JOURNAL OF BACTERIOLOGY, Feb. 2007, p. 1145–1149 Vol. 189, No. 3
0021-9193/07/$08.00+0 doi:10.1128/JB.01061-06


# NOTES

## RamB, the Transcriptional Regulator of Acetate Metabolism in *Corynebacterium glutamicum*, Is Subject to Regulation by RamA and RamB[▽]

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Received 19 July 2006/Accepted 5 November 2006

**In *Corynebacterium glutamicum*, the transcriptional regulator RamB negatively controls the expression of genes involved in acetate metabolism. Here we show that RamB represses its own expression by direct interaction with a 13-bp motif in the *ramB* promoter region. Additionally, *ramB* expression is subject to carbon source-dependent positive control by RamA.**

*Corynebacterium glutamicum* is an aerobic gram-positive soil bacterium well known for its use in large-scale biotechnological production of L-glutamate and lysine (10–14). We are interested in acetate metabolism and its regulation in this organism (7), and we recently identified and characterized two regulatory proteins, designated *r*egulator of *a*cetate *m*etabolism *A* and *B* (RamA and RamB, respectively) from *C. glutamicum* ATCC 13032 (4, 6). RamA binds to single or tandem stretches of A/C/TG$_{4-6}$T/C or AC$_{4-5}$A/G/T and thereby acts as an activator of the *pta*, *ack*, *aceA*, and *aceB* genes, encoding phosphotransacetylase, acetate kinase, isocitrate lyase, and malate synthase, all involved in acetate metabolism of *C. glutamicum*. RamA was also shown to activate transcription of the surface layer protein gene (*cspB*) in *C. glutamicum* ATCC 14067 (9). Furthermore, RamA was shown to bind to the promoter region of its own gene and to act as a repressor there (3). The RamB protein specifically binds to a highly conserved 13-bp motif (AA/GAACTTTGCAAA) and represents a repressor of the *pta*, *ack*, *aceA*, and *aceB* genes when *C. glutamicum* is grown on glucose as a carbon and energy source (6). Inspection of the *ramB* promoter region revealed the presence of putative RamA and RamB binding sites, and this observation prompted us to study expression of *ramB* in cells growing in media containing glucose and/or acetate and to test for a regulatory function of RamA and RamB in *ramB* expression.

The bacterial strains and the plasmids, their relevant characteristics and sources, and the oligonucleotides used in this study are given in Table 1. The media used and the methods not outlined explicitly (DNA preparation, promoter binding assays with hexahistidyl-tagged RamA and RamB fusion proteins, enzyme assays, RNA preparation and identification of the transcriptional start site by the RACE [rapid amplification of cDNA ends] method, generation of polyclonal antibodies, and Western blotting) were described previously (3, 4, 6).

Western blot experiments with RamB-specific antibodies and crude extracts from *C. glutamicum* wild-type (WT) cells grown in minimal medium containing glucose, glucose plus acetate, and acetate showed that RamB is present in cells grown on either carbon source (Fig. 1). In agreement with the results of DNA affinity chromatography experiments (4), the largest amounts of RamB were observed in the cells grown on glucose alone.

To test for *ramB* promoter activities in *C. glutamicum* cells grown in minimal media with glucose and/or acetate, we constructed reporter gene fusions by cloning a 593-bp *ramB* promoter fragment (covering the region 521 bp upstream to 72 bp downstream of the *ramB* start codon) into the promoter test vector pET2, resulting in plasmid p*ramB*p1. This vector was transformed into *C. glutamicum* WT, and the plasmid-bound *ramB* promoter activity of *ramB* was then tested in *C. glutamicum* (p*ramB*p1) by measuring the specific activities of the reporter gene product chloramphenicol acetyltransferase (CAT). As shown in Table 2, *C. glutamicum* (p*ramB*p1) showed about fourfold-lower specific CAT activity when grown in medium containing acetate or a mixture of both carbon sources instead of glucose. To exclude copy number or titration effects, we also tested the *ramB*p1-*cat* fusion after monocopy integration into the chromosome of WT *C. glutamicum*. For this purpose, the PCR-generated fragment *ramB*p1 (Fig. 2A) was ligated into plasmid pRIM2, and the resulting plasmid was integrated into the chromosome as described elsewhere (17). As in the case of the multicopy *ramB*p1-*cat* fusion, the specific CAT activity was about fourfold lower when the cells were grown on glucose instead of acetate (0.004 and 0.001 U/mg protein, respectively). These result show the presence of a promoter on the *ramB*p1 fragment and indicates transcriptional regulation of *ramB*, i.e., induction or derepression when cells are grown on glucose as the sole carbon and energy source and/or repression when acetate is present in the growth medium. Taken together, the

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▽ Published ahead of print on 17 November 2006.

