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Identification of AcnR, a TetR-type Repressor of the Aconitase Gene *acn* in *Corynebacterium glutamicum**

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In *Corynebacterium glutamicum*, the activity of aconitase is 2.5–4-fold higher on propionate, citrate, or acetate than on glucose. Here we show that this variation is caused by transcriptional regulation. In search for putative regulators, a gene (*acnR*) encoding a TetR-type transcriptional regulator was found to be encoded immediately downstream of the aconitase gene (*acn*) in *C. glutamicum*. Deletion of the *acnR* gene led to a 5-fold increased *acn*-mRNA level and a 5-fold increased aconitase activity, suggesting that AcnR functions as repressor of *acn* expression. DNA microarray analyses indicated that *acn* is the primary target gene of AcnR in the *C. glutamicum* genome. Purified AcnR was shown to be a homodimer, which binds to the *acn* promoter in the region from −11 to −28 relative to the transcription start. It thus presumably acts by interfering with the binding of RNA polymerase. The *acn-acnR* organization is conserved in all corynebacteria and mycobacteria with known genome sequence and a putative AcnR consensus binding motif (CAGNACnnncGTACTG) was identified in the corresponding *acn* upstream regions. Mutations within this motif inhibited AcnR binding. Because the activities of citrate synthase and isocitrate dehydrogenase were previously reported not to be increased during growth on acetate, our data indicate that aconitase is a major control point of tricarboxylic acid cycle activity in *C. glutamicum*, and they identify AcnR as the first transcriptional regulator of a tricarboxylic acid cycle gene in the *Corynebacterianeae*.

Corynebacterium glutamicum is a non-pathogenic, aerobic Gram-positive soil bacterium that was described in 1957 (1) as an L-glutamate-excreting bacterium. It has gained considerable interest because of its use in the large scale biotechnological production of L-glutamate (>1 million tons per year) and L-lysine (~0.6 million tons per year) (2–5) and because of its emerging role as a model organism for the *Corynebacterineae*, a suborder of the actinomycetes, which also includes the genus *Mycobacterium* (6).

The tricarboxylic acid cycle is of central importance for the metabolism of *C. glutamicum* because it provides energy and biosynthetic precursors and therefore the flux through this cycle is an important aspect for the production of amino acids of the aspartate and glutamate family. Thus, it is not surprising that several tricarboxylic acid cycle enzymes of *C. glutamicum* have been studied biochemically and/or genetically in the past, *i.e.* citrate synthase (*gltA*, Ref. 7), isocitrate dehydrogenase (*icd*, Ref. 8), 2-oxoglutarate dehydrogenase (9), malate:quinone oxidoreductase (*mqo*, Ref. 10), and malate dehydrogenase (*mdh*, Ref. 11). However, no information is available hitherto on the genetic regulation of this pathway in *C. glutamicum*, although there is evidence that it differs from that of the model bacteria *Escherichia coli* and *Bacillus subtilis*, since *e.g.* glucose and acetate are consumed in parallel rather than successively (12).

No studies have been performed yet in *C. glutamicum* on aconitase (EC 4.2.1.3), which catalyzes the stereospecific and reversible isomerization of citrate to isocitrate via *cis*-aconitate in the tricarboxylic acid cycle and in the glyoxylate cycle. It is an unusual enzyme in that it contains a [4Fe-4S] cluster, which is not involved in electron transfer, but in binding of the substrate (13). Besides their catalytic function, a certain class of aconitases can also have a regulatory function by binding to certain mRNAs and inhibiting or increasing their translation into protein (14–18). This regulatory function is carried out by a catalytically inactive form of aconitase, which is formed under conditions of iron starvation or oxidative stress, when the iron-sulfur cluster is disassembled and the apoprotein is formed.

In many bacteria, expression of the aconitase genes is controlled by transcriptional regulators (see "Discussion"). Here, we provide evidence that this is also the case for the *C. glutamicum* aconitase gene (*acn*)[1] and describe the identification and characterization of the TetR-type repressor protein AcnR.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—All strains and plasmids used in this work are listed in Table I. *C. glutamicum* strain ATCC13032 was used as wild type in this study. Strain Δ*acnR* is a derivative containing an in-frame deletion of the *acnR* gene. For cultivation of *C. glutamicum* in liquid media, 5 ml of CGIII medium (19) or brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI), both supplemented with 2% (w/v) glucose, were inoculated with colonies from a fresh Luria-Bertani (LB) agar plate (20) and incubated overnight at 30 °C and 170 rpm. This first preculture was used to inoculate 60 ml of CGXII minimal medium (21) in a 500-ml shake flask with two baffles. The second preculture was incubated overnight at 30 °C and 120 rpm and then used to inoculate the main culture, for which the same conditions were applied as for the second preculture. The CGXII minimal medium contained as a carbon source either 222 mM glucose, 244 mM sodium acetate, 50 mM sodium citrate, or 104 mM sodium propionate. When citrate was used as carbon source, the medium contained in addition 100 mM $MgCl_2$. When appropriate, the medium was supplemented with 25 μg of kanamycin/ml. *C. glutamicum* strains carrying plasmid pEKEx2 and derivatives thereof were cultivated in the pres-

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This article is dedicated to Prof. Rudolf K. Thauer on the occasion of his 65th birthday.

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[1] The abbreviations used are: *acn*, *C. glutamicum* aconitase gene; RT-PCR, reverse transcriptase-PCR; AcnR, TetR-type repressor protein.

TABLE I
Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source or Ref.
Strains		
C. glutamicum		
ATCC13032	Biotin-auxotrophic wild type	(1)
13032Δ*acnR*	In-frame deletion of the *acnR* gene	This work
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
BL21(DE3)	*ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)	(22) Novagen
Plasmids		
pK19*mobsacB*	Kan^R; vector for allelic exchange in *C. glutamicum*; (pK18 *oriV*$_{E.c.}$, *sacB, lacZ*α)	(27)
pK19*mobsacB*-Δ*acnR*	Kan^R; *pK19mobsacB* derivative containing a crossover PCR product covering the up- and downstream regions of *acnR*	This work
pET24b	Kan^R; vector for overexpression of genes in *E. coli*, adding a C-terminal hexahistidine affinity tag to the synthesized protein (pBR322 *oriV*$_{E.c.}$, P_{T7}, *lacI*)	Novagen
pET24b-Streptag	Kan^R; pET24b derivative adding a C-terminal StrepTag-II to the synthesized protein	Meyer and Bott[a]
pET24b-*acnR*-C	Kan^R; pET24b-Streptag derivative for over-production of AcnR with a C-terminal StrepTag-II	This study
pET28a	Kan^R; vector for overexpression of genes in *E. coli*, adding an N-terminal or a C-terminal hexahistidine tag to the synthesized protein (pBR322 *oriV*$_{E.c.}$, P_{T7}, *lacI*)	Novagen
pET28a-Streptag	Kan^R; pET28a derivative adding an N-terminal StrepTag-II to the synthesized protein	(29)
pET28a-*acnR*-N	Kan^R; pET28a-Streptag derivative for over-production of AcnR with an N-terminal StrepTag-II	This study
pEKEx2	Kan^R; *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression (P_{tac}, *lacI*^{Q}, pBL1 *oriV*$_{C.g.}$, pUC18 *oriV*$_{E.c.}$)	(63)
pEKEx2-*acnR*	Kan^R; pEKEx2 derivative containing the *acnR* gene from *C. glutamicum* under the control of the *tac* promoter	This study
pEKEx2-*acnR*60	Kan^R; pEKEx2 derivative containing a shortened version of the *acnR* gene (first 180 bp) from *C. glutamicum* under the control of the *tac* promoter	This study
pEKEx2-*acnR*89	Kan^R; pEKEx2 derivative containing a shortened version of the *acnR* gene (first 267 bp) from *C. glutamicum* under the control of the *tac* promoter	This study
pEKEx2-*acnR*-C-Strep	Kan^R; pEKEx2 derivative containing the *acnR* gene with a C-terminal StrepTag-II from *C. glutamicum* under the control of the *tac* promoter	This study

[a] Unpublished data.

ence of 1 mM isopropyl-1-thio-β-D-galactopyranoside. For cloning purposes, *E. coli* DH5α (Invitrogen Life Technologies, Inc.) was used and grown at 37 °C in LB medium (20). *E. coli* BL21(DE3) (22) was used for overproduction of the *C. glutamicum* AcnR protein with expression plasmids based on pET24 or pET28 (Novagen, Madison, WI).

