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Involvement of the Global Regulator GlxR in 3-Hydroxybenzoate and Gentisate Utilization by *Corynebacterium glutamicum*

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Corynebacterium glutamicum **is an industrially important producer of amino acids and organic acids, as well as an emerging model system for aromatic assimilation. An IclR-type regulator GenR has been characterized to activate the transcription of *genDFM* and *genKH* operons for 3-hydroxybenzoate and gentisate catabolism and represses its own expression. On the other hand, GlxR, a global regulator of the cyclic AMP (cAMP) receptor protein-fumarate nitrate reductase regulator (CRP-FNR) type, was also predicted to be involved in this pathway. In this study, electrophoretic mobility shift assays and footprinting analyses demonstrated that GlxR bound to three sites in the promoter regions of three *gen* operons. A combination of site-directed mutagenesis of the biding sites, promoter activity assay, and GlxR overexpression demonstrated that GlxR repressed their expression by binding these sites. One GlxR binding site (DFMx) was found to be located −13 to +8 bp upstream of the *genDFM* promoter, which was involved in negative regulation of *genDFM* transcription. The GlxR binding site R-KHx01 (located between positions −11 to +5) was upstream of the *genKH* promoter sequence and involved in negative regulation of its transcription. The binding site R-KHx02, at which GlxR binds to *genR* promoter to repress its expression, was found within a footprint extending from positions −71 to −91 bp. These results reveal that GlxR represses the transcription of all three *gen* operons and then contributes to the synchronization of their expression for 3-hydroxybenzoate and gentisate catabolism in collaboration with the specific regulator GenR.**

The gentisate (2,5-dihydroxybenzoate [GEN]) pathway is one of the important ring cleavage pathways in the catabolism of various aromatic compounds, including 3-hydroxybenzoate (3-HBA) (1, 2), naphthalene (3, 4), salicylate (5, 6), 3,6-dichloro-2-methoxybenzoate (7), and xylenol (8). The initial reaction of this pathway is the ring cleavage oxidation of gentisate, catalyzed by gentisate 1,2-dioxygenase (9). Subsequently, the ring fission product maleylpyruvate is degraded via either direct hydrolysis to maleate and pyruvate (10, 11) or isomerization to fumarylpyruvate before hydrolysis to fumarate and pyruvate (1, 4, 9, 12). Two LysR-type transcriptional regulators, NagR in *Ralstonia* sp. strain U2 (13) and MhbR in *Klebsiella pneumoniae* M5a1 (14), have been reported to activate the expression of enzymes involved in naphthalene and 3-HBA catabolism via gentisate pathways.

Corynebacterium glutamicum, a Gram-positive bacterium with a high G+C content, plays a prominent role for the microbial production of amino acids, organic acids, and alcohols (15–20). Derivative strains of *Corynebacterium* were used to produce 2.5 million tons of glutamate as monosodium glutamate per year as well as several thousand tons of other amino acids such as lysine, isoleucine, tryptophan, and threonine (16, 21). Recently, *C. glutamicum* ATCC 13032 has been found to utilize a number of aromatic compounds as its sole carbon and energy source, including 3-HBA and GEN (22). Its catabolic pathway of 3-HBA degradation via GEN was catalyzed by *genD*-, *genF*-, *genM*-, and *genH*-encoded enzymes (1, 23–25). In addition to these catabolic genes, GenK was identified to actively transport gentisate to facilitate GEN utilization (23). More recently, IclR-type regulator GenR has been characterized to be a dual-function protein that acts as an activator of the transcription of *genDFM* and *genKH* operons for 3-HBA and GEN catabolism in response to 3-HBA and GEN and as a repressor of its own expression (25).

It has been recognized that expression of genes in *C. glutamicum*, including those involved in aromatic catabolism, is generally regulated by pathway-specific regulators (22, 26). However, the presence of global regulators has also been observed when GlxR binding sites were found in the potential operator regions involved in aromatic degradation (27). GlxR is a major global transcriptional regulator of *C. glutamicum*, belonging to the family of cyclic AMP (cAMP) receptor protein-fumarate nitrate reductase regulators (CRP-FNR). It was first characterized as a factor repressing the promoter activity of a gene coding for an enzyme in the glyoxylate pathway and presenting as dimers (28). Subsequently, a total of 215 potential GlxR binding sites were predicted bioinformatically and 77 sites were experimentally confirmed (29). Recently, *in silico* detection and chromatin immunoprecipitation in conjunction with microarray (ChIP-chip) analyses detected 209 GlxR binding sites in the *C. glutamicum* genome, and 84 regions were previously described (30). Among these 209 binding sites, 72 of them were verified by *in vitro* binding assays to be associated with regulons consisting of genes for carbon metabolism, nitrogen metabolism, respiration, resuscitation, cell wall formation, and cell division (27, 29, 30). More recently, GlxR was demonstrated to positively regulate expression of genes for aerobic respiration, ATP synthesis, and glycolysis and to be necessary for expression of genes for cell separation and mechanosensitive channels (30). Interestingly, negative regulation of GlxR was found in the expression of a citrate uptake gene (30). Even though

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TABLE 1 Bacteria and plasmids involved in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacteria		
Corynebacterium glutamicum		
ATCC 13032	Wild type, plasmid free, Nx^r	ATCC
RES167	Restriction-deficient mutant of ATCC 13032, Δ(*cglIM-cglIR-cglIIR*)	55
Escherichia coli		
DH5α	λ− ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Gibco BRL
Rosetta(DE3)pLysS	Cam^r, F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3), pLysSRARE vector (Cam^r)	Novagen
*Trans*1-T1 phage resistant	F^- ϕ80(*lacZ*)ΔM15 Δ*lacX74 hsdR*(r_K^- m_K^+)Δ*recA1398 endA1 tonA*	TransGen Biotech
Plasmids		
pMD18-T	Amp^r, *lacZ*α, cloning vector for sequencing	TaKaRa
pET-28a(+)	Expression vector, Kan^r, C/N-terminal His tag/thrombin/T7 tag, T_7 *lac* promoter, T7 transcription start, f1 origin, *lacI*	Novagen
pZWHJ024	Kan^r, 681-bp PCR fragment of *glxR* gene ORF in *C. glutamicum* ATCC 13032 genome cloned into pET-28a(+) NdeI/EcoRI site, His tag banding C-terminal GlxR	This study
pZWHJ002	Cam^r, pBL1 *ori*V_{Cg}, pK18, *ori*V_{Ec}, t_{rrnB} of *E. coli*, *lacZ* promoterless, derivative of pXMJ19 with *lacZ* digested with the enzyme combination NarI/HindIII, deletion of *Ptac lac*^{q}, shuttle vector	25
pZWHJ006	Cam^r, derivative of pZWHJ002 with 463-bp promoter of *genDFM* operon for detecting promoter activation	25
pZWHJ007	Cam^r, derivative of pZWHJ002 with 457-bp promoter of *genR* operon for detecting promoter activation	25
pZWHJ008	Cam^r, derivative of pZWHJ002 with 427-bp promoter of *genKH* operon for detecting promoter activation	25
pZWHJ025	Cam^r, pZWHJ006 with site DFMx mutated	This study
pZWHJ026	Cam^r, pZWHJ007 with site R-KHx01 mutated	This study
pZWHJ027	Cam^r, pZWHJ007 with site R-KHx02 mutated	This study
pZWHJ028	Cam^r, pZWHJ007 with sites R-KHx01 and R-KHx02 mutated	This study
pZWHJ029	Cam^r, pZWHJ008 with site R-KHx01 mutated	This study
pZWHJ030	Cam^r, pZWHJ008 with site R-KHx02 mutated	This study
pZWHJ031	Cam^r, pZWHJ008 with sites R-KHx01 and R-KHx02 mutated	This study
pZWHJ033	Cam^r, pBL1 *ori*V_{Cg}, pK18, *ori*V_{Ec}, t_{rrnB} of *E. coli*, carrying *glxR* gene, derivative of pXMJ19 with *glxR* from RES167 chromosome, digested with the enzyme combination SalI and EcoRI, shuttle vector	This study

