Regulation of the nitrate reductase operon *narKGHJI* by the cAMP-dependent regulator GlxR in *Corynebacterium glutamicum*

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The *Corynebacterium glutamicum* anaerobic nitrate reductase operon *narKGHJI* is repressed by a transcriptional regulator, ArnR, under aerobic conditions. A consensus binding site of the cAMP receptor protein (CRP)-type regulator, GlxR, was recently found upstream of the ArnR binding site in the *narK* promoter region. Here we investigated the involvement of GlxR and cAMP in expression of the *narKGHJI* operon *in vivo*. Electrophoretic mobility shift assays showed that the putative GlxR binding motif in the *narK* promoter region is essential for the cAMP-dependent binding of GlxR. Promoter-reporter assays showed that mutation in the GlxR binding site resulted in significant reduction of *narK* promoter activity. Furthermore, a deletion mutant of the adenylate cyclase gene *cyaB*, which is involved in cAMP synthesis, exhibited a decrease in both *narK* promoter activity and nitrate reductase activity. These results demonstrated that *C. glutamicum* GlxR positively regulates *narKGHJI* expression in a cAMP-dependent manner.

INTRODUCTION

Corynebacterium glutamicum, a non-pathogenic high-GC Gram-positive bacterium, has been widely used for industrial production of various amino acids and nucleic acids (Kinoshita, 1985; Terasawa & Yukawa, 1993). In addition, this species is of increasing interest as a suitable model organism for *Corynebacterineae*, a suborder of the *Actinomycetes*, which includes the medically important pathogenic species *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis* (Brune *et al.*, 2005; Mishra *et al.*, 2007). *C. glutamicum* has long been regarded as an aerobic bacterium because there have been few reports of its growth by anaerobic respiration or fermentation, despite the fact that most other coryneform bacteria are facultative anaerobes (Liebl, 2001). Recently, anaerobic growth of *C. glutamicum* by nitrate respiration was reported (Nishimura *et al.*, 2007; Takeno *et al.*, 2007). This property is attributed to the presence of a *narKGHJI* operon. The *narK* and *narGHJI* genes encode a putative nitrate/nitrite transporter and a membrane-bound nitrate reductase complex, respectively. These components of nitrate respiration are conserved in a broad range of bacteria (Moreno-Vivián *et al.*, 1999; Zumft, 1997). Nitrite is excreted from the cell as an end product of anaerobic nitrate reduction in *C. glutamicum*, and neither nitrate nor nitrite is utilized as the sole nitrogen source for its

anaerobic or aerobic growth, suggesting that this micro-organism lacks an efficient enzymic apparatus to reduce nitrite further (Nishimura *et al.*, 2007; Takeno *et al.*, 2007). This property is distinct from that of well-studied nitrate-reducing bacteria such as *Escherichia coli* and *Bacillus subtilis*, which are able to metabolize nitrate as a nitrogen source to nitrite and further to ammonia (Cole, 1996; Nakano & Zuber, 1998), and of denitrifiers such as *Pseudomonas aeruginosa* and *Paracoccus denitrificans* (Zumft, 1997). *Mycobacterium bovis* BCG and *M. tuberculosis*, which are closely related to *C. glutamicum*, also possess anaerobic nitrate reductase encoded by a *narGHJI* gene cluster (Sohaskey & Wayne, 2003; Weber *et al.*, 2000), although these bacteria are unable to grow in the absence of oxygen. The nitrate reductase activity of *M. tuberculosis* cells is strongly induced during chronic infection of mice and under *in vitro* conditions of gradual oxygen depletion, which are the conditions established to provide a model of the dormant state with latent tuberculosis (Shi *et al.*, 2005; Sohaskey & Wayne, 2003). It has been suggested that nitrate respiration plays a role in the survival of the pathogen during the inhibition of aerobic respiration (Sohaskey, 2008). Notably, a *narG* mutant of *M. bovis* BCG showed reduced virulence and lung damage in both immunocompetent and immunodeficient SCID mice (Fritz *et al.*, 2002; Weber *et al.*, 2000).

