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Identification of RamA, a Novel LuxR-Type Transcriptional Regulator of Genes Involved in Acetate Metabolism of *Corynebacterium glutamicum*

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In *Corynebacterium glutamicum*, the acetate-activating enzymes phosphotransacetylase and acetate kinase and the glyoxylate cycle enzymes isocitrate lyase and malate synthase are coordinately up-regulated in the presence of acetate in the growth medium. This regulation is due to transcriptional control of the respective *pta-ack* operon and the *aceA* and *aceB* genes, brought about at least partly by the action of the negative transcriptional regulator RamB. Using cell extracts of *C. glutamicum* and employing DNA affinity chromatography, mass spectrometry, and peptide mass fingerprinting, we identified a LuxR-type transcriptional regulator, designated RamA, which binds to the *pta-ack* and *aceA*/*aceB* promoter regions. Inactivation of the *ramA* gene in the genome of *C. glutamicum* resulted in mutant RG2. This mutant was unable to grow on acetate as the sole carbon and energy source and, in comparison to the wild type of *C. glutamicum*, showed very low specific activities of phosphotransacetylase, acetate kinase, isocitrate lyase, and malate synthase, irrespective of the presence of acetate in the medium. Comparative transcriptional *cat* fusion experiments revealed that this deregulation takes place at the level of transcription. By electrophoretic mobility shift analysis, purified His-tagged RamA protein was shown to bind specifically to the *pta-ack* and the *aceA*/*aceB* promoter regions, and deletion and mutation studies revealed in both regions two binding motifs each consisting of tandem A/C/TG$_{4-6}$T/C or AC$_{4-5}$A/G/T stretches separated by four or five arbitrary nucleotides. Our data indicate that RamA represents a novel LuxR-type transcriptional activator of genes involved in acetate metabolism of *C. glutamicum*.

Corynebacterium glutamicum is a nonpathogenic, aerobic gram-positive soil bacterium that is widely used for the large-scale production of amino acids such as L-glutamate and L-lysine (21, 24, 28, 30, 35). In addition, the organism has gained increasing interest as a suitable model organism for the *Corynebacterineae*, a suborder of the actinomycetes which also includes the medically important genus *Mycobacterium* (51).

C. glutamicum is able to grow on a variety of carbohydrates and organic acids as single or combined sources of carbon and energy, and among these substrates are glucose and acetate (31, 34). Based on biochemical, genetic, and regulatory studies; on quantitative determination of metabolic fluxes during utilization of acetate and/or glucose; and on genome-wide comparative expression analyses, there is considerable knowledge of enzymes and genes involved in acetate metabolism of *C. glutamicum* (reviewed in references 9 and 17). The utilization of acetate involves its uptake and subsequent activation to acetyl coenzyme A (CoA) and, when acetate is the sole carbon substrate, also requires the operation of the glyoxylate cycle as anaplerotic pathway. The key enzymes of acetate activation, acetate kinase (AK) and phosphotransacetylase (PTA), and those of the glyoxylate cycle, isocitrate lyase (ICL) and malate synthase (MS), have been intensively studied with respect to their biochemical properties as well to their regulation, and all four enzymes have been shown to be essential for the growth of *C. glutamicum* on acetate as the sole carbon and energy source (43, 44, 45, 49). The specific activities of AK, PTA, ICL, and MS in *C. glutamicum* are much higher in the presence of acetate in the growth medium than in its absence, and, as shown by Northern blot hybridization and transcriptional fusion experiments, as well as by DNA microarray technology, these differences in activity can be explained almost completely by coordinated transcriptional regulation of the respective genes (17, 20, 40, 45, 55). The AK and PTA genes (*ack* and *pta*, respectively) form an operon with *pta* upstream of *ack* (*pta-ack* operon [45]) and with transcriptional initiation at 159 and 47 bp (TS1 and TS2, respectively) upstream of the proposed *pta* translational start codon (17). The ICL and MS genes (*aceA* and *aceB*, respectively) are not located in the vicinity of the *pta-ack* operon; they are clustered on the chromosome, separated by 597 bp and transcribed in divergent directions (44). Kim et al. (29) recently described the GlxR protein, belonging to the cyclic AMP (cAMP) receptor protein family of transcriptional regulators and able to bind to the *aceA*/*aceB* intergenic region in the presence of cAMP. The overexpression of the *glxR* gene in *C. glutamicum* led to 10- to 15-fold-lower specific activities of ICL and MS when the cells were grown on acetate. Although the growth on acetate probably does not require a down-regulation of the glyoxylate cycle genes, these observations indicate that GlxR represents a repressor for the *aceA* and *aceB* genes. In accordance with coordinated expression control of the *pta-ack* operon, *aceA*, and *aceB* by a common regulator protein(s), we recently identified and characterized a novel transcriptional regulator (designated RamB) which, during growth of *C. glutamicum* in the absence of acetate,

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represses the expression of the four genes by specific binding to highly conserved 13-bp motifs (AA/GAACTTTGCAAA) in the *pta-ack* and the *aceA*/*aceB* promoter/operator regions (16). However, destruction of the 13-bp motif and, thus, prevention of the binding of the RamB protein to the *aceA*/*aceB* promoter region did not lead to complete deregulation of the respective promoter activities, and in a RamB-deficient mutant, there was still some residual regulation of the *aceA* and *aceB* genes (16). Moreover, cosubstrate experiments with glucose and with lactate in addition to acetate (55) revealed that aside from a negative effect of glucose and of lactate in the growth medium, there is a positive effect of acetate on the specific AK, PTA, ICL, and MS activities. These results suggested that in *C. glutamicum*, aside from RamB, an activator is involved in transcriptional control of the enzymes of acetate metabolism.

In the present study, we identified a novel LuxR-type transcriptional regulator, designated RamA, which is essential for growth of *C. glutamicum* on acetate and which activates the expression of the AK, PTA, ICL, and MS genes during growth in the presence of this carbon source. We show binding of purified RamA protein to the respective promoter/operator regions and, by deletion and mutation studies, present evidence for a consensus binding motif of RamA.

MATERIALS AND METHODS

Bacteria, plasmids, oligonucleotides, and culture conditions. *Escherichia coli* DH5α (19), *E. coli* BL21(DE3) (8), and the wild-type (WT) strain *C. glutamicum* ATCC 13032 were employed. Plasmids, their relevant characteristics and sources, and oligonucleotides used in this study are given in Table 1. The minimal medium used for *C. glutamicum* has been described previously (10) and contained 1% (wt/vol) acetate and/or 1% (wt/vol) glucose. Tryptone-yeast extract medium (2×) (46) was used as complex medium for *C. glutamicum* and for *E. coli*. When appropriate, kanamycin (50 μg ml^{-1}) was added to the medium. If not stated otherwise, *C. glutamicum* was grown aerobically at 30°C, *E. coli* at 37°C, as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Growth of the bacteria was followed by measuring the optical density at 600 nm (OD_{600}).

DNA preparation and transformation. The isolation of chromosomal DNA and of plasmids from *C. glutamicum* was performed as described previously (11), and plasmid isolation from *E. coli* was carried out according to the method of Birnboim (3). DNA transfer into *C. glutamicum* was performed by electroporation, and recombinant strains were selected on Luria-Bertani–brain heart infusion–sorbitol agar plates containing kanamycin (15 μg ml^{-1}) (52). Transformation of *E. coli* was carried out with competent cells according to the method of Inoue et al. (26).

PCR techniques. PCR experiments were performed in a Biometra Personal Cycler (Biotron, Göttingen, Germany). Amplification of DNA was carried out with Vent polymerase (New England Biolabs, Schwalbach, Germany). Buffers and deoxynucleoside triphosphates were taken from MBI-Fermentas (St. Leon-Rot, Germany). Oligonucleotides (primers) were obtained from MWG-Biotech (Ebersberg, Germany) or from biomers.net GmbH (Ulm, Germany). Cycling times and temperatures were chosen according to fragment length and primer constitution. PCR products were separated on agarose gels and purified with the Nucleospin extract kit (Macherey Nagel, Düren, Germany).

DNA manipulation and Southern hybridization. Restriction enzymes, T4 DNA ligase, calf intestinal phosphatase, RNase A, proteinase K, and *Taq* polymerase were obtained from MBI-Fermentas and used as instructed by the manufacturer. DNA purification after restriction digestion was performed by separation on agarose gels and purification with the Nucleospin extract kit (Macherey Nagel). DNA hybridization experiments were performed as previously described (43). The complete *ramA* gene was amplified and labeled with digoxigenin-dUTP by PCR and used as a probe. Labeling, hybridization, washing, and detection were performed with the nonradioactive DNA Labeling and Detection kit and the instructions from Roche Diagnostics (Penzberg, Germany).

DNA affinity purification. The purification of DNA-binding proteins was performed essentially as described previously (14). Briefly, the *pta-ack* promoter/operator probe and the *aceA*/*aceB* promoter/operator probe were generated by PCR using plasmid pRob19 and oligonucleotides *pta*-bio and *pta*-16 (*pta-ack* probe) and plasmid pRob10 and oligonucleotides *aceb*1-bio and *aceAB*-rev (*aceA*/*aceB* probe). The primers *pta*-bio and *aceb*1-bio were tagged with biotin via a TEG linker (MWG-Biotech). Unincorporated oligonucleotides were removed by twofold ultrafiltration of the sample with Microcon-30 concentrators (Amicon, Witten, Germany). About 100 pmol of biotin-labeled PCR product was coupled to 3 mg of Dynabeads streptavidin (Dynal, Oslo, Norway), and uncoupled DNA was removed by magnetic separation according to the manufacturer's protocol. The coupled Dynabeads were stored at 4°C for a maximum of 1 week. Directly before incubation with the *C. glutamicum* crude extracts (see below), the coupled Dynabeads were equilibrated with 300 μl of binding buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol [DTT], 100 mM NaCl, 10% [vol/vol] glycerol, 0.05% [vol/vol] Triton X-100 [pH 8.0]) for 2 min.