Determination of Aconitase Activity—For the determination of the aconitase activity, cells from the CGXII main culture (~25 ml) were harvested with ~25 g of crushed ice (precooled to −20 °C) by centrifugation at 4,000 × *g* for 5 min. The cell pellet was resuspended in 900 μl of Tris-HCl (90 mM; pH 8.0), and the cells were mechanically disrupted by 3× 20 s bead beating with 1 g of zirconia-silica beads (diameter 0.1 mm; Roth, Karlsruhe, Germany) using a Silamat S5 (Vivadent, Ellwangen, Germany). After centrifugation (5 min; 18,320 × *g*; 4 °C), the supernatant was used immediately for the enzyme assay. Aconitase activity was assayed by following the formation of *cis*-aconitate from isocitrate (23) at 20 °C. The assay mixture contained 950–995 μl of 90 mM Tris-HCl pH 8.0 containing 20 mM DL-trisodium isocitrate. The reaction was started by the addition of 5–50 μl of cell extract, and *cis*-aconitate formation was determined by measuring the absorbance increase at 240 nm using a Jasco V560 spectrophotometer. An extinction coefficient for *cis*-aconitate of 3.6 mM^{-1} cm^{-1} at 240 nm was used. One unit of activity corresponds to 1 μmol of isocitrate converted to *cis*-aconitate per min.

Recombinant DNA Work—The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany) or New England Biolabs (Frankfurt, Germany). The oligonucleotides used in this study were obtained from MWG Biotech (Ebersberg, Germany) and are listed in Table II. Routine methods like PCR, restriction, or ligation were carried out according to standard protocols (20). Chromosomal DNA from *C. glutamicum* was prepared as described (7). Plasmids from *E. coli* were isolated with the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). *E. coli* was transformed by the RbCl method (24), *C. glutamicum* by electroporation (25). DNA sequencing was performed with a Licor 4200 sequencer (Licor Inc., Lincoln, NB). Sequencing reactions were carried out with the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Biosciences, Freiburg, Germany).

An in-frame *acnR* deletion mutant of *C. glutamicum* was constructed via a two-step homologous recombination procedure as described previously (26). The *acnR* up- and downstream regions (~500 bp each) were amplified using the oligonucleotide pairs tetR-A-for/tetR-B-rev and tetR-C-for/tetR-D-rev, respectively, followed by a crossover PCR with oligonucleotides tetR-A-for and tetR-D-rev. The resulting PCR product of ~1 kb was digested with EcoRI and HindIII and cloned into pK19*mobsacB* (27) cut with the same enzymes. DNA sequence analysis confirmed that the cloned PCR product did not contain spurious mutations. Transfer of the resulting plasmid pK19*mobsacB*-Δ*acnR* into *C. glutamicum* and screening for the first and second recombination event was performed as described previously (26). The genomic structure of the mutant was first checked by PCR analysis of chromosomal DNA with the primer pair tetR-amp-for/tetR-amp-rev (Table II). The PCR products obtained with wild-type DNA (1.6 kb) and Δ*acnR* mutant DNA (1.1 kb) had the expected sizes. Furthermore, the mutant was controlled by Southern blot analysis (26) using EcoRI-digested DNA and a digoxigenin-labeled PCR product covering the *acnR* gene and the 500-bp up- and downstream DNA as probe. The expected fragment sizes were obtained for wild type (1.8 kb) and the Δ*acnR* mutant (1.3 kb), confirming the successful *acnR* deletion (data not shown).

For the purification of AcnR with a C-terminal StrepTag-II (28), the *acnR* coding region was amplified using the Expand High Fidelity polymerase mix (Roche Diagnostics) and oligonucleotides that introduced an NdeI restriction site overlapping the start codon (acnR-FP-for) and an XhoI restriction site preceding the stop codon (acnR-FP-24rev). The purified PCR product was cloned into the expression vector pET24b-Streptag cut with NdeI and XhoI. The AcnR protein encoded by the pET24 derivative (AcnR-C) contains ten additional amino acid residues (LEWSHPQFEK) at the C terminus. For the purification of AcnR with an N-terminal StrepTag-II, the *acnR* coding region was amplified with the primers acnR-FP-for and acnR-FP-28rev, and the PCR product was cloned into pET28a-Streptag (29) cut with NdeI and XhoI. The AcnR protein encoded by the pET28 derivative (AcnR-N) contains 14 additional amino acid residues (MASWSHPQFEKGAH) at the N terminus. DNA sequence analysis confirmed that the AcnR coding sequence of the resulting plasmids pET24b-acnR-C and pET28a-acnR-N did not contain spurious mutations. For the overproduction of the streptagged AcnR derivatives, the plasmids were transferred into *E. coli* BL21(DE3).

In order to construct plasmids pEKEx2-*acnR*, pEKEx2-*acnR*60, pEKEx2-*acnR*89, and pEKEx2-*acnR*-C-Strep, the *acnR* fragments were amplified with oligonucleotide acnR-for as forward primer and acnR-rev, acnR-rev-60, acnR-rev-89, or acnR-strep-rev, respectively, as reverse primers. After digestion with BamHI and EcoRI, the purified PCR products were cloned into the vector pEKEx2 cut with the same enzymes. All PCR-derived parts of the resulting plasmids were checked by

TABLE II
Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′) and properties[a]
tetR-A-for	TAT ATA GAA TTC TGC AGT TCC CTG CAG GCG AAT C (EcoRI)
tetR-B-rev	*CCC ATC CAC TAA ACT TAA ACA* GCC TGC CGC TAC GGA CAC AAT G
tetR-C-for	*TGT TTA AGT TTA GTG GAT GGG* GAT CTG GTC GAG GGA ACT GTC CG
tetR-D-rev	TAT ATA AAG CTT GTT AGC AAC ACT GTG GTG GCG CC (HindIII)
tetR-amp-for	CTA AGG GCA CTA ACC TGC TCG G
tetR-amp-rev	TTG CCA ACA CCA CGT GTC CAG G
acnR-FP-for	TAT ATA CAT ATG TCC GTA GCG GCA GGC GAC AAA C (NdeI)
acnR-FP28-rev	TAT ATA CTC GAG TTA GTC GCG TTT ACG GAC AGT TC (XhoI)
acnR-FP24-rev	TAT ATA CTC GAG GTC GCG TTT ACG GAC AGT TC (XhoI)
acn-PEK-for	CCT TCA TTA GTG TCG GGC TCA CG
acn-PEK-rev	GGA TTT CGA TGC TTG GAT CGG AAG
acn-PE1*	IRD800-GGG TGC TCT TAG CAT TGA AGG AG
acn-PE3*	IRD800-GTG AGA TTT CGC GAC GGC GTC TG
acn-FP1*	IRD800-GAA AGT CAC ATC ACG CAC GTA CC
RT-1-for	CAG GCG ACA AAC CAA CAA ATA GCC
RT-1-rev	CTC GAC CAG ATC CAA TAC TTC GG
RT-2-for	CGA GAC TCC TAA GAC TGT CAA GG
RT-2-rev	GTT GCT TCT TCC AGT CGG CGT AC
RT-3-for	CAG AAC CAG CTG GTT GAC ATC GC
RT-3-rev	CAT CTG ACG CAG CAC GTA CTG C
acnR-for	TAT ATA GGA TCC AAG GAG ATA TAG ATG TGT CCG TAG CGG CAG GCG AC (BamHI)
acnR-rev	TAT ATA GAA TTC GTC ACG TCG CTC AGC TGC GGC (EcoRI)
acnR-strep-rev	TAT ATA GAA TTC TTA CTT CTC GAA CTG CGG GTG GCT CCA CTC GAG GTC GCG TTT ACG GAC AGT TCC (EcoRI)
acnR-rev-60	TAT ATA GAA TTC TAG GAA CAG GTT TTC TTT GTC ACC G (EcoRI)
acnR-rev-89	TAT ATA GAA TTC ATC TTC CAG CAT TCC TCG CAT C (EcoRI)
RT-dnaE-fw	TGC CCT TCC GGC GAT GTG CAA
RT-dnaE-rv	CTG GAA CCA TGT CGT CCC AGA G
BS-1-for	TCC GAC AAA ACC GCT GCC TAG G
BS-1-rev	GGC GTC TGC TTC GGC TGA GCC
BS-2-for	AGA CGT AGG GTC CTT TTC CAC AG
BS-2-rev	TCA TTA TCC TAA CAG TAC AAG CGT T
BS-3-for	CAA CTT TCC CGC CAG AAC GCT TG
BS-3-rev	GGG TTC TTG GCG CGT CAA TAA CG
BS-4*-for	CAA CTT TCC CGC CAG ATG GCT TCA ACT GTT AGG A
BS-5*-for	CAA CTT TCC CGC GTG AAC GCT TGT ACA CTT AGG ATA AT
BS-6*-for	CAA CTT TCG GGC CAG AAC GCT TGT ACT GTT TCG ATA ATG AAG
BS-7*-for	CAA CTT TCC CGC CAG AAC CGA AGT ACT GTT AG

[a] In some cases oligonucleotides were designed to introduce recognition sites for restriction endonucleases (recognition sites underlined, restriction endonucleases indicated in parentheses) or complementary 21mer sequences for generating crossover PCR products (printed in italics).

