

Involvement of the LuxR-Type Transcriptional Regulator RamA in Regulation of Expression of the *gapA* Gene, Encoding Glyceraldehyde-3-Phosphate Dehydrogenase of *Corynebacterium glutamicum*^{∇†}

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SugR, RamA, GlxR, GntR1, and a MarR-type transcriptional regulator bind to the promoter region of the *gapA* gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), essential for glycolysis in *Corynebacterium glutamicum*. We previously showed that SugR, a transcriptional repressor of phosphotransferase system genes for the sugar transport system, is involved in the downregulation of *gapA* expression in the absence of sugar. In this study, the role of RamA in the expression of the *gapA* gene was examined. Comparing the *gapA* expression and GAPDH activity of a *ramA* mutant with those of the wild type revealed that RamA is involved in upregulation of *gapA* expression in glucose-grown cells. DNase I footprint analyses and electrophoretic mobility shift assays revealed that RamA binds with different affinities to three sites in the *gapA* promoter. *lacZ* reporter assays with mutated RamA binding sites in the *gapA* promoter showed that the middle binding site is the most important for RamA to activate *gapA* expression and that binding of RamA to the *gapA* promoter activates the gene expression not only in glucose-grown cells but also in acetate-grown cells. Furthermore, RamA also directly activates *sugR* expression, indicating that two global regulators, RamA and SugR, are coordinately involved in the complex regulation of *gapA* expression in *C. glutamicum*.

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalyzing the oxidation of D-glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate by using NAD⁺ as a coenzyme, is a key enzyme of glycolysis and plays a crucial role in catabolic and anabolic carbohydrate metabolism due to the reversibility of its catalysis. Regulation of the GAPDH-encoding genes in bacteria has been studied most in *Escherichia coli* and *Bacillus subtilis*. In *E. coli*, the expression of the GAPDH-encoding gene, *gapA*, is upregulated in the presence of glucose and this induction depends upon the EII^{Glc} glucose-permease protein, a component of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) (3). *E. coli gapA* expression is repressed by catabolite repressor activator (Cra), and its repression is relieved in the presence of sugar metabolite fructose-1-phosphate or fructose-1,6-bisphosphate (36, 39). The *B. subtilis* GAPDH-encoding gene, *gapA*, is induced in the presence of sugar (29), and this induction indirectly depends on catabolite control protein A (CcpA), the major transcriptional regulator for catabolite repression (11, 29, 30). In addition, *B. subtilis gapA* expression is repressed by central glycolytic gene regulator (CggR), and the repression by CggR is relieved by fructose-1,6-bisphosphate (7, 29). Thus, *gapA* expression in both these organisms is repressed by transcriptional regulators sensing sugar phosphate and upregulated by alleviation of the repression in the presence of sugar.

Corynebacterium glutamicum is a high-GC gram-positive bacterium widely used in the industrial production of amino acids, e.g., L-glutamate and L-lysine, as well as organic acids (18, 22, 25, 27, 42). *C. glutamicum* additionally produces organic acids and ethanol under oxygen-deprived conditions, under which cells retain the ability to excrete product despite cessation of growth (20, 21, 34). Glycolysis is one of the most important targets to improve the yield of production from sugar. *C. glutamicum* possesses two GAPDHs, GapA and GapB. GapA is essential for glycolysis, and GapB is dispensable for glycolysis and important for gluconeogenesis (35). The susceptibility of GapA activity to the intracellular NADH/NAD⁺ ratio (8) suggests that the enzyme may well catalyze the rate-limiting step of glycolysis.

The expression of *gapA* is controlled at the transcriptional level in *C. glutamicum*. It is upregulated in glucose-grown cells compared to that in acetate-grown cells (15, 17, 33). The *gapA* expression level is increased at the onset of the stationary phase during growth on glucose and remains high upon oxygen deprivation (21). SugR, a transcriptional repressor of genes involved in PTS (10, 13, 41), acts as a repressor of *gapA* expression in the absence of sugar (43). This repression of *gapA* is relieved by fructose-1-phosphate and fructose-1,6-bisphosphate (43), a pattern similar to the regulation of *E. coli gapA* expression by Cra and *B. subtilis gapA* by CggR. However, *gapA* expression in a *sugR* deletion mutant is still upregulated in the presence of glucose at the onset of the stationary phase, as it is in the wild-type strain, suggesting the involvement of other transcriptional regulators (43). GlxR, a cyclic AMP receptor protein-type transcriptional regulator involved in many physiological reactions in *C. glutamicum* (23, 24, 26, 28), binds to the *gapA*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i>		
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	Takara
JM110	<i>dam dcm supE44 hsdR17 tih leu rpsL lacy galK galT ara tonA thr tsx Δ(lac-proAB)/F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	37
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3)</i>	40
<i>C. glutamicum</i>		
R	Wild-type strain	45
KT1	R with deletion in <i>sugR</i>	43
KT2	R with <i>gapA-lacZ</i>	43
KT3	KT1 with <i>gapA-lacZ</i>	43
KT6	R with deletion in <i>ramA</i>	This study
KT7	R with deletion in <i>glsR</i>	This study
KT8	R with deletion in <i>gntR1</i>	This study
KT9	R with deletion in <i>marR</i>	This study
KT10	R with deletions in <i>ramA</i> and <i>sugR</i>	This study
KT11	KT6 with <i>gapA-lacZ</i>	This study
KT12	KT10 with <i>gapA-lacZ</i>	This study
KT13 derivatives	R with <i>gapA-lacZ</i> with mutations in the RamA binding sites in the <i>gapA</i> promoter	This study
Plasmids		
pCold I	Ap ^r ; cold-inducible expression vector	Takara
pCRC604	Ap ^r ; pColdI with an 846-bp fragment of the <i>ramA</i> gene	This study
pCRA725	Km ^r ; the suicide vector containing the <i>B. subtilis sacB</i> gene	19
pCRC605	Km ^r ; pCRA725 with 2.3-kb XbaI-SacI PCR fragment containing the <i>ramA</i> gene	This study
pCRC606	Km ^r ; pCRA604 with a 1.6-kb fragment containing a truncated <i>ramA</i> gene	This study
pCRA741	Km ^r ; pCRA725 with a 2.0-kb PCR fragment from strain-specific island 7 and a 3.1-kb PCR fragment containing the <i>E. coli lacZ</i> gene	21
pCRC602	Km ^r ; pCRA741 with a 0.6-kb PCR fragment containing the <i>C. glutamicum</i> R <i>gapA</i> promoter region	43
pCRC607	Km ^r ; pCRA741 with a 0.6-kb PCR fragment containing the <i>gapA</i> promoter region with mutations in RamA binding site P	This study
pCRC608	Km ^r ; pCRA741 with a 0.6-kb PCR fragment containing the <i>gapA</i> promoter region with mutations in RamA binding site M	This study
pCRC609	Km ^r ; pCRA741 with a 0.6-kb PCR fragment containing the <i>gapA</i> promoter region with mutations in RamA binding site D	This study
pCRC610	Km ^r ; pCRA741 with a 0.6-kb PCR fragment containing the <i>gapA</i> promoter region with mutations in RamA binding sites P and D	This study
pCRC611	Km ^r ; pCRA741 with a 0.6-kb PCR fragment containing the <i>gapA</i> promoter region with mutations in RamA binding sites M and D	This study
pCRC612	Km ^r ; pCRA741 with a 0.6-kb PCR fragment containing the <i>gapA</i> promoter region with mutations in RamA binding sites P and D	This study
pCRC613	Km ^r ; pCRA741 with a 0.6-kb PCR fragment containing the <i>gapA</i> promoter region with mutations in all RamA binding sites	This study

promoter region, although its physiological function remains to be elucidated (15).

