Citrate Utilization by *Corynebacterium glutamicum* Is Controlled by the CitAB Two-Component System through Positive Regulation of the Citrate Transport Genes *citH* and *tctCBA*[∇]

Melanie Brocker, Steffen Schaffer,[†] Christina Mack, and Michael Bott^{*}

Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany

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In this work, the molecular basis of aerobic citrate utilization by the gram-positive bacterium *Corynebacterium glutamicum* was studied. Genome analysis revealed the presence of two putative citrate transport systems. The permease encoded by *citH* belongs to the citrate-Mg²⁺:H⁺/citrate-Ca²⁺:H⁺ symporter family, whereas the permease encoded by the *tctCBA* operon is a member of the tripartite tricarboxylate transporter family. The expression of *citH* or *tctCBA* in *Escherichia coli* enabled this species to utilize citrate aerobically, indicating that both CitH and TctABC are functional citrate transporters. Growth tests with the recombinant *E. coli* strains indicated that CitH is active with Ca²⁺ or Sr²⁺ but not with Mg²⁺ and that TctABC is active with Ca²⁺ or Mg²⁺ but not with Sr²⁺. We could subsequently show that, with 50 mM citrate as the sole carbon and energy source, the *C. glutamicum* wild type grew best when the minimal medium was supplemented with CaCl₂, but that MgCl₂ and SrCl₂ also supported growth. Each of the two transporters alone was sufficient for growth on citrate. The expression of *citH* and *tctCBA* was activated by citrate in the growth medium, independent of the presence or absence of glucose. This activation was dependent on the two-component signal transduction system CitAB, composed of the sensor kinase CitA and the response regulator CitB. CitAB belongs to the CitAB/DcuSR family of two-component systems, whose members control the expression of genes that are involved in the transport and catabolism of tricarboxylates or dicarboxylates. *C. glutamicum* CitAB is the first member of this family studied in *Actinobacteria*.

Citrate is a ubiquitous natural compound which can be utilized as a carbon and energy source by many bacterial species. The anaerobic catabolism of citrate, which occurs, e.g., in enterobacteria like *Klebsiella pneumoniae* and *Escherichia coli* (7) and in lactic acid bacteria (14), usually involves the key enzyme citrate lyase (EC 4.1.3.6), which catalyzes the cleavage of citrate into acetate (the end product) and oxaloacetate (8, 47, 48). The subsequent catabolism of oxaloacetate can occur via different pathways, leading to, e.g., acetate and succinate as end products. Aerobic citrate utilization by bacteria possessing a complete tricarboxylic acid cycle usually requires only a citrate uptake system. Presently, at least five families of citrate transporters in bacteria have been characterized according to the classification system introduced by Saier (44): (i) the metabolite:H⁺ symporter family (transporter classification [TC] no. 2.A.1.6) within the major facilitator superfamily (TC no. 2.A.1), represented by CitH of *K. pneumoniae* (53, 54); (ii) the citrate-Mg²⁺:H⁺/citrate-Ca²⁺:H⁺ symporter (CitMHS) family (TC no. 2.A.11), represented by CitM and CitH of *Bacillus subtilis* (6, 30, 31); (iii) the citrate:cation symporter family (TC no. 2.A.24), represented by CitS and CitW of *K. pneumoniae* (24, 41); (iv) the divalent anion:Na⁺ symporter family (TC no. 2.A.47), represented by CitT of *E. coli* (42); and (v) the tripartite tricarboxylate transporter (TTT) family (TC no. 2.A.80),

represented by TctABC of *Salmonella enterica* serovar Typhimurium (63). Whereas the transporters of the former four families consist of a single protein, the TctABC system is composed of three different subunits: two integral membrane proteins with presumably 12 (TctA) and 4 (TctB) transmembrane helices, plus a periplasmic citrate binding protein (TctC).

For most citrate transporter genes studied so far with respect to regulation, expression is induced in the presence of the substrate. In many bacteria, the transcription of genes for citrate uptake and catabolism is activated by two-component signal transduction systems (TCS) consisting of a membrane-bound histidine kinase which controls the phosphorylation status of a soluble response regulator and thereby its activity as a transcriptional regulator. Examples are the CitA-CitB TCS of *K. pneumoniae* and *E. coli* (9, 34) and the CitS-CitT TCS of *B. subtilis* (64), which belong to the CitAB/DcuSR family of TCS (23). The periplasmic domains of the CitA histidine kinases from *K. pneumoniae* and *E. coli* were shown to bind citrate with high specificities and affinities (between 0.1 and 10 μM at neutral pH) (22, 23). A previous mutagenesis study identified several amino acid residues critical for citrate binding, three of which are highly conserved within the CitAB/DcuSR subfamily (18). The direct involvement of these residues in citrate binding was confirmed by the first crystal structure of a periplasmic domain in a histidine kinase, i.e., that of *K. pneumoniae* CitA in a complex with citrate (43). More recently, a structural comparison between citrate-free and citrate-bound states of the periplasmic CitA domain became possible, revealing that ligand binding causes considerable contraction of the sensor domain. This contraction may represent the molecular switch that activates transmembrane signaling in the receptor (49).

^{*} Corresponding author. Mailing address: Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany. Phone: 49-2461-615515. Fax: 49-2461-612710. E-mail: m.bott@fz-juelich.de.

[†] Present address: Evonik-Degussa GmbH, Science-to-Business Center Bio, D-45764 Marl, Germany.

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Besides induction by citrate, the expression of citrate permease genes is sometimes also subject to carbon catabolite repression, which can at least partially override citrate induction, if other carbon sources are available. The expression of *citH* from *B. subtilis* is subject to control by the central regulator CcpA (56) and by a product of arginine metabolism (57). The expression of the *tctCBA* genes of *S. enterica* serovar Typhimurium and of *citS* from *K. pneumoniae* is controlled by the cyclic AMP receptor protein-cyclic AMP complex system (33, 62).

Whereas a wealth of information on citrate transporters and their transcriptional regulation in proteobacteria and gram-positive bacteria with low GC contents (*Firmicutes*) is available, not much is known about these systems in gram-positive bacteria with high GC contents (*Actinobacteria*). Here, we describe citrate utilization and its regulation in *Corynebacterium glutamicum*, a predominantly aerobic soil bacterium belonging to the *Corynebacterium-Mycobacterium-Nocardia* group of actinomycetes. This species is of interest due to its biotechnological importance as a producer of L-glutamate and L-lysine and its potential to serve as a nonpathogenic model organism for studying selected features common to corynebacteria and pathogenic mycobacteria. Overviews of *C. glutamicum* biology, genetics, physiology, and biotechnology can be found in two recent monographs (11, 15).

