Genome-Wide Identification of *In Vivo* Binding Sites of GlxR, a Cyclic AMP Receptor Protein-Type Regulator in *Corynebacterium glutamicum*[▽][†]

Koichi Toyoda, Haruhiko Teramoto, Masayuki Inui, and Hideaki Yukawa*

Research Institute of Innovative Technology for the Earth (RITE), 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan

Corynebacterium glutamicum GlxR is a cyclic AMP (cAMP) receptor protein-type regulator. Although over 200 GlxR-binding sites in the *C. glutamicum* genome are predicted *in silico*, studies on the physiological function of GlxR have been hindered by the severe growth defects of a *glxR* mutant. This study identified the GlxR regulon by chromatin immunoprecipitation in conjunction with microarray (ChIP-chip) analyses. In total, 209 regions were detected as *in vivo* GlxR-binding sites. *In vitro* binding assays and promoter-reporter assays demonstrated that GlxR directly activates expression of genes for aerobic respiration, ATP synthesis, and glycolysis and that it is required for expression of genes for cell separation and mechanosensitive channels. GlxR also directly represses a citrate uptake gene in the presence of citrate. Moreover, ChIP-chip analyses showed that GlxR was still able to interact with its target sites in a mutant with a deletion of *cyaB*, the sole adenylate cyclase gene in the genome, even though binding affinity was markedly decreased. Thus, GlxR is physiologically functional at the relatively low cAMP levels in the *cyaB* mutant, allowing the *cyaB* mutant to grow much better than the *glxR* mutant.

Cyclic AMP (cAMP) receptor proteins (CRPs) are global transcriptional regulators that are broadly distributed in bacteria (44). CRP family proteins have diverse cellular functions, including in carbohydrate metabolism (44, 91), development of competence for transformation (16), growth phase-dependent regulation of gene expression (1), modulation of virulence gene expression and pathogenesis (20, 71, 87), resuscitation (64), and germination and morphological development (21, 22).

The best-studied member of the family is the CRP of *Escherichia coli*. *E. coli* CRP activated by cAMP specifically binds to the consensus sequence 5′-TGTGA-N_6-TCACA-3′ in target promoters, thereby recruiting RNA polymerase and promoting transcription at the promoter via protein-protein interactions (13). Intracellular cAMP levels in *E. coli* cells increase in response to glucose starvation via activation of adenylate cyclase, an enzyme which catalyzes the formation of cAMP from ATP. The interaction between the phosphotransferase system for glucose uptake and adenylate cyclase is involved in the control of synthesis of cAMP (44, 46). The presence of glucose lowers intracellular cAMP levels and deactivates CRP (44), allowing CRP to mediate preferential utilization of the fast-metabolizable carbon source, a phenomenon called carbon catabolite repression (23). Based on transcriptome or *in silico* analyses (29, 65, 75, 89, 90), *E. coli* CRP directly affects the expression of up to 200 genes.

Corynebacterium glutamicum is a Gram-positive bacterium with a high G+C content which has been used in the biotechnological production of amino acids, organic acids, and alcohols (35, 36, 39, 48, 72, 85). In addition, because it is nonpathogenic and grows fast, *C. glutamicum* is an emerging model system for members of the *Corynebacterineae*, an *Actinomycetes* suborder, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis. In the course of extensive genome-wide analyses, a transcriptional regulatory network model composed of an increasing number of known regulators has been constructed and updated (8, 9, 56, 68). GlxR, which is a CRP-type transcriptional regulator, is one of the most important regulators of *C. glutamicum*. First characterized as a factor repressing the promoter activity of a gene coding for a glyoxylate pathway enzyme (41), GlxR is involved in the regulation of several other genes. Like *E. coli* CRP, it binds to the consensus site 5′-TGTGA-N_6-TCACA-3′ in a cAMP-dependent manner *in vitro* (32, 33, 40, 41, 49). *In silico* analyses have detected more than 200 potential binding sites for GlxR in the *C. glutamicum* genome, and binding to 72 of the sites has been verified by *in vitro* binding assays, revealing that the regulon includes genes for carbon metabolism, nitrogen metabolism, respiration, resuscitation, cell wall formation, and cell division (42, 43). However, whether GlxR acts as a transcriptional activator or repressor of most of these genes is difficult to evaluate, not only because construction of a *glxR* deletion mutant is difficult but because any mutant that has been successfully constructed shows severe growth defects (41, 49, 59, 79). The role of GlxR has been assessed only by *in vitro* binding assays and overexpression studies *in vivo* (14, 32, 33, 40, 49), although GlxR-dependent repression of genes involved in the glyoxylate pathway and glutamate uptake system was recently confirmed by experiments using a *glxR* mutant (59). Therefore, the physiological function of and environmental signal(s) sensed by GlxR remain poorly understood.

In contrast to the case for *E. coli*, cAMP levels in *C. glu-*

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
JM109	recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F′ (traD36 proAB⁺ lacI�q lacZΔM15) dam dcm supE44 hsdR17 thi leu rpsL	Takara
JM110	lacY galK galT ara tonA thr tsx Δ(lac-proAB)/F′ (traD36 proAB⁺ lacI�q lacZΔM15)	67
BL21(DE3)	F⁻ ompT gal dcm lon hsdS_B (r_B⁻ m_B⁻) λ(DE3)	74
C. glutamicum		
R	Wild-type strain	88
KT7	R with deletion in glxR	79
KT23	R with Strep-tag II-tagged glxR	This study
ΔcyaB mutant	R with deletion in cyaB	57
KT25	ΔcyaB mutant with Strep-tag II-tagged glxR	This study
Plasmids		
pCold	Apʳ; cold-inducible expression	Takara
pGEM T-Easy	Vector	Promega
pCRA725	Kmʳ; suicide vector containing the B. subtilis sacB gene	36
pCRA741	Kmʳ; pCRA725 with a 2.0-kb PCR fragment from strain-specific island 7 and a 3.1-kb PCR fragment containing the E. coli lacZ gene	38
pCRC619	Kmʳ; pCRA725 with a 2.4-kb fragment containing a Strep-tag II-tagged glxR gene	This study
pCRC620	Apʳ: pColdI with a 684-bp fragment containing the glxR gene	This study

tamicum are higher during growth on glucose than during growth on acetate (41). In addition, *C. glutamicum* can simultaneously utilize different carbon sources with glucose (11, 18, 24, 77, 86), apart from glutamate and ethanol, consumption of which is almost completely repressed in the presence of glucose (2, 45, 47). That the repressed expression of a glutamate transporter gene in the presence of glucose is relieved in a *glxR* mutant suggests the involvement of GlxR in carbon catabolite repression (59). However, cAMP levels in cells grown in the presence of glutamate or ethanol have not been determined, and effects of cAMP levels on GlxR function *in vivo* are not known. Although the *C. glutamicum* genome carries only a single putative adenylate cyclase gene, *cyaB*, cAMP is still detected in a *cyaB* mutant (15). In addition, the *cyaB* mutant is able to grow on any carbon source much better than a *glxR* mutant (15, 59), implying that GlxR may be physiologically functional even in the *cyaB* mutant background.

Chromatin immunoprecipitation in conjunction with microarray (ChIP-chip) analysis has been used to identify *in vivo* target sites and physiological states of bacterial transcriptional regulators in recent years (12, 17, 27, 30, 31, 52, 60, 66). The regions bound by the transcriptional regulator are enriched by ChIP, and the degrees of the enrichment reflect the ability of the regulator to bind to the regions in the cells under the conditions tested. Therefore, in response to an environmental signal that alters binding of the regulator, the degree of enrichment of target regions in ChIP-chip analysis is changed (17, 25, 27, 66). Addition of glucose to the culture eliminates enrichment of target regions by CRP in *E. coli* (31).

In this study, we employed ChIP-chip analyses to investigate the target genes and binding ability of GlxR *in vivo* at the genome-wide level. The binding sites identified are validated by *in vitro* binding assays and *in vivo* promoter assays, demonstrating that GlxR directly activates expression of genes with various cellular functions. Furthermore, we used ChIP-chip

analyses with a *cyaB* mutant to show that even in the absence of a known adenylate cyclase, GlxR still binds to the sites *in vivo*, albeit with lower affinity than in the wild type, suggesting that GlxR in the *cyaB* mutant is physiologically active.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and growth conditions. The strains and plasmids used in this study are listed in Table 1. The oligonucleotides used are listed in Table S1 in the supplemental material. For genetic manipulation, *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium. *C. glutamicum* strains were grown at 33°C in nutrient-rich A medium (37) with 4% glucose. When appropriate, the media were supplemented with antibiotics. The final antibiotic concentrations for *E. coli* were 50 μg of ampicillin ml⁻¹ and 50 μg of kanamycin ml⁻¹; for *C. glutamicum*, kanamycin (50 μg ml⁻¹) was used. For promoter assays, *C. glutamicum* strains chromosomally carrying a promoter-*lacZ* fusion were grown in A medium containing 1% glucose or acetate to the stationary phase (8 h). When *citH* promoter activity was determined, A medium containing 5 mM CaCl₂ was used as basal medium for efficient uptake of citrate (11).