TABLE III
Influence of the carbon source on the aconitase activity of C. glutamicum wild type and the ΔacnR mutant strain

C. glutamicum wild type and Δ*acnR* mutant were grown in CGXII minimal medium containing either 222 mM glucose, 50 mM sodium citrate (+ 100 mM $MgCl_2$), 244 mM sodium acetate, or 104 mM sodium propionate as carbon source. In the exponential growth phase at an OD_{600} of about 5, cells were harvested, washed, and disrupted by bead beating. After centrifugation at 15,000 × *g*, the supernatant (cell extract) was immediately used for the aconitase assay. Aconitase activity was determined with DL-trisodium isocitrate as substrate by measuring the formation of *cis*-aconitate at 240 nm.

Carbon source	Aconitase activity	
	13032 wild type	13032 Δ*acnR*
	units/mg of protein	
Glucose	0.20 ± 0.05	1.04 ± 0.06
Citrate	0.53 ± 0.08	1.78 ± 0.40
Acetate	0.82 ± 0.08	1.54 ± 0.08
Propionate	0.48 ± 0.13	2.38 ± 0.21

DNA sequence analysis in order to exclude unwanted mutations.

Preparation of Total RNA—Cultures of the wild type and the Δ*acnR* mutant were grown in CGXII minimal medium containing 4% (w/v) glucose. In the exponential growth phase at an OD_{600} of 5–6, 25 ml of the cultures were used for the preparation of total RNA as described previously (30, 31). Isolated RNA samples were analyzed for quantity and quality by UV spectrophotometry and denaturing formaldehyde agarose gel electrophoresis (20), respectively, and stored at −70 °C until use.

DNA Microarray Analyses—The generation of whole genome DNA microarrays (32), synthesis of fluorescent-labeled cDNA from total RNA, microarray hybridization, washing, and data analysis were per-

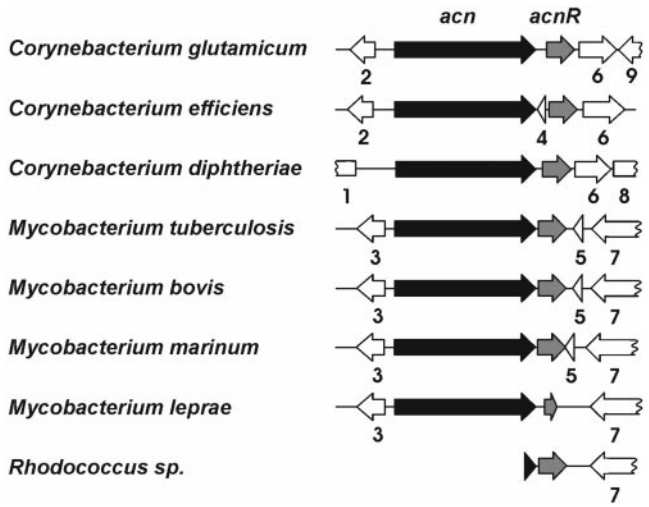

FIG. 1. **Genomic locus of *acn* from *C. glutamicum* and other species of the suborder *Corynebacterineae*.** In all cases except *Mycobacterium leprae*, a gene encoding a transcriptional repressor was identified downstream of *acn*. This gene (shown in *dark gray*) was named *acnR*. In the case of *Rhodococcus sp.*, only the 3′-terminal part of the *acn* gene was available. Other genes were numbered and annotated as follows: *1*, invasin; *2*, *3*, *4*, and *8*, hypothetical proteins; *5*, hypothetical cytosolic protein; *6*, putative GMP synthase-glutamine amidotransferase domain; *7*, ABC transporter, ATP-binding protein; *9*, putative NAD^+-dependent dehydrogenase. Data were taken from the National Center for Biotechnology Information (NCBI) and the bioinformatics software ERGO (Integrated Genomics).

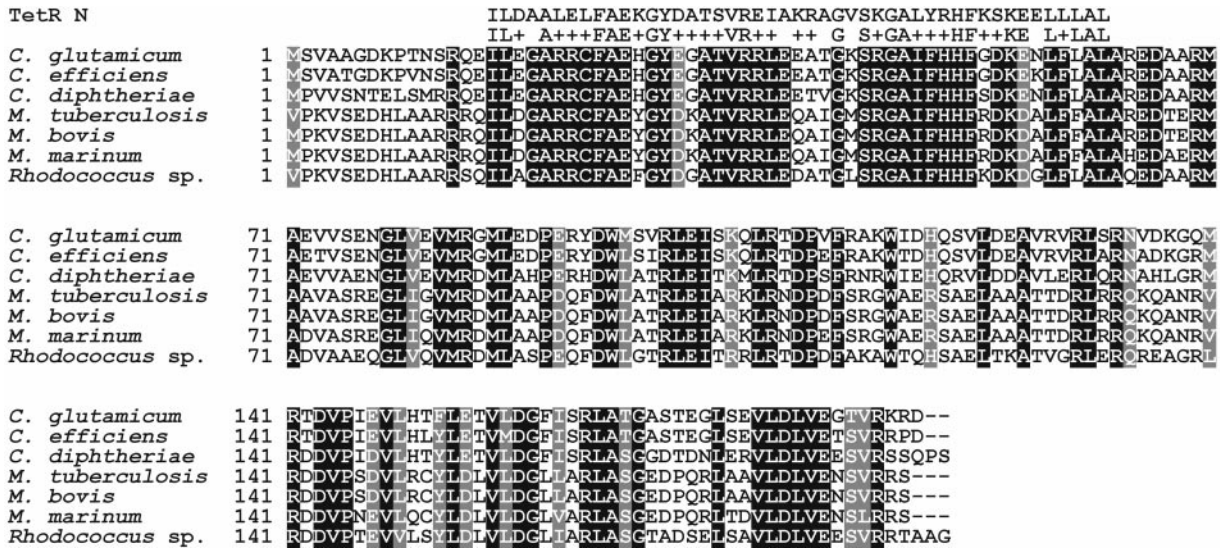

FIG. 2. **Sequence alignment of AcnR proteins from different *Corynebacterineae* species.** Amino acids identical in all sequences are shaded in *black*, other conserved amino acids in *gray*. The N-terminal region of the AcnR proteins (amino acids 16–62) shows clear similarity to the N-terminal part of the TetR family of bacterial regulatory proteins (TetR-N; PFAM00440). The consensus sequence of TetR-N and the amino acids identical to the consensus in the AcnR proteins are shown *above* the *alignment*.

formed as described previously (33–35). Genes that exhibited significantly changed mRNA levels ($p < 0.05$ in a Student's t test) by at least a factor of three were determined in four series of DNA microarray experiments: (i) 10 comparisons of the wild type and the Δ*acnR* mutant cultivated in regular CGXII minimal medium with 4% (w/v) glucose (contains 36 μM iron before autoclaving and ~8 μM iron after autoclaving); (ii) four comparisons of the wild type and the Δ*acnR* mutant cultivated in CGXII-glucose medium with an elevated iron concentration (addition of 100 or 500 μM $FeSO_4$ after autoclaving); (iii) three comparisons of the wild type grown with ~8 μM iron and with 508 μM iron; (iv) three comparisons of the Δ*acnR* mutant grown with ~8 μM iron and with 508 μM iron. The 34 genes that were differentially expressed in one or more of these series of experiments were subjected to a hierarchical cluster analysis (36) as described previously (33, 35).

Primer Extension Analysis—Non-radioactive primer extension analysis was performed using the IRD800-labeled oligonucleotides acn-PE1* and acn-PE3* (Table II) and 15 μg of total RNA as described previously (29). The length of the primer extension products was determined by running the four lanes of a DNA sequencing reaction set up with the same oligonucleotide as used for primer extension alongside the primer extension products on the denaturing polyacrylamide gel. A PCR product obtained with the primer pair acn-PEK-fw/acn-PEK-rev was used as template for the DNA sequencing reaction.