In an early study, Kimura (26) reported that *C. glutamicum* cannot grow on citrate unless the medium is supplemented with corn steep liquor. Later, Birnbaum and Demain (3) presented evidence that the function of corn steep liquor is to supply energy for the induction of a citrate transport system. They showed that resting cells of *C. glutamicum* pregrown on glucose with citrate take up citrate and convert it to extracellular glutamate but that cells pregrown on glucose without citrate do not or do so very slowly. Quite recently, we characterized citrate utilization in *C. glutamicum* by transcriptome and proteome analyses (39). In that study, it was shown that genes for two putative citrate uptake systems (*citM*, which has been renamed *citH* based on the studies presented here, and *tctCBA*) are strongly activated during growth on citrate and that several genes for the transport of alternative carbon sources, such as glucose, fructose, saccharose, and gluconate, show decreased mRNA levels in the presence of citrate. In addition, genes of the tricarboxylic acid cycle, of the respiratory chain, of F_1F_0 ATP synthase, and of gluconeogenesis show increased expression during growth on citrate (39).

In the present study, we have analyzed the molecular basis of citrate utilization by *C. glutamicum* in more detail. We provide evidence that both CitH (previously named CitM) and TctABC are in fact functional citrate transporters but with differing cation specificities. Citrate-dependent activation of *citH* and *tctCBA* was found to be dependent on the CitAB two-component system.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *C. glutamicum* wild-type strain ATCC 13032 and the $\Delta citAB$, $\Delta citH$, $\Delta tctCBA$, and $\Delta citH \Delta tctCBA$ deletion mutants (Table 1) were cultivated aerobically on a rotary shaker (120 rpm) at 30°C in CGXII minimal medium (25) containing 30 mg/liter 3,4-dihydroxybenzoate as an iron chelator and carbon sources and salts as described in Results. For strain construction, plates of BHIS agar (brain heart infusion agar [Difco,

Detroit, MI] with 0.5 M sorbitol) were used. Precultivation for growth experiments was usually performed in CGXII medium containing 50 mM sodium citrate, 50 mM glucose, and either 5 mM $CaCl_2$ or 100 mM $MgCl_2$. For cloning purposes, *E. coli* DH α (Bethesda Research Laboratories) was cultivated aerobically on a rotary shaker (120 rpm) at 37°C in Luria-Bertani medium (45). In order to test *E. coli* strains for aerobic growth on citrate as the sole carbon source, the strains were streaked onto Simmons citrate agar plates (Merck). For testing the cation specificity of the citrate transport systems CitH and TctABC, *E. coli* DH α carrying either pXMJ19-*citH* or pXMJ19-*tctCBA* was cultivated in M9 minimal medium (45) supplemented with 1 g/liter Casamino Acids. For selection purposes, chloramphenicol (10 μ g/ml for *C. glutamicum* and *E. coli*) and kanamycin (25 μ g/ml for *C. glutamicum* and 50 μ g/ml for *E. coli*) were used.

Construction of *C. glutamicum* deletion mutants. *C. glutamicum* mutants with in-frame deletions of *citAB* ($\Delta citAB$), *citH* ($\Delta citH$), *tctCBA* ($\Delta tctCBA$), and *citH* and *tctCBA* ($\Delta citH \Delta tctCBA$) were constructed via a two-step homologous recombination procedure as described previously (37). The regions up- and downstream (approximately 450 bp each) of the gene/operon to be deleted were amplified using pairs of oligonucleotides designated Delta-gene/operon-1 and Delta-gene/operon-2 and Delta-gene/operon-3 and Delta-gene/operon-4 (Table 2), respectively, and the products served as templates for crossover PCR with oligonucleotides designated Delta-gene/operon-1 and Delta-gene/operon-4. The resulting PCR product of ~0.9 kb was digested with the restriction enzymes indicated in the primer table (Table 2) and cloned into pK19mobsacB cut with the same enzymes (46). DNA sequence analysis confirmed that the cloned PCR products did not contain spurious mutations. The transfer of the resulting plasmids, designated pK19mobsacB- Δ gene/operon, into *C. glutamicum* and selection for the first and second recombination events were performed as described previously (37). Kanamycin-sensitive and saccharose-resistant clones were tested by colony PCR analysis with a primer pair designated Delta-gene/operon-out-fw and Delta-gene/operon-out-rv (Table 2). Clones which had the desired in-frame deletion of the gene/operon revealed a 1-kb fragment in which all nucleotides except for the first 6 codons and the last 12 codons were replaced by a 21-bp tag.

Plasmid construction. For the construction of pXMJ19-*citAB*, the entire *citAB* operon was amplified using oligonucleotides citAB-OE-fw-SalI and citAB-OE-rv-EcoRI. For the construction of pXMJ19-*citH* and pXMJ19-*tctCBA*, the *citH* gene and the *tctCBA* operon were amplified using the oligonucleotide pairs citH-OE-fw-PstI/citH-OE-rv-EcoRI and tct-OE-fw/tct-OE-rv, respectively. Since the plasmid pXMJ19 does not carry a ribosomal binding site, regions of 81, 52, and 70 bp upstream of *citA*, *citH*, and *tctC*, respectively, were also amplified with these primers. The PCR products were digested using the appropriate restriction enzymes (Table 2) and cloned into pXMJ19 (20). For the overproduction and purification of CitB with an amino-terminal histidine tag, the *citB* coding region was amplified using oligonucleotides that introduce an NdeI restriction site including the start codon (cg-citB-for) and an XhoI restriction site after the stop codon (cg-citB-rev). The purified 698-bp PCR product was digested with NdeI and XhoI and cloned into the expression vector pET28b cut with the same enzymes, resulting in plasmid pET28b-CgCitB-NHis₆. The CitB protein encoded by this plasmid (238 amino acids; 25.6 kDa) contains a stretch of 20 additional amino acids (MGSSHHHHHHSSGLVPRGSH) at the amino terminus, including a hexahistidine tag and a thrombin cleavage site.