Construction of a GlxR-Strep-tag II strain. The Strep-tag II-coding sequence was introduced into the 3′ end of the *glxR* gene by overlapping PCR using overlapping primer pair glxRstrepFW-glxRstrepRV, together with primers glxRFW and glxRRV and genomic DNA from strain R as a template. The overlapping PCR products were purified and used as a template for PCR using primers glxRFW and glxRRV. The resulting fragment was cloned into pCRA725, a suicide vector for markerless gene disruption (36), yielding pCRC619. This was isolated as nonmethylated DNA from *E. coli* JM110 for efficient gene introduction into *C. glutamicum* R (84). *C. glutamicum* R (wild type) (79) and the *cyaB* mutant strain (57) were transformed by electroporation with pCRC619, and screening for the mutants was performed as described previously (36). Introduction of the tag into the *glxR* gene on the chromosome was confirmed by direct sequencing of a PCR product, which was amplified using primers glxRFW and glxRRV and genomic DNA extracted from the strains obtained as a template.

ChIP-chip analysis. Exponentially growing *C. glutamicum* cultures (35 ml) at an optical density at 610 nm (OD₆₁₀) of up to 2.5 were treated with formaldehyde (at a final concentration of 1%) and incubated for 20 min at room temperature. The cross-linking was quenched by addition of glycine (at a final concentration of 125 mM) and incubation for 10 min at room temperature. Cells were then collected by centrifugation, washed twice with Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl), and stored at −80°C. Pellets were resuspended in

2 ml IP buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, Roche Antiprotease mini). The cells were mechanically disrupted using a FastPrep FP120 instrument (Bio 101, Thermo Savant) as described previously (78), and the supernatant after centrifugation was sonicated on ice to shear DNA to an average size of 600 to 1,000 bp. A 50-μl fraction of the supernatant was saved for later analysis (reference DNA). The remainder was subjected to immunoprecipitation with 100 μl of magnetic beads coated with protein G (Invitrogen), which was coupled to the monoclonal anti-Strep-tag II antibody (Qiagen). The mixture was incubated overnight on a rotating platform at 4°C. The beads were washed once with IP buffer, twice with IP buffer containing 400 mM NaCl, eight times with radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES [pH 7.6], 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% sodium deoxycholate), and twice with Tris-EDTA (TE) buffer with 50 mM NaCl. Immunoprecipitated complexes were eluted from the beads by treatment with 210 μl elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS) at 65°C for 20 min. Cross-links of immunoprecipitated samples and of total DNA samples were reversed by incubation overnight at 65°C. Samples were then treated with RNase A and proteinase K for 2 h at 55°C. DNA was extracted with phenol-chloroform and purified with a QIAquick PCR purification minElute kit (Qiagen). DNA samples were blunted with T4 DNA polymerase, ligated to linkers, and amplified by PCR. Amplified DNAs from reference DNA and immunoprecipitated DNA were differentially labeled with Cy3 and Cy5, respectively, by using a CGH labeling kit (Invitrogen) according to the manufacturer's instructions. Hybridization to microarrays and array scanning were done as described previously (38). The arrays were spotted with 3,056 duplicate PCR products corresponding to *C. glutamicum* open reading frames (ORFs) (38). Data were normalized so that the mean of the Cy5/Cy3 intensity ratio of all of the features except the flagged ones was equal to 1, using GenePix 5.0 software. The enrichment factor for a given gene was calculated as the $\log_2$ ratio of hybridization of immunoprecipitated DNA to reference DNA. The entire procedure was carried out at least three times, and the results were averaged. A result was considered significant when the mean value was higher than 0.5 and with an associated *P* value of lower than 0.05.

Overexpression and purification of His-tagged GlxR protein. The *glxR* gene was amplified from chromosomal DNA of *C. glutamicum* R by PCR with primers GlxRHisFW and GlxRHisRV. The PCR product was cloned into the expression vector pColdI (Takara), yielding pCRC620. His-tagged GlxR was overexpressed in *E. coli* BL21(DE3) by cold shock and purified by affinity chromatography as described previously (80). The concentration of the purified protein was determined by a Bio-Rad protein assay (Bio-Rad Laboratories) using bovine serum albumin (BSA) as a standard.

EMSA. DNA fragments of interest were obtained by PCR amplification using promoter-*lacZ* fusion plasmids, which were constructed as described below, as templates and cloned into pGEM-T Easy (Promega). The sequence and direction of the cloned fragments were confirmed, and the cloned fragments were labeled with Cy3 by PCR amplification using primers SP6Cy3 and T7 (see Table S1 in the supplemental material). The probe containing the *gapA* promoter region was prepared by PCR amplification using primers PgapA RV3Cy3 and PgapAFW5NaeI (see Table S1 in the supplemental material). The resulting fragments were purified with the QIAquick PCR purification kit (Qiagen). Electrophoretic mobility shift assay (EMSA) was performed as described previously (80), except that His-tagged GlxR was incubated with 0.5 mM cAMP in EMSA buffer for 10 min before addition of the labeled DNA fragment. DNA and DNA-protein complexes were visualized with a Typhoon Trio variable-mode imager (GE Healthcare Bioscience).

Construction of promoter-*lacZ* fusions. The regions containing putative GlxR targets were amplified by PCR from *C. glutamicum* R chromosomal DNA using primers listed in Table S1 in the supplemental material. Mutations in GlxR-binding sites were introduced by PCR using mutated primers (indicated with "mut" in Table S1 in the supplemental material). When the GlxR-binding site was located within 200 bp from the initiation codon of the target gene, mutations were introduced by overlapping PCR as described previously (78). Fragments were phosphorylated and cloned upstream of the *lacZ* gene in pCRA741 (38). The direction and sequence of the inserted fragment were confirmed by DNA sequencing. The plasmids isolated as nonmethylated DNA from *E. coli* JM110 were introduced into *C. glutamicum* R and were subsequently integrated into strain-specific island 7 (SSI7) on the chromosome of *C. glutamicum* R by markerless gene insertion methods as described previously (36).

β-Galactosidase assay. *C. glutamicum* cells carrying the promoter-*lacZ* fusion were harvested, washed once with Z buffer (51), resuspended with the same buffer, and treated with toluene. The permeabilized cells were then incubated with *o*-nitrophenyl-β-galactoside, and activity was measured in Miller units as previously described (51).

5′-RACE-PCR. For the identification of the transcriptional start points (TSPs) of *mscL* and cgR_1596, 5′ rapid amplification of cDNA ends (RACE)-PCR analyses were carried out. Total RNA was extracted using the RNeasy Minikit (Qiagen) from wild-type *C. glutamicum* grown in A medium containing 1% glucose and treated with DNase I (Takara) as described previously (38). The RNA extracted was poly(A) tailed using a poly(A) tailing kit (Life Technologies) according to the manufacturer's instructions. After phenol-chloroform extraction, the RNA with a poly(A) tail was purified by ethanol precipitation. cDNA was synthesized using the SMARTer RACE cDNA amplification kit (Clontech, CA) with a supplied oligo(dT)-anchored primer and 1 μg of the tailed RNA prepared as described above. The cDNA was amplified with universal primer A (supplied with the kit) and gene-specific primers (see Table S1 in the supplemental material). The resulting PCR products were cloned into a pGEM-T Easy vector (Promega). At least 10 clones for each 5′ RACE-PCR product were sequenced.

cAMP assays. Intracellular cAMP levels were measured using the Amersham cAMP Biotrak enzyme immunoassay (EIA) system (GE Healthcare). Wild-type *C. glutamicum* and a *cyaB* deletion mutant were grown in A medium with 1% glucose or acetate at 33°C. Cells equivalent to an OD_{610} of 1.5 to 3 were harvested, resuspended in lysis buffer 1B (supplied with the kit) (200 μl for the wild type and 300 μl for the *cyaB* mutant), and incubated at 100°C for 15 min with intermittent mixing. The supernatants after centrifugation were stored at −20°C. Samples from the wild type were appropriately (from 5- to 40-fold) diluted in lysis buffer 1B, while those from the *cyaB* mutant were directly assayed. One hundred microliters of the samples from three independent experiments was assayed in duplicate according to the manufacturer's instructions.

Microarray data accession number. The ChIP-chip data from this study have been deposited at the Gene Expression Omnibus database under accession number GSE26870.