In this study, we show that RamA, which is involved in various physiological reactions, including acetate metabolism (6), synthesis of a cell surface layer protein (16), and the expression of resuscitation-promoting factor (23), binds to three sites in the *gapA* promoter region with different affinities and that RamA is important for upregulation of *gapA* expression at the onset of the stationary phase. Finally, we show that RamA directly activates *sugR* expression. These results indicate a complex regulation of *gapA* expression by two different types of global regulators in *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1.

Culture conditions. 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 A medium (21) with 4% glucose. When appropriate, the medium was 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. Growth experiments with *C. glutamicum* were performed using A medium containing 1% glucose or acetate as described previously (43).

DNA manipulations. The oligonucleotides used in this study (see Table S1 in the supplemental material) were obtained from Gene Design (Osaka, Japan). Plasmid DNA was isolated with a QIAprep spin miniprep kit (Qiagen). Chromosomal DNA was isolated from *C. glutamicum* with Genomic prep (GE Healthcare Bioscience), modified by using 4 mg ml⁻¹ lysozyme at 37°C for 2 h. Restriction endonucleases were purchased from Takara (Osaka, Japan) and used according to the manufacturer's instructions. PCR was performed with a GeneAmp PCR system (Applied Biosystems) using LA *Taq* polymerase (Takara) or Phusion polymerase (New England Biolabs). The resulting PCR fragments were purified with a QIAquick PCR purification kit (Qiagen). *E. coli* was transformed by the CaCl₂ procedure (37). *C. glutamicum* was transformed by electroporation as described previously (44). The nucleotide sequence of cloned DNA fragments was determined with a genetic analyzer 3130xl (Applied Biosystems).

DNA affinity purification. DNA affinity purification of proteins binding to the *gapA* promoter region and N-terminal sequencing of the proteins were performed as described previously (43).

Construction of *ramA* and *ramA-sugR* mutants. A DNA fragment containing the *ramA* gene was amplified by using primer ramAFWXba and primer ramARVSac and genomic DNA from *C. glutamicum* strain R as template. The PCR product was cloned into pCRA725, a suicide vector for markerless gene disruption (19), yielding pCRC605. An internal 759-bp segment of the *ramA* gene was removed by inverse PCR using primers ramAinvMunFW and ramAinvMunRV and pCRC605 as template. The PCR product was digested with MunI and self-ligated, yielding pCRC606. Plasmid pCRC606 was isolated as nonmethylated DNA from *E. coli* JM110 for efficient gene introduction into *C. glutamicum* R (44). *C. glutamicum* R and the *sugR* mutant strain KT1 (43) were transformed by electroporation with pCRC606, and screening for the deletion mutants was performed as described previously (19). Deletion of the *ramA* gene was checked by PCR using primers ramAFWXba and ramARVSac. The *ramA* and *ramA-sugR* mutant strains obtained were designated KT6 and KT10, respectively. Similarly, the *gntR1*, *glxR*, and *marR* deletion mutants were constructed and designated KT7, KT8, and KT9, respectively. The *gntR1*, *glxR*, or *marR* gene was amplified by using primer pair gntRFW-gntRRV, glxRFW-glxRRV, or marRFW-marRRV, respectively. The PCR products were cloned into pCRA725. The cloned gene was truncated by inverse PCR using the following primer pairs: gntRinvFW-gntRinvRV, glxRinvFW-glxRinvRV, and marRinvFW-marRinvRV. The PCR product was digested with appropriate restriction enzymes and self-ligated. *C. glutamicum* R was transformed by electroporation with the resulting plasmids as described above.

qRT-PCR. Total RNA was extracted from *C. glutamicum* cells by using a Qiagen RNeasy mini kit (Qiagen) as described previously (43). Isolated RNA samples were checked for purity by both agarose gel electrophoresis and spectrophotometric analysis and stored at -80°C . Quantitative reverse transcription-PCR (qRT-PCR) was performed using an Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems) and power SYBR green PCR master mix with MuLV reverse transcriptase and RNase inhibitor from a GeneAmp RNA PCR kit (Applied Biosystems) as described previously (43). Specific primers (see Table S1 in the supplemental material) were designed by using Primer Express Software version 3.0 (Applied Biosystems). The specificity of the amplicons was checked by qRT-PCR dissociation curve analysis. The comparative threshold cycle method (7500 Fast System SDS software version 1.4.0.; Applied Biosystems) was used to quantify relative expression.

GAPDH assay. *C. glutamicum* cells were harvested by centrifugation, washed with 50 mM Tris-HCl buffer, pH 7.5, and resuspended with buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and 10% (vol/vol) glycerol. The cells were mechanically disrupted by using a FastPrep FP120 instrument (BIO 101; Thermo Savant) as described previously (43), and the supernatant after centrifugation was used as crude extract for the enzyme assay. The activity of GAPDH was determined as described previously (35).

Overexpression and purification of His-tagged RamA protein. The *ramA* gene was amplified from chromosomal DNA of *C. glutamicum* R by PCR with primers RamAHisFW and RamAHisRV. The PCR product was cloned into the expression vector pColdI (Takara), yielding pCRC604. pCRC604 was used to transform *E. coli* BL21(DE3), and the resulting strain was grown at 37°C in LB medium to an optical density at 600 nm of 0.5. The culture was incubated for 30 min at 15°C , and then expression of the His-tagged RamA was induced by the addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The culture was shaken overnight at 15°C . The cells were harvested by centrifugation and frozen at -80°C until ready for use. The His-tagged protein was purified by affinity chromatography on Ni-nitrilotriacetic acid agarose (Qiagen) according to the instruction manual. For desalting, the eluted protein was loaded to a PD-10 column (GE Healthcare Bioscience) and eluted with buffer A (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol). The concentration of the purified protein was determined by a Bio-Rad protein assay (Bio-Rad Laboratories) using bovine serum albumin as a standard.