Cultures (500 ml) of *C. glutamicum* were grown in acetate and glucose minimal medium, harvested at an OD_{600} of about 5, washed with 1 volume of TN buffer (50 mM NaCl, 50 mM Tris-HCl [pH 7.6]), and suspended in 6 ml of disruption buffer (50 mM Tris-HCl [pH 7.6], 1 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 10% [vol/vol] glycerol, 10 μM phenylmethylsulfonyl fluoride). Aliquots (1 ml) of the cell suspension were added to 2-ml screw-cap vials together with 250 mg of glass beads (150 to 212 μm; Sigma-Aldrich) and subjected six times for 25 s to mechanical disruption with a RiboLyser (setting, 6.5; Hybaid, Heidelberg, Germany) at 4°C with intermittent cooling on ice for 2 min. After disruption, glass beads and cellular debris were removed by two consecutive centrifugation steps at 13,000 × *g* and 4°C for 10 min and at 45,000 × *g* and 4°C for 60 min. The supernatant was concentrated by incubation (20 to 30 min) on solid polyethylene glycol 20000 in Visking dialysis tubes (Serva, Heidelberg, Germany) with a pore size of 25 Å. The dialyzed crude extract (about 500 μl), together with 10 μl of competitor DNA (herring sperm DNA, 10 mg/ml), was incubated with the coupled and equilibrated (see above) Dynabeads for 2 h at room temperature with shaking at 350 rpm. Unbound proteins were removed by magnetic separation with a magnet particle concentrator (Dynal) and two washes with 300 μl of binding buffer (see above). Subsequent elution of the DNA-bound proteins was done with binding buffer containing 0.3 and 1 M NaCl (20 μl each; magnetic separation was performed after each step). Eluted fractions were collected, and 10 μl of each was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the technique of Laemmli (33). Gels were subsequently stained with the colloidal blue Coomassie staining kit (Novex, Frankfurt/Main, Germany).

Construction of plasmids for the synthesis and preparation of His-tagged RamA fusion protein. Vector pET28a was used for the synthesis of the hexahistidyl (His)-tagged RamA fusion protein. The *ramA* gene was amplified from chromosomal DNA of WT *C. glutamicum* by PCR with primers *ramA*f and *ramA*r. The PCR product was digested by NdeI and HindIII, ligated into the NdeI/HindIII-restricted plasmid pET28a, and transformed into *E. coli* BL21(DE3). The synthesis of the RamA protein carrying the His tag at its N terminus was induced in recombinant *E. coli* BL21(DE3) (pET28-RamAx6His) by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) after the culture had reached an OD_{600} of 0.6. The cells were grown for 4 h to an OD_{600} of about 5, harvested, and disrupted with a French pressure cell. Purification of the fusion protein was performed with Ni-nitrilotriacetic acid affinity chromatography according to the instructions of QIAGEN (Hilden, Germany). For desalting, the RamA fusion protein was dialyzed overnight against 30% (wt/vol) glycerol in water with Visking dialysis tubes (Serva) with a pore size of 25 Å. The fusion protein was then used directly for the promoter/operator binding assay.

Promoter/operator binding assays with His-tagged RamA fusion protein. The binding of the His-tagged RamA protein to the *pta-ack* and *aceA*/*aceB* promoter/operator regions was tested by DNA electrophoretic mobility shift assay (EMSA) using the DNA fragments or double-stranded oligonucleotides indicated and shown in Results. The DNA fragments were generated by PCR and purified by the Nucleospin extract kit (Macherey Nagel). For generating native and mutated double-stranded oligonucleotides, the two complementary primers were mixed and heated to 95°C for 10 min in TE buffer (10 mM Tris [pH 7.8], 1 mM EDTA) and allowed to anneal by slow cooling at room temperature. In the binding assays, 50 to 100 ng of the fragments or oligonucleotides was incubated with various amounts of RamA (0 to 2 μg) in 20 μl of 10 mM Tris-HCl reaction buffer, pH 7.6, containing 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% (wt/vol) glycerol, and 1 μg of poly(dI-dC) for 20 min at room temperature. When indicated, acetate, acetyl-P, acetyl-CoA, free CoA, cAMP, cGMP, 2-oxoglutarate, NAD, NADH, ADP, and ATP were added at the final concentrations indicated in Results. Subsequently, the mixture was separated on a 2% agarose gel in 1× TAE buffer (200 mM Tris-HCl [pH 7.5], 100 mM acetate, 5 mM EDTA) at 70 V and 80 mA and stained with ethidium bromide.

TABLE 1. Plasmids and oligonucleotides used in this study

Plasmid or oligonucleotide	Relevant characteristics or sequence[a]	Source/reference or purpose[b]
Plasmids		
pET2	Promoter probe vector carrying the promoterless *cat* gene; Km^r	53
pRob19	pET2 containing the 895-bp insert of the *pta-ack* promoter region	16
pRob32	pET2 containing the 895-bp *pta-ack* promoter fragment with mutagenized 13-bp motifs (see Fig. 4)	16
pRob1	pET2 containing the 605-bp *aceA* promoter fragment	16
pRob10	pET2 containing the 584-bp *aceB* promoter fragment	16
pRob26	pET2 containing the 605-bp *aceA* with mutagenized 13-bp motif (see Fig. 5)	16
pET28a	Km^r; f1 origin; 6× histidyl fusion vector	Calbiochem-Novabiochem, Schwalbach, Germany
pET28-RamAx6His	pET28 containing the *ramA* structural gene	This work
pK19mobsacB	Km^r; mobilizable (*oriT*); *oriV*	47
pK19Δ*ramA*	pK19mobsacB carrying a 1,159-bp insert with a truncated *ramA* gene, shortened by 364 bp	This work
Oligonucleotides		
pta-bio	5′-biotin-TEG-CAGATTTGTCACGCTGCG-3′	Biotinylated forw primer for *pta-ack* promoter/operator probe
pta-16	5′-TTCGTCCGCCTTGCGTACAG-3′	Rev primer for *pta-ack* promoter/operator probe
*aceb*1-bio	5′-biotin-TEG-TCCTTAAGTGCTGATTCG-3′	Biotinylated forw primer for *aceA*/*aceB* promoter/operator probe
aceAB-rev	5′-TACTAAGTCATTACCCCCGC-3′	Rev primer for *aceA*/*aceB* promoter/operator probe
*ramA*f	5′-GGAATTCCATATGGATACCCAGCGGATTAAAGAT GACG-3′	Forw primer for pET28-RamAx6His (NdeI)
*ramA*r	5′-CCCAAGCTTGGGCTTTTTAAGGCAGTGCGC-3′	Rev primer for pET28-RamAx6His (HindIII)
*ramA*1	5′-CCCAAGCTTGGGTACACTGTACCCTTGTC-3′	Primer for *ramA* deletion (HindIII)
*ramA*2	5′-CGCGGATCCCGCATCAGGAACGCCATTGC-3′	Primer for *ramA* deletion (BamHI)
*ramA*3	5′-CGGGATCCGCGGGTTTTCATCTTTTTCCG-3′	Primer for *ramA* deletion (BamHI)
*ramA*4	5′-CCGGAATTCCGAAGATCTATACGCGAACC-3′	Primer for *ramA* deletion (EcoRI)
1 forw	5′-CCTCCATGATACGTGGTAAG-3′	Forw primer for fragment 1, 1mut 1a, 1a1, 1a9–1a12
1 rev	5′-ATGACACTTCAGGCTTGTGCCT-3′	Rev primer for fragment 1, 1mut, 1b
1a rev	5′-CACGCTTTGAGCAGCACA-3′	Rev primer for fragment 1a, 1a2, 1a3, 1a4
1b forw	5′-TGTGCTGCTCAAAGCGTG-3′	Forw primer for fragment 1b
1a1 rev	5′-GTACTTTACAGCTCTTCA-3′	Rev primer for fragment 1a1
1a2 forw	5′-TGAAGAGCTGTAAAGTAC-3′	Forw primer for fragment 1a2
1a3 forw	5′-CGCTTTCATAGACCCCATTA-3′	Forw primer for fragment 1a3, 1a5–1a8
1a4 forw	5′-ATGGGGGGTGAAGAGCTGTA-3′	Forw primer for fragment 1a4
1a5 rev	5′-AATGCTTATTGCCGTAGA-3′	Rev primer for fragment 1a5
1a6 rev	5′-AAACAATACGGAGCGGTTAC-3′	Rev primer for fragment 1a6
1a7 rev	5′-AAGTTGCGAAGCACCCTTTG-3′	Rev primer for fragment 1a7
1a8 rev	5′-GAAAGTTTTTAGCGGTACTT-3′	Rev primer for fragment 1a8
1a9 rev	5′-TAATGGGGTCTATGAAAGCG-3′	Rev primer for fragment 1a9
1a10 rev	5′-CGCAGCGTGACAAATCTGAC-3′	Rev primer for fragment 1a10
1a11 rev	5′-CCCAGCAAAGCCCCCAATAC-3′	Rev primer for fragment 1a11
1a12 rev	5′-TCCCCGACTGGCCTTTATTAG-3′	Rev primer for fragment 1a12
2 forw	5′-CAAAGTCACTTCCTTAAGTG-3′	Forw primer for fragment 2, 2mut, 2a, 2a1–2a5
2 rev	5′-CTCCTTTTAAAGCATGGG-3′	Rev primer for fragment 2, 2mut and 2b
2a rev	5′-TACTAAGTCATTACCCCCGC-3′	Rev primer for fragment 2a and 2a12
2b forw	5′-ACTTAGTAAAGTTCGCAAAC-3′	Forw primer for fragment 2b
2a1 rev	5′-CCAAACCGATTGACGCACCA-3′	Rev primer for fragment 2a1
2a2 rev	5′-CCCCGCACAAACTTAAAAAC-3′	Rev primer for fragment 2a2
2a3 rev	5′-TCACAACAGGGGTGACCAC-3′	Rev primer for fragment 2a3
2a4 rev	5′-AGCCAGAACTTTGCAAACT-3′	Rev primer for fragment 2a4
2a5 rev	5′-CCGCCCACTTTTTCTGCGA-3′	Rev primer for fragment 2a5
2a6 forw	5′-TCGGTTTGCGTGGTGGCGTG-3′	Forw primer for fragment 2a6
2a6 rev	5′-ATCCGTACTAGCAACTC-3′	Rev primer for fragment 2a6–2a11
2a7 forw	5′-GTTCACACATTGCTCCATCG-3′	Forw primer for fragment 2a7
2a8 forw	5′-GGCATTGGTGCGTCAATCGG-3′	Forw primer for fragment 2a8