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

| Strain, plasmid, or oligonucleotide | Relevant characteristics or sequence[a] | Source, reference, or purpose |
|---|---|---|
| Strains | | |
| *E. coli* DH5 | *supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | 8 |
| *E. coli* BL21 (DE) | *ompT hsdS*$_B$ ($r_B^-$ $m_B^-$) *gal dcm* (DE3) | 5 |
| *C. glutamicum* WT | Wild-type strain ATCC 13032 | American Type Culture Collection |
| *C. glutamicum* RG1 | WT *C. glutamicum* with truncated *ramB* gene, shortened by 775 bp | 6 |
| *C. glutamicum* RG2 | WT *C. glutamicum* with truncated *ramA* gene, shortened by 364 bp | 4 |
| Plasmids | | |
| pET2 | Multicopy promoter probe vector carrying the promoterless *cat* gene; Km$^r$ | 17 |
| pRIM2 | Integrative promoter probe vector carrying the promoterless *cat* gene; Km$^r$ | 17 |
| pET28-RamAx6His | pET28 containing the *ramA* structural gene | 4 |
| pET29-RamBx6His | pET29 containing the *ramB* structural gene | 6 |
| p*ramB*p1 | pET2 containing the 593-bp *ramB* promoter fragment | This work |
| p*ramB*p3 | pET2 containing the 370-bp *ramB* promoter fragment | This work |
| p*ramB*p3b | pET2 containing the 211-bp *ramB* promoter fragment | This work |
| p*ramB*p3c | pET2 containing the 135-bp *ramB* promoter fragment | This work |
| pRIM-*ramB*p1 | pRIM containing the 593-bp *ramB* promoter fragment | This work |
| pRIM-*ramB*p3c | pRIM containing the 135-bp *ramB* promoter fragment | This work |
| pDrive | Cloning vector; Km$^r$ Amp$^r$ *lacZ*α *ori*f1 *ori*-pUC | QIAGEN GmbH, Hilden, Germany |
| pDrive *ramB*pRACE | pDrive derivative plasmid containing the *ramB* PCR-amplified fragment from the RACE assay | This work |
| Oligonucleotides[b] | | |
| *ramB*pLF forw | 5′-ACGCGTCGACCTAACAGTCATGGCACCTCCAGTGTGG-3′ | *ramB*p1and *ramB*p5 |
| *ramB*pLF rev | 5′-CGCGGATCCCAAGGGTTGCTGCTAAGGATGCCTG-3′ | *ramB*p1, *ramB*p3, *ramB*p3a, *ramB*p3b, *ramB*p3c |
| *ramB*p forw | 5′-AGCGAAAATCAACAAGTTTGCAACACCTCAGT-3′ | *ramB*p 13-bp motif |
| *ramB*p rev | 5′-ACTGAGGTGTTGCAAACTTGTTGATTTTCGCT-3′ | *ramB*p 13-bp motif |
| *ramB*pKF forw | 5′-ACGCGTCGACGCTTCCTCACAGGATACCGA-3′ | *ramB*p3 |
| *ramB*p3a forw | 5′-CAGGGAGCAACTTTGCGCAG-3′ | *ramB*p3a |
| *ramB*p3b forw | 5′-ACGCGTCGACGATGTGGCCCGACCACGCCG-3′ | *ramB*p3b |
| *ramB*p3c forw | 5′-ACGCGTCGACCTCAGTGCCAAGAGTGGTTA-3′ | *ramB*p3c |
| *ramB*p5 rev | 5′-CGCAGGTAGAGCACACTCAAT-3′ | *ramB*p5 |
| CM4 | 5′-GAAAATCTCGTCGAAGCTCG-3′ | cDNA synthesis; 5′-RACE *ramA* and *ramB* transcriptional start site determination |
| Oligonucleotide dT anchor primer | 5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV-3′ | Amplification of dA-tailed cDNA (Roche, 5′/3′ RACE kit) |