EMSA. DNA fragments of various lengths covering the *gapA* promoter region (P1 to P6) were obtained by amplifying regions of the *gapA* promoter from chromosomal DNA using the following primer pairs: pgapAFW2-pgapARV3Cy3, pgapAFW3-pgapARV3Cy3, pgapAFW5-pgapARV3Cy3, pgapAFW5Cy3-pgapARV4, pgapAFW5Cy3-pgapARV5, and pgapAFW5Cy3-pgapARV6. DNA fragments P1, P3, and P4 containing mutations in the RamA binding sites were amplified by PCR using the following primer pairs and plasmids as template: pgapAFW5-pgapARV3Cy3, pgapAFW3-pgapARV3Cy3, and pgapAFW5Cy3-pgapARV4 and pCRC607, pCRC608, pCRC609, and pCRC610 containing the *gapA* promoter region with mutations in the RamA binding sites as described for construction of the *lacZ* fusion. The DNA fragments were purified with a QIAquick

column (Qiagen). Electrophoretic mobility shift assay (EMSA) was performed using the purified His-tagged RamA as described previously (43). The resulting DNA-protein complex was loaded onto a 5% polyacrylamide gel in $0.5\times$ Tris-borate-EDTA and separated at 125 V for 40 min. The gel was stained with ethidium bromide and visualized with a UV transilluminator. When Cy3-labeled probes were used, DNA and DNA-protein complexes were visualized by using a Typhoon TRIO variable mode imager (GE Healthcare Bioscience).

DNase I footprinting. Labeled DNA fragments were obtained by amplification with 5'-IRD700-labeled oligonucleotides. The *gapA* promoter region was amplified by using the following primer pairs: gapAIRD FW and pgapA RV2 (coding strand), and gapAIRD RV and pgapA RV (noncoding strand). An IRD700-labeled DNA fragment (4 nM) was mixed with purified RamA in a total volume of 50 μl of binding buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 3 mM MgCl₂, 5 mM CaCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% [vol/vol] glycerol, and 1.5 $\mu\text{g}/\mu\text{l}$ of bovine serum albumin). The mixture was incubated for 25 min at room temperature. The reaction mixture volume was increased to 100 μl with $1\times$ binding buffer, and then DNase I (Takara) was added at 35 mU, and incubation was continued for 1 min at 25°C . The mixture was treated with phenol, precipitated with ethanol, and resuspended with IR2 stop solution (Li-Cor, Lincoln, NE). The dissolved sample was separated on a 5.5% KB plus gel matrix (Li-Cor) using a Li-Cor 4300 DNA analyzer. The DNA-sequencing reaction mixtures were set up using the same IRD-700-labeled primers and a DYEnamic direct cycle sequencing kit with 7-deaza-dGTP (GE Healthcare/Bioscience).

Construction of the *gapA* promoter-*lacZ* fusions. The DNA fragment containing the *gapA* promoter region was generated by PCR using primers pgapAFW5 and pgapARV and cloned upstream of the *lacZ* gene in pCRA741 (21), yielding pCRC602. The direction and sequence of the inserted fragment were confirmed by DNA sequencing. Plasmid pCRC602 was isolated as nonmethylated DNA from *E. coli* JM110 for efficient gene introduction into *C. glutamicum* R (44) and was subsequently integrated into the chromosome of *C. glutamicum* R, the *ramA* mutant strain KT6, and the *ramA-sugR* mutant strain KT10 by markerless gene insertion methods, as described previously (19), yielding the resulting strains KT2 (43), KT11, and KT12, respectively. Mutations in RamA binding sites were constructed by overlapping PCR using overlapping primer pairs ramAmutPFW-ramAmutPRV, ramAmutMFW-ramAmutMRV, and ramAmutDFW-ramAmutDRV for the proximal, middle, and distal sites, respectively, together with primers pgapAFW5 and pgapARV and genomic DNA from strain R as a template. The overlapping PCR products were purified and used as template for PCR using primer pgapAFW5 and primer pgapARV. The resulting fragments were cloned into pCRA741, generating pCRC607, pCRC608, and pCRC609 containing mutations in the proximal, middle, and distal sites in the *gapA* promoter, respectively, hereafter referred to as sites P, M, and D. Double and triple mutations in the RamA binding sites were introduced by overlapping PCR using the primer pairs described above and plasmids pCRC607 to pCRC609 instead of genomic DNA as template, and the resulting fragments were cloned into pCRA741, generating pCRC610, pCRC611, pCRC612, and pCRC613. Plasmids from pCRC607 to pCRC613 were individually integrated into the chromosome of *C. glutamicum* R. The resulting strains were designated KT13P, KT13M, KT13D, KT13PM, KT13MD, KT13PD, and KT13PMD, where the last letter(s) indicates the RamA binding site mutated. The mutations introduced in the *gapA* promoter-*lacZ* fusion on the chromosome were confirmed by direct sequencing of a PCR fragment which was amplified using a primer specific for *lacZ* and one specific for the *gapA* promoter (pgapAFW5), with genomic DNA extracted from the mutants obtained as a template.

β -Galactosidase assay. *C. glutamicum* cells carrying the *gapA* promoter-*lacZ* fusion were harvested, washed once with Z buffer (32), resuspended with the same buffer, and treated with toluene. The permeabilized cells were then incubated with *o*-nitrophenyl- β -galactoside, and activity was measured in Miller units as previously described (32).

RESULTS

Transcriptional regulatory proteins binding to the *gapA* promoter region of *C. glutamicum*. To identify proteins binding to the *gapA* promoter region, we previously performed DNA affinity purification with magnetic streptavidin beads (43). A 334-bp biotinylated promoter region (positions between -304 and $+30$ relative to the transcriptional start point [TSP] of *gapA*) was linked to streptavidin-coated magnetic beads and incubated with cell extracts from *C. glutamicum* grown on

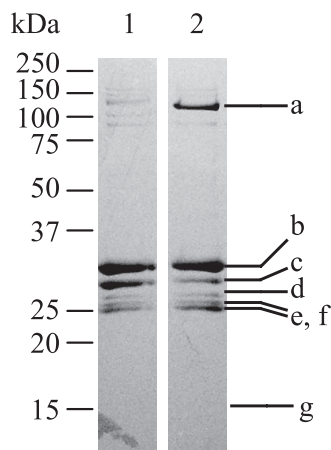


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins binding to the *gapA* promoter region. Cell extracts of *C. glutamicum* R grown in minimal medium containing 1% acetate (lane 1) or glucose (lane 2) were incubated with magnetic beads linked to biotin-labeled *gapA* promoter DNA fragment, and bound proteins were eluted with 1 M NaCl. The N-terminal amino acid sequence of the proteins in bands a to g was analyzed: band a, termination factor Rho (CgR1278); band b, RamA (CgR2464); band c, SugR (CgR1761); band d, GntR1 (CgR2434); bands e and f, GlxR; band g, MarR-type transcriptional regulator (CgR2877). A molecular mass standard is shown to the left.

minimal medium containing glucose or acetate as the sole carbon source. Figure 1 shows the proteins eluted from the beads. We identified the proteins by comparing the N-terminal amino acid sequence of the proteins (Fig. 1, a to g) with the entire genome sequence of *C. glutamicum* R (45). In addition to SugR, which was previously identified and shown to act as a repressor of *gapA* expression (43), four other transcriptional regulatory proteins were identified in this study (Fig. 1). Three of them, RamA (CgR2464), GntR1 (CgR2434), and GlxR (CgR0377), were previously identified as regulators of genes involved in various physiological reactions other than glycolysis (6, 10, 12, 13, 16, 23, 24, 28, 41). The last one, a MarR-type transcriptional regulator (CgR2877), has not been fully characterized to date.