[a] *ori*V_{Cg}, *C. glutamicum oriV*; *ori*V_{Ec}, *E. coli oriV*.

in vitro binding assays indicated its possible involvement in regulating the expression of the *paa* gene cluster for phenylacetic acid catabolism, whether or not the GlxR was involved in aromatic metabolism in this strain still remains unknown (31). The study of the global regulatory factor GlxR will help to improve the ability of *C. glutamicum* in industrial production as well as aromatic degradation. In this article, we show that GlxR represses the transcription of *gen* operons and is involved in 3-HBA and GEN catabolism in *C. glutamicum* by assays of mobility shift electrophoresis, footprinting, overexpression, and promoter activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used are listed in Table 1 and primers in Table 2. *Escherichia coli* was grown aerobically on a rotary shaker (200 rpm) at 37°C in lysogeny broth (LB) or on an LB plate with 1.5% (wt/vol) agar. *C. glutamicum* strains were grown in LB medium or in mineral salt medium (MM) supplemented with 2 g liter^{-1} of glucose on a rotary shaker (200 rpm) at 30°C (32). Aromatic compounds were added at a final concentration of 2 mM as carbon and energy sources in MM and supplemented with 0.05 g liter^{-1} of yeast extract to meet the requirement of vitamins for the strains. According to a previous report (1), the overnight cultures of *C. glutamicum* strains in MM with 2 mM 3-HBA are usually controlled within 10 h of growth for enzyme assays and gene transcription measurements. Brain heart infusion broth medium (BHIBM) was used for generation of mutants and maintenance of *C. glutamicum* (33). When required for selection, antibiotics were added at the following concentrations: kanamycin (Kan), 50 μg ml^{-1} for *E. coli* and 25 μg ml^{-1} for *C. glutamicum*; chloramphenicol (Cam), 30 μg ml^{-1} for *E. coli* and 10 μg ml^{-1} for *C. glutamicum*; ampicillin, 100 μg ml^{-1} for *E. coli*; and nalidixic acid (Nx), 50 μg ml^{-1} for *C. glutamicum*.

Construction of plasmids and strains. DNA manipulation was carried out as described previously (34). *E. coli* and *C. glutamicum* were transformed by electroporation according to a method described previously (33).

The *glxR* gene was amplified using primers glxre01 and glxre02 and was digested with NdeI and EcoRI before being inserted into pET28a(+) to produce pZWHJ024. N-terminal His-tagged GlxR (His_6-GlxR) was expressed in *E. coli* Rosetta(DE3)pLysS carrying pZWHJ024. The *glxR* gene amplified using primers glxre08 and glxre09 was cloned into SalI and EcoRI sites of plasmid pXMJ19 to generate plasmid pZWHJ033. The *glxR* gene was overexpressed in *C. glutamicum* carrying pZWHJ033.

The GlxR binding sites DFMx, R-KHx01, and R-KHx02 were mutated

TABLE 2 Primers used in this study

Use and published name	Plasmid or probe(s)	Sequence (5′–3′)
For construction of the GlxR expression plasmid		
glxre01	pZWHJ024	GGAATTCCATATGGAAGGTGTACAGGAGATCCTG
glxre02		CCGGAATTCTTATCGAGCGCGACGTGCC
glxre08	pZWHJ033	ACGCGTCGACAAAGGAGGACAACCAAAGCAGTGGAAGGTGTACA GGAGATCCTG
glxre09		CCGGAATTCTTATCGAGCGCGACGTGCC
For mutated GlxR binding sites		
pDFMxmut01	DFMax	GTGACGTGGAACATATTGGGCTTTGAAAGGTGACTC
pDFMxmut02		TATGTTCCACGTCACAAAGTGTTCAAGGGCTAGACGTATGC
pR-KHxmut01	R-KHax01	CACAGCCAAGTTGTGATCAAAGGGATTCCGCTATCTG
pR-KHxmut02		CACAACTTGGCTGTGCTCGATTATGTGTTTATGAC
pR-KHxmut03	R-KHax02	CCGAGACACGACTCTGTGTGAAGTCCTCCCGATTCTGGTGCG
pR-KHxmut04		AGAGTCGTGTCTCGGAGCATCGAATAGCTCCCAAAC
For EMSA of His_6-GlxR		
pgenDFM04	DFMan and DFMaxm	CCCAAGCTTGCGCCCATTGTTGGAGTCC
pgenDFM09		CGCGGATCCATGGGGGGAATTTTCAGAGCTG
pgenR04	R-KHan	CCCAAGCTTGGCGACGTTATCCATAATC
pgenKH07		CCCAAGCTTGCCAATGAGCACCGCAGCCAAG
pgenR02	R-KHax01 and R-KHax01 m	CCCAAGCTTGCACCGGAAATGGTCAAAC
pgenR03		CGCGGATCCTCTGGTGCGTGTGATGTC
pgenKH11	R-KHax02 and R-KHax02 m	CGCGGATCCTATGACATCACACGCACCAG
pgenKH09		CCCAAGCTTAAGTTCCACTCCTTAGCCAG

using the In-Fusion Advantage PCR cloning method with the templates of pZWHJ006, pZWHJ007, and pZWHJ008, respectively, as described previously (25). To mutate site DFMx, 5-bp substitutions (GTGN^{8}TTC to CACN^{8}GTG) were generated using the mutagenic primers pDFMxmut01 and pDFMxmut02 to generate pZWHJ025. In the same manner, constructs pZWHJ026 and pZWHJ029 were made by replacing **TGT**AACTT GGCT**CAC**C with **CAC**AACTTGGCT**GTG**C at the R-KHx01 sites on pZWHJ007 and pZWHJ008, respectively (substitutions are in boldface). Constructs pZWHJ027 and pZWHJ030 were made by replacing T**GTG**A CAGAGTC**AAC**TCTCGG with T**CAC**ACAGAGTC**GTG**TCTCGG at the R-KHx02 site on pZWHJ007 and pZWHJ008, respectively. Constructs pZWHJ028 and pZWHJ031, each containing both mutated sites R-KHx01 and R-KHx02, were respectively obtained by replacing T**GTG** ACAGAGTC**AAC**TCTCGG with T**CAC**ACAGAGTC**GTG**TCTCGG at the R-KHx02 site on pZWHJ026 and pZWHJ029. The promoters with or without mutation are represented in Fig. 1.