Transcription of *C. glutamicum narKGHJI* is considerably induced by the transition from aerobic to anaerobic nitrate respiratory conditions, as seen in other nitrate-reducing

Abbreviations: CRP, cAMP receptor protein; EMSA, electrophoretic mobility shift assay.

bacteria (Nishimura *et al.*, 2007). Our previous study demonstrated that a novel transcriptional regulator, ArnR, directly represses expression of the *narKGHJI* operon under aerobic conditions (Nishimura *et al.*, 2008). The repression is relieved by a shift to anaerobic nitrate respiratory conditions, resulting in elevated expression of *nar* genes. In addition to the *narKGHJI* operon, the *hmp* gene encoding flavohaemoglobin was identified as an additional target of ArnR (Nishimura *et al.*, 2008). *C. glutamicum* flavohaemoglobin may be involved in detoxifying nitric oxide during anaerobic nitrate respiration, as has been reported for *E. coli* (Moreno-Vivián *et al.*, 1999; Poole & Hughes, 2000; Poole *et al.*, 1996). ArnR's function as an aerobic repressor of the *narKGHJI* and *hmp* contrasts with that of the oxygen-sensing global regulator FNR, which activates the expression of many of the genes involved in anaerobic metabolism, including nitrate respiration in *E. coli* (Bonnefoy & Demoss, 1994; Gunsalus & Park, 1994). *E. coli* FNR constitutes the paradigm of oxygen-sensing regulators, which have been found in a variety of Gram-negative and a few Gram-positive bacteria (Körner *et al.*, 2003).

GlxR, a member of the CRP/FNR family, was first identified in *C. glutamicum* as a cAMP-dependent regulator which represses transcription of the glyoxylate shunt genes *aceA* and *aceB* (Kim *et al.*, 2004). The GlxR regulon has been predicted to comprise more than 400 genes covering diverse cellular functions (Kohl & Tauch, 2009; Kohl *et al.*, 2008). Deletion of *glxR* indeed results in severe growth defects (Toyoda *et al.*, 2009), implying pleiotropic negative effects. This makes it difficult to examine involvement of GlxR in the transcriptional regulation of individual target genes. Signal transduction and the physiological role of the GlxR–cAMP-mediated regulation are still largely unknown. It was recently reported that GlxR binds to the promoter region of the *narKGHJI* operon in the presence of cAMP (Kohl *et al.*, 2008). However, *in vivo* regulation of *narKGHJI* expression by GlxR remains to be elucidated. How GlxR is involved in anaerobic metabolism of *C. glutamicum* is of particular interest.

In this study, we investigated the involvement of GlxR and cAMP in the regulation of *narKGHJI* expression in *C. glutamicum*. We present *in vivo* experimental evidence indicating that the positive regulation of *narKGHJI* expression by GlxR depends on cAMP.

METHODS

Bacterial strains, media and culture conditions. Strains used in this study are listed in Table 1. *C. glutamicum* R (FERM P-18976) (Yukawa *et al.*, 2007) and its derivative strains were pre-cultured at 33 °C overnight in nutrient-rich medium (A medium; Inui *et al.*, 2004b) with 1% (w/v) glucose. The cells were harvested by centrifugation and were subsequently washed once with mineral salts medium (BT medium; Inui *et al.*, 2004b). For aerobic cultivation, the washed cells were grown at 33 °C in 100 ml BT medium containing

1% (w/v) glucose in a 500 ml flask with vigorous shaking (200 r.p.m.). For anaerobic cultivation, anaerobic BT medium was prepared in advance by bubbling with argon for 5 min. Cells from the same batch as those used for aerobic cultivation were subsequently transferred to the anaerobic BT medium in an anaerobic chamber (Coy Laboratory Products) in which the gas composition was kept constant at 95% nitrogen and 5% hydrogen. The cells were grown at 33 °C in 50 ml BT medium containing 0.5% (w/v) glucose in a 50 ml culture bottle with gentle stirring. Potassium nitrate was added to a concentration of 30 mM when necessary. Kanamycin and chloramphenicol were used for cultivation of *C. glutamicum* strains carrying various plasmids (Table 1), at 50 and 5 µg ml⁻¹, respectively.

E. coli JM109 (Takara) was used as a host for all genetic manipulations. *E. coli* BL21(DE3) (Novagen) was used for protein expression. *E. coli* strains were cultivated at 37 °C in LB medium, supplemented with ampicillin, kanamycin or chloramphenicol (all at 50 µg ml⁻¹) when necessary.