Continued on following page

TABLE 1—*Continued*

Plasmid or oligonucleotide	Relevant characteristics or sequence[a]	Source/reference or purpose[b]
2a9 forw	5′-TTTGGGTTTTTAAGTTTTGT-3′	Forw primer for fragment 2a9
2a10 forw	5′-GCGGGGGTGGTCACCCCTG-3′	Forw primer for fragment 2a10
2a11 forw	5′-TGTGGAAGTTTGCAAAGTTCT-3′	Forw primer for fragment 2a11
2a12 forw	5′-GAGTTGCTAGTACGGAT-3′	Forw primer for fragment 2a12–2a14
2a12 rev	5′-TACTAAGTCATTACCCCCGC-3′	Rev primer for fragment 2a12
2a13 rev	5′-CCCGCCTAACCCCGACTTT-3′	Rev primer for fragment 2a13
2a14 rev	5′-CGACTTTTATCTAGGTCACA-3′	Rev primer for fragment 2a14
2a15 forw	5′-CGGGGTTAGGCGGGGGTAATG-3′	Forw primer for fragment 2a15
2a16 forw	5′-ACTTAGTAAAGTTCGCAAAC-3′	Forw primer for fragment 2a16
2a15 rev	5′-ATCTAGTGCCACTCTTC-3′	Rev primer for fragment 2a15, 2a16
ace oligo1 forw	5′-TGTGCGGGGGTGGTCACCCCTGTTG-3′	Forw oligonucleotide for fragment *ace-1*
ace oligo1 rev	5′-CAACAGGGGTGACCACCCCCGCACA-3′	Rev oligonucleotide for fragment *ace-1*
ace oligo2 forw	5′-AAAGTCGGGGTTAGGCGGGGGTAATGACTTAG-3′	Forw oligonucleotide for fragment *ace-2*
ace oligo2 rev	5′-CTAAGTCATTACCCCCGCCTAACCCCGACTTT-3′	Rev oligonucleotide for fragment *ace-2*
pta oligo1 forw	5′-GAGTATTGGGGGCTTTGCTGGGGTCAGAT-3′	Forw oligonucleotide for fragment *pta-1*
pta oligo1 rev	5′-ATCTGACCCCAGCAAAGCCCCCAATACTC-3′	Rev oligonucleotide for fragment *pta-1*
pta oligo2 forw	5′-TCATAGACCCCATTAATGGGGGGTGAAGAG-3′	Forw oligonucleotide for fragment *pta-2*
pta oligo2 rev	5′-CTCTTCACCCCCCATTAATGGGGTCTATGA-3′	Rev oligonucleotide for fragment *pta-2*
ace oligo1 muta forw	5′-TGTGATCGTCAGGTCACCCCTGTTG-3′	Forw oligonucleotide for fragment *ace-1*-ma
ace oligo1 muta rev	5′-CAACAGGGGTGACCTGACGATCACA-3′	Rev oligonucleotide for fragment *ace-1*-ma
ace oligo1 mutb forw	5′-TGTGCGGGGGTGGTCGTAGTTGTTG-3′	Forw oligonucleotide for fragment *ace-1*-mb
ace oligo1 mutb rev	5′-CAACAACTACGACCACCCCCGCACA-3′	Rev oligonucleotide for fragment *ace-1*-mb
ace oligo1 mutab forw	5′-TGTGATCGTCAGGTCGTAGTTGTTG-3′	Forw oligonucleotide for fragment *ace-1*-mab
ace oligo1 mutab rev	5′-CAACAACTACGACCTGACGATCACA-3′	Rev oligonucleotide for fragment *ace-1*-mab
ace oligo2 muta forw	5′-AAAGTATCATGTAGGCGGGGGTAATGACTTAG-3′	Forw oligonucleotide for fragment *ace-2*-ma
ace oligo2 muta rev	5′-CTAAGTCATTACCCCCGCCTACATGATACTTT-3′	Rev oligonucleotide for fragment *ace-2*-ma
ace oligo2 mutb forw	5′-AAAGTCGGGGTTAGGACGTACGAATGACTTAG-3′	Forw oligonucleotide for fragment *ace-2*-mb
ace oligo2 mutb rev	5′-CTAAGTCATTCGTACGTCCTAACCCCGACTTT-3′	Rev oligonucleotide for fragment *ace-2*-mb
ace oligo2 mutab forw	5′-AAAGTATCATGTAGGACGTACGAATGACTTAG-3′	Forw oligonucleotide for fragment *ace-2*-mab
ace oligo2 mutab rev	5′-CTAAGTCATTCGTACGTCCTACATGATACTTT-3′	Rev oligonucleotide for fragment *ace-2*-mab
pta oligo1 muta forw	5′-GAGTATACAGCCTTTTGCTGGGGTCAGAT-3′	Forw oligonucleotide for fragment *pta-1*-ma
pta oligo1 muta rev	5′-ATCTGACCCCAGCAAAAGGCTGTATACTC-3′	Rev oligonucleotide for fragment *pta-1*-ma
pta oligo1 mutb forw	5′-GAGTATTGGGGGCTTTGCACGTAGCAGAT-3′	Forw oligonucleotide for fragment *pta-1*-mb
pta oligo1 mutb rev	5′-ATCTGCTACGTGCAAAGCCCCCAATACTC-3′	Rev oligonucleotide for fragment *pta-1*-mb
pta oligo1 mutab forw	5′-GAGTATACAGCCTTTTGCACGTAGCAGAT-3′	Forw oligonucleotide for fragment *pta-1*-mab
pta oligo1 mutab rev	5′-ATCTGCTACGTGCAAAAGGCTGTATACTC-3′	Rev oligonucleotide for fragment *pta-1*-mab
pta oligo2 muta forw	5′-TCATAGGTACAATTAATGGGGGGTGAAGAGCTGT-3′	Forw oligonucleotide for fragment *pta-2*-ma
pta oligo2 muta rev	5′-ACAGCTCTTCACCCCCCATTAATTGTACCTATGA-3′	Rev oligonucleotide for fragment *pta-2*-ma
pta oligo2 mutb forw	5′-TCATAGACCCCATTAAGCTAGCCAGAAGAGCTGT-3′	Forw oligonucleotide for fragment *pta-2*-mb
pta oligo2 mutb rev	5′-ACAGCTCTTCTGGCTAGCTTAATGGGGTCTATGA-3′	Rev oligonucleotide for fragment *pta-2*-mb
pta oligo2 mutab forw	5′-TCATAGGTACAATTAAGCTAGCCAGAAGAGCTGT-3′	Forw oligonucleotide for fragment *pta-2*-mab
pta oligo2 mutab rev	5′-ACAGCTCTTCTGGCTAGCTTAATTGTACCTATGA-3′	Rev oligonucleotide for fragment *pta-2*-mab

[a] Restriction sites in the oligonucleotides are underlined; mutagenized nucleotides are double underlined.
[b] forw, forward; rev, reverse.

Construction of a *ramA* mutant. To construct a *ramA* mutant of *C. glutamicum*, two DNA fragments were generated by PCR using the oligonucleotide pairs *ramA*1/*ramA*2, creating a 588-bp fragment covering 87 bp upstream of the *ramA* start codon and 478 bp of the 5′ end of *ramA*, and *ramA*3/*ramA*4, creating a 582-bp fragment covering the region between 58 bp and 618 bp downstream of the TGA stop codon. The two fragments were ligated with PCR-generated BamHI restriction sites, thereby creating a fragment containing a 3′-truncated *ramA* gene (lacking 364 bp). The deduced polypeptide of this truncated *ramA* gene should be devoid of a functional helix-turn-helix (HTH) motif. Using the flanking 5′ HindIII and 3′ EcoRI restriction sites of the fragment, the construct was ligated into pK19*mobsacB* and transformed into *C. glutamicum* by electroporation. The truncated *ramA* gene then was introduced into the *C. glutamicum* genome by homologous recombination (double crossover) according to a protocol described by Schäfer et al. (47). The deletion in the resulting strain, *C. glutamicum* RG2, was confirmed by PCR (using primers *ramA*1 and *ramA*4; data not shown) and by Southern blot analysis. For the latter, BamHI-restricted chromosomal DNA from WT *C. glutamicum* and *C. glutamicum* RG2 was hybridized to a labeled *ramA* probe covering the entire open reading frame, resulting in a signal of about 5.3 kb with DNA from the *ramA* mutant and a signal of about 6.8 kb with DNA from the WT strain. According to the restriction map of the *ramA* gene region, these sizes were expected.

Construction of *cat* fusions. The construction and sequence validation of *cat* fusions with the *pta-ack*, *aceA*, and *aceB* promoter regions in plasmid pET2, i.e., of plasmids pRob19, pRob1, and pRob10, were described previously (16).

Enzyme assays. To determine enzyme activities in cell extracts, *C. glutamicum* cells were grown in minimal medium to the exponential growth phase, washed twice in 20 ml of 50 mM Tris-HCl buffer, pH 7.8, and resuspended in 1 ml of the same buffer containing 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT (except for determination of chloramphenicol acetyltransferase [CAT] activity), and 30% (vol/vol) glycerol. The cell suspension was added to 2-ml screw-cap vials together with 250 mg of glass beads (150 to 212 μm; Sigma-Aldrich), and the cells were disrupted with the RiboLyser as described above. After disruption, glass beads and cellular debris were removed by two consecutive centrifugation steps (13,000 × *g*, 4°C, 10 min, and 45,000 × *g*, 4°C, 60 min) and the supernatant was used for the assays. The Biuret method (18), with bovine serum albumin as the standard, was used to determine protein concentrations.

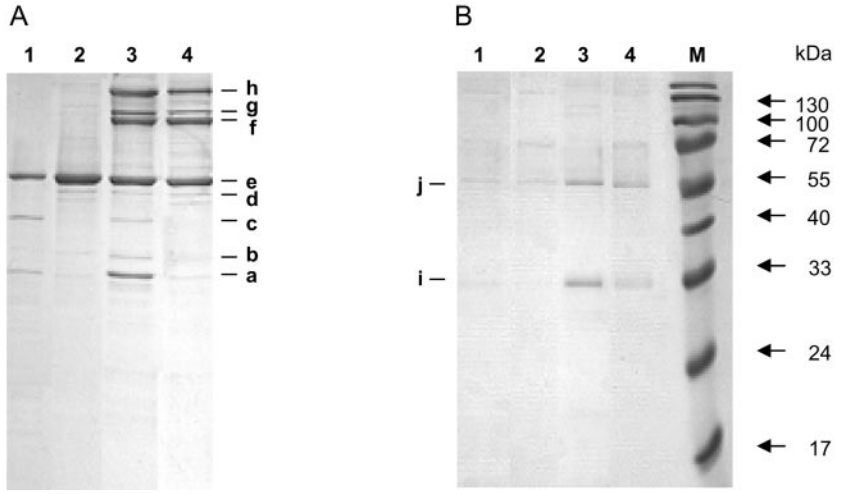

FIG. 1. SDS-PAGE of *C. glutamicum* proteins eluted from a DNA affinity chromatography experiment using *pta-ack* (A) and *aceA*/*aceB* (B) promoter/operator probes. The proteins in lanes 1 and 2 were eluted with 0.3 M NaCl, and those in lanes 3 and 4 were eluted with 1 M NaCl. The protein fractions applied to lanes 1 and 3 were obtained with cell extracts from acetate-grown cells of WT *C. glutamicum*, and those applied to lanes 2 and 4 were from glucose-grown cells. The proteins in bands a to h in panel A and i and j in panel B were identified by MALDI-TOF MS and peptide mass fingerprint analysis, and the assignments are given in the text. The molecular mass standard (lane M) is given to the right.