GlxR overexpression and purification. N-terminal His-tagged GlxR (His_6-GlxR) was expressed in *E. coli* Rosetta(DE3)pLysS carrying pZWHJ024. The cells were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.4 in LB supplemented with 50 μg ml^{-1} kanamycin. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to a final concentration of 0.1 mM, and the cultures were incubated at 30°C for another 5 h. His_6-tagged GlxR was purified using Ni-nitrilotriacetic acid (Ni-NTA) agarose chromatography (Novagen) before being stored in glycerol at 4°C, and its purity was monitored by SDS-PAGE.

EMSAs. DNA probes containing both wild-type GlxR binding sites and their mutants (illustrated in Fig. 1) were prepared as BamHI-HindIII fragments with 5′ overhanging ends by PCR amplification using primers described in reference 25 or in Table 2. The probes were treated with calf intestine alkaline phosphatase (Promega, Beijing, China) and then labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Promega) to fill the 5′ recessed ends. The specific competitors were the nonradiolabeled probes. The nonspecific competitor (155 bp) from the *genD* gene was amplified using primers ptgenIKL01 and pgenDFM01 (25) from the *C. glutamicum* genome. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (35). GlxR-DNA complexes were generated in 20-μl reaction mixtures, which were incubated for 30 min at 25°C. Each reaction mixture contained 2 μl 10× binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol [DTT]; pH 7.5), 2 ng (1.2 to 1.6 nM) of each probe, and various amounts of purified His_6-GlxR. The EMSAs were run at 4°C on 5.0% native polyacrylamide gels for 1 h. Gels were dried and exposed in a cassette (Yuehua, Shantou, China) using a phosphor storage screen (PerkinElmer, Boston, MA, USA). Images of radioactive filters were obtained and quantified with a Cyclone Plus storage phosphor system and OptiQuant image analysis software (PerkinElmer).

DNase I footprinting. Footprinting assays were performed as described previously (36), and the primers were the ones used in a DNase I footprinting study with GenR (25). The probe was prepared by labeling the 5′ end of the primers with [γ-^{32}P]ATP and T4 polynucleotide kinase before PCR. The footprinting reaction mixture contained 200 ng labeled DNA probe and different concentrations of His_6-GlxR in 100 μl EMSA reaction buffer. After incubation of the mixture at 25°C for 30 min, 11 μl DNase reaction buffer and different concentrations of RNase-free DNase (Promega) were added to the binding mixture and incubated at 25°C for 1 min. The reaction was stopped by the addition of 10 μl stop solution [200 mM EGTA (Sigma), pH 8.0] and100 μl nuclease-free water. An equal volume of a 1:1 mixture of Tris-saturated phenol-chloroform was then added to the above-described solution. DNA fragments in the aqueous phase were precipitated by adding DNAmate (TaKaRa, Dalian, China) and directly suspended in 10 μl formamide loading dye (80% deionized formamide, 10 mM NaOH, 1.25 mM EDTA, 0.1% xylene cyanol FF, and 0.1% bromophenol blue). Samples were then denatured at 95°C for 5 min and run on 6% polyacrylamide-urea sequencing gels next to the corresponding sequencing ladder of the AccuPower DNA sequencing kit (Bioneer, Seoul, South Korea). After electrophoresis, the gels were dried and exposed to Kodak X-ray film.

Enzyme assays. β-Galactosidase activity was determined in Miller units (37, 38). *C. glutamicum* cells were grown to an OD_{600} of 1.2 and then harvested and washed once in 1 ml Z buffer (0.06 M $Na_2HPO_4{\cdot}12H_2O$, 0.04 M $NaH_2PO_4{\cdot}2H_2O$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercap-