Construction of *arnR* and *cyaB* deletion mutants. Plasmids and primers used in this study are listed in Table 1 and Table 2, respectively.

DNA fragments containing the *arnR* and *cyaB* genes were amplified using primer pairs *arnR* FWSph/*arnR* RVXba and *cyaB* FWXba/*cyaB* RVSpH, respectively. The PCR products were cloned into pCRA725 (Inui *et al.*, 2004a), a suicide vector with the *sacB* gene for markerless gene disruption, yielding pCRB622 and pCRC619, respectively. An internal 269 bp fragment of the *arnR* gene was removed by inverse PCR using primer pair *arnR*inV FWBgIII/*arnR*inV RVBgIII, with plasmid pCRB622 as a template. Likewise, an internal 1425 bp deletion of the *cyaB* gene was done using primer pair *cyaB*inV FWSal/*cyaB*inV RVSal, with pCRC619 as a template. The PCR products were digested with BglII and SalI, and self-ligated, yielding pCRB623 and pCRC620, respectively. *C. glutamicum* R was transformed by electroporation with pCRB623 and pCRC620, and screening for the deletion mutants was performed as described previously (Inui *et al.*, 2004a). Deletion of the *arnR* and *cyaB* genes was checked by PCR. The mutant strains obtained were designated TN01 (Δ *arnR*) and TN06 (Δ *cyaB*).

Construction of *narK* promoter–*lacZ* fusions and a plasmid for GlxR overexpression. The DNA fragment containing the *narK* promoter region was generated by PCR using primer pair *pnarK* FSmall/*pnarK* RSmall, and cloned upstream of the *lacZ* gene in pCRA741 (Inui *et al.*, 2007), yielding pCRB626. The direction of the inserts was confirmed by DNA sequencing. Plasmid pCRB626 was isolated as non-methylated DNA from *E. coli* JM110 for efficient gene introduction into *C. glutamicum* R (Vertès *et al.*, 1993), and was subsequently integrated into the chromosome of *C. glutamicum* R, TN01 and TN06 by markerless gene insertion methods, as described previously (Inui *et al.*, 2007), yielding strains TN02, TN04 and TN07, respectively. Mutation in the GlxR binding site was generated by inverse PCR using primer pair *glxR*mut FW/*glxR*mut RV, with plasmid pCRB626 as a template. The resulting PCR product was phosphorylated with T4 polynucleotide kinase (Takara Bio) and self-ligated to generate pCRB627. Plasmid pCRB627 was integrated into the chromosome of *C. glutamicum* R and TN01, yielding strains TN03 and TN05, respectively.

Plasmid pCRB621 for GlxR overexpression in the TN02 strain was constructed as described below. The DNA fragment covering the *glxR* promoter and coding region was amplified from chromosomal DNA of *C. glutamicum* R by PCR with primer pair *glxR* FWEco/*glxR* RVKpn. The PCR product was cloned into the vector pCRB1, yielding pCRB621. Plasmids pCRB1 and pCRB621 were introduced into the TN02 strain.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>C. glutamicum</i>		
R	Wild-type	FERM P-18976
TN01	R with deletion of <i>arnR</i>	This study
TN02	R with <i>PnarK-lacZ</i>	This study
TN03	R with <i>PnarK-lacZ</i> with mutation in the GlxR binding site in <i>narK</i> promoter	This study
TN04	TN01 with <i>PnarK-lacZ</i>	This study
TN05	TN01 with <i>PnarK-lacZ</i> containing mutation in the GlxR binding site in <i>narK</i> promoter	This study
TN06	R with deletion of <i>cyaB</i>	This study
TN07	TN06 with <i>PnarK-lacZ</i>	This study
<i>E. coli</i>		
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F'[traD36 proAB⁺ lacI^f lacZΔM15]</i>	Takara
JM110	<i>dam dcm supE44 hsdR17 thi leu rpsL lacy galK galT ara tonA thr tsx Δ(lac-proAB)/F'[traD36 proAB⁺ lacI^f lacZΔM15]</i>	Sambrook <i>et al.</i> (1989)
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdSB(r_B⁻ m_B⁻) λBL21(DE3)</i>	Novagen
Plasmids		
pColdI	Ap ^r , cold-inducible expression vector	Takara
pCRB1	Cm ^r , <i>α-lac</i> multicloning site; <i>E. coli</i> – <i>Corynebacterium</i> sp. shuttle vector	Nakata <i>et al.</i> (2003)
pCRA725	Km ^r ; suicide vector containing the <i>B. subtilis</i> <i>sacB</i> gene	Inui <i>et al.</i> (2004b)
pCRA741	Km ^r ; pCRA725 with a 2.0 kb PCR fragment from ISSs 7 and a 3.1 kb PCR fragment containing the <i>E. coli</i> <i>lacZ</i> gene	Inui <i>et al.</i> (2007)
pCRC621	Ap ^r , pColdI with <i>glxR</i> coding region	This study
pCRB621	Cm ^r ; pCRB1 with a 1.1 kb fragment containing <i>glxR</i> promoter and coding region	This study
pCRB622	Km ^r ; pCRA725 with a 4.8 kb <i>SphI</i> – <i>XbaI</i> PCR fragment containing the <i>arnR</i> gene	This study
pCRB623	Km ^r ; pCRB622 with a 4.5 kb fragment containing a truncated <i>arnR</i> gene	This study
pCRC619	Km ^r ; pCRA725 with a 3.4 kb <i>XbaI</i> – <i>SphI</i> PCR fragment containing the <i>cyaB</i> gene	This study
pCRC620	Km ^r ; pCRC619 with a 2.0 kb fragment containing a truncated <i>cyaB</i> gene	This study
pCRB626	Km ^r ; pCRA741 with a 0.3 kb PCR fragment containing the <i>C. glutamicum</i> R <i>narK</i> promoter region	This study
pCRB627	Km ^r ; pCRA741 with a 0.3 kb PCR fragment containing the <i>narK</i> promoter region with mutations in the GlxR binding site	This study