[a] Restriction sites in the oligonucleotides are underlined. V represents an A, C, or G.
[b] forw., forward; rev, reverse.

results indicate that the amount of RamB protein in *C. glutamicum* is dependent on the carbon source in the growth medium and that the respective control is due to transcriptional regulation of the *ramB* gene.

To identify the *ramB* transcriptional initiation site (TS) and to localize the respective promoter, total RNA from *C. glutamicum* (p*ramB*p1) was isolated, and cDNA of the 5′ end of the *ramB* transcript was generated with reverse transcriptase. Subsequently, this cDNA was amplified and subcloned into the vector pDrive, and the resulting plasmid, pDrive *ramB*pRACE,

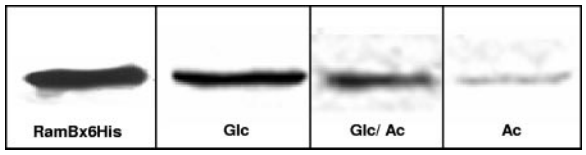


FIG. 1. Western blot using specific antibodies against RamB and cell extracts of *C. glutamicum* WT (100 μg total protein) grown in minimal medium containing glucose (Glc), glucose and acetate (Glc/Ac), or acetate (Ac). The presence of RamB in the extracts was tested by incubation with specific mouse antibodies followed by incubation with peroxidase-conjugated goat anti-mouse immunoglobulin G Fab fragments and visualization by chemiluminescence. Purified RamB protein (RamBx6His; 0.2 μg) was used as a control.

TABLE 2. Specific CAT activities of *C. glutamicum* grown in minimal medium (MM) containing glucose and/or acetate as the carbon and energy source

| *C. glutamicum* strain | CAT sp act (U/mg protein)[a] on MM with: | | |
|---|---|---|---|
| | Glucose | Glucose + acetate | Acetate |
| WT(p*ramB*p1) | 0.28 ± 0.02 | 0.07 ± 0.01 | 0.06 ± 0.01 |
| RG1(p*ramB*p1) (Δ*ramB*) | 0.45 ± 0.03 | 0.21 ± 0.01 | 0.15 ± 0.02 |
| RG2(p*ramB*p1) (Δ*ramA*) | 0.08 ± 0.01 | 0.08 ± 0.01 | NG |
| WT(p*ramB*p3) | 0.28 ± 0.03 | 0.09 ± 0.01 | 0.10 ± 0.01 |
| WT(p*ramB*p3b) | 0.14 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 |
| WT(p*ramB*p3c) | 0.45 ± 0.02 | 0.22 ± 0.01 | 0.16 ± 0.02 |

[a] Values are means ± standarsd deviations obtained from at least three independent cultivations and two determinations per experiment. NG, no growth.

