletion and a decreased cAMP concentration, as it could be reverted by plasmidborne *cyaB* and caused by decreased cAMP concentrationby supplementation of the medium with cAMP.

The growth defect was observed in acetate, but not in glucose medium although the cAMP concentration is higher in glucose grown cells (Cha et al., 2009; Kim et al., 2004). Furthermore, activities of enzymes essential for growth on acetate or ethanol (Arndt et al., 2008; Arndt and Eikmanns, 2007), could be varified in Δ*cyaB*. PTA (Phosphotransacetylase) and AK (Acetate kinase) converting acetate to acetyl-CoA showed in Δ*cyaB* the same activity as in the wild type (data not shown). Also, MS (malate synthase) and ICL (isocitrate lyase) essential components of the glyoxylate cycle, showed levels of activity akin to the wildtype in the Δ*cyaB*-mutant (Cha et al., 2009).

Beside acetate, the gluconeogenic substrates propionate and ethanol were examined. Both propionate and acetate are transported into the cell by the monocarboxylic acid transporter (MctC) (Jolkver et al., 2009). Ethanol is oxidised to acetate in two steps by alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH), and subsequently activated to acetyl-CoA. Therefore, the enzymes AK, PTA, ICL, and MS, which are components of the acetate metabolism, are also essential for growth on ethanol. Growth of the Δ*cyaB* mutant was inhibited on propionate but not on ethanol, indicating that the enzymes involved in acetate metabolism were active. The reason for growth defects on acetate must be the presence of extracellular acetate or an inhibited import of acetate. Although acetate and propionate are transported by the same uptake carrier MctC, it is unlikely that the growth defect is caused by a

Table 5
Comparison of mRNA levels (Δ*cyaB*/WT) of predicted or experimentally verified GlxR target genes in *C. glutamicum*

Gene ID	Gene name	Annotation	mRNA ratios Glc/Ace	Glc
Central carbohydrate metabolism				
cg0791	*pyc*	pyruvate carboxylase	0.75	1.09
cg1142		Na+/proline. Na+/panthothenate symporter	1.43	1.14
cg1143		putative GntR-family transcriptional regulator	1.06	-
cg1537	*ptsG*	glucose-specific enzyme II BC component of PTS	4.16	1.56
cg2117	*ptsI*	phosphoenolpyruvate:sugar phosphotransferase system enzymei	3.84	1.60
cg2119	*pfkB*	1-phosphofructokinase protein	6.61	1.24
cg2118	*fruR*	transcriptional regulator of sugar metabolism. DeoR family	3.41	1.49
cg2560	*aceA*	isocitrate lyase	1.33	0.90
cg3107	*adhAe*	Zn-dependent alcohol dehydrogenase	26.18	1.79
cg3219	*ldh*	L-lactate dehydrogenase	0.38	1.60
cg3220		hypothetical protein cg3220	-	-
cg0949	*gltA*	citrate synthase	1.54	0.93
cg1737	*acn*	aconitate hydratase	1.49	1.04
cg2837	*sucC*	succinyl-CoA synthetase subunit beta	80.41	1.08
cg0445	*sdhC*	succinate dehydrogenase CD	0.96	1.24
cg2559	*aceB*	malate synthase	1.84	1.03
cg2732	*gntK*	putative gluconokinase	-	-
cg3216	*gntP*	gluconate permease	2.11	2.36
cg1145	*fum*	fumarate hydratase	0.77	1.04
cg2846	*pstS*	ABC-type phosphate transport system. secreted component	0.66	0.53
cg3169	*pck*	phosphoenolpyruvate carboxykinase (GTP)	0.32	0.53
cg3226		putative L-lactate permease	0.13	1.95
Aromatic compound degradation				
cg1226	*pobB*	pobB. 4-hydroxybenzoate 3-monooxygenase	1.81	1.37
cg1309		3-(3-hydroxyphenyl)propionate hydroxylase	-	-
cg2616	*vanA*	vanillate demethylase. oxygenase subunit	-	3.28
cg2631	*pcaH*	protocatechuate dioxygenase beta subunit	-	1.16
cg2636	*catA*	catechol 1.2-dioxygenase	2.55	1.15
cg2637	*benA*	benzoate 1.2-dioxygenase alpha subunit	-	-
cg2642	*benK*	putative benzoate transport protein	-	-
cg2953	*xylC*	benzaldehyde dehydrogenase	0.58	1.06
cg2965		AraC-type transcriptional regulator	-	0.31
cg2966		phenol 2-monooxygenase	-	3.71
cg3351	*nagI*	gentisate 1.2-dioxygenase	7.65	0.93
cg3352	*nagR*	transcriptional regulator of gentisate pathway	-	-
cg3353	*nagT*	gentisate transporter	-	-
cg3388		bacterial regulatory proteins. IclR family		1.41
Aerobic and anaerobic respiration				
cg1345	*narK*	putative nitrate/nitrite transporter	0.24	1.17
cg1656	*ndh*	NADH dehydrogenase	0.85	1.27
cg1766	*mptB*	Mannosyltransferase	-	-
cg2406	*ctaE*	cytochrome C oxidase subunit 3	0.67	0.81
cg2409	*ctaC*	cytochrome C oxidase chain II	0.63	1.08
cg2780	*ctaD*	probable cytochrome C oxidase polypeptide subunit	0.69	0.99

Glutamate uptake and nitrogen assimilation				
cg0229	*gltB*	glutamine 2-oxoglutarate aminotransferase large SU	32.03	-
cg2136	*gluA*	glutamate uptake system ATP-binding protein	0.46	1.07
cg2280	*gdh*	glutamate dehydrogenase	1.53	1.04
cg2429	*glnA*	glutamine synthetase I	1.69	0.90
Fatty acid biosynthesis				
cg0344	*fabG1*	3-oxoacyl-(acyl-carrier protein) reductase	25.97	-
cg0812	*dtsr1*	Racetyl/propionyl-CoA carboxylase beta chain	1.62	1.12
cg0814	*birA*	biotin--protein ligase	0.74	0.97
cg0951	*accDA*	acetyl-coenzyme A carboxylase carboxyl transferase	0.58	0.93
cg0957	*fas-IB*	fatty acid synthase	0.89	0.88
cg2743	*fas-IA*	fatty acid synthase	0.93	0.83
Other cellular processes				
cg0878	*whcE*	positive role in survival under (heat and oxidative) stress	4.75	0.96
cg0879		membrane protein	1.62	-
cg0936	*rpf1*	resuscitation promoting factor	1.07	0.95
cg1595	*uspA2*	universal stress protein UspA or related nucleotide-binding protein	0.11	0.44
cg1956	*recJ*	single-stranded-DNA-specific exonuclease	-	-
cg2402	*nlpC*	putative secreted cell wall peptidase	1.04	1.19
cg2781	*nrdF*	ribonucleotide-diphosphate reductase beta subunit	1.24	0.91
cg1037	*rpf2*	resuscitation promoting factor	0.86	1.10
cg3096	*aldA*	alcohol degyhdrogenase	8.97	2.83
Regulators				
cg0350	*glxR*	cAMP-dependent transcriptional regulator	1.42	1.03
cg0444	*ramB*	transcriptional regulator. involved in acetate metabolism	2.31	0.84

The mRNA ratios represent mean values of at least two DNA microarray analyses starting from independent cultures. Only ratios with a p-value ≤ 0.05 are shown. The strains were cultivated either in CGXII minimal medium with 200 mM glucose or in CGXII minimal medium with 100 mM glucose and 100 mM acetate. RNA was isolated in the exponential growth phase at an OD_{600} of 5.

malfunctioning carrier in the Δ*cyaB* mutant since a Δ*mctC* mutant is able to grow on acetate (Jolkver et al., 2009). If the carrier is not able to operate in the Δ*cyaB* mutant, acetate would still diffuse into the cells. Another possibility might be that extracellular acetate affects the proton motive force in the Δ*cyaB* mutant and therefore growth is inhibited.

We also tested the Δ*cyaB* mutant for growth on substrate mixtures containing acetate. Growth of Δ*cyaB* mutant on the single carbon sources glucose, ethanol or gluconate was not effected (Fig. 2), but addition of acetate inhibited growth of the Δ*cyaB* mutant in all cases (Fig. 3). The strongest effect was observed on acetate/ethanol mixture. No growth or carbon source consumption could be detected for Δ*cyaB*. The growth defect of Δ*cyaB* on acetate/glucose mixture went along with inhibited glucose consumption, in particular during the first 12 hours The effects on growth and substrate consumption of the second carbon source which is standardly metabolised if solely present, show that extracellular acetate seems to be sensed and triggers the observed effects.

In *E. coli* as well as in a broad range of other bacteria a cAMP-binding transcriptional regulator (CRP or CRP homologous) regulates gene expression depending on intracellular cAMP level (Botsford and Harman, 1992). In *C.*

glutamicum the CRP homologous GlxR is known to be a global transcriptional regulator with a theoretical regulon covering 14 % of the annotated genes (Kohl and Tauch, 2009). cAMP as effector molecule regulating carbon metabolism, has been shown for the glyoxylate bypass and the TCA cycle (Bott, 2007; Bussmann, 2009; Kim et al., 2004). By using a Δ*cyaB* mutant with a defect in cAMP-syntheses we have presented transcriptome comparisons with the wild type for glucose and acetate-glucose grown cells. The transcriptome comparisons of glucose-grown cells showed that only 25 genes had an altered mRNA level in the Δ*cyaB* mutant (mRNA-ratio Δ*cyaB*/WT either ≥2 or ≤0.5). Considering that the cAMP levels of glucose-grown cells are higher compared to the cAMP-levels of acetate grown cells, a higher influence on gene expression in glucose-grown cells was expected. However, growth of the Δ*cyaB* mutant is inhibited in acetate containing medium, but not in glucose medium. 9 out of 25 differently expressed genes could be identified as GlxR targets, of which the majority code for proteins of central metabolism.

In the transcriptome comparison of acetate-glucose grown cells many more genes showed an altered mRNA level in the Δ*cyaB* mutant.

From the batch of GlxR-targets, genes of the PTS components are upregulated in the Δ*cyaB* mutant, including *ptsG* which codes for the EII protein for glucose uptake (Table 4). In contrast to our expectations the glucose consumption in Δ*cyaB* cells was inhibited during the first 12 hours of growth although *ptsG* was upregulated (Fig. 3). This indicates that GlxR acts as a repressor of the *pts* genes and that inhibited glucose consumption is not related to a *ptsG* repression on transcriptional level. Therefore, PtsG activities must be influenced by the absence of CyaB in combination with the extracellular acetate.

GntP (gluconate permease) and *gluC* (glutamate permease) represent further target genes of GlxR involved in transport processes which showed altered expressionpatterns. *C. glutamicum* wild type cells grown on citrate show a higher intracellular cAMP concentration and an inverse regulation of *gntP*, *gluC* and *pts* genes compared to our results, indicating the direct influence of GlxR by changed cAMP level (Polen et al., 2007).

Other genes which are experimentally proven targets of GlxR as for example genes of the glyoxylate bypass *aceA*, *aceB* or *sdhCAB* of the TCA cycle (Bussmann, 2009; Kim et al., 2004) are unexpectedly not in the evaluable list of genes in the transcriptome comparisons.

The results of this study show that the Δ*cyaB* mutant should be capable to metabolize acetate or propionate, since all enzymes which are essential for acetate metabolism seem to be active because Δ*cyaB* grows on ethanol. On transcriptome level, no genes of acetate or propionate metabolism are significantly downregulated. The results suggest that in the absence of the adenylate cyclase extracellular acetate inhibits growth and glucose consumption of *C. glutamicum*. In order to determine the biochemical processes of the adverse effect on glucose uptake and growth further investigation is required. Target genes of the cAMP-binding transcriptional regulator GlxR were mostly represented in the list of genes coding for proteins involved in central metabolism. The characterized GlxR regulon covers more than 10 % of the annotated genes in *C. glutamicum* (Kohl and Tauch, 2009) Therefore a similar role in carbon catabolite regulation, as it is reported for its homologous CRP in. *E. coli,* is assumed.

However, there is still little information on cAMP regulation in *C. glutamicum* which needs further investigation in order to determine the regulatory network of cAMP. Moreover, studies on an extracellular signal which modulates CyaB activity is expect to give a better understanding which environmental conditions affect intracellular cAMP level.

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RosR (Cg1324), a redox-sensing MarR-type transcriptional regulator of *Corynebacterium glutamicum*

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The cg1324 gene (*rosR*) of *Corynebacterium glutamicum* encodes a MarR-type transcriptional regulator. By a comparative transcriptome analysis with DNA microarrays of a Δ*rosR* mutant and the wild type and subsequent electrophoretic mobility shift assays (EMSAs) with purified RosR protein, direct target genes of RosR were identified. The *narKGHJI* operon, which encodes a nitrate/nitrite transporter and the nitrate reductase complex, was found to be activated by RosR. All other target genes were found to be repressed by RosR. They encode four putative monooxygenases, two putative FMN reductases, a protein of the glutathione S-transferase family, a putative polyisoprenoid-binding protein and RosR itself. The DNA binding site of RosR was characterized as a 20-bp inverted repeat with the consensus sequence WTTGTTGAYR-YRTCAACWAA (RosR box). The *in vitro* DNA binding activity of RosR was inhibited by the oxidant H_2O_2. Mutational analysis of the three cysteine residues present in RosR (Cys64, Cys92, Cys151) showed that these are essential for redox-dependent DNA-binding of RosR. A disruptant of cg1322 lacking the putative polyisoprenoid-binding protein showed an increased sensitivity to H_2O_2, supporting a role of RosR in the oxidative stress response of *C. glutamicum*.

Corynebacterium glutamicum is a predominantly aerobic, nonpathogenic, biotin-auxotrophic Gram-positive soil bacterium that was isolated by Kinoshita and co-workers (1) in a screen for bacteria that excrete L-glutamate, which as monosodium glutamate is used as a flavor enhancer. Over the past 45 years, different strains of this species have been used for the industrial production of L-glutamate (2) and several other amino acids, in particular the feed additive L-lysine. Today, more than two million tons of amino acids are produced annually with *C. glutamicum*.

Because of its importance in industrial biotechnology, the genome sequence of *C. glutamicum* has been determined several times independently (3,4) and the species became a model organism for systems biology (5). In this context, we and others started to study the function of transcriptional regulators in this species. Based on the genome sequence, 127 one-component DNA-binding transcriptional regulators, 13 two-component signal transduction systems and 7 sigma factors were annotated (6,7). Within the past years, we analysed five of the one-component regulators, i.e. ClgR (8-10), AcnR (11), RipA (12), DtxR (13), GntR1 and GntR2 (14) and three of the two-component systems, MtrAB (15,16), PhoRS (7,17)) and CitAB (18).

In this work, we performed a comprehensive analysis of a previously uncharacterized MarR-type regulator of *C. glutamicum*. With nine so far uncharacterized representatives, the MarR family is one of the most prevalent families of transcriptional regulators in *C. glutamicum* (6). The MarR (multiple

antibiotic resistance regulator) family includes proteins that are critical for control of virulence factor production, response to environmental stresses, or can be involved in regulation of catabolic pathways for aromatic compounds (19). In general these regulators exist as homodimers in free and DNA-bound state. Most members of the MarR family are transcriptional repressors and often bind to the -10 or -35 region in the promoter causing a steric inhibition of RNA polymerase binding (19).

The MarR-type regulator studied here is encoded by the gene cg1324. Our results indicate that the Cg1324 protein is involved in the response of *C. glutamicum* to oxidative stress and therefore we named the corresponding protein RosR, an acronym for *regulator of oxidative stress response*.

EXPERIMENTAL PROCEDURES

Bacterial strains, media and growth conditions – All strains and plasmids used in this work are listed in Table 1. The *C. glutamicum* type strain ATCC 13032 (1) was used as wild type. Strains Δ*rosR* and Δcg1322 are derivatives with in-frame deletions of the *rosR* gene and the cg1322 gene, respectively. All *C. glutamicum* strains were routinely grown at 30°C. For growth experiments, chloramphenicol acetyltransferase (CAT) assays and RNA isolations, 5 ml of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA) was inoculated with colonies from a fresh BHI agar plate and incubated for 6 h on an orbital shaker at 120 rpm. After washing, the cells of this first preculture were used to inoculate a 500-ml baffled shake flask containing 60 ml of CGXII minimal medium (Eggeling and Reyes, Corynebuch Chapter 23) with 4% (w/v) glucose. This second preculture was incubated overnight at 120 rpm and then used to inoculate the main culture (CGXII minimal medium with 4% (w/v) glucose) to an optical density at 600 nm (OD_{600}) of ~1. The trace elements were always added after autoclaving. If appropriate, kanamycin was added to a final concentration of 25 µg/ml. For anaerobic cultivation of *C. glutamicum*, an 5-ml aerobic preculture in LB medium was inoculated with cells from a fresh BHI agar plate and cultivated overnight at 120 rpm. After washing with BT medium (20), cells were used to inoculate 100 ml anoxic BT medium containing 0.5% (w/v) glucose, 30 mM potassium nitrate as terminal electron acceptor and 1 µg/ml resazurin as oxygen indicator. The medium was prepared in an 200-ml bottle and oxygen was removed by bubbling with 100% nitrogen gas for 2 h. Subsequently, the bottle was closed with a rubber stopper. After inoculation with a syringe, the bottles were gently shaken at 120 rpm and 30°C. For all cloning purposes, *Escherichia coli* DH5α was used. Overproduction of RosR with plasmid pET16b-rosR was performed with *E. coli* BL21(DE3)/pLysS. The *E. coli* strains were cultivated in Luria Bertani (LB) medium (21) at 37°C or at room temperature for RosR overproduction. If appropriate, ampicillin (100 µg/ml), or kanamycin (50 µg/µl) was added.

General DNA techniques and sequence analysis - Standard methods such as PCR, restriction digestion or ligation were carried out according to established protocols (22). *E. coli* was transformed as described (23). DNA sequencing was performed with a 3100-Avant genetic analyser (Applied Biosystems, Darmstadt, Germany). Sequencing reactions were carried out with the ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Alternatively, sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). All oligonucleotides used in this study are listed in Table S1.

Mapping of transcriptional start sites by primer extension analysis - To determine the transcriptional start site of the genes cg1322, cg1426 and cg3084, primer extension analysis was performed as described previously (8) using the IRD800-labelled oligonucleotides cg1322-1, cg1322-2, cg1426-1, cg1426-2, cg3084-1, cg3084-2 listed in Table S1. Total RNA was isolated as described previously (24) from *C. glutamicum* Δ*rosR* cells grown in

glucose minimal medium up to the exponential growth phase (OD_{600} 5–6). The length of the primer extension products was determined by running the four lanes of a DNA sequencing reaction set up using the same IRD800-labelled oligonucleotide as for reverse transcription alongside the primer extension products. As templates for the sequencing reactions PCR products were used that were obtained with the oligonucleotide pairs cg1322-A/cg1322-B, cg1426-A/cg1426-B and cg3084-A/cg3084-B (starting upstream and downstream of the respectively start codon, Table S1) and chromosomal DNA of *C. glutamicum* wild type as template.