Overproduction and purification of CitB. *E. coli* BL21(DE3) carrying plasmid pET28b-CgCitB-NHis₆ was grown at 30°C in 500 ml of Luria-Bertani medium with 50 μ g/ml kanamycin to an optical density at 600 nm (OD₆₀₀) of ~1 before the addition of 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). After cultivation for another 3 h, cells were harvested by centrifugation, washed once, and stored at -20°C. For cell extract preparation, thawed cells were resuspended in 10 ml of TNIG5 (20 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, 5% [vol/vol] glycerol, pH 7.9). After the addition of 1 mM diisopropylfluorophosphate and 1 mM phenylmethylsulfonyl fluoride, the cells were disrupted using a French pressure cell (SLM Aminco; Spectronic Instruments, Rochester, NY). Intact cells and cell debris were removed by centrifugation (20 min at 5,000 $\times$ g and 4°C), and the cell extract was subjected to ultracentrifugation (1 h at 150,000 $\times$ g and 4°C). CitB, present in the supernatant from the ultracentrifugation step, was purified by Ni²⁺-chelate affinity chromatography using His-Bind resin (Novagen; Merck KGaA, Darmstadt, Germany). After the column was washed with 10 bed volumes of TNIG20 buffer and 10 bed volumes of TNIG100 buffer (the same as TNIG5 but containing 20 and 100 mM imidazole, respectively), CitB protein was eluted with TNIG400 buffer (containing 400 mM imidazole). Fractions containing CitB were pooled, and the elution buffer was exchanged for BS buffer (50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 10% [vol/vol] glycerol, pH 7.5) by gel filtration using PD-10 columns (Amersham Biosciences). Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description and/or genotype ^a	Source or reference
<i>C. glutamicum</i> strains		
ATCC 13032	Biotin-auxotrophic wild-type strain	1
ATCC 13032 Δ <i>citAB</i>	Derivative of ATCC 13032 with a <i>citAB</i> deletion	27
ATCC 13032 Δ <i>citH</i>	Derivative of ATCC 13032 with an in-frame deletion of the <i>citH</i> gene	This work
ATCC 13032 Δ <i>tctCBA</i>	Derivative of ATCC 13032 with an in-frame deletion of the <i>tctCBA</i> operon	This work
ATCC 13032 Δ <i>citH</i> Δ <i>tctCBA</i>	Derivative of ATCC 13032 with an in-frame deletion of the <i>citH</i> gene and in the <i>tctCBA</i> operon	This work
<i>E. coli</i> strains		
MG1655	Wild-type strain	5
DH5 α	F [−] ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>U169 endA1 recA1 hsdR17</i> (r _K [−] m _K ⁺) <i>deoR thi-1 phoA supE44</i> λ [−] <i>gyrA96 relA1</i>	Invitrogen
BL21(DE3)	<i>ompT hsdS_B</i> (r _B [−] m _B [−]) <i>gal dcm</i> (DE3)	Novagen
Plasmids		
pXMJ19	Cm ^r ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for IPTG-inducible gene expression (pBL1 <i>oriV_{C.g.}</i> pK18 <i>oriV_{E.c.}</i> <i>lacI P_{lac}</i>)	20
pXMJ19- <i>citAB</i>	Cm ^r ; pXMJ19 carrying the <i>C. glutamicum citAB</i> genes	This work
pXMJ19- <i>citH</i>	Cm ^r ; pXMJ19 carrying the <i>C. glutamicum citH</i> gene	This work
pXMJ19- <i>tctCBA</i>	Cm ^r ; pXMJ19 carrying the <i>C. glutamicum tctCBA</i> operon	This work
pET28b	Km ^r ; vector for overexpression of genes in <i>E. coli</i> under the control of a T7 promoter and for addition of an N-terminal or C-terminal hexahistidine tag to the synthesized protein (pBR322 <i>oriV_{E.c.}</i> <i>P_{T7}</i> <i>lacI</i>)	Novagen
pET28b-CgCitB-NHis ₆	Km ^r ; pET28b derivative for overproduction of the <i>C. glutamicum</i> response regulator CitB with an N-terminal hexahistidine tag	This work
pK19 <i>mobsacB</i>	Km ^r ; vector for allelic exchange in <i>C. glutamicum</i> (pK18 <i>oriV_{E.c.}</i> <i>sacB lacZ</i> α)	46
pK19 <i>mobsacB</i> - Δ <i>citH</i>	Km ^r ; pK19 <i>mobsacB</i> derivative containing a crossover PCR product covering the regions up- and downstream of <i>citH</i>	This work
pK19 <i>mobsacB</i> - Δ <i>tctCBA</i>	Km ^r ; pK19 <i>mobsacB</i> derivative containing a crossover PCR product covering the region upstream of <i>tctC</i> and regions downstream of <i>tctA</i>	This work
pK19 <i>mobsacB</i> - Δ <i>citAB</i>	Km ^r ; pK19 <i>mobsacB</i> derivative containing a crossover PCR product covering the region upstream of <i>citA</i> and regions downstream of <i>citB</i>	27

^a *oriV_{C.g.}*, *C. glutamicum oriV*; *oriV_{E.c.}*, *E. coli oriV*.

standard. The purity of the protein preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent protein detection with Gel Code blue staining reagent (Pierce, Rockford, IL).

Electrophoretic mobility shift assays (EMSAs). For testing the binding of CitB to putative target promoters, purified CitB protein (0 to 1.4 μ M) was mixed with 100-ng DNA fragments (219 to 336 bp; final concentrations, 22 to 35 nM) in a total volume of 20 μ l. The binding buffer contained 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, and 10% (vol/vol) glycerol. The reaction mixture was incubated at room temperature for 30 min and then loaded onto a native 15% polyacrylamide gel. Electrophoresis and DNA detection with Sybr green I were performed as described before (59). The DNA fragments were generated by PCR, purified with a PCR purification kit (Qiagen, Hilden, Germany), and eluted in EB buffer (10 mM Tris-HCl, pH 8.5).

Analysis of glucose and citrate. The quantitation of glucose and citrate in the supernatants of cultures was performed by high-performance liquid chromatography (HPLC) using a Merck Hitachi HPLC system equipped with an Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad). Isocratic elution was performed with 6 mM H₂SO₄ at 80°C for 20 min at a flow rate of 0.6 ml/min. Citric acid was detected with a LaChrom L-7400 UV detector (Merck Hitachi) at 215 nm. Glucose was detected with a LaChrom L-7490 refractive index detector (Merck Hitachi). Quantification was performed using calibration curves obtained with external standards.

Primer extension analysis. Nonradioactive primer extension analysis was performed as described previously (16). Each transcriptional start site was determined using at least two different digoxigenin-labeled oligonucleotides: citH-PE-1, citH-PE-2, and citH-PE-4 for *citH*, tct-PE-2 and tct-PE-3 for *tctCBA*, and citAB-PE-1 and citAB-PE-2 for *citAB* (Table 2).

DNA microarray analysis. The preparation of RNA and the synthesis of fluorescently labeled cDNA were carried out as described previously (35). Custom-made DNA microarrays printed with 70-mer oligonucleotides for *C. glutamicum* ATCC 13032 were obtained from Operon (Cologne, Germany) and are based on the genome sequence entry with accession no. NC_006958 (21). Hy-

bridization and stringent washing of the microarrays were performed according to the instructions of the supplier. Hybridization was carried out for 16 to 18 h at 42°C by using a microarray user interface hybridization system (BioMicro Systems, Salt Lake City, UT). After being washed, the microarrays were dried by centrifugation (5 min at 1,600 $\times$ g) and the levels of fluorescence at 532 nm (for Cy3-dUTP) and 635 nm (for Cy5-dUTP) were determined with 10- μ m resolution by using a GenePix 6000 laser scanner (Axon Instruments, Sunnyvale, CA). Quantitative image analysis was carried out using GenePix image analysis software (GenePix Pro 6.0; Axon Instruments), and the results were saved as GenePix results files. For data normalization, GenePix results files were processed using the Bioconductor/R packages limma and marray (<http://www.bioconductor.org>). Processed and normalized data, as well as experimental details (minimum information about a microarray experiment) (10), were stored in the in-house microarray database for further analysis (40).