RESULTS

Identification of binding sites for GlxR *in vivo*. To elucidate the *in vivo* role of GlxR, we performed ChIP-chip analyses. We constructed strain KT23 whose *glxR* gene was modified to encode GlxR with a C-terminal Strep-tag II. Western blot analyses of total protein from strain KT23 and its parent *C. glutamicum* wild-type strain, probed with anti-Strep-tag II, detected the tagged GlxR protein with the expected size from only strain KT23 (data not shown). Although an in-frame deletion mutant of *glxR* is known to show severe growth defects (59, 79), strain KT23 grew as well as the wild type did (see Fig. S1 in the supplemental material), demonstrating that the tagged GlxR is as functional *in vivo* as the native one. Strain KT23 cells were grown in the presence of glucose for ChIP-chip analyses, bearing in mind that intracellular cAMP levels in *C. glutamicum* during growth on glucose are higher than that those during growth on acetate (41). ChIP and microarray analyses were performed as described in Materials and Methods. Genes exhibiting enrichment factors of higher than 0.5 with associated *P* values lower than 0.05 were chosen as possible targets of GlxR. Complete data sets are shown in Table S2 in the supplemental material. Since the microarrays were spotted with PCR products corresponding to coding sequences but not intergenic regions, it was not necessarily apparent which ORF was under the control of GlxR, especially in the case of the GlxR-binding site located between divergently transcribed genes. Therefore, when divergently transcribed ORFs were identified in ChIP-chip analyses, we regarded the intergenic region and the intragenic region of the gene with a higher enrichment factor as one putative GlxR-binding region (e.g., cgR_1326 and cgR_1327) (Table 2). When two consecutive genes transcribed in the same direction were identified, the intergenic region and the intragenic region of the upstream gene were considered one GlxR-binding region (e.g.,

TABLE 2. Binding sites identified by ChIP-chip which were used for validation in this study

Promoter region detected	Gene ID	Gene name	Direction	Wild type		cyaB mutant		Function	Binding site (5' → 3')	Distance[b]	Prediction[c]
				EF[a]	P value	EF	P value				
cgR_0087 and cgR_0088	cgR_0087	citH	−	0.15	3.41E−01	0.01	9.59E−01	Citrate transporter	CGTGACACACGCCACC	−76 (−20)	Novel
	cgR_0088	citA	+	0.89	3.89E−03	0.59	3.60E−02	Sensory histidine kinase	GGTGGCGTGTGTCACG	−132	Novel
cglR0323 and cgR_0324	cgR_0323	leuA	−	0.55	1.79E−02	0.48	2.78E−02	2-Isopropylmalate synthase	TGTGATTTCAGACACA	−294 (−159)	Novel
	cgR_0324		+	0.01	9.01E−01	−0.05	6.78E−01	Hypothetical protein	TGTGTCTGAAATCACA	−345	Novel
cgR_0992	cgR_0992	mscL	−	0.39	1.74E−01	−0.14	5.29E−01	Large-conductance mechanosensitive channel	TGTGACAAACGTCACT	−389 (−349)	Novel
	cgR_0993		−	3.14	5.77E−03	2.28	2.99E−03	Putative secreted protein			
cgR_1284	cgR_5018	atpB	−	1.73	2.30E−04	0.83	5.01E−02	Hypothetical protein	TGTGACACACATAAACA	−326 (−118)	Novel
	cgR_1284		+	0.24	7.52E−02	0.06	5.15E−01	F_oF_1 ATP synthase a chain			
cgR_1326 and cgR_1327	cgR_1326		−	2.36	2.95E−03	1.74	2.58E−02	Putative membrane protein	TGTGGTGTGCTGCACA	−79	Predicted
	cgR_1327	pfk	+	1.26	4.24E−03	0.78	2.17E−03	6-Phosphofructokinase	TGTGCAGCACCACA	−166 (−166)	Predicted
cgR_1596	cgR_1596		−	0.21	1.29E−01	0.51	1.62E−02	Secreted cell wall-associated hydrolase	AGTGATAAACATCACA	−411 (−132)	Predicted
	cgR_1597		−	1.40	3.08E−03	0.92	1.04E−02	Putative membrane protein			
cgR_1636	cgR_1636	gapA	−	0.77	2.61E−03	0.68	3.10E−02	Glyceraldehyde-3-phosphate dehydrogenase	TGTGAGTTGCATCACA	−427 (−244)	Predicted
	cgR_1637		−	1.34	2.66E−03	0.77	2.96E−02	Putative transcriptional regulator, WhiA homolog			
cgR_2076 and cgR_2077	cgR_2076	ctaC	−	0.81	1.33E−02	0.74	2.95E−03	Cytochrome c oxidase subunit II	TGTGACGTGGCATACA	−215 (−162)	Predicted
	cgR_2077	ltsA	+	0.13	7.99E−03	0.29	2.28E−02	Glutamine-dependent amidotransferase	TGTATGCCACGTCACA	−303	Predicted
cgR_2120	cgR_2119		−	2.35	1.90E−02	1.54	1.45E−02	Hypothetical protein	TGAGAGAAACATCACA	−337 (−219)	Novel
	cgR_2120	aceE	+	−0.09	6.77E−02	0.09	1.46E−01	Pyruvate dehydrogenase			
cgR_2431	cgR_2431	ctaD	−	0.13	3.42E−01	0.23	1.34E−01	Cytochrome c oxidase subunit I	TGTGAACCCCTTCACA	−227 (−171)	Predicted
	cgR_5047		−	2.46	6.49E−03	1.77	1.77E−02	Hypothetical protein			

[a] EF, enrichment factor in strain KT23 or strain KT25 (cyaB mutant); values are means from at least three independent experiments.
[b] Distance from the translational start point of the gene to the center of the binding site identified. In parentheses, the distance from the transcriptional start point of the gene to the center of the binding site is indicated. Except for mscL and cgR_1596, information on the transcriptional start points was obtained from literature data.
[c] Predicted, sites detected by both ChIP-chip and in silico analyses; novel, sites newly identified by ChIP-chip analysis.

FIG. 1. GlxR consensus sequences. A conserved motif was identified using the MEME algorithm by analyzing the regions with enrichment factors of ≥1 in the ChIP-chip experiment. The size of each letter indicates the relative abundance at the respective position in the consensus matrix generated with MEME.

cgR_1636 and cgR_1637) (Table 2). In this way, 268 ORFs which corresponded to 209 regions of the chromosome were identified as possible GlxR target regions *in vivo* under the conditions used.

A MEME (Multiple Em for Motif Elicitation) (6) alignment of the upstream sequences of the genes with relatively high enrichment factors (≥1) produced a consensus sequence of 5′-TGTGNNNNNNNNCACA-3′ (Fig. 1). The consensus motif obtained is almost the same as the previously reported 5′-TGTGANNTANNTCACA-3′ (42, 43, 49). It was further confirmed that, with the notable exception of one region, the consensus sequence is present in all other regions with enrichment factors of between 0.5 and 1 (see Table S2 in the supplemental material).

The regions identified by ChIP-chip analyses in this study were compared with the regions detected by previous *in silico* analyses in *C. glutamicum* ATCC 13032 (42, 43). The ChIP-chip analyses detected almost half of the sites predicted by *in silico* analyses (84/170). If the cutoff threshold was relaxed from 0.5 to 0.2, 34 sites were additionally identified. When the predicted sites that were not detected or were detected with low enrichment factors (<0.5) in the ChIP-chip analyses were compared with the motif obtained in the current study (Fig. 1), the GTG (position 2 to 4) and/or CAC (position 13 to 15) were not perfectly conserved (data not shown). In the motif obtained (Fig. 1), the sequences GTG and CAC are most conserved, implying that the nucleotides at these positions are important for the GlxR-DNA interactions.

Based on the results of ChIP-chip analyses and the consensus motif identified, 94 GlxR-binding regions that have not been previously predicted were newly identified here (Table 2; see Table S2 in the supplemental material). Of these, 53 sites are located in the coding region or the intergenic region between two convergently oriented genes.

Validation of the identified GlxR-binding sites. To validate the GlxR-binding sites identified in the upstream regions of genes, we examined effects of mutations within the sites on the binding of GlxR *in vitro*. We exchanged the nucleotides corresponding to the positions of GTG and CAC in the consensus motif (Fig. 1) to CAC and GTG, respectively (5′-T<u>GTG</u>-N$_8$-<u>CACA</u>-3′ → 5′-T<u>CAC</u>-N$_8$-<u>GTGA</u>-3′). We chose the upstream regions of the genes involved in fundamental cellular functions for further investigation (Table 2): aerobic respiration (cytochrome *c* oxidase subunits, *ctaC* [cgR_2076] and *ctaD*

[cgR_2431]), ATP synthesis (F$_o$F$_1$ ATPase subunit A, *atpB* [cgR_1284]), carbon metabolism (2-isopropylmalate synthase, *leuA* [cgR_0323]; phosphofructokinase, *pfk* [cgR_1327]; glyceraldehyde-3-phosphate dehydrogenase, *gapA* [cgR_1636]; and E1 component of pyruvate dehydrogenase, *aceE* [cgR_2120]), transport of carbon sources (citrate transporter, *citH* [cgR_0087]), cell division (secreted cell wall-associated hydrolase, cgR_1596), and the stress response (large-conductance mechanosensitive channel, *mscL* [cgR_0992]). The GlxR-binding sites located in the upstream regions of *leuA*, *aceE*, *mscL*, and *citH* were newly identified by ChIP-chip analyses in the current study.

The results of EMSA showed that GlxR binds to the corresponding regions in a cAMP-dependent manner (Fig. 2). In addition, the mutations introduced abolished the GlxR binding (Fig. 2), demonstrating that the binding sites identified are essential for GlxR binding and that the ChIP-chip was able to detect interaction of GlxR with target sites at genome-wide level *in vivo*.

GlxR bound to the sites upstream of cgR_1596 and *mscL* in the absence of cAMP, although the affinity was much lower than that in its presence. The strong affinity of these two sites may be attributed to the fact that the two sites share the same nucleotides at 12 positions (5′-tGTGAnAAACnTCACa-3′; shared nucleotides are capitalized) out of 16 positions in the consensus motif (Fig. 1).