Mapping of transcriptional start sites by rapid amplification of cDNA ends (RACE) - To determine the transcriptional start site of the genes *rosR,* cg1150*,* cg2329 and cg1848, 5´- RACE analysis was performed using the gene-specific primers cg1324-sp1, cg1324-sp2, cg2329-sp1, cg2329-sp2, cg1150-sp1, cg1150-sp2, cg1848-sp1, cg1848-sp2 (Table S1) and the "5´/3´ RACE Kit, 2nd Generation" (Roche Diagnostics, Mannheim, Germany). Total RNA was isolated as described previously (24) from *C. glutamicum* Δ*rosR* cells grown in glucose minimal medium up to the exponential growth phase (OD_{600} 5–6). Subsequent PCR amplification of the 5´-junction and sequencing identified transcriptional start sites.

Construction of strains and plasmids - In-frame deletion mutants of *C. glutamicum* lacking either the *rosR* gene or gene cg1322 were constructed *via* a two step homologous recombination procedure as described previously (25). The oligonucleotides used for this purpose (D1-1324, D2-1324, D3-1324 and D4-1324 for the *rosR* deletion, D1-cg1322, D2-cg1322, D3-cg1322 and D4-cg1322 for the cg1322 deletion) are listed in Table S1. The chromosomal deletions were confirmed by PCR using oligonucleotide pairs D1324-fw/D1324-rv and Dcg1322-fw/Dcg1322-rv.

For construction of the expression plasmid pET16b-rosR which codes for a RosR derivative with an N-terminal decahistidine tag, the *rosR* coding region was amplified by PCR using oligonucleotides 1124-NdeN and 1124-XhoC, which included *Nde*I and *Xho*I restriction sites for cloning purposes (Table S1). After digestion with *Nde*I and *Xho*I, the PCR product was cloned into the expression vector pET16b (Novagen). The PCR-derived part of the resulting pET16b-rosR plasmid and the ligation sites were sequenced in order to exclude unwanted mutations. The RosR protein encoded by this plasmid contained 21 additional amino acids (MGHHHHHHHHHHSSGHIEGRH) at the amino terminus, including a factor Xa cleavage site (SGHIEGR). Plasmids pET16b-rosR-C64S, -C92S, -C151S, -C64,92S, -C64,151S, -C92,151S and -C64,92,151S, which code for RosR muteins in which the three cysteine residues C64, C92 and C151 were replaced by serine residues, were constructed using the QuickChange XL Site Directed Mutagenesis Kit (Stratagene) using pET16b-rosR as template and the oligonucleotides c1-for, c1-rev, c2-for, c2-rev, c3-for and c3-rev (Table S1). All recombinant plasmids were sequenced in order to exclude unwanted mutations.

For construction of the pET2-rosR plasmid which was used for chloramphenicol acetyltransferase assays, a 282 bp promoter fragment of *rosR* (extending from position +10 to –272 with respect to the *rosR* transcriptional start site) was amplified by PCR with oligonucleotides containing *Bam*HI and *Sac*I restriction sites for cloning (pET2-1324-for and pET2-1324-rev; Table S1). After digestion with *Bam*HI and *Sac*I, the PCR product was cloned into the promoter-probe plasmid pET2 (26). The PCR-derived part of the resulting pET2-rosR plasmid and the ligation sites were sequenced in order to exclude unwanted mutations.

Overproduction and purification of RosR - For overproduction of RosR and its mutated derivatives, *E. coli* BL21(DE3)/pLysS was transformed with expression plasmid pET16b-rosR and cultivated in LB medium at 37°C to an

TABLE I
Bacterial strains and plasmids used in this study

Strains / plasmids	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (r_k^- , m_k^+) *supE44 thi-1 gyrA96 relA1 phoA*, host for cloning purposes	Invitrogen
E. coli BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^-, m_B^-) *gal dcm* (DE3); host for overproduction of GlxR, RamA and RamB	(58)
C. glutamicum ATCC 13032	Biotin-auxotrophic wild-type strain	American Type Culture Collection
C. glutamicum Δ*rosR*	Strain with an in-frame deletion of the *rosR* (cg1324) gene	This work
Plasmids		
pK19mobsacB	Kan^R; vector for allelic exchange in *C. glutamicum* (pK18 *oriV*$_{E.c.}$, *sacB*, *lacZ*α)	(59)
pK19mobsacB-ΔrosR	Kan^R; pK19mobsacB derivative containing an overlap-extension PCR product composed of the up- and downstream regions of *rosR*	This work
pET16b	Amp^R; plasmid for overexpression of genes in *E. coli,* adding a C-terminal decahistidine tag to the target protein (pBR322 *oriV*$_{E.c.}$, *P*$_{T7}$, *lacI*)	Novagen
pET16b-rosR	Kan^R; pET16b derivative for overproduction of RosR with an N-terminal decahistidine tag	This work
pET16b-rosR-$_{C64S}$	pET16b-rosR derivative coding for a RosR mutein with a Cys64→Ser exchange	This work
pET16b-rosR$_{C92S}$	pET16b-rosR derivative coding for a RosR mutein with a Cys92→Ser exchange	This work
pET16b-rosR$_{C151S}$	pET16b-rosR derivative coding for a RosR mutein with a Cys151→Ser exchange	This work
pET16b-rosR$_{C64,92S}$	pET16b-rosR derivative coding for a RosR mutein with Cys64,92→Ser exchanges	This work
pET16b-rosR$_{C64,151S}$	pET16b-rosR derivative coding for a RosR mutein with Cys64,151→Ser exchanges	This work
pET16b-rosR$_{C92,151S}$	pET16b-rosR derivative coding for a RosR mutein with Cys92,151→Ser exchanges	This work
pET16b-rosR$_{C64,92,151S}$	pET16b-rosR derivative coding for a RosR mutein with Cys64,92,151→Ser exchanges	This work
pAN6	Kan^R; *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression; derivative of pEKEx2 (*P*$_{tac}$, *lacI*^{q}, pBL1 *oriV*$_{C.g.}$, pUC18 *oriV*$_{E.c.}$)	(14)
pAN6-rosR	Kan^R; pAN6 derivative carrying the *rosR* gene under the control of an IPTG-inducible *tac* promoter	This work
pET2	Kan^R; promoter probe plasmid for *C. glutamicum*	(26)
pET2-rosR	Kan^R; pET2-derivative with a 284 bp fragment covering the *C. glutamicum rosR* promoter	This work

OD_{600} of 0.5. Then expression of the target gene was induced by addition of 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and the culture was incubated for another 3 h at room temperature. After the cells were harvested, purification of RosR by Ni^{2+}-NTA affinity chromatography was performed essentially as described previously for the RipA protein (12). The RosR-containing elution fractions were desalted by size-exclusion chromatography using PD10 columns (GE Healthcare) and binding buffer containing 20 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5% (v/v) glycerol, and 0.5 mM EDTA.

Global gene expression analysis using DNA microarrays - Preparation of RNA and synthesis of fluorescently labelled cDNA were carried out as described (35). Custom-made DNA microarrays for *C. glutamicum* ATCC 13032 printed with 70mer oligonucleotides were obtained from Operon (Cologne, Germany) and are based on the genome sequence entry NC_006958 (21). Hybridization and stringent washing of the microarrays were performed according to the instructions of the supplier. Hybridization was carried out for 16–18 h at 42°C using a MAUI hybridization system (BioMicro Systems, Salt Lake City, USA). After washing the microarrays were dried by centrifugation (5 min, 1600 *g*) and fluorescence was determined at 532 nm (Cy3-dUTP) and 635 nm (Cy5-dUTP) with 10 µm resolution using an Axon GenePix 6000 laser scanner (Axon Instruments, Sunnyvale, USA). Quantitative image analysis was carried out using GenePix image analysis software and results were saved as GPR-file (GenePix Pro 6.0, Axon Instruments). For data normalization, GPR-files were processed using the BioConductor/R packages limma and marray (http://www.bioconductor.org). Processed and normalized data as well as experimental details (MIAME, (10)) were stored in the in-house microarray database for further analysis (40).

Chloramphenicol acetyltransferase assay - For analysing the expression of the *rosR* gene, *C. glutamicum* wild type and the deletion mutant Δ*rosR* were transformed with plasmid pET2-*rosR*, which is based on the corynebacterial promoter-probe vector pET2 (26) and contains the *rosR* promoter region in front of a promoter-less *cat* (chloramphenicol acetyltransferase) gene. The promoter activity test was performed as described previously by measuring the formation of 5-thio-2-nitrobenzoate photometrically at 412 nm and 37°C (27,28). 1 Unit of enzyme activity is defined as the turnover of 2 µmol acetyl-CoA per min.

Gene expression analysis using real-time PCR - Equal amounts of 500 ng RNA were transcribed into cDNA using specific primers for the genes in question. The product was quantified via real-time PCR using a LightCycler instrument 1.0 (Roche Diagnostics, Mannheim, Germany) with SYBR Green I as the fluorescence dye following the instructions of the supplier (Qiagen, Hilden, Germany). The accuracy of the PCR was checked by melting curve analysis. To quantify the amount of cDNA, a calibration curve was generated from known concentrations of template DNA (eight for each gene) that were processed in parallel via real-time PCR. These reference samples were generated by PCR using chromosomal DNA of *C. glutamicum* as a template and KOD DNA polymerase (Invitrogen, Karlsruhe, Germany). Expression values were given in relation to the *ddh* gene (cg2900) encoding diaminopimelate dehydrogenase, which was used as a reference gene with constitutive expression.

Electrophoretic mobility shift assay (EMSA) - The binding of RosR protein or its mutated derivatives to putative target promoters was tested as described previously for RipA (12). Purified protein was incubated with DNA fragments (100–700 bp, final concentration 8–20 nM) in a total volume of 20 µl. The binding buffer contained 20 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5% (v/v) glycerol, and 0.5 mM EDTA. 1 mM DTT was freshly added to the reaction mixture before incubation. Promoter fragments of putative

non-target genes (~13 nM) of RosR were used as negative controls. The reaction mixtures were incubated at room temperature for 20 min and then loaded onto a 15% native polyacrylamide gel. Electrophoresis was performed at room temperature and 170 V using 1x TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) as electrophoresis buffer. The gels were subsequently stained with SybrGreen I according to the instructions of the supplier (Sigma-Aldrich, Taufkirchen, Germany) and photographed. All PCR products used in the gel shift assays were purified with the PCR purification kit (Qiagen, Hilden, Germany) and eluted in water.

DNase I footprinting assays with purified RosR – The binding site of purified RosR protein in the *narK* promoter region was analyzed by DNase I footprinting as described previously (8). A PCR product covering the *narK* promotor region from position -341 to + 91 relative to the *narK* transcriptional start site was obtained with the oligonucleotides narK-for and narK-rev-IRD (labelled template strand). Mixtures containing RosR and IRD800-labelled DNA were treated with DNase I and the reaction products were separated by denaturing PAGE using a Long Read IR DNA sequencer (Licor, Bad Homburg, Germany). The regions that were protected from DNase I digestion were localized by running besides the footprinting reactions the four lanes of a DNA sequencing reaction set up with the oligonucleotide narK-rev-IRD and a PCR product covering the *narK* promoter region as template.

TABLE 2

Genome-wide comparison of mRNA levels in C. glutamicum wild type and the ΔrosR mutant using DNA microarrays

Accession no.	Gene	Annotation	mRNA ratio Δ*rosR*/WT
		Genes with a decreased mRNA level in strain ΔrosR	
cg0416		secreted protein carrying a eukaryotic domain	0.24
cg0415	*ptpA2*	low molecular weight phosphotyrosine protein phosphatase	0.32
cg1109	*porB*	anion-specific porin precursor	0.46
cg1324*	*rosR*	transcriptional regulator of MarR family	0.00
cg1345*	*narK*	putative nitrate/nitrite antiporter	0.34
cg1344*	*narG*	respiratory nitrate reductase, α-subunit	0.31
cg1343*	*narH*	respiratory nitrate reductase, β-subunit	0.43
cg1341*	*narI*	respiratory nitrate reductase, γ-subunit	0.42
cg1382	*glgE*	putative α-amylase	0.49
cg1671		putative membrane-associated GTPase	0.42
cg2136	*gluA*	ABC transporter for glutamate, ATP-binding protein	0.39
cg2137	*gluB*	ABC transporter for glutamate, secreted glutamate-binding protein	0.36
cg2138	*gluC*	ABC transporter for glutamate, permease	0.37
cg2649		secreted penicillin-binding protein	0.39
cg2773		uncharacterized protein with SCP/PR1 domain	0.32
		Genes with an increased mRNA level in strain ΔrosR	
cg0072		membrane protein of unknown function	189.00
cg0144		putative arabinitol or ribitol transporter	2.25
cg0292	*tnp16a*	transposase (ISCg16a)	2.17
cg0364		membrane protein	3.51

cg0384	*rluC1*	ribosomal large subunit pseudouridine synthase C	2.57
cg0408		membrane protein	2.82
cg0823		putative monooxygenase	69.95
cg0824	*tnp5a*	transposase (ISCg5a)	3.10
cg1001	*mscL*	large conductance mechanosensitive channel	2.24
cg1150*		putative NAD(P)H-dependent FMN reductase	169.08
cg1255		HNH endonuclease	3.48
cg1322*		putative polyisoprenoid-binding protein	20.66
cg1426*		protein of glutathione S-transferase family	26.02
cg1848*		luciferase-like monooxygenase	22.24
cg1850*		putative FMN reductase	419.73
cg2329*		luciferase-like monooxygenase (monocistronic)	140.65
cg2426	*tnp2d*	transposase (ISCg2d)	4.14
cg2485	*phoD*	secreted alkaline phosphatase precursor	2.31
cg2504		protein of unknown function	4.93
cg2599		pirin-related protein	3.68
cg2759	*tnp15b*	transposase (ISCg15b)	2.87
cg2844	*pstA*	ABC transporter for phosphate uptake, permease component	3.51
cg2914	*tnp5b*	transposase (ISCg5b)	3.51
cg2953	*(xylC)*	putative benzaldehyde dehydrogenase	2.13
cg3004	*(gabD2)*	putative succinate semialdehyde dehydrogenase	2.34
cg3084*		putative flavin-containing monooxygenase	40.64
cg3085*		luciferase-like monooxygenase	2.46
cg3258	*rluC2*	putative pseudouridine synthase	2.38
cg3272		membrane protein (putative Fe^{2+}/Mn^{2+} transporter)	2.67
cg3273		protein of unknown function	2.38
cg3274		site-specific recombinase (resolvase) fragment	3.69
cg3284	*cgtS9*	two component histidine kinase CgtS9	198.14
cg3288		hypothetical protein predicted by Glimmer	2.58
cg3289		putative thiol-disulfide isomerase	2.46
cg3291		putative transcriptional regulator of Crp/FNR family	95.66
cg3293		protein of unknown function	3.17
cg3295		probable cation-transporting P-type ATPase	4.19
cg3329		member of uncharacterized protein family UPF0027 (homolog of *E. coli* RtcB)	2.06
cg3413	*(azlC)*	putative branched-chain amino acid permease (azaleucine resistance)	2.21
cg3418		putative secreted protein	3.21

The mRNA ratios represent mean values from three independent microarray experiments starting from independent cultures (see Experimental procedures). The strains were cultivated in CGXII minimal medium with 4% (w/v) glucose and mRNA was isolated in the exponential growth phase. The table includes those genes which showed a ≥2-fold changed mRNA level (increased or decreased) in at least two of the three experiments and which had a *P*-value ≤ 0.05. The genes are ordered according to their position on the genome.

Size exclusion chromatography - The native molecular mass of purified RosR was estimated by size exclusion chromatography using a Hi-Load 16/60 Superdex 200 prep grade column (Amersham Biosciences) integrated into an ÄKTA FPLC™ system (Amersham Biosciences). The column was equilibrated with 20 mM HEPES buffer pH 8.0 containing 500 mM NaCl. After application

of 0.5–1 mg of purified protein, chromatography was performed at 15°C with a flow rate of 1 ml/min. The column was calibrated with a premixed protein molecular mass marker (MWGF-200, Sigma).

2D gel electrophoresis and protein identification by MS - Analysis

2D gel electrophoresis were performed as described previously (29). For the analysis 300 µg protein extracts of the wild type and the deletion mutant Δ*rosR* were used. Extracts were prepared from cells grown in glucose minimalmediumor to the exponential growth phase (OD600 = 5–6). Identification of proteins from Coomassie-stained 2D SDS-polyacrylamide gels were performed by peptide mass fingerprinting of tryptic digests as described by Schaffer *et al.* (2001), except that peptides were extracted by addition of 0.2% (v/v) trifluoroacetic acid in 30% (v/v) acetonitrile instead of 0.1% (v/v) trifluoroacetic acid in 30% (v/v) acetonitrile. MALDI-TOF-MS was performed with an Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The MASCOT software (30) was used to compare the peptide mass patterns obtained with those of all proteins from the theoretical *C. glutamicum* proteome. The molecular weight search (MOWSE) scoring scheme (31) with a cut-off value of 50 was used for unequivocal identification of proteins.

RESULTS

Influence of a rosR deletion on global gene expression - The gene cg1324 of *C. glutamicum* ATCC 13032 encodes a protein of 162 amino acids (mass 18 641 Da) which has been annotated as a putative transcriptional regulator of the MarR family (4). The role of this protein, which we named RosR, has not been studied hitherto. In order to get clues on the function, we searched for putative RosR target genes by analyzing the influence of a deletion of the *rosR* gene on global gene expression. For this purpose, strain *C. glutamicum* Δ*rosR* was constructed which contains an in-frame deletion of the *rosR* gene. Global gene expression of the Δ*rosR* mutant was compared with that of the wild type by transcriptome analysis with DNA microarrays. For this purpose, the two strains were cultivated in minimal medium with 4% (w/v) glucose as carbon and energy source. No differences in growth behaviour were observed under these conditions: both strains grew with a growth rate of 0.39 h^{-1} and reached a final OD_{600} of ~60. For the transcriptome comparisons, cells were harvested in the exponential growth phase (OD_{600} ~5) and total RNA was isolated. Three microarray experiments were performed, each starting from independent cultures. In total 56 genes showed an at least twofold change in mRNA concentrations with a p-value ≤ 0.05 (Table 2).