RESULTS

Use of *E. coli* to test whether the *C. glutamicum* genes *citH* and *tctCBA* encode functional citrate transporters. Our previous study on citrate utilization by *C. glutamicum* had shown that the genes for two putative citrate transport systems, *citH* (cg0088; previously designated *citM*) and *tctCBA* (cg3127, cg3126, and cg3125), were the ones most strongly induced in the presence of citrate in the medium (39). CitH belongs to the CitMHS family (TC no. 2.A.11), whereas TctABC is a member of the TTT family (TC no. 2.A.80) (63). The CitH protein shows 42 and 43% sequence identity to the *B. subtilis* citrate transporters CitM and CitH (6, 30). The first of several putative start codons for *citH* (Fig. 1A) leads to a protein consisting

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'→3') ^a	Restriction endonuclease
citAB-OE-fw-SalI	TAT AGT CGA CCT TCA CGC TCT GCT GAT AAT CGC	SalI
citAB-OE-rv-EcoRI	TAT AGA ATT CAT GCT GAC CGA GGT GGA CAT CC	EcoRI
citH-OE-fw-PstI	TAT ACT GCA GCA GAA CAC TCG GGA TCT CAA AGT TTC G	PstI
citH-OE-rv-EcoRI	TAT AGA ATT CGG GGT TTG GCG TCG AAA AGC	EcoRI
tct-OE-fw	TAT ATC TAG ACC CAA GCA CTT AGG CAT CAA ACA TTC	XbaI
tct-OE-rv	TAT ACC CGG GAT GGT CTC TAA GCG ACT TCG AGG	XmaI
cg-citB-for	CGC CGC CAT ATG GAT CAA ACA CTT AAA GTT TTA GTA	NdeI
cg-citB-rev	ATA ATC CTC GAG CTA GAG CAG TGG CTT TGA ATA	XhoI
citH-PE-1	GTG GCT GAG CGT GTA GTC AGC GGC TAA TGC CG	
citH-PE-2	CTT TCT GTG TTT CTC GAA ACT TTG AGA TCC CGA G	
citH-PE-4	TAA TGC CGT GAC TTG TCC TGA AG	
tct-PE-2	TGC GGG TTC TGC TCC TAC TTC GGC C	
tct-PE-3	AAT CCC TCT CGG TCG GAC TTT TG	
citAB-PE-1	AAA TAG CTA CCA CCA ACG CGA CGG TAG CCA C	
citAB-PE-2	TCC ATC ATC AAA ACT GCG AAA ATT CCG GTG C	
Delta-citAB-1	TAT ATC TAG AGA GCG TGT AGT CAG CGG CTA ATG	XbaI
Delta-citAB-2	CCC ATC CAC TAA ACT TAA ACA CAA AAC TGC GAA AAT TCC GGT GC	
Delta-citAB-3	TGT TTA AGT TTA GTG GAT GGG GGG CGA CCA GAA CAT CTA TAT TC	
Delta-citAB-4	ATA TGA ATT CAC CAT GTG GTG TTG TGC GTA CC	EcoRI
Delta-citAB-out-fw	GAC TAC CAG GAT GCC ATC TGA G	
Delta-citAB-out-rv	TAT ATC CAA CCT GCA CCA AGT AC	
Delta-citM-1	TAT ACT GCA GAT GAA GAT AAG CCC GAC TCC CC	XbaI
Delta-citM-2	CCC ATC CAC TAA ACT TAA ACA GTA GCC CTC TTC ATC GGC GTC G	
Delta-citM-3	TGT TTA AGT TTA GTG GAT GGG GAG GAT CAT GGC GAA GCC AAG	
Delta-citM-4	ATA TGA ATT CAC CAT GTG GTG TTG TGC GTA CC	EcoRI
Delta-citM-out-fw	CGC CCG CAT ATG AAC CAA TAG C	
Delta-citM-out-rv	TGA ATC ACC AGT ATT CGG GTA GC	
Delta-tct-1	TAT ATC TAG ACG AGC GCT ACG TGA CTT CTT TGA	XbaI
Delta-tct-2	CCC ATC CAC TAA ACT TAA ACA TGC TCC TAC TTC GGC CAT TTT GGG GG	
Delta-tct-3	TGT TTA AGT TTA GTG GAT GGG AAG CAC CTG ACT TCT CAG CTC GAA ACC	
Delta-tct-4	TAT ACC CGG GGG TTT AGC AAG CGG GAC GCT TTC G	XmaI
Delta-tct-out-fw	GGG TTT GAT TTT GAG GTG ACA ACG G	
Delta-tct-out-rv	TTC CTA GGC TGC CCG CTT GGG CT	
P_citH_fw	TTT CCA GTC GGA TCC ACC AAC AG	
P_citH_rv	GTT TCT CGA AAC TTT GAG ATC CCG	
P_citH_control_fw	CGG GAT CTC AAA GTT TCG AGA AAC	
P_citH_control_rv	CGA AGA TGG TGG GGA CCA ACA G	
P_tct_fw	GAC AAC AAG GGG GAG TCC TAG C	
P_tct_rv	GGT TCT GCT CCT ACT TCG GCC	
P_cg2836_control_fw	GGT TAG CGC GCC GCC CTG TC	
P_cg2836_control_rv	CGA CTT CTC AAA GGC GAC TTC AC	

^a In some cases, oligonucleotides were designed to introduce recognition sites for restriction endonucleases (recognition sites are underlined, and the corresponding restriction endonucleases are listed) and complementary 21-mer sequences for generating crossover PCR products (indicated by italics) into the resulting PCR products.

of 526 amino acid residues. The *tctCBA* genes (Fig. 1B) presumably form an operon, since the last nucleotides of the *tctC* and *tctB* stop codons correspond to the first nucleotides of the start codons of *tctB* and *tctA*, respectively. Downstream of *tctA*, a Rho-independent transcriptional terminator structure ($\Delta G^\circ = -128.5$ kJ/mol) was identified. TctA (510 amino acid residues) and TctB (188 residues) are integral membrane proteins with 12 and 4 predicted transmembrane helices, respectively. TctC (334 residues) is presumably a periplasmic citrate binding protein that contains a putative transmembrane helix (residues 19 to 39) in the N-terminal region. This transmembrane helix may either tether the protein to the cytoplasmic membrane or be cleaved off by signal peptidase. TctA, TctB, and TctC from *C. glutamicum* show 38, 29, and 27% sequence identity to the corresponding proteins from *S. enterica* serovar Typhimurium, the first and most carefully studied members of the TTT transporter family (50–52, 60–62).

In order to test whether the *C. glutamicum* CitH and

TctABC proteins are in fact citrate transporters, the corresponding genes were cloned and expressed in the heterologous host *E. coli* MG1655. This strain does not synthesize a citrate transporter under aerobic conditions and consequently cannot grow on citrate. It is therefore a suitable host to test the functions of putative citrate transporters (42). The recombinant strains were tested for their abilities to utilize citrate as the sole carbon source under aerobic conditions by being streaked onto Simmons citrate agar supplemented with 10 μ g/ml chloramphenicol. The formation of colonies and a color change of the pH indicator bromthymol blue from green to blue indicated that MG1655/pXMJ19-*citH* and MG1655/pXMJ19-*tctCBA*, but not the control strain, MG1655/pXMJ19, were able to utilize citrate (data not shown). The color change is observed only with cells that are able to metabolize citrate, which leads to the alkalinization of the medium. These results clearly indicate that both CitH and the TctABC complex of *C. glutamicum* are functional citrate transporters.