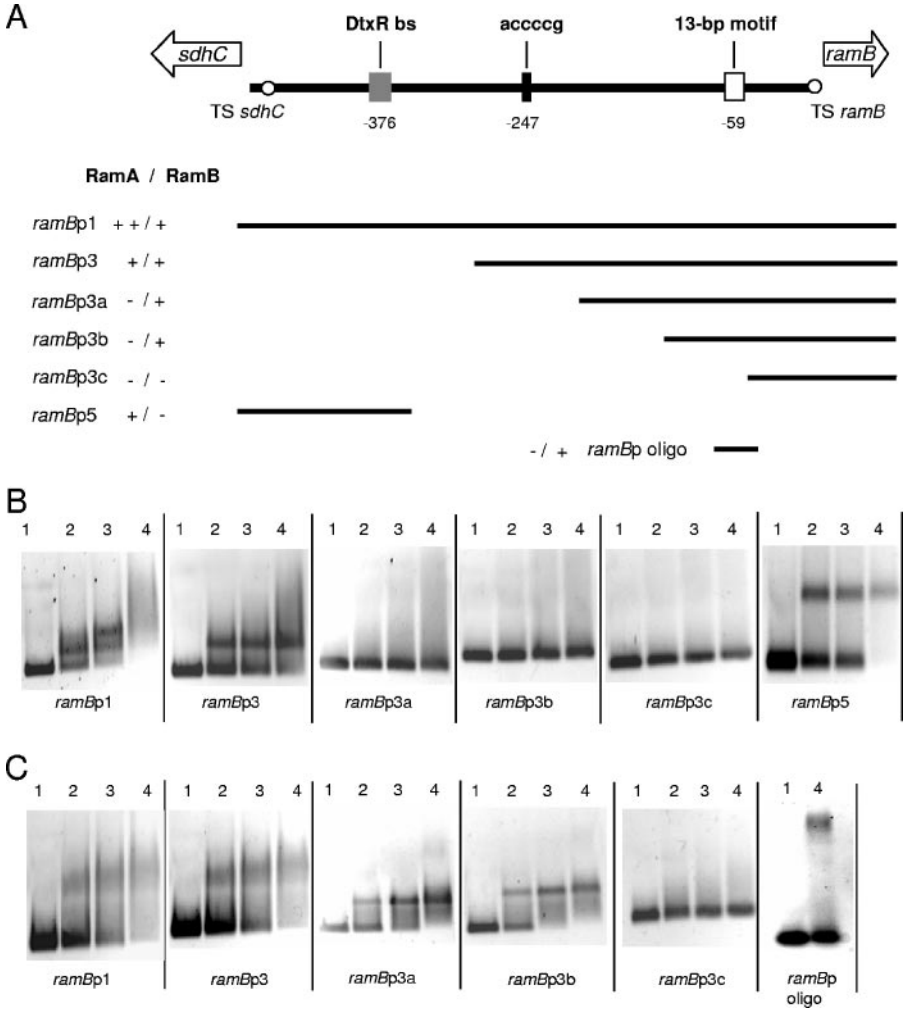


FIG. 2. Genomic locus of the *ramB* promoter region and DNA fragments used for mapping the relevant RamA and RamB binding loci (A) and representative EMSAs using various DNA fragments and RamA protein (B) and RamB protein (C). *sdhC* codes for a protein annotated as a membrane anchor protein of the succinate dehydrogenase. The transcriptional start sites for the *ramB* gene and the *sdhC* gene are designated TS *ramB* and TS *sdhC*, respectively. (A) The RamB binding site is designated as a 13-bp motif. The nucleotide sequence of the putative RamA binding site is given above the black box. The putative DtxR binding site (DtxR bs) is indicated by a dark gray box. The numbers indicate the positions of the centers of these sites relative to the *ramB* TS site. The fragments used for the binding assays are shown as narrow bars. Also indicated are binding (+) and lack of binding (−) of RamA and RamB. Lanes 1 to 4 (B and C) show EMSAs using 0, 0.25, 0.5, and 1.0 μg of RamA or RamB, respectively.

was sequenced, leading to the identification of the TS for *ramB*. In four independently obtained pDrive *ramB*pRACE clones, the TS of *ramB* was found to be an A residue which is identical to the translational start site proposed (11) (NCBI accession number NC_006958). Such leaderless transcripts are not uncommon in *C. glutamicum* and other actinomycetes (16). Inspection of the upstream region of the TS led to the identification of a potential −10 box (TATAGT) which is conserved in five of six nucleotides relative to the −10 consensus sequence described for corynebacteria (TA[C/T]AAT) (15). No apparent −35 region (TTGCCA) could be recognized, which also is a common feature of *C. glutamicum* promoters (15, 16).