***In vivo* function of GlxR.** Even though some GlxR-binding sites listed in Table 2 have been previously found and shown to be bound by GlxR *in vitro*, those functions *in vivo* have not been investigated. To test the *in vivo* roles of GlxR in regulation of target gene expression, each of the same DNA fragments as those tested by EMSA was fused to the promoterless *lacZ* gene, and the resulting construct was integrated into the wild-type chromosome as described in a previous study (78). It should be noted that a strain chromosomally carrying an empty vector, pCRA741, had no detectable β-galactosidase activity. As we and others have shown that the expression levels of the glycolytic genes, *pfk*, *gapA*, and *aceE*, in cells grown on glucose are higher than those in cells grown on acetate (33, 34, 53), we first tested whether GlxR is involved in the carbon source-dependent regulation of these genes. The promoter activities of the *pfk*, *gapA*, and *aceE* genes exhibited similar patterns during growth in nutrient-rich A medium containing 1% either glucose or acetate; they were increased more than 2-fold at the onset of the stationary phase (6 h) of growth on glucose, while the increase was not as drastic on acetate (Fig. 3). Introduction of the mutations into the GlxR-binding sites in the promoters suppressed the increase of the activity on glucose, while the activity on acetate was slightly reduced by the mutations. Thus, GlxR activates genes for the glycolytic enzymes, especially during growth on glucose.

The intracellular cAMP levels of *C. glutamicum* during growth on glucose are higher than those on acetate (15, 41), suggesting that the relatively high cAMP levels in the presence of glucose activate GlxR to increase the glycolytic gene expression. Under the growth conditions used in this study, the intracellular cAMP levels were comparable in glucose-grown cells and acetate-grown cells at the exponential phase, whereas, at the stationary phase, the cAMP levels in glucose-grown cells were up to 5-fold higher than those in acetate-
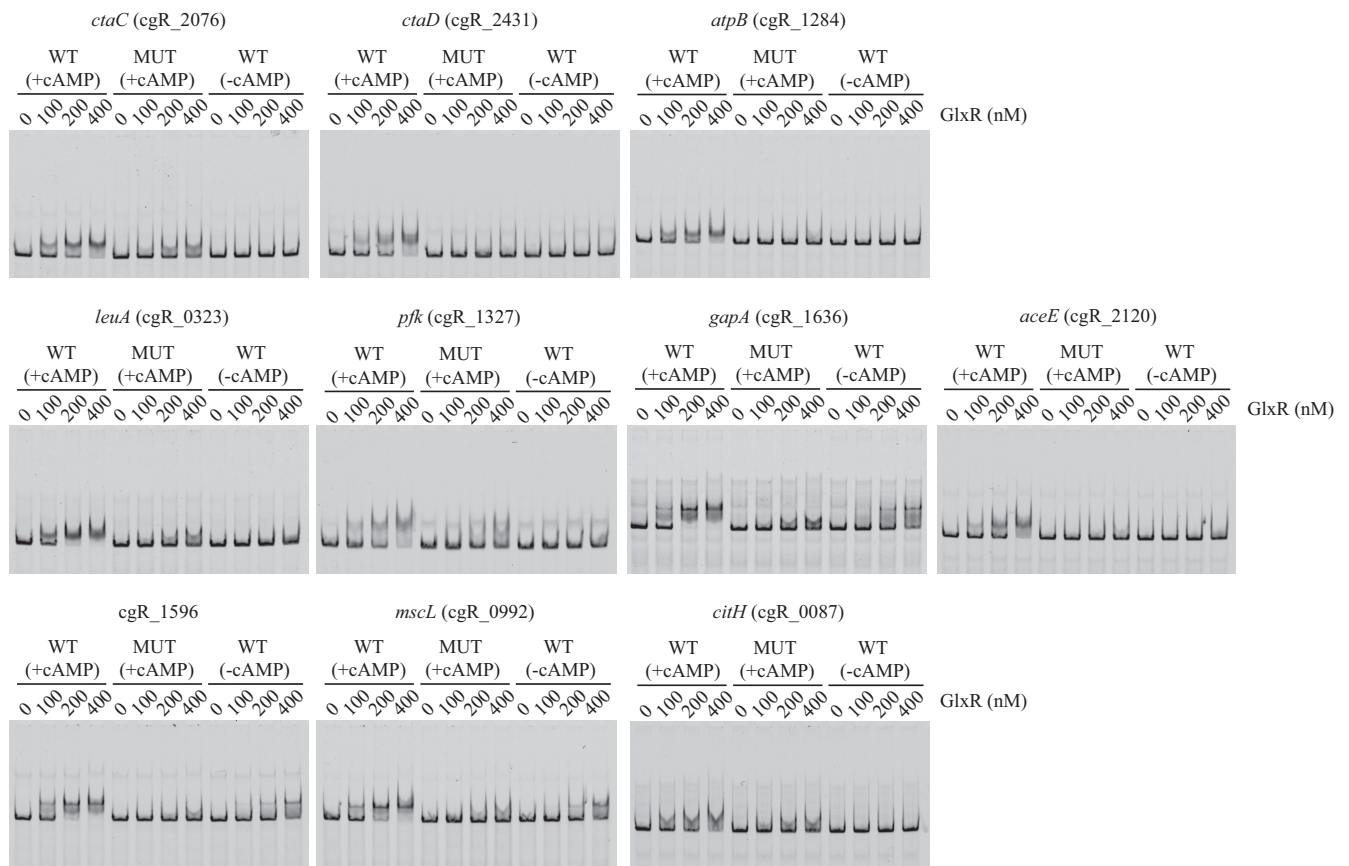
FIG. 2. EMSAs of representative *in vivo* GlxR-binding promoter regions detected by ChIP-chip analyses. The regions containing a wild-type (WT) or mutated (MUT) GlxR site were amplified by PCR using primers listed in Table S1 in the supplemental material and were labeled with Cy3 as described in Materials and Methods. The DNA probe was incubated with the purified GlxR at the indicated concentrations in the presence (+cAMP) or absence (−cAMP) of 0.5 mM cAMP and analyzed by nondenaturing PAGE. The target genes are indicated at the tops of the gels. Each well contained 10 nM DNA fragment.

grown cells (see Fig. S2A in the supplemental material). Thus, the increase in promoter activity of the glycolytic genes was likely to be correlated with the increase in the intracellular cAMP levels.

In contrast, promoter activity of the other genes, except *citH*, was not affected by the growth conditions examined (Fig. 4), implying that the changes in the intracellular cAMP levels had little effect on expression of these genes. However, mutations in the GlxR site resulted in a decrease of the activities of the *ctaC*, *ctaD*, and *atpB* promoters, with less effect on the *ctaD* promoter, indicating that GlxR activates expression from these promoters (Fig. 4). Moreover, promoter activity of cgR_1596 and *mscL* was lost by the mutation in the GlxR-binding site under the conditions used (Fig. 4), indicating that expression of these genes is highly dependent on GlxR. The TSPs of cgR_1596 and *mscL* were determined by 5′-RACE to be located at 279 nucleotides (nt) and 40 nt upstream of the start codon, respectively. Since the GlxR-binding sites are located at positions −132 and −349 with respect to the TSPs of cgR_1596 and *mscL*, respectively, the mutations introduced in the binding sites are unlikely to affect the basal promoter activity. Taken together, the results indicate that GlxR is important for upregulation of genes underlying various cellular functions but that the regulation is not entirely dependent on the cAMP levels.

The *leuA* promoter showed higher activity during growth on acetate than during growth on glucose. The activity was not affected by the mutations in the GlxR-binding site under the experimental conditions used (Fig. 4). Promoter activity during growth in minimal medium containing either glucose or acetate was almost the same as that in nutrient-rich medium and was not affected by the mutations in the GlxR-binding site (data not shown). Three ORFs (cgR_0324, cgR_0325, and cgR_0326), which are not conserved in the *C. glutamicum* ATCC 13032 genome, are present upstream of *leuA* in the *C. glutamicum* R genome (88). The GlxR-binding site found in the upstream region of *leuA* is also not conserved. Therefore, GlxR binding to the site may affect expression of the cgR_0324 gene, which is transcribed divergently from *leuA* and encodes a protein of unknown function.

The *citH* gene and the *tctCBA* operon encode citrate transporters with different cation specificities, and their expression is induced by the CitAB two-component system in the presence of citrate (11). Here, the ChIP-chip analyses identified GlxR-binding sites upstream of both *citH* and *tctC* (cgR_2710), suggesting that GlxR is involved in regulation of citrate uptake. We tested how GlxR is involved in induction of *citH* expression in the presence of citrate. The activity of the *citH* promoter in the presence of citrate was 5-fold higher than that in its ab-
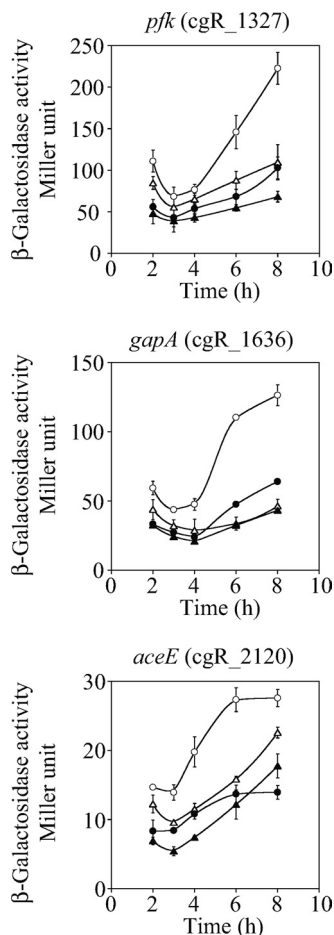
FIG. 3. GlxR activates expression of *pfk* (cgR_1327), *gapA* (cgR_1636), and *aceE* (cgR_2120). The β-galactosidase activities of *C. glutamicum* strains chromosomally carrying promoters of the genes, which were fused with the *lacZ* reporter gene, were measured. Activities derived from the native (open symbols) or GlxR-binding site-mutated (closed symbols) promoters during growth in nutrient-rich A medium with 1% glucose (circles) or acetate (triangles) are shown. The activity is the mean value from at least three independent cultivations. Error bars indicate standard deviations.