Overproduction and purification of the hexahistidyl-tagged GlxR protein. The *glxR* gene was amplified from chromosomal DNA of *C. glutamicum* R by PCR with primer pair *glxR* His FWNde/*glxR*

His RVSal. The PCR product was cloned into the expression vector pColdI (Takara), yielding pCRC621. *E. coli* BL21(DE3) transformed with pCRC621 was grown at 37 °C in LB medium to an OD₆₁₀ of 0.5.

Table 2. Oligonucleotide primers used in this study

Name	Sequence (5'–3')
<i>glxR</i> His FWNde	GAGAAAACATATGGAAGGTGTACAGGAGATCC
<i>glxR</i> His RVSal	GCGCTTGTCTGACTTATCGAGCGCGACGTGCCAAATG
<i>pnarK</i> FWSmaI	TCCCCCGGCATCAATTTCTCCTCAAGTGG
<i>pnarK</i> RVSmaI	TCCCCCGGTTTGGTGTGAGTTGTGTCAC
<i>glxR</i> mut FW	TGCATAAACCCATTACGACACAA
<i>glxR</i> mut RV	GGTAAACGAATTTGTGCTTATATC
<i>glxR</i> FWEco	GGAATTCGCTGCCTAAATAAGGGTTATG
<i>glxR</i> RVKpn	GGGGTACCGTGGCTATTCGTTACCTGCA
<i>arnR</i> FWSph	ACATGCATGCGGATGCGCACCCATACTGG
<i>arnR</i> RVXba	GCTCTAGAGTCTATAGTGGCTAGGTACC
<i>arnR</i> inv FWBglII	GAAGATCTGACGATGACGCAATGCACAA
<i>arnR</i> inv RVBglII	GAAGATCTTGGGCTGAGTTTGTAGGACT
<i>cyaB</i> FWXba	CGATCTAGATTCTTACGGCTTCGAGTTCGGCTTC
<i>cyaB</i> RVSpH	GAGCGCATGCCAAGGAAGCAGCAGATGGCGGAG
<i>cyaB</i> inv FWSal	AGCATAGTCGACCCACGATGTGCCCCACAGCCATTTTC
<i>cyaB</i> inv RVSal	TAGAACGTCGACCGGCCGACGTTGGCGGACAGG

The culture was incubated for 30 min at 15 °C, then expression of the His-tagged GlxR was induced by addition of 0.5 mM IPTG. The culture was shaken overnight at 15 °C. The cells were harvested by centrifugation and frozen at –80 °C until ready for use. The His-tagged protein was purified by affinity chromatography on Ni-NTA agarose (Qiagen) according to the instruction manual. For desalting, the eluted protein was loaded onto a PD-10 column (GE Healthcare Bioscience) and eluted with buffer A (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 1 mM EDTA and 1 mM DTT). The concentration of the purified protein was determined by a Bio-Rad protein assay using BSA as a standard.