Overproduction and Purification of Streptagged AcnR—E. coli BL21(DE3) carrying pET24b-*acnR*-C was cultivated in 100 ml of LB medium containing 50 μg/ml kanamycin in a 500-ml Erlenmeyer flask at 30 °C until the OD_{600} reached a value between 0.3 and 0.5. Synthesis of the AcnR-C was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM, and the culture was incubated for another 3 h. Cells (final OD_{600} ~1) were washed once and resuspended in 10 ml of buffer W (100 mM Tris-HCl, pH 8.0, 1 mM EDTA). After addition of 1 mM diisopropylfluorophosphate and 1 mM phenylmethylsulfonyl fluoride, the cell suspension was passed three times through a French pressure cell (SLM Aminco, Spectronic Instruments, Rochester) at 207 MPa. Intact cells and cell debris were removed by centrifugation (20 min, 5,000 × *g*, 4 °C). The cell-free extract was subjected to ultracentrifugation (1 h, 150,000 × *g*, 4 °C) and the supernatant applied to a StrepTactin Sepharose column with a bed volume of 1 ml (IBA, Göttingen, Germany). The column was washed with 15 ml of buffer W and streptagged AcnR was eluted with 10 × 1 ml buffer W containing 15 mM desthiobiotin (Sigma-Aldrich). Fractions containing AcnR-C were pooled, and the elution buffer was exchanged against 40 mM Tris-HCl buffer, pH 7.5, containing 10% (v/v) glycerol by gel filtration with Sephadex G-25 (PD-10 column, Amersham Biosciences). Protein concentrations were determined with the BCA protein assay kit (Pierce) using bovine serum albumin as standard. The purity of the protein preparation was assessed by SDS-polyacrylamide gel electrophoresis (37) and subsequent protein detection with Gel Code blue stain reagent (Pierce). Using this protocol, about 1 mg of AcnR-C was purified to apparent homogeneity. The same procedure as described above was used for the isolation of AcnR with an N-terminal StrepTag-II using *E. coli* BL21(DE3)/pET28a-*acnR*-N.

Size Exclusion Chromatography—The size of purified AcnR-C and AcnR-N was estimated by size exclusion chromatography using a Hi-Load 26/60 Superdex 200 prep grade column (Amersham Biosciences) integrated into an Äkta Explorer system (Amersham Biosciences). The column was equilibrated with 20 mM HEPES-buffer pH 8.0 containing 300 mM NaCl and 1 mM dithiothreitol. After application of 0.5–1 mg of purified protein, elution was performed at 4 °C with a flow rate of 2 ml/min. The column was calibrated with a premixed protein molecular mass marker (MWGF-200, Sigma).

DNase I Footprinting Assays—The binding site of purified AcnR protein at the *acn* promoter was analyzed by DNase I footprinting as described previously (29). PCR products covering the *acn* promotor region from position −346 to +52 or from position −268 to +241 relative to the proposed TTG start codon were obtained with the primer pairs acn-PEK-for/acn-PE1* (labeled non-template strand) and acn-FP1*/acn-PEK-rev (labeled template strand) respectively. The primers with an asterisk were IRD800-labeled at the 5′-end. The DNA-AcnR mixtures were treated with DNase I, and the reaction products were separated by denaturing PAGE. The regions, which were protected from DNase I digestion, were localized by running besides the footprinting reactions the four lanes of a DNA sequencing reaction set up with the oligonucleotide acn-PE1* or acn-FP1* and a PCR product obtained with the primer pair acn-PEK-for/acn-PEK-rev as template.

Gel Shift Assays—Purified AcnR-C (500 nM) was mixed with ~50 nM DNA fragments (80–300 bp) covering the *acn* promoter region in a total volume of 20 μl. The buffer contained 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5% (v/v) glycerol, 0.5 mM dithiothreitol, 0.005% (v/v) Triton X-100, 50 mM NaCl, 5 mM $MgCl_2$, and 2.5 mM $CaCl_2$. After incubation for 20 min at room temperature, the samples were separated by agarose gel electrophoresis with Tris acetate/EDTA buffer (20) at 4 °C and 75 V. The gel was subsequently stained with ethidium bromide and photographed.

RESULTS

Influence of the Carbon Source on Aconitase Activity of C. glutamicum—Previous studies revealed that in *C. glutamicum* the carbon flux through the tricarboxylic acid cycle varies with the carbon source (12). In cells growing on acetate, the tricarboxylic acid cycle flux was about 0.4 μmol min^{-1} mg $protein^{-1}$ and thus 4-fold higher than in cells growing on glucose. Remarkably, the activities of citrate synthase (0.5–0.8 units (mg of protein)$^{-1}$; Ref. 7) and isocitrate dehydrogenase (0.9–1.1 units (mg of protein)$^{-1}$; Ref. 8) as measured in cell extracts were reported to be similar in glucose- and acetate-grown cells and are sufficient to allow the maximally measured carbon fluxes. We

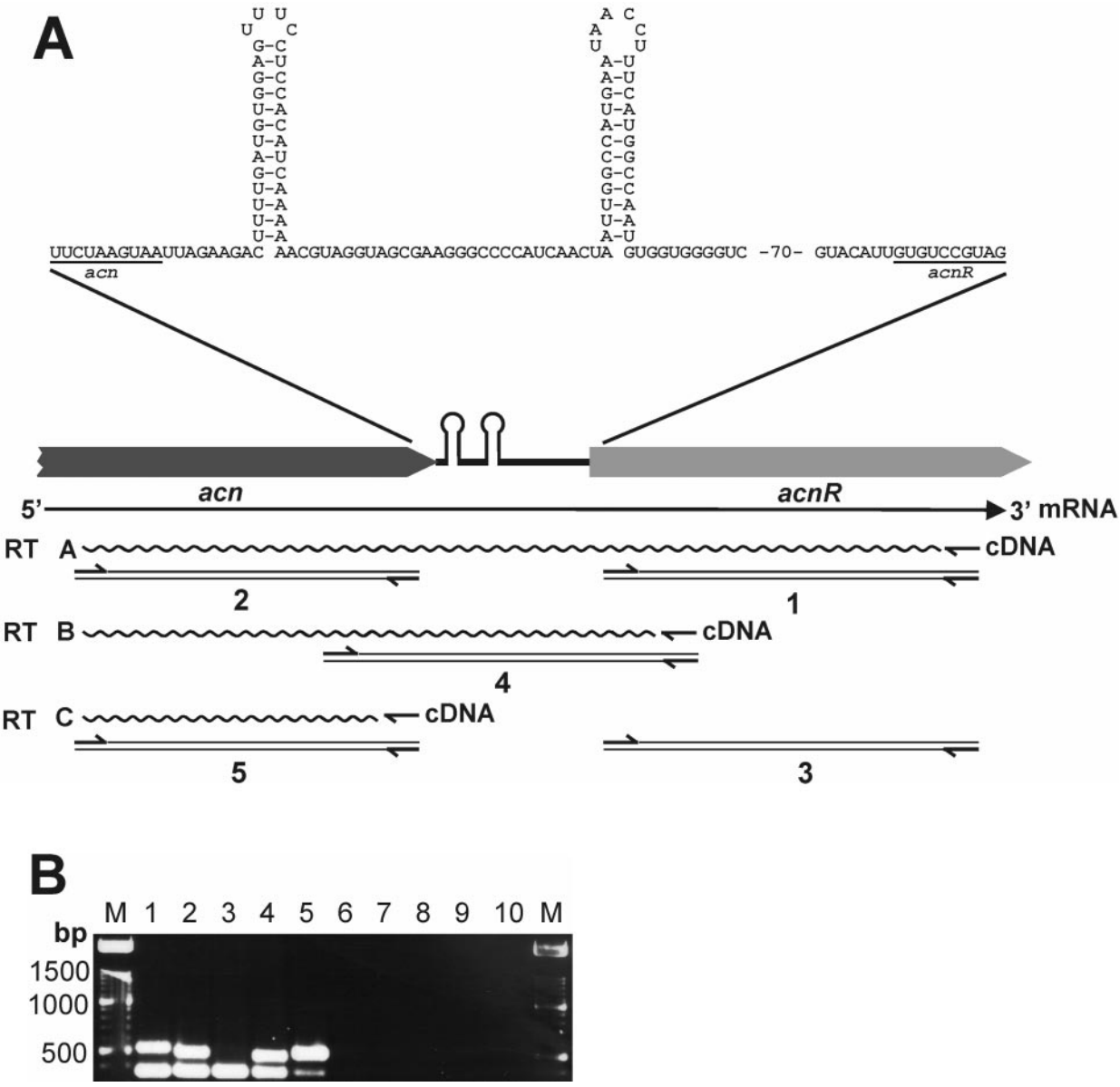

FIG. 3. **Transcriptional organization of the *acn-acnR* locus in *C. glutamicum* analyzed by RT-PCR.** *A*, *scheme* showing the *acn-acnR* intergenic region containing two putative stem loop structures. *Below* the RT-PCR, reactions used to determine co-transcription of *acn* and *acnR* are shown schematically. RNA from *C. glutamicum* wild type was transcribed into cDNA with three different primers in the reverse transcriptase reactions *A–C*. Afterward these cDNAs were used as templates for the PCR reactions labeled *1–5*. *B*, results from the RT-PCR analyses described above. The lower DNA fragment visible in *lanes 1–5* represents *dnaE*, and RT-PCR of *dnaE* served as positive control in all reactions. The *upper bands* correspond to the products of the PCR reactions 1–5 indicated in *A*. *Reactions 6–10* represent controls confirming the absence of DNA in the RNA preparation. The reactions were identical to the PCR reactions as shown in *lanes 1–5* except that reverse transcriptase was omitted in reactions (*A–C*).

therefore became interested to determine whether the activity of aconitase does or does not vary with different carbon sources and measured its activity in extracts of cells cultivated on glucose, citrate, acetate, or propionate. As shown in Table III, the aconitase activity of glucose-grown cells was 0.2 units/mg of protein, whereas the activity of citrate-, acetate-, and propionate-grown cells was 0.5, 0.8, and 0.5 units/mg protein, respectively, *i.e.* up to 4-fold higher. Thus, aconitase activity is regulated and might represent a rate-limiting step in the tricarboxylic acid cycle flux. The increased activity on acetate correlates with the increased tricarboxylic acid cycle flux on this carbon source (12), which might also occur with citrate. Propionate is metabolized in *C. glutamicum* via the methylcitrate cycle (38), in the course of which methylcitrate is converted to methylisocitrate. In *E. coli*, which also degrades propionate via the methylcitrate cycle (39), this conversion requires two enzymes, *i.e.* methylcitrate dehydratase (PrpD) and aconitase (AcnB) (40). This could explain the increased aconitase activity in *C. glutamicum* during growth on propionate.