16 genes showed a 2- to 4-fold decreased mRNA concentration in the Δ*rosR* mutant. This group included e. g. the *narKGHJI* operon coding for a nitrate/nitrite transporter (NarK) and the dissimilatory nitrate reductase (NarGHJI), which allows restricted anaerobic growth of *C. glutamicum* by nitrate respiration (20,32) or the *gluABCD* operon coding for a ABC-type glutamate uptake system (33).

40 genes were found to have a ≥2-fold increased mRNA concentration in the Δ*rosR* mutant, with 11 of them showing increases between 20- and 420-fold. The genes with a strongly increased expression code for a membrane protein of unknown function (cg0072), a putative polyisoprenoid-binding protein (cg1322, gene upstream of and divergent to *rosR*), a sensory histidine kinase (cgtS9), a putative transcriptional regulator of Crp/FNR family (cg3291), a protein of the glutathione S-transferase family (cg1426), two putative FMN reductases (cg1150, cg1850), and four putative monooxygenases (cg0823, cg1848, cg2329, cg3084). Besides the multiplicity of monoxygenase genes, another peculiar feature was that many genes of the HGC1 (high G+C content) region of the *C. glutamicum* genome, which

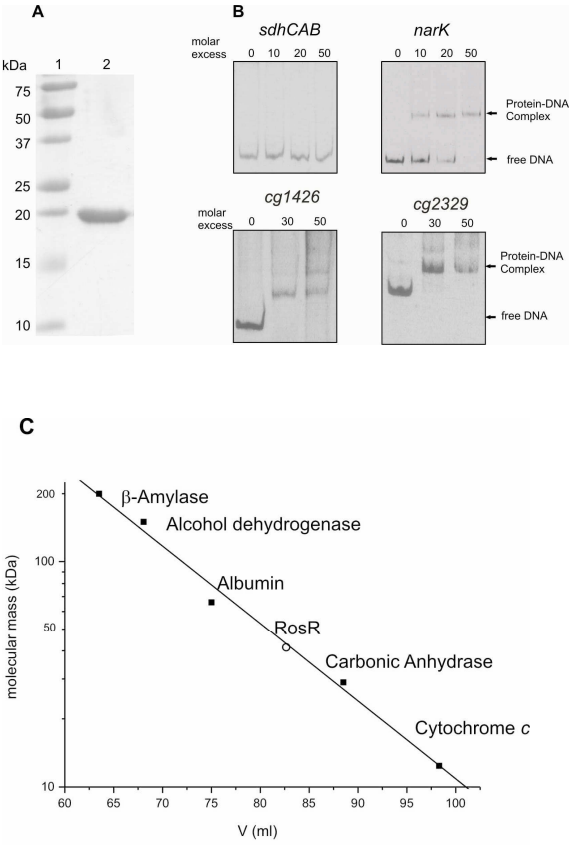

FIG. 1. **Purification of RosR and EMSAs with selected promoter regions.** A. Coomassie-stained SDS polyacrylamide gel showing RosR-His (lane 2) after purification by Ni^{2+}-NTA affinity chromatography from *E. coli* BL21(DE3)/pLysS carrying the expression plasmid pET16b-RosR. Lane 1 contains protein molecular mass standards. B. Result of size exclusion chromatography of purified RosR-His using a HiLoad 16/60 Superdex 200 column (Amersham Biosciences). The column was equlibrated with 20 mM HEPES buffer pH 7.5 containing 500 mM NaCl and elution was performed with the same buffer at 1 ml/min. Calibration was performed with cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). C. EMSAs with purifed RosR-His and DNA fragments (15 nM each) covering the promoter regions of *sdhCAB*, *narKGHJI,* cg1426 and cg2329. The DNA fragments were incubated either without RosR-His or with 150 nM, 300 nM, 450 nM or 750 nM RosR-His for 20 min at room temperature. Then the samples were separated by native polyacrylamide gel electrophoresis (15%) and the DNA was stained with SybrGreen I.

extends from cg3267 to cg3295 and is flanked by defective insertion sequences (Kalinowski 2005), showed an increased mRNA level in Δ*rosR* mutant.

To verify the results obtained by the DNA microarray experiments, quantitative real-time PCR was performed for some of the genes with altered mRNA levels in the Δ*rosR* mutant, namely *narK* and *narG* as examples for downregulated genes and cg1426 and cg2329 as representatives for upregulated genes. The mRNA ratios in relation to the reference gene *ddh* were 0.35 for *narK*, 0.63 for *narG*, 27.7 for cg1426, and 58.6 for cg2329 and thus confirmed the results of the DNA microarray data (Table 2).

Purification of RosR and binding to the promoter regions of narKGHJI, cg1426, and cg2329 - To determine which of the genes showing a different expression level in the DNA microarray experiments are direct target genes of the transcriptional regulator RosR, electrophoretic mobility shift assays (EMSAs) were performed. For this purpose, a RosR derivative with an N-terminal decahistidine tag (calculated mass 21161.8 Da) was overproduced in *Escherichia coli* BL21(DE3)/pLysS using plasmid pET16b-rosR and purified by Ni^{2+}-chelate affinity chromatography (Fig. 1A). The native mass of RosR-His as determined by size exclusion chromatography using a Superdex 200 column (Amersham Biosciences) was found to be 40 kDa (Fig. 1C). This indicates that RosR forms a homodimer, similar to other MarR type regulators like MarR or MexR (19).

For the EMSAs, RosR-His was routinely used, as a control experiment in which the His tag had been cleaved off by the endoproteinase factor Xa had shown that the tag has no influence on the DNA-binding properties of RosR (data not shown). In the first series of EMSAs, the *narKGHJI*, *sdhCAB*, cg1426 and cg2329 were tested. DNA fragments covering the promoter regions of the *narKGHJI* operon (-440 bp to +70 bp with respect to the translational start site of *narK*), *sdhCAB* (-490 bp to +110 bp), cg1426 (-500 bp to +120 bp) and cg2329 (-500 bp to +89 bp) were amplified by PCR. As shown in Fig.

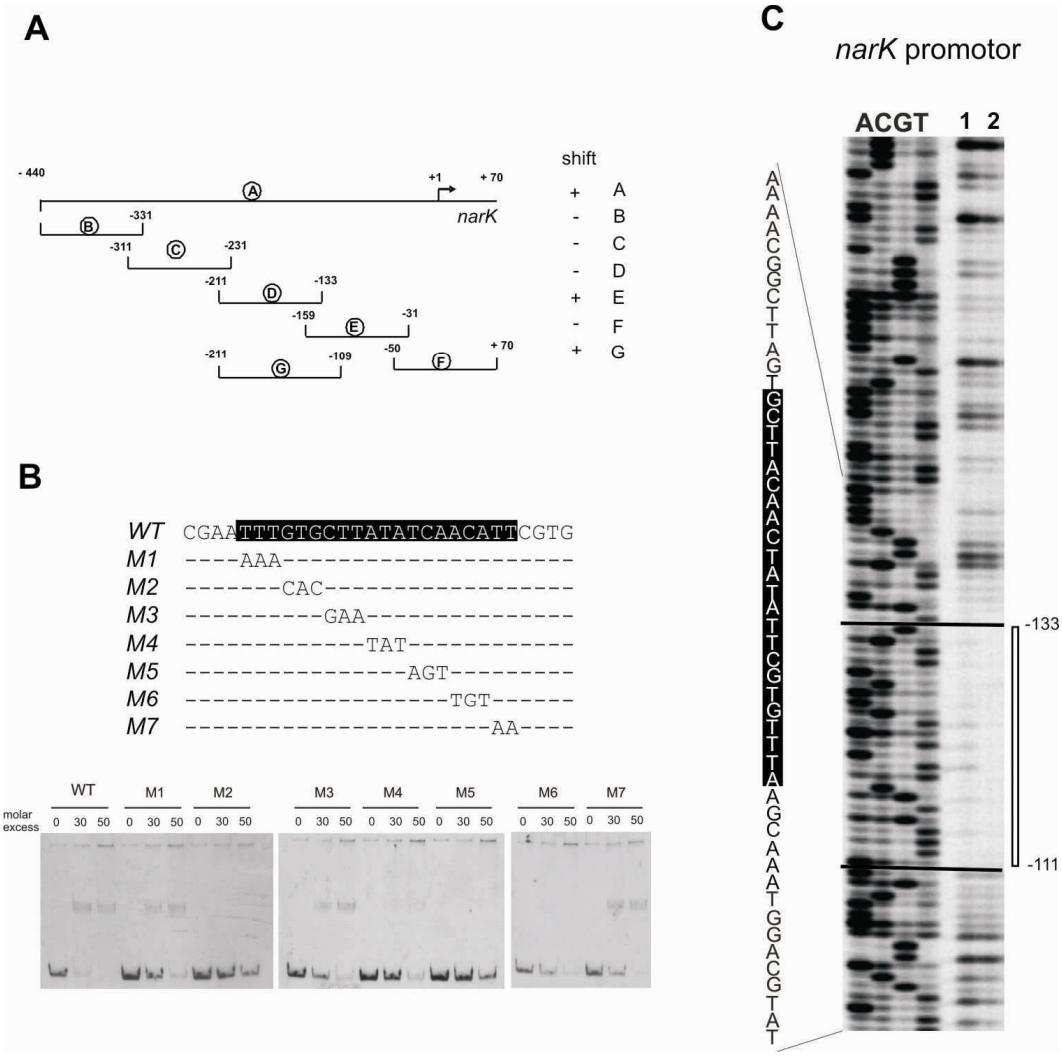

FIG. 2. **Identification of the RosR binding site in the promoter region of the *narKGHJI* operon.** (A) Localization of the RosR binding site using *narKGHJI* promoter fragments (designated A-G) and purified RosR in EMSAs. The numbers show the position of the fragments relative to the transcription start site (+1). "+" and "-" indicate if the fragment was shifted by RosR or not. (B) Mutational analysis of the RosR binding site (shaded in black) in the *narKGHJI* promoter region. The mutations M1-M7 were introduced by PCR and are shown below the wild-type sequence. The corresponding DNA fragments were analysed in EMSAs with purified RosR. (C) DNase I footprint analysis using *narKGHJI* promoter region and isolated RosR. 2 nM IRD800-labelled *narK* template strand was incubated without (lane 1) or with 60 nM RosR (lane 2). DNA regions protected by RosR are indicated by the bar on the right and the numbers show the distance to the *narK* transcription start site. The DNA sequencing reactions were set up using the same IRD-800-labeled fragment.

1B, the promoter fragments of cg1426 and cg2329 were completely shifted with 450 nM RosR-His, that of *narKGHJI* at 750 nM RosR-His. In contrast, no shift was observed for the promoter region of *sdhCAB*, even at 750 nM RosR-His. According to these results, *narKGHJI*, cg1426 and cg2329 represent direct target genes of RosR. In further EMSAs the promoter regions of the *gluABCD* operon and of cg3291 were tested, but no binding of RosR-His was observed (data not shown). Thus, even genes which are strongly upregulated in the Δ*rosR* mutant not necessarily are direct target genes of RosR.

Identification of the RosR binding motif - In order to locate the binding site(s) of RosR in the promoter region of the *narKGHJI* operon, the 510-bp fragment used for the initial binding studies was split into several subfragments, which were synthesized by PCR and analysed in

EMSAs for binding of RosR. As shown in Fig. 2A, only subfragments E and G were bound by RosR. As subfragment E differs from subfragment D only by 22 additional bp at the promoter-proximal site, the most important part of the RosR binding site was assumed to be located within these 22 bp, which extend from position -109 to -133 with respect to the *narK* transcriptional start site determined previously (20). The relevance of this region was analyzed by mutational analysis. Seven mutated fragments were amplified by PCR (Fig. 2B), each with two or three nucleotides exchanged, and tested again in EMSAs. In the case of fragments M2, M4, M5 and M6, mutations prevented the binding of RosR, indicating that the corresponding nucleotides are required for binding (Fig. 2B). In an independent approach, the RosR binding site within the *narK* promoter regions was analyzed by DNase I footprinting. When using a labelled template strand, a protected region was detected that extends from position -111 to -133 with respect to the *narK* transcriptional start site (Fig. 2C). This region overlaps with the binding motif identified by the EMSAs.

Importantly, DNA sequence motifs similar to those identified in the *narK* promoter regions were also identified in the promoter regions of cg1426 (ATTGTTGACATATCATCTAA) and cg2329 (ATTGTTGATATATCTAC-AAA). To confirm their relevance for RosR binding, promoter fragments including or lacking the proposed binding site (cg1426: -49 bp to +120 bp and -20 bp to +120 bp; cg2329: -64 bp to +89 bp and -36 bp to +89 bp with respect to the translational start sites) were synthesized by PCR. EMSAs showed that only the DNA fragments containing the motif were shifted by RosR (Fig. 3A).

Identification of further RosR target genes – The identification of the RosR binding sites in the promoter regions of *narKGHJI*, cg1426 and cg2329 allowed to derive a first generation RosR consensus binding motif ANTGTTGANA-TANNNNCNAA, which was used for a genome-wide *in silico* search for similar sequences (allowing two

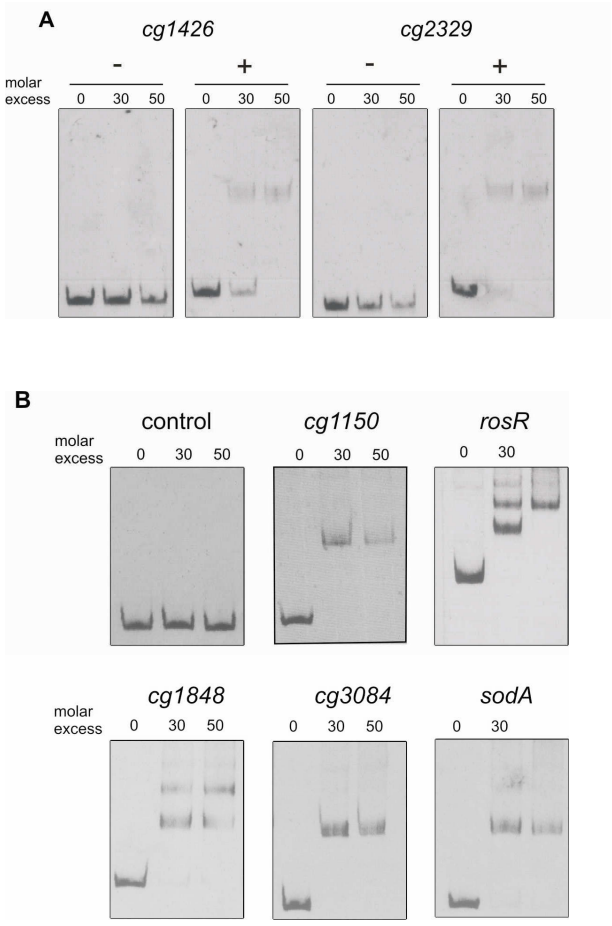

FIG. 3. **EMSAs with purified RosR protein.** (A) Requirement of the predicted RosR binding sites in the promoter regions of cg2329 and cg1426 for RosR binding. DNA fragments (15 nM) of the cg2329 and cg1426 promoter regions containing (+) or missing (-) the predicted RosR binding site were incubated either without or with 30- or 50-fold molar excess (450 nM, 750 nM) of purified RosR. (B) Binding of RosR to the promoter regions of target genes predicted from *in silico* search. DNA fragments (15 µM) covering the promoter regions of the putative target genes cg3237 (superoxide dismutase), cg1150 (putative FMN reductase), cg1426 (putative protein of the glutathione S-transferase family), cg2329 (putative monooxygenase), cg1848 (putative monooxygenase), *rosR* and cg3084 (putative monooxgenase) were incubated for 20 min at room temperature with a 0- to 50-fold molar excess (450 nM, 750 nM) of purified RosR. The negative control contained a fragment covering the promoter region of *gluABCD* (ABC transporter for glutamate). Samples were separated by native PAGE (15 %) and stained with SybrGreen I.

mismatches) in the genome of *C. glutamicum* ATCC 13032 using ERGO software (Integrated Genomics Inc.). In total 67 matches were obtained, 17 of which were located between open reading frames and thus within putative promoter regions. This group included the already known target promoters of *narKGHJI*, cg1426 and cg2329. The remaining 14 promoter regions were amplified and tested for binding of RosR in EMSAs. A shift in the presence of purified RosR (Fig. 3B) was observed for five fragments, which represent the promoter regions of cg1150 (putative NADPH-dependent FMN reductase), cg1848 (luciferase-like monooxygenase), cg3084 (putative flavin-containing monooxygenase), *sodA* (cg3237, superoxide dismutase), and *rosR* itself. The latter result indicates that RosR is subject to negative autoregulation, a feature known for MarR-type transcriptional regulators (19). In the case of the promoter fragments of *rosR* and cg1848, two DNA-RosR comlexes were formed, indicating the presence of two binding motifs in these regions (Fig. 3B). Inspection of the cg1848 promoter sequence revealed the presence of two RosR binding motifs in close neighbourhood. Also in the intergenic region between *rosR* and the divergent gene cg1322, two motifs could be identified, one of which overlaps with the translational start site of *rosR*. The fact that cg1322 (codes for a putative polyisoprenoid-binding protein) was strongly upregulated in the Δ*rosR* mutant (Table 2) indicates that this gene is also repressed by RosR.

Except for the *sodA* gene, all other newly identified RosR target genes showed significantly increased mRNA ratios in Δ*rosR*/wt transcriptome comparison (cg1150, 169.1; cg1322, 20.7; cg1848, 22.2; cg3084, 40.64). Inspection of the corresponding genomic regions indicated that the cg1848 gene presumably forms an operon with cg1849 and cg1850 (putative FMN reductase), the latter of which also showed a 420-fold increased mRNA level in the Δ*rosR* mutant. Also the cg1849 gene coding for a hypothetical protein showed at least 3-fold increased mRNA levels in all three experiments, but in this case the p-value was above the threshold of 0.05. The cg3084 gene is presumably cotranscribed with cg3085 (luciferase-like mono-oxygenase), which showed a 2.5-fold increased expression in the Δ*rosR* mutant.

Alignment of the DNA-binding sites of RosR in the promoter regions *narKGHJI*, cg1150, *rosR*, cg1426, cg1848, cg2329, cg3084, and *sodA* led to the definition of a 2nd generation consensus binding motif of 20 bp forming an inverted repeat (Fig. 4B).