RamA is involved in upregulation of *gapA* expression in the presence of glucose. To clarify *in vivo* roles of the transcriptional regulators identified, the corresponding genes were inactivated by in-frame deletion. Deletion mutants of each of the *ramA*, *glxR*, *gntR1*, and *cgR2877* genes were grown on nutrient-rich A medium containing glucose or acetate. We consequently compared the expression level of *gapA* of each of the mutant strains with that of the wild-type strain by qRT-PCR. Total RNA was extracted from the cells grown on glucose or acetate at the mid-exponential phase (3 h) and the onset of the stationary phase (6 h) (43). Deletion of the *gntR1* or *cgR2877* gene had no effect on growth and *gapA* expression under the conditions tested (data not shown). In contrast, the *glxR* mutant grew too slowly to analyze. The *ramA* mutant strain grew at a rate similar to that of the wild type in the presence of glucose, although the final cell density of the mutant culture was slightly lower than that of the wild type (Fig. 2A), which is consistent with the results of a previous study (6). As the *ramA* mutant strain is unable to grow on acetate as the sole carbon source

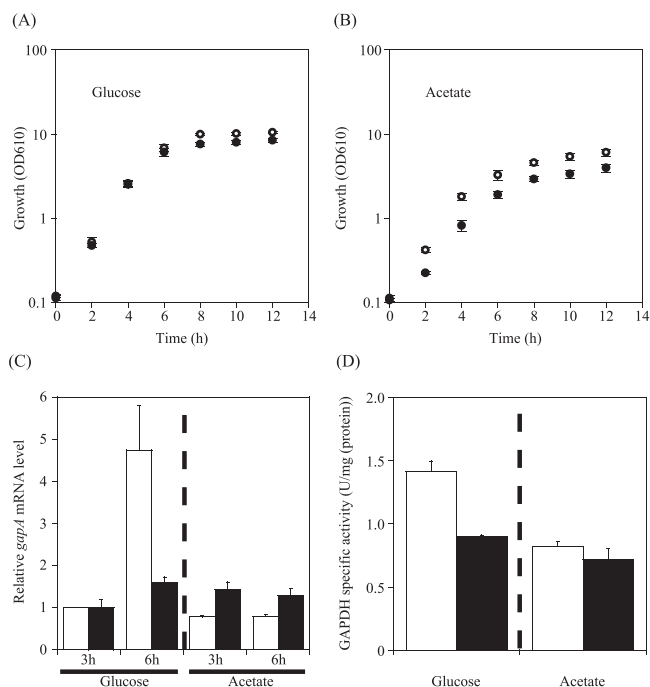


FIG. 2. Effect of deletion of the *ramA* gene on growth (A, B), *gapA* expression (C), and GAPDH activity (D). (A, B) Growth of the wild-type R (white circles) and the *ramA* mutant KT6 (black circles) in nutrient-rich A medium containing 1% glucose (A) or acetate (B). Mean values from at least four independent cultures are shown, with standard deviations. OD₆₁₀, optical density at 610 nm. (C) The level of *gapA* mRNA in the wild-type R (white) and *ramA* mutant KT6 (black) during growth in A medium with 1% glucose or acetate at the exponential (3 h) and the stationary (6 h) phase was analyzed by qRT-PCR, and the levels relative to that in the wild type grown in the presence of glucose at 3 h were determined. Mean values from three independent cultures are shown, with standard deviations. (D) Activities of GAPDH in the wild-type R (white) and the *ramA* mutant KT6 (black) grown in A medium with 1% glucose or acetate. The activity was determined at the stationary (6 h) phase. Mean values from at least three independent cultures are shown, with standard deviations.

(6), it grew more slowly than the wild type in A medium with acetate (Fig. 2B).

The level of *gapA* mRNA of the wild-type strain grown on glucose was comparable to that of cells grown on acetate at the exponential phase (Fig. 2C). In the presence of glucose, the level of *gapA* mRNA in the wild type was increased nearly fivefold at the onset of the stationary phase (21), while the level was constant throughout growth in the presence of acetate (Fig. 2C). In the presence of glucose, the level of *gapA* mRNA in the *ramA* mutant was almost the same as that in the wild-type strain at the exponential phase, while *gapA* expression was downregulated in the *ramA* mutant by 60% compared to that in the wild type strain at the onset of the stationary phase (Fig. 2C). In the presence of acetate, the level of *gapA* mRNA in the *ramA* mutant was relatively low compared with that in the presence of glucose throughout growth (Fig. 2C). As observed with the mRNA level, the corresponding activity of GAPDH encoded by the *gapA* gene in the *ramA* mutant was lower than that of the wild type in the glucose-grown cells at the onset of the stationary phase (Fig. 2D). In the presence of acetate, the GAPDH activity of the *ramA* mutant was comparable to that

of the wild type. These results suggested that, in the presence of glucose, RamA is involved in upregulation of *gapA* expression at the onset of the stationary phase.

RamA binds to three distinct sites within the *gapA* promoter region. To examine the binding of RamA to the *gapA* promoter region, we performed EMSAs with a DNA fragment containing the *gapA* promoter region, which encompasses positions +57 to -373 relative to the TSP of *gapA*, and the recombinant RamA protein (Fig. 3A). The results of the EMSAs showed that RamA specifically bound to the *gapA* promoter (Fig. 3B, P1 fragment). Three shifted bands were observed when 800 nM of RamA was added to the reaction mixture, suggesting that there are three RamA binding sites in the *gapA* promoter. To identify the RamA binding sites in the *gapA* promoter region, we performed EMSAs with a series of the *gapA* promoter fragments from P2 to P6 (Fig. 3B). The results showed that RamA bound to P3 and P4 but not to P2 or P6, indicating that RamA specifically binds to the two regions between positions -86 and -148 and positions -152 and -197 relative to the TSP of the *gapA* gene. We found a putative RamA consensus binding site consisting of tandem A/C/TG₄₋₆T/C or AC₄₋₅A/G/T in each region (Fig. 3C) (6). The two sites are located between positions -77 and -91 and positions -160 and -174 relative to the TSP. The significance of these sites for RamA binding was tested by mutational analysis, in which G/C stretches were changed (Fig. 3C). RamA failed to bind to the mutated P3 fragment (Fig. 3D), whereas RamA was still able to bind to the mutated P4 fragment but only one shifted band was observed, unlike in the case of the wild-type fragment (Fig. 3D, P4 fragments). RamA binds to not only tandem but also single GC-rich stretches (5). We found a single GC-rich stretch, ACGGGGT, similar to the RamA binding site, at position -198 relative to the TSP (Fig. 3C), and showed that this sequence was sufficient for RamA binding (Fig. 3B, P5 fragment). Simultaneous mutation in each of these three sites diminished the binding of RamA to the *gapA* promoter (Fig. 3D, P1 fragments). These results demonstrated that RamA binds to the three sites centered at positions -84, -168, and -198 with respect to the TSP (sites P, M, and D, respectively) in the *gapA* promoter.