Electrophoretic mobility shift assay (EMSA). The wild-type DNA probe containing the *narK* promoter region was amplified by PCR using primer pair pnarK F5mal/pnarK R5mal and *C. glutamicum* R genomic DNA as a template. The DNA probe with mutation in the GlxR binding site was amplified by PCR using plasmid pCRB627 as a template.

Binding reactions were performed using a solution containing 10 mM Tris/HCl (pH 7.4), 50 mM KCl and 0.1 mM EDTA. A total of 10 nM specific DNA fragment and 0–800 nM (0–80 molar excess) purified GlxR-His₆ protein were added to a final volume of 10 µl and incubated at room temperature for 15 min. The samples were immediately loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed at 150 V in 0.5 × TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA). The gel was stained using an EMSA kit (Invitrogen).

β-Galactosidase assay. Exponentially growing *C. glutamicum* cells harbouring the *narK* promoter–*lacZ* fusion were harvested, and washed with Z-buffer (Miller, 1972). Toluene treatment and β-galactosidase assays using *o*-nitrophenyl β-D-galactopyranoside were performed as described by Miller (1972). The values presented are the means of at least three independent experiments.

Nitrate reductase enzyme assay. To prepare crude cell extracts for nitrate reductase enzyme assay, exponentially growing *C. glutamicum* cells were harvested, and washed with phosphate buffer (pH 7.2). The pellet was resuspended in the same buffer. A 1 ml volume of the cell suspension was mixed with 0.5 g 0.1 mm zirconia/silica beads. The cells were mechanically disrupted with a Multi-Beads Shocker (Yasui Kikai) at 2500 r.p.m. for 10 periods of 30 s at 4 °C. The resultant lysate was centrifuged for 10 min at 2000 g and 4 °C and the supernatant was used for nitrate reductase enzyme assay. Nitrate reductase activity was measured as described previously (MacGregor & Schnaitman, 1974). Nitrite concentration was determined by using an NO₂/NO₃ Assay kit-C II (Colorimetric) – Griess Reagent kit (Dojindo).

RESULTS

A consensus motif essential for cAMP-dependent specific binding of GlxR to the *narK* promoter region

In vitro cAMP-dependent binding of GlxR to the 40 bp upstream region of the *narKGHJI* operon was shown previously, and a putative 16 bp consensus motif for the GlxR binding was identified in its upstream region (Kohl *et al.*, 2008). To verify specific interaction of GlxR with the putative binding site (AGTGAATTACGACACA) in the *narK* promoter region, we performed mutational analysis using EMSAs. In the presence of cAMP, purified GlxR bound to the wild-type *narK* promoter region, which

covers the region –199 through +82 bp relative to the transcriptional start site of the *narKGHJI* operon, while binding was not detected in the absence of cAMP (Fig. 1). The cAMP-dependent interaction was completely inhibited by introduction of a mutation in the putative binding motif (ACCCCATACGACACA), indicating that the motif is essential for GlxR binding.

GlxR is involved in activation of *narKGHJI* expression

It was previously reported that growth of a *glxR* deletion mutant is too slow to be analysed (Toyoda *et al.*, 2009). To verify the involvement of GlxR in expression of the *narKGHJI* operon, the influence on promoter activity of mutation of the GlxR binding motif, as tested in EMSAs, was examined using a *lacZ* reporter system. The wild-type *narK* promoter–*lacZ* fusion (*PnarK*–*lacZ*) and the mutant *PnarK*–*lacZ* were integrated into the chromosome of the *C. glutamicum* wild-type strain, yielding strains TN02 and

Fig. 1. (a) Genomic locus of the *narK* promoter region with the GlxR and ArnR binding sites, and the DNA probe covering the *narK* promoter region for EMSAs (*PnarK*). Numbers adjacent to the GlxR and ArnR binding sites indicate the first position of the binding site relative to the transcriptional start site (+1). The GlxR consensus binding site (TGTGAnnTAnnTCACA) and the mutation (Mut) introduced into the DNA probe are shown above and below the wild-type (WT) sequence, respectively. (b) EMSAs were carried out using the wild-type and the mutant DNA probes (WT *PnarK* and Mut *PnarK*, respectively) and purified His-tagged GlxR protein with (+) or without (–) 0.2 mM cAMP. GlxR protein was added at a molar excess of 0, 40 or 80 to 10 nM DNA probe. Free DNA and GlxR–cAMP complex are indicated with open and filled arrowheads, respectively.

