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 regulation of gene expression (1), modulation of virulence (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-N6-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 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 members of the family and grows fast, physiological 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 glyoxylyte 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-N6-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 glyoxylyte 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-
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, albeit with lower affinity than in the wild type, suggesting that GlxR in the cyA 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 cyaH 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 glxRStrepF-glxRStrepR, together with primers glxRFw and glxRRv. The resulting fragment was cloned into pCRC619, a suicide vector for markerless gene disruption (36), yielding pCRC620. This was isolated as nonmethylated DNA from E. coli JM110 for efficient gene introduction into C. glutamicum R (wild type) (79) and the cyA 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

ChiP-chip analyses with a cyA 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 cyA mutant is physiologically active.

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

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
</table>
| **E. coli**
| JM109 | recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)F' (traD36 proAB)T lacP lacZΔM15 lacY galK galT ara tonA thi lux hsdSH (rB mB) λ (DE3) | Takara |
| JM110 | lacY galK galT ara tonA thi lux Δ(lac-proAB)F' (traD36 proAB') lacP lacZΔM15 lacTΔM15 | 67 |
| BL21(DE3) | F' ompT gal dcm lon hsdR (rB mB) 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 |
| ΔcyA mutant | R with deletion in cyA | 57 |
| KT25 | ΔcyA mutant with Strep-tag II-tagged glxR | This study |
| **Plasmids**
| pCold | Ap'; cold-inducible expression | Takara |
| pGEM T-Easy Vector | Promega |
| pCRA725 | Km'; suicide vector containing the B. subtilis sacB gene | 36 |
| pCRA741 | Km'; 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 | Km'; pCRA725 with a 2.4-kb fragment containing a Strep-tag II-tagged glxR gene | This study |
| pCRC620 | Ap'; pColdI with a 684-bp fragment containing the glxR gene | This study |
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 DNA was blotted on nylon membranes, 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 spots minus the flagged ones was equal to 1, using GenePix 5.0 software. The enrichment factor for a given gene was calculated as the log2 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 by PCR with primer GlxRHisFW and GlxRHisRV. The PCR product was cloned into the expression vector pCOLD1 (Takara), yielding pCRCS62. His-tagged GlxR was overexpressed in E. coli BL21(DE3) at 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 labeled DNA fragments were confirmed, and the labeled fragments were labeled with Cy3 by PCR amplification using primers SP6Cy3 and T7 (see Table S1 in the supplemental material). The probe containing the gap4′ promoter region was prepared by PCR amplification using primers PgapA RV3Cy3 and PgapB RV (Table S1 in the supplemental material). The amplified 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 with primer GlxRPrim1 (data not shown). The sequences and direction of the amplified DNA were confirmed, and the amplified fragments were cloned with Cy3 by PCR amplification using primers SP6Cy3 and T7 (see Table S1 in the supplemental material). The probe containing the gap4′ promoter region was prepared by PCR amplification using primers PgapA RV3Cy3 and PgapB RV (Table S1 in the supplemental material). The amplified 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).

