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Involvement of Regulatory Interactions among Global Regulators GlxR, SugR, and RamA in Expression of *ramA* in *Corynebacterium glutamicum*

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The central carbon metabolism genes in *Corynebacterium glutamicum* are under the control of a transcriptional regulatory network composed of several global regulators. It is known that the promoter region of *ramA*, encoding one of these regulators, interacts with its gene product, RamA, as well as with the two other regulators, GlxR and SugR, *in vitro* and/or *in vivo*. Although RamA has been confirmed to repress its own expression, the roles of GlxR and SugR in *ramA* expression have remained unclear. In this study, we examined the effects of GlxR binding site inactivation on expression of the *ramA* promoter-*lacZ* fusion in the genetic background of single and double deletion mutants of *sugR* and *ramA*. In the wild-type background, the *ramA* promoter activity was reduced to undetectable levels by the introduction of mutations into the GlxR binding site but increased by *sugR* deletion, indicating that GlxR and SugR function as the transcriptional activator and repressor, respectively. The marked repression of *ramA* promoter activity by the GlxR binding site mutations was largely compensated for by deletions of *sugR* and/or *ramA*. Furthermore, *ramA* promoter activity in the *ramA-sugR* double mutant was comparable to that in the *ramA* mutant but was significantly higher than that in the *sugR* mutant. Taken together, it is likely that the level of *ramA* expression is dynamically balanced by GlxR-dependent activation and repression by RamA along with SugR in response to perturbation of extracellular and/or intracellular conditions. These findings add multiple regulatory loops to the transcriptional regulatory network model in *C. glutamicum*.

Corynebacterium glutamicum is a high-G+C-content Gram-positive soil bacterium which is used in biotechnological production of amino acids, organic acids, and alcohols (1–6). Since the complete genome sequence of *C. glutamicum* became available (7–9), a number of transcriptional regulators controlling genes involved in central carbon metabolism have been characterized (10, 11). Genome-wide studies reveal that a global transcription regulatory system for carbon source-dependent regulation in *C. glutamicum* is quite different from the well-established systems in *Escherichia coli* and *Bacillus subtilis*. A major control system for utilization of carbon sources, namely, carbon catabolite repression, is mediated by the cyclic AMP (cAMP) receptor protein (CRP) via intracellular cAMP levels in *E. coli*, while in *B. subtilis*, it is mediated by carbon control protein A (CcpA) via phosphorylation states of HPr, a component of a phosphotransferase system. In contrast to the situation in *E. coli*, where the phosphotransferase system for glucose uptake modifies adenylate cyclase activity to decrease the intracellular cAMP levels in the presence of glucose (12, 13), the *C. glutamicum* intracellular cAMP levels are increased in the presence of glucose by an unknown mechanism(s) (14–16). Besides, no HPr kinase/phosphatase system is found in the *C. glutamicum* genome. These differences in the molecular characteristics are likely reflected in the capacity of *C. glutamicum* to cometabolize glucose and a variety of carbon sources, including sugars, organic acids, and aromatic compounds without catabolite repression (17–24), expect for a few cases (25–27).

Carbon source-dependent regulation of gene expression in *C. glutamicum* has been investigated by comparing the expression profile in glucose-grown cells to that in acetate-grown cells (28–30). These studies reveal that genes for sugar uptake and metabolism, including the phosphotransferase system and glycolysis, are upregulated in the presence of glucose. In contrast, genes for enzymes involved in the tricarboxylic acid cycle, glyoxylate shunt, and gluconeogenesis are upregulated in the presence of acetate. Multiple transcription regulators are involved in the expression of these genes. For example, expression of the *gapA-pgk-tpi* operon, encoding the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and triose phosphate isomerase, respectively, is coordinately regulated by SugR, RamA, and GlxR (15, 31, 32). SugR is a global repressor of genes for sugar uptake and metabolism, including phosphotransferase systems, glycolysis, and fermentative lactate dehydrogenase (32–37). As sugar phosphates, e.g., fructose-1-phosphate and fructose-1,6-bisphosphate, act as negative effectors of SugR, the SugR regulon is derepressed in the presence of sugar. GlxR, a cAMP-responsive regulator, and RamA, a LuxR-type regulator, were first identified as the transcriptional repressor and activator, respectively, of the *aceB* gene, encoding malate synthase of the glyoxylate cycle (14, 38). Both regulators not only activate *gapA* expression (15, 31) but are also involved in expression of a number of genes for various physiological functions, including carbon and nitrogen metabolism, respiration, SOS and stress responses, and cell division (15, 39–41). However, in contrast to the mechanism by which SugR switches gene expression, how GlxR and RamA switch expression