Table 3. Involvement of the GlxR binding site and *cyaB* gene in regulation of *narKGHJI* expression

The wild-type and its *arnR* and *cyaB* deletion mutants ($\Delta arnR$ and $\Delta cyaB$), expressing wild-type or mutant *narK* promoter–*lacZ* gene fusion (WT *PnarK*–*lacZ* or Mut *PnarK*–*lacZ*), were grown to exponential phase in BT minimal medium under aerobic or anaerobic nitrate respiratory conditions as described in Methods. They were then subjected to β -galactosidase assay. The results are reported as means $\pm$ SD of at least three independent experiments.

Strain (relevant genotype)	Reporter gene fusion	β -Galactosidase activity (Miller units)	
		Aerobic	Anaerobic nitrate respiration
TN02 (wild-type)	WT <i>PnarK</i> – <i>lacZ</i>	8.6 $\pm$ 0.6	97.8 $\pm$ 9.2
TN03 (wild-type)	Mut <i>PnarK</i> – <i>lacZ</i>	1.5 $\pm$ 0.4	32.8 $\pm$ 2.3
TN04 ($\Delta arnR$)	WT <i>PnarK</i> – <i>lacZ</i>	54.9 $\pm$ 4.9	147.5 $\pm$ 9.3
TN05 ($\Delta arnR$)	Mut <i>PnarK</i> – <i>lacZ</i>	23.3 $\pm$ 1.0	68.5 $\pm$ 0.6
TN07 ($\Delta cyaB$)	WT <i>PnarK</i> – <i>lacZ</i>	<0.1	14.3 $\pm$ 3.3

TN03, respectively. β -Galactosidase activity of these strains grown under aerobic and anaerobic nitrate respiratory conditions was measured (Table 3). The *PnarK*–*lacZ* expression observed in the TN02 strain was markedly higher under anaerobic nitrate respiratory conditions than under aerobic conditions. The induction of *narK* promoter activity in response to anaerobiosis is consistent with findings of a previous study (Nishimura *et al.*, 2008). Under anaerobic conditions, the mutant *PnarK*–*lacZ* expression observed in the TN03 strain was significantly lower than the wild-type *PnarK*–*lacZ* expression observed in the TN02 strain. Under aerobic conditions, the *narK* promoter activity of the TN02 strain was repressed at a low basal level; however, mutation of the GlxR binding site resulted in further decreased activity, as seen in the TN03 strain. Effects of the GlxR binding-site mutation on the *narK* promoter activity was also investigated in the *arnR* deletion mutant strains ($\Delta arnR$) (Table 3). The wild-type *narK* promoter activity was derepressed by *arnR* disruption especially under aerobic conditions, as seen in the TN04 strain. This is consistent with our previous study indicating that ArnR functions as an aerobic repressor of the *narKGHJI* operon (Nishimura *et al.*, 2008). The mutant *PnarK*–*lacZ* expression of the $\Delta arnR$ strain (TN05) was significantly lower than the wild-type promoter–*lacZ* expression of the $\Delta arnR$ strain (TN04) under both aerobic and anaerobic nitrate respiratory conditions. These results suggest that binding of GlxR is responsible for upregulation of the *narK* promoter activity, regardless of ArnR function.

To buttress the role of GlxR as an activator of *narKGHJI* expression, the influence of GlxR overexpression on *narK* promoter activity was examined. We introduced a multi-copy plasmid carrying the *glxR* promoter and coding region (pCRB621) into the wild-type strain expressing *PnarK*–*lacZ* (TN02), and measured its β -galactosidase activity (Table 4). The *narK* promoter activity of the TN02 strain carrying pCRB621 was approximately 1.4- and 2.0-fold higher than that of a strain carrying empty vector (pCRB1) under aerobic and anaerobic nitrate respiratory

conditions, respectively. This result is consistent with that observed with mutational analysis of the GlxR binding site in the *narK* promoter.