Identification of transcriptional start sites of RosR target genes – According to the results described above, RosR functions as a transcriptional activator for the *narKGHJI* operon and as a transcriptional repressor for all other known target genes. In many cases, the function as transcriptional activator or repressor correlates with a binding site upstream of the -35 region or within or downstream of the -10/-35 regions of the promoter, respectively. In order to analyse whether such a correlation is also true for RosR, we determined the transcriptional start sites (TS) of all known target genes except for the *narKGHJI* operon. Based on the fact that the *narK* upstream sequence of *C. glutamicum* ATCC 13032 is completely identical to the one of strain R, it was assumed that also the TS of *narK* in strain ATCC 13032 is identical to the one determined previously for strain R (20). In the *narK* promoter the RosR binding motif, which is also completely conserved in strain R, stretches from position -131 to -112 with respect to the TS (position +1).

The TS of cg1322, cg1426 and cg3084 were determined by primer extension using two different primers for each gene (see Table S1). As shown in Fig. S3, the TS were found to be located 32 bp, 34 bp and 7 bp upstream of the proposed translational start sites of cg1322, cg1426 and cg3084, respectively. The TS of *rosR,* cg1150, cg2329 and cg1848 were determined by 5'-RACE-PCR (for primers see Table S1). In each case, 10 clones containing the

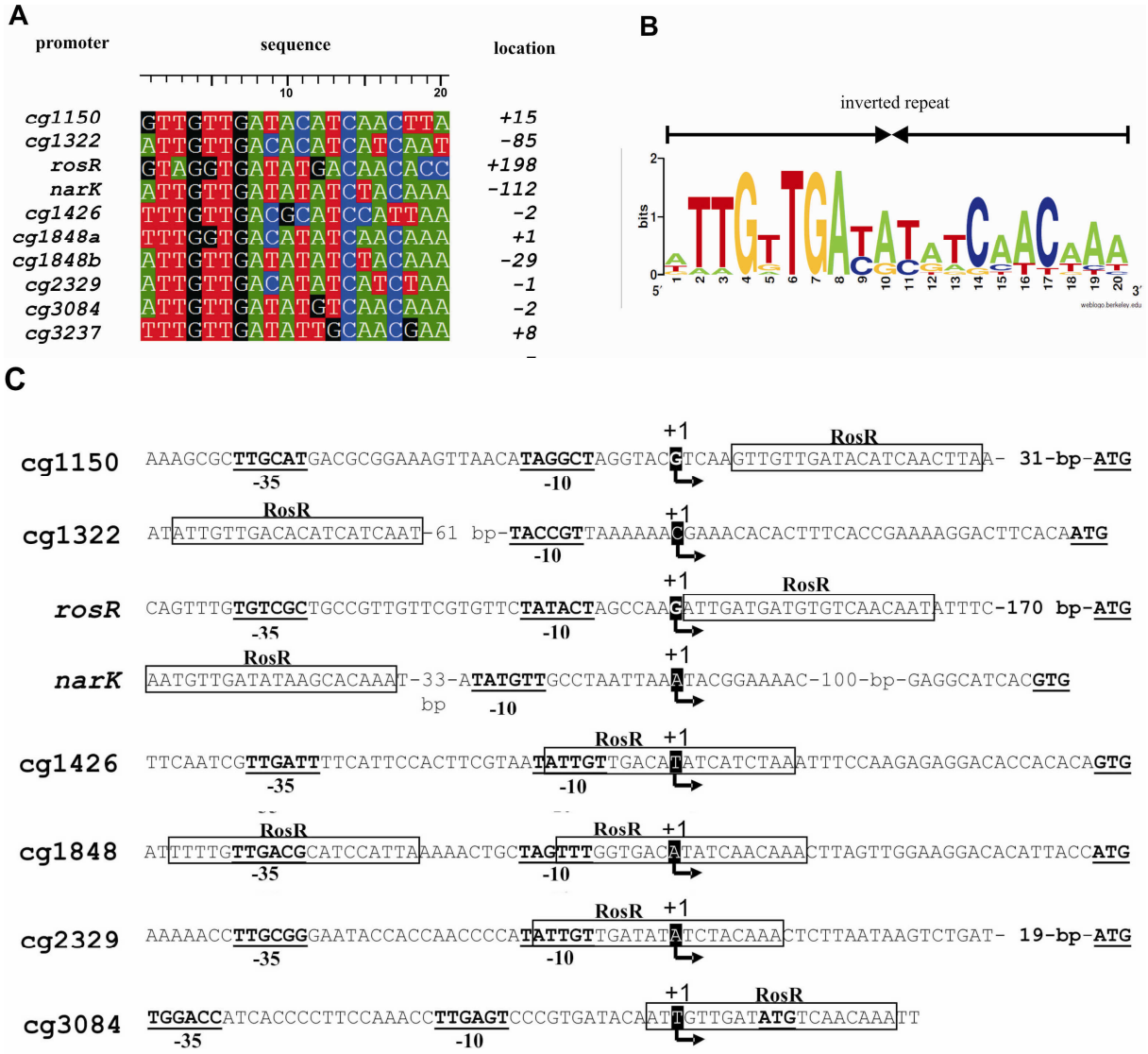

FIG. 4. **Definition of a RosR consensus DNA binding site and location to determined transcriptional start sites.** (A) Alignment of the experimentally identified RosR binding sites. The location of the 11th nucleotide of the 20 bp binding sites is indicated with respect to the transcriptional start site of the target genes. (B) DNA sequence logo representing the RosR binding site in *C. glutamicum*. The verified binding sites were used as input for WebLogo (60). The sequence logo represents the information content of the alignment of RosR DNA binding sites, showing the sequence conservation (overall height at each position) and the relative frequency of each nucleotide at each position (nucleotide hight). (C) Transcriptional start sites of RosR target genes. Sequences show the transcriptional start site (+1 and arrow) identified by primer extension or 5´-RACE experiments. The RosR binding site is indicated as box, the -10 and -35 regions are indicated in bold letters and are underlined.

amplified cDNA were sequenced to determine the 5'-ends of the mRNA. A transcriptional start site was assumed when at least 3 of the 10 clones showed the same 5'-end and when the other clones contained non-identical shorter transcripts. The TS were found to be located 57 bp, 196 bp, 32 bp and 45 bp upstream of the proposed translational start sites of cg1150, *rosR*, cg1848 and cg2329, respectively.

In Fig. 4C the promoter sequences based on the newly identified TS are shown. In all cases except for cg1322, the RosR binding site either overlaps with the -35 or -10 regions or is located at or immediately downstream of the TS, indicating that repression is achieved by inhibition of RNA polymerase binding of or initiation complex formation. In the case of cg1322, RosR binding sites are centred

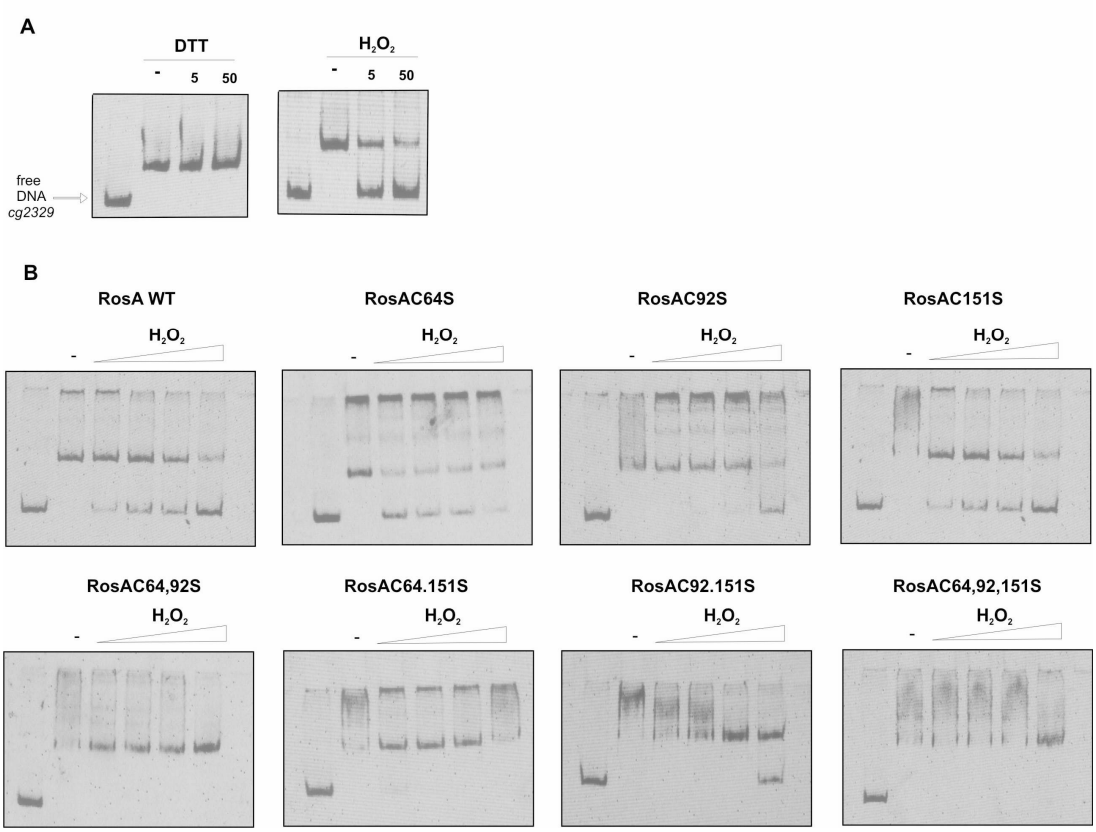

Fig. 5. Influence of H_2O_2 on the DNA-binding capability of RosR. (A) The promoter region of cg2329 (15 nM) was incubated without RosR or with a 30-fold molar (450 nM) excess of RosR. DTT or H2O2 were added to RosR-containing samples at 5 mM or 50 mM. After incubation for 20 min at room temperature, the samples were separated by native PAGE (15 %) and stained with SybrGreen I. (B) Influence of the cysteine residues in RosR on its DNA-binding activity. Binding of wild-type RosR and the muteins RosR-C64S, RosR-C92S; RosR-C151S, RosR-C64,92S, RosR-C64,151S, RosR-C92,151S and RosR-C64,92,151S (each 450 nM) to the promoter region of cg2329 (15 nM) in the absence and presence of H_2O_2 (1, 2, 5, 50 mM) as oxidant. The samples were incubated for 20 min at room temperature and then separated by native PAGE (15 %) and stained with SybrGreen I.

86 bp and 247 bp upstream of the TS. In this case, the mechanism of repression is unclear. It might involve the blockage of RNA polymerase binding to the cg1322 promoter by the stalled RNA polymerase at the *rosR* promoter, as the -35 regions of the two divergent promoters are separated by only five bp.

Negative autoregulation of rosR - The EMSAs showed a positive binding of RosR to its own promoter region and the two RosR binding sites were located immediately downstream of the TS and overlapping the proposed translational start site (Fig. 4C), indicating a negative autoregulation of RosR. To investigate this possibility, the *rosR* promoter region (extending from position –272 to +12) was cloned in front of a promoterless chloramphenicol acetyltransferase (CAT) gene in the promoter probe vector pET2. The resulting plasmid pET2-rosR was transferred into *C. glutamicum* wild type and the Δ*rosR* mutant. CAT activity was measured for cells cultivated in CGXII minimal medium with 4% (w/v) glucose and harvested in the exponentially growth phase. The experiments were performed in triplicate and revealed a specific CAT activity of 52 ± 2 nmol/min/mg protein for wild-type cells and of 318 ± 15 nmol/min/mg protein for Δ*rosR* cells. This result confirmed that *rosR* expression is negatively autoregulated.

DNA-binding of RosR is inhibited by H_2O_2 and dependent on the oxidation status of cysteine residues – Based on the fact that several target genes encode putative monooxygenases and FMN reductases that might act in concert, we considered the possibility that some kind of oxidative stress could be sensed by RosR. To test this possibility, the effects of the oxidant H_2O_2 and of the reductant DTT on the DNA-binding properties of RosR were tested by EMSAs. As shown in Fig. 5A, binding of RosR to the cg2329 promoter fragment was not influenced by addition of up to 50 mM DTT, whereas H_2O_2 clearly inhibited binding. A similar effect was observed when cumene hydroperoxide (CHP) was added in concentrations of 5 and 25 mM (data not shown). In a control experiment we tested the effect of up to 50 mM H_2O_2 on the binding of the response regulator MtrA of *C. glutamicum* to its target promoter *nlpC* (Brocker and Bott, 2006) and found no inhibition (data not shown). This shows that there is no general inhibition of protein-DNA interaction by H_2O_2.

Inspection of the RosR amino acid sequence revealed three cysteine residues located at position 64, 92 and 151, which might be responsible for the inhibitory effect of H_2O_2 on DNA-binding. To test this assumption, the cysteine residues were exchanged by site-directed mutagenesis either individually or in all possible combinations to serine residues. The resulting seven different RosR variants were overproduced in *E. coli*, purified and used for EMSAs. As shown in Fig. 5B, all of the seven RosR variants were still binding-competent in the absence of H_2O_2, as the cg2329 promoter fragment was completely shifted at a 30-fold molar excess of these proteins. Thus, none of the cysteine residues is essential for DNA-binding or dimerization of RosR. In the presence of increasing concentrations of H_2O_2, the DNA-binding properties of the RosR derivatives varied. The muteins RosR-C64S and RosR-C151S behaved similar to wild-type RosR, meaning that DNA-binding was inhibited already in the presence of 1 mM H_2O_2. In contrast, the DNA-binding of RosR-C92S was not diminished up to 5 mM H_2O_2, but only at 50 mM of the oxidant. This indicates that oxidation of C92 is important for inhibition of DNA-binding by H_2O_2. The mutein RosR-C92,151S showed similar DNA-binding properties as RosR-C92S, whereas in the case of RosR-C64,92S even 50 mM H_2O_2 did not prevent DNA-binding. The same was true for the triple mutein RosR-C64,92,151S. Suprisingly, RosR-C64,151S also showed no inhibition of DNA-binding by up to 50 mM H_2O_2, although the single muteins RosR-C64S and RosR-C151S were sensitive to H_2O_2. Thus, the combined

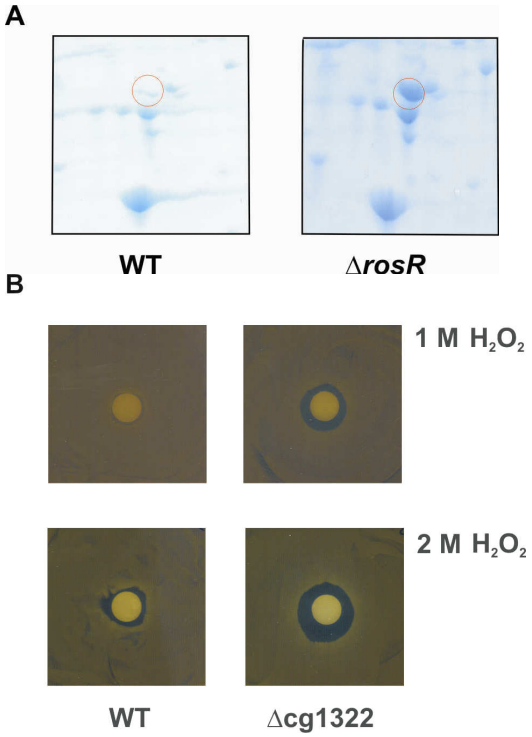

Fig. 6. (A) Section of Coomassie-stained 2D gels (pH 4 – 7) showing the strong induction of the Cg1322 protein (circled) in the Δ*rosR* mutant compared to the wild type (WT). The cells were grown in glucose minimal medium and used in the exponential growth phase for preparation of cell extracts. Cg1322 was identified by peptide mass fingerprinting. (B) Agar plate assays showing the increased sensitivity of the Δcg1322 mutant to hydrogen peroxide. A paper disc impregnated with 70 µl of either 0.5 M or 1 M H_2O_2 was placed on BHI plates containing either Δcg1322 or wild-type cells. The plates were incubated at 30°C for 24 h. Experiments were performed in triplicate starting with independent precultures.

oxidation of both residues also can lead to H_2O_2 sensitivity. The above results show that the inhibition of DNA-binding by H_2O_2 is caused by the oxidation of cysteine residues, in particular C92, and that H_2O_2-insensitive, but still binding-competent RosR derivatives can be obtained by exchange of the cysteine residues to serines.

Evidence for a role of Cg1322 in H_2O_2 resistance – In a proteome comparison of *C. glutamicum* wild type and the Δ*rosR* mutant using 2D gel electrophoresis, Cg1322 was the only protein that showed a highly increased abundance in the Δ*rosR* mutant (Fig. 6A). To investigate the role of the Cg1322 protein in the response of *C. glutamicum* to oxidative stress, the in-frame deletion mutant Δcg1322 was constructed and analysed in agar diffusion assays. A paper disc containing 1 M or 2 M H_2O_2 was placed on a freshly plated lawn of Δcg1322 and wild-type cells (Fig. 6B). The inhibition zone around the paper disc was about twofold larger in the case of Δcg1322 cells (1 M H_2O_2: 2.6 ± 1 mm; 2 M H_2O_2: 4.75 ± 0.43 mm) than in the case of wild-type cells (1 M H_2O_2: 1.25 ± 0.82 mm; 2 M H_2O_2: 2.8 ± 1.4 mm), indicating that the absence of cg1322 causes a significantly increased H_2O_2 sensitivity.

DISCUSSION

In this work we have analysed the function of the as yet uncharacterized MarR-type transcriptional regulator RosR (Cg1324) of *Corynebacterium glutamicum*. By combining a comparative transcriptome analysis with DNA microarrays of *C. glutamicum* wild type and a Δ*rosR* mutant and DNA-interaction studies with purified RosR, direct target genes of RosR could be identified and a 20-bp consensus DNA-binding site could be defined. Based on these data, RosR functions as activator of the *narKGHJI* operon and as repressor of at least nine genes.

The *narKGHJI* operon codes for a nitrate-nitrite antiporter (NarK) and dissimilatory nitrate reductase (NarGHIJ) and allows *C. glutamicum* to reduce nitrate to nitrite and to grow under anaerobic conditions using nitrate as final electron acceptor (2,20). Since genes for nitrite reductase or for denitrification are absent in *C. glutamicum* (Bott and Niebisch, 2003), nitrite cannot be further metabolized and accumulates (2,20). Although the activation of *narKGHJI* expression by RosR is missing in the Δ*rosR* mutant, this strain showed the same growth behavior under anaerobic conditions with nitrate as terminal electron acceptor as the wild type (data not shown). This might be explained by the assmuption that activation of the *narKGHJI* operon by RosR is not strong enough to effect growth or occurs primarily under aerobic conditions. The reduced expression of the *nar* operon under stress conditions that inactivate RosR might help to avoid additional stress by the accumulation of nitrite or products derived from nitrite. The *nar* operon of *C. glutamicum* was previously shown to be transcriptionally regulated also by ArnR, GlxR and RipA (12,34,35).