Inspection of the *ramB* promoter region revealed the presence of a typical 13-bp RamB binding motif (ACAAGTTTG CAAC; mismatches underlined), centered 59 bp upstream of the *ramB* TS. Furthermore, a nucleotide sequence resembling the RamA binding site was also found, centered 247 bp upstream of the *ramB* TS (Fig. 2A). Further possible RamA binding sites are located in the region near the *sdhC* transcriptional start site (not shown in Fig. 2A). To test whether expression of *ramB* in *C. glutamicum* in fact is subject to transcriptional control by RamA and/or RamB, the *ramB* promoter activities were determined in the *ramB* and *ramA* deletion mutants *C. glutamicum* RG1 and RG2. For this purpose, plasmid p*ramB*p1 (see above) was transformed into both mutants, and after growth in minimal medium containing glucose and/or acetate, the *ramB* promoter activities were tested by measuring the specific CAT activity and compared to that of *C. glutamicum* WT (p*ramB*p1). The *C. glutamicum* RG2 derivative did not grow on minimal medium containing acetate as the sole carbon source, a phenotype which was previously found for the parental strain RG2 (4).

*C. glutamicum* RG1 (p*ramB*p1) cells showed about twofold-higher specific CAT activities than *C. glutamicum* WT (p*ramB*p1) cells on glucose and about threefold-higher activities in the presence of acetate (Table 2). On the other hand, only very low CAT activity was observed in *C. glutamicum* RG2 (p*ramB*p1) cells independent of the presence or absence of glucose. These results indicate that (i) *ramB* transcription is negatively autoregulated by RamB under all conditions tested and (ii) *ramB* expression is positively regulated by RamA when glucose is the sole carbon and energy source. Since the *ramB* mutant RG1 (p*ramB*p1), in spite of a deregulation, showed different CAT activities when grown in medium with glucose alone, glucose plus acetate, or acetate alone (Table 2), it must be concluded that either RamA is responsible for the induction (or derepression) of the *ramB* gene in the presence of glucose or another regulatory factor is involved.

Wennerhold and Bott (18) and Brune et al. (2) identified a binding site for the iron regulator DtxR centered 376 bp upstream of the *ramB* TS. However, although this binding site is closer (105 bp) to the TS of the neighboring succinate dehydrogenase subunit C gene *sdhC* (Fig. 2A), we speculated about a function of this motif for control of *ramB* or *sdhC* (18). To test for involvement of DtxR in the regulation of *ramB* expression, we determined CAT activities in WT *C. glutamicum* (p*ramB*p1) and in *C. glutamicum* Δ*dtxR* (17) transformed with plasmid p*ramB*p1. Both strains showed identical CAT activities in glucose minimal medium under iron limitation (1 μM) and iron excess (100 μM) conditions (data not shown). These results suggest that DtxR is not involved as a transcriptional regulator in the control of *ramB* expression.

Electrophoretic mobility shift assays (EMSAs) with hexahistidyl-tagged RamA and RamB fusion proteins and a series of *ramB* promoter fragments (Fig. 2A) were performed to assay for direct binding of RamA and/or RamB to the putative RamA and RamB binding sites observed within the *ramB* promoter region. Different amounts of purified RamA or RamB protein were incubated with the *ramB* promoter fragments and separated on an agarose gel. The relevant results of these EMSAs with the *ramB* fragments and RamA and RamB protein are shown in Fig. 2B and C, respectively.

The promoter fragments *ramB*p1, *ramB*p3, and *ramB*p5 were retarded effectively by RamA (Fig. 2B). Retardation in all three cases was complete with 1 μg of RamA protein, corresponding to molar excesses (protein/DNA) of about 50. The fragment *ramB*p1 formed two RamA/DNA complexes, while the fragments *ramB*p3 and *ramB*p5 formed only one RamA/DNA complex. No retardation was observed with fragments lacking the putative RamA binding sites (i.e., *ramB*p3a, *ramB*p3b, and *ramB*p3c). These results show relatively tight binding of RamA to the *ramB* promoter region.

As shown in Fig. 2C, the *ramB* promoter fragments containing the putative 13-bp motif (i.e., *ramB*p1, *ramB*p3, *ramB*p3a, and *ramB*p3b) were retarded by RamB, while the fragment *ramB*p3c (without this motif) showed no retardation. A 32-bp oligonucleotide covering the 13-bp motif also revealed retardation, corroborating our conclusion that this motif in fact is responsible for the specific binding of RamB to the *ramB* promoter.