The three RamA binding sites have different affinities for RamA. To confirm the RamA binding site in the *gapA* promoter, DNase I footprinting assays were performed. A DNA fragment encompassing the *gapA* promoter (from position +56 to -316 with respect to the TSP) was labeled on one strand or the other, incubated with an increasing quantity of RamA, and then hydrolyzed by using DNase I. RamA protected three regions on either strand (Fig. 4). The regions that were protected were positions -74 to -93, -155 to -176, and -187 to -203 on the coding strand, and -71 to -97, -156 to -181, and -186 to -205 on the noncoding strand. These regions included the three RamA binding sites described above (Fig. 3C). As indicated by the appearance of DNase I-hypersensitive sites in the P and M sites and between the two sites, conformational changes might occur in the DNA upon binding of RamA (Fig. 4). At low concentrations, RamA preferentially bound to the region containing the M site, while simultaneous protection of the other binding sites was observed at increasing concentrations (Fig. 4). The results suggested that RamA has the highest affinity for the M site.

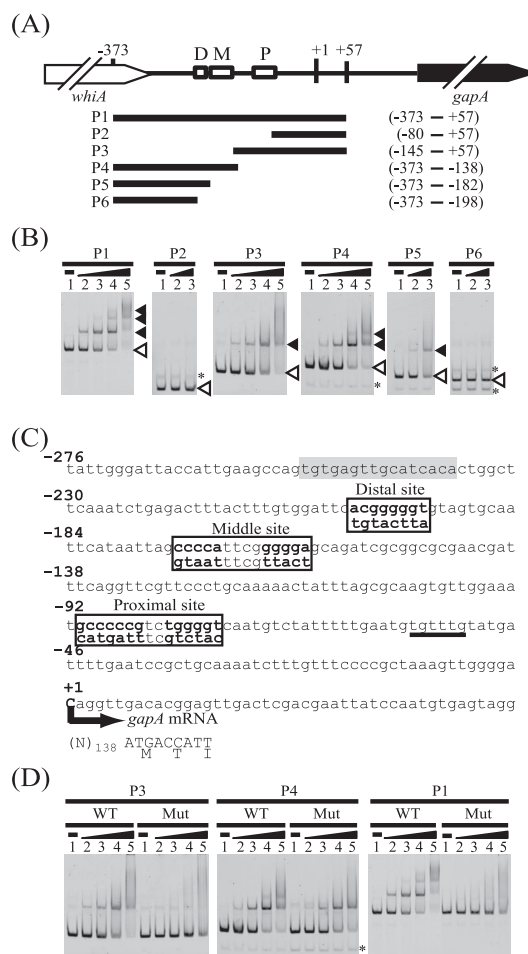


FIG. 3. Binding of RamA to the *gapA* promoter region. (A) DNA fragments (P1 to P6) used to determine the location of the RamA binding site in the *gapA* promoter are shown. The numbers above the diagram indicate the position relative to the TSP (+1) of the *gapA* gene. The regions included in the DNA fragments are indicated, with positions with respect to the TSP at the right side. The *gapA* gene is indicated with a black arrow. The *whiA* gene, which encodes a WhiA-like protein, is located upstream of *gapA* with the same direction and is indicated with a white arrow. Three RamA binding sites (P, M, and D sites) are indicated with white boxes. (B) Results of EMSAs using the *gapA* promoter region and His-tagged RamA. The DNA fragments (P1 to P6) used are indicated at the top of the gels. Each well contained 10 nM of DNA fragment. Lanes 1 to 5 show EMSA results using 0, 200, 400, 800, and 1,600 nM of RamA protein, respectively. Probe DNA and DNA-RamA complexes are indicated with white and black arrowheads, respectively. Nonspecific bands are indicated with asterisks. (C) DNA sequence of the *gapA* promoter showing the putative RamA binding sites. The TSP (+1) is indicated with a bold letter and an arrow. The numbers indicate the position relative to the TSP (+1). The coding region of *gapA* is indicated with capital letters and the amino acid sequence. Three RamA binding sites (P, M, and D sites) and the mutated sequences shown under the sites are boxed. The mutated sequences are indicated with bold letters. The binding sites of SugR and GlxR are underlined and shaded in gray, respectively. (D) The results of EMSAs using DNA fragments without (WT) or with (Mut) the mutations in the RamA binding site are shown. The DNA fragments (P1, P3, and P4) used are indicated at the top of the gels. Each well contained 10 nM of DNA fragment. Lanes 1 to 5 show results of EMSAs using 0, 200, 400, 800, and 1,600 nM of RamA protein, respectively. The mutated P3 fragment contains the mutated P binding site. The mutated P4 fragment contains the mutated middle and native distal binding sites. The mutated P1 fragment contains mutations in all the binding sites. A nonspecific band is indicated with an asterisk.

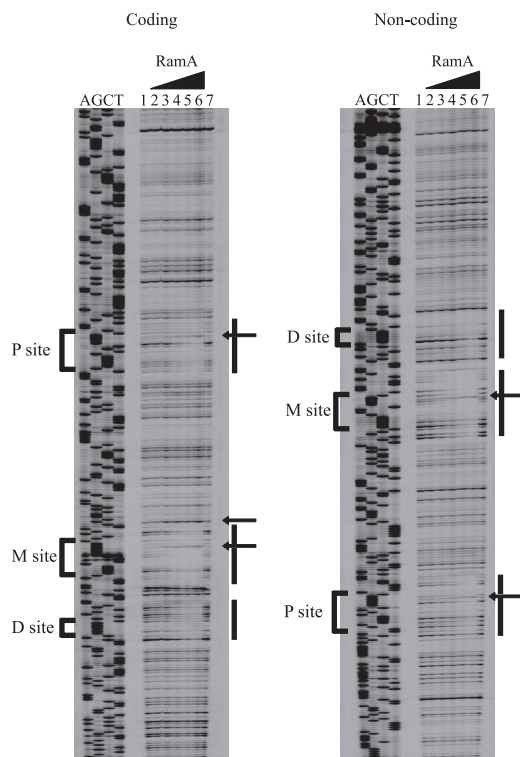


FIG. 4. DNase I footprint analysis of the interaction between RamA and the *gapA* promoter regions examined on the coding and the noncoding strands. A DNA fragment (4 nM) was incubated with different concentrations of RamA: lanes 1 and 7, no protein; lane 2, 200 nM; lane 3, 400 nM; lane 4, 800 nM; lane 5, 1,600 nM; lane 6, 3,200 nM. The three RamA binding sites are indicated with brackets on the left side of each panel. Protected regions are indicated by bars, and hypersensitive sites are indicated by arrows. The DNA sequencing reactions were set up using the same labeled primer and plasmid as for generating labeled footprinting probes.