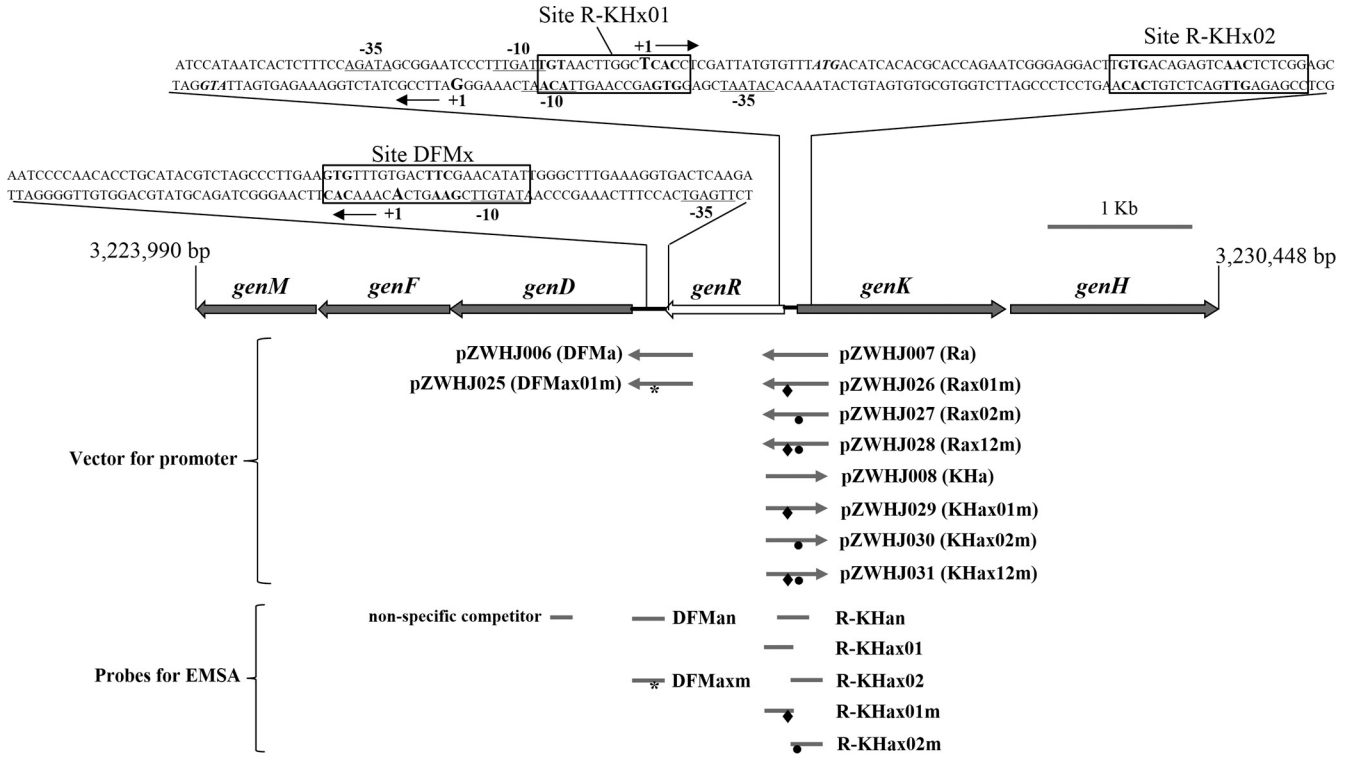


FIG 1 Organization of the *gen* cluster, physical locations of promoters for activity detection, and probes for EMSAs. The region of the DNA fragment in the genome of *Corynebacterium glutamicum* ATCC 13032 is indicated (54). The promoter or probes below the *gen* cluster contained the following mutated GlxR binding sites: mutated site DFMx (from GTGN^{8}TTC to CACN^{8}GTG), indicated with an asterisk; mutated site R-KHx01 (from TGTN^{9}CAC to CACN^{9}GTG), indicated with a rhombus (◆); mutated site R-KHx02 (from GTGN^{8}AAC to CACN^{8}GTG), indicated with a filled circle (●).

toethanol) before being resuspended with the same buffer and treated with 2% toluene for 10 min at 37°C (38). The permeabilized cells were then incubated with *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma-Aldrich, St. Louis, MO) at 30°C, and β-galactosidase activity was determined in Miller units as previously described (37). The 3-HBA 6-hydroxylase activity (39) and maleylpyruvate isomerase activity (40) were determined as described previously. Protein concentration was determined according to the Bradford method (41).

RNA preparation and transcription analysis. Total RNA from *C. glutamicum* was isolated using the hot-phenol method (42). For transcription analysis, total RNA was digested with 1 U μg^{-1} recombinant DNase I (TaKaRa) for 1 h at 37°C, and 1 μg RNA was reversely transcribed with PrimeScript reverse transcriptase (TaKaRa). The resulting cDNA was amplified using quantitative real-time PCR (RT-qPCR), using the primers from a previous study (25). RT-qPCR was performed in a CFX Connect real-time PCR detection system (Bio-Rad) in a 25-μl reaction volume using iQ SYBR green Supermix (Bio-Rad). All samples were run in triplicate in three independent experiments. Relative expression levels were estimated using the $2^{-\Delta\Delta C_T}$ method (where C_T is the threshold cycle), and the 16S rRNA gene served as a reference for normalization (43).

Statistical analysis. Statistical analysis was performed with SPSS version 20.0.0 software. Paired-samples tests were used to calculate probability values (*P*) for the transcription of *genDFM*, *genR*, and *genKH* and promoter activity containing DFMa or DFMax01m. And one-way analysis of variance (ANOVA) was used to calculate probability values (*P*) for β-galactosidase activity analyses of Ra, KHa promoter containing GlxR binding sites, or their corresponding mutants. *P* values of <0.05 and <0.01 were considered to be statistically significant and greatly statistically significant, respectively.

RESULTS AND DISCUSSION

GlxR binds to the promoter regions of the *gen* operons. Although *in silico* detection and ChIP-chip analyses have indicated that GlxR binds to the promoter regions of 281 operons comprising 439 genes in this strain, including those of the *gen* cluster for 3-HBA and GEN catabolism (30), studies on the biochemical and physiological roles of GlxR are very limited.

To investigate whether GlxR directly regulates the transcription of the *gen* cluster for 3-HBA and GEN catabolism, recombinant GlxR was purified for analysis of its ability to bind to the DNA fragments encompassing the promoter regions of *genDFM*, *genR*, and *genKH* operons (DFMan and R-KHan in Fig. 1) in EMSA. The purified His_6-GlxR was incubated with fragments of DNA spanning approximately −180 to +120 bp relative to their translational start sites of target genes. The addition of His_6-GlxR to reaction mixtures caused a shift in the mobility of the promoter DNA fragments at protein concentrations ranging from 7.5 to 180 nM (Fig. 2A and C). A saturating concentration of 80 nM His_6-GlxR, at which a retarded and stable protein-DNA complex was formed, was used to further examine its specificity of binding to probes. After addition of excess specific cold probe, the shift was found to be abrogated (Fig. 2B and D), indicating a specific interaction between GlxR and promoter DNA fragments. The nonspecific competitor is also able to decrease GlxR binding ability to promoter regions, although this competition is relatively weak. Unlike the specific regulator GenR, the global regulator GlxR, with more than 200 DNA binding sites in *C. glutamicum* (27, 30), may

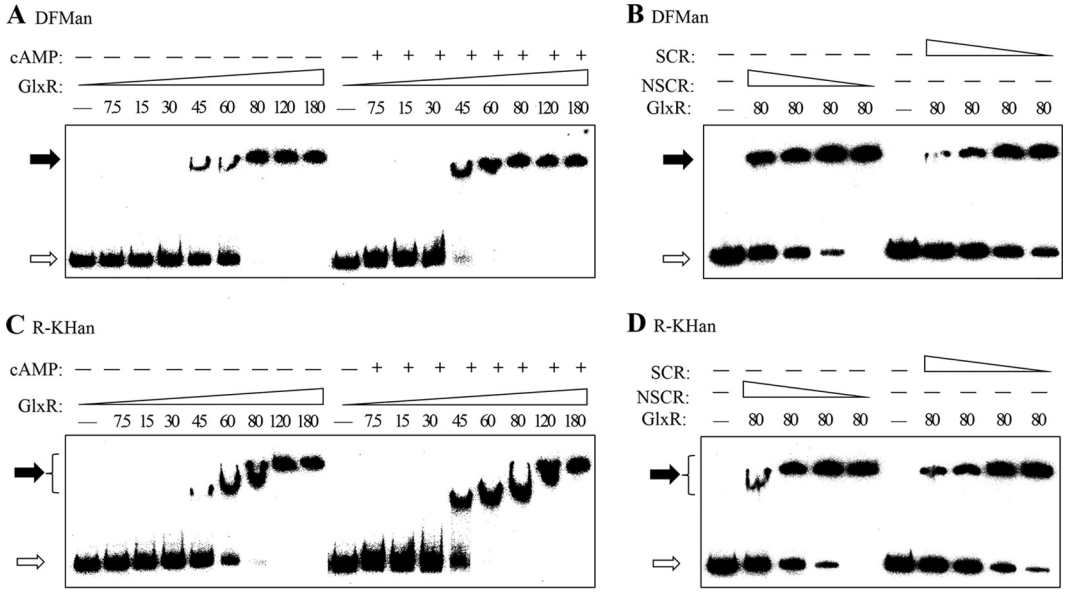


FIG 2 Electrophoretic mobility shift assays for determination of GlxR binding sites upstream of *genDFM*, *genR*, and *genKH* operons. (A and C) Effects of cAMP on the binding affinity of GlxR for the upstream regions of *genDFM*, *genR*, and *genKH* operons. The probes for *genDFM* (DFMan in panel A), *genR*, and *genKH* operons (R-KHan in panel C) (25) were incubated with increasing amounts of His_6-GlxR, while the amounts of cAMP were constant. The concentrations (nM) of purified His_6-GlxR and the presence of 500 nM cAMP (+) or absence of cAMP (−) are indicated above the lanes. Glucose served as a control. (B and D) Competition assays using unlabeled specific competitor (SCR) and nonspecific competitor (NSCR) DNA. Labeled probe and unlabeled competitor of 25-fold, 50-fold, and 100-fold more in amount were incubated with His_6-GlxR for 30 min at 25°C. Each lane contained 0.5 ng (0.3 to 0.4 nM) of ^{32}P-labeled DFMan and R-KHan probes. The free probes are indicated by open arrows and the retarded DNA fragments by solid arrows.