Although none of the proteins repressed by RosR (except for RosR itself) has been studied experimentally, their bioinformatic analysis revealed some noticeable features. Four of the repressed proteins (Cg1848, Cg2329, Cg3084 and Cg3085) might be active as monooxygenases and three of them (Cg1848, Cg2329 and Cg3085) belong to the PFAM family PF00296 which harbors luciferase-like monooxygenases (36). These are flavin monooxygenases that catalyze the oxidation of long-chain aldehydes using reduced flavin as second substrate: $RCHO + FMNH_2 + O_2 \rightarrow RCOO^- + H^+ + FMN + H_2O$ + light. This family also includes nonfluorescent flavoproteins such as alkanesulphonate monooxygenase, which catalyzes the following reaction: $R\text{-}CH_2\text{-}SO_3H + FMNH_2 + O_2 \rightarrow R\text{-}CHO + FMN + sulfite + H_2O$ (37). In agreement with the requirement of these enzymes for reduced FMN, two of the proteins repressed by RosR encode putative FMN reductases (Cg1150 and Cg1850). A better knowledge of the substrates of the

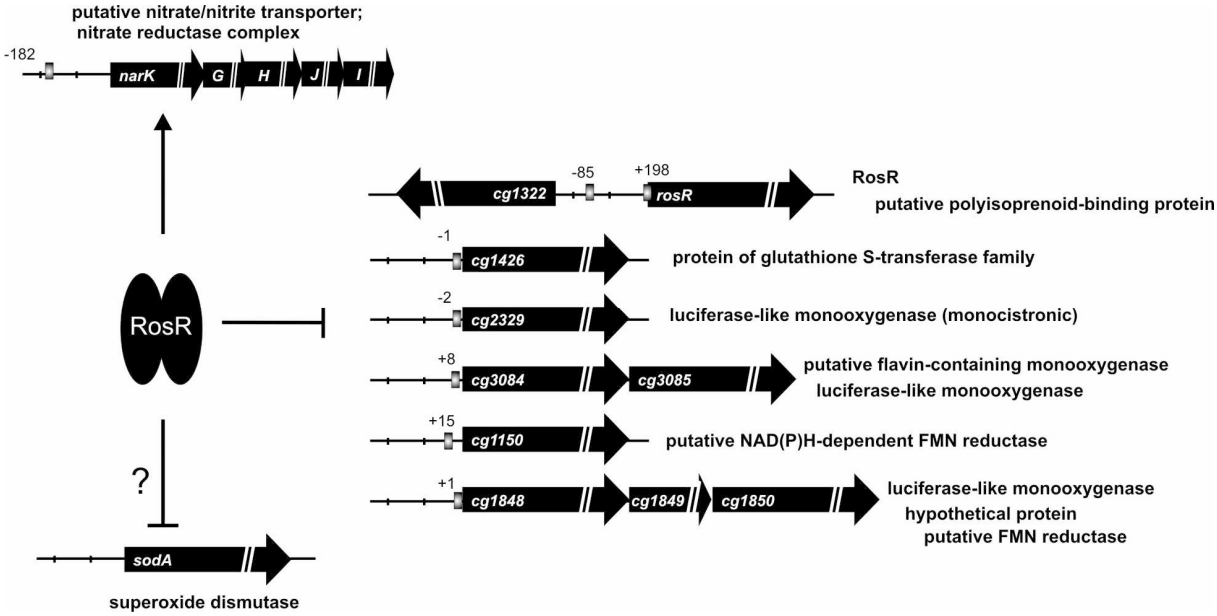

Fig. 7. Overview on the RosR regulon. Under reducing conditions, RosR is active and binds to the 20-bp sequence motifs present in the target promoters (indicated as boxes; the numbers show the distance to TS). Under oxidizing conditions, RosR is inactivated, causing the derepression of the repressed genes and loss of *narKGHJI* activation.

putative monooxygenases would help to understand the function of RosR.

The protein encoded by cg1426 belongs to the glutathione-S-transferase family (PFAM family PF00043). The members of this family have a variety of functions, such as the detoxification of electrophilic compounds by catalyzing their conjugation to glutathione or biodegradative metabolism (38,39). Corynebacteria, mycobacteria and related genera contain mycothiol instead of glutathione (40,41). Therefore, Cg1426 might form a conjugate of mycothiol and compounds such as cumene hydroperoxide.

Cg1322, which is encoded by the gene divergent to *rosR*, is a member of the YceI family (PFAM family PF04264). In *E. coli*, YceI is induced by alkaline pH and salt stress (42,43) and proposed to be a secreted protein. Recently, the crystal structure of a member of the YceI protein family, TT1927b (TTHA0802) from *Thermus thermophilus* HB8, has been solved (44). The structure consists of an extended, eight-stranded, antiparallel □-barrel. In the hydrophobic pore of the barrel, the ligand octaprenylpyrophosphat was identified, which was unintentionally copurified with the protein from *E. coli*. The polyisoprenoid chain was bound by hydrophobic interactions. As the amino acid residues that contact the ligand are largely conserved in *C. glutamicum* Cg1322, it is likely that also this protein binds octaprenylphosphate or a closely related polyisoprenoid. In *E. coli*, octaprenylphosphate is synthetisized by octaprenylpyrophosphate synthase IspB (45) and forms the side chain of the respiratory quinones menaquinone-8 (MK-8), demethylmenaquinone-8 (DMK8) and ubiquinone-8 (UQ-8). It is attached to 1,4-dihydroxy-2-naphthoic acid (DHNA), resulting in the formation of DMK-8, which is then methylated to MK-8. The former step is catalyzed by the DHNA octaprenyltransferase encoded by the *menA* gene (46), the latter one by the DMK methyltransferase MenG using S-adenosyl-methionine as methyl group donor. In *C. glutamicum*, a similar pathway for the synthesis of menaquinone is present (47). Interestingly, a *C. glutamicum* Δcg1322 mutant showed an increased sensitivity to hydrogen peroxide, indicating that the Cg1322 protein is involved in the response to oxidative stress. Strong binding of octaprenylpyrophosphate by Cg1322 might lead to a decreased synthesis of MK. It was previously proposed that the adventitious

autoxidation of reduced menaquinone in the cytoplasmic membrane releases a steady flux of superoxide into the periplasm of *E. coli*, as mutations that eliminated menaquinone synthesis eradicated the superoxide formation, while mutations in genes encoding respiratory complexes affected it only insofar as they are likely to affect the redox state of menaquinone (48).

Besides the genes mentioned above, also the *sodA* gene encoding superoxide dismutase was identified as putative RosR target in the *in silico* search based on the 1st generation consensus binding site. Although a specific DNA-binding of RosR to the *sodA* promoter region could be demonstrated (Fig. 3B), the effect on *sodA* expression remains unclear. In the DNA microarray studies comparing wild type and Δ*rosR* mutant, the *sodA* mRNA level was not changed. In additional DNA microarray experiments comparing a RosR overproducing strain with the wild type, the *sodA* transcript level was two-fold decreased in the overproducing strain, suggesting a repressing effect of RosR (data not shown). However, enzyme activity tests with a strain overexpressing *rosR* and the Δ*rosR* mutant revealed no changes in superoxide dismutase activity compared to the wild type (data not shown). Consequently, further studies are required to clarify the role of RosR in *sodA* expression.

Hydrogen peroxide was found to inhibit the DNA-binding ability of RosR, indicating that this regulator is redox-active. The inhibition by H_2O_2 could be partially or completely prevented by exchange of one (C92), two (C64 and C92, C64 and C151, C92 and C151) or all three cysteine residues (C64, C92, C151) present in the protein to serine residues. None of the three cysteine residues is *per se* required for DNA-binding. Therefore it is assumed that H_2O_2 causes the formation of intra- or intermolecular disulfide bridges in the RosR dimer which are accompanied by conformational changes that attenuate or prohibit DNA binding. The formation of a disulfide bond is well-known to serve as mechanism for activation or inactivation of redox- response transcriptional regulators.

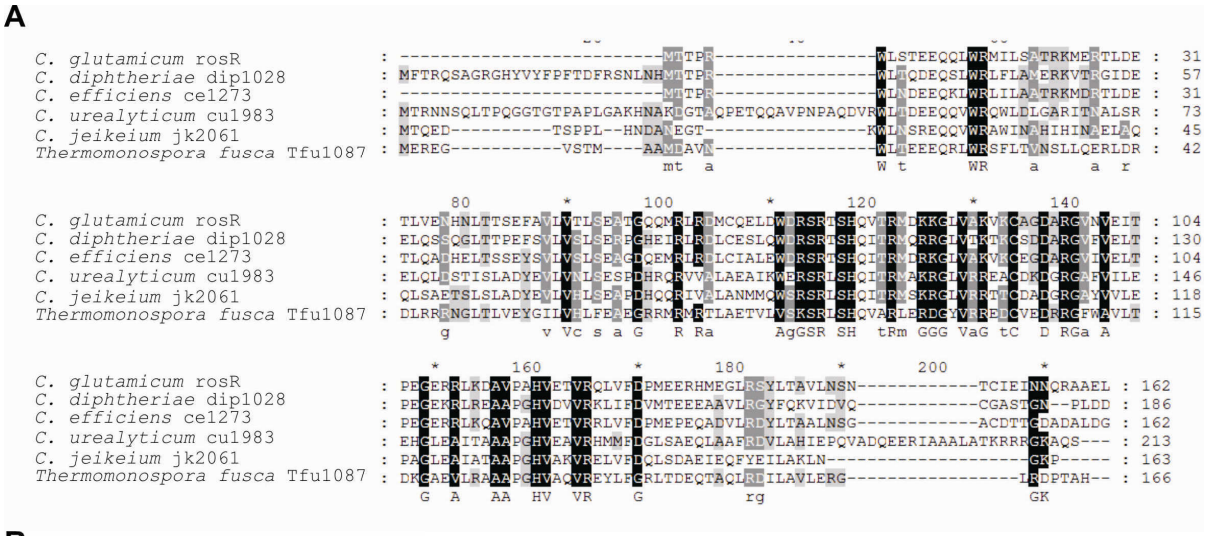

Fig. 8. (A) Amino acid sequence alignment of RosR homologs and (B) Organisation of *rosR* and cg1322 homologs.

The best studied example is the LysR-type regulator OxyR of *E. coli*, which is activated by H_2O_2 through the formation of a disulfide bond between C199 and C208 and then activates transcription of genes necessary for the defense againgst this type of oxidative stress (49,50). An example for inactivation by disulfide bond formation provides the transcriptional regulator CprK of *Desulfitobacterium dehalogenans*, which controls genes involved in dehalorespiration (51,52). Although the inactivation of RosR by the formation of disulfide bonds is an attractive model, other mechanisms such as S-thiolation cannot be excluded.

Some members of the MarR family were previously shown to be involved in the response to oxidative stresses. *Bacillus subtilis* OhrR is an organic peroxide sensor that represses expression of an inducible peroxiredoxin, OhrA. The DNA-binding activity of OhrR is regulated by the oxidation status of its sole cysteine residue C15. After oxidation to a sulfenic acid intermediate, which retains DNA-binding activity, further reactions generate either a mixed disulfide or a protein sulfenamide, both of which prevent DNA-binding and cause derpession of OhrA (53). MhqR (YkvE) of *B. subtilis* represses genes encoding multiple dioxygenases/glyoxylases, oxidoreductases and an azoreductas (54). MhqR target proteins confer resistance to 2-methylhydroquinone (MQH) and catechol, as shown by the fact that a Δ*mhqR* mutant is hyperresistant to these compounds whereas mutants lacking target genes such as *azoR2* are sensitive to these compounds. Binding of MhqR to its target genes is not affected by thiol-reactive compounds such as MQH, catechol, diamide or methylglyoxal and hydrogen peroxide. YodB of *B. subtilis* represses the *yocJ* (*azoR1*) encoding an azoreductase and its DNA-binding activity is directly inhibited by thiol-reactive compounds and H_2O_2 (55).

In *C. glutamicum* strain R, the redox-sensing transcriptional regulator QorR was recently described (56,57). QorR represses the *qor2* gene located upstream and divergent to *qorR* and its own structural gene. The *qor2* gene encodes a quinone oxidoreductase, which is involved in diamide resistance. The DNA-binding activity of QorR is inhibited by oxidants such as diamide, H_2O_2 and cumene hydroperoxide *in vitro* and its only cysteine residues, C17, is essential for redox-responsive regulation of QorR. However, the *qor2* transcript level did not change in the presence of hydrogen peroxide stress, but under diamide stress (57). This might be due to the fact that *C. glutamicum* is highly resistant to hydrogen peroxide.

In summary, RosR was identified as a novel redox-responsive transcriptional regulator in *C. glutamicum* whose DNA-binding activity can be inhibited by hydrogen peroxide *in vitro*. Except for the *narKGHJI* operon, all other currently known target genes, such as cg1322 are repressed by RosR (Fig. 7). Deletion of cg1322, which encodes a putative polyisoprenoid-binding protein, caused increased sensitivity to hydrogen peroxide, supporting a role of Cg1322 and RosR in the oxidative stress response. Homologs of RosR and Cg1322 are found in some other species of the order Actinomycetales, where the corresponding genes show the same divergent organisation as in *C. glutamicum* (Fig. 8A). Amino acid sequence alignments of the RosR homologs reveal the conservation of the cysteine residue Cys92, which found to be redox-sensitive.

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4. Discussion

4.1 TCA cycle regulation in *C. glutamicum*

Knowledge on TCA cycle regulation in the industrial amino acid producer *C. glutamicum* is important because this cycle provides oxaloacetate and 2-oxoglutarate, which are the precursors for the major products synthesized with this organism, L-lysine and L-glutamate. In this work the genetic control of the TCA cycle in *C. glutamicum* was studied and it was shown that the transcriptional regulator GlxR is involved in this regulation.

Depending on the provided carbon source a regulation of TCA cycle and glyoxylate cycle genes is required in *C. glutamicum*. When acetate is present the glyoxylate cycle is required, which is not present if glucose is used as carbon source (Emer et al., 2008; Wendisch et al., 2000). Furthermore the flux through the TCA cycle is elevated on acetate (~ 300 mU/mg protein for acetate; ~ 100 mU/mg protein for glucose)(Wendisch et al., 2000), which is also realized by an altered expression of the TCA cycle genes *gltA, acn, sucCD, sdhCAB* and *fum* (Han et al., 2008b). A similar regulation was reported in *Bacillus subtilis* (Nakano et al., 1998). The results obtained by providing different carbon sources suggest a specific genetic control of the TCA cycle genes by transcriptional regulators responding directly or indirectly to the different carbon sources present in the culture medium (Gerstmeir et al., 2003; Han et al., 2008b).

The transcriptional regulators of the TCA cycle and the glyoxylate cycle in *C. glutamicum* which have been identified so far are AcnR (Krug et al., 2005), RipA (Wennerhold et al., 2005), DtxR (Wennerhold and Bott, 2006), RamB (Gerstmeir et al., 2004), RamA (Cramer et al., 2006) and GlxR (Kim et al., 2004). AcnR, RipA and RamA are all directly involved in the regulation of *acn* (aconitase) expression (Fig. 4.1.B).

Transcriptional regulation of *acn* is a nice example for the complexity of regulatory systems controlling genes of the central metabolism and similar situations can be expected for other TCA cycle genes. *E. coli* contains two aconitases, the first one encoded by *acnB*, is the major TCA cycle enzyme and the second, encoded by *acnA* is an aerobic stationary phase enzyme induced by iron and redox stress (Gruer and Guest, 1994). The *acnB* gene is also regulated in a complex manner by several transcriptional regulators such as CRP.

4.1.1 GlxR, a transcriptional regulator of TCA cycle genes

The transcriptional regulator GlxR was first described as a cAMP-dependent DNA-binding protein which is involved in the regulation of the glyoxylate bypass gene *aceB* in *C. glutamicum* (Kim et al., 2004). Experiments using cGMP as a replacement of cAMP showed the specificity of GlxR for cAMP (Sorger, 2006). The first hint regarding a regulation of TCA cycle genes by GlxR was an *in silico* identification of a DNA-binding motif similar to the *E. coli* CRP motif $TGTGAN_6TCACA$ in the promoter region of the succinate dehydrogenase operon *sdhCAB* (Bott, 2007). Because no GlxR consensus sequence had been identified at this time, a motif similar to that bound by CRP was assumed to be recognized by GlxR.

GlxR regulates *sdhCAB* expression

A cAMP-specific binding of GlxR to the promoter region of *sdhCAB* was reported by Han et al. (2008), but had previously also been shown in our laboratory. In order to identify transcriptional regulators of the succinate dehydrogenase (SDH) operon, *sdhCAB* DNA affinity chromatography experiments were performed using crude extracts of *C. glutamicum* wild-type cells grown on glucose or acetate. In these experiments GlxR could be identified as an enriched transcriptional regulator next to the other transcriptional regulators RamA, RamB and DtxR. GlxR was enriched much more strongly from glucose-grown cells than from acetate-grown cells, which could be explained by the presence of more active GlxR protein in glucose-grown cells due to a higher cAMP level (Kim et al., 2004). Although the GlxR regulon in *C. glutamicum* has been published, the effect of GlxR on expression of *sdhCAB* or other TCA cycle genes has not been reported (Han et al., 2008b; Kohl et al., 2008). In our work, the results of the DNA affinity chromatography were confirmed by showing a specific binding of purified GlxR to the motif TGTGAGGAAGCTCACC located 200 bp upstream of the TS of the *sdhCAB* promoter. A strain overproducing GlxR showed decreased expression of *sdhCAB* and decreased SDH activity (both 30% reduced) and these effects were dependent on the GlxR binding motif. Therefore, GlxR acts not only as repressor of the glyoxylate genes *aceA* and *aceB* and the gluconate catabolism genes *gntK* and *gntP*, but also of the TCA cycle genes *sdhCAB* (Kim et al., 2004; Letek et al., 2006).

The distance of the GlxR DNA-binding site 200 bp upstream of the mapped TS of *sdhCAB* is quite long for a repressor binding site (Fig. 4.1C). Usually repressor binding sites are found in short distance to the TS, often between -30 and +10 bp with respect to the TS

(Collado-Vides et al., 1991). The described GlxR binding sites in Kohl et al. (2008) show in several cases a distance of more than 200 bp with respect to the translational start site and 6 out of 20 GlxR binding sites are located more than 40 bp upstream of the TS of genes with known TS. The repressory effect on *sdhCAB* expression might be a result of a competitive interplay with the binding of a yet unknown transcriptional activator. Another explanation could be the existence of a second TS that has not yet been identified. A second TS is also present in the case of the *gltA* gene (citrate synthase) of *C. glutamicum*, which is regulated by GlxR, too. Here, only the long transcript is repressed by GlxR. In *E. coli* next to the *gltA* gene also the *sdhCDBA* operon is controlled by two promoters (Wilde and Guest, 1986).