The fragments *ramB*p3, *ramB*p3b, and *ramB*p3c were also tested for *ramB* promoter activity. For this purpose, we constructed respective reporter gene fusions in the promoter test vector pET2, resulting in plasmids p*ramB*p3, p*ramB*p3b, and p*ramB*p3c. The promoter activities of these fragments were tested in *C. glutamicum* WT by measuring specific CAT activity. As shown in Table 2, p*ramB*p3 conferred the same promoter activity to *C. glutamicum* as p*ramB*p1, on all media tested. This result shows that the RamA bindings site located near the *sdhC* TS site has no influence on *ramB* expression. Plasmid p*ramB*p3b, lacking the RamA binding site 247 bp upstream of the *ramB* TS, conferred about twofold-lower promoter activity in glucose medium than p*ramB*p1 and p*ramB*p3, and the activities of WT *C. glutamicum* carrying p*ramB*p3b were comparable to those of the RamA-negative mutant RG2(p*ramB*p1). These results indicate that RamA activates *ramB* expression in *C. glutamicum* by direct binding to the *ramB* promoter region when cells are grown in glucose medium.

The lack of the RamB binding site in plasmid p*ramB*p3c [WT *C. glutamicum*(p*ramB*p3c)] resulted in high *ramB* promoter activities similar to those observed in the *ramB* mutant *C. glutamicum* RG1(p*ramB*p1). In accordance, the specific CAT activity of a *ramB*p3c-*cat* fusion after monocopy integration into the chromosome of *C. glutamicum* WT (tested by ligating fragment *ramB*p3c [Fig. 2A] into plasmid pRIM2 and integration) was significantly higher than that of the *ramB*p1-*cat* fusion, independent of the carbon source used (0.014 and 0.004 U/mg protein on glucose and 0.007 and 0.001 U/mg protein on acetate as the carbon sources). These results show that RamB negatively autoregulates its expression by direct binding to the 13-bp motif located 59 bp upstream of the *ramB* TS.

In conclusion, we provide evidence that expression of the *ramB* gene as well as the amount of RamB protein is significantly higher in glucose-grown cells than in acetate-grown cells, that both RamA and RamB bind to the *ramB* promoter region, and that *ramB* expression is subject to negative control by RamB and to carbon source-dependent positive control by RamA. Since RamA negatively controls the expression of its own gene (3) and additionally positively influences the expression of *ramB*, it can be concluded that RamA is a master regulator of acetate metabolism in *C. glutamicum*. A model summarizing the present knowledge on the regulation network involving RamA and RamB in *C. glutamicum* is shown in Fig. 3. It is interesting that genes encoding orthologs of *ramA* and *ramB* have been found in other corynebacteria, such as *C. efficiens*, *C. diphtheriae*, and *C. jeikeium* (1). In the former two species, the *ramB* ortholog is preceded by a motif resembling the 13-bp motif (centered 59 and 275 bp, respectively, upstream of the postulated translational start codons) and by several motifs similar to the *C. glutamicum* RamA binding sites. These observations may indicate similar regulation of *ramB* expression by RamB itself and by RamA in *C. efficiens* and *C. diphtheriae*.

We thank J. Kalinowski (University of Bielefeld) for helpful comments on our manuscript.

The support of the BMBF (grants 031U213D and 0313105 "Genome research on bacteria relevant for agriculture, environment and biotechnology") and of the Degussa AG is gratefully acknowledged.

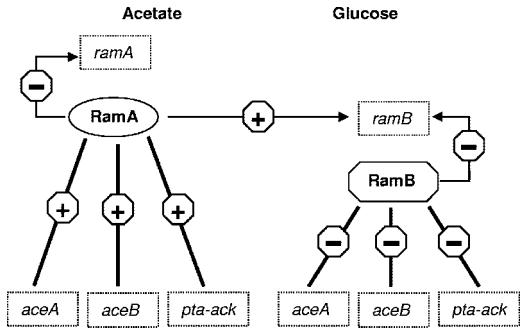


FIG. 3. Model of the regulatory network involving RamA and RamB in *C. glutamicum* grown in medium containing either glucose or acetate as the carbon source. The model is based on previous data (3, 4, 6, 7) and on data obtained here. Activation and repression are indicated by plus and minus signs, respectively. The thickness of the lines give a rough indication of the strength of activation/repression.

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