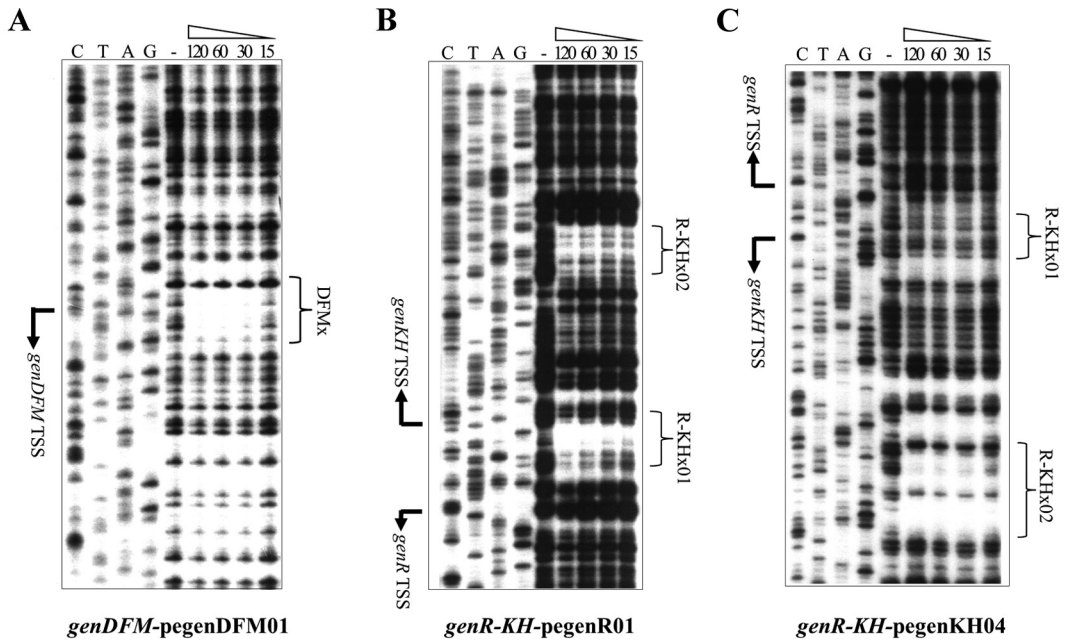
have a lower specificity in the binding to the promoter region of *gen* operons.

GlxR has been found to be involved in the regulation of many genes by binding to partial region of promoters in a cAMP-dependent manner *in vitro* (27, 29). However, recent findings indicated that its regulation is not entirely dependent on the cAMP level, and the cAMP level required for GlxR binding to DNA varies depending on the target site (30). In the current study, although GlxR was found to bind to the corresponding regions regardless of the presence or absence of cAMP (Fig. 2A and C), cAMP could enhance the affinity of GlxR for these promoters to some degrees.

It has been demonstrated that creating a *glxR* deletion mutant is problematic in several cases, or generated mutants showed severe growth defects (28, 30, 44). Attempts were also made in the current study, but no such mutant was obtained. The involvement of GlxR in regulating physiological function, including aromatic degradation, in *C. glutamicum* was generally performed by *in vitro* binding and promoter-reporter assays (27, 29, 30). Following *in vitro* binding assays in this study, specific GlxR binding sites with promoters of *gen* operons were subsequently determined.

Determination of the GlxR binding sites. The transcriptional start sites (TSSs) of *gen* operons have been determined in previous work (25). To further determine the regulatory role of GlxR in the expression of *gen* operons, the DNA binding sites of each operon were identified by DNase I footprinting analyses. For *genDFM*, His_6-GlxR was found to protect a region (designated DFMx) from −13 to +8 bp relative to TSS (Fig. 3A). The footprint on the R-KH promoter region covered two regions, one (designated R-KHx01) from −24 to −9 bp relative to the *genR* TSS and from −11 to +5 bp relative to the *genKH* TSS, and the other (designated R-KHx02) from −71 to −91 bp relative to the *genR* TSS and from +52 to +72 bp relative to the *genKH* TSS, as shown in Fig. 3B with primer pegenR01 and in Fig. 3C with primer pegenKH04, respectively. Sites DFMx and R-KHx01 were almost the same as those previously reported (27, 29, 30). A novel site, R-KH02, detected in the footprinting analysis of this study was not identified by *in silico* detection (27, 29) or ChIP-chip analysis (30). In addition, the probe R-KHan contained two GlxR binding sites (Fig. 1). The regulators binding two sites through higher-order complex formation have been reported (45, 46), although most of the regulator proteins bind two sites without change in the configuration as indicated in the review articles (47, 48). There is a possibility that the GlxR-DNA complex in this study is formed in a variety of formations. Unfortunately, we have not captured the significant changes by *in vitro* experiments, including EMSA (Fig. 2C) and DNase I footprinting (Fig. 3B and C).

In order to find a consensus sequence from the above-described three DNA regions of *gen* promoters interacting with GlxR, bioinformatics analysis was performed using the motif-based sequence analysis tool MEME (multiple Em for motif elicitation) (49). A consensus sequence in the form of an imperfect palindrome ([T]GTGN^{8}T[A]T[A]C) (where bases in square brackets are occasionally present) was therefore revealed as shown in Fig. 4A. This imperfect palindrome was obtained from the following: (i) the imperfect palindrome in site DFMx is GTGN^{8}TTC; (ii) the imperfect palindrome of the complementary sequence for site R-KHx01 (GGTGAGCCAAGTTACA) is GTGN^{8}TAC, but the perfect palindrome GTGN^{9}ACA of this site matches better with the reported palindrome; (iii) site R-KHx02 is TGTGN^{8}AAC. The



D *genDFM*

pegenDFM01 →

TGGTGTCCAGCTCTGCCTGCTCTTCTGGAGTTGCGGGTGGGACTTCTTGCTTGACGTGTTCAGTTGCGTAATCGTTTTTA
ACCACAGGTCGAGACGGACGAGAAGACCTCAACGCCCACCCTGAAGAACGAACTGCACAAGTCAACGCATTAGCAAAAAT