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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} *lacZ*ΔM15]	TaKaRa
JM110	*dam dcm supE44 hsdR17 tih leu rpsL lacy galK galT ara tonA thr tsx* Δ(*lac-proAB*) [F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	46
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3)	47
C. glutamicum		
R (JCM 18229)	Wild-type strain	9
KT27	R with mutations in the GlxR binding site in the *ramA* promoter region on the chromosome	This study
Plasmids		
pCold	Ap^r; cold-inducible expression vector	TaKaRa
pGEM-T Easy	Ap^r; TA cloning vector	Promega
pCRA725	Km^r; suicide vector containing the *B. subtilis sacB* gene	2
pCRA741	Km^r; pCRA725 with a 2.0-kb PCR fragment from SSI7 and a 3.1-kb PCR fragment containing the *E. coli lacZ* gene	48
pCRC621	Km^r; pCRA725 with a 2.65-kb fragment containing a mutated *ramA* promoter	This study
pCG1	3,069-bp plasmid from *C. glutamicum*	49
pKK223-3	Ap^r; expression vector under the control of the *tac* promoter	Pharmacia
pDW363	Ap^r; source of *lacI*^{q}	NBRP (NIG, Japan)
pCRB12iP	Km^r *lacI*^{q} P*tac*, IPTG-inducible vector based on pCG1	This study
pCRC622	Km^r; pCRB12iP with a 909-bp PCR fragment containing the-*ramA* coding region	This study

of genes involved in glucose and acetate metabolism in response to a carbon source provided is not fully understood. This is because the identity of an effector molecule that controls RamA activity and understanding how intracellular cAMP levels are controlled remain elusive.

Furthermore, carbon source-dependent regulation is complicated by a hierarchical and/or interactive transcriptional control of the transcription regulators. SugR, RamA, and GlxR act as transcription repressor of their own genes, *sugR*, *ramA*, and *glxR*, respectively (42–44). Expression of the *sugR* gene is directly activated by RamA (31). Expression of the *ramA* gene is upregulated by an unknown factor in the presence of acetate (43). Thus, the carbon metabolism genes in *C. glutamicum* are under the control of a complex hierarchical regulatory network consisting of transcription factors responding to various environmental and/or physiological signals.

In this study, to unveil a new hierarchical interaction in the regulatory network, we focus on regulation of the *ramA* gene. It has been reported that four transcription regulators, RamA, SugR, GlxR, and RamB, bind to the *ramA* promoter *in vitro* and/or *in vivo* (15, 35, 43). RamA is subject to negative autoregulation, as described above. RamB is involved in repression of the acetate metabolism genes in the presence of glucose (45), but disruption of its gene, *ramB*, has no effect on expression of *ramA* (43). However, the involvement of SugR and GlxR in *ramA* expression has not been investigated so far. Here, the effects of mutations in the GlxR binding site in combination with deletion of *ramA*, *sugR*, and *ramB* on *ramA* promoter activity are described.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions. The strains and plasmids used in this study are listed in Table 1. The oligonucleotide primers 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 (50), which contains 0.2% yeast extract and 0.7% Casamino Acids 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 was used at 50 μg ml^{-1}. For promoter-reporter assays, *C. glutamicum* strains chromosomally carrying a promoter-*lacZ* fusion were grown in A medium containing 1% glucose or acetate.

EMSA. GlxR was expressed with an N-terminal His tag and purified by affinity chromatography as described previously (15). DNA fragments containing the *ramA* promoter region with a native or mutated GlxR binding site were amplified by PCR using primers PramAFW and PramARV and promoter-*lacZ* fusion plasmids, which were constructed as described below, as the templates and cloned into the pGEM-T Easy vector (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 resulting fragments were purified with a QIAquick PCR purification kit (Qiagen). Electrophoretic mobility shift assay (EMSA) was performed as described previously (51). DNA and DNA-protein complexes were visualized by a Typhoon TRIO variable-mode imager (GE Healthcare Bioscience).

Construction of promoter-*lacZ* fusions. The *ramA* promoter region from positions −474 to +15 and the *cysK* promoter region from positions −595 to +15 with respect to the translational start point were amplified from *C. glutamicum* R chromosomal DNA by PCR using primers PramAFW-PramARV and PcysKFW-PcysKRV, respectively (see Table S1 in the supplemental material). Mutations in the GlxR binding site were introduced by overlapping PCR using primers PramAmutFW and PramAmutRV, as described below. The fragments amplified were phosphorylated and cloned upstream of the *lacZ* gene in pCRA741 (48). The direction and sequence of the inserted fragment were confirmed by DNA sequencing. The plasmids were isolated as nonmethylated DNA from *E. coli* JM110, introduced into *C. glutamicum*, and subsequently integrated into a strain-specific island 7 (SSI7) on the chromosome of *C. glutamicum* R by markerless gene insertion methods, as described previously (2). The integration was confirmed by PCR using primers specific for the promoter region and the SSI7 region.

Construction of a genetically modified strain. To modify the GlxR binding site on the chromosome, the GlxR binding site and both flanking

FIG 1 Binding sites for transcriptional regulators in the *ramA* promoter region. While the intergenic region between *ramA* and *cysK* is indicated with lowercase letters, coding regions of the genes are indicated with uppercase letters. Primers used for amplification of the *ramA* promoter region are indicated with arrows. The transcriptional start points of *ramA* and *cysK* are indicated with +1. The binding sites for GlxR and RamB are boxed and indicated with a dotted line, respectively. The putative binding sites for RamA and SugR are indicated with bold italic letters and underlines, respectively.

regions were amplified by PCR using primer pairs ramA_integ_FW/PramAmutRV and ramA_integ_RVxba/PramAmutFW. The two fragments were used as a template for PCR using primers ramA_integ_FW and ramA_integ_RV. The resulting fragment with mutations in the GlxR binding site was digested with SalI-XbaI and cloned into pCRA725, a suicide vector for markerless gene disruption (2), yielding pCRC621. *C. glutamicum* R (wild type [WT]) was transformed with pCRC621, which was isolated as nonmethylated DNA from *E. coli* JM110 for efficient gene introduction into *C. glutamicum* (52), by electroporation. Screening for the mutants was performed as described previously (2). Introduction of the mutations into the site on the chromosome was confirmed by direct sequencing of a PCR product, which was amplified using primers PramAFW and PramRV and genomic DNA extracted from the strains obtained as the template.