FIG. 4. Involvement of the GlxR-binding sites in regulation of *in vivo* expression of the target genes. The β-galactosidase activity of *C. glutamicum* strains chromosomally integrated with the target promoter fused with the *lacZ* reporter gene was measured. Activities derived from the native (white bars) or GlxR-binding site-mutated (black bars) promoters at the stationary phase of growth in nutrient-rich A medium with 1% glucose (Glc) or acetate (Ace) are shown. The activity is the mean value from at least three independent cultivations. Error bars indicate standard deviations. ND, not detected.

sence (Fig. 5, white bars). In the presence of both glucose and citrate, the *citH* promoter activity was slightly downregulated compared with that in the presence of citrate alone. Mutations introduced in the GlxR-binding site enhanced the promoter activity in the presence of citrate by 5-fold (Fig. 5, black bars). As they had no effect on the activity in the absence of citrate and did not affect the putative −10 and −35 regions (11), the possibility that the mutations affected the basal promoter activity was excluded. These results suggested that GlxR is involved in attenuation of induction of *citH* by CitAB.

Deletion of the *cyaB* gene decreases affinity of GlxR for the target sites *in vivo*. Although the *C. glutamicum* genome carries only a single putative adenylate cyclase gene, *cyaB*, cAMP was still detected in a *cyaB* mutant (15). The cAMP levels in the *cyaB* mutant were 25- and 45-fold lower than those in the wild type at the exponential and the onset of the stationary phase of growth on glucose, respectively (see Fig. S2B in the supplemental material). To examine binding of GlxR *in vivo* in the
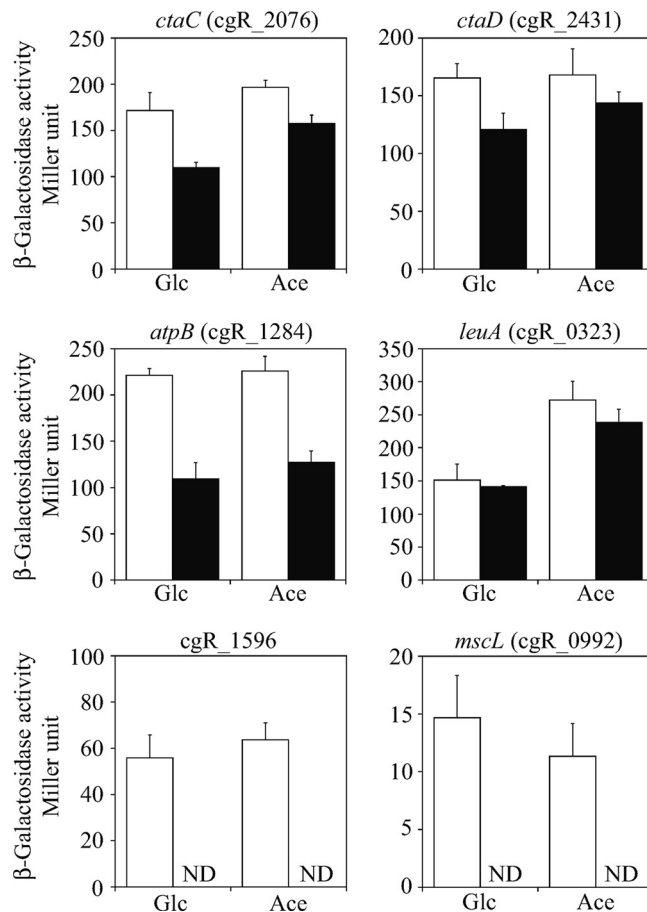
cyaB mutant background, we performed ChIP-chip analyses using strain KT25 expressing a GlxR-Strep-tag II in the background of the *cyaB* deletion mutant as described above. The number of ORFs with an enrichment factor of ≥0.5 was reduced from 268 in the wild type to 108 in the *cyaB* mutant (see Table S2 in the supplemental material). In addition, the signal intensities of more than 80 probes which exhibited an enrichment factor of ≥0.5 in the wild type decreased more than 1.5-fold in the *cyaB* mutant background. These results indicated that affinity of GlxR for its target sites *in vivo* was decreased in the mutant but that the *cyaB* gene was dispensable for GlxR binding to many target regions.

DISCUSSION

In this study, we used ChIP-chip technology to investigate the genome-wide binding sites of GlxR *in vivo*. The ChIP-chip experiments identified 209 GlxR-binding regions. Of these, 84 regions have been previously predicted by *in silico* analyses (42,
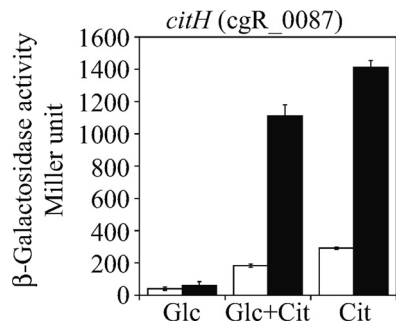
citH (cgR_0087)

FIG. 5. GlxR attenuates upregulation of *citH* (cgR_0087) expression by CitAB in the presence of citrate. The β-galactosidase activities of *C. glutamicum* strains chromosomally integrated with the *citH* promoter fused with the *lacZ* reporter gene were measured. Activities derived from the native (white bars) or GlxR-binding site-mutated (black bars) promoters at the exponential phase of growth in nutrient-rich A medium with 1% glucose (Glc), citrate (Cit), or glucose-citrate (Glc+Cit) are shown. The activity is the mean value from at least three independent cultivations. Error bars indicate standard deviations.

43). Among the remaining 125 regions, 94 were newly identified, 31 of which are specific to *C. glutamicum* R. Fifty-two other regions predicted by previous *in silico* analyses were not detected by the ChIP-chip analyses in this study. These discrepancies are probably caused by differences in the methods used to detect the binding sites and by differences in the regions used to search for the binding sites. While *in silico* analyses can identify all the possible binding sites based on the sequence similarity, ChIP-chip can identify binding sites where a protein of interest, GlxR in this study, actually binds *in vivo*. As the cAMP levels in *C. glutamicum* are dependent on the carbon source used and growth phase (41), GlxR may be unable to interact with some sites at the sampling point under the conditions used in this study. While *in silico* analyses searched for putative binding sites within intergenic regions of annotated ORFs (42), the current study used microarrays which were spotted with PCR products corresponding to coding sequences but not intergenic regions. Therefore, the GlxR-binding sites located far from the coding region could not be detected by the microarray, as has been described by others (27, 52). False negatives may also occur in ChIP-chip experiments due to sequestration of the transcription factor in nucleoprotein complexes, rendering them inaccessible to the specific test antibody used for the IP reaction (12, 30). Of the binding sites not detected by ChIP-chip analyses in this study, 21 have been shown to bind to GlxR in EMSA (42, 43). However, GlxR did not bind *in vitro* to two sites predicted by *in silico* analyses (42), suggesting that GlxR does not actually bind to these sites *in vivo*.

The ChIP-chip analyses also identified binding sites located within coding regions. Although the binding sites in the regions may be fortuitous and have no regulatory role, the ChIP-chip study of CRP from *E. coli* has suggested that, in addition to regulating transcription initiation, CRP contributes to the compaction of the *E. coli* chromosome by binding throughout the genome (31). The analyses also suggested that GlxR is involved in expression of strain-specific genes. Of 60 strain-specific genes in the *C. glutamicum* R genome, 41, corresponding to 31 regions, were identified by the ChIP-chip analyses.

The results of promoter-reporter fusion studies indicated that GlxR positively regulates expression of genes for various cellular functions, although it has been characterized as a repressor for some genes, such as *gltA*, *aceB*, *gluABCD*, and *sdhCAB*, encoding citrate synthase, malate syntheses, the glutamate uptake system, and succinate dehydrogenase, respectively (14, 59, 83). *ctaC* and *atpB* are the first genes of operons coding for cytochrome *c* oxidase and ATP synthase, respectively. No transcriptional regulator has been reported to be involved in expression of these genes, although *atpB* may be activated by SigH, which is a sigma factor that promotes expression of genes in response to various stress involving heat and oxidative stress (26), under alkaline conditions (7). The activity of promoters of the genes was not affected by either the carbon source or growth phase (Fig. 4 and data not shown). The loss of the GlxR-binding site resulted in a decrease of the promoter activity regardless of carbon source used, suggesting that these promoters are constitutively activated by GlxR and not affected by changes in cAMP levels under the conditions used here.