CCTGGGGCGCCCATTGTTGGAGTCCTCCTTTACGGATGTTGTAGATGCGTATGAAGGGTGTGATTGCCCTTGAGATAAGC
GGACCCCGCGG***GTA***ACAACCTCAGGAGGAAATGCCTACAACATCTACGCATACTTCCCACACTAACGGGAACTCTATTCG

genDFM ←
+108

Site DFMx

AATCCCCAACACCTGCATACGTCTAGCCCTTGAAGTGTTTGTGACTTCGAACATATTGGGCTTTGAAAGGTGACTCAAGA
TTAGGGGTTGTGGACGTATGCAGATCGGGAACTTCACAAACACTGAAGCTTGTATAACCCGAAACTTTCCACTGAGTTCT

← **+1** **-10** **-35**

TAATTCCGCTATGTGGAATCTAAGTATTCCCCTCAGGAAACGGGGGTGTGACAGCTCTGAAAATTCCCCCCATTCACCCT
ATTAAGGCGATACACCTTAGATTCATAAGGGGAGTCCTTTGCCCCCACACTGTCGAGACTTTTAAGGGGGGTA***AGT***GGGA

Site DFMn01 Site DFMn02

E *genR-KH*

pegenR01 →

CACGGAGCAGCAAAATGAGCTGCAATGCAATGTCGACAGAGCTTAGAAATTCTTTCGGGGGTAACCCCTGGGTGGGGGCG
GTGCCTCGTCGTTTTACTCGACGTTACGTTACAGCTGTCTCGAATCTTTAAGAAAGCCCCCATTGGGGACCCACCCCCGC

genKH →

Site R-KHn01 **-35** **-10** Site R-KHx01 **+1** → Site R-KHxu

ACGTTATCCATAATCACTCTTTCCAGATAGCGGAATCCCTTTGATTGTAACTTGGCTCACCTCGATTATGTGTTT***ATG***AC
TGCAATAG***GTA***TTAGTGAGAAAGGTCTATCGCCTTAGGGAAACTAACATTGAACCGAGTGGAGCTAATACACAAATACTG

genR ← ←**+1** **-10** **-35**

Site R-KHx02

ATCACACGCACCAGAATCGGGAGGACTTGTGACAGAGTCAACTCTCGGAGCATCGAATAGCTCCCAAACAATTGAAAATA
TAGTGTGCGTGGTCTTAGCCCTCCTGAACACTGTCTCAGTTGAGAGCCTCGTAGCTTATCGAGGGTTTGTTAACTTTTAT

Site R-KHn02

AAGGCCTCACCATCTTGGGCATCAGCGGCCGACGCTTGGCTGCGGTGCTCATTGGCTGGTTTTTTGTCATTTTCGACGGC
TTCCGGAGTGGTAGAACCCGTAGTCGCCGGCTGCGAACCGACGCCACGAGTAACCGACCAAAAAACAGTAAAAGCTGCCG

← pegenKH04

FIG 3 Identification of the GlxR binding sites upstream of *genDFM* (A), *genR* (B), and *genKH* (C) operons by DNase I footprinting analysis. The reaction mixtures contained approximately 200 ng of end-labeled PCR products. These were amplified with prnagRD02 primer and ^{32}P-labeled pegenDFM01 primer (A), pgenR01 and ^{32}P-labeled pegenR01 (B), or pgenKH02 and ^{32}P-labeled pegenKH04 (C) (25). Before DNase I treatment, labeled DNA was preincubated with His_6-GlxR for 30 min at 25°C. Standards were generated by sequencing with ^{32}P-labeled primers pegenDFM01(A), pegenR01 (B), and pegenKH04 (C). The concentrations (μM) of purified His_6-GlxR and the GlxR-protected sequences are indicated. The nucleotide sequence around the TSS (+1) (25) is shown by solid arrows, and the GlxR binding sites are represented by brackets. Arrows indicate the direction of transcription. (D and E) Organization of the upstream region of the *genDFM* operon (D) and the *genR-genKH* promoter region (E) is shown in detail. The first codon of each gene is given in italics and boldface. The sequences in the dotted boxes are site R-KHxu as proposed previously (30), and the regulator GlxR binding sites are showed in the box. The TSSs are shown by +1 (boldface) and arrows and the putative −10 and −35 promoter sequences are underlined. The regulator GenR binding sites are highlighted in gray.

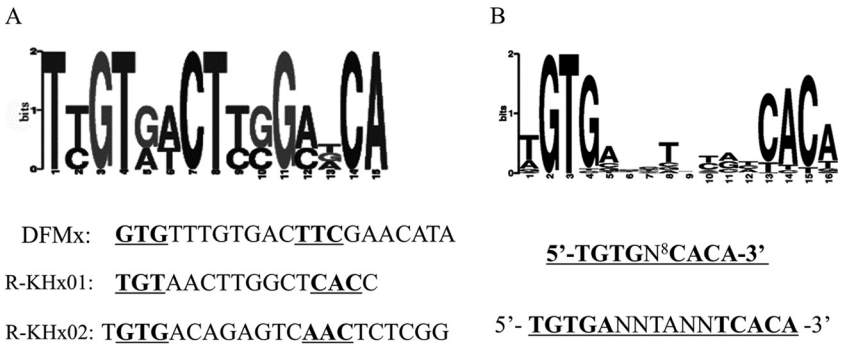


FIG 4 Sequence logos of motifs identified in GlxR binding sites by MEME (multiple Em for motif elicitation). (A) Motifs identified in GlxR binding sites for 3-HBA/GEN catabolic genes by MEME. (B) Data modified from references 27, 29, 30, and 44.

consensus motif obtained is almost the same as the previously reported 5′-TGTGAN^{6}TCACA-3′ (27, 29, 44, 50–52) and 5′-TGTGN^{8}CACA-3′ (30) for GlxR in *C. glutamicum* (Fig. 4B). However, site R-KHxu, identified by ChIP-chip, was not detected in footprinting analysis in this study (Fig. 3). It is worth noting that our conclusion of this consensus motif of GlxR binding sites was based on footprinting analysis rather than *in silico* detection or ChIP-chip analysis used in previous determinations.

Role of the conserved sequence in GlxR binding and transcriptional regulation of *genDFM*, *genR*, and *genKH* operons. To further confirm the GlxR binding sites identified in the upstream regions of *gen* operons, the effects of mutations within the three GlxR binding sites were examined *in vitro*. Toyoda et al. have examined effects of mutations within the GlxR binding sites *in vivo* and *in vitro* by exchanging the nucleotides corresponding to the positions of GTG and CAC in the consensus motif to CAC and GTG, respectively (5′-TGTG-N^{8}-CACA-3′ to 5′-TCAC-N^{8}-GTGA-3′) (30). According to this reported method, the underlined and bolded sequences in Fig. 4A have been used for further study. The nucleotides in the consensus motif of DFMx (**GTG**N^{8}**TTC**), R-KHx01 (**TGT**N^{9}**CAC**), and R-KHx02 (**GTG**N^{8}**AAC**) (Fig. 4A) were changed to form DFMxm (**CAC**N^{8}**GTG**), R-KHx01m (**CAC**N^{9}**GTG**), and R-KHx02m (**CAC**N^{8}**GTG**), respectively. Probes containing both wild-type and mutated sites (Fig. 1) were used with purified His$_6$-GlxR in EMSAs. As shown in Fig. 5, the introduced mutations abolished the GlxR binding, indicating that the consensus sequences [T]GTGN^{8}T[A]T[A]C and GTGN^{9}ACA were an essential determinant of GlxR binding activity.