An adenylate cyclase gene, *cyaB*, is involved in the activation of *narKGHJI* expression

EMSAs showed that GlxR is unable to interact with the binding motif in the *narK* promoter region without cAMP *in vitro* (Fig. 1). The intracellular cAMP level in *C. glutamicum* has been previously shown to decrease upon deletion of the adenylate cyclase gene *cyaB* (Cha *et al.*, 2010). Here, we examined the effects of *cyaB* deletion on *narK* promoter activity. Wild-type *PnarK*–*lacZ* was integrated into the chromosome of a *cyaB* deletion strain ($\Delta cyaB$), and its β -galactosidase activity measured (Table 3). The *narK* promoter activity of the $\Delta cyaB$ strain (TN07) was markedly lower than that of the wild-type strain (TN02) under aerobic and anaerobic nitrate respiratory conditions. The effect of *cyaB* deletion on *narK* promoter activity was relatively larger than that of mutation of the GlxR binding site. It should be noted that *cyaB* deletion

Table 4. Effects of overexpression of the *glxR* gene on *narK* promoter activity

Strain TN02 harbouring control vector pCRB1 or plasmid pCRB621 for GlxR overexpression was grown to exponential phase in BT minimal medium under aerobic or anaerobic nitrate respiratory conditions. β -Galactosidase activity was then determined. The data are means $\pm$ SD of at least three independent experiments.

Strain (plasmid)	β -Galactosidase activity (Miller units)	
	Aerobic	Anaerobic nitrate respiration
TN02 (pCRB1)	7.8 $\pm$ 1.8	58.7 $\pm$ 11.2
TN02 (pCRB621)	11.0 $\pm$ 0.6	117.9 $\pm$ 8.8

Table 5. Influence of *cyaB* deletion on nitrate reductase activity

The wild-type R strain and its *cyaB* deletion mutant ($\Delta cyaB$, strain TN06) were grown to the exponential phase in BT minimal medium under aerobic and anaerobic nitrate respiratory conditions, and their nitrate reductase activity was determined as described in Methods. The data are the means $\pm$ SD of at least three independent experiments.

Strain (relevant genotype)	Nitrate reductase activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	Aerobic	Anaerobic nitrate respiration
R (wild-type)	4.8 $\pm$ 1.2	17.1 $\pm$ 0.9
TN06 ($\Delta cyaB$)	3.0 $\pm$ 0.6	9.1 $\pm$ 0.4

may affect a variety of cAMP-dependent cellular functions, including the wide physiological role of GlxR.

To confirm the positive effect of cAMP on the *narK* promoter activity, nitrate reductase enzyme (NAR) activity of the $\Delta cyaB$ strain was evaluated (Table 5). The NAR activity of the $\Delta cyaB$ strain (TN06) was 40–50 % lower than that of the wild-type strain under aerobic and anaerobic nitrate respiratory conditions, although the influence of *cyaB* disruption was relatively weak when compared with that on the *narK* promoter activity (Table 3). The positive regulation of the promoter activity by GlxR and cAMP, in conjunction with the *in vitro* GlxR binding to the *narK* promoter region only in the presence of cAMP, indicates that *in vivo* activation of the *narKGHJI* expression by GlxR is cAMP-dependent.

DISCUSSION

This study showed that mutation of a putative GlxR binding motif located in the *narK* promoter region of *C. glutamicum* resulted in inhibition of the *in vitro* binding of GlxR and a significant decrease in the *narK* promoter activity *in vivo*. Furthermore, deletion of the *cyaB* gene caused considerable reduction of the *narK* promoter activity. These results indicate that the CRP-type regulator GlxR positively regulates *narKGHJI* expression in a cAMP-dependent manner in *C. glutamicum*. This is supported by the finding that GlxR overexpression using a multi-copy plasmid enhanced the *narK* promoter activity *in trans*. The highly induced *narK* promoter activity under anaerobic conditions was shown to be repressed several-fold by GlxR binding site mutation and *cyaB* disruption, suggesting that GlxR contributes to the maintenance of high-level *narKGHJI* expression. However, *narKGHJI* expression repressed at a low basal level under aerobic conditions was further decreased by those genetic modifications. The transcriptional regulator ArnR strongly represses *narKGHJI* expression under aerobic conditions (Nishimura *et al.*,