Construction of an IPTG-inducible expression vector and overexpression of *ramA*. To construct a *ramA*-overexpressing strain, we constructed an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector, pCRB12iP. This plasmid carries the LacI repressor gene *lacI*^{q} and the *tac* promoter, which is followed by the *rrnB1* and *rrnB2* terminators. The DNA fragment containing *lacI*^{q} was amplified by PCR using pDW363 as the template, while the DNA fragments containing the *tac* promoter and the terminators were amplified by PCR using pKK223-3 as the template. The coding region of *ramA* was amplified by PCR using primers ramAFWKpn and ramARVKpn and the genomic DNA as the template and cloned into pCRB12iP, yielding pCRC622. Overexpression of *ramA* in strains carrying pCRC622 was induced by supplementation of 0.5 mM IPTG.

β-Galactosidase assay. *C. glutamicum* cells carrying the promoter-*lacZ* fusion were harvested, washed once with Z buffer (53), resuspended in 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 (53).

qRT-PCR. Total RNA was extracted from *C. glutamicum* cells using an RNeasy minikit (Qiagen) as described previously (32). Isolated RNA samples were checked for purity using an Agilent RNA 6000 nanokit on an Agilent 2100 bioanalyzer (Agilent Technologies, CA) and stored at −80°C. Quantitative reverse transcription-PCR (qRT-PCR) was performed using an Applied Biosystems 7500 Fast real-time PCR system (Life Technologies, CA) and Power SYBR green PCR master mix with murine leukemia virus reverse transcriptase and the RNase inhibitor of the GeneAmp RNA PCR kit (Life Technologies) as described previously (32). Specific primers (see Table S1 in the supplemental material) were designed using Primer Express software (version 3.0; Life Technologies). The specificity of the amplicons was checked by DNA dissociation curve analysis. The comparative threshold cycle method (Life Technologies) was used to quantify relative expression.

5′ RACE-PCR. For the identification of the transcriptional start points (TSPs) of *cysK*, 5′ rapid amplification of cDNA ends (RACE)-PCR analyses were carried out. Total RNA was extracted as described above. 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 the poly(A) tail was purified by ethanol precipitation. cDNA was synthesized using a 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 kit) and gene-specific primers (see Table S1 in the supplemental material). The resulting PCR products were cloned into the pGEM-T Easy vector (Promega). At least 10 clones of the 5′ RACE-PCR product were sequenced.

RESULTS

GlxR positively regulates *ramA* expression. Our previous chromatin immunoprecipitation (ChIP) chip analysis identified the GlxR binding site at position −290 with respect to the transcriptional start point (TSP) of the *ramA* gene (15) (Fig. 1). To examine GlxR binding to the site, EMSA using purified His-tagged GlxR was performed as described previously (15). The results of EMSA demonstrated that GlxR actually binds to the 489-bp *ramA* promoter region in the presence of cAMP *in vitro* (Fig. 2). The GlxR binding was abolished by mutations (underlined) in the GlxR binding site (5′-AGTGTTCTATGACACT-3′ → 5′-ACACTTCT ATGAGTGT-3′) (Fig. 2), confirming that the site identified is the sole binding site in this region. The binding was also abolished by removing cAMP from the reaction, demonstrating that the binding is cAMP dependent.

Because a *glxR* mutant shows severe growth defects (31, 54), a

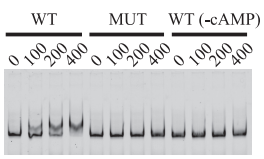

FIG 2 Binding of GlxR to the *ramA* promoter region. DNA fragments covering the *ramA* promoter region with the wild-type (WT) or mutated (MUT) GlxR site were incubated with the purified GlxR at the indicated concentrations (nM) in the presence of 0.5 mM cAMP or in the absence of cAMP (−cAMP) and analyzed by nondenaturing PAGE. Each well contained 10 nM DNA fragment.

TABLE 2 Activity of the *ramA* promoter with or without mutations in the GlxR binding site in the genetic background of the wild type and *ramA*, *sugR*, *ramA-sugR*, and *ramB* mutants[a]

Genotype	GlxR site[b]	β-Galactosidase activity (Miller units)[c]	
		Glucose	Acetate
WT	WT	25.16 ± 3.75	61.32 ± 6.14
	Mut	BLD	BLD
Δ*sugR*	WT	64.18 ± 8.77	123.8 ± 20.6
	Mut	39.84 ± 7.42	75.97 ± 7.06
Δ*ramA*	WT	474.2 ± 34.4	637.8 ± 68.1
	Mut	245.7 ± 6.5	330.9 ± 33.3
Δ*ramA*-Δ*sugR*	WT	393.0 ± 1.5	634.8 ± 10.0
	Mut	215.6 ± 20.4	349.8 ± 10.7
Δ*ramB*	WT	18.34 ± 1.30	52.08 ± 1.55
	Mut	BLD	BLD

