JOURNAL OF BACTERIOLOGY, May 2002, p. 2439–2446 Vol. 184, No. 9
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.9.2439–2446.2002

Identification of a Gene Cluster in *Klebsiella pneumoniae* Which Includes *citX*, a Gene Required for Biosynthesis of the Citrate Lyase Prosthetic Group

Karin Schneider,[1] Christopher N. Kästner,[1] Margareta Meyer,[1] Mirja Wessel,[2] Peter Dimroth,[1] and Michael Bott[2]*

Institut für Mikrobiologie, Eidgenössische Technische Hochschule Zürich, CH-8092 Zürich, Switzerland,[1] and Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany[2]

Received 21 August 2001/Accepted 7 February 2002

The biosynthesis of the 2′-(5″-phosphoribosyl)-3′-dephospho-coenzyme A (CoA) prosthetic group of citrate lyase (EC 4.1.3.6), a key enzyme of citrate fermentation, proceeds via the initial formation of the precursor 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA and subsequent transfer to apo-citrate lyase with removal of pyrophosphate. In *Escherichia coli*, the two steps are catalyzed by CitG and CitX, respectively, and the corresponding genes are part of the citrate lyase gene cluster, *citCDEFXG*. In the homologous *citCDEFG* operon of *Klebsiella pneumoniae*, *citX* is missing. A search for *K. pneumoniae citX* led to the identification of a second genome region involved in citrate fermentation which comprised the *citWX* genes and the divergent *citYZ* genes. The *citX* gene was confirmed to encode holo-citrate lyase synthase, whereas *citW* was shown to encode a citrate carrier, the third one identified in this species. The *citYZ* genes were found to encode a two-component system consisting of the sensor kinase CitY and the response regulator CitZ. Remarkably, both proteins showed ≥40% sequence identity to the citrate-sensing CitA-CitB two-component system, which is essential for the induction of the citrate fermentation genes in *K. pneumoniae*. A *citZ* insertion mutant was able to grow anaerobically with citrate, indicating that CitZ is not essential for expression of citrate fermentation genes. CitX synthesis was induced to a basal level under anaerobic conditions, independent of citrate, CitB, and CitZ, and to maximal levels during anaerobic growth with citrate as the sole carbon source. Similar to the other citrate fermentation enzymes, CitX synthesis was apparently subject to catabolite repression.

Many species of enterobacteria, such as *Klebsiella pneumoniae* and *Escherichia coli*, are able to utilize citrate under anoxic, fermentative conditions. Whereas *K. pneumoniae* can grow with citrate as the sole carbon and energy source (for a review, see reference 6), *E. coli* is dependent on the presence of an oxidizable cosubstrate (18), due to the lack of oxaloacetate decarboxylase. The initial step in all known citrate fermentation pathways is the Mg^{2+}-dependent cleavage of citrate to acetate and oxaloacetate, a reaction catalyzed by citrate lyase (2, 10, 33).

In *K. pneumoniae*, the structural genes for citrate lyase are part of the *citCDEFG* operon, which is located divergent to *citS* (Fig. 1). The proteins deduced from *citC*, *citD*, *citE*, and *citF* are citrate lyase ligase and the γ, β, and α subunits of citrate lyase, respectively (7). The *citC* operon is induced under anoxic conditions in the presence of citrate and Na^+ ions. Its expression is strictly dependent on the citrate-sensing CitA-CitB two-component regulatory system (8, 16, 20) and is subject to catabolite repression, presumably by the cyclic AMP receptor protein (19).

In *E. coli*, a *citCDEFXG* gene cluster between 13.9 and 14.2 min (Fig. 1) that exhibited high similarity to the *K. pneumoniae* citrate lyase cluster (5) but differed by the presence of an additional gene, designated *citX* (25), was identified. Like in *K. pneumoniae*, expression of the *E. coli citC* operon is regulated by a two-component system named either CitA-CitB (25) or DpiB-DpiA (14), which shows high similarity to the corresponding proteins of *K. pneumoniae*.