FIG. 2. Testing the cation dependency of the citrate transporters CitH and TctABC of *C. glutamicum* by using the growth of *E. coli* on citrate as the indicator. *E. coli* MG1655 transformed with pXMJ19-citH (●), pXMJ19-tctCBA (■), or pXMJ19 (▲) was grown in a 500-ml Erlenmeyer flask containing 50 ml of M9 minimal medium supplemented with 1 g/liter Casamino Acids, 50 mM sodium citrate, and none or one of the following salts: 5 mM CaCl_2 , 5 mM MgCl_2 , 5 mM ZnCl_2 , 5 mM NiCl_2 , and 5 mM SrCl_2 . The cultures were incubated at 37°C on a rotary shaker at 120 rpm. The growth curves shown here are representative of those from three independent growth experiments with comparable results.

the fact that *C. glutamicum* possesses two functional citrate transporters, we tested whether the organism is able to grow on citrate as the sole carbon and energy source. In initial studies, wild-type cells pregrown in standard CGXII minimal medium with 50 mM glucose and 50 mM sodium citrate showed only minimal growth (from an OD_{600} of 0.9 to an OD_{600} of 3) when they were subsequently cultivated in standard CGXII minimal medium containing 50 mM sodium citrate as the sole carbon source (Fig. 3). Standard CGXII minimal medium contains 0.09 mM Ca^{2+} and 1 mM Mg^{2+} , indicating that the poor growth may be due to insufficient concentrations of the cations that were found to be required for citrate utilization via CitH and TctABC in *E. coli*. Therefore, we supplemented the standard CGXII medium with additional CaCl_2 , MgCl_2 , or SrCl_2 . As shown in Fig. 3A, *C. glutamicum* grew at a rate of 0.31 h^{-1} to an OD_{600} of about 12 on citrate as the sole carbon source with 2 mM CaCl_2 . Increased CaCl_2 concentrations up to 20 mM did not further stimulate either the growth rate or the growth yield. Even higher Ca^{2+} concentrations led to the precipitation of salt, presumably as calcium phosphate. Growth on citrate was accompanied by an increase of the pH of the me-

FIG. 3. Influence of Ca^{2+} (A), Mg^{2+} (B), or Sr^{2+} (C) on the growth of the *C. glutamicum* wild type with citrate as the sole carbon and energy source. Cells pregrown in CGXII minimal medium with 50 mM glucose, 50 mM citrate, and 20 mM CaCl_2 , 50 mM MgCl_2 , or 5 mM SrCl_2 , respectively, were used to inoculate 50 ml of CGXII medium (containing 0.09 mM CaCl_2 , 1 mM MgSO_4 , and 0 mM SrCl_2) containing 50 mM citrate as the sole carbon and energy source. The cultures were incubated at 30°C on a rotary shaker at 120 rpm. The growth curves shown here are representative of those from three independent growth experiments with comparable results.

dium from an initial pH of 7 to 8.5 to 8.7 at the end of growth (data not shown). Growth behavior in the presence of MgCl_2 was different, as the maximal growth rate (0.20 h^{-1}) was obtained only with 100 mM (Fig. 3B) and then decreased with even higher concentrations (data not shown). In the case of SrCl_2 , the maximal growth rate (0.14 h^{-1}) and cell yield were obtained with 10 mM (Fig. 3C). Higher concentrations of dissolved SrCl_2 could not be achieved. In the subsequent growth experiments with *C. glutamicum* on citrate, the medium was usually supplemented with 5 mM CaCl_2 .

Testing the roles and cation specificities of CitH and TctABC for the growth of *C. glutamicum* on citrate. Since *C. glutamicum* possesses two functional citrate transporters, the question arises of whether both are required for growth on citrate or if each one alone is sufficient for growth. Moreover, the cation specificities determined for the two transporters in *E. coli* should also be tested in *C. glutamicum*. For this purpose,

FIG. 4. Cation dependency of the growth of the *C. glutamicum* wild type (A), the Δ *citH* strain (B), and the Δ *tctCBA* strain (C) in CGXII medium with 50 mM citrate as the sole carbon and energy source. The medium contained either no additional salts ($\diamond$) or 5 mM CaCl₂ (●), 5 mM MgCl₂ (▲), or 5 mM SrCl₂ (■). Cells were pregrown in CGXII minimal medium with 50 mM glucose and 50 mM citrate. The cultures were incubated at 30°C on a rotary shaker at 120 rpm. The growth curves shown here are representative of those from three independent growth experiments with comparable results.

three deletion mutants of *C. glutamicum*, the Δ *citH*, Δ *tctCBA*, and Δ *citH* Δ *tctCBA* strains, were constructed as described in Materials and Methods. The three strains and, for comparison, the wild type were cultivated in CGXII minimal medium with 50 mM citrate as the sole carbon and energy source, with and without the addition of 5 mM CaCl₂, MgCl₂, or SrCl₂. In the case of MgCl₂ and SrCl₂, the concentration of 5 mM is below the concentration required for maximal growth but still sufficient to allow growth.

As shown in Fig. 4A, the wild type grew best in the presence of Ca²⁺, followed by Mg²⁺ and Sr²⁺. In the presence of Sr²⁺, the wild type reached only about one-third of the OD₆₀₀ obtained with calcium or magnesium ions (a final OD₆₀₀ of 4 compared to a final OD₆₀₀ of 12). The Δ *citH* mutant grew well in the presence of magnesium ions, worse with additional calcium ions, and not at all with added strontium ions (Fig. 4B). The Δ *tctCBA* mutant grew equally well in the presence of calcium and strontium ions but did not grow in the presence of magnesium ions (Fig. 4C). This behavior reflects the cation specificity determined for the TctCBA system (Ca²⁺ and

FIG. 5. Simultaneous consumption of carbon sources during the growth (□) of *C. glutamicum* on citrate and glucose. The wild type was grown in CGXII medium containing 50 mM glucose, 50 mM sodium citrate, and 5 mM CaCl₂. Cells were precultivated in CGXII minimal medium with 50 mM glucose and 50 mM citrate. The consumption of citrate (▲) and glucose (●) was determined via HPLC as described in Materials and Methods. The curves shown here are representative of those from three independent growth experiments with comparable results.

Mg²⁺) and CitH (Ca²⁺ and Sr²⁺) in *E. coli*. A remarkable result becomes obvious by a comparison of Fig. 4A and C. In the absence of TctABC, Sr²⁺ allows growth on citrate at the same rate and to the same final OD₆₀₀ as Ca²⁺ (Fig. 4C). In the presence of TctABC, however, both the growth rate and the final OD₆₀₀ obtained with Sr²⁺ are much lower than those obtained with Ca²⁺ (Fig. 4A). This result indicates that the simultaneous presence of TctABC and Sr²⁺ ions inhibits growth on citrate in a yet unknown manner. The much better growth of the Δ *citH* strain with Mg²⁺ than with Ca²⁺ may indicate that Mg²⁺ is the preferred cation for citrate transport by the TctCBA system.

The Δ *citH* Δ *tctCBA* double mutant did not grow on citrate under any tested conditions during the first 10 h of incubation; however, in a few cases, growth up to an OD₆₀₀ of 10 to 12 after 20 to 30 h was observed (data not shown).