To investigate the affinity of RamA for the binding sites in detail, the effects of mutations in the binding sites on the binding of RamA to the *gapA* promoter fragment were compared by EMSAs (Fig. 5). With the wild-type promoter (WT) fragment, a shifted band was observed at 100 nM of RamA and a lower-mobility complex was observed at 400 nM of RamA. RamA bound to a fragment containing mutations in the P site with the same affinity as it did to the WT fragment (Fig. 5, WT and P). Mutations in the M site, however, drastically reduced the ability of RamA to bind to the *gapA* promoter, but the lower-mobility complex was more clearly observed compared with that observed in the WT fragment at 400 nM of RamA (Fig. 5, WT and M). Mutations in the D site also reduced the ability of RamA to bind to the *gapA* promoter, but the inhibitory effect was smaller than that observed for mutations in the M site (Fig. 5, WT and D). These results supported the results of the footprint analysis, in which the region containing the M site was protected from DNase I at lower concentration of RamA than the other sites. When fragments containing two mutated binding sites were used, the lower-mobility complex was not observed. The effects of the mutations in each binding site on the binding of RamA seemed to be additive, i.e., the affinity of RamA for the fragment with

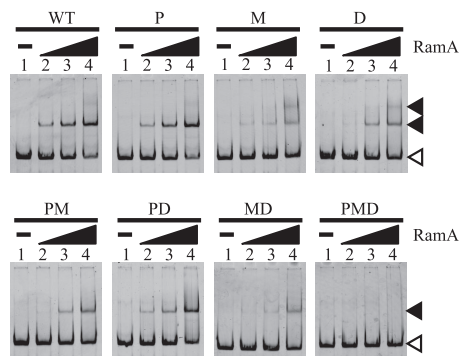


FIG. 5. RamA has different affinities for the three binding sites in the *gapA* promoter region. The *gapA* promoter fragment without (WT) or with mutated RamA binding sites was incubated with different concentrations of RamA: lane 1, no protein; lane 2, 100 nM; lane 3, 200 nM; lane 4, 400 nM. The binding sites mutated are indicated at the top of the gels. Probe DNA and DNA-RamA complexes are indicated with white and black arrowheads, respectively.

mutations in both the P and D sites (PD) was lower than that for a fragment with mutations in only the P site, and the affinity of RamA for the fragment with mutations in both the M and D sites was lower than that for a fragment with mutations in only the M site (Fig. 5; compare MD and M).

Effects of mutations of the RamA binding sites on *gapA* promoter activity. To investigate roles of the three RamA binding sites in the regulation of *gapA* expression in vivo, the effects of mutations in the RamA binding sites (Fig. 3C) on the *gapA* promoter activity were examined by using the *lacZ* reporter assay system (Fig. 6). We integrated the *gapA* promoter-*lacZ* translational fusion (*PgapA-lacZ*), containing mutations in each site; in both the P and M, P and D, and M and D sites; or in all sites, into the chromosome of *C. glutamicum* R and designated the resulting strains KT13P, KT13M, KT13D, KT13PM, KT13PD, KT13MD, and KT13PMD, respectively (Fig. 6A). In the presence of glucose (Fig. 6B), mutations in the M site decreased the β -galactosidase activity to 30% of that of the wild type. Mutations in the P site also elicited a negative effect on the β -galactosidase activity, but the effect was much smaller than that elicited by mutations in the M site. Mutations in the D site had almost no discernible effect on the β -galactosidase activity. The effects of mutations in the RamA binding sites on the *gapA* promoter activity were not additive, in contrast to those on the binding of RamA (Fig. 5). The β -galactosidase activity of strain KT13PM, containing mutations in both the P and M sites, was higher than that of KT13M but lower than that of KT13P. The combination of the D site mutation with the mutations of other KT13 derivatives had no effect on β -galactosidase (Fig. 6B, KT13PD, KT13MD, and KT13PMD). Similar effects of mutations in the RamA binding sites on the *gapA* promoter activity were also observed in acetate-grown cells, although the activity was lower than that in glucose-grown cells (Fig. 6C). These results suggested that the *gapA* promoter activity is affected by the coordination of multiple RamAs binding to the M and P sites in the promoter under the conditions tested and that the binding of RamA to the *gapA* promoter activates the gene expression not only in glucose-grown cells but also in acetate-grown cells.

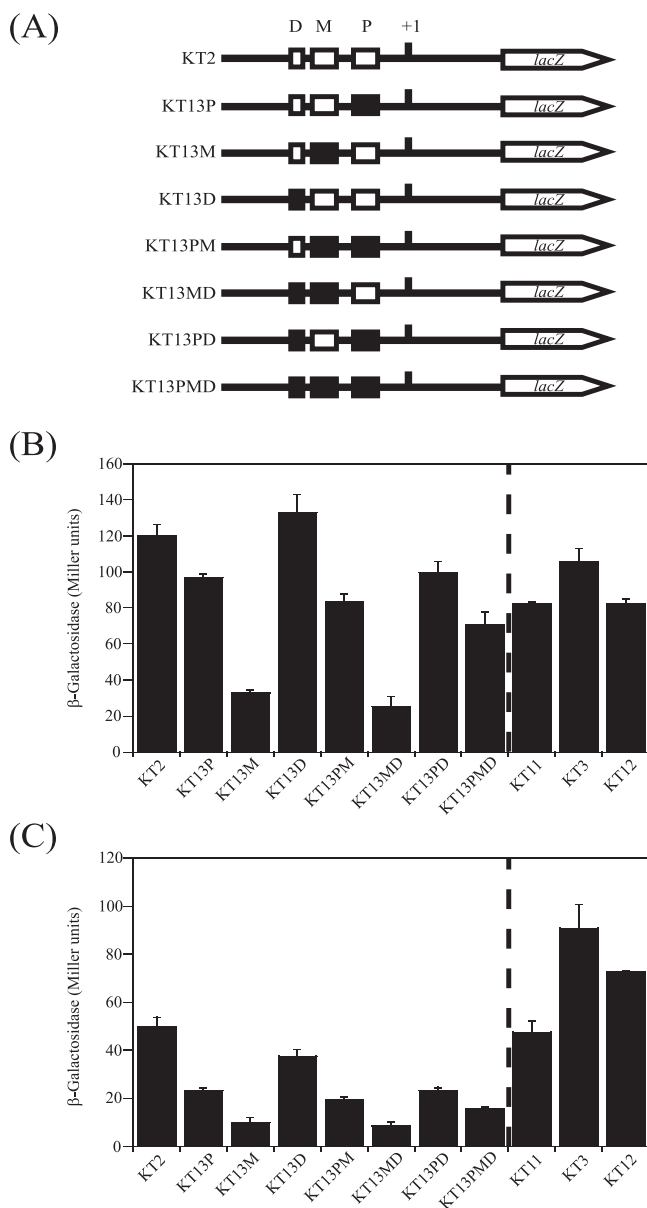


FIG. 6. Effects of mutation of the RamA binding sites and deletion of the *ramA* and *sugR* genes on expression of the *gapA* promoter-*lacZ* translational fusion. (A) The *gapA* promoter-*lacZ* fusion constructs are indicated. The strains carrying the fusion constructs in their genome are indicated at the left side. The TSP of the *gapA* gene is indicated with +1. The three RamA binding sites (P, M, and D) without or with mutations are indicated with white or black boxes, respectively. The mutated *gapA* promoter-*lacZ* fusions, containing RamA binding sites mutated as shown in Fig. 3C, were introduced into the wild type, yielding KT13 derivatives (KT13P, KT13M, KT13D, KT13PM, KT13MD, KT13PD, and KT13PMD). The *gapA* promoter-*lacZ* fusion was also introduced into the wild-type strain (KT2), the *ramA* mutant (KT11), the *sugR* mutant (KT3), and the *ramA-sugR* double mutant (KT12). (B, C) β -Galactosidase activity in each strain grown in nutrient-rich A medium containing 1% glucose (B) or acetate (C) at the onset of the stationary phase. Mean values from at least three independent cultures are shown, with standard deviations.