Citrate lyase contains the prosthetic group 2′-(5″-phosphoribosyl)-3′-dephospho-coenzyme A (CoA) that is attached via phosphodiester linkage to a serine residue of the γ subunit (4, 9, 22, 26, 31), which serves as an acyl carrier protein (ACP) (11). It was recently demonstrated (29, 30) that synthesis and attachment of the prosthetic group involve two reactions catalyzed by CitG and CitX (Fig. 2). In the first step, CitG catalyzes an unusual α-1,2-glycosidic linkage between ATP and dephospho-CoA, forming the prosthetic group precursor 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA and adenine as products. CitG therefore functions as triphosphoribosyl-dephospho-CoA synthase (systematic name, ATP:dephospho-CoA 5′-triphosphoribosyl transferase). In the second step, the precursor is transferred to apo-ACP by CitX, resulting in the formation of holo-ACP and pyrophosphate. CitX thus functions as holo-citrate lyase synthase [systematic name, 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA:apo-citrate lyase 2′-(5″-phosphoribosyl)-3′-dephospho-CoA transferase]. The mechanism for the biosynthesis of the 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group is not unique for citrate lyase but, as shown in a parallel work, follows a very similar route in the case of malonate decarboxylase (13).

In *E. coli* the genes encoding CitX and CitG are part of the citrate lyase gene cluster. Therefore, expression of the plasmid-encoded *citCDEFXG* genes in *E. coli* led to the formation of a functional holo-citrate lyase (29). In contrast, expression of the *K. pneumoniae* cluster *citCDEFG*, which lacks *citX*, resulted in

* 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.

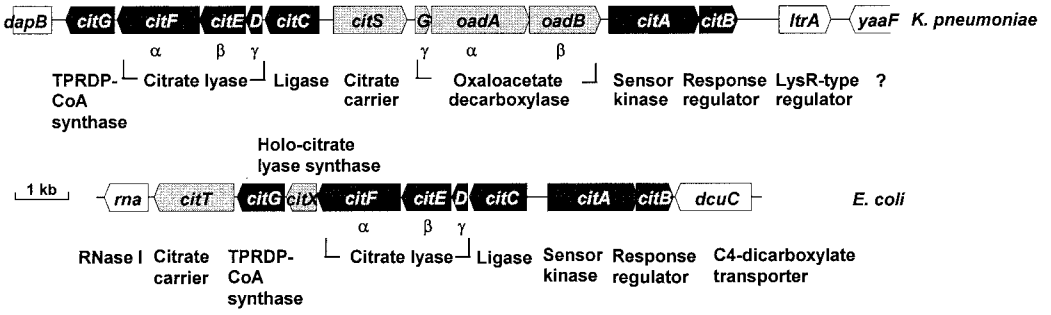


FIG. 1. Physical map of the citrate fermentation gene clusters in *K. pneumoniae* and *E. coli*. Genes in black are those present in both clusters, whereas genes in gray are present in only one. All other genes indicated presumably do not participate in citrate fermentation. TPRDP-CoA, 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA.

the synthesis of an inactive citrate lyase because only the apoform of the ACP was formed (7). This deficiency could be complemented, however, by coexpression of the *E. coli citX* gene (29), which strongly indicated the existence of a *citX* homologous gene in *K. pneumoniae*. The fact that it is not part of the *citCDEFG* cluster might be incidental, but it could also reflect a transcriptional regulation different from that of the *citC* operon.

In this work, the *K. pneumoniae citX* gene was identified and its function was verified. Remarkably, *citX* clustered with three genes which presumably are also involved in citrate fermentation. One of these, *citW*, was shown to encode a citrate carrier, the third one to be identified, in addition to CitH (34, 35) and CitS (36, 37). The two other genes clustered with *citX*, named *citY* and *citZ*, encode a two-component regulatory system with high homology to the CitA-CitB system, which was previously shown to be essential for expression of the *citC* and *citS* operons (8).