Identification of a Transcriptional Repressor Gene Downstream of the Aconitase Gene—Since previous DNA microarray experiments had shown that the mRNA level of *acn* is 4-fold higher in acetate-grown cells (41), we assumed that the *acn* gene is transcriptionally regulated. As in bacteria genes encoding transcriptional regulators are often located in the vicinity of their target genes, we inspected the *C. glutamicum* genome region encoding aconitase. In contrast to *e.g. E. coli*, *C. glutamicum* contains only a single aconitase gene (42) encoding a protein of 943 amino acid residues with a calculated molecular mass of 102,168 Da (43). It shows 58.8% sequence identity to AcnA of *E. coli* (44) (45) but less than 20% identity to AcnB of *E. coli* (46) and thus belongs to the bacterial AcnA group of

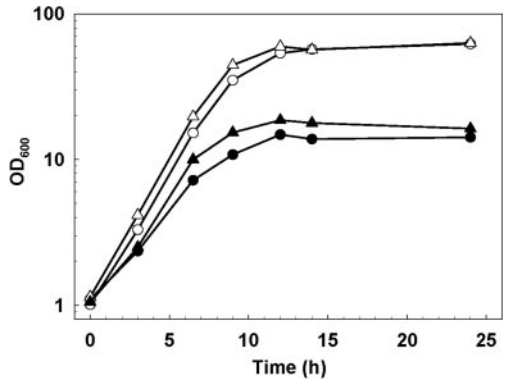

FIG. 4. **Growth of *C. glutamicum* wild type (*circles*) and the Δ*acnR* mutant (*triangles*) on glucose or acetate minimal medium.** The strains were cultivated at 30 °C and 120 rpm in CGXII minimal medium containing either 222 mM glucose (*open symbols*) or 244 mM sodium acetate (*filled symbols*) as carbon source.

aconitases, which are homologous to the iron-responsive proteins (IRPs) of eukaryotes (47).

As shown in Fig. 1, immediately downstream of *acn*, a gene encoding a putative transcriptional regulator of 188 amino acid residues (21,184 Da) was identified, which we named *acnR*. Interestingly, the same gene organization was also found in *C. efficiens*, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *M. bovis*, *M. marinum*, and *Rhodococcus* strain IG124 (Fig. 1). In *M. leprae*, the *acnR* gene is disrupted by frameshifts and stop codons, as found for many other genes of this organism (48). An amino acid sequence alignment of the AcnR homologs (Fig. 2) revealed that the primary sequences are highly conserved, particularly in the N-terminal part from position 16 to 62, where nearly 75% of the amino acids are identical. *In silico* analysis revealed that this conserved N-terminal part of the AcnR proteins belongs to the TetR family of bacterial regulatory proteins (PFAM family PF00440; (49). Members of this family contain a multihelical DNA binding domain at their N-terminal end and a highly divergent C terminus, which is involved in the binding of an inducer molecule (50).

Operon Structure of the acn-acnR Genes—The transcriptional organization of the *acn-acnR* locus was analyzed by RT-PCR (see Fig. 3 and "Experimental Procedures"). Total RNA isolated from *C. glutamicum* wild type was transcribed into cDNA using three different primers (RT-1-rev, RT-2-rev, and RT-3-rev) in the reverse transcriptase reactions A, B, and C. The resulting products were then used for the PCR tests no. 1–5 (Fig. 3*A*). As shown in Fig. 3*B*, evidence was obtained that *acn* and *acnR* are co-transcribed, since a cDNA created with a primer annealing at the 3′-end of *acnR* (RT reaction A) allowed not only the amplification of an *acnR* fragment (no. 1), but also of an *acn* fragment (no. 2). As an internal control in the RT-PCR assays we used *dnaE*, which encodes a subunit of DNA polymerase. Besides the control reaction no. 3, where no *acnR* product could be obtained with a cDNA covering only *acn*, five additional control reactions (no. 6–10) were performed which were identical to reactions no. 1–5, respectively, except that reverse transcriptase was omitted from the initial reactions A-C. The fact that no PCR products were obtained in these reactions confirmed that the RNA was not contaminated with chromosomal DNA.

Aconitase Activity and Growth of a ΔacnR Mutant—Since AcnR was found to be a member of the TetR family, we suggested that it functions as transcriptional repressor of the neighboring aconitase gene. In order to test this hypothesis, an in-frame deletion mutant of *C. glutamicum* was constructed (13032 Δ*acnR*) in which codons 7–177 of *acnR* were replaced by a 21-bp sequence tag. The genomic structure of the mutant was confirmed by PCR and Southern blot analysis (51; see "Experimental Procedures"). In a first set of experiments, the influence of the *acnR* deletion on the aconitase activity was determined. Cells were cultivated in CGXII minimal medium with different carbon sources and used in the early exponential growth phase (OD_{600} ~6) for the determination of enzyme activity. As shown in Table III, the aconitase acitivity in glucose-grown cells was about 1 unit/mg protein and thus 5.3-fold higher than in the wild type. This result clearly supported the proposed function of AcnR as repressor of *acn* expression. In Δ*acnR* mutant cells grown on citrate, acetate, or propionate, the aconitase activity was 3.4-fold, 1.9-fold, and 5.0-fold higher than in wild-type cells grown with the same carbon sources and 1.7-fold, 1.5-fold, and 2.3-fold higher than in Δ*acnR* cells grown on glucose. This indicates that the increased aconitase activity of wild-type cells grown on acetate is partially because of a lower repression by AcnR and that an additional transcriptional regulator besides AcnR or another regulatory mechanism for aconitase might exist.

In the cultivations for the determination of aconitase activity it was observed that in most cases the Δ*acnR* mutant grew somewhat faster than the wild type on glucose and acetate minimal medium, sometimes also in citrate minimal medium. In glucose medium, for example, the growth rate of the Δ*acnR* mutant was 0.40 ± 0.02 h^{-1} and that of the wild type 0.36 ± 0.01 h^{-1} (values from seven replicates). This behavior was also evident when the cells were allowed to grow to stationary phase (Fig. 4) and might be caused by an increased tricarboxylic acid cycle activity in the mutant.

Transcriptome Analyses—To determine the effect of the *acnR* deletion on global gene expression and in particular on *acn* expression, the transcriptomes of the Δ*acnR* mutant and the wild type were analyzed by DNA microarray analyses. RNA was isolated from cells growing exponentially in CGXII minimal medium with 4% (w/v) glucose (OD_{600} ~6). In ten comparisons with RNA from five independent cultivations (*experiments A01–A10*, Fig. 5) the *acn* mRNA level was always higher in the Δ*acnR* mutant than in the wild type (4.2-fold ±2.3), confirming that the increased aconitase activity of the mutant is caused by increased *acn* expression. Besides *acn*, several other genes showed mRNA levels that were on average ≥3-fold increased in the Δ*acnR* mutant, most of which are presumably involved in the uptake of heme and iron siderophores (Fig. 5). This indicates that the Δ*acnR* mutant faces iron limitation, a situation which might be due to the increased production of aconitase, a [4Fe-4S] protein. Indeed, after supplementation of the medium with 100 μM or 500 μM $FeSO_4$ these genes showed no longer an increased mRNA level in the Δ*acnR* mutant, whereas the *acn* mRNA ratio was still raised (5.8-fold ±0.6) in the mutant (*experiments B01–B04*, Fig. 5). These data indicate that *acn* is the only gene whose mRNA level is significantly increased in the Δ*acnR* mutant of *C. glutamicum*.

Further microarray experiments, in which the influence of different iron concentrations (508 μM *versus* 8 μM) on global gene expression was analyzed separately for the wild type (*experiments C01–C03*, Fig. 5) and the Δ*acnR* mutant (*experiments D01–D03*, Fig. 5), revealed that the *acn* mRNA level was increased under iron excess, both in the wild type (3.0-fold ±1.0) and in the Δ*acnR* mutant (4.8-fold ±0.7). Thus, AcnR is not directly involved in iron regulation. Besides *acn*, several other genes encoding iron-containing proteins (52) showed increased mRNA levels under iron excess, indicating that the synthesis of these iron-containing proteins is to some extent controlled by the availability of iron in *C. glutamicum*.