In addition to the evidence that three GlxR binding sites in *gen* operons were bound by GlxR *in vitro* (Fig. 3), their roles *in vivo* were also investigated subsequently. The same mutation on consensus sequence for these binding sites used in EMSA was also introduced in the *genDFM* promoter of pZWHJ006, the *genR* promoter of pZWHJ007, and the *genKH* promoter of pZWHJ008 (Fig. 1). The resulting seven constructs were transformed into strain RES167 for β-galactosidase activity assays. As shown in Fig. 6B, the activity in the presence of *genDFM* promoter containing mutated site DFMax had an approximately 2-fold increase under the GEN-induced condition and a 1.5-fold increase under the 3-HBA condition in comparison with its wild-type promoter. For the *genR* promoter containing the mutated site R-KHx01m, its activity was similar to that in its wild-type counterpart. However, a 2-fold β-galactosidase activity was observed with the *genR* promoter containing mutated site R-KHx02m or sites R-KHx12m with a double mutation. On the other hand, the transcription of *genKH* was increased 1.5-fold in the presence of promoter containing mutant site R-KHx01m or double mutated site R-KHx12m, but it was not af-

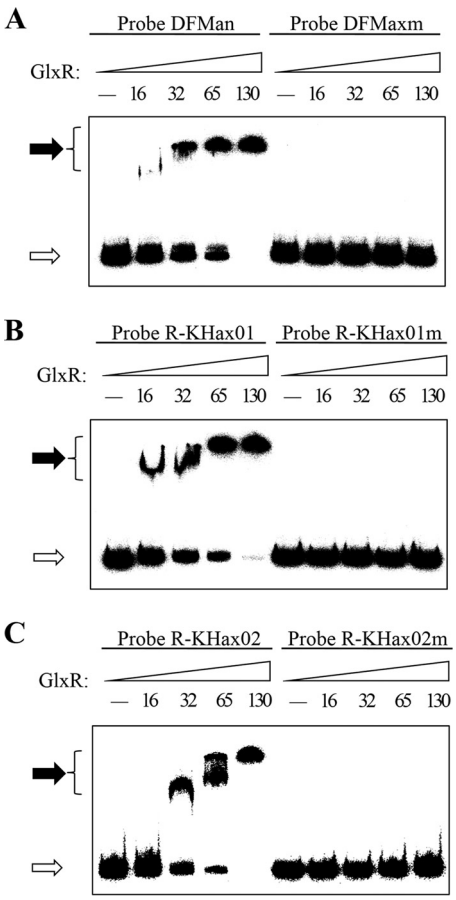


FIG 5 Mutational analyses of the four GlxR binding sites. EMSAs using the wild-type and mutated DNA fragments. Probes DFMan, R-KHax01, and R-KHax02 contained the intact GlxR binding sites DFMx, R-KHx01, and R-KHx02, respectively. Probes DFMaxm, R-KHax01m, and R-KHax02m contained the mutated GlxR binding sites described in Materials and Methods. The amounts (nM) of His$_6$-GlxR used in lanes 1 to 5 are indicated. The free probes are indicated by open arrows, and the retarded DNA fragments are indicated by solid arrows.