To examine the roles of the two transcriptional regulators, RamA and SugR, in *gapA* expression, the *gapA* promoter activities in the *ramA*, *sugR*, and *ramA-sugR* mutants were determined (Fig. 6). In the presence of glucose, the β -galactosidase

activities in the *ramA* (KT11) and *ramA-sugR* mutants (KT12) were decreased to the same level, which was lower than that in the wild type (KT2). In the presence of acetate, the β -galactosidase activity of the *ramA* mutant was the same as that of the wild-type strain. The *gapA* promoter activity of the *ramA-sugR* mutant was higher than that of the wild type and lower than that of the *sugR* mutant strain (KT3). These results suggested that RamA acts as a primary positive regulator of *gapA* expression regardless of the carbon source used, while SugR acts as a negative regulator of *gapA* expression in the absence of glucose.

RamA activates *sugR* expression. The *PgapA-lacZ* activity of the *ramA* mutant (KT11) was higher than that of the wild type harboring mutated RamA binding sites (KT13PMD) in the presence of acetate but similar in the presence of glucose (Fig. 6). This discrepancy might be attributed not only to differences in growth characteristics between the wild type and the *ramA* mutant but also to differences in the expression of other regulators binding to *gapA* promoters in the two strains. Thus, we compared the expression of the *sugR* gene in the wild type with that in the *ramA* mutant by qRT-PCR. The level of *sugR* mRNA in the wild-type strain grown on acetate was twice that of the glucose-grown cells at the exponential phase (Fig. 7A). At the onset of the stationary phase, the level of *sugR* mRNA in the wild-type strain grown on either glucose or acetate was lower than the level at the exponential phase. The level of *sugR* mRNA in the *ramA* mutant was less than 20% or 30% of that of the wild-type strain grown on glucose or acetate, respectively, at the exponential phase (Fig. 7A). At the onset of the stationary phase, the level of *sugR* mRNA of the *ramA* mutant grown on either glucose or acetate was lower than that of the wild type. These results suggested that RamA positively regulates *sugR* expression.

To examine whether RamA regulates *sugR* expression directly or indirectly, we performed EMSA using the upstream region of the *sugR* gene, which was amplified with the primers indicated in Fig. 7B. When using the DNA fragment amplified with primer pair *psugR* FW1/*psugR* RV1 (Fig. 7B), two shifted bands were observed in the presence of RamA, suggesting that RamA is able to bind to the upstream region of *sugR* and that at least two RamA binding sites are located in the promoter (Fig. 7C). We found two putative RamA binding sites comprised of tandem GC-rich stretches at positions -110 and -222 with respect to the translational start point of the *sugR* gene (Fig. 7B). When using the *sugR* promoter fragment containing one or no RamA binding site, one or no shifted band was observed, respectively (Fig. 7C), confirming that RamA bound to the two RamA binding sites. These results demonstrated that RamA directly activates *sugR* expression.

DISCUSSION

In this study, we identified the LuxR-type transcriptional regulator, RamA, as a protein binding to the *gapA* promoter and showed that RamA positively regulates *gapA* expression in *C. glutamicum*. RamA was first identified as a positive transcriptional regulator of the expression of genes involved in acetate metabolism, including the *pta-ack* operon, *aceA*, and *aceB*, encoding phosphotransacetylase and acetate kinase, isocitrate lyase, and malate synthase, respectively, and

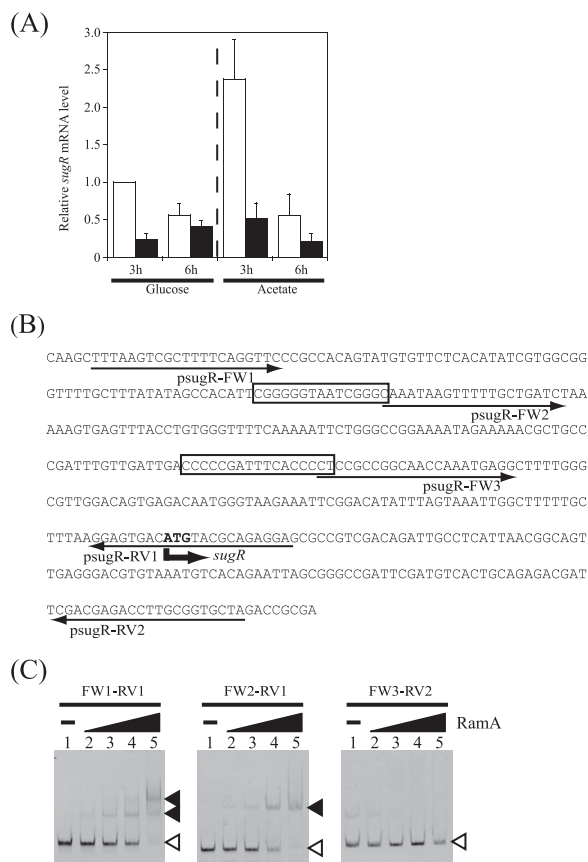


FIG. 7. RamA directly regulates *sugR* expression. (A) The levels of *sugR* mRNA in the wild-type R (white) and the *ramA* mutant KT6 (black) during growth in nutrient-rich A medium with 1% glucose or acetate at the exponential (3 h) and the stationary (6 h) phases were analyzed by qRT-PCR, and the levels relative to that in the wild type grown in the presence of glucose at 3 h were determined. Mean values from three independent cultures are shown, with standard deviations. (B) DNA sequence of the upstream region of *sugR* of *C. glutamicum*. The proposed RamA binding sites are boxed. The start codon of *sugR* is indicated by a bold arrow and bold letters. Thin arrows indicate the primers used to prepare probes for EMSAs. (C) The *sugR* promoter fragments prepared by PCR using primers indicated at the top of the gels were incubated with different concentrations of RamA: lane 1, no protein; lane 2, 200 nM; lane 3, 400 nM; lane 4, 800 nM; lane 5, 1,600 nM. Probe DNA and DNA-RamA complexes are indicated with white and black arrowheads, respectively.

the *ramA* mutant is incapable of growing on acetate as the sole carbon source (6). RamA is also involved in the regulation of genes encoding PS2 protein (*cspB*), a component of the surface layer of *C. glutamicum* cells (16); RamB (*ramB*), a repressor of genes involved in acetate metabolism (4, 14); alcohol dehydrogenase (*adhA*) (1); and resuscitation promoting factor 2 (*rpf2*) (23). Thus, RamA is a global regulator rather than an acetate-specific regulator. Our current study expanded the RamA regulon to sugar metabolism. It has been previously implied, by the fact that the final cell density of the *ramA* mutant was lower than that of the wild type during growth on glucose, that RamA is involved in glucose metabolism (6). As the *gapA* gene constitutes an operon with the *pgk* and *tpi* genes, encoding phosphoglycerate kinase and triosephosphate isomerase, respectively (38), these glycolytic genes are under the control of the

gapA promoter. The negative effect of *ramA* inactivation on growth on glucose might be attributed in part to the decrease in expression of the *gapA* operon.