Next to the described regulation of expression by GlxR it was shown that RamA activates *sdhCAB*. This activation is dependent on the presence of acetate as carbon source and the growth phase. A 2-3-fold higher *sdhCAB* promoter activity is found in acetate grown cells compared to glucose grown cells and a 4-fold lower promoter activity was found in Δ*ramA* cells grown on glucose or glucose plus acetate. Both RamA binding sites, which are located 57 bp and 235 bp upstream of the TS, were bound by RamA and were shown to be of relevance for the regulation of *sdhCAB* expression (Fig. 4.1C). The regulation of *sdhCAB* and *acn* expression by RamA indicates that RamA plays an essential role in the regulation of TCA cycle genes in cells grown on acetate. As mentioned previously the results of the DNA affinity chromatography revealed a binding of the RamA antagonist RamB to the intergenic region between *sdhCAB* and *ramB*. Although a specific binding by RamB was confirmed in EMSAs, further analyses revealed that RamB apparently does not have any function in regulating *sdhCAB* expression under the tested conditions. The RamB binding site, which is located 423 bp upstream of the TS of *sdhCAB*, might have only an influence on the control of *ramB* expression (Cramer et al., 2007).

Another transcriptional regulator enriched by DNA affinity experiments is the global regulator of iron homeostasis DtxR (Wennerhold and Bott, 2006). Decreased mRNA level of *sdhCAB* in a *dtxR* deletion mutant suggested that DtxR acts as transcriptional activator (Brune et al., 2006; Wennerhold and Bott, 2006). Furthermore, the transcriptional regulator RipA also represses *sdhCAB* expression and is itself repressed by DtxR. Taken together, the transcriptional control of *sdhCAB* shows a similar complexity as the regulation of *acn* expression and involves many of the same players. One can however exclude the regulation of other TCA cycle genes by RipA and DtxR due to missing DNA-binding motifs and the fact that succinate dehydrogenase and aconitase are the only iron containing enzymes in the TCA cycle of *C. glutamicum* (Wennerhold et al., 2005).

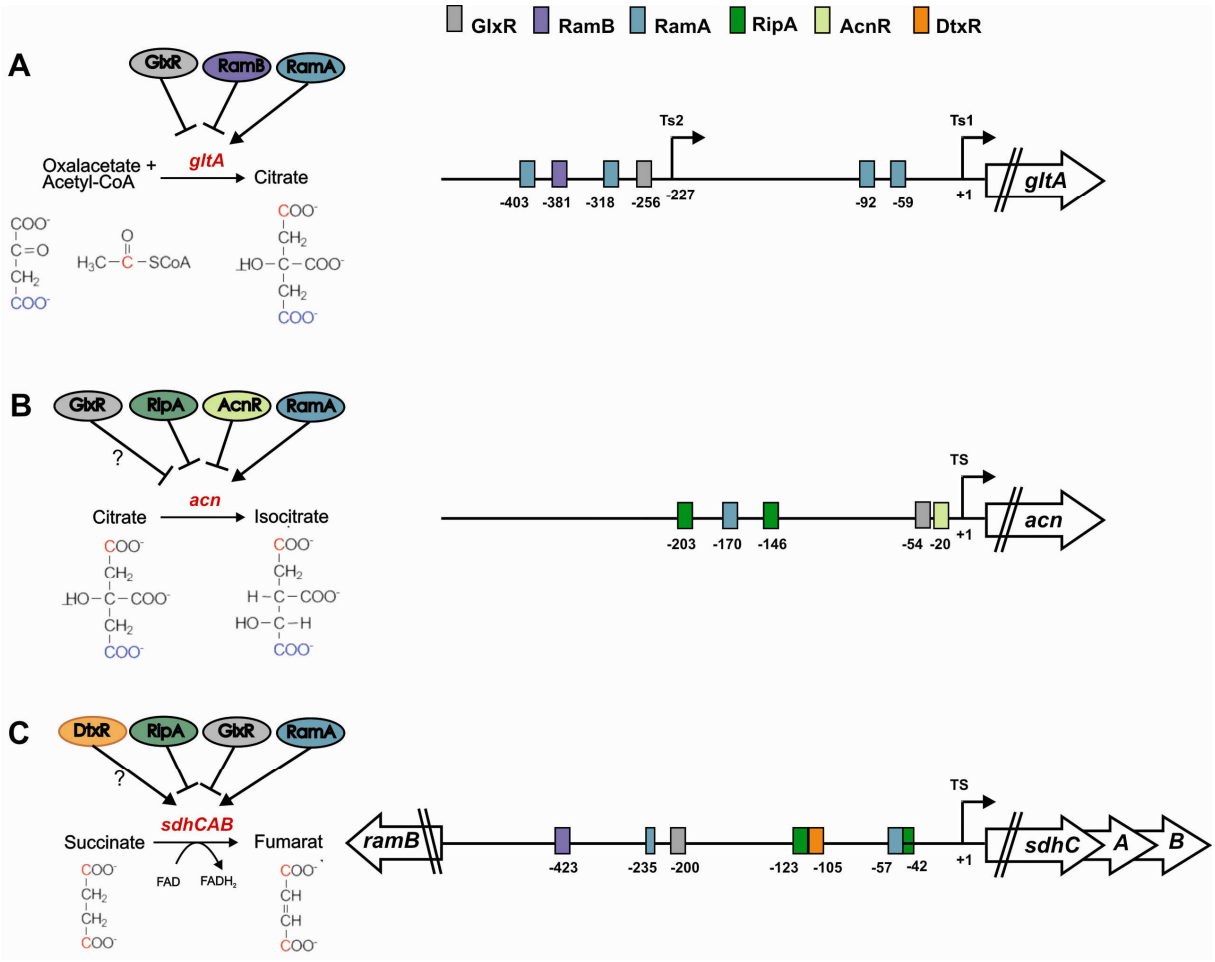


Fig. 4.1. Overview on the regulation of *gltA* (A), *acn* (B) and *sdhCAB* (C) expression in *C. glutamicum*. Directly involved transcriptional regulators and known DNA-binding motifs are indicated. The numbers give the distance between the center of binding motis and the transcriptional start site. Activation is indicated by arrows, repression by blunt ended lines.

GlxR regulates *gltA* expression

Next to the binding of GlxR to the *sdhCAB* promoter the binding to the citrate synthase (*gltA*) promoter region has been reported by Han et al., (2008b) and also previously shown in our laboratory. Due to a long distance of the DNA-binding motif to the TS it was assumed that GlxR acts as activator of *gltA* expression (Han et al., 2008b). The results of the work described here indicate that the motif 357 bp upstream of the TS is recognized by GlxR and that CS activity decreases in a GlxR overproducing strain grown on glucose or acetate. The reduction of CS activity by 41% in glucose-grown cells compared to 25% in acetate-grown cells might be caused again by the higher cAMP level mesured in glucose-grown cells (Kim et al., 2004). The weak effects of GlxR on *gltA* (40-25% reduction) and *sdhCAB* (30%

reduction) expression indicate a function in fine-tuning the expression of TCA cycle genes. Results obtained by Jan van Ooyen showed that the monocistronic *gltA* gene is controlled by two promoters (P1 and P2) resulting in two different transcripts. The recognized GlxR motif is now located 19 bp upstream of the second identified TS and thus in a position typical for repressors. Northern blot analyses confirmed that GlxR represses only the long *gltA* transcript in glucose-grown cells (data not shown).

The dominance of the short *gltA* transcript in acetate-grown cells shows that not only GlxR is involved transcriptional regulation. RamA was demonstrated to exert a positive control on both promoters and RamB repressed expression from both promoters. The transcriptional regulators GlxR, RamA and RamB were originally isolated as regulators of the glyoxylate bypass genes *aceA* and *aceB* (Cramer et al., 2006; Gerstmeir et al., 2004; Kim et al., 2004). They control in addition the expression of the TCA cycle genes *acn*, *gltA* and *sdhCAB* which also act in the glyoxylate cycle and RamB is involved in the control of *gltA* expression (Fig. 4.1; Fig. 4.2). Thus the global regulators GlxR, RamA and RamB show a coordinated expression of their target genes in central metabolism. Activation of RamA during growth on acetate and varying cAMP concentrations depending on the carbon source provided indicate a carbon catabolite regulation in *C. glutamicum* by GlxR and RamA.

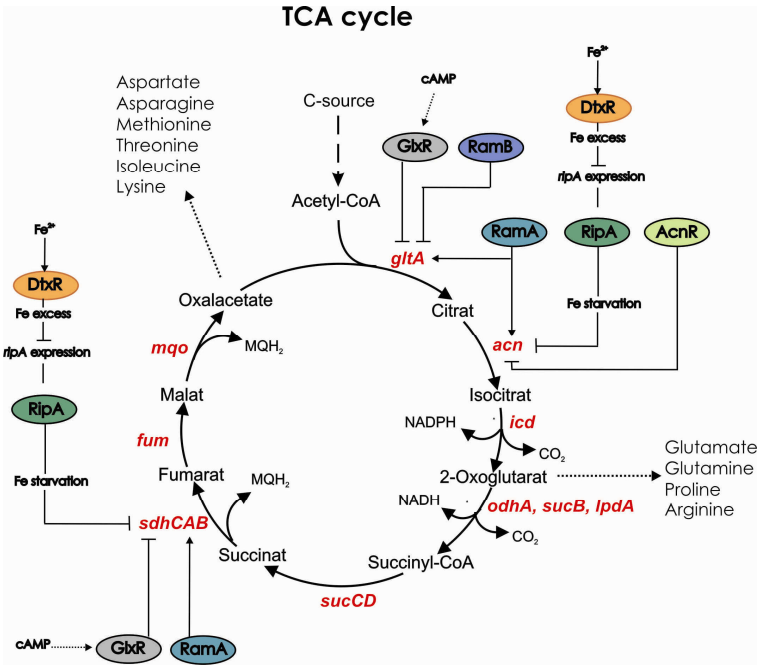
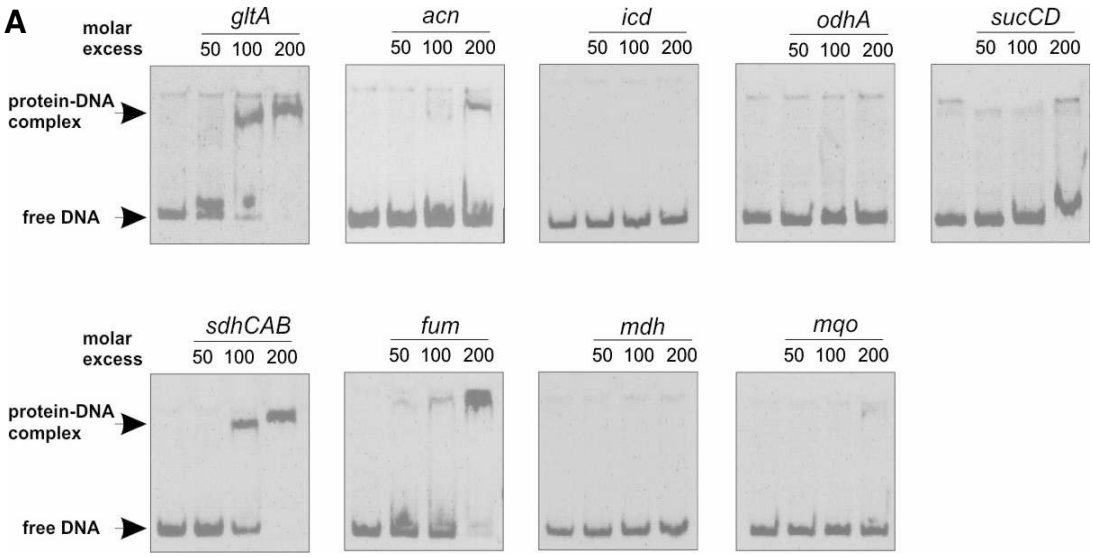


Fig. 4.2. Transcriptional regulation of the TCA cycle in *C. glutamicum*. The genes indicated encode the following enzymes: *gltA*: citrate synthase, *acn*: aconitase, *icd*: isocitrate dehydrogenase, *odhA*, *sucB, lpdA*: 2-oxoglutarate dehydrogenase, *sucCD*: succinyl-CoA synthetase, *sdhCAB*: succinate dehydrogenase, *fum*: fumarase, *mqo*: malate:menaquinone oxidoreductase. The amino acids derived from oxaloacetate and 2-oxoglutarate and the currently known transcriptional regulators including their proven target genes are indicated. Lines emanating from transcriptional regulators ending with arrows indicate transcriptional activation and the lines ending blunt indicate transcriptional repression. Adapted from Bott (2007).

GlxR as global transcriptional regulator

In additional experiments which are not shown in the publication and the manuscripts, EMSAs using purified GlxR protein revealed that GlxR binds to further promoters of TCA cycle genes, namely that of *acn*, *fum* and *sucCD*. Similar results have been described by Han et al. (2008), but they show EMSAs using only a single GlxR concentration, which gives the impression that GlxR binds with similar affinity to these promoters. In our work the EMSAs were carried out using three different GlxR concentrations and showed that the promoter regions of *gltA* and *sdhCAB* have the highest affinity to GlxR (Fig. 4.3A). The corresponding promoter fragments were partially or nearly completely shifted using a 100-fold molar excess of GlxR and a complete shift was obtained at a 200-fold molar excess. The promoter fragments of *sucCD* and *fum* were only completely shifted at a 200-fold molar excess and the



B

TGTGA--N6--ACACT

TGTGCACCAGTCCACT	$gltA_a$
TGTGTCGAGTAACAAA	$gltA_b$
TGTGGAAAAGGACCCT	*acn*
TGTGGTCACCAGCATT	*icd*
TGTGAAGTCTAACACT	*sucC*
TGTGAGGAAGCTCACC	*sdhC*
TATTAACCTAGACAAT	fum_a
CCTTATTTCTCACACT	fum_b
GTTGTCGCGAAACACT	fum_c
TATTACCCACCTCACT	fum_d
TATCAAAGATAACACC	*mdh*
TGTGCGCGAGTCGACT	*mqo*

Fig. 4.3. (A) . GlxR binding to promoter probes of TCA cycle genes. (A) DNA fragments (15 nM) covering the promoter region of the TCA cycle genes *gltA* (citrate synthase), *acn* (aconitase), *icd* (isocitrate dehydrogenase), *odhA* (2-oxoglutarate dehydrogenase), *sucCD* (succinyl CoA synthetase), *sdhCAB* (succinate dehydrogenase), *fum* (fumarase), *mdh* (malate dehydrogenase) and *mqo* (malate quinone oxidoreductase) were incubated for 20 min at room temperature with 0.2 mM cAMP and 0.75 – 3 mM purified GlxR protein. Samples were separated by native PAGE (15 %) and stained with SybrGreen. (B) Putative GlxR-binding motifs present in the promoter regions of *C. glutamicum* TCA cycle genes. The letters in bold and underlined indicate nucleotides identical to the consensus motif shown on top

acn fragment was shifted only partially at a 200-fold molar excess of purified GlxR. Promoter regions of *icd*, *mdh* and *mqo* were not shifted by GlxR. The results revealed different affinities of GlxR to the respective promoters, due to differences in the binding motifs (Fig. 4.3B). The motifs present in the completely shifted DNA fragments share the inverted repeat GTG and CAC, which are also present in the *E. coli* CRP motif (Lawson et al., 2004). An exception is fum_c containing TTG and CAC. The differences in the inverted repeat seem to cause a varying affinity of GlxR to the promoter fragments. The reason for such a diversity of the motifs in the promoter regions of TCA cycle genes could be a hierarchically regulation based on active GlxR concentration. This might have the consequence that only the promoter regions with the highest affinity are bound by GlxR.

Beside the DNA-binding experiments, which indicated further GlxR targets in the TCA cycle of *C. glutamicum*, fumarase activity measurements using a GlxR-overproducing strain howed a 50% increased activity in the overproducing strain. This indicates that GlxR might function as an activator of *fum* expression. Including the other relatively weak effects on *gltA* and *sdhCAB* a function of GlxR in fine-tuning expression of TCA cycle genes is assumed. The question why GlxR activates *fum* expression but represses *sdhCAB* expression might be answered by a hierarchically control dependent on cAMP or GlxR concentration.

The results show that GlxR appears to control expression of more than half of the TCA cycle genes, but strong effects as were described for RamA control are absent under the tested conditions. DNA-binding of GlxR as a global transcriptional regulator is reported for 64 promoters of genes or operons involved in different cellular processes (Han et al., 2008a; Han et al., 2008b; Jungwirth et al., 2008; Kim et al., 2004; Kohl et al., 2008; Kohl and Tauch, 2009; Letek et al., 2006). These cellular processes can be divided in central carbohydrate metabolism, aromatic compound degradation, aerobic and anaerobic respiration, glutamate uptake and nitrogen assimilation, fatty acid biosynthesis and others. Comparison of the CRP regulon of *E. coli* and the GlxR regulon of *C. glutamicum* led to the identification of 23 orthologous genes, indicating a significant overlap between the regulons of these cAMP-dependent regulators (Kohl et al., 2008). This group contains 6 genes of central carbohydrate metabolism including *aceA, gntK, gntP*, *ptsG, ptsI*, two respiratory genes and 6 genes involved in aromatic compound catabolism. Based on a genome-scale prediction of GlxR DNA-binding motifs, a direct regulation of about 14% of annotated *C. glutamicum* genes is assumed (Kohl and Tauch, 2009). Thus, a physiological function as a global transcriptional regulator co-ordinately controlling various cellular processes can be envisaged for GlxR similar to Crp of *E. coli*.

In *E. coli* CRP also regulates succinate dehydrogenase (*sdhCDBA*) expression (Nam et al., 2005; Takeda et al., 1999). The expression of the *E. coli sdhCDBA* operon is complexly regulated in response to growth conditions, such as anaerobiosis and carbon sources. The regulatory mechanisms of anaerobic repression have been well documented, involving both the ArcA-ArcB two-component system and Fnr as global anaerobic regulator (Park et al., 1995; Perrenoud and Sauer, 2005). CRP, the key mediator of carbon catabolite repression, which itself is directly regulated in a cAMP-dependent manner, regulates glucose repression of *sdhCDBA* (Nam et al., 2005). Furthermore, expression of *gltA* is controlled by CRP. In the promoter region of *gltA* four CRP DNA-binding motifs are present (Wilde and Guest, 1986) and like in *C. glutamicum* the *E.coli gltA* gene is expressed from two promoters (Wilde and Guest, 1986). The redox-sensing ArcA-ArcB two-component system also controls *gltA* expression in *E. coli* (Park et al., 1994). Further TCA cycle genes regulated negatively by ArcAB in anaerobic catabolism are *icdA, sdhCDBA, fumA* and *mdh* (Perrenoud and Sauer, 2005).