[a] The strains carrying the *ramA* promoter-*lacZ* fusion in the chromosome were cultured on either glucose or acetate for 8 h (in the stationary phase). The activities are the mean values from at least three independent cultivations with standard deviations.
[b] The GlxR binding site was mutated (Mut) or not mutated (WT).
[c] BLD, below the level of detection.

role of GlxR in expression of *ramA* was assessed by promoter-reporter assays. The same DNA fragments as those examined in EMSA were fused to the promoter-less *lacZ* gene, and the fusions (P*ramA*-*lacZ*) yielded were integrated into the wild-type chromosome. The β-galactosidase activity driven by the native and mutated promoters during growth in nutrient-rich medium (A medium) containing either 1% glucose or 1% acetate was determined. We used the medium because the *ramA* deletion mutant strains described in the following section are incapable of growing on acetate as the sole carbon source, as has been reported (38). The activities were largely unchanged during growth, although they slightly increased in some of these strains used in the later growth phase. The promoter activities in the cells cultured for 8 h (in the stationary phase) are summarized in Table 2. The activity of the wild-type promoter was higher in the cells grown on acetate than those grown on glucose (Table 2), which is consistent with previous results (43). The promoter carrying the above-described mutations in the GlxR binding site showed no detectable activity during growth under these conditions (Table 2), suggesting that GlxR plays an essential role in *ramA* expression.

The GlxR binding site is not implicated in expression of *cysK* transcribed divergently from *ramA*. The GlxR binding site in the *ramA* promoter region is located 336 bp upstream of the translational start codon of *cysK*, which is transcribed divergently from *ramA*. The *cysK* gene encodes cysteine synthase. The TSP of *cysK* was determined by 5′ RACE and sequencing to be an adenine located 99 bp upstream of the translational start codon; the GlxR binding site was centered at position −237 with respect to the TSP (Fig. 1). To examine whether GlxR regulates *cysK* expression, we investigated the effect of the mutations in the GlxR binding site on the promoter activity of the 595-bp upstream region of the *cysK* gene using the promoter-reporter assay as described above. The results obtained from the analyses showed that the mutations in the GlxR binding site had no effect on *cysK* expression under the conditions used (see Fig. S1 in the supplemental material), indicating that GlxR bound to the site regulates only *ramA* expression.

***ramA* expression is repressed by SugR.** SugR interacts with the *ramA* promoter region *in vivo* and *in vitro* (35). As one of the two deduced binding sites is found between positions −8 and −21 with respect to the TSP, SugR is predicted to act as a repressor of *ramA* (Fig. 1) (35). The other site is located between positions −55 and −68. To experimentally demonstrate a role of SugR in expression of *ramA*, the *ramA* promoter-*lacZ* fusion described above was integrated into the chromosome of a *sugR* mutant. The activity of the *ramA* promoter in the *sugR* mutant was 2-fold higher than that in the wild type under the same conditions described earlier (Table 2). These results confirmed that SugR represses *ramA* expression.

Repression by SugR is relieved in the *ramA* mutant. Next, we examined whether RamA has a role in expression of the *ramA* gene, using a *ramA* mutant carrying the *ramA* promoter-*lacZ* fusion in the chromosome. The *ramA* promoter activity in the *ramA* mutant background was almost 10- to 20-fold higher than that in the wild-type background (Table 2). It is consistent with a previous finding that RamA represses its own expression (43), although the extent of derepression observed in our experiment was higher than that reported in the study (up to a 5-fold increase by *ramA* deletion). The promoter activity in the *ramA* mutant background was higher than that in the *sugR* mutant background in both glucose- and acetate-grown cells, indicating that the degree of repression by RamA is greater than that by SugR. Furthermore, the effects of disruption of both *sugR* and *ramA* on the *ramA* promoter activity were investigated. The activity of the *ramA* promoter in the *sugR-ramA* double mutant background was comparable to that in the *ramA* single mutant background (Table 2). These results indicate that SugR has no influence on the *ramA* promoter activity in the *ramA* mutant background, influence that it has in the wild-type background. As RamA directly activates *sugR* expression (31), it is conceivable that RamA is required for SugR to repress *ramA* expression.

GlxR activates *ramA* expression in *ramA* and *sugR* mutants. The *ramA* promoter with the mutations in the GlxR binding site showed no activity in the wild type, as described earlier (Table 2). We examined the effects of the mutations on the promoter activity derepressed in the three genetic backgrounds, i.e., *sugR*, *ramA*, and *sugR-ramA* deletion mutants. The GlxR binding site mutations reduced 2-fold the *ramA* promoter activity in all the deletion mutants grown under the conditions used (Table 2), indicating that GlxR positively regulates *ramA* expression. It should be noted that the apparent essential role of GlxR in the *ramA* promoter activity observed in the wild-type background was compensated for to a large extent by inactivation of RamA and/or SugR.