The specific enzyme activities of CAT, AK, PTA, ICL, and MS were determined exactly as described before (16).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). For peptide mass fingerprinting, the protein bands of interest (each approximately 5 by 1.5 by 1 mm in size) were excised from colloidal Coomassie-stained gels with a scalpel and subjected to in-gel digestion with trypsin as described previously (16). Peptides were extracted by sequential addition of 12 μl of water and 10 μl of 0.1% (vol/vol) trifluoroacetic acid (TFA) in 30% (vol/vol) acetonitrile (ACN); 0.5 μl of the resulting peptide solution was mixed on a stainless steel sample plate with 0.5 μl of a saturated α-cyano-4-hydroxy-*trans* cinnamic acid solution in 50% (vol/vol) ACN–0.1% (vol/vol) TFA. Extraction of the peptides and close external calibration of each sample were also performed as described before (16). Samples were analyzed manually in positive reflector mode with 20-kV accelerating voltage and 63% grid voltage, with the delay time set at 125 ns. Data acquisition and analysis were performed with Voyager Control Panel software, version 5.0, and Voyager Data Explorer software, version 3.5 (Applied Biosystems). The generated mass lists were used to search the nonredundant NCBI database using MS-Fit (6).

Computational analysis. The search for protein domains was performed by using SMART (Simple Modular Architecture Research Tool) (48), NPS@ (Network Protein Sequence Analysis) (7), and the Pfam protein families database (2). Alignments were carried out by using BLAST (basic local alignment search tool) (1).

RESULTS

Isolation and identification of RamA. In the course of previous studies on the regulation of the *pta-ack* operon and the *aceA* and *aceB* genes and on the characterization of the transcriptional regulator RamB (see the introduction), we speculated about an additional regulatory element(s) involved in expression control of these genes in *C. glutamicum* (16). To identify this factor(s), we attempted to enrich a *C. glutamicum* regulatory protein(s) binding specifically to the promoter/operator regions by DNA affinity purification with magnetic streptavidin beads. For this purpose, a 221-bp biotinylated *pta-ack* promoter/operator probe (located 61 to 282 bp upstream of the *pta* start codon) and a 355-bp biotinylated *aceA*/*aceB* promoter/operator probe (intergenic region between *aceA* and *aceB*, located 10 to 365 bp upstream of the *aceA* start codon and 237 to 592 bp upstream of the *aceB* start codon) were linked to streptavidin-coated magnetic beads and incubated with crude extracts from *C. glutamicum* cells grown on minimal medium containing acetate or glucose. To remove unspecifically bound proteins from the promoter/operator probes, we performed several low-salt washing steps (100 mM NaCl) with subsequent magnetic separation. We then eluted specifically bound proteins with buffers containing 0.3 M and 1 M NaCl. The eluted fractions were analyzed by SDS-PAGE. As shown in Fig. 1A (*pta-ack* probe) and Fig. 1B (*aceA*/*aceB* probe), several proteins were highly enriched (especially with the *pta-ack* probe) from the extracts of both acetate- and glucose-grown cells. The protein bands labeled a to h in Fig. 1A and those labeled i and j in Fig. 1B were isolated and subjected to MALDI-TOF MS and peptide mass fingerprint analysis. Comparison of the results with the nonredundant NCBI database resulted in the identification of nine *C. glutamicum* proteins. Protein bands b and c correspond to the 50S ribosomal protein L2 RplB (Cg0598) and the stress-sensitive restriction system protein 1 CgllR (Cg1997), respectively. The proteins in bands d, f, g, and h were identified as elongation factor Tu (EF-Tu) (Cg0587), ppGpp synthetase GpsI (Cg2166), an unknown hypothetical protein (Cg2160), and transcription termination factor Rho (Cg1354), respectively. The prominent bands e and j eluted from the *pta-ack* and *aceA*/*aceB* probes, respectively, both correspond to the transcriptional regulator RamB (encoded by Cg0444 [characterized in reference 16). Finally, the proteins of about 33 kDa in bands a and i correspond to a protein annotated "predicted transcriptional regulator" (NP_601759 and CAF21222.1, encoded by *cg2831*). This protein was highly enriched with both the *pta-ack* and *aceA*/*aceB* probes and with extracts from acetate-grown cells of *C. glutamicum*. Significantly smaller amounts of the protein were observed with extracts from glucose-grown cells. Moreover, it is obvious that this protein mainly eluted with buffers containing the higher concentration of NaCl (1 M). The corresponding gene, *cg2831*, was sequenced in the course of the determi-

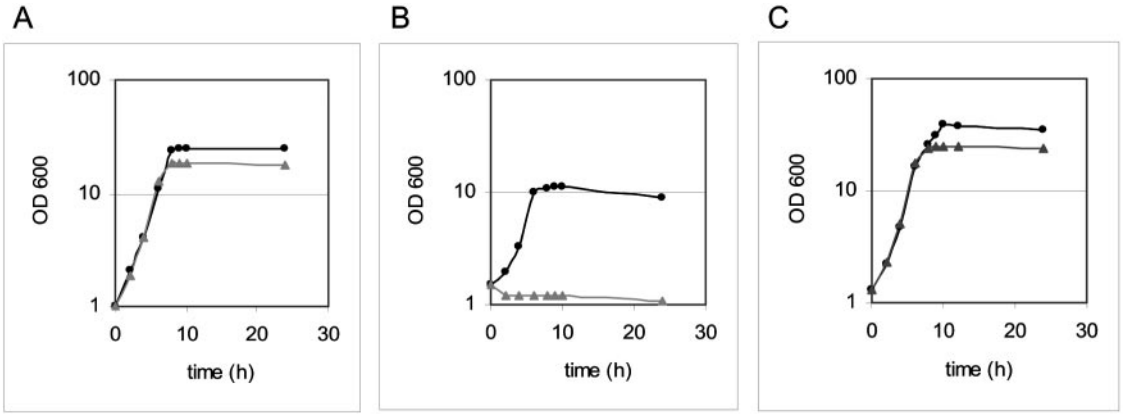

FIG. 2. Growth of WT *C. glutamicum* (black circles) and the *ramA* mutant RG2 (gray triangles) in minimal medium containing 1% glucose (A), 1% acetate (B), or 1% glucose plus 1% acetate (C). Substrate concentrations are as indicated in Materials and Methods.

nation of the genome sequence of *C. glutamicum* (NC_003450 and NC_006958 [25, 27]). It has a length of 846 bp, is preceded by a ribosomal binding site (AGGAGGA), and is followed by a sequence resembling a Rho-independent transcriptional terminator centered 19 bp downstream of the TAA stop codon (ΔG [25°C] = −14.8 kcal mol^{-1}). The deduced protein consists of 281 amino acids and has a predicted molecular mass of 30.8 kDa, which corresponds well with the experimentally determined mass of the protein isolated by DNA affinity chromatography (Fig. 1). A putative HTH motif identified at the C terminus (amino acid positions 214 to 274) shows a high degree of amino acid sequence similarity with HTH motifs from known LuxR-like regulators (see Discussion). Therefore, the protein likely represents a LuxR-type transcriptional regulator. As the protein controls the expression of the *pta-ack* operon and of the *aceA and aceB* genes (see below), we designated it RamA (for "regulator of acetate metabolism A"), with the corresponding gene designated *ramA*.

Inspection of the *C. glutamicum* genomic locus of *ramA* revealed that it is located upstream of and in an orientation opposite that of the genes *cysK* (*cg2833*) and *cysE* (*cg2834*), putatively coding for *O*-acetylserine (thiol)-lyase and serine *O*-acetyltransferase, respectively. Downstream of, and also in an orientation opposite that of, *ramA*, the *pduO* gene (*cg2830*) is located, putatively coding for adenosylcobalamin-dependent diol dehydratase.

Inactivation of *ramA* in *C. glutamicum* and effect on growth. To functionally analyze the RamA protein of *C. glutamicum*, we constructed the *ramA* mutant *C. glutamicum* RG2. In this mutant, the *ramA* gene is shortened by 364 bp and encodes a 3′-truncated RamA protein without the HTH motif. Successful deletion within the *ramA* gene was confirmed by PCR and by Southern blot analysis (see Materials and Methods).

C. glutamicum RG2 and the parental WT strain were tested for growth on different media (Fig. 2). The mutant showed a growth rate similar to that of the WT (μ, 0.53 h^{-1} versus 0.5 h^{-1}) during exponential growth on minimal medium containing glucose (Fig. 2A); however, it entered the stationary phase earlier than the wild-type and the final OD_{600} was lower (18 versus 24). In minimal medium containing acetate as the sole substrate, the *ramA* mutant RG2 was not able to grow (Fig. 2B). In minimal medium containing glucose plus acetate, the mutant initially showed the same growth rate as the WT strain (μ, 0.52 h^{-1}). After having reached an OD_{600} of about 17, the growth rate of the mutant decreased and it entered the stationary phase. The final cell density of the mutant was lower than that of the WT (OD_{600}, 23 versus 35) (Fig. 2C). These results show that the RamA protein is essential for growth of *C. glutamicum* on acetate and suggest that it is also directly or indirectly involved in metabolic pathways that are relevant for glucose catabolism.