The results of EMSAs and DNase I footprint analyses showed that RamA binds to three sites containing a single or tandem putative consensus sequences, a stretch of four to five G or C residues flanked by A, C, or T (6), in the *gapA* promoter. The three RamA binding sites in the *gapA* promoter were centered at positions -84 (P), -168 (M), and -198 (D) with respect to the TSP of the *gapA* gene (Fig. 3C). The RamA binding site varies in location and number in the promoter of its target genes, with one common feature being that almost all sites are located upstream of the -35 region where RNA polymerase binds; one to four sites are located in the region between position -50 and -247 relative to the TSP of the target genes (1, 2, 4, 6, 14, 16). The fact that a transcriptional regulator binding to a region upstream of -35 in the target promoter generally functions as an activator is consistent with the role of RamA in regulation of *gapA* expression (31). However, it should be noted that the location of the binding sites of RamA in the promoter of target genes, e.g., -247 and -176 with respect to the TSP of *ramB* and *cspB*, respectively, seems to be more upstream than the sites of typical bacterial transcriptional activators.

The mutations in the M binding site in the *gapA* promoter reduced the promoter activity to 30% of that of the native one, while the mutations in all three sites decreased the activity to 70% of that of the native one, as observed for deletion of the *ramA* gene (Fig. 6, strains KT2, KT13M, KT13PMD, and KT11). This suggests that the M site is the most important for RamA to activate the *gapA* promoter activity. The reason why the activity in KT13PM was higher than that in KT13M is not clear so far. Since RamA has the highest affinity for the M site (Fig. 4 and 5), RamA probably binds to the P and/or D sites after binding to the M site. When the M site is occupied by RamA, binding of RamA to the P site is likely to have a positive effect on the *gapA* promoter. On the other hand, when the M site is not occupied (which could not occur in vivo except in strains KT13M and KT13MD), binding of RamA to the P site would have a negative effect on the *gapA* promoter (Fig. 6, KT13M and KT13MD). In fact, *PgapA-lacZ* with mutations in the M site had higher activity in the *ramA* mutant background than in the wild type (data not shown). The mutations in the P site had little effect on the binding of RamA and a small effect on the *gapA* promoter activity (Fig. 5 and 6, KT13P), suggesting that the P site may function properly only in combination with the M site to let RamA alter the *gapA* promoter conformation and activate *gapA* expression. The binding of RamA to the D site does not seem to affect the promoter activity, at least under the conditions tested. The binding sites of RamA are almost perfectly conserved in the *gapA* promoter region of *C. glutamicum* ATCC 13032 (data not shown), indicating that RamA also binds to the same regions in this strain and the importance of the alignment of the RamA binding sites. As far as we know, this is the first instance in which multiple binding sites of RamA in the target promoter were separately investigated for in vivo functions. However, it remains to be investigated how RamA activates transcription of the target genes.

It is still unknown what environmental conditions and physiological signals RamA senses (2, 6). The genes regulated by

RamA are involved in acetate metabolism, whose gene expression in the presence of glucose is downregulated relative to that in the presence of acetate. *gapA* expression, on the other hand, is upregulated in the presence of glucose compared with that in the presence of acetate (15, 17, 33). These findings suggest that effector molecules, which control RamA activity, may be present in both glucose- and acetate-grown cells. The expression of the glyoxylate shunt genes, *aceA* and *aceB*, and that of the alcohol dehydrogenase gene, *adhA*, is increased in response to the presence of inducer substrates, i.e., acetate and ethanol, respectively, but the expression levels of these genes in the *ramA* mutant are lower than those in the wild type regardless of the carbon source used (1, 6). Similarly, the activity of the *gapA* promoter with mutations in the RamA binding sites was lower than that of native promoter in both glucose- and acetate-grown cells (Fig. 6). These findings suggest that RamA is required for basal upregulation of the expression of the target genes rather than carbon source-dependent regulation. This would enable the cell to maintain *gapA* expression at a high level. *gapA* expression is controlled by SugR in response to the carbon source used. Further studies are required to address an effector regulating RamA activity.

We showed that RamA directly activates the expression of *sugR*, suggesting that *gapA* expression is under the control of a complex transcriptional regulatory network. The expression of the *gapA* gene in *E. coli* and *B. subtilis* is negatively regulated by Cra and CggR, respectively (7, 29, 39). A component of PTS is required for upregulation of *gapA* in *E. coli* (3), while CcpA is indirectly involved in upregulation of *gapA* in *B. subtilis* (11, 30). As CcpA is required for upregulation of PTS in *B. subtilis*, the uptake of sugar is inhibited in the *ccpA* mutant (28). Repression of *gapA* expression by Cra and CggR is relieved in the presence of sugar phosphate involving fructose-1,6-bisphosphate, which is generated upon the uptake of sugar. In *C. glutamicum*, *gapA* expression is repressed by SugR in the absence of sugar, and the repression is also relieved in the presence of fructose-1-phosphate and fructose-1,6-bisphosphate (43). Taken together, derepression of *gapA* expression in these organisms requires functional PTS and efficient uptake of sugar. On the other hand, it is noteworthy that in *C. glutamicum* a different type of global regulator, RamA, which regulates the expression of the sugar-sensing global regulator SugR, is involved in complex regulation of the glycolytic gene. This complex regulatory mechanism may be advantageous for *C. glutamicum* to control *gapA* expression, a key element of the glycolytic pathway, in response to changes in physiological conditions. Very recently it was shown that SugR also represses genes encoding glycolytic enzymes 6-phosphofructokinase, fructose-1,6-bisphosphate aldolase, enolase, and pyruvate kinase (9).

In summary, we identified transcriptional regulators involved in *gapA* expression. *C. glutamicum gapA* expression is negatively regulated by SugR during growth in the absence of sugar and positively regulated by RamA regardless of the carbon source used. We obtained other transcriptional regulators binding to the *gapA* promoter in this study (Fig. 1). Although deletion of *gntR1* or *cgR2877* had no effect on *gapA* expression under the conditions tested, these regulators may be involved in control of *gapA* expression under other conditions. It should be noted that GntR1 is involved in upregulation of the *ptsG*

gene encoding a glucose-specific component of PTS, EII^{Glc} (12). GlxR has been reported to act as a repressor for various genes, including *aceB*, *gntP*, and *gntV* (23, 24, 28). The whole binding sites of GlxR on the genome were predicted by in silico analysis and confirmed by EMSAs, suggesting that GlxR is involved in the activation and repression of the expression of numerous genes (26). The binding site of GlxR in the *gapA* promoter region, centered at position -245 with respect to the TSP (15), seems to be far upstream with respect to the TSP to repress *gapA* expression, suggesting that GlxR may positively regulate *gapA* expression. A study to identify a function of these regulators is under way in our laboratory.

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