The growth of *C. glutamicum* on citrate is not subject to catabolite repression. Previous studies (3, 19, 55) had indicated that citrate and glucose are catabolized simultaneously by *C. glutamicum*. To confirm this hypothesis, we monitored the consumption of the substrates by a culture growing with 50 mM citrate and 50 mM glucose (supplemented with 5 mM CaCl₂). As shown in Fig. 5, the substrates were consumed simultaneously, indicating that the genes required for citrate utilization are not subject to catabolite repression. Interestingly, citrate consumption started before glucose consumption, indicating that citrate initially had some inhibitory effect on glucose utilization. Nevertheless, the two substrates were consumed after the same time period of about 10 h.

The CitAB two-component system is required for citrate utilization by *C. glutamicum*. *C. glutamicum* possesses genes for 13 TCS (27). One of these TCS, encoded by the *citAB* genes, belongs to the CitAB/DcuSR subfamily (23). The *C. glutamicum* sensor kinase CitA (551 amino acid residues) contains two putative transmembrane helices separated by an extracytoplasmic domain extending from residues 48 to 188. It displays 24 and 29% overall sequence identity to the CitA histidine kinases from *K. pneumoniae* and *E. coli*, respectively, which were previously shown to function as citrate receptors (22, 23). The *C. glutamicum* response regulator CitB (218 amino acid residues)

FIG. 6. Growth of the *C. glutamicum* wild type (squares) and the mutant strain Δ *citAB* (triangles) in CGXII minimal medium containing either 50 mM glucose (filled symbols) or 50 mM citrate (hollow symbols) as the sole carbon and energy source. Cells were pregrown in CGXII minimal medium with 50 mM glucose and 50 mM citrate. The medium was supplemented with 5 mM CaCl_2 . The cultures were incubated at 30°C on a rotary shaker at 120 rpm. The growth curves shown here are representative of those from three independent growth experiments with comparable results.

shows 32 and 35% sequence identity to the CitB proteins from *K. pneumoniae* and *E. coli*, respectively. The affiliation with the CitAB/DcuSR subfamily and the fact that the *citAB* genes are located immediately upstream of and divergent from the *citH* gene (Fig. 1A) suggested an involvement of the *C. glutamicum* CitAB TCS in the regulation of citrate uptake genes. This suggestion was confirmed by the fact that a *C. glutamicum* mutant lacking the *citAB* genes (the Δ *citAB* strain) was unable to grow with citrate as the sole carbon and energy source within the first 20 h of cultivation but grew like the wild type on glucose (Fig. 6) or pyruvate (data not shown). The growth defect could be abolished by the transformation of the deletion mutant with the *citAB* expression plasmid pXMJ19-*citAB* (data not shown). Interestingly, the growth of the Δ *citAB* mutant on citrate occurred after prolonged incubation (about 30 h in the experiment with results shown in Fig. 6). When such cultures were used to inoculate fresh citrate minimal medium, cells grew either immediately or with a shortened lag phase, indicating that a suppressor mutation had presumably allowed the expression of either *citH* or *tctCBA*.

Transcriptome comparisons reveal that CitAB is responsible for the activation of *citH* and *tctCBA*. To analyze the influence of the *citAB* deletion on genomewide gene expression in the presence of citrate, the transcriptomes of the Δ *citAB* mutant and the wild type were compared using DNA microarrays. For this purpose, the cells were grown in CGXII minimal medium with 50 mM citrate, 50 mM glucose, and 50 mM MgCl_2 . Four sets of comparisons, each starting with independent cultures, were made. Sixty genes showed alterations in mRNA levels of more than threefold in at least three of four experiments ($P \leq 0.05$). Thirty-six of these genes showed decreases and 29 genes showed increases in mRNA levels in the Δ *citAB* mutant compared to those in the wild type. As shown in Table 3, the functions of the encoded proteins are highly diverse, and only those genes showing the most drastic changes in mRNA levels will be dealt with here. The *citH* and *tctCBA* mRNA levels in the Δ *citAB* mutant were 25- to 100-fold lower than those in the wild type, indicating that the CitAB TCS is responsible for the activation of these genes. Only two other genes, namely, *cg2380* and *ssuC*, showed mRNA levels that were changed to the same extent and in the same manner

(Table 3). The former gene encodes a protein of 14,084 Da that contains a single transmembrane helix extending from amino acid residue 65 to 85, with the N-terminal region located extracytoplasmically. Homologs are present in many other corynebacteria, mycobacteria, and other genera of the actinomycetes; however, the function is still unknown. The *ssuC* gene is part of the *ssuDICBA* operon encoding a sulfonase (*ssuDI*) and an ABC transporter for aliphatic sulfonates (28). The other genes of this operon, as well as further genes involved in sulfonate metabolism (*ssuD2*, *ssuI*, and *seuA*), also showed altered mRNA levels (Table 3). The reason for the changed expression patterns of these genes in the comparison of the Δ *citAB* mutant and the wild type is unclear. In contrast to the genes mentioned above, *cg3195*, encoding a flavin-containing monooxygenase, showed a 27-fold increase in the mRNA level in the Δ *citAB* mutant compared to that in the wild type. Again, an obvious reason for this change is not apparent.

Requirement of citrate for CitAB-dependent activation of *citH* and *tctCBA*. In order to confirm the microarray data and to test whether the presence of citrate is required for *citH* and *tctCBA* induction, primer extension experiments were performed. As shown in Fig. 7, primer extension products were detectable for *citH* and *tctC* RNAs of the wild type cultivated on citrate or citrate plus glucose, but not for RNAs of the wild type cultivated on glucose or the Δ *citAB* mutant cultivated on glucose or glucose plus citrate. From these results, three conclusions can be drawn: (i) the expression of *citH* and *tctCBA* is activated in the presence of citrate, and in the absence of citrate, there is no or very weak expression; (ii) glucose does not inhibit the expression of *citH* and *tctCBA*, since the primer extension products for RNAs from cells grown on citrate and those grown on citrate plus glucose were of equal strengths; and (iii) citrate-inducible expression of both *citH* and *tctCBA* is strictly dependent on the CitAB TCS.

In the case of *citH*, one major and two minor primer extension products were observed. The major transcription start site was located 56 bp upstream of the first putative start codon of *citH*, and the minor ones were located 55 and 53 bp upstream of the start codon (Fig. 1A and 7). The same start sites were also obtained with a second primer annealing at a different position (data not shown). The -10 region (Fig. 1A) deduced from the transcription start site (5'-TATACT-3') is very similar to the -10 consensus core hexamer reported previously for *C. glutamicum*, 5'-TA(C/T)AAT-3' (38). In the case of *tctC*, a single primer extension product, which ended 98 bp upstream of the predicted start codon of *tctC*, was identified (Fig. 1B and 7). Again, the same transcription start site was identified with a second primer annealing at a different position (data not shown), and the sequence of the deduced -10 region (5'-TTTAAT-3') was similar to the consensus sequence listed above.