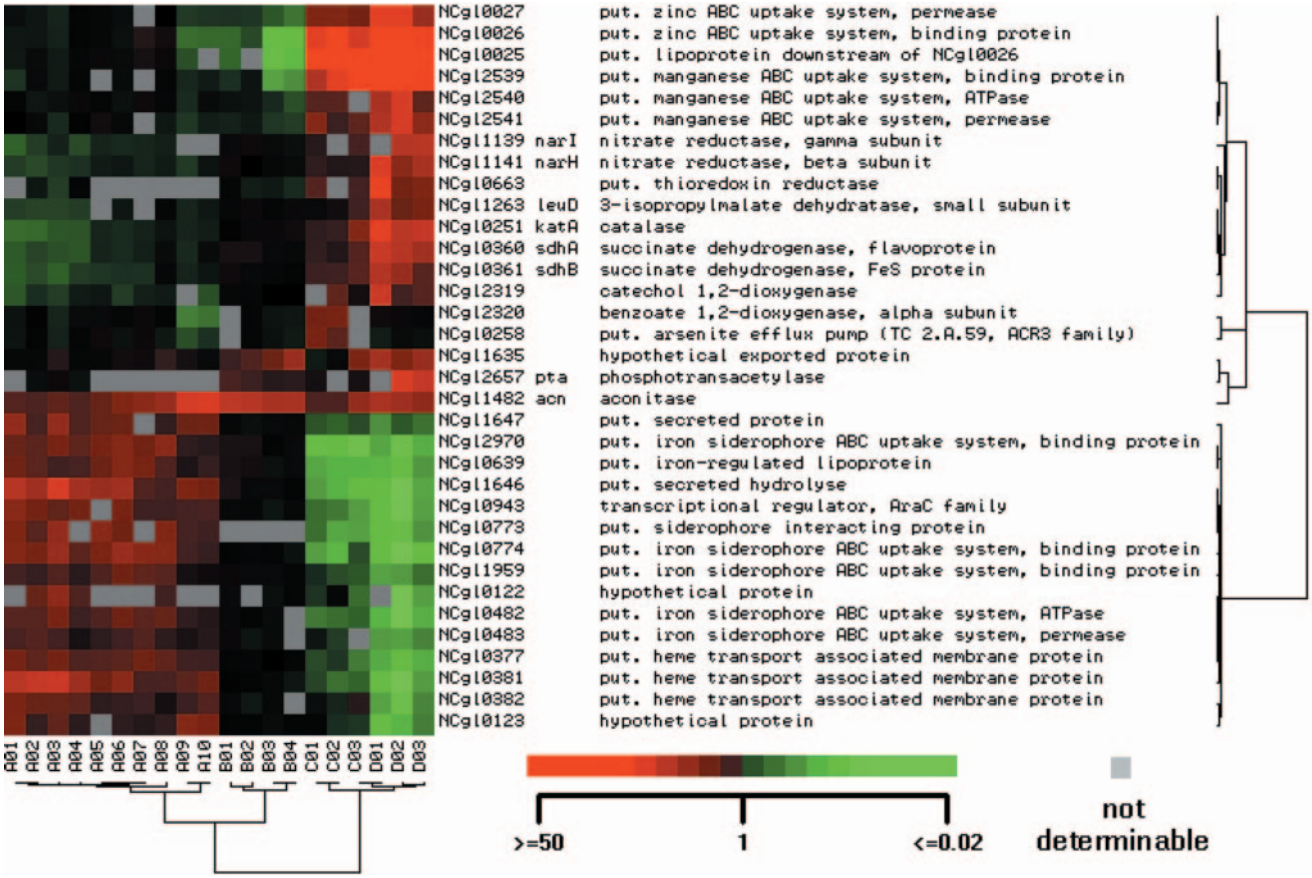

FIG. 5. **Hierarchical cluster analysis of gene expression changes observed in four series of DNA microarray experiments.** The cluster includes those genes, whose average mRNA level was changed ≥3-fold or ≤3-fold in at least one of four series of microarray experiments (totally 20). The following series of experiments were performed: *A01–A10*, Δ*acnR versus* wild type cultivated in CGXII-glucose medium with ~8 μM iron; *B01–B04*, Δ*acnR versus* wild type cultivated in CGXII-glucose medium with 108 μM iron (*B01*, *B02*) or 508 μM iron (*B03*, *B04*); *C01–C03*, wild type with 508 μM iron *versus* wild type with 8 μM iron; *D01–D03*, Δ*acnR* with 508 μM iron *versus* Δ*acnR* mutant with 8 μM iron. The *scale bar* indicates the color coding of the relative RNA ratios.

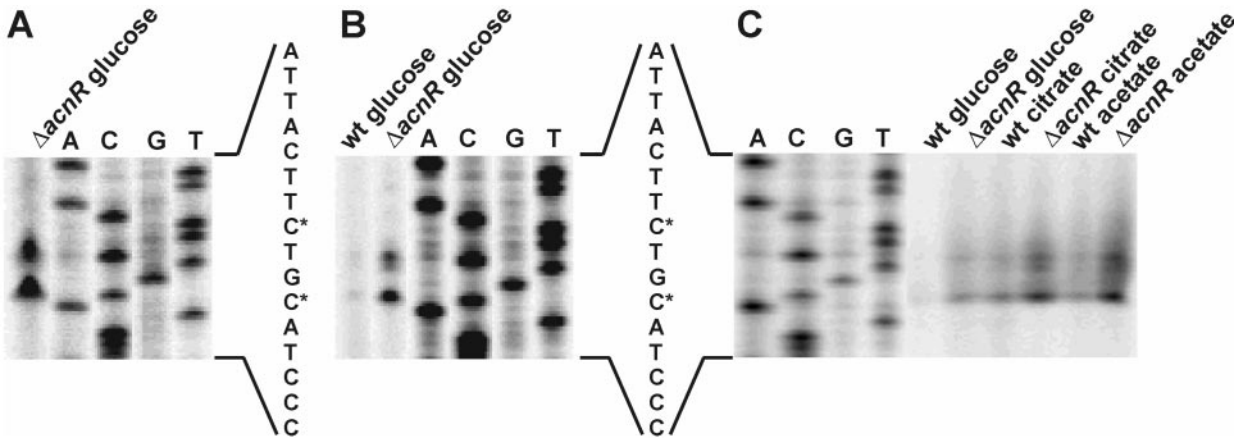

FIG. 6. **Determination of the transcriptional start sites of the *C. glutamicum acn* gene and expression analysis.** 12.5 μg (*A* and *B*) or 15 μg (*C*) of total RNA isolated from cells of *C. glutamicum* wild type and strain Δ*acnR* cultivated in CGXII minimal medium with different carbon sources was used for primer extension analysis with the oligonucleotides acn-PE1* (*A* and *C*) and acn-PE3* (*B*). The transcriptional start sites are indicated by *asterisks*. The sequencing reactions were generated with a PCR product covering the corresponding DNA region as template and the same IRD-800-labeled oligonucleotide as in the primer extension reaction.

Primer Extension Analysis of the acn Promoter—In order to confirm the results of the DNA microarray experiments with an alternative method and to locate the *acn* promoter, primer extension was performed using total RNA samples of wild type and Δ*acnR* mutant, reverse transcriptase and two different primers, acn-PE1* (Fig. 6, *A* and *C*) and acn-PE3* (Fig. 6*B*). With both primers, the major transcriptional start point of *acn* was localized 110 bp upstream of the proposed start codon. In addition, a weaker transcriptional start point was observed 113 bp upstream of the TTG start codon. The intensity of the primer extension products was stronger in the Δ*acnR* mutant than in the wild type, in accordance with the DNA microarray data (Fig. 6*B*). This was true not only for RNA isolated from glucose-grown cells, but also for RNA isolated from citrate- and acetate-grown cells (Fig. 6*C*), which qualitatively agrees with the aconitase activity measurements (Table III).

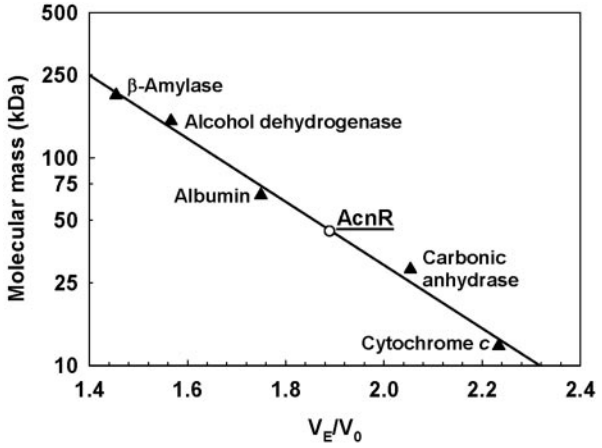

FIG. 7. **Determination of the native molecular mass of AcnR by size exclusion chromatography.** Purified AcnR protein containing either a C-terminal or an N-terminal StrepTag-II was separated on a HiLoad 26/60 Superdex 200 prep grade column using 20 mM HEPES buffer (pH 8.0) containing 300 mM NaCl, and 1 mM dithiothreitol for elution. For calibration, a premixed protein molecular mass marker containing the following proteins was used: cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa). V_0 was determined with blue dextran (2,000 kDa).