In contrast, the promoter activities of *gapA*, *pfk*, and *aceE*, which are involved in central carbon metabolism, were enhanced by the presence of glucose and the GlxR-binding site. The intracellular cAMP levels that are increased by the presence of glucose are likely to stimulate GlxR activity. Previously, RamA and SugR have been shown to be involved in upregulation and downregulation, respectively, of *gapA* expression (78, 79). RamA is a global regulator which controls expression of a number of genes and is essential for acetate and ethanol utilization (2–4, 14, 19, 69, 83), while SugR is a sugar-responsive transcriptional regulator that negatively controls genes involved in sugar uptake and catabolism in the absence of sugar (27, 28, 76, 78, 81). Although the physiological signal sensed by RamA is unknown, RamA is required for upregulation of *gapA* expression regardless of the carbon source (79). These current and previous findings suggest that, besides basal upregulation by RamA, *gapA* expression is enhanced in the presence of glucose by inactivating SugR and activating GlxR with sugar metabolites and cAMP, respectively. While *pfk* is negatively regulated by SugR and RamA (4, 27), *aceE* is positively controlled by RamB, a repressor of acetate utilization genes, under nutrient-rich conditions (10). The binding sites of these regulators in the promoter regions of *gapA*, *pfk*, and *aceE* do not overlap the GlxR-binding sites, eliminating the possibility that the mutations in the GlxR-binding sites affect binding of these regulators. GlxR acts as a transcriptional repressor of genes encoding tricarboxylic acid (TCA) cycle enzymes, including *gltA* and *sdhCAB* (14, 83), whose expression is also controlled by RamA. Thus, it is likely that GlxR contributes to the synchronization of expression of the central metabolic enzymes in collaboration with other transcription factors.

Although the promoter activities of cgR_1596 and *mscL* were not affected by the carbon source, the GlxR-binding sites identified are likely essential for activity of these promoters (Fig. 3). *C. glutamicum* possesses at least two cell wall hydrolases (cgR_1596 and cgR_2070) involved in separation of two daughter cells during cell division (82) and two mechanosensitive channels (MscL [cgR_0992] and MscS [cgR_1346]). cgR_2070 encodes a protein carrying the same catalytic cell wall hydrolase domain as that of the cgR_1596 protein and

plays a minor or supportive role in cell separation in addition to the cgR_1596 protein (82). *In silico* analyses detected the GlxR-binding site upstream of cgR_2070 (43), and the site is highly similar to that in the upstream region of cgR_1596 (see Table S2 in the supplemental material). Therefore, GlxR is probably involved in control of cgR_2070 expression. The low enrichment factor derived from the GlxR-binding site upstream of cgR_2070 is likely to be because the site is far from flanking ORFs (cgR_2070 and cgR_2071). MscS (cgR_1346) is a mechanosensitive channel with small conductance. MscL and MscS are required to adapt to hypoosomotic stress (58). MscS is also involved in glutamate excretion by *C. glutamicum* (55), suggesting the importance of these channels in view of biotechnological production. *In silico* analyses identified the GlxR-binding site in the upstream region of *mscS* only, while the ChIP-chip analyses identified the sites in the upstream regions of both *mscL* and *mscS*. Hence, both channels are likely to be under the control of GlxR. To our knowledge, this is the first report on transcriptional regulation of genes encoding cell wall hydrolases and mechanosensitive channels in *C. glutamicum*. Interestingly, *E. coli* CRP is also involved in regulation of one of the mechanosensitive channel genes, although binding to the promoter has not been examined (89). Taken together, these results indicate that the essential role of GlxR in expression of genes related to maintenance of cell morphology may be largely responsible for the severe growth defect of the *glxR* mutant.

The promoter assays in the current study indicated that GlxR attenuated the induction of *citH* expression by CitAB in response to the presence of citrate. This is in contrast to expression of the citrate transporter (CitS) of *Klebsiella pneumoniae* (50). *K. pneumoniae citS* is induced by the two-component system, CitAB, like *citH* of *C. glutamicum* but is positively regulated by the CRP homolog and repressed by the presence of glucose. The binding site of *K. pneumoniae* CRP in the *citS* promoter is centered at position -41.5 with respect to the *citS* TSP. This position was previously demonstrated to be one of the optimal sites for transcriptional activation by *E. coli* cAMP-CRP (13). The GlxR sites in the upstream regions of *citH* and *tctCBA*, another type of citrate transporter activated by CitAB, are located near positions -10 and $+10$ with respect to the TSP, respectively, implying that GlxR downregulates these genes. The attenuation by GlxR was similarly observed irrespective of the presence of glucose, indicating that GlxR does not mediate the glucose repression that is observed for *K. pneumoniae* CRP. This is supported by the facts that cAMP levels in citrate-grown cells are higher than those in glucose-grown cells (62) and that *C. glutamicum* is able to utilize glucose and citrate simultaneously (11). The attenuation of *citH* induction by GlxR may be required for achievement of an appropriate expression level.

In *M. tuberculosis*, which is phylogenetically closely related to *C. glutamicum*, a CRP homolog plays an important role in survival in macrophages and resuscitation (64). Despite its high amino acid sequence identity (79%) to GlxR, the *M. tuberculosis* CRP homolog, which is encoded by Rv3676, binds to DNA even in the absence of cAMP *in vitro* (5, 64), although its affinity to DNA is slightly increased upon forming a complex with cAMP (63, 73). Deletion of Rv3676 causes a growth defect and affects expression of several genes during exponen-

tial phase (64). The consensus binding site of Rv3676, 5′-GT GNNANNNNNCACA-3′, is almost identical to that for GlxR (5, 64). *In silico* analyses detected 55 intergenic regions that contained the consensus site (5). Of the genes with altered expression in the Rv3676 mutant or those flanking the consensus sites detected by *in silico* analyses, nearly 10 orthologs of *C. glutamicum* genes, including cgR_1596, *rpfA* (cgR_1010), and *whiB1* (*whcE*), were detected in our ChIP-chip analyses. *rpfA* encodes resuscitation-promoting factor, which is involved in reviving dormant bacteria (54). The *rpfA* orthologs in *M. tuberculosis* and *C. glutamicum* are directly regulated by Rv3676 and GlxR, respectively (40, 64). This may suggest that the two CRP homologs are in part functionally conserved in the two bacteria. On the other hand, almost all the remaining genes in the putative Rv3676 regulon encode proteins specific to *M. tuberculosis*, suggesting that GlxR- and Rv3676-dependent regulatory systems have diversified to adapt to the habitat of each organism over the evolutionary history. This may also be represented by the low response of Rv3676 to cAMP and high intracellular cAMP levels in *M. tuberculosis*, possessing 17 adenylate cyclase genes in the genome (70).

The current study demonstrated that GlxR was able to bind to target sites *in vivo* in the background of the *cyaB* mutant. This is in agreement with the facts that cAMP was still detected in the *cyaB* deletion mutant and its growth phenotype was not consistent with that of the *glxR* mutant (see Fig. S2 in the supplemental material) (15, 59). In *E. coli*, a cAMP-free intracellular environment can be achieved by deletion of the sole adenylate cyclase gene, *cya*, and the phenotype of the *cya* mutant is basically comparable to that of a *crp* mutant (61). In addition, the binding of *E. coli* CRP to the genome *in vivo* was completely inhibited in the presence of glucose, which lowered the cAMP level (31). As *cyaB* is the sole gene annotated as encoding adenylate cyclase in the genome, *C. glutamicum* is likely to possess a novel enzyme and/or pathway to synthesize cAMP. However, it should be noted that the possibility that the GlxR binding detected in the *cyaB* mutant is derived from not only the cAMP-GlxR complex but also cAMP-free GlxR cannot be ruled out. The results of EMSAs showed that GlxR was able to bind to the upstream regions of cgR_1596, *mscL*, and *gapA* even in the absence of cAMP, with much lower affinity than in its presence (Fig. 2). Furthermore, the binding of GlxR was not evenly affected by the depletion of cAMP in the *cyaB* mutant (see Table S2 in the supplemental material). These results indicate that the cAMP levels required for GlxR to bind to DNA vary depending on the target site. Finding an additional enzyme(s) involved in synthesis of cAMP and conditions which deplete intracellular cAMP would provide additional clues to understand the highly versatile physiological function of GlxR.

REFERENCES

1. Agari, Y., A. Kashihara, S. Yokoyama, S. Kuramitsu, and A. Shinkai. 2008. Global gene expression mediated by *Thermus thermophilus* SdrP, a CRP/FNR family transcriptional regulator. Mol. Microbiol. **70:**60–75.

2. **Arndt, A., and B. J. Eikmanns.** 2007. The alcohol dehydrogenase gene *adhA* in *Corynebacterium glutamicum* is subject to carbon catabolite repression. J. Bacteriol. **189:**7408–7416.

3. **Auchter, M., A. Arndt, and B. J. Eikmanns.** 2009. Dual transcriptional control of the acetaldehyde dehydrogenase gene *ald* of *Corynebacterium glutamicum* by RamA and RamB. J. Biotechnol. **140:**84–91.

4. **Auchter, M., et al.** 2011. RamA and RamB are global transcriptional regulators in *Corynebacterium glutamicum* and control genes for enzymes of the central metabolism. J. Biotechnol. **154:**126–139.

5. **Bai, G., L. A. McCue, and K. A. McDonough.** 2005. Characterization of *Mycobacterium tuberculosis* Rv3676 (CRPMt), a cyclic AMP receptor protein-like DNA binding protein. J. Bacteriol. **187:**7795–7804.

6. **Bailey, T. L., and C. Elkan.** 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc. Int. Conf. Intell. Syst. Mol. Biol. **2:**28–36.

7. **Barriuso-Iglesias, M., C. Barreiro, F. Flechoso, and J. F. Martín.** 2006. Transcriptional analysis of the F₀F₁ ATPase operon of *Corynebacterium glutamicum* ATCC 13032 reveals strong induction by alkaline pH. Microbiology **152:**11–21.