Binding of the response regulator CitB to the promoter regions of *citH* and *tctCBA*. To support the assumption that CitB functions as a direct transcriptional activator of *citH* and *tctCBA*, EMSAs were performed with purified CitB and the promoter regions of *citH* and *tctC*. For this purpose, the CitB protein containing an amino-terminal hexahistidine tag was overproduced in *E. coli* BL21(DE3) and purified to apparent homogeneity by affinity chromatography (data not shown). DNA fragments covering the promoter regions of *citH* (bp -179 to $+41$ with respect to the major *citH* transcriptional

TABLE 3. Genes with ≥ 3 -fold change in mRNA levels^a

cg designation	NCgl designation	Gene name	Description of (putative) product	Avg mRNA ratio ($\Delta citAB$ strain mRNA/wt mRNA)
cg0089	NCgl0067	<i>citA</i>	Sensor histidine kinase	0.00
cg0090	NCgl0068	<i>citB</i>	Response regulator	0.00
cg3125	NCgl2724	<i>tctA</i>	Tricarboxylate transport membrane protein	0.04
cg3126	NCgl2725	<i>tctB</i>	Tricarboxylate transport membrane protein	0.01
cg3127	NCgl2726	<i>tctC</i>	Tricarboxylate binding protein	0.02
cg2380	NCgl2088		14-kDa membrane protein	0.03
cg0088	NCgl0066	<i>citH</i>	Citrate transporter	0.04
cg1376	NCgl1173	<i>ssuD1</i>	Sulfonatase (alkanesulfonate monooxygenase)	0.10
cg1377	NCgl1174	<i>ssuC</i>	Aliphatic sulfonate transmembrane ABC transporter protein	0.04
cg1379	NCgl1175	<i>ssuB</i>	Aliphatic sulfonate ATP binding ABC transporter protein	0.10
cg1380	NCgl1176	<i>ssuA</i>	Aliphatic sulfonate binding protein	0.20
cg3226	NCgl2816		L-Lactate permease	0.05
cg3227	NCgl2817	<i>lldD</i>	Quinone-dependent L-lactate dehydrogenase	0.15
cg3308	NCgl2881	<i>rpsF</i>	30S ribosomal protein S6	0.09
cg0810	NCgl0676		Hypothetical protein	0.09
cg1628	NCgl1383		Hydrolase of the α/β superfamily	0.12
cg1581	NCgl1341	<i>argJ</i>	Bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	0.14
cg1582	NCgl1342	<i>argB</i>	N-Acetylglutamate kinase	0.18
cg1583	NCgl1343	<i>argD</i>	Acetylornithine aminotransferase	0.27
cg3026	NCgl2635	<i>mrpD</i>	Subunit D of multisubunit Na ⁺ /H ⁺ antiporter	0.19
cg3027	NCgl2636	<i>mrpE</i>	Subunit E of multisubunit Na ⁺ /H ⁺ antiporter	0.15
cg3028	NCgl2637	<i>mrpF</i>	Subunit F of multisubunit Na ⁺ /H ⁺ antiporter	0.24
cg3029	NCgl2638	<i>mrpG</i>	Subunit G of multisubunit Na ⁺ /H ⁺ antiporter	0.23
cg1156	NCgl0975	<i>ssuD2</i>	Monooxygenase for sulfonate utilization	0.19
cg1451	NCgl1235	<i>serA</i>	Phosphoglycerate dehydrogenase	0.22
cg0576	NCgl0471	<i>rpoB</i>	Subunit β of DNA-directed RNA polymerase	0.23
cg2894	NCgl2523		Drug resistance-related transcriptional repressor	0.24
cg1364	NCgl1161	<i>atpF</i>	Subunit B of ATP synthase	0.26
cg2157	NCgl1892	<i>terC</i>	Tellurium resistance membrane protein	0.30
cg1151	NCgl0972	<i>seuA</i>	Monooxygenase for sulfonate ester utilization	0.30
cg1386	NCgl1182	<i>fixA</i>	Subunit β of putative electron transfer flavoprotein	0.30
cg0653	NCgl0538	<i>rpsK</i>	30S ribosomal protein S11	0.31
cg1147	NCgl0968	<i>ssuI</i>	Flavin mononucleotide binding protein required for sulfonate and sulfonate ester utilization	0.32
cg1349	NCgl1147		Membrane protein containing CBS domain	0.33
cg0825	NCgl0689	<i>fabG</i>	3-Ketoacyl-(acyl carrier protein) reductase	0.35
cg0882	NCgl0738		Hypothetical protein	0.36
cg3420	NCgl2983	<i>sigM</i>	RNA polymerase σ factor of ECF subfamily	2.37
cg2069	NCgl1774	<i>psp1</i>	Putative secreted protein	2.50
cg3351	NCgl2920	<i>nagI</i>	Gentisate 1,2-dioxygenase	2.56
cg2630	NCgl2314	<i>pcaG</i>	Subunit α of protocatechuate dioxygenase	2.57
cg2966	NCgl2588		Phenol 2-monooxygenase	2.71
cg1255	NCgl1060		HNH endonuclease	2.87
cg0638	NCgl0524		HD superfamily hydrolase	3.09
cg3216	NCgl2808	<i>gntP</i>	Gluconate permease	3.10
cg2504	NCgl2201		Hypothetical protein	3.14
cg2181	NCgl1915		Secreted component of ABC-type peptide transport system	3.26
cg2182	NCgl1916		Permease component of ABC-type peptide transport system	3.46
cg2183	NCgl1917		Permease component of ABC-type peptide transport system	3.71
cg2184	NCgl1918		ATPase component of ABC-type peptide transport system; contains duplicated ATPase domains	4.41
cg3151	NCgl2131	<i>tnp2b</i>	Transposase	3.56
cg0759		<i>prpD2</i>	Methylcitrate dehydratase	3.63
cg0760	NCgl0629	<i>prpB2</i>	Methylisocitrate lyase	9.11
cg0762	NCgl0630	<i>prpC2</i>	Methylcitrate synthase	11.05
cg2938	NCgl2563		Permease component of ABC-type dipeptide/oligopeptide/nickel transport system	3.93
cg2940	NCgl2565		ATPase component of ABC-type transport system; contains duplicated ATPase domains	3.69
cg2836	NCgl2476	<i>sucD</i>	Subunit α of succinyl-CoA synthetase	4.92
cg2837	NCgl2477	<i>sucC</i>	Subunit β of succinyl-CoA synthetase	3.82
cg3047	NCgl2656	<i>ackA</i>	Acetate kinase	4.00
cg3048	NCgl2657	<i>pta</i>	Phosphotransacetylase	5.43
cg0961	NCgl0805	<i>metA</i>	Homoserine O-acetyltransferase	4.89
cg2426		<i>tnp2d</i>	Transposase	5.32
cg1612	NCgl1368		Acetyltransferase	5.69
cg2610	NCgl2294		Secreted component of ABC-type dipeptide/oligopeptide/nickel transport system	6.19
cg3107	NCgl2709	<i>adhA</i>	Zn-dependent alcohol dehydrogenase	9.21
cg3195	NCgl2787		Flavin-containing monooxygenase	26.82

^a For the genes listed, the mRNA level in the $\Delta citAB$ strain was altered ≥ 3 -fold compared to the mRNA level in the wild type (wt) in at least three of four independent experiments after the growth of the strains in CGXII minimal medium containing 50 mM citrate and 50 mM glucose as carbon sources. The table includes those genes for which the mean mRNA ratio showed at least a twofold change (increase or decrease) and had a *P* value of ≤ 0.05 . The mRNA ratios shown are mean values from three to four independent DNA microarray experiments starting from independent cultures. mRNA was prepared from cells in the exponential growth phase. CBS, cystathionine- β -synthase; ECF, extracytoplasmic function; succinyl-CoA, succinyl coenzyme A.