Effect of *ramA* inactivation on specific PTA, AK, ICL, and MS activities. To test for an involvement of RamA in the expression control of the *pta-ack* operon and the *aceA* and *aceB* genes, the respective specific activities of PTA, AK, ICL, and MS were measured in crude extracts of WT *C. glutamicum* and *C. glutamicum* RG2 after growth of the cells in minimal medium containing acetate, glucose plus acetate, or glucose alone (Fig. 3). The specific activities of PTA and AK in WT *C. glutamicum* were four- to fivefold higher after growth on acetate than after growth on glucose. In cells grown in the presence of both carbon sources, the specific activities were also higher than in cells grown on glucose, although they were reduced by about one-third compared to the activities of acetate-grown cells. In contrast, cells of the mutant *C. glutamicum* RG2 grown on the mixture showed specific activities of PTA and AK that were in the same range or even lower than those measured in cells grown on glucose. The specific ICL and MS activities in WT *C. glutamicum* cells also were much higher when grown on acetate instead of glucose (Fig. 3). When grown on a mixture of both carbon sources, the specific activities in the cells were again reduced by about one-third compared to those in cells grown on acetate alone. In *C. glutamicum* RG2 cells, however, only very low basal level activities of ICL and MS were measured, independent of the presence or absence of acetate in the medium. These results lead us suggest that RamA is a transcriptional activator of the *C. glutamicum* *pta-ack* operon and of the *aceA* and *aceB* genes, activating their expression during growth in the presence of acetate and thus leading to higher specific PTA, AK, ICL, and MS activities under these conditions. Furthermore, the data suggest that transcription of the *aceA* and *aceB* genes, but not of the *pta-ack*

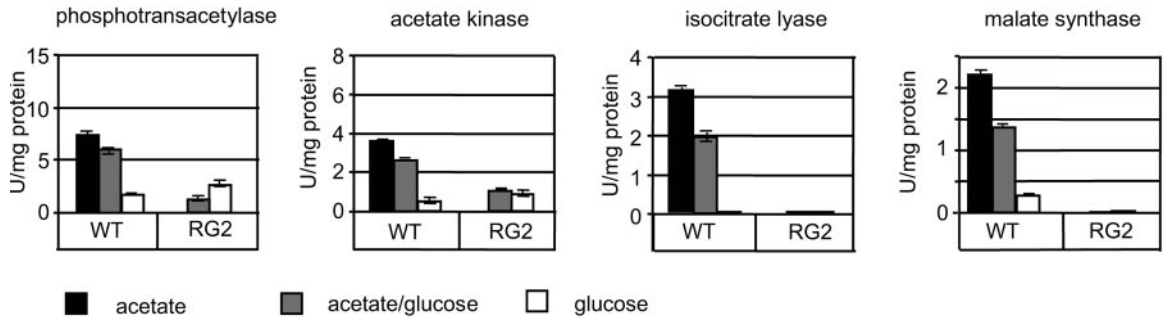

FIG. 3. Specific activities of PTA, AK, ICL, and MS of WT *C. glutamicum* and the *ramA* mutant *C. glutamicum* RG2 in crude extracts of cells grown in minimal medium containing acetate (black bars), glucose and acetate (gray bars), or glucose (white bars). The activities given represent mean values ± standard deviations for at least three independent cultivations and two determinations per experiment.

operon, in WT *C. glutamicum* is even completely dependent on the presence of RamA. The complete lack of ICL or MS activity in *C. glutamicum* RG2 explains the inability of *C. glutamicum* RG2 to grow on acetate as the sole carbon source.

Effect of *ramA* inactivation on *pta-ack* and *aceA* and *aceB* promoter activities. To test whether the higher specific activities of AK, PTA, ICL, and MS in *C. glutamicum* cells grown in the presence of acetate are in fact due to transcriptional control of the respective genes by the RamA protein, comparative promoter fusion assays were carried out with WT *C. glutamicum* and the *ramA* mutant RG2. For this purpose, the promoter-reporter fusion plasmid pET2 and its derivatives pRob19 (*pta-ack* promoter), pRob1 (*aceA* promoter), and pRob10 (*aceB* promoter) were transformed in both strains and, after growth in minimal medium containing glucose plus acetate or glucose alone, the respective promoter activities were tested by measuring the specific activity of the reporter gene product CAT. Plasmid pET2 did not confer CAT activity to either strain, independent of the carbon source used. In WT *C. glutamicum* cells, the pRob19 fragment conferred about 3-fold-higher and the pRob1 and pRob10 fragments more than 50-fold-higher CAT reporter gene expression when they were grown in medium containing acetate plus glucose instead of medium containing glucose alone (Table 2). This result is in agreement with the *pta-ack*, *aceA*, and *aceB* expression ratios observed in previous studies (reviewed in references 16 and 17) and indicates induction or derepression of these genes in the presence of acetate in the growth medium. In *C. glutamicum* RG2(pRob19) cells, however, CAT activity was low and independent of the presence of acetate in the growth medium; in RG2(pRob1) and RG2(pRob10) cells, no CAT activity at all was detected (Table 2). These results correspond well to the (very) low specific AK, PTA, ICL, and MS activities of the *ramA* mutant in the presence of acetate (Fig. 3). Taken together, the results indicate that RamA activates the transcription of the *pta-ack* operon and of the *aceA* and *aceB* genes in the presence of acetate; thus, the RamA protein is a novel positive transcriptional regulator of genes involved in acetate metabolism of *C. glutamicum*.

TABLE 2. Specific CAT activities of WT *C. glutamicum* and *C. glutamicum* RG2 cells carrying the *pta-ack*, *aceA*, and *aceB* promoter fragments in plasmids pRob19, pRob1, and pRob10, respectively, and grown in minimal medium containing acetate and glucose or glucose alone as the carbon and energy source

Strain	Specific CAT activity (U/mg protein)[a]	
	Acetate + glucose	Glucose
C. glutamicum WT (pRob19)	0.31	0.10
C. glutamicum WT (pRob1)	3.27	0.02
C. glutamicum WT (pRob10)	2.38	0.04
C. glutamicum RG2 (pRob19)	0.12	0.12
C. glutamicum RG2 (pRob1)	<0.01	<0.01
C. glutamicum RG2 (pRob10)	<0.01	<0.01

[a] The promoter activities given are averages of at least three independent cultivations and two determinations per experiment. The standard deviations were in all cases below 10%.

Binding of purified RamA protein to the *pta-ack* and *aceA*/*aceB* promoter/operator regions and identification of the RamA binding sites. To test for the binding of RamA to the *pta-ack* and the *aceA* and *aceB* promoter regions, we performed EMSAs with a series of *pta-ack* and *aceA*/*aceB* promoter fragments. For this purpose, RamA was synthesized as a hexahistidyl-tagged fusion protein in *E. coli* BL21(DE3) and purified by affinity chromatography. Different amounts of purified RamA protein were then incubated separately with the promoter fragments shown in Fig. 4A and Fig. 5A (*pta-ack* and *aceA*/*aceB* promoter regions, respectively) and separated on agarose gels. The relevant results of these EMSAs with the *pta-ack* fragments are shown in Fig. 4B, and those with the *aceA*/*aceB* fragments are shown in Fig. 5B; binding or nonbinding of RamA is indicated by +/++ or −, respectively, in Fig. 4A and 5A.

From the left-hand panels of Fig. 4B and Fig. 5B, it can be seen that the 359-bp *pta-ack* fragment 1 and the 593-bp *aceA*/*aceB* fragment 2 were retarded (almost) completely by incubation with 1.0 μg of RamA, which corresponds to molar excesses (protein/DNA) of about 70 for *pta-ack* and 100 for *aceA*/*aceB*. In both cases, apparently two RamA/DNA complexes were formed, suggesting that there are two RamA binding sites in both promoter fragments. Incubation of the fragments with 2 μg of unspecific protein (i.e., bovine serum albumin) did not result in retardation (lanes 5 in the left-hand panels of Fig. 4B and 5B). Conversely, unspecific DNA (i.e., an