Many Gram-positive bacteria such as *Bacillus subtilis* do not possess cAMP and use a different CCR mechanism. Also in these bacteria the citrate synthase gene *citZ* is subject to CCR. In *B. subtilis* the transcriptional regulator CcpA controls the expression of *citZ* directly and indirectly (Kim et al., 2002b). CcpA binds to the catabolite-responsive element (cre) which is present in the *citZ* promoter and also in the promoter of the transcriptional regulator CcpC that negatively regulates genes of the TCA cycle and thus regulates directly or indirectly dozens of target genes in *B. subtilis* (Fujita, 2009; Kim et al., 2002a; Kim et al., 2002b).

4.2. The adenylate cyclase CyaB in *C. glutamicum*

4.2.1 Characteristics of a *cyaB* deletion mutant

In order to understand the physiological role of GlxR, it is important to understand the regulation of the cellular cAMP concentration, which is determined by activity of the enzymes synthesizing and degrading cAMP. In this context, the adenylate cyclase CyaB was studied in this work. The first unexpected result was the successful construction of the deletion mutant Δ*cyaB*. As our attempts to delete *glxR* failed and as CyaB is the only annotated adenylate cyclase in *C. glutamicum*, it had been expected that also cannot be deleted. The adenylate cyclase CyaB of *C. glutamicum* consists of a CHD (cyclase homolog domain) at the C-terminus and six transmembrane helices at the N-terminus. These domains

are separated by a cytoplasmic HAMP (found in histidin kinase, adenylyl cyclase, methyl binding proteins and phosphatases) domain, which is assumed to serve as transmitter domain to transfer extracellular signals to cytosolic effectors. HAMP domains are found in bacterial sensors and connect extracellular sensory with intracellular signaling domains in over 7500 proteins (Hulko et al., 2006). Due to the fact that adenylate cyclases are active as dimers (Tesmer et al., 1997; Zhang et al., 1997), a resulting twelve transmembrane helix anchor could function as sensor or as transporter (Fig. 4.4). However, a signal or ligand is not known yet.

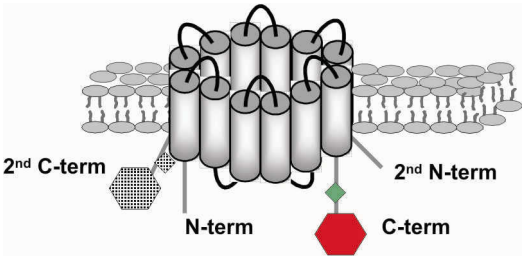


Fig. 4.4. Model of CyaB dimer as putative sensor or transproter in *C. glutamicum*. One CyaB monomer consists of 6 transmembrane helices and a HAMP-domain (green square) followed by a cyclase catalytic domain (red hexagon) at the C-terminus.

The characterization of Δ*cyaB* revealed a phenotype with growth defects in minimal medium containing acetate or propionate as sole carbon source and acetate/glucose, acetate/gluconate or acetate/ethanol as carbon source mixtures. A similar phenotype was very recently described by Cha et al. (2009). That the growth defects were indeed caused by the *cyaB* deletion was confirmed by their reversal with plasmid-encoded CyaB. Also the provision of extracellular cAMP could reverse the growth defect, indicating that an altered cAMP concentration is the reason for it. Afterwards the question arose why Δ*cyaB* has a growth defect in acetate minimal medium or acetate mixtures. Both carbon sources, acetate and propionate, are transported by the monocarboxylic acid transporter MctC (Jolkver et al., 2009). However, a changed transporter activity due to *cyaB* deletion can not be the reason for the growth defect, because a *mctC* deletion mutant grew in acetate medium like the WT (Jolkver et al., 2009). In this case acetate diffuses into the cell or is transported additionally by an unknown transporter, which could be CyaB. Another reasonable explanation would be a defect in proton-motive force in Δ*cyaB,* which is essential for growth on acetate.

Growth of Δ*cyaB* was not inhibited in ethanol minimal medium, although ethanol is oxidized to acetate by alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH), which afterwards is activated to acetyl-CoA by acetate kinase (AK) and phosphotransacetylase (PTA). All these enzymes are essential for growth on acetate or ethanol (Arndt and Eikmanns, 2007). The results show that growth is only inhibited when

acetate is present extracellularly. Growth on ethanol and the measured PTA and AK activities (PTA [U/mg protein]: 7.3 ± 0.6 (WT), 7.7 ± 0.2 (Δ*cyaB*); AK [U/mg protein]: 3 ± 0.2 (WT), 2.7 ± 0.1 (Δ*cyaB*)) show that enzymes required to metabolize acetate are active in strain Δ*cyaB*. Furthermore, Cha et al. (2009) reported that malate synthase (MS) and isocitrate lyase (ICL) had similar activities in a *cyaB* mutant and in the wild type.

Our results showed a slightly decreased cAMP concentration in glucose-grown cells of strain Δ*cyaB* and a very strong decrease in Δ*cyaB*-cells grown in an acetate/glucose minimal medium. The fact that cAMP was still detectable in strain Δ*cyaB* suggests that an unknown source of cAMP or another enzyme with adenylate cyclase activity exists in *C. glutamicum*.

4.2.2 Decreased cAMP level effects regulation by GlxR

In the DNA microarray experiments 29 genes with a twofold altered mRNA level in Δ*cyaB* cells grown in glucose minimal medium and 408 genes with twofold altered mRNA level grown in acetate/glucose mixture have been identified. Focused on GlxR target genes (Han et al., 2008a; Kim et al., 2004; Kohl et al., 2008; Letek et al., 2006), 47% of them showed a minimally twofold altered mRNA level in Δ*cyaB* cells grown in acetate/glucose minimal medium. In Δ*cyaB* cells grown in glucose minimal medium, just 2% of GlxR target genes showed a twofold altered mRNA level. Due to higher cAMP levels in *C. glutamicum* cells grown in glucose compared to acetate grown cells, a higher influence on the transcriptome was assumed. In the DNA microarray experiments from acetate/glucose grown cells, the genes *eno* (enolase), *pgm* (phosphoglycerate mutase), *pck* (PEP carboxykinase) acting in glycolysis or anaplerosis were found to have reduced mRNA levels in Δ*cyaB*, which might also explain the decreased growth rate.

Taken together, it was shown that some genes with altered mRNA level in the DNA microarray comparisons are GlxR target genes and show a different mRNA level due to missing regulation by GlxR caused by a lower cAMP concentration. The results confirm the suggestion that GlxR plays a similar role like *E. coli* CRP in carbon catabolite repression. However, an identification of the extracellular signal recognized by CyaB will show which environmental conditions cause an altered cAMP concentration in *C. glutamicum*.

In *C. glutamicum* and *E. coli* as nonpathogenic organisms, AC and the cAMP level is subject to carbon catabolite repression. In pathogenic organisms, such as *Bordetella pertussis* the situation differs. Here the Class II ACs are secreted toxins (Agarwal and Bishai, 2009;

Dautin et al., 2002). In *M. tuberculosis*, a relative of *C. glutamicum*, cAMP regulation seems to be much more complex. Several ACs are present in *M. tuberculosis* and biochemically distinct, which shows that cAMP production is driven by different ACs under different conditions. These conditions, such as low pH, detergents, bicarbonate/CO_2 or fatty acids can modulate activities of mycobacterial ACs (Abdel Motaal et al., 2006; Cann et al., 2003; Castro et al., 2005; Ketkar et al., 2006; Linder et al., 2004; Linder et al., 2002; Tews et al., 2005). Furthermore *M. tuberculosis* possesses 10 NMP-binding proteins (or cNMP), including Rv3676 as CRP homologues binding cAMP. However, less is known about Rv3676 regulation. It was only demonstrated that Rv3676 regulates mycobacterial gene expression in a cAMP dependent manner (Rickman et al., 2005). In mycobacterial pathogenicity it is assumed that also cAMP plays an important role. *M. microti* for example utilizes cAMP secretion to prevent phago-lysosomal fusion (Lowrie et al., 1979; Lowrie et al., 1975). *M. tuberculosis* also secretes cAMP and a cAMP-based pathogenicity is assumed (Agarwal et al., 2009). However the multiplicity of ACs in *M. tuberculosis* makes it difficult to address the question of cAMP- based pathogenesis (Dittrich et al., 2006; Guo et al., 2005).

4.3 Regulation of oxidative stress response

With the advent of O_2 in the earth´s atmosphere, organisms had to evolve sophisticated protection mechanisms to encounter the novel problem of oxidative stress. The raising oxygen concentration led to the formation of harmful oxygen species, resulting e.g. from the Fenton reaction, in which Fe^{2+} reacts with hydrogen peroxide to yield Fe^{3+}, OH^-, and the highly reactive hydroxyl radical (·OH). Another radical is the superoxide (O_2^-), which is also produced as a byproduct of respiration and highly reactive resulting in the damage of all macromolecules (proteins, DNA, and lipids). Living organisms have to build defense systems against oxidative stress, with enzymes such as superoxide dismutase or catalase, small proteins like glutaredoxin or thioredoxin, and molecules such as glutathione. Sophisticated regulatory systems have evolved to control this important response. *E. coli* possesses a defense program against peroxides, mediated by the transcriptional activator OxyR, and another one against superoxide, controlled by the SoxRS system. OxyR is reversibly activated by the formation of an intramolecular disulfide bond in the presence of H_2O_2 and then activates expression of genes for e.g. catalase or glutaredoxins (Aslund et al., 1999; Zheng et al., 1998). SoxR is a homodimer, with each monomer containing a $[2Fe2S]^{2+}$ cluster, which is oxidized by the superoxide radical to a $[2Fe2S]^{3+}$ cluster. This oxidation is proposed to cause a structural alteration that activates SoxR (Gaudu et al., 1995). In enteric bacteria, the target gene of SoxR is *soxS*, which encodes a transcription factor that promotes

expression of more than a dozen genes playing roles in mitigating oxidative stress (Amàbile-Cuevas and Demple, 1991). In the *C. glutamicum* genome, a homolog of OxyR is encoded (cg2109), which is assumed to have a similar function as the *E. coli* protein, but has not yet been characterized. SoxRS homologoues genes are not present in the *C. glutamicum* genome.

4.3.1 RosR, a bifunctional transcriptional regulator in *C.glutamicum*

RosR (Cg1324) is the first member of the MarR family of transcriptional regulator that has been studied in *C. glutamicum*. Members of this family exist usually as homodimers in free and DNA-bound state and often positively regulate gene expression by acting as derepressors (Amàbile-Cuevas and Demple, 1991; Heroven et al., 2004; Nasser et al., 1999; Wyborn et al., 2004). RosR in *C. glutamicum* was also shown to be a homodimer and to act as a repressor of nearly all of its target genes. This set of genes consists of two genes encoding FMN-reductases (cg1850, cg1150), three luciferase-like monooxygenases (cg2329, cg3085, cg1848), one putative flavin-containing monooxygenase (cg3084), one protein of glutathione S-transferase family (cg1426), on hypothetical protein (cg1849) and cg1322 which encodes a putative polyisoprenoid-binding protein. In all cases the physiological function of the proteins is unknown. Pfam analysis of Cg1322, whose gene cg1322 lies divergently to *rosR*, revealed that it is a YceI-like protein. The YceI protein of *E. coli* was shown to be induced under pH or osmotic stress (Nakunst et al., 2007; Weber et al., 2006). The crystal structure of the TT1927b protein, a YceI homolog of *Thermus thermophilus*, revealed that it bound octaprenylpyrophosphate and therefore was proposed to have a role in isoprenoid quinone metabolism and/or storage (Handa et al., 2005). Cg3084 belongs to the FAD-dependent pyridine nucleotide reductases (FADPNR) which use the isoalloxazine ring of FAD to shuttle reducing equivalents from NAD(P)H to a cysteine residue that is usually a part of a redox-active disulfide bridge, including glutathione reductase or thioredoxin reductase. In combination with Cg1426, annotated as a protein of the glutathione S-transferase (GST) family, Cg3084 might be involved in the oxidative stress response in *C. glutamicum*. Bacterial GSTs of known function often have a specific, growth-supporting role in biodegradative metabolism, for example epoxide ring opening (Vuilleumier, 1997). Although Cg1426 belongs to the glutathione S-transferase family, *C. glutamicum* does not posses glutathione, but instead mycothiol (Feng et al., 2006; Ordònez et al., 2009).

One target that is activated by RosR is the operon *narKGHIJ* which codes for a nitrate/nitrite antiporter (*narK*) and the dissimilatory nitrate reductase *(narGHIJ*). This

enzyme allows *C. glutamicum* to use nitrate as terminal electron acceptor during anaerobic growth (Nishimura et al., 2007; Takeno et al., 2007). Anaerobic growth of the *rosR* deletion mutant and a *rosR* overexpressing strain was tested, but no differences to the wild type were observed (data not shown), indicating a more important role of other transcriptional regulators known to regulatoe *narKGHIJ* expression, such as RipA, ArnR or GlxR (Kohl et al., 2008; Nishimura et al., 2008; Wennerhold et al., 2005).

Another target of RosR which was identified in the *in silico* search using the identified DNA-binding motif was *sodA*, encoding superoxide dismutase (SOD). SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and would fit to a role of RosR in the oxidative stress response. Although the *sodA* promoter region was positively shifted by RosR in EMSAs, DNA microarray experiments and enzyme activity tests showed no influence of RosR on *sodA* expression and SOD activity. Therefore, the role of RosR in *sodA* regulation remains unclear.

The DNA-binding motif recognized by RosR in the promoter regions of the target genes was identified by DNase I footprint analyses and EMSAs using the *narK* promoter. The motif AATGTTGATATAAGCACAAA in the *narK* promoter is reasonably conserved in the RosR regulon and consists of an inverted repeat ($TTGTTGAN_4TCAACAA$), suggesting that each half is bound by one of the monomers of the RosR dimer, as is typical for several transcriptional regulators such as CRP of *E. coli* (Lawson et al., 2004).

Binding of MarR family proteins often cause a steric inhibition of RNA polymerase binding, resulting in the repression of gene expression (Wilkinson and Grove, 2006). In this case the DNA-binding motif is usually located near the -10 or -35 elements of the promoter. Identification of the TS of RosR target genes close to the DNA-binding motifs suggests a similar inhibition of the RNA polymerse by RosR. The only exception is cg1322. Here the TS is located 85 bp downstream to the RosR-binding site. In this case RosR might block an unknown activator of cg1322 expression or the repression by RosR is caused by a DNA-loop between the two DNA-binding motifs in the intergenic region of cg1322 and *rosR* due to interacting RosR dimers. The identification of the *rosR* TS directly upstream of the RosR-binding motif indicated a negative autoregulation of RosR. Promoter activity tests confirmed the assumption of a negative autoregulation, which is observed for more than 50% of MarR family members (Wilkinson and Grove, 2006).

In additional DNA microarray experiments *C. glutamicum* wild type was subjected to cumene hydroperoxide (CHP) stress (data not shown). The results revealed that cg1322, cg1848 and cg1426 are upregulated under CHP stress, indicating an involvement in CHP

resistance. In general, the response to peroxides seems to vary in *C. glutamicum* due to missing *ahpCF* genes which are part of the peroxide stimulon in *B. subtilis* or *E. coli*. AhpC and AhpF are proteins reducing various alkyl hydroperoxides (Bsat et al., 1996). The assumption that Cg1322 plays a role in peroxide resistance was confirmed by a cg1322 deletion mutant, which was more sensitive towards hydrogen peroxide. Taken together, RosR acts as bifunctional transcriptional regulator and binds to a specific 20-bp motif in the promoter regions of its target genes, which seem to be involved in the oxidative stress response of *C. glutamicum*.

4.3.2 RosR as redox-sensitive transcriptional regulator

Transcriptional regulators involved in the oxidative stress response often sense the stresses by changes in their redox status, which cause an activation or inactivation of the regulator. The transcriptional regulator OhrR, which also is a member of the MarR family, is inactivated by oxidation of a single conserved cysteine residue to the corresponding cysteine-sulfenic acid (Fuangthong and Helmann, 2002). The already mentioned OxyR and SoxR are regulators which sense hydrogen peroxide or superoxide radicals using either thiol disulfide exchanges or redox labile 2Fe-2S clusters, respectively (Pomposiello and Demple, 2001; Zheng and Storz, 2000). EMSAs with RosR showed that the protein is sensitive to hydrogen peroxide as it loses its DNA-binding ability in the presene of this compound. RosR contains three cysteine residues, of which Cys-92 is conserved among the RosR homologs in the *Corynebacterineae*. The role of the three cysteine residues in RosR sensitivity to hydrogen peroxide was demonstrated in mutational analyses. Therefore, the cysteines (Cys-64, Cys-92, Cys-151) were exchanged to serine. In EMSAs experiments it was shown that the hydrogen peroxide sensitivity is primarily due to Cys-92. It is assumed that during oxidation intermolecular disulfide bridges are formed in the RosR dimer resulting in a conformational change which affects the DNA-binding activity. The transcriptional regulator RosR-C64,92,151S, which contains no cystein residues anymore and is insensitive to hydrogen peroxide, was used for *in vivo* experiments under CHP stress. Strain Δ*rosR* was transformed with a plasmid encoding RosR-C64,92,151S, which was assumed to repress the RosR target genes also under oxidizing conditions. This strain showed a weakly increased sensitivity to CHP on agar plates and in liquid cultures. Under CHP stress, the RosR target genes seem to be involved in the defence, which was also reflected by the upregulation of three genes of the

RosR regulon in the presence of CHP. A putative higher sensitivity against hydrogen peroxide was also tested but revealed no clear data.

One reason for non-reproducible data might be that *C. glutamicum* is capable to deal with high concentrations of hydrogen peroxide (Nakunst et al., 2007). Hydrogen peroxide is degraded by catalase (*katA*) to prevent the formation of highly toxic hydroxyl radicals in the Fenton reaction (Green and Paget, 2004). Catalase appears to be present not only in the cytoplasm, but also secreted into the medium by the SecA2 system, which is an essential translocase in *C. glutamicum* (Caspers, 2006). This would allow to degrade hydrogen peroxide before it can enter the cell. In *M. tuberculosis* it is assumed that secreted superoxide dismutase and catalase are protecting the cells against reactive oxygen intermediates (ROI), the antimicrobial defence in host macrophages (Daffe and Etienne, 1999). Although *C. glutamicum* is not pathogenic and has not to compete with ROIs of macrophages, it has to deal with ROIs formed during aerobic metabolism and with hydrogen peroxide, since bacterial membranes are permeable for hydrogen peroxide (Green and Paget, 2004; Storz and Imlay, 1999). Therefore, a secreted catalase serves as a better protection against hydrogen peroxide and might be one explanation why the intracellular oxidative stress response is not induced by hydrogen peroxide. It was also shown that the *qor2* transcript level, which is regulated by the hydrogen peroxide-sensitive regulator QorR, did not change under hydrogen peroxide stress, but under diamide stress in *C. glutamicum* (Ehira et al., 2009; Nakunst et al., 2007).