The level of *ramA* mRNA expression is not affected by GlxR binding site mutation. To examine the effects of the negative autoregulation of *ramA* on the positive regulation by GlxR, the *ramA* promoter at the original locus in the wild type was modified with the aforementioned GlxR binding site mutations, yielding strain KT27. Expression of *ramA* driven by the mutated promoter in strain KT27 grown under the conditions described earlier was compared to that in the wild type by qRT-PCR analysis. The *ramA* mRNA levels in these two strains were comparable (see Fig. S2 in the supplemental material). Moreover, expression of the representative RamA regulon members *sugR* and *aceA* in strain KT27 was also comparable to that in the wild type (data not shown). As

RamA is essential for expression of *aceA* encoding isocitrate lyase in the glyoxylate shunt, the *ramA* mutant shows a growth defect on acetate (38). In contrast, the growth of strain KT27 on acetate was comparable to that of the wild type (data not shown). Taken altogether, these findings suggest that the level of *ramA* expression is maintained without the GlxR-dependent activation in strain KT27. This is probably due to the strong negative autoregulation of *ramA*.

We also compared the *cysK* mRNA levels between strain KT27 and the wild type, showing that the *cysK* mRNA level was not affected by the mutations in the GlxR binding site (data not shown). This is consistent with the results of the promoter-reporter assay that GlxR is not involved in *cysK* expression (see Fig. S1 in the supplemental material).

Overexpression of *ramA* shuts off *ramA* promoter activity. To assess the functional significance of the negative autoregulation of *ramA*, we examined the effects of overexpression of *ramA* on the activity of the P*ramA-lacZ* fusion. A plasmid carrying the *ramA* gene under the control of the *tac* promoter and LacI repressor (pCRC622) was introduced into the *ramA* and *ramA-sugR* mutants that chromosomally carry the native P*ramA-lacZ* fusion. Since the expression of the *ramA* gene in the resulting strain (the Δ*ramA* P*ramA-lacZ*/pCRC622 and Δ*ramA-sugR* P*ramA-lacZ*/pCRC622 strains) was derived only from the IPTG-inducible promoter on the plasmid, the autoregulation of *ramA* expression was eliminated. The cells exponentially growing on glucose (4 h after the start of culture) were supplemented with IPTG. Alterations in the levels of *ramA* and *lacZ* mRNA after the IPTG supplementation were analyzed by qRT-PCR. The mRNA levels relative to the value obtained from the wild type carrying the native P*ramA-lacZ* fusion (the WT P*ramA-lacZ* strain) before IPTG supplementation (0 h) are shown in Fig. 3. After the addition of IPTG, the level of *ramA* mRNA in the WT P*ramA-lacZ* strain was nearly unchanged for 2 h, although a slight decrease was observed (Fig. 3, top, circles). Before the addition of IPTG, the level of *ramA* expression in the plasmid-carrying strains was 4-fold lower than that in the WT P*ramA-lacZ* strain (Fig. 3, top, squares and triangles). The level of *ramA* mRNA in these strains increased 400-fold within 0.5 h of IPTG supplementation and slightly increased in the subsequent 1.5 h (Fig. 3, top, open symbols). Even in the absence of IPTG, the level of *ramA* mRNA in the Δ*ramA* P*ramA-lacZ*/pCRC622 strain gradually increased (Fig. 3, top, filled squares), indicating leaky expression of *ramA* from the plasmid. At the end of the sampling period (2 h), the level of *ramA* mRNA in this strain was about 2-fold higher than that in the WT P*ramA-lacZ* strain but 80-fold lower than the IPTG-induced level (Fig. 3, top, filled and open squares). These results confirmed the IPTG-inducible overexpression of *ramA* in the Δ*ramA* P*ramA-lacZ*/pCRC622 strain.

qRT-PCR analysis revealed that the level of *lacZ* expression in the WT P*ramA-lacZ* strain was unchanged during the period (Fig. 3, middle, circles). Before the addition of IPTG, the levels of *lacZ* expression in the Δ*ramA* P*ramA-lacZ*/pCRC622 and Δ*ramA-sugR* P*ramA-lacZ*/pCRC622 strains were 6- and 8-fold higher, respectively, than the level in the WT P*ramA-lacZ* strain (Fig. 3, middle, squares and triangles). The level of *lacZ* expression in both the plasmid-carrying strains decreased about 60-fold within 0.5 h of IPTG supplementation (Fig. 3, middle, open symbols). When the Δ*ramA* P*ramA-lacZ*/pCRC622 strain was cultured in the absence of IPTG throughout, the level of *lacZ* expression remained 6-fold higher than that in the WT P*ramA-lacZ* strain during the first 1 h and then decreased 2-fold in the subsequent 1 h (Fig. 3, middle, filled squares). This downregulation of P*ramA-lacZ* expression in the absence of IPTG may be due to the leaky expression of *ramA* from the plasmid. These expression profiles of *ramA* and *lacZ* in the *ramA* mutant background in the absence of IPTG were comparable to those in the *ramA-sugR* mutant background (data not shown). These results indicated that RamA overexpressed from pCRC622 strongly repressed the *ramA* promoter activity.