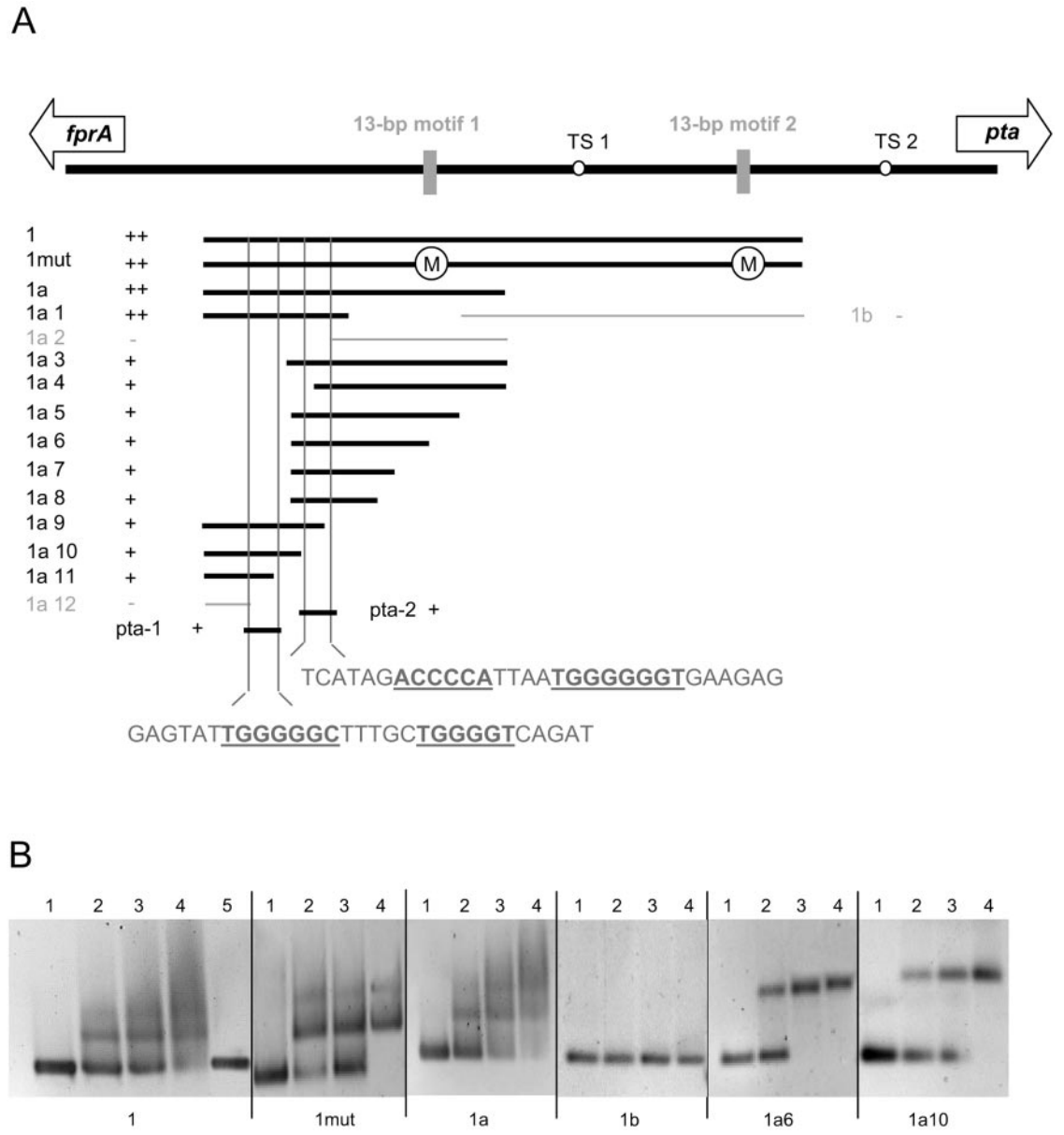

FIG. 4. Genomic locus of the *pta-ack* promoter region and DNA fragments used for mapping of the RamA binding sites (A) and representative EMSAs using RamA protein and different DNA fragments (B). (A) Transcriptional start sites for the *pta-ack* operon are designated TS 1 and TS 2, and the two RamB binding sites are designated 13-bp motif 1 and 13-bp motif 2. The fragments used for the binding assays are given as bars and designated as indicated to the left. Also indicated are binding (+, ++, and thicker bars) and nonbinding (− and thinner bars) of the respective fragments. + and ++ indicate the observation of one or two RamA/DNA complexes, respectively. The circled M's in fragment 1mut indicate that the 13-bp motifs were destroyed by mutation. At the bottom of the panel, the nucleotide sequences of fragments *pta-1* and *pta-2* are given. *fprA* represents a gene encoding a protein with similarity to ferredoxin NADP reductase from *Mycobacterium tuberculosis*. (B) The fragments used in the EMSAs are indicated below the different parts of the gels. Lanes 1 to 4 show EMSAs using 0, 0.25, 0.5, and 1 μg of RamA protein, respectively, and lane 5 shows an EMSA using 2 μg of bovine serum albumin. For details of the EMSAs, see Materials and Methods.

internal fragment of *aceA*) was not shifted by up to 2 μg of RamA protein (data not shown). These results indicate that the binding of RamA to the *pta-ack* and *aceA*/*aceB* target promoters is specific.

To test for involvement of the RamB binding site (i.e., the 13-bp motifs indicated in Fig. 4A and 5A) in binding between the purified RamA and the *pta-ack* and *aceA*/*aceB* promoter regions, we performed EMSAs using respective promoter fragments with mutagenized 13-bp motifs, i.e., fragments 1mut and 2mut in Fig. 4A and 5A, respectively. As shown in Fig. 4B and 5B, the RamA protein was retarded by both of these probes, indicating that RamA binds to sites different from the RamB binding sites.

Successive fragmentation and shortening of the *pta-ack* and *aceA*/*aceB* promoter fragments and employment of these fragments in EMSAs led to the identification of shorter promoter

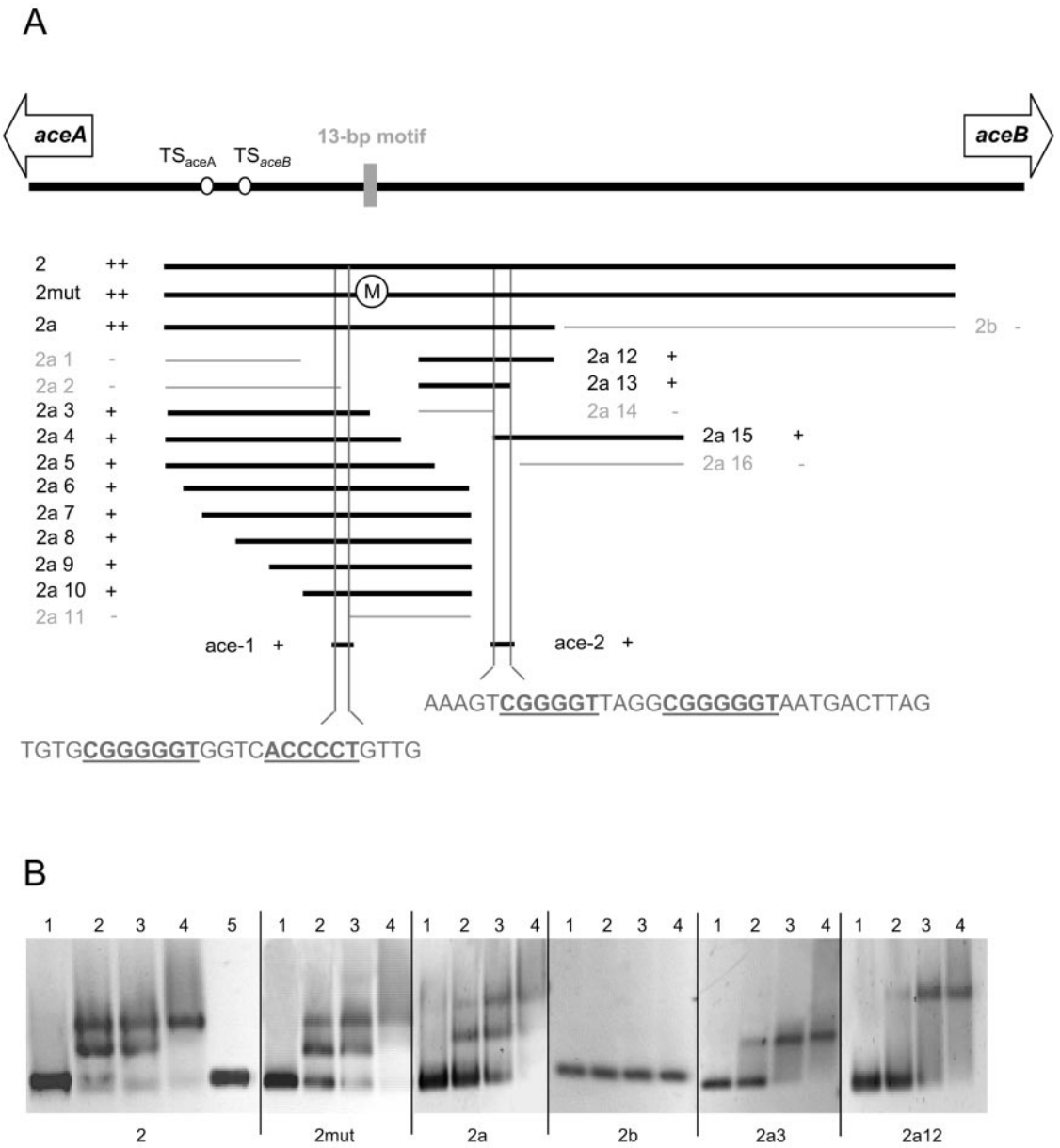

FIG. 5. Genomic locus of the *aceA*/*aceB* intergenic promoter region and DNA fragments used for mapping of the RamA binding sites (A) and representative EMSAs using RamA protein and different DNA fragments (B). (A) Transcriptional start sites of *aceA* and *aceB* are designated TS$_{aceA}$ and TS$_{aceB}$, respectively, and the RamB binding site is designated the 13-bp motif. The fragments used for the binding assays are given as bars and designated as indicated to the left. Also indicated are binding (+, ++, and thicker bars) and nonbinding (− and thinner bars) of the respective fragments. + and ++ indicate the observation of one or two RamA/DNA complexes, respectively. The circled M in fragment 2mut indicates that the 13-bp motif was destroyed by mutation. At the bottom of the panel, the nucleotide sequences of fragments *ace-1* and *ace-2* are given. (B) The fragments used in the EMSAs are indicated below the different parts of the gels. Lanes 1 to 4 show EMSAs using 0, 0.25, 0.5, and 1 μg of RamA protein, respectively; lane 5 shows an EMSA using 2 μg of bovine serum albumin. For details of the EMSAs, see Materials and Methods.

regions still forming two RamA/DNA complexes (e.g., fragments 1a and 2a in Fig. 4B and 5B, respectively) and of promoter regions forming only one RamA/DNA complex (e.g., fragments 1a6 and 1a10 in Fig. 4B and fragments 2a3 and 2a12 in Fig. 5B). The results indicated that the former regions carry two RamA binding sites and that the latter carry only one RamA binding site. The use of PCR-generated DNA fragments finally led to the identification of (i) two separate DNA fragments of the *pta-ack* promoter region (fragments *pta-1* and *pta-2* in Fig. 4A) and (ii) two separate DNA fragments of the *aceA*/*aceB* promoter region (fragments *ace-1* and *ace-2* in Fig. 5A), all four of which are shifted by the RamA protein (Fig. 6B, left-hand panels) and form a single RamA/DNA complex. Fragments *pta-1* and *pta-2* are separated by only 18 bp and centered 135 and 85 bp upstream of the distal transcriptional start site (TS 1 in Fig. 4A) of the *pta-ack* operon. Fragments