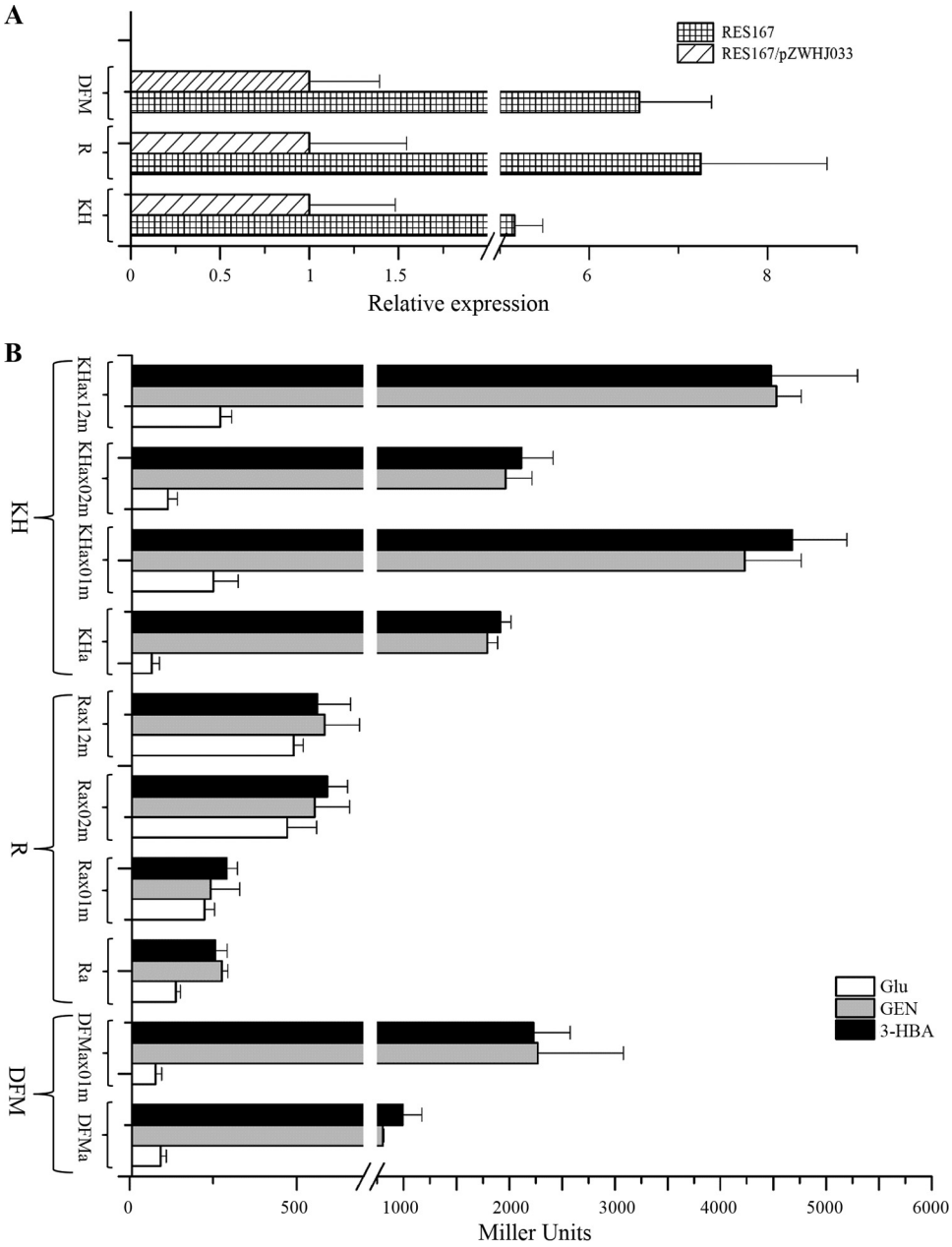


FIG 6 GlxR attenuates upregulation of the transcription of *genDFM*, *genR*, and *genKH* operons. (A) qRT-PCR analyses examining the transcription of *genDFM*, *genR*, and *genKH* in strains RES167 and RES167/pZWHJ033. RNA samples were isolated from strains RES167 and RES167/pZWHJ033 grown on MM with 2 mM 3-HBA overnight. The levels of gene expression in each sample were calculated as the fold expression ratio after normalization to the 16S rRNA gene transcript. The values are averages of two independent RT-qPCR experiments. Error bars indicate standard deviations. These data are derived from at least three independent measurements. There was a significant difference in the transcription of *genDFM*, *genR*, and *genKH* between strains RES167 and RES167/pZWHJ033 ($P < 0.001$, paired-samples test). (B) β-Galactosidase activity driven by *genDFM*, *genR*, and *genKH* promoters with their corresponding GlxR binding sites in strain RES167. DFMa, Ra, and KHa containing GlxR binding sites are the GenR binding sites reported as previously (25). DFMax01m, Rax01m, Rax02m, Rax12m, KHax01m, KHax02m, and KHax12m are the constructs containing the mutated GlxR binding sites described in Materials and Methods and shown in Fig. 1. The β-galactosidase activity analyses were performed as given in the text. The data are derived from at least three independent measurements, and error bars indicate standard deviations. There was a significant difference in promoter activity between GlxR binding sites and its mutated sites from strains RES167 grown on MM with GEN or 3-HBA ($P < 0.001$, one-way ANOVA).

fected by the mutated site R-KHx02m. These results suggested that the transcription of *genDFM*, *genR*, and *genKH* was negatively regulated by the GlxR binding to sites DFMx, R-KHx02, and R-KHx01, respectively. And the consensus sequences [T]GTGN^{8}T[A]T[A]C and GTGN^{9}ACA were essential for the negative regulation. It is worth noting that GlxR binding site R-KHx01 overlaps each of the −10 regions of *genR* and *genKH* promoters. In the mutation procedure, the nucleotides in the consensus motif of R-KHx01 (TGTN^{9}CAC) were changed to form R-KHx01m (CACN^{9}GTG). The two −10 regions have been changed in the following manner: −10 region of

genR, from TACAAT to TTGTAT; −10 region of *genKH*, from TT GATT to TTGATC. However, higher activities were detected from promoters of *genR* and *genKH* operons containing the mutated GlxR binding site. Therefore, the mutations in the GlxR binding sites have no effect on the −10 region of *genR* and *genKH* operons.

Previous work has identified three GlxR binding sites in the *gen* cluster by bioinformatics analysis or ChIP-chip analyses (27, 30), but one of them was not among the three sites identified in this study. In footprinting analyses for two directions of the *genR-KH* region (Fig. 3B and C), the sequencing results demonstrated that GlxR bound to three sites at the promoter regions of *gen* operons (Fig. 3). Based on the mutation and β-galactosidase assays, the GlxR binding site DFMx identified is similar to the GlxR binding site obtained by *in silico* detection (27) and ChIP-chip analysis (30), which is necessary for negative regulation of the transcription of *genDFM*. A single binding site, R-KHx01, required for repression of the transcription of *genKH*, matches the GlxR binding site of *in silico* detection (27). However, GlxR binding site R-KHx02, involved in negative regulation of the activity of the *genR* promoter for the expression of GenR regulator from this study, was not detected by *in silico* and ChIP-chip (27, 30). In contrast, site R-KHxu, identified by ChIP-chip, was not detected in footprinting analysis of this study (Fig. 3). Our study has demonstrated that the prediction of two GlxR binding sites for the *gen* cluster by *in silico* analysis and ChIP-chip was valuable for experimental studies of its regulation, although one of three predicted sites did not match the experimental data.

GlxR represses the expression of *gen* operons involved in the 3-HBA and GEN pathway. It has been predicted that global regulator GlxR may be involved in the 3-hydroxybenzoate and gentisate pathways by activating the expression of *genDFM* genes in *C. glutamicum*, based on bioinformatics and ChIP-chip analyses (29, 30). In order to analyze the involvement of GlxR in the expression of genes in the *gen* cluster, the biochemical activity of enzymes encoded by *genH* in the *genKH* operon and *genM* in the *genDFM* operon was assayed in the *glxR*-overexpressed recombinant strain RES167/pZWHJ033, with 3-HBA induction. *genH*-encoded 3-HBA 6-hydroxylase activity of 0.31 ± 0.01 U/mg protein was present in wild-type strain RES167, but a much lower activity of 0.05 ± 0.01 U/mg protein was found in the *glxR*-overexpressed recombinant strain RES167/pZWHJ033. For *genM*-encoded maleylpyruvate isomerase, strain RES167 exhibited an activity of 1.88 ± 0.04 U/mg protein and the *glxR*-overexpressed recombinant strain showed a lower activity (0.80 ± 0.02 U/mg protein). To further assess the effects of GlxR on the expression of the 3-HBA and GEN catabolic operons, transcription of *gen* operons was also analyzed in the *glxR*-overexpressed recombinant strain RES167/pZWHJ033 by RT-qPCR. As shown in Fig. 6A, transcriptional levels of *genDFM*, *genR*, and *genKH* operons were one-fifth, one-seventh, and one-sixth, respectively, in recombinant RES167/pZWHJ033, in comparison with that in the wild-type strain under the 3-HBA-induced conditions. No enzyme activity for 3-hydroxybenzoate/gentisate catabolism and promoter activity (β-galactosidase assays) of *gen* operons was found to be present in the absence of 3-hydroxybenzoate and gentisate in strain RES167 (1, 25). These results indicated that the overexpression of *glxR* repressed all three operons to significant degrees. Recently, a unique recombineering method for constructing a mutant strain has been established in *Corynebacterium glutamicum* to study its biological function *in vivo* (21). If the mutation of GlxR binding sites in the *glxR* overexpression strain had been performed using this method, the relationship between the effects of the binding site mutations on gene expression and the binding of GlxR would have been made much more direct.

In *C. glutamicum*, a dual-function protein, GenR of the IclR family, has previously been identified to positively regulate the expression of *genDFM* and *genKH* operons for degradation of 3-HBA and GEN and repress the expression of its own encoding gene (*genR*) (25). We have now demonstrated that a CRP/FNR-type GlxR is an additional regulator for repressing the expression of three *gen* operons for 3-HBA and GEN catabolism. It is likely that GlxR contributes to the synchronization of expression of *gen* operons for 3-HBA and GEN catabolism in collaboration with the specific regulator GenR. Despite the fact that GlxR has been predicted to be involved in the activation or repression of considerable genes of this strain (30, 53), no thorough study on this global regulator has been conducted for its regulation of a catabolic pathway until this one.

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