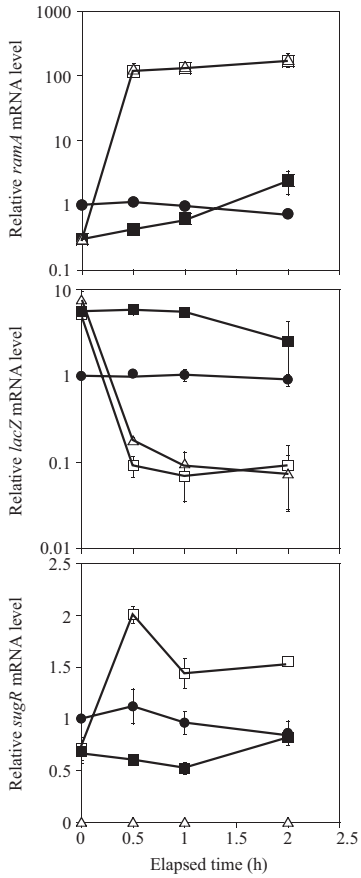

FIG 3 Overexpression of *ramA* represses the *ramA* promoter activity. The exponentially growing cells were supplemented with IPTG (0 h). The strains used carried the P*ramA-lacZ* fusion in the chromosome in the genetic background of the wild type (filled circles), *ramA* mutant transformed with pCRC622 for IPTG-inducible *ramA* expression (open squares), and *ramA-sugR* mutant transformed with pCRC622 (open triangles). Changes in the levels of *ramA* (top), *lacZ* (middle), and *sugR* (bottom) mRNAs were analyzed using qRT-PCR. The mRNA levels in the *ramA* mutant carrying pCRC622 cultured in the absence of IPTG throughout are also shown (filled squares). The mRNA levels are presented relative to the value obtained from the wild type carrying P*ramA-lacZ* before IPTG supplementation (0 h). Mean values obtained from at least three independent cultivations are shown with standard deviations.

We also examined the effects of overexpression of *ramA* on the expression profile of *sugR* under the same conditions. After the addition of IPTG, the level of *sugR* mRNA in the WT P*ramA-lacZ* strain was nearly unchanged for 2 h (Fig. 3, bottom, circles). Before the addition of IPTG, the level of *sugR* expression in the Δ*ramA* P*ramA-lacZ*/pCRC622 strain was lower than that in the WT P*ramA-lacZ* strain (Fig. 3, bottom, squares). The level of *sugR* mRNA in the Δ*ramA* P*ramA-lacZ*/pCRC622 strain increased 2.5-fold within 0.5 h of IPTG supplementation, and the level remained

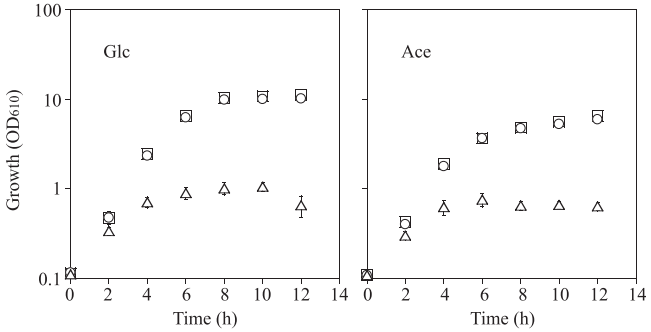

FIG 4 Growth profiles of the *ramA* mutant carrying pCRC622 in A medium containing either 1% glucose (Glc) or 1% acetate (Ace) without (squares) or with IPTG. IPTG was added either 0 h (triangles) or 4 h (circles) after the start of culture. Mean values obtained from at least three independent cultivations are shown with standard deviations. OD_{610}, optical density at 610 nm.

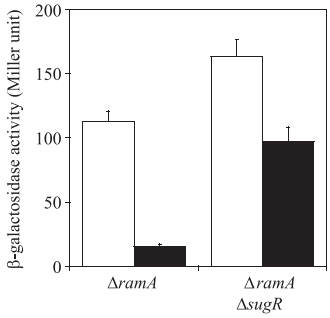

FIG 5 Activity of the *ramA* promoter with (black bars) or without (white bars) mutations in the GlxR binding site in the *ramA* and *ramA-sugR* mutants carrying pCRC622 during growth on glucose without IPTG. The β-galactosidase activity of these strains carrying the P*ramA-lacZ* fusion cultured for 8 h (in the stationary phase) was measured. Mean values obtained from at least three independent cultivations are shown with standard deviations.

higher than that in the WT P*ramA-lacZ* strain in the subsequent 1.5 h (Fig. 3, bottom, open squares). When the Δ*ramA* P*ramA-lacZ*/pCRC622 strain was cultured in the absence of IPTG during this period, the level of *sugR* mRNA remained lower than that in the WT P*ramA-lacZ* strain in the first 1 h (Fig. 3A, bottom, filled squares). No difference in the level of *sugR* expression between these two strains was observed at the end of the period, probably due to the gradual increase in the level of *ramA* mRNA in the Δ*ramA* P*ramA-lacZ*/pCRC622 strain under the noninducing conditions. Thus, the changes in the level of *sugR* expression in the pCRC622-carrying strain seemed to be correlated with those in the level of *ramA* expression. This is consistent with the finding that RamA directly activates *sugR* expression (31).

Addition of IPTG to the cell cultures in the exponential growth phase had no effect on the growth of these strains tested. However, when IPTG was supplemented at the start of culture, growth of the *ramA* mutant carrying pCRC622 was significantly retarded, and the final cell density was markedly lower than the density of cells cultured in the absence of IPTG (Fig. 4). The *sugR-ramA* mutant carrying pCRC622 showed the same growth profiles as the *ramA* mutant carrying pCRC622 (data not shown). These results indicate that overexpression of *ramA* is detrimental to the growth of *C. glutamicum* cells.