2008). It is noted that the *narK* promoter activity was clearly repressed by the GlxR binding motif mutation even under conditions where the repression is released by deletion of the *arnR* gene. Therefore, ArnR plays a key regulatory role in response to anaerobiosis, and GlxR probably acts as an activator of the *narKGHJI* operon irrespective of the ArnR function. Concerning residual induction of *narKGHJI* expression in the *arnR* deletion mutant (Table 3), it is possible that additional regulators are involved in anaerobic induction of expression. Consequently, it is suggested that GlxR is not directly associated with the sensing mechanism for the anaerobiosis-responsive induction of *narKGHJI* expression. The regulation of *narKGHJI* expression by ArnR and CRP-type GlxR regulators in *C. glutamicum* is quite different from the well-known FNR-dependent anaerobic induction of the nitrate reductase gene expression in *E. coli* and *B. subtilis* (Bonney & Demoss, 1994; Reents *et al.*, 2006). In *Shewanella oneidensis* MR-1, a metal reducer that uses more than 14 terminal electron acceptors for respiration, instead of EtrA (an FNR homologue), CRP is involved in positive regulation of a variety of anaerobic respiration including nitrate, DMSO, fumarate and Fe(III) reduction (Saffarini *et al.*, 2003). Expression of the relevant genes has been suggested to be anaerobically induced by CRP in a cAMP-dependent manner (Charania *et al.*, 2009), although control of the intracellular cAMP level in response to anaerobiosis remains unclear.

The degree of anaerobic induction of *C. glutamicum* nitrate reductase activity was relatively small compared with that of the *narKGHJI* transcription level. It has been previously reported that nitrate reductase activity was not influenced by the oxygen and nitrate levels, although aerobically growing *C. glutamicum* cells exhibited no significant nitrite production in the presence of oxygen (Takeno *et al.*, 2007). This may be explained by preferential electron flow to oxygen due to the higher electron-accepting affinity of cytochrome oxidase than nitrate reductase enzymes. The current findings suggest that unknown factors at post-transcriptional and translational levels in addition to the transcriptional level affect nitrate reductase expression in *C. glutamicum*. It is possible that *C. glutamicum* nitrate reductase may function to some extent using nitrate as an additional or alternative electron acceptor under low oxygen tension as well as anaerobic conditions. In *M. tuberculosis*, *narGHJI* expression is constitutive irrespective of the presence of oxygen. Instead, the whole-cell nitrate reductase activity is regulated by control of the transcriptional level of the putative nitrate/nitrite transporter gene *narK2* (Sohaskey & Wayne, 2003). In contrast to *C. glutamicum*, *M. tuberculosis* is able to utilize nitrate aerobically as a nitrogen source (Malm *et al.*, 2009). Further investigation is required to understand the physiological role of nitrate reductase under changing environmental conditions in *C. glutamicum*.

The intracellular cAMP level of *C. glutamicum* during aerobic growth is significantly higher in the presence of

glucose than that in acetate-grown cells (Kim *et al.*, 2004), which is opposite to the situation in *E. coli* (Crother & Steitz, 1992). GlxR–cAMP is presumably involved in acetate-responsive transcriptional induction of the glyoxylate bypass genes *aceA* and *aceB*, and that of the TCA cycle genes *sdhCAB* and *gltA* (Bussmann *et al.*, 2009; Kim *et al.*, 2004; van Ooyen *et al.*, 2010), although the regulatory mechanism of cAMP level is still unclear. It should be noted that GlxR–cAMP probably regulates expression of genes with a variety of cellular functions, including metabolism not only of carbon but also of nitrogen and phosphate, iron–sulfur homeostasis and the stress response (Kohl & Tauch, 2009; Panhorst *et al.*, 2010). Therefore, it remains unknown what signal GlxR–cAMP responds to and what regulatory role GlxR–cAMP plays in *C. glutamicum*. In this context, it should be noted that the *cydB* disruptant still has a certain level of intracellular cAMP, as reported in a recent study (Cha *et al.*, 2010), and that the phenotypes of the *glxR* and *cydB* deletion mutants are not comparable (Cha *et al.*, 2010; Park *et al.*, 2010). Further studies on the regulatory mechanism of GlxR–cAMP may provide insight into new aspects of the role of cAMP in global genetic regulation in bacteria.

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