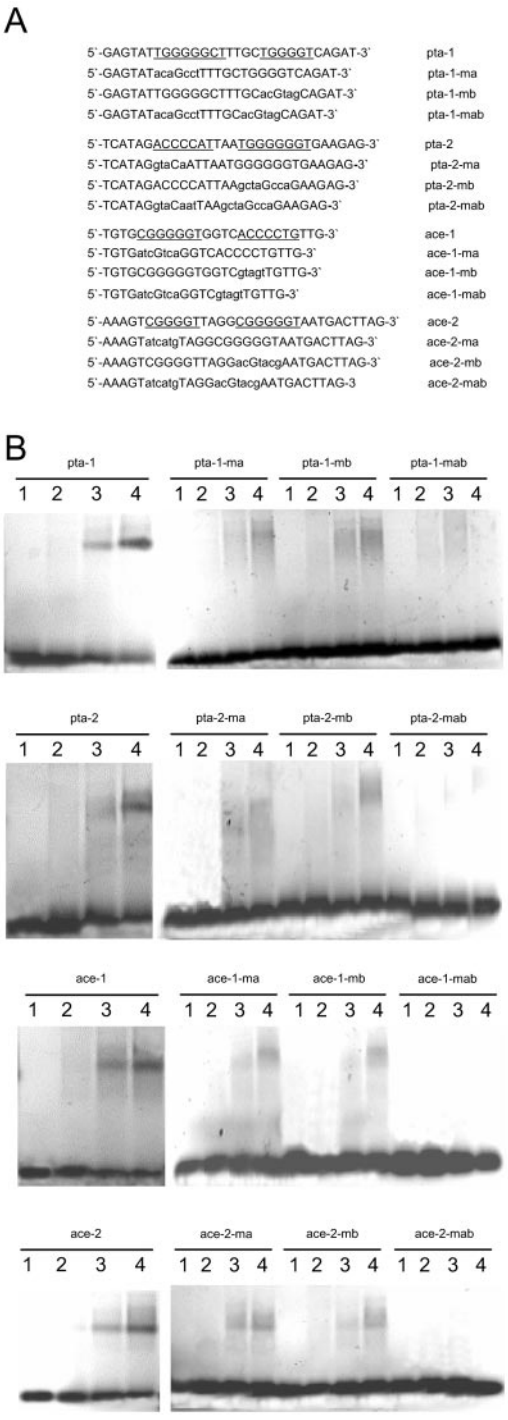

FIG. 6. Alignment of the native *pta-1*, *pta-2*, *ace-1*, and *ace-2* fragments with their derivatives possessing base substitutions in either one or both of the G/C stretches (A) and EMSAs using RamA protein and these DNA fragments (B). In panel A, the native G/C stretches are underlined and the base substitutions in the derivatives are shown in lowercase letters. In panel B, the DNA fragments used are indicated above the different parts of the gels. Lanes 1 to 4 show EMSAs using 0, 0.25, 0.5, and 1 μg of RamA protein, respectively.

ace-1 and *ace-2* are separated by 137 bp and centered 72 bp and 235 bp upstream of the *aceA* transcriptional start site and 52 and 215 bp downstream of the *aceB* transcriptional start site (TS_{aceA} and TS_{aceB} in Fig. 5A). All four fragments contained a common stretch of four to six G residues flanked by T or C residues and, within a distance of four or five nucleotides, a further stretch of four to five G or C residues flanked by A, C, or T residues (Fig. 4A and 5A).

To test for the significance of the C/TGGGG(G)(G)T/C and ACCCC(C)A/T/G motifs indicated above, EMSAs were performed using the native *pta-1*, *pta-2*, *ace-1*, and *ace-2* fragments and identical probes with mutagenized (base-substituted) G or C stretches (Fig. 6). In all four cases, partial retardation of RamA was still observed when one of the two G/C stretches was mutagenized. However, RamA was not retarded by any of the four fragments when both G/C stretches were destroyed. These results indicate that RamA specifically and most favorably binds to tandem G_{4-6} and/or C_{4-6} stretches flanked by specific residues (A or C for the G stretch and A, T, or G for the C stretch) and separated by four or five undefined nucleotides.

The *aceA*/*aceB* fragment 2a12, containing the *ace-2* RamA binding site, was employed to test for possible physiological effectors influencing the binding of RamA. For this purpose, EMSAs were performed using 0 to 1.0 μg of RamA incubated with 0.2 mM acetate, acetyl phosphate, acetyl-CoA, free CoA, 2-oxoglutarate, cAMP, cGMP, NAD, NADH, or 3 mM ATP or ADP. As shown in Fig. 7, none of the metabolites tested led to abolition or to a drastic increase of RamA binding to the fragment. However, it should be noted that shifting of the DNA fragment reproducibly required slightly higher concentrations of RamA protein when incubated with acetyl-CoA and slightly lower concentrations of RamA when incubated with ATP and ADP (Fig. 7). Although the latter results may hint at slight influences of acetyl-CoA, ATP, and ADP on RamA binding to its operator region, our results suggest that none of the metabolites tested is a major effector for expression control by the RamA protein.

DISCUSSION

For growth on acetate and for metabolic adaptation to the presence of this carbon source in the growth medium, *C. glutamicum* requires the acetate-activating enzymes AK and PTA as well as the glyoxylate cycle enzymes ICL and MS (43, 44, 45). All four enzymes are widely distributed among microorganisms; however, in recent years it has become evident that the genomic organization and carbon source-dependent expression control of the respective genes in *C. glutamicum* are different from those of other bacteria (reviewed in references 9 and 17). In a first step toward elucidating the molecular mechanisms of coordinated transcriptional regulation of the four genes in *C. glutamicum*, we recently identified and functionally characterized the RamB protein (16). This protein was shown to bind specifically to conserved 13-bp motifs and thereby to function as a repressor for the *pta-ack* operon and for the *aceA* and *aceB* genes when *C. glutamicum* grows on glucose (16). A second regulatory protein, the glyoxylate bypass regulator GlxR, has been isolated and shown to bind in a cAMP-dependent manner to the *aceA*/*aceB* intergenic region

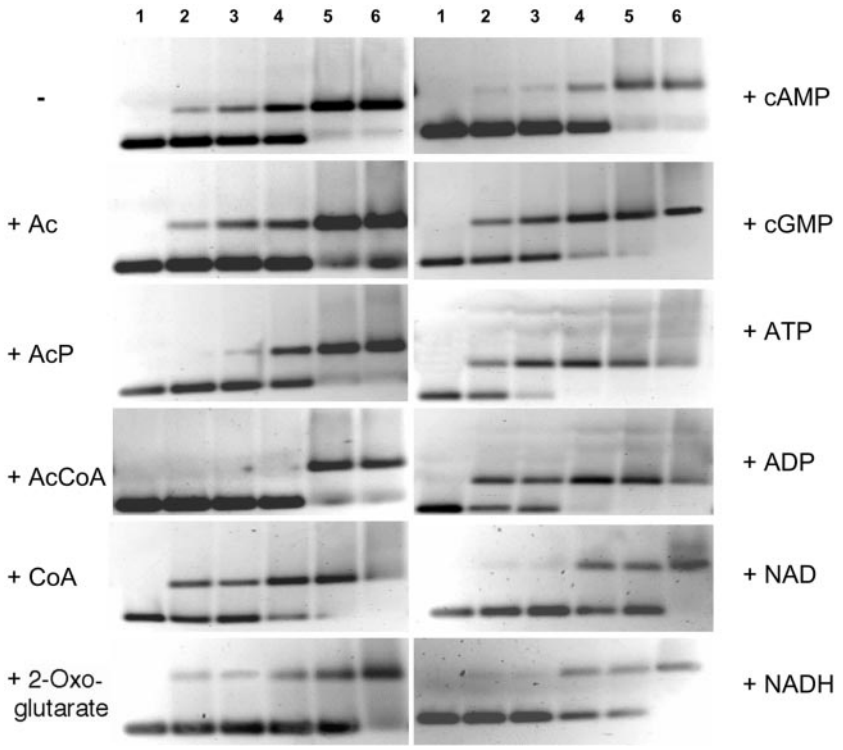

FIG. 7. EMSAs using the *aceA*/*aceB* fragment 2a12 and RamA protein incubated in the absence and presence of 200 μM acetate (Ac), acetyl phosphate (AcP), acetyl-CoA (AcCoA), free CoA (CoA), 2-oxoglutarate, cAMP, cGMP, NAD, NADH, or 3 mM ATP or ADP. Lanes 1 to 6 indicate EMSAs using 0, 0.06, 0.125, 0.25, 0.5, and 1.0 μg of RamA protein, respectively.

and to repress the *aceB* gene (29). Here we provide evidence for RamA as a further transcription factor involved in controlling expression of the *aceA*, *aceB*, and *pta-ack* operons in a manner that is dependently on the presence or absence of acetate in the growth medium. As evidenced by comparative AK, PTA, ICL, and MS activity studies; by comparative reporter gene (*cat*) expression analyses using promoter fusions in WT *C. glutamicum* and the *ramA* mutant RG2; and by binding studies using His-tagged RamA protein and the respective promoter regions, we here show that RamA acts as a positive regulatory protein for expression control of *pta-ack*, *aceA*, and *aceB.* Thus, RamA is a novel transcriptional activator of genes involved in proper adaptation of *C. glutamicum* to acetate as the carbon and energy source.

As evident from our comparative growth experiments with WT *C. glutamicum* and RG2, RamA is essential for growth of this organism on acetate. The inability of the *ramA* mutant to grow on acetate as the sole carbon and energy source can be explained by the almost complete loss of ICL and MS activities. However, strain RG2 also showed a lower final OD_{600} when cultivated on glucose minimal medium. Since ICL and MS are not essential for growth on glucose (43, 44), the growth phenotype of the *ramA* mutant under these conditions cannot be explained by the lack of the two enzymes. Instead, it can be assumed that RamA somehow controls the expression of enzymes involved in glucose metabolization. However, there is so far no experimental evidence of RamA-directed control of glucose metabolism, and further studies are necessary to clarify the significance of RamA for expression of genes other than *aceA*, *aceB*, and *pta-ack*.

BLAST databank analyses with the deduced amino acid sequence of RamA revealed significant identity of this protein with putative regulatory proteins from other corynebacteria, i.e., with CE2445 from *C. efficiens* (95% identity), DIP1889 from *C. diphtheriae* (81%), and JK0397 from *C. jeikeium* (76%). RamA also shows 45% sequence identity to a putative LuxR-type regulator (BAC69743) from *Streptomyces avermitilis* and to a putative response regulator (SCO6194; CAB36602) from *Streptomyces coelicolor*. However, none of the proteins with similarity to RamA have yet been functionally characterized; thus, the regulatory role of any of these proteins remains speculative. BLAST searches, as well as SMART analysis, revealed the presence of a typical LuxR-type HTH motif at the C terminus of RamA (amino acids 214 to 274) showing 30 to 50% identity to the HTH motifs of LuxR-type transcriptional regulators such as LuxR from *Vibrio fischeri*, *Rhodoferax ferrireducens*, *Chloroflexus aurantiacus*, and other bacteria; GerE from *Bacillus subtilis*; and MalT and MalT-like proteins from *E. coli* and other prokaryotes. In these transcriptional activators, which belong to the LuxR-FixJ protein family, the HTH motif in general is located at the C terminus of the respective proteins and has been shown to be important for DNA binding (54). Due to the presence of the LuxR-type HTH motif in the C-terminal domain, and in agreement with the HTH position/function relationship postulated by Perez-Rueda et al. (42), the *C. glutamicum* RamA protein, with its HTH motif at its C

terminus, likely would represent an activator protein. This assumption has been verified here by characterization of the *ramA* mutant *C. glutamicum* RG2 and by the transcriptional fusion experiments.