GlxR is important for the activation of *ramA* expression against repression by both SugR and RamA. The level of expression of *lacZ* fused to P*ramA* in the *ramA* and *ramA-sugR* mutants carrying pCRC622 in the absence of IPTG was much higher than that in the wild type, probably due to the low level of expression of *ramA* (Fig. 3). Therefore, we expected that the effect of the mutations in the GlxR binding site on *ramA* promoter activity could be evaluated quantitatively using these pCRC622-carrying strains, in contrast to the observation for the wild type, in which the activity of the mutated *ramA* promoter was undetectable. pCRC622 was introduced into the *ramA* and *ramA-sugR* mutants that chromosomally carry the P*ramA-lacZ* fusion with the mutated GlxR binding site. The β-galactosidase activity of the resulting strains during growth on glucose in the absence of IPTG was determined as described earlier. The activity of the mutated *ramA* promoter in the *ramA* mutant carrying pCRC622 was more than 7-fold lower than that of the native one (Fig. 5). Meanwhile, the activity of the mutated *ramA* promoter in the *ramA-sugR* mutant carrying pCRC622 was less than 2-fold lower than that of the native one (Fig. 5). It was confirmed by qRT-PCR that there was no difference in the level of *ramA* mRNA between the strains carrying the *lacZ* gene under the control of the native and the mutated *ramA* promoters in the same genetic backgrounds (data not shown). These findings suggested that the GlxR-mediated activation has less of a contribution to the derepressed *ramA* expression. This is consistent with the observation that the same mutations reduced the *ramA* promoter activity to undetectable levels in the wild-type background but only by 2-fold in the genetic background of single and double deletion mutants of *sugR* and *ramA* (Table 2).

RamB is not implicated in *ramA* expression. It has been reported that the *ramA* promoter activity in the *ramB* mutant is comparable to that in the wild type (43). However, as the RamB binding site flanks the GlxR binding site on the *ramA* promoter region (Fig. 1), we posited that GlxR bound to the *ramA* promoter prevents RamB from binding to the adjacent site, which masks the effects of *ramB* deletion on *ramA* expression. However, this hypothesis was excluded because the activity of the *ramA* promoter with or without mutations in the GlxR binding site in the *ramB* mutant was comparable to that in the wild type (Table 2).

DISCUSSION

The current study shows that expression of *ramA* encoding a LuxR-type transcription regulator of various genes involved in central carbon metabolism is negatively and positively regulated by SugR and GlxR, respectively, in addition to negative autoregulation (43). Since both regulators' activity is modulated with biomolecules whose concentrations varied with the carbon sources used, it is conceivable that SugR and GlxR are involved in the carbon source-dependent regulation of *ramA*. However, it is interesting to note that the effects of inactivation of these regulators in any combinations tested on the *ramA* promoter activity appeared to be unaffected by the carbon source used. Because SugR represses genes involved in sugar uptake and metabolism in the absence of sugar and its repressor activity is inhibited by sugar metabolites (32, 35, 36, 55), the degree of derepression of most SugR target genes in the *sugR* mutant is higher in the absence of sugar than in its presence. However, the degree of derepression of *ramA* promoter activity in the *sugR* mutant in the presence of glucose was comparable to that in the presence of acetate. The discrepancy in the derepression levels in the *sugR* mutant between *ramA* and other SugR targets may be due to the negative autoreg-

ulation of *ramA*; the derepressed RamA in the *sugR* mutant in turn represses its own gene, which may lead to underestimation of the degree of derepression of *ramA* by relief of SugR repression, especially in the presence of acetate. It is also conceivable that relief of the RamA-mediated repression of the *ramA* promoter-*lacZ* fusion irrespective of the carbon source masks the effects of SugR inactivation on the *ramA* promoter activity in the *ramA-sugR* double mutant.

GlxR binds to its target sites in a cAMP-dependent manner *in vitro*. The intracellular cAMP levels of *C. glutamicum* are higher on glucose than on acetate (14–16). Although GlxR bound to the *ramA* promoter in a cAMP-dependent manner (Fig. 2), the *ramA* promoter activity in any of the strains examined in this study was decreased by the mutations in the GlxR binding site irrespective of the carbon source (Table 2). We previously showed that GlxR mediates not only glucose-dependent upregulation of the glycolytic genes *gapA* and *pfk*, encoding glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase, respectively, but also the carbon source-independent upregulation of genes for ATP synthase and cytochrome *c* oxidase (15). It has also been reported that the glyoxylate pathway genes *aceA* and *aceB*, which encode isocitrate lyase and malate synthase, respectively, are negatively regulated by GlxR in the presence of either glucose or acetate (54). These findings indicate that the intracellular cAMP levels and the regulatory roles of GlxR are not necessarily correlated. An unidentified regulator other than SugR and GlxR may be responsible for the upregulation of *ramA* observed in the presence of acetate compared to the level of regulation observed in the presence of glucose. Although previous DNA affinity purification with the *ramA* promoter region as a ligand identified neither SugR nor GlxR, it identified, in addition to RamA and RamB, a GntR-type transcriptional regulator (cg0764), which has not yet been characterized (43).