start site, defined as bp +1) and *tctC* (bp -216 to +120 with respect to the *tctC* transcriptional start site) were incubated with four different concentrations of CitB and separated by native polyacrylamide gel electrophoresis. As negative controls, a fragment extending from bp +17 to +248 with respect

to the major *citH* transcriptional start site and a promoter fragment of the arbitrarily chosen gene cg2836 (bp -557 to -226 with respect to the translational start site of cg2836) were tested. As shown in Fig. 8, the *citH* and *tct* promoter fragments in the presence of only a 10-fold molar excess of CitB were

FIG. 7. Expression analyses and determination of the transcriptional start sites of the *C. glutamicum citH* gene (A) and *tctCBA* operon (B) by primer extension analyses with the oligonucleotides *citH*-PE-1 and *tct*-PE-3. Results for genes from the following strains cultivated under the indicated conditions are shown: lanes 1, wild type grown on glucose; lanes 2, wild type grown on citrate; lanes 3, wild type grown on citrate and glucose; lanes 4, Δ *citAB* mutant grown on glucose; and lanes 5, Δ *citAB* mutant grown on citrate and glucose. RNAs from the strains were isolated after cultivation in CGXII minimal medium with 50 mM $MgCl_2$ and a 25 mM concentration of the indicated carbon source. In all primer extension experiments for which results are shown, equivalent amounts of total RNA (20 μ g) were used. The transcriptional start sites are indicated by asterisks.

partially shifted, and complete retardation in the presence of a 20- or 30-fold molar excess of CitB was observed. No shift in the two DNA fragments serving as negative controls was observed, even at the highest CitB concentration used. In the case of the *citH* and *tct* promoter regions, a second protein-DNA

complex was formed with increasing CitB concentrations, indicating either the presence of a second binding site or the binding of further CitB molecules to the initial protein-DNA complex. An inspection of the *citH* and *tctC* promoter regions did not reveal a highly conserved sequence motif that might represent the CitB binding site.

FIG. 8. Binding of CitB to the promoter regions of *citH* and *tctCBA*. DNA fragments (219 to 336 bp; final concentrations, 22 to 35 nM) covering the promoter regions of *citH* and *tctCBA* and two DNA fragments serving as negative controls (see the text) were incubated for 30 min at room temperature without CitB (far left lanes) or with a 10- to 40-fold molar excess of purified CitB protein, as indicated, before separation by native polyacrylamide (15%) gel electrophoresis and staining with Sybr green I.

DISCUSSION

In this study, we have unraveled the molecular basis for citrate utilization and its regulation in *C. glutamicum*. As the expression of the *C. glutamicum* genes *citH* and *tctCBA* in *E. coli* MG1655 enabled growth on citrate, both CitH and TctCBA must be able to catalyze citrate uptake into the cell (Fig. 2). However, the two transporters differ in their cation dependency. CitH is active with Ca^{2+} and Sr^{2+} but not with Mg^{2+} , and TctABC is active with Ca^{2+} and Mg^{2+} but not with Sr^{2+} . In *B. subtilis*, the citrate transporters CitM and CitH, which show 42 and 43% sequence identity to *C. glutamicum* CitH, have complementary metal ion specificities. CitM of *B. subtilis* transports citrate in complexes with Mg^{2+} , Ni^{2+} , Mn^{2+} , Co^{2+} , and Zn^{2+} and CitH in complexes with Ca^{2+} , Sr^{2+} , and Ba^{2+} (30). As the metal ion specificity of the *C. glutamicum* homolog clearly resembles that of *B. subtilis* CitH, the original designation of CitM was changed to CitH. For *B. subtilis* CitH, apparent K_m values of 33 and 40 μ M for the uptake of Ca-citrate and Sr-citrate complexes into *E. coli* cells were determined previously (30), and similar values can be expected for *C. glutamicum* CitH. In the case of the TctABC transporter, a number of physiological and biochemical studies were performed with the system of *S. enterica* serovar Typhimurium (reviewed in reference 63). However, no clear picture of the cation dependency of this system exists. Our results suggest that Ca^{2+} or Mg^{2+} is required for citrate transport catalyzed by *C. glutamicum* TctABC. Since high concentrations of these cations were required for growth on citrate by using the Tct

FIG. 9. Colocalization of genes encoding two-component systems of the CitAB/DcuSR family with genes for tricarboxylate or dicarboxylate transporters in selected *Actinobacteria*. Gene identifiers for all sensor kinases are indicated. DAACS, dicarboxylate/amino acid:cation (Na^+ or H^+) symporter; SSS, solute:sodium symporter.

system, a different cation may be used by this transporter in the natural habitat.

Birnbaum and Demain (3) suggested that citrate transport by *C. glutamicum* is inducible. The findings from our previous DNA microarray analyses (39) and the primer extension studies in this work clearly show that both *citH* and *tctCBA* are expressed only when citrate is present in the medium, and the transcriptome data indicate that the *tctCBA* operon is activated much more strongly than *citH*. The reason for this difference and its physiological meaning are not yet clear. The activation of *citH* and *tctCBA* in the presence of citrate is strictly dependent on the CitAB TCS, and a ΔcitAB deletion mutant of *C. glutamicum* was unable to grow on citrate as the sole carbon and energy source within the first ~30 h of cultivation (see below). These results indicate (i) that the histidine kinase CitA may function as a citrate sensor, as shown previously for the orthologous CitA proteins from *K. pneumoniae* (23) and *E. coli* (22), and (ii) that the response regulator CitB presumably functions as a transcriptional activator of *citH* and the *tctCBA* operon. The latter hypothesis was supported by results from EMSAs, which showed that purified CitB binds to the promoter regions of *citH* and *tctC*. According to these results, the transcriptional control of citrate permease genes by a TCS of

the CitAB/DcuSR family occurs not only in gram-negative bacteria and low-GC-content gram-positive bacteria, but also in high-GC-content gram-positive bacteria and thus is distributed in all major lines of the eubacteria. BLAST searches (2) with completed genome sequences of *Actinobacteria* revealed homologs of CitA/DcuS and CitB/DcuR in many members of this class of bacteria, such as *Mycobacterium smegmatis*, *Nocardia farcinica*, and *Streptomyces coelicolor*, and in nearly all cases, the corresponding genes were localized next to genes for a tricarboxylate or dicarboxylate transporter (Fig. 9).

Whereas citrate utilization is subject to catabolite repression in many bacteria (see the introduction), *C. glutamicum* uses citrate and glucose simultaneously (Fig. 5). In accord with this observation, the activation of *citH* and *tctCBA* was not influenced by the presence of glucose in the growth medium (39) (Fig. 7). Cometabolism of different carbon sources with glucose is typical for *C. glutamicum* and was shown previously for, e.g., fructose (13), acetate (58), propionate (12), serine (36), vanillate and protocatechuate (32), and gluconate (17).

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