8. **Baumbach, J.** 2007. CoryneRegNet 4.0—a reference database for corynebacterial gene regulatory networks. BMC Bioinformatics **8:**429.

9. **Baumbach, J., and L. Apeltsin.** 2008. Linking Cytoscape and the corynebacterial reference database CoryneRegNet. BMC Genomics **9:**184.

10. **Blombach, B., A. Cramer, B. J. Eikmanns, and M. Schreiner.** 2009. RamB is an activator of the pyruvate dehydrogenase complex subunit E1p gene in *Corynebacterium glutamicum*. J. Mol. Microbiol. Biotechnol. **16:**236–239.

11. **Brocker, M., S. Schaffer, C. Mack, and M. Bott.** 2009. Citrate utilization by *Corynebacterium glutamicum* is controlled by the CitAB two-component system through positive regulation of the citrate transport genes *citH* and *tctCBA*. J. Bacteriol. **191:**3869–3880.

12. **Bucca, G., et al.** 2009. Development and application of versatile high density microarrays for genome-wide analysis of *Streptomyces coelicolor*: characterization of the HspR regulon. Genome Biol. **10:**R5.

13. **Busby, S., and R. H. Ebright.** 1999. Transcription activation by catabolite activator protein (CAP). J. Mol. Biol. **293:**199–213.

14. **Bussmann, M., et al.** 2009. Transcriptional control of the succinate dehydrogenase operon *sdhCAB* of *Corynebacterium glutamicum* by the cAMP-dependent regulator GlxR and the LuxR-type regulator RamA. J. Biotechnol. **143:**173–182.

15. **Cha, P. H., et al.** 2010. Characterization of an adenylate cyclase gene (*cyaB*) deletion mutant of *Corynebacterium glutamicum* ATCC 13032. Appl. Microbiol. Biotechnol. **85:**1061–1068.

16. **Chandler, M. S.** 1992. The gene encoding cAMP receptor protein is required for competence development in *Haemophilus influenzae* Rd. Proc. Natl. Acad. Sci. U. S. A. **89:**1626–1630.

17. **Cho, B. K., C. L. Barrett, E. M. Knight, Y. S. Park, and B. Ø. Palsson.** 2008. Genome-scale reconstruction of the Lrp regulatory network in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **105:**19462–19467.

18. **Cocaign-Bousquet, M., C. Monnet, and N. D. Lindley.** 1993. Batch kinetics of *Corynebacterium glutamicum* during growth on various carbon substrates: use of substrate mixtures to localise metabolic bottlenecks. Appl. Microbiol. Biotechnol. **40:**526–530.

19. **Cramer, A., R. Gerstmeir, S. Schaffer, M. Bott, and B. J. Eikmanns.** 2006. Identification of RamA, a novel LuxR-type transcriptional regulator of genes involved in acetate metabolism of *Corynebacterium glutamicum*. J. Bacteriol. **188:**2554–2567.

20. **de Crecy-Lagard, V., et al.** 1990. A *Xanthomonas campestris* pv. campestris protein similar to catabolite activation factor is involved in regulation of phytopathogenicity. J. Bacteriol. **172:**5877–5883.

21. **Derouaux, A., et al.** 2004. Crp of *Streptomyces coelicolor* is the third transcription factor of the large CRP-FNR superfamily able to bind cAMP. Biochem. Biophys. Res. Commun. **325:**983–990.

22. **Derouaux, A., et al.** 2004. Deletion of a cyclic AMP receptor protein homologue diminishes germination and affects morphological development of *Streptomyces coelicolor*. J. Bacteriol. **186:**1893–1897.

23. **Deutscher, J.** 2008. The mechanisms of carbon catabolite repression in bacteria. Curr. Opin. Microbiol. **11:**87–93.

24. **Dominguez, H., M. Cocaign-Bousquet, and N. D. Lindley.** 1997. Simultaneous consumption of glucose and fructose from sugar mixtures during batch growth of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **47:**600–603.

25. **Efromovich, S., D. Grainger, D. Bodenmiller, and S. Spiro.** 2008. Genome-wide identification of binding sites for the nitric oxide-sensitive transcriptional regulator NsrR. Methods Enzymol. **437:**211–233.

26. **Ehira, S., H. Teramoto, M. Inui, and H. Yukawa.** 2009. Regulation of *Corynebacterium glutamicum* heat shock response by the extracytoplasmic-function sigma factor SigH and transcriptional regulators HspR and HrcA. J. Bacteriol. **191:**2964–2972.

27. **Engels, V., S. N. Lindner, and V. F. Wendisch.** 2008. The global repressor SugR controls expression of genes of glycolysis and of the L-lactate dehydrogenase LdhA in *Corynebacterium glutamicum*. J. Bacteriol. **190:**8033–8044.

28. **Gaigalat, L., et al.** 2007. The DeoR-type transcriptional regulator SugR acts as a repressor for genes encoding the phosphoenolpyruvate:sugar phosphotransferase system (PTS) in *Corynebacterium glutamicum*. BMC Mol. Biol. **8:**104.

29. **Gosset, G., Z. Zhang, S. Nayyar, W. A. Cuevas, and M. H. Saier, Jr.** 2004. Transcriptome analysis of Crp-dependent catabolite control of gene expression in *Escherichia coli*. J. Bacteriol. **186:**3516–3524.

30. **Grainger, D. C., H. Aiba, D. Hurd, D. F. Browning, and S. J. Busby.** 2007. Transcription factor distribution in *Escherichia coli*: studies with FNR protein. Nucleic Acids Res. **35:**269–278.

31. **Grainger, D. C., D. Hurd, M. Harrison, J. Holdstock, and S. J. W. Busby.** 2005. Studies of the distribution of *Escherichia coli* cAMP-receptor protein and RNA polymerase along the *E. coli* chromosome. Proc. Natl. Acad. Sci. U. S. A. **102:**17693–17698.

32. **Han, S. O., M. Inui, and H. Yukawa.** 2008. Effect of carbon source availability and growth phase on expression of *Corynebacterium glutamicum* genes involved in the tricarboxylic acid cycle and glyoxylate bypass. Microbiology **154:**3073–3083.

33. **Han, S. O., M. Inui, and H. Yukawa.** 2007. Expression of *Corynebacterium glutamicum* glycolytic genes varies with carbon source and growth phase. Microbiology **153:**2190–2202.

34. **Hayashi, M., et al.** 2002. Transcriptome analysis of acetate metabolism in *Corynebacterium glutamicum* using a newly developed metabolic array. Biosci. Biotechnol. Biochem. **66:**1337–1344.

35. **Hermann, T.** 2003. Industrial production of amino acids by coryneform bacteria. J. Biotechnol. **104:**155–172.

36. **Inui, M., H. Kawaguchi, S. Murakami, A. A. Vertès, and H. Yukawa.** 2004. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. J. Mol. Microbiol. Biotechnol. **8:**243–254.

37. **Inui, M., et al.** 2004. Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J. Mol. Microbiol. Biotechnol. **7:**182–196.

38. **Inui, M., et al.** 2007. Transcriptional profiling of *Corynebacterium glutamicum* metabolism during organic acid production under oxygen deprivation conditions. Microbiology **153:**2491–2504.

39. **Inui, M., M. Terasawa, and H. Yukawa.** 1999. Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. Wiley, New York, NY.

40. **Jungwirth, B., et al.** 2008. Triple transcriptional control of the resuscitation promoting factor 2 (*rpf2*) gene of *Corynebacterium glutamicum* by the regulators of acetate metabolism RamA and RamB and the cAMP-dependent regulator GlxR. FEMS Microbiol. Lett. **281:**190–197.

41. **Kim, H. J., T. H. Kim, Y. Kim, and H. S. Lee.** 2004. Identification and characterization of *glxR*, a gene involved in regulation of glyoxylate bypass in *Corynebacterium glutamicum*. J. Bacteriol. **186:**3453–3460.

42. **Kohl, T. A., J. Baumbach, B. Jungwirth, A. Pühler, and A. Tauch.** 2008. The GlxR regulon of the amino acid producer *Corynebacterium glutamicum*: *in silico* and *in vitro* detection of DNA binding sites of a global transcription regulator. J. Biotechnol. **135:**340–350.

43. **Kohl, T. A., and A. Tauch.** 2009. The GlxR regulon of the amino acid producer *Corynebacterium glutamicum*: detection of the corynebacterial core regulon and integration into the transcriptional regulatory network model. J. Biotechnol. **143:**239–246.

44. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749–795.

45. **Kotrbova-Kozak, A., P. Kotrba, M. Inui, J. Sajdok, and H. Yukawa.** 2007. Transcriptionally regulated *adhA* gene encodes alcohol dehydrogenase required for ethanol and n-propanol utilization in *Corynebacterium glutamicum* R. Appl. Microbiol. Biotechnol. **76:**1347–1356.

46. **Krin, E., O. Sismeiro, A. Danchin, and P. N. Bertin.** 2002. The regulation of enzyme IIA^Glc expression controls adenylate cyclase activity in *Escherichia coli*. Microbiology **148:**1553–1559.

47. **Kronemeyer, W., N. Peekhaus, R. Krämer, H. Sahm, and L. Eggeling.** 1995. Structure of the *gluABCD* cluster encoding the glutamate uptake system of *Corynebacterium glutamicum*. J. Bacteriol. **177:**1152–1158.