Another reason why *C. glutamicum* secretes catalase for a better protection could be that too high intracellular catalase concentrations have a negative effect. KatB activity in *M. tuberculosis* for example is also dangerous (Master et al., 2001). KatB has also a function as

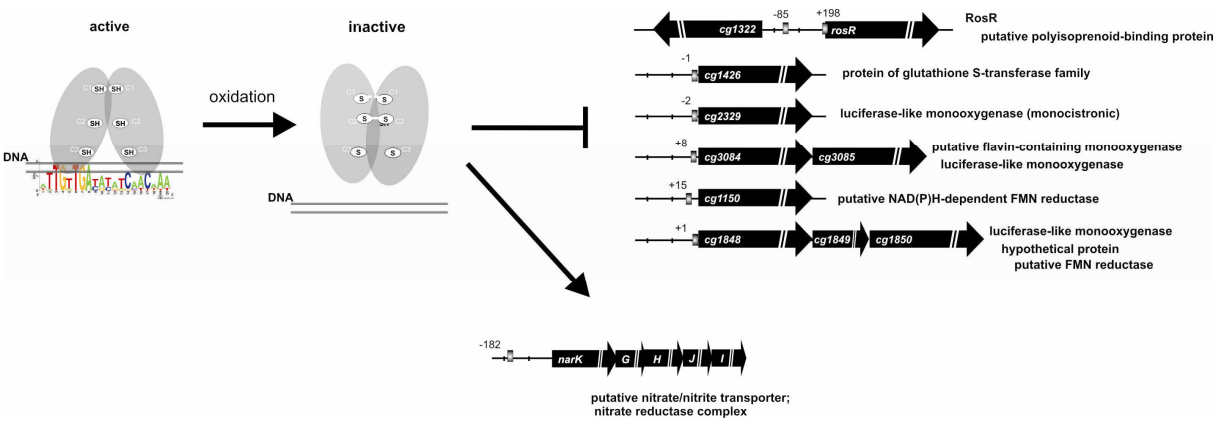


Fig. 4.5. Model of RosR function in *C. glutamicum*. The boxes indicate the RosR binding motif and the numbers indicate the distance to the mapped TS.

peroxidase which converts isonicotinic acid hydrazid (INH) to toxic isonicotinic acid (Slayden and Barry, 2000). However, *katA* in *C. glutamicum* is not homologues to *katB* in *M. tuberculosis.*

In summary, RosR is a redox-sensitive transcriptional regulator which represses and activates genes under reducing conditions. Upon exposure to hydrogen peroxide, RosR becomes oxidized resulting in an inactive regulator and the subsequent derepression or deactivation of its target genes (Fig. 4.5). However, the role of the proteins encoded by the RosR target genes in detoxification of ROIs is still unclear and needs further investigation. In the soil as the natural environment *of C. glutamicum* anaerobic areas or oxygen deprivation conditions exist. Under these conditions RosR is active and might activate expression of the *narKGHIJ* operon which is essential for anaerobic growth (Nishimura et al., 2007; Takeno et al., 2007).

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6. Appendix

6.1 Supplemental Material-CyaB

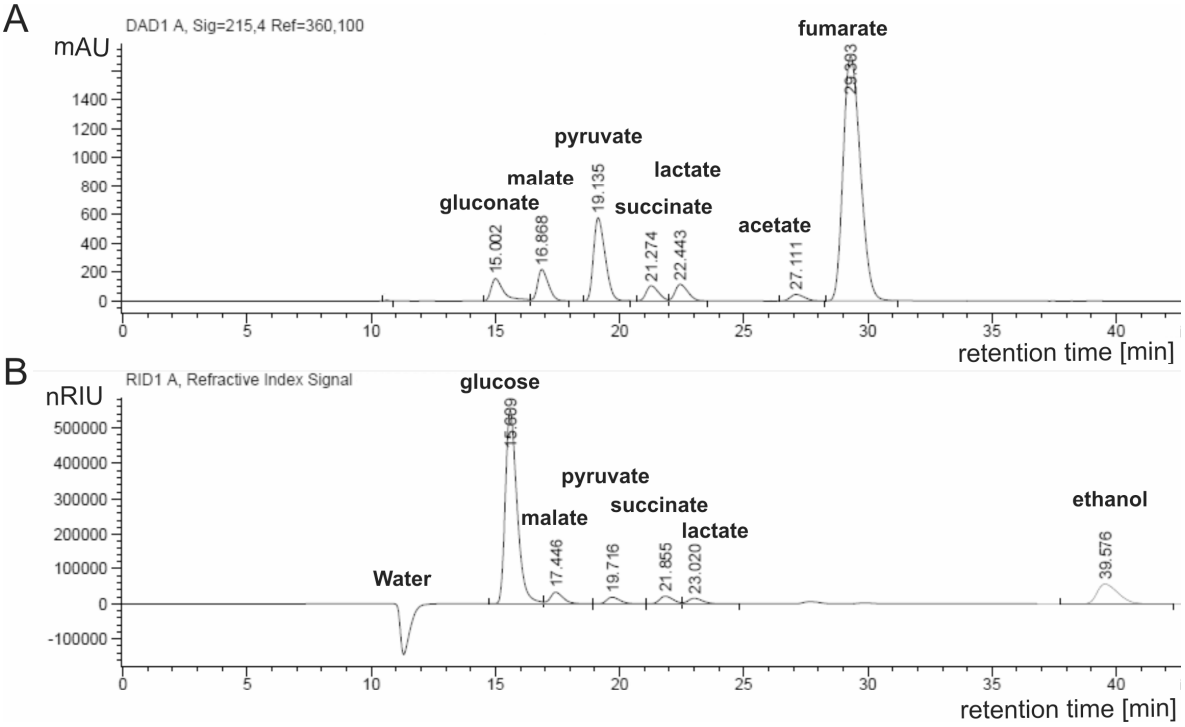

Fig. S1. Ion-exchange chromatography elution chromatogram of external standards. The standart mixture contained 1 mM fumarate; 10 mM pyruvate. lactate. succinate. acetate. malate. and gluconte; 100 mM glucose and 200 mM ethanol and was loaded on an Agilent 1100 LC system (Agilent Technologies. Waldbronn. Germany) equipped with an kation exchange column (Organic acid Refil-column. 300 x 8 mm. CS-Chromatography GmbH). Substances were eluted with a flow rate of 0.4 ml min^{-1} for 42 min at 40°C with 100 mM H_2SO_4. Figure A shows the elution chromatogram of the Diod Array detector (DAD)-detector at a wavelength of 215 nm (mAU = milli absorbance units). Figure B shows the elution chromatogram of the Refractive Index Detector (RID) with an optical unit temperature of 35°C (nRIU = nano refractive units). To quantify the consumed carbon source and secreted aminio acids at once. this new HPLC method was developed. One particular problem in the past was the separation of fumarat and acetate. Fumarate is very sensitively detected by DAD detector in low concentrations due to the C=C double bond. Therefore it was important that the fumarate peak does not interfere with other peaks. especially acetate.

The new developed method realizes the separation of all known acids secreted *by C. glutamicum*. The elution with 100 mM H_2SO_4 produces stable signals of each substance. Additionally to organic acids detected in DAD detector. sugars or ethanol can be detected in the RI-detector in one single run.

6.2 Supplemental Material-RosR

TABLE S1

Oligonucleotides used in this study

Oligonucleotide	Sequence (5´ to 3´); restriction sites underlined	Relevant characteristics
pET2-1324-for	AATAGGATCCGTCCTTTTCGGTGAAAG TGTG	*rosR* promotor region
pET2-1324-rev	AATAGAGCTCGTGTTGTCAAATCACCT ACCCTAG	for CAT assay
narK-RT-1-for	GGTTTCTGCCATCGCTCCCC	Real-time PCR
narK-RT-2-rev	GTTCGGGCGGGCGTAGTAGA	*narK*
narK-RT-3-for	GTGGCTTCTCACACTCGCTG	1+2 for standard realtime PCR
narK-RT-4-rev	CTGAATTATCGAGACGCCGAG	reaction
narG-RT-1-for	CACCGTCGGTTCTGAAGGCC	Realtime PCR
narG-RT-2-rev	CGGTGCCTTCAGGAATGCGG	*narG*
narG-RT-3-for	GCGCTGACCTCTATGTGTGG	1+2 for standard real time PCR
narG-RT-4-rev	CAGTGGTGATCATCTGACGTG	reaction
gluS-1-for	CGTCATCCGATTGGGCAGGC	Realtime PCR
gluS-2-rev	CTTCGCCCGCAGGAATTGGG	cg1426
gluS-3-for-LC	CGCGGCATTACTGTCCCAGC	1+2 for standard real time PCR
gluS-4-rev-LC	CTCCGCGGGGTAGAGGTTAG	reaction
2329-1-for	GTCTCCTTCGGCCTCGACAC	Realtime PCR
2329-2-rev	GCCTGCTTGGTCAAGATGTCC	cg2329
2329-3-for-LC	CTCGACACCTTCGGCGACAAC	1+2 for standard real time PCR
2329-4-rev-LC	GTATTCCTCACGGTGGTGCTC	reaction
narK-for-IRD	GATCCCGGCGCGCCAACGCC	DNase I footprint
narK-rev-IRD	GCTTTCCGTCAAGCCCGCCC	with *narK* promotor region
NCgl0971-for	CAAATGCGTTGAACAGGATTTTCCG	promotor region
NCgl0971-rev	GATGATGGCGATCTTGCTC	cg1150
NCgl2686-for	CGATCCGTTCGGTTGGCAGAC	promotor region
NCgl2686-rev	GTCCAGCCTGACCTGCTCC	cg3084
NCgl2826-for	GCGCCTCATCAGCGGTAACC	promotor region

NCgl2826-rev	GCTCGAGAGCGTCGTATGCG	cg3237
NCgl1580-for	GGGAACGGAATACGTGGCAC	promotor region
NCgl1580-rev	CTTCGGTGGGGGCTTTGCCG	cg1848
cg1324-for	GCCCTTAACGTGTGCATCGC	promotor region
cg1324-rev	GAGTTGTTGCTCTTCAGTGGAG	cg1324
coe420-for	CGACGAAGACCTGGTCTCATG	promotor region
coe420-rev	CGAAGTGTTTGTGCAGGGG	cg2329
glut-S_for	GTCGCGTTGCAGGATGTCGTTG	promotor region
glut-S_rev	GGATCCCGCTGGAACGTCTG	cg1426
nr2-x1	CGAATTTGTGCTTATATCAACATTCGTG	mutation of the
nr2-mut1	CGAAAAAGTGCTTATATCAACATTCGTG	*narK* promotor
nr2-mut2	CGAATTTCACCTTATATCAACATTCGTG	region
nr2-mut3	CGAATTTGTGGAAATATCAACATTCGTG	
nr2-mut4	CGAATTTGTGCTTTATTCAACATTCGTG	
nr2-mut5	CGAATTTGTGCTTATAAGTACATTCGTG	
nr2-mut6	CGAATTTGTGCTTATATCATGTTTCGTG	
nr2-mut7	CGAATTTGTGCTTATATCAACAAACGTG	
nr2-x1	CGAATTTGTGCTTATATCAACATTCGTG	
nr-2 -A-rev	CATTCACCGTCGTGGAGGAG	fragmentation of
nr-2-B-for	CTCCTCCACGACGGTGAATG	*narK*
nr-2-B-rev	GTCATCGTTGTGTCTGATCG	promotor region
nr-2-C-for	CGATCAGACACAACGATGAC	
nr-2-C-rev	CGTGATTCGGCAAAATTAATTAAACTG	
nr-2-D-for	CAGTTTAATTAATTTTGCCGAATCACG	
nr-2-D-rev	CCCGCATGTTTTTCAACGGG	
nr-2-E-for	CCCGTTGAAAAACATGCGGG	
nr-2-E-rev	GTTGTGTCACGTGATGCCTC	
nr-2-F-for	GAGGCATCACGTGACACAAC	
gluSt- -Mot-fw	CCAAGAGAGGACACCACACAG	fragmentation of
gluSt+ Mot-fw	GTAATATTGTTGACATATCATCTAAATTTCC	cg1426 promotor region
Coe420--Mot-fw	CTTTTAATAAGTCTGATCAACAACGTG	fragmentation of cg2329 promotor region
Coe420 + Mot-fw	CCCCATATTGTTGATATATCTACAAAC	
D1-1124	GGTGTTCTAGATGAATTCACC	construction of
D2-1124	CCCATCCACTAAACTTAAGTATTCAGTGGAGAGCCATCGTG	pK19mobsacB-ΔrosR
D3-1124	TACTTAAGTTTAGTGGATGGGCTCACCGCAGTGTTGAACTC	for *rosR* deletion
D4-1124	GCGATTTTCCCGGGATGGG	
D1124-fw	GGAAGCGGTCTTGATAACAACC	colony PCR for
D1124-rv	GGTGTCGCGCTGCTTCGCC	verifying *rosR* deletion
D1-cg1322	AATTCTAGAGCATTTTCGGGTTGATTTCC	construction of
D2-cg1322	CCCATCCACTAAACTTAAGTAGACGGCTCCGCAATCAAGG	pK19mobsacB-Δcg1322 for cg1322
D3-cg1322	TACTTAAGTTTAGTGGATGGGGAGGGTCCAGGTGCCAGTC	deletion
D4-cg1322	AATCCCGGGGTTCTTGGCACATGTCTCG	

Dcg1322-fw	GGTTCTACTGCGGTCCCAAT	colony PCR for verifying cg1322 deletion
Dcg1322-rv	CAATTCCCGTTCTACGCAG	
1124-NdeN	GGTAGGTCATATGACAACACC	construction of pET16b-rosR
1124-XhoC	TAAAAACCCTCGAGAAAACGC	
c1-for	GCGCCTGCGAGACATGTCCCAAGAAC TAGATTGGGAC	side directed mutagenesis of Cys64, 92, 151 to Ser using pET16b-rosR
c1-rev	GTCCCAATCTAGTTCTTGGGACATGTC TCGCAGGCGC	
c2-for	CTTAGTGGCCAAGGTTAAATCCGCAG GTGACGCACGAGG	
c2-rev	CCTCGTGCGTCACCTGCGGATTTAAC CTTGGCCACTAAG	
c3-for	GTTGAACTCCAACACCTCCATTGAGA TCAACAACCAAC	
c3-rev	GTTGGTTGTTGATCTCAATGGAGGTGT TGGAGTTCAAC	
cg1322-A	GTGGTCAGGTTGTGGTTTTC	primer extension
cg1322-B	GTGTACTCGGTGAATTCACC	
cg1426-A	GTCGCGTTGCAGGATGTCGT	
cg1426-B	GAACGTCTGCGACGATGCG	
cg3084-A	CGATCCGTTCGGTTGGCAGA	
cg3084-B	GAGGACGAGAAAATCTTTTCC	
cg1324-sp1	GAAAGAGTAACTAGTACTGC	5´ RACE
cg1324-sp2	ATTCTGAAGTGGTCAGGTTG	
cg2329-sp1	CTCCGATGCCGATGATATCC	
cg2329-sp2	CCCCGACTTTGTCTGCCATC	
cg1150-sp1	CGTCGAGGACGGGGAAGTTG	
cg1150-sp2	AATCGGCGATATCAACGAGC	
cg1848-sp1	GTGCTCGCCGGTGGCAAAGAC	
cg1848-sp2	CATCGAGGCCTACTTCTTCAG	

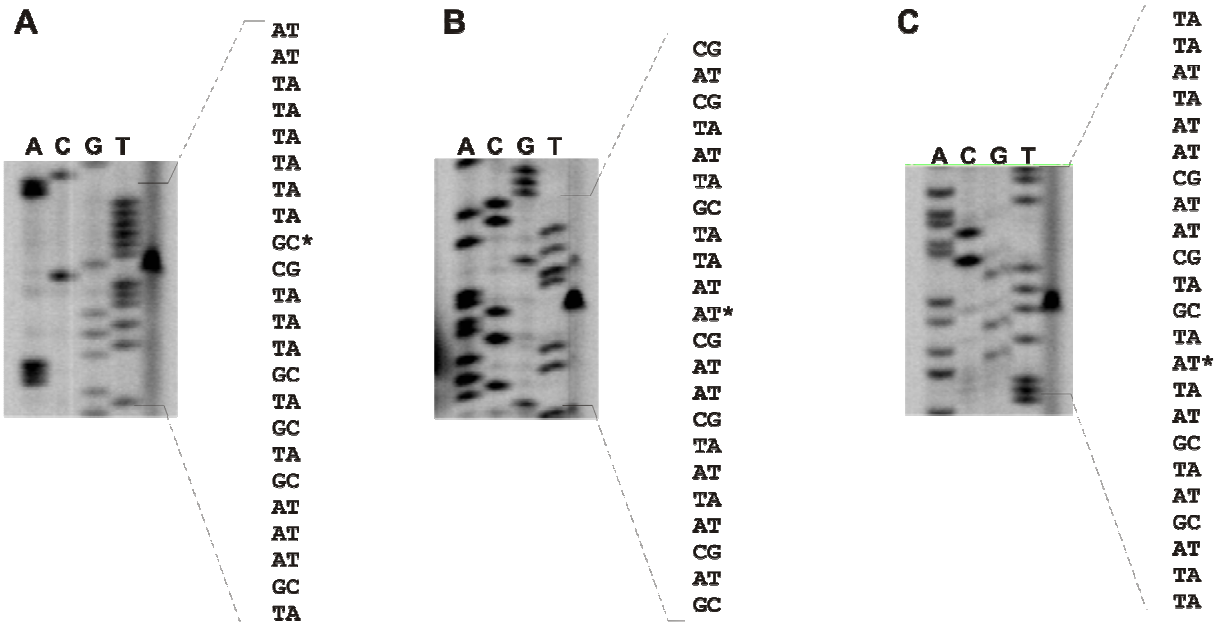

Fig. S1. Determination of the transcriptional start sites of cg1322 (A), cg3084 (B) and cg1426 (C) using oligonucleotides cg1322A, cg3084A and cg1426A and total RNA isolated from *C. glutamicum* Δ*rosR* cells grown on glucose minimal medium. The residues marked with the star represent the identified transcription start site.

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Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Neuss, den 16.11.2009