Further analysis of the RamA protein sequence using the Pfam HMM database revealed that the N terminus (amino acids 8 to 146) of RamA shows similarity to GAF domains found in cGMP-specific phosphodiesterases in prokaryotes and eucaryotes, in cyanobacterial and plant phytochromes, in adenylyl cyclase from *Anabaena*, and in the formate hydrogen lyase transcriptional activator FhlA from *E. coli* (13, 22, 23, 38). The GAF domains are known to bind small molecules such as cAMP and cGMP (22, 38, 50), formate (23), and/or 2-oxoglutarate (36, 39). Since in many bacteria cyclic nucleotides are involved as signaling molecules in the regulation of gene expression in response to environmental stimuli (including the carbon source) (4, 6, 12, 15, 37), and since 2-oxoglutarate has been implicated as a key metabolic signal of carbon status (41), cAMP, cGMP, and 2-oxoglutarate were attractive candidates as potential metabolic effector(s) controlling the activity of RamA. However, we did not observe any significant effect of cAMP, cGMP, or 2-oxoglutarate on the DNA-binding activity of RamA; thus, it remains unclear whether the GAF domain in RamA has a function in triggering its regulatory function.

Since our DNA affinity chromatography revealed the presence of RamA in both glucose- and acetate-grown cells of *C. glutamicum*, it can be expected that RamA function is triggered by an effector which is specific for the one or the other growth condition. To identify this effector for RamA activity (or inactivity), we further tested acetate, acetyl phosphate, acetyl-CoA, free CoA, NAD, NADH, ATP, and ADP for their effect on the DNA-binding activity of RamA. Except for NAD and NADH, all of these metabolites are involved in acetate activation during growth of *C. glutamicum* on acetate and thus were possible effector candidates. However, under the conditions employed, none of these candidates showed a significant positive or negative effect on the DNA binding of RamA, indicating that none of them represents the direct physiological trigger of the transcriptional regulation brought about by RamA.

As is the case with RamA, the RamB repressor protein binds to the promoter/operator regions of the *pta-ack* operon and of *aceA*/*aceB*, and so far, we had been unable to identify a metabolite preventing or increasing its binding activity (16). In contrast, the glyoxylate bypass regulator GlxR contains a domain with similarity to cAMP binding motifs, and in fact, cAMP was shown to be essential for binding of GlxR to the *aceA*/*aceB* intergenic region (29). Since *C. glutamicum* showed a higher intracellular cAMP level during growth in glucose medium than during growth in acetate medium, Kim et al. speculated that GlxR may repress the glyoxylate bypass genes in the presence of glucose; however, overexpression of the *glxR* gene in *C. glutamicum* had no effect on the specific ICL and MS activities when grown in glucose medium but surprisingly resulted in ten- to fifteenfold reductions of these activities when the cells were grown in acetate medium (29). This obvious repression of the ICL and MS genes in acetate-grown cells of *C. glutamicum* certainly does not reflect the physiological situation, and Kim et al. explained the result by the multicopy effects of *glxR*. Unfortunately, so far it has not been possible to obtain and analyze a *glxR* mutant (29). Thus, although the available data suggest that GlxR represents a cAMP-triggered repressor for expression of the *aceA* and *aceB* genes, the physiological function and the mechanism of expression control by GlxR remain to be clarified.

Using subfragments and mutational analysis, two RamA binding sites were identified upstream of the *pta-ack* operon and two in the *aceA*/*aceB* intergenic region. Alignment of the corresponding sequences revealed a minimal consensus sequence consisting of tandem A/C/TG_{4-6}T/C or AC_{4-5}A/G/T stretches separated by four or five arbitrary nucleotides. Although we also observed partial retardation in our EMSAs with fragments mutated in one of the stretches, we regard the tandem stretches as the physiological binding site, since complete retardation was observed only when the fragments contained two intact half sites. The two RamA binding sites within the promoter/operator regions are separated by different distances, and all of the sites vary in location with respect to the transcriptional start site(s) of the target genes. In the cases of the *pta-ack* operon and of *aceA*, the identified binding sites are located upstream of the transcriptional start sites (that of *pta-ack* is centered 134 and 87 bp upstream of TS1 and 246 and 199 bp upstream of TS2; that of *aceA* is centered 236 and 72 bp upstream of the transcriptional start), whereas they are located downstream (50 and 218 bp) of the transcriptional start site in the case of *aceB* (Fig. 4A and 5A). Therefore, the question of how RamA activates expression of the target gene, despite this variance in binding location, arises. One possibility is that RamA somehow interacts with (DNA-bound) RamB and/or with an additional, hitherto unidentified protein. Such an interaction might lead to tertiary DNA structures facilitating (or preventing) access of the RNA polymerase and thus allowing (or preventing) gene expression. A point in favor of an interaction between RamA and RamB may be that the respective binding sites are located close to one another (Fig. 4A and 5A). However, we have so far no experimental evidence for a protein-protein interaction involving RamA. Another possibility is the formation of tertiary DNA structures brought about by binding of RamA to two or more binding sites. The presence of two binding sites in both the *pta-ack* and *aceA*/*aceB* promoter regions may allow the formation of DNA loop structures, and these again may have an influence on the binding and/or activity of the RNA polymerase.

With respect to the nature and location of the consensus motif proposed here for the *C. glutamicum* RamA binding site, the relatively low but significant similarity (30% identity) of the RamA C terminus and the LuxR-type HTH of the MalT proteins from *E. coli* and from *Klebsiella pneumoniae* must be mentioned again (see also above). MalT is a transcriptional LuxR-type activator for maltose-inducible operons, it is activated by maltotriose and ATP, and it recognizes the nucleotide sequence 5′-GGGGAT/GGAGG-3′ as a binding site (5, 54). All promoters of the *K. pneumoniae* maltose regulon contain pairs of this binding site in direct repeat and separated by three nucleotides (54). This tandem organization of the motifs, the variation of the binding sites in location and orientation with respect to the transcriptional start sites of target genes, and the fact that the consensus sequence of the MalT binding site shares the long stretch of G residues with the RamA binding

motif may indicate that the regulatory mechanisms of MalT and RamA are somehow related.

Recent DNA microarray experiments using RNA from *C. glutamicum* cells grown on glucose or acetate revealed, aside from the *pta-ack* operon and the *aceA* and *aceB* genes, about 55 genes with different amounts of transcript (17, 20, 40). Among these genes/operons were those for several enzymes of the central metabolism of *C. glutamicum*, e.g., for some of the tricarboxylic acid cycle enzymes and for some involved in sugar metabolism of this organism. For 11 of these genes belonging to the acetate stimulon, we showed the 13-bp RamB binding motif (with up to five mismatches) to be present in their promoter regions, and we therefore suggested that RamB might have broader significance in controlling the central metabolism of *C. glutamicum* (16). Since it is reasonable to speculate that RamA is also involved in expression control of genes belonging to the acetate stimulon, we analyzed the 800-bp upstream regions of all of those candidate genes for the presence of the pair of $NG_{4-6}TN$ or $NC_{4-5}N$ stretches, allowing a separation of up to six nucleotides. This analysis revealed the presence of tandem motifs upstream of the succinate dehydrogenase operon *sdhCAB* ($TG_5T\ N_2\ TG_5A$, centered 71 bp upstream of the start codon), of the aconitase gene *acn* ($TG_5\ N_5\ AG_5T$, centered 280 bp upstream of the start codon and 170 bp upstream of the main *acn* transcriptional start site) (32), and of the phosphoenolpyruvate carboxykinase gene *pck* ($TG_4T\ N_4\ TG_4A$, centered 602 bp upstream of the start codon). The promoter regions of all three genes, *sdhCAB*, *acn*, and *pck*, also contain a typical RamB binding site (16), suggesting that these genes, together with the *pta-ack* operon and the *aceA* and *aceB* genes, are controlled by coordinated action of RamA and RamB. However, further studies are required to experimentally prove the functionality of the putative RamA and RamB binding sites in front of the *sdhCAB*, *acn*, and *pck* genes and to clarify the relevance of the RamA and RamB proteins for expression control of these genes.

By comparison of the expression profiles of the WT of *C. glutamicum* and of a mutant defective in the regulator-of-iron proteins (RipA), Wennerhold et al. (56) recently found that the mutant contained increased levels of *pta* mRNA under iron starvation. Moreover, these authors showed binding of the RipA protein to two conserved RipA binding motifs in the *pta-ack* promoter region. These binding motifs are centered at positions −111.5 and +156.5 with respect to the transcriptional start site TS1 of the *pta*-ack operon and thus do not interfere with either the RamA or the RamB binding site. Although we used a *pta-ack* promoter fragment containing one of the RipA binding sites, we did not observe RipA in our DNA affinity enrichments. In fact, we did not expect to enrich the RipA protein, since we grew our cultures under an excess of iron and it had been shown that expression of the *ripA* gene is repressed under iron excess (by the global iron repressor DtxR) and derepressed under iron starvation (32, 56). However, although not relevant under the conditions applied here, all of the findings show that the operon encoding AK and PTA is not only influenced by the carbon source (i.e., under control of RamA and RamB) but also regulated by the iron content of the medium, i.e., under indirect and direct control of DtxR and RipA, respectively.

From the results of this and other very recent studies of the regulation of the acetate metabolism of *C. glutamicum*, it becomes evident that the genes encoding AK, PTA, ICL, and MS are under the control of a variety of transcriptional regulators, i.e., RamA, RamB, GlxR, DtxR, and RipA (16, 29, 56). These regulators obviously permit the adaptation of this industrially important organism to specific extracellular and intracellular nutritional environments. However, many questions about the molecular mechanism of activation and repression, the signals involved, and the interplay of the regulatory proteins remain to be answered by further investigations.

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ADDENDUM IN PROOF

After this article was accepted for publication, Hansmeier et al. (N. Hansmeier, A. Albersmeier, A. Tauch, T. Damberg, R. Ros, D. Anselmetti, A. Pühler, and J. Kalinowski, Microbiology, in press) provided further information on the RamA regulator. These authors showed convincingly that it activates transcription of the *cspB* gene, which encodes the S-layer protomer PS2 in *C. glutamicum* ATCC 14067.

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