TABLE IV
Aconitase activity in different C. glutamicum strains

All strains were cultivated under the same conditions in CGXII medium with 222 mM glucose. For strains harboring pEKEx2 or derivatives the medium contained in addition 50 μg/ml kanamycin and 1 mM isopropyl-1-thio-β-D-galactopyranoside.

C. glutamicum strain	Aconitase activity
	units/mg of protein
13032 (wild type)	0.20 ± 0.05
13032/pEKEx2	0.24 ± 0.01
13032/pEKEx2-*acnR*	0.09 ± 0.01
13032/pEKEx2-*acnR* C-Strep	0.08 ± 0.02
13032/pEKEx2-*acnR* 60	0.24 ± 0.02
13032/pEKEx2-*acnR* 89	0.25 ± 0.01
Δ*acnR*	1.04 ± 0.06
Δ*acnR*/pEKEx2	1.17 ± 0.21
Δ*acnR*/pEKEx2-*acnR*	0.16 ± 0.13

Purification of AcnR and Determination of Its Native Mass—The increased level of *acn* mRNA and of aconitase activity in the Δ*acnR* mutant indicated that AcnR represses *acn* transcription. In order to identify the operator region, the AcnR protein containing either an N-terminal or a C-terminal StrepTag-II was overproduced in *E. coli* and purified by Strep-Tactin affinity chromatography to apparent homogeneity as described under "Experimental Procedures." The apparent mass on an SDS gel corresponded to the calculated mass of 22.5 kDa. Similar results were obtained for AcnR-N (data not shown). The native molecular mass of the purified AcnR proteins was determined by size exclusion chromatography. AcnR-C (1 mg) or AcnR-N (0.5 mg) were chromatographed on a Superdex 200 column (Amersham Biosciences) calibrated with five proteins. As shown in Fig. 7, both AcnR proteins were found to have a molecular mass of 44 kDa, indicating a homodimeric structure, which was also found for other members of the TetR family, *e.g.* TetR (53), CamR (50), QacR (54), or EthR (55). Complementation studies with the streptagged AcnR showed that this tag did not interfere with the repressing activity of AcnR (Table IV).

Identification of the AcnR Binding Site within the acn Promoter Region—The binding site of AcnR at the *acn* promoter was determined with DNase I footprint assays. As shown in Fig. 8, the protected region on the template strand extended

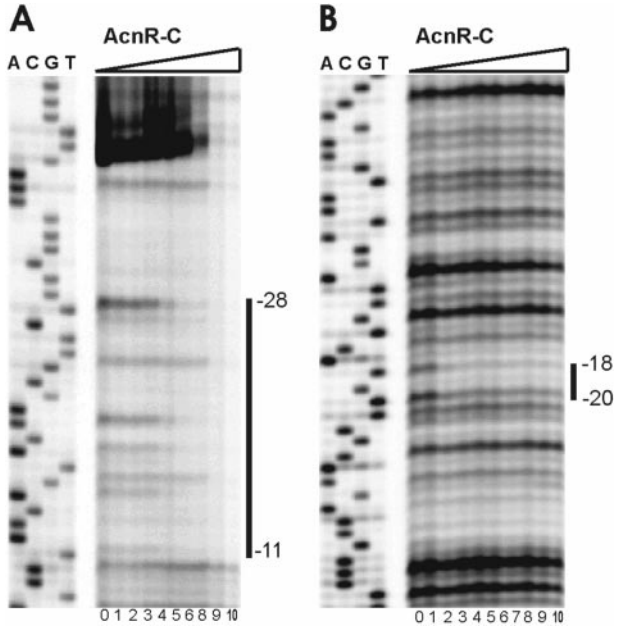

FIG. 8. **DNase I footprinting analysis with AcnR-C and the *acn* promoter region.** 0.95 nM labeled *acn* template strand (*panel A*) or 0.75 nM labeled *acn* non-template strand (*panel B*) were incubated with increasing concentrations of AcnR-C: *lane 0*, no protein; *lane 1*, 5 nM monomeric AcnR; *lane 2*, 10 nM; *lane 3*, 25 nM; *lane 4*, 50 nM; *lane 5*, 100 nM; *lane 6*, 250 nM; *lane 7*, 500 nM (not in *panel A*); *lane 8*, 1.0 μM; *lane 9*, 2.5 μM; *lane 10*, 5 μM. Regions protected from digestion by DNase I are indicated by a *black bar*. The DNA sequencing reactions were set up with the IRD-800-labeled oligonucleotides used for generating the labeled PCR fragments for footprinting. Unlabeled PCR fragments, identical in sequence to the footprinting probes, were used as template DNA.

from position −11 to −28 relative to the first transcription start site (Fig. 8*A*), whereas on the non-template strand, protection was observed only in the region −18 to −20. Inspection of the upstream regions of the *acn* genes in *C. diphtheriae*, *C. efficiens*, and four *Mycobacterium* species revealed that they all contained sequence motifs similar to the one protected by AcnR in *C. glutamicum*. According to the alignment shown in Fig. 9, a putative AcnR consensus sequence could be derived, which contains an imperfect inverted repeat separated by four nucleotides: CAGNACaagcGTACTG. A binding motif of this type and this size is typical for TetR-type transcriptional regulators like TetR or CamR, although larger motifs have also been found, *e.g.* for QacR (56) or EthR (55).

In order to confirm the footprint data and the proposed AcnR consensus sequence, gel shift assays were performed. Experiments with a varying AcnR/DNA ratio revealed that a 4–6-fold molar excess of AcnR is sufficient for a shift (data not shown). In the experiment shown in Fig. 10, the shift of nine different DNA fragments was analyzed, in each case with a 10-fold excess of AcnR. The results obtained with fragments 1–5 confirmed that the binding site is located within a 40-bp region, which includes the binding site proposed from the footprints. Fragments containing this region (nos. 1, 2, and 5) were completely shifted, whereas those lacking this region (nos. 3 and 4) were not shifted at all. Fragments 6–9 represent derivatives of fragment 5 with mutations within or outside the proposed binding motif. Exchange of the two inner (fragment 6) or the two outer (fragment 7) bases of the imperfect inverted repeat completely inhibited the shift, as did an exchange of the four bases separating the inverted repeat (fragment 9). In contrast, exchange of four bases outside the proposed binding site (fragment 8) did not prevent the shift. These data provide strong support for the AcnR consensus binding site proposed above.

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                  -35                          -10          *  *
C. glutamicum     CTTTCCCGCCAGAACGCTTGTACTGTTAGGATAATGAAGACGTAGGG -104- TTGGAGCTCACTG
C. efficiens      CGTACCCAGCAGAACGATCGTACTGTTACGATAATGGGGACGTAGGG -100- TTGGAGCTCACTG
C. diphtheriae    GGTAGGCAACATTACAACCGTACTGTTAGAATGTGCGTCAGGCGACG -135- GTGGAGCTCACTG
M. tuberculosis   GCAACAAAGCAGGACAAGCGTACTGCTAGGCTGACACCACGCCGTCG - 45- GTGACTAGCAAAT
M. bovis          GCAACAAAGCAGGACAAGCGTACTGCTAGGCTGACACCACGCCGTCG - 45- GTGACTAGCAAAT
M. marinum        AACGCAATGCAGGACAAGCGTACTGTTAATGTTGCTTTGCGCATTTG - 44- TTGGGAGTTGATG
M. leprae         AATGCAATGCAGGACAAGCGTACTGTTAACGTGGCAGGGCGCCGTCG - 45- GTGATAAGTAGCA
                        gCAggACaagcGTACTGtTAgg T
                        CAGNACnnncGTACTG
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FIG. 9. **Sequence of the *acn* promoter region of *C. glutamicum* aligned to putative *acn* promoter regions from other *Corynebacterium* and *Mycobacterium* species.** Indicated are the start of the *acn* coding region (start codons *bold* and *underlined*), the transcriptional start sites (*) of the *C. glutamicum acn* gene, putative −10 (*underlined* and *bold*) and −35 regions (*bold*), and the AcnR binding site as determined by DNase I footprints (shaded in *gray*) for the *C. glutamicum acn* gene. As shown by the alignment, also the other species possess putative AcnR binding sites in the *acn* upstream region. The binding site represents an imperfect inverted repeat with the consensus sequence CAGNACnnncGTACTG.