Deletion of *ramA* had the most prominent effects on the *ramA* promoter activity (Table 2), indicating that of the four global regulators investigated, expression of *ramA* is primarily regulated by RamA itself. The large increase in the *ramA* promoter activity in the *ramA* mutant background is due to relief of repression mediated not only by RamA but also by SugR. This is supported by the findings that the activity of the *ramA* promoter in the *ramA* mutant background was comparable to that in the *sugR-ramA* mutant background (Table 2). In this context, it should be noted that RamA acts as a transcription activator of *sugR* and *sugR* mRNA is markedly downregulated by *ramA* deletion (31). We further confirmed that β-galactosidase activity of the *sugR* promoter-*lacZ* fusion in the *ramA* mutant background was undetectable (data not shown). In this context, it is noteworthy that the overexpression of *ramA* markedly repressed the expression of the *ramA* promoter-*lacZ* fusion and enhanced *sugR* expression to some extent (Fig. 3). These findings suggest that *C. glutamicum* cells need to maintain *ramA* expression levels below a certain threshold under the strict negative control of RamA along with SugR. This idea may be supported by our observation that overexpression of *ramA* had a negative effect on growth (Fig. 4).

The strong direct and indirect negative autoregulation may explain the discrepancy between the effects of the GlxR binding site mutations on the level of *ramA* mRNA expression in strain KT27 and their effects on expression of the *ramA* promoter-*lacZ* fusion. Given that GlxR activates *ramA*, the decrease in *ramA* expression by the loss of GlxR binding in strain KT27 was possibly compensated for by alleviation of the repression by RamA itself, resulting in the wild-type level of *ramA* expression. On the other hand, in the promoter-reporter assays, the loss of GlxR binding to the *ramA* promoter that is fused to *lacZ* had no effect on expression of *ramA* at the original chromosomal locus, resulting in the undetectable level of β-galactosidase activity by RamA-mediated repression. Taken together, it is likely that the level of *ramA* expression is dynamically balanced by the GlxR-dependent activation and the RamA- and/or SugR-dependent repression in response to perturbation of extracellular and/or intracellular conditions. It is interesting to note that the GlxR-mediated activation appears to have a relatively minor role in *ramA* expression when the repression by either SugR or RamA is relieved, as shown in this study (Table 2 and Fig. 5). The detailed molecular mechanism of this regulation mediated by the three regulators is a subject for future studies.

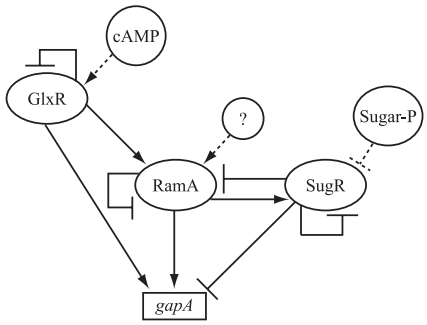

FIG 6 Model of the transcriptional regulatory network involved in expression of *gapA* in *C. glutamicum*. Arrows indicate transcriptional activation, while T bars indicate repression. Effector molecules controlling the activity of transcriptional regulators are shown with circles and dotted lines. An unknown effector for RamA is indicated with a question mark. Sugar-P, sugar phosphate.

The current study demonstrated a new regulatory connection between GlxR and RamA, which has a great impact on the *C. glutamicum* genome-wide transcriptional regulatory network structure (56), because the connection not only expands the GlxR regulon by including the RamA regulon but also newly creates multiple feed-forward loops (FFLs) in the regulatory cascades. The FFL, which is one of the most significant network motifs found in a transcriptional regulatory network in both *E. coli* (57) and *Saccharomyces cerevisiae* (58), comprises two cascaded transcription factors that jointly regulate a common gene (59). The coherent FFL, in which the direct effect of the upper regulator on target gene expression is consistent with the indirect effect of that via regulation of the lower regulator, has been reported to contribute to sense signal persistency, thereby filtering noise or fluctuations in the environmental input signal (60). In contrast, the incoherent FFL, in which the direct and indirect effects of the upper regulator are opposite, accelerates the response of the regulatory system (61). In *E. coli*, genes for nonglucose sugar metabolism are under the control of the FFL composed of CRP and the carbon-specific transcriptional regulator to respond to a combination of the respective signals (59, 61). In the case of *C. glutamicum gapA* for a key glycolytic enzyme, one incoherent FFL composed of RamA and SugR and two coherent FFLs based on the newly identified regulatory interactions among GlxR, SugR, and RamA are involved in gene regulation (Fig. 6). On the basis of the presumed

physiological function of each type of FFL, the coherent FFLs formed with GlxR-RamA and SugR-RamA are predicted to play a role in maintaining *gapA* expression levels in response to fluctuations in cAMP and sugar phosphate levels, whereas the incoherent FFL composed of RamA-SugR may be involved in a rapid response to physiological and/or environmental changes, which are sensed by RamA. Moreover, the FFLs formed by GlxR and RamA are possibly involved in regulation of other central carbon metabolism genes, i.e., *ptsF*, *gltA*, *acn*, *sdhCAB*, *aceA*, and *aceB*, encoding the fructose uptake phosphotransferase system, citrate synthase, aconitase, succinate dehydrogenase, isocitrate lyase, and malate synthase, respectively (35, 38, 45, 54, 62–65). Further insights into the biological roles of these complex regulatory connections will be provided by elucidation of the environmental signals to which RamA and RamB respond and how the intracellular cAMP levels are controlled. These important issues need to be addressed in future studies.

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