48. **Kumagai, H.** 2000. Microbial production of amino acids in Japan. Adv. Biochem. Eng. Biotechnol. **69:**71–85.

49. **Letek, M., et al.** 2006. Characterization and use of catabolite-repressed promoters from gluconate genes in *Corynebacterium glutamicum*. J. Bacteriol. **188:**409–423.

50. **Meyer, M., P. Dimroth, and M. Bott.** 2001. Catabolite repression of the citrate fermentation genes in *Klebsiella pneumoniae*: evidence for involvement of the cyclic AMP receptor protein. J. Bacteriol. **183:**5248–5256.

51. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

52. **Molle, V., et al.** 2003. The Spo0A regulon of *Bacillus subtilis*. Mol. Microbiol. **50:**1683–1701.

53. **Muffler, A., et al.** 2002. Genome-wide transcription profiling of *Corynebacterium glutamicum* after heat shock and during growth on acetate and glucose. J. Biotechnol. **98:**255–268.

54. Mukamolova, G. V., et al. 2002. The *rpf* gene of *Micrococcus luteus* encodes an essential secreted growth factor. Mol. Microbiol. **46:**611–621.

55. Nakamura, J., S. Hirano, H. Ito, and M. Wachi. 2007. Mutations of the *Corynebacterium glutamicum* NCgl1221 gene, encoding a mechanosensitive channel homolog, induce L-glutamic acid production. Appl. Environ. Microbiol. **73:**4491–4498.

56. Neuweger, H., et al. 2007. CoryneCenter—an online resource for the integrated analysis of corynebacterial genome and transcriptome data. BMC Syst. Biol. **1:**55.

57. Nishimura, T., H. Teramoto, K. Toyoda, M. Inui, and H. Yukawa. 2011. Regulation of nitrate reductase operon *narKGHJI* by cyclic AMP-dependent regulator GlxR in *Corynebacterium glutamicum*. Microbiology **157:**21–28.

58. Nottebrock, D., U. Meyer, R. Krämer, and S. Morbach. 2003. Molecular and biochemical characterization of mechanosensitive channels in *Corynebacterium glutamicum*. FEMS Microbiol. Lett. **218:**305–309.

59. Park, S. Y., M. W. Moon, B. Subhadra, and J. K. Lee. 2010. Functional characterization of the *glxR* deletion mutant of *Corynebacterium glutamicum* ATCC 13032: involvement of GlxR in acetate metabolism and carbon catabolite repression. FEMS Microbiol. Lett. **304:**107–115.

60. Partridge, J. D., D. M. Bodenmiller, M. S. Humphrys, and S. Spiro. 2009. NsrR targets in the *Escherichia coli* genome: new insights into DNA sequence requirements for binding and a role for NsrR in the regulation of motility. Mol. Microbiol. **73:**680–694.

61. Perrenoud, A., and U. Sauer. 2005. Impact of global transcriptional regulation by ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc on glucose catabolism in *Escherichia coli*. J. Bacteriol. **187:**3171–3179.

62. Polen, T., D. Schluesener, A. Poetsch, M. Bott, and V. F. Wendisch. 2007. Characterization of citrate utilization in *Corynebacterium glutamicum* by transcriptome and proteome analysis. FEMS Microbiol. Lett. **273:**109–119.

63. Reddy, M. C., et al. 2009. Structural insights into the mechanism of the allosteric transitions of *Mycobacterium tuberculosis* cAMP receptor protein. J. Biol. Chem. **284:**36581–36591.

64. Rickman, L., et al. 2005. A member of the cAMP receptor protein family of transcription regulators in *Mycobacterium tuberculosis* is required for virulence in mice and controls transcription of the *rpfA* gene coding for a resuscitation promoting factor. Mol. Microbiol. **56:**1274–1286.

65. Robison, K., A. M. McGuire, and G. M. Church. 1998. A comprehensive library of DNA-binding site matrices for 55 proteins applied to the complete *Escherichia coli* K-12 genome. J. Mol. Biol. **284:**241–254.

66. Sala, C., et al. 2009. Genome-wide regulon and crystal structure of BlaI (Rv1846c) from *Mycobacterium tuberculosis*. Mol. Microbiol. **71:**1102–1116.

67. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

68. Schröder, J., and A. Tauch. 2010. Transcriptional regulation of gene expression in *Corynebacterium glutamicum*: the role of global, master and local regulators in the modular and hierarchical gene regulatory network. FEMS Microbiol. Rev. **34:**685–737.

69. Seibold, G. M., et al. 2010. The transcriptional regulators RamA and RamB are involved in the regulation of glycogen synthesis in *Corynebacterium glutamicum*. Microbiology **156:**1256–1263.

70. Shenoy, A. R., K. Sivakumar, A. Krupa, N. Srinivasan, and S. S. Visweswariah. 2004. A survey of nucleotide cyclases in Actinobacteria: unique domain organization and expansion of the class III cyclase family in *Mycobacterium tuberculosis*. Comp. Funct. Genomics **5:**17–38.

71. Skorupski, K., and R. K. Taylor. 1997. Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*. Proc. Natl. Acad. Sci. U. S. A. **94:**265–270.

72. Smith, K. M., K. M. Cho, and J. C. Liao. 2010. Engineering *Corynebacterium glutamicum* for isobutanol production. Appl. Microbiol. Biotechnol. **87:**1045–1055.

73. Stapleton, M., et al. 2010. *Mycobacterium tuberculosis* cAMP receptor protein (Rv3676) differs from the *Escherichia coli* paradigm in its cAMP binding and DNA binding properties and transcription activation properties. J. Biol. Chem. **285:**7016–7027.

74. Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.

75. Tan, K., G. Moreno-Hagelsieb, J. Collado-Vides, and G. D. Stormo. 2001. A comparative genomics approach to prediction of new members of regulons. Genome Res. **11:**566–584.

76. Tanaka, Y., H. Teramoto, M. Inui, and H. Yukawa. 2008. Regulation of expression of general components of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) by the global regulator SugR in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **78:**309–318.

77. Teramoto, H., M. Inui, and H. Yukawa. 2009. Regulation of expression of genes involved in quinate and shikimate utilization in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **75:**3461–3468.

78. Toyoda, K., H. Teramoto, M. Inui, and H. Yukawa. 2008. Expression of the *gapA* gene encoding glyceraldehyde-3-phosphate dehydrogenase of *Corynebacterium glutamicum* is regulated by the global regulator SugR. Appl. Microbiol. Biotechnol. **81:**291–301.

79. Toyoda, K., H. Teramoto, M. Inui, and H. Yukawa. 2009. Involvement of the LuxR-type transcriptional regulator, RamA, in regulation of expression of the *gapA* gene encoding glyceraldehyde-3-phosphate dehydrogenase of *Corynebacterium glutamicum*. J. Bacteriol. **191:**968–977.

80. Toyoda, K., H. Teramoto, M. Inui, and H. Yukawa. 2009. The *ldhA* gene, encoding fermentative L-lactate dehydrogenase of *Corynebacterium glutamicum*, is under the control of positive feedback regulation mediated by LldR. J. Bacteriol. **191:**4251–4258.

81. Toyoda, K., H. Teramoto, M. Inui, and H. Yukawa. 2009. Molecular mechanism of SugR-mediated sugar-dependent expression of the *ldhA* gene encoding L-lactate dehydrogenase in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **83:**315–327.

82. Tsuge, Y., H. Ogino, H. Teramoto, M. Inui, and H. Yukawa. 2008. Deletion of cgR_1596 and cgR_2070, encoding NlpC/P60 proteins, causes a defect in cell separation in *Corynebacterium glutamicum* R. J. Bacteriol. **190:**8204–8214.

83. van Ooyen, J., et al. 2011. Citrate synthase in *Corynebacterium glutamicum* is encoded by two *gltA* transcripts which are controlled by RamA, RamB, and GlxR. J. Biotechnol. **154:**140–148.

84. Vertès, A. A., M. Inui, M. Kobayashi, Y. Kurusu, and H. Yukawa. 1993. Presence of *mrr*- and *mcr*-like restriction systems in coryneform bacteria. Res. Microbiol. **144:**181–185.

85. Wendisch, V. F., M. Bott, and B. J. Eikmanns. 2006. Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. Curr. Opin. Microbiol. **9:**268–274.

86. Wendisch, V. F., A. A. de Graaf, H. Sahm, and B. J. Eikmanns. 2000. Quantitative determination of metabolic fluxes during coutilization of two carbon sources: comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. J. Bacteriol. **182:**3088–3096.

87. Wolfgang, M. C., V. T. Lee, M. E. Gilmore, and S. Lory. 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev. Cell **4:**253–263.

88. Yukawa, H., et al. 2007. Comparative analysis of the *Corynebacterium glutamicum* group and complete genome sequence of strain R. Microbiology **153:**1042–1058.

89. Zhang, Z., et al. 2005. Functional interactions between the carbon and iron utilization regulators, Crp and Fur, in *Escherichia coli*. J. Bacteriol. **187:**980–990.

90. Zheng, D., C. Constantinidou, J. L. Hobman, and S. D. Minchin. 2004. Identification of the CRP regulon using *in vitro* and *in vivo* transcriptional profiling. Nucleic Acids Res. **32:**5874–5893.

91. Zubay, G., D. Schwartz, and J. Beckwith. 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Natl. Acad. Sci. U. S. A. **66:**104–110.