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 glucose are higher than 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>
<thead>
<tr>
<th>Promoter region detected</th>
<th>Gene ID</th>
<th>Gene name</th>
<th>Direction</th>
<th>Wild type</th>
<th>cyaB mutant</th>
<th>Function</th>
<th>Binding site (5' → 3')</th>
<th>Distance</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>cgR_0087 and cgR_0088</td>
<td>cgR_0087</td>
<td>citH</td>
<td>−</td>
<td>0.15</td>
<td>0.01</td>
<td>Citrate transporter</td>
<td>CGTGACACAGCGCACCC</td>
<td>−76 (−20)</td>
<td>Novel</td>
</tr>
<tr>
<td></td>
<td>cgR_0088</td>
<td>citA</td>
<td>+</td>
<td>0.89</td>
<td>0.01</td>
<td>Sensory histidine kinase</td>
<td>GGTGCGTGTGTGACCC</td>
<td>−132</td>
<td>Novel</td>
</tr>
<tr>
<td>cgR0323 and cgR_0324</td>
<td>cgR_0323</td>
<td>leuA</td>
<td>−</td>
<td>0.55</td>
<td>0.48</td>
<td>2-Isopropylmalate synthase</td>
<td>TGTGATTTCAAGCACCA</td>
<td>−294 (−159)</td>
<td>Novel</td>
</tr>
<tr>
<td></td>
<td>cgR_0324</td>
<td></td>
<td>+</td>
<td>0.01</td>
<td>−0.05</td>
<td>Hypothetical protein</td>
<td>TGTGCTGGAATCCACCA</td>
<td>−345</td>
<td>Novel</td>
</tr>
<tr>
<td>cgR_0992</td>
<td>cgR_0992</td>
<td>mscL</td>
<td>−</td>
<td>0.39</td>
<td>−0.14</td>
<td>Large-conductance mechanosensitive channel</td>
<td>TGTGACAAACGTACCA</td>
<td>−389 (−349)</td>
<td>Novel</td>
</tr>
<tr>
<td></td>
<td>cgR_0993</td>
<td></td>
<td></td>
<td>3.14</td>
<td>2.28</td>
<td>Putative secreted protein</td>
<td>TGTGACACATAACCA</td>
<td>−326 (−118)</td>
<td>Novel</td>
</tr>
<tr>
<td>cgR_1284</td>
<td>cgR_5018</td>
<td>atpB</td>
<td>−</td>
<td>1.73</td>
<td>0.83</td>
<td>Hypothetical protein</td>
<td>TGTGACACATAACCA</td>
<td>−294 (−159)</td>
<td>Novel</td>
</tr>
<tr>
<td></td>
<td>cgR_1284</td>
<td></td>
<td>+</td>
<td>0.24</td>
<td>0.06</td>
<td>F₁,F₂ ATP synthase a chain</td>
<td>TGTGACACATAACCA</td>
<td>−326 (−118)</td>
<td>Novel</td>
</tr>
<tr>
<td>cgR_1326 and cgR_1327</td>
<td>cgR_1326</td>
<td></td>
<td></td>
<td>2.36</td>
<td>1.74</td>
<td>Putative membrane protein</td>
<td>TGTGTTGCTGACCA</td>
<td>−79</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>cgR_1327</td>
<td>pfk</td>
<td>+</td>
<td>1.26</td>
<td>0.78</td>
<td>6-Phosphofructokinase</td>
<td>TGTGCAGCACACCA</td>
<td>−166 (−166)</td>
<td>Predicted</td>
</tr>
<tr>
<td>cgR_1596</td>
<td>cgR_1596</td>
<td></td>
<td>−</td>
<td>0.21</td>
<td>0.51</td>
<td>Secreted cell wall-associated hydrolase</td>
<td>AGTGATAAACATCCAC</td>
<td>−411 (−132)</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>cgR_1597</td>
<td></td>
<td>−</td>
<td>1.40</td>
<td>0.92</td>
<td>Putative membrane protein</td>
<td>TGTGAGTGACCATCA</td>
<td>−427 (−244)</td>
<td>Predicted</td>
</tr>
<tr>
<td>cgR_1636</td>
<td>cgR_1636</td>
<td>gapA</td>
<td>−</td>
<td>0.77</td>
<td>0.68</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>TGTGAGTGACCATCA</td>
<td>−427 (−244)</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>cgR_1637</td>
<td></td>
<td>−</td>
<td>1.34</td>
<td>0.77</td>
<td>Putative transcriptional regulator, WhiA homolog</td>
<td>TGTGAGTGACCATCA</td>
<td>−427 (−244)</td>
<td>Predicted</td>
</tr>
<tr>
<td>cgR_2076 and cgR_2077</td>
<td>cgR_2076</td>
<td>ctaC</td>
<td>−</td>
<td>0.81</td>
<td>0.74</td>
<td>Cytochrome c oxidase subunit II</td>
<td>TGTGACGTGGTACCA</td>
<td>−215 (−162)</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>cgR_2077</td>
<td>ltsA</td>
<td>+</td>
<td>0.13</td>
<td>0.29</td>
<td>Glutamine-dependent amidotransferase</td>
<td>TGTATGCCGCTACCA</td>
<td>−303</td>
<td>Predicted</td>
</tr>
<tr>
<td>cgR_2120</td>
<td>cgR_2119</td>
<td>aceE</td>
<td>+</td>
<td>2.35</td>
<td>1.54</td>
<td>Hypothetical protein</td>
<td>TGAGAGCAACATCCAC</td>
<td>−337 (−219)</td>
<td>Novel</td>
</tr>
<tr>
<td></td>
<td>cgR_2120</td>
<td></td>
<td>−</td>
<td>0.09</td>
<td>0.09</td>
<td>Pyruvate dehydrogenase</td>
<td>TGAGAGCAACATCCAC</td>
<td>−337 (−219)</td>
<td>Novel</td>
</tr>
<tr>
<td>cgR_2431</td>
<td>cgR_2431</td>
<td>ctaD</td>
<td>−</td>
<td>0.13</td>
<td>0.23</td>
<td>Cytochrome c oxidase subunit I</td>
<td>TGTGACCCCTTCACCA</td>
<td>−227 (−171)</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>cgR_5047</td>
<td></td>
<td>−</td>
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<td>1.77</td>
<td>Hypothetical protein</td>
<td>TGTGACCCCTTCACCA</td>
<td>−227 (−171)</td>
<td>Predicted</td>
</tr>
</tbody>
</table>

* EF, enrichment factor in strain KT25 or strain KT25 (cyaB mutant); values are means from at least three independent experiments.

* 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.

* Predicted, sites detected by both ChIP-chip and in silico analyses; novel, sites newly identified by ChIP-chip analysis.
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'-TGTG-N6-CACA-3' → 5'-TCAC-N6-GTGA-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_6,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'-TGTG-AnAACnTCACa-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 ace...
expression of these genes is highly dependent on GlxR. The TSPs of these genes are 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-
In the presence of both glucose and citrate, the $\text{citH}$ 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 $\text{H11002}$ and $\text{H11002}$ 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 $\text{citH}$ by CitAB.

Deletion of the $\text{cyaB}$ gene decreases affinity of GlxR for the target sites in vivo. Although the $\text{C. glutamicum}$ genome carries only a single putative adenylate cyclase gene, $\text{cyaB}$, cAMP was still detected in a $\text{cyaB}$ mutant (15). The cAMP levels in the $\text{cyaB}$ 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 $\text{cyaB}$ mutant background, we performed ChIP-chip analyses using strain KT25 expressing a GlxR-Strep-tag II in the background of the $\text{cyaB}$ 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 $\text{cyaB}$ 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 $\text{cyaB}$ mutant background. These results indicated that affinity of GlxR for its target sites in vivo was decreased in the mutant but that the $\text{cyaB}$ 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,
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.

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, glaABC, and sdhCAB, encoding citrate synthase, malate synthases, the glutamate uptake system, and succinate dehydrogenase, respectively (14, 59, 83). citA 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 promoters in the regulator 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 mseL 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 (MseL [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 hypoosmotic 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-
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