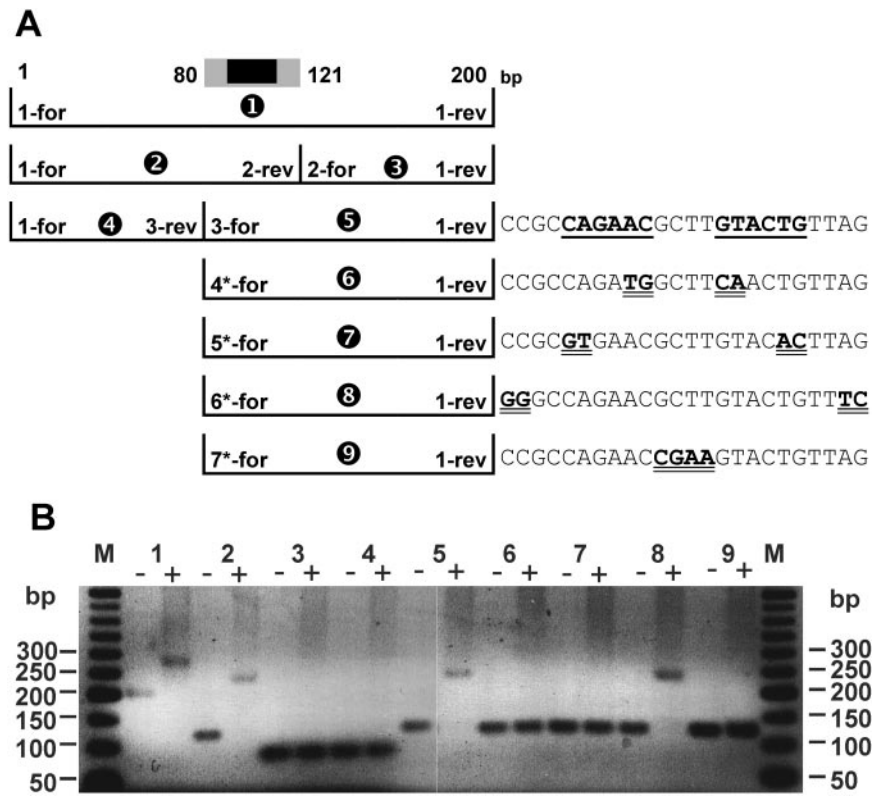

FIG. 10. **Gel shift experiments with purified AcnR-C.** *A*, DNA fragments used for gel shift analysis (*1–9*) are indicated by the *white numbers* in *black circles*. The *gray bar* indicates the region required for a shift and the *black bar* indicates the proposed position of the AcnR binding site. Fragments 6–9 are derivatives of fragment 5 containing mutations that were introduced with the primers 4*-for, 5*-for, 6*-for, and 7*-for. The 24-bp sequence shown beside these fragments includes the proposed AcnR consensus binding site (*bold* and *underlined*) and four base pairs up- and downstream. The mutated bases are indicated in *bold* and *double underlined*. *B*, approximately 50 nM DNA fragments nos. 1–9 were incubated either without AcnR-C (*lanes* labeled with −) or with 500 nM AcnR-C (*lanes* labeled with +) for 20 min at room temperature. Subsequently, the samples were separated on a 2.5% agarose gel at 4 °C and 75 V in 1× Tris acetate/EDTA buffer, and the gel was stained with ethidium bromide. The lanes labeled *M* contain a DNA size standard (50-bp ladder, Fermentas, St. Leon-Rot, Germany).

Effect of Overexpression of acnR and Derivatives on Aconitase Activity—Since deletion of *acnR* had caused derepression of *acn*, overexpression of *acnR* was supposed to lead to an enhanced *acn* repression. As shown in Table IV, overexpression of the unmodified *acnR* gene by the expression plasmid pEKEx-*acnR* caused an ~2-fold decreased aconitase activity. A comparable decrease was observed upon overexpression of an *acnR* derivative encoding an AcnR protein with a C-terminal StrepTag-II. Therefore, the tag does not interfere with the repressing activity of AcnR. If two shortened derivatives of *acnR* were overexpressed, which contained only the N-terminal 60 or 89 amino acid residues of AcnR, there was no effect on aconitase activity. Thus, the N-terminal DNA binding region of AcnR is not sufficient for its repressing activity, but requires the C-terminal effector binding region. Most likely, the C-terminal domain is essential for the dimerization of AcnR, as shown for TetR (53) and QacR (56). When unmodified *acnR* was overexpressed in the Δ*acnR* mutant, wild-type levels of aconitase activity were obtained (Table IV).

DISCUSSION

In this study the first regulator of a tricarboxylic acid cycle gene in *C. glutamicum* was identified. The *acnR* gene located downstream of the aconitase gene *acn* of *C. glutamicum* was shown to encode a repressor of *acn* expression. Deletion of *acnR* led to a 5-fold increased *acn* mRNA level and to a 5-fold increased aconitase activity. Repression is most likely caused by binding of the dimeric AcnR protein to the *acn* promoter in the region located 14 to 29 bp upstream of the transcriptional start point, where it interferes with the binding of RNA polymerase. By comparing the experimentally determined AcnR binding site within the *C. glutamicum acn* promoter (CAGAACGCTTTGTACTG) with the *acn* upstream regions of other *Corynebacterium* and *Mycobacterium* species, a putative consensus sequence CAGNACnnnnGTACTG containing an imperfect inverted repeat was found. Analysis of the *C. glutamicum* genome sequence revealed that this motif occurs only three times in the entire genome, once within the *acn* promoter, once within the coding region for the ribosomal protein L31, and

once within the coding region of a gene of unknown function. This suggests that *acn* is the only target gene of AcnR. Further support for this assumption was obtained by comparing the transcriptomes of the Δ*acnR* mutant and the wild type with whole genome DNA microarrays. In these studies, only the *acn* gene showed reproducibly a ≥3-fold increased mRNA level in the Δ*acnR* mutant. Experiments with different iron concentrations revealed that the *acn* mRNA level is higher under iron sufficiency than under iron deficiency. A regulation of the aconitase level by the iron availability was previously also reported for *M. tuberculosis* (57).

Overexpression of the *acnR* gene by a plasmid-encoded copy under the control of the *tac* promoter caused a 2-fold decrease of the aconitase activity in glucose-grown wild-type cell extracts. Previous experience with other genes that were cloned into the same vector (pEKEx2) and cultivated under the same growth conditions used for *acnR* overexpression indicates that at least 10-fold higher AcnR levels should be obtained. Therefore, the question arises why there is only a 2-fold reduction of aconitase activity. Besides other explanations one obvious reason could be that increased *acn* repression leads to an increase of the concentration of a metabolite, which binds to AcnR, triggers the dissociation of the AcnR-operator complex and thus counteracts the increased repression. Such a mechanism would ensure that aconitase expression is never completely repressed by AcnR, which would be harmful to the cell. In order to identify the proposed metabolite we tested a variety of metabolites of central carbon metabolism with respect to their effects on AcnR binding to the *acn* promoter using the gel shift assay. However, we could not yet find a metabolite that inhibits binding of AcnR to its operator. Thus, the question of the ligand recognized by AcnR is still open.

Regulation of aconitase gene expression is widespread in bacteria. As mentioned before, *E. coli* contains two distinct aconitases (46), AcnB, which is the major citric acid cycle enzyme and AcnA, an aerobic stationary phase enzyme induced by iron and redox stress. The *acnB* gene is activated by CRP and repressed by ArcA, FruR and Fis and *acnA* expression is activated directly or indirectly by CRP, FruR, Fur, and SoxRS and repressed by ArcA and FNR (58). In *B. subtilis*, expression of the aconitase gene *citB* is subject to repression by the LysR-type regulator CcpC and repression can be relieved by citrate (59, 60). Besides CcpC, also CodY and AbrB were reported to be involved in *citB* expression (61). Similar to *E. coli* and *B. subtilis*, we also obtained evidence that *acn* expression in *C. glutamicum* might be controlled by more than one regulator. As shown in Table III, the aconitase activity of the Δ*acnR* mutant was 1.5–2.3-fold higher on acetate, citrate, and propionate compared with glucose. Regulators other than AcnR or other regulatory mechanisms might be responsible for this increase. Recently, RamB was identified as a negative transcriptional regulator of genes involved in acetate metabolism of *C. glutamicum* (62). The *acn* gene belongs to the acetate stimulon (41) and two putative RamB binding sites were identified at positions −492 to −480 (+ strand) and −456 to −444 (−strand) upstream of the proposed *acn* start codon (62). Thus, RamB might represent a second regulator of *acn* expression besides AcnR. Our future studies will focus on the search for additional regulators of *acn* expression, for the inducer molecule recognized by AcnR, and for the answer to the question whether the *C. glutamicum* aconitase also has a regulatory function.

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