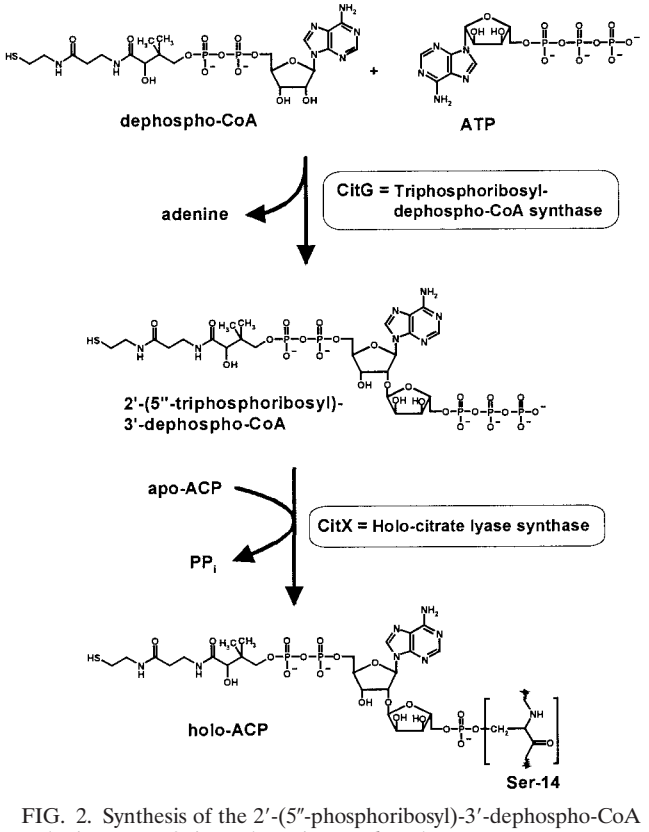


FIG. 2. Synthesis of the 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group of citrate lyase in *E. coli* and *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and culture media. *E. coli* and *K. pneumoniae* strains were routinely grown in Luria-Bertani (LB) medium at 37°C (27). *E. coli* DH5α (Bethesda Research Laboratories) was used for general cloning purposes. *E. coli* BL21(DE3) containing the T7 RNA polymerase gene under the control of a *lacUV5* promoter (32) served as the host for overproduction of the CitX protein from pET expression plasmids. *E. coli* SM10λpir (21) served as the host during construction and mobilization of pKNG101-′citZ′.

For anaerobic growth, the *K. pneumoniae* cells were cultivated in a minimal medium which contained 100 mM potassium phosphate buffer (pH 7.0), 50 mM NaCl, 10 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$ and 0.4% trace element solution (180 mM $CaCl_2$, 0.77 mM $CoCl_2$, 0.51 $MnCl_2$, 0.41 mM Na_2MoO_4, and 7.2 mM $FeSO_4$). Carbon sources were added to a final concentration of 20 mM (potassium citrate or glucose) or 46 mM (glycerol). For analyzing the synthesis of CitX under anaerobic growth conditions, cells were cultivated in 16-ml tubes completely filled with medium and closed with screw caps containing a silicon septum. After 19 h of incubation at 37°C without agitation, when the cultures were in the stationary phase and citrate had been completely consumed, the cells were used for Western blot analysis of CitX. For comparing the growth properties of the *citZ* insertion mutant KS1 with those of the wild type and the *citB* deletion mutant KM4 (8), cells were grown at 37°C in serum bottles closed with rubber septa under nitrogen (150 kPa). Growth was monitored by measuring the optical density at 600 nm. The cells used for inoculation were routinely precultured aerobically on LB medium and washed in 100 mM potassium phosphate buffer (pH 7.0) prior to inoculation. For selective growth of *K. pneumoniae* and for testing the ability of *E. coli* recombinants to grow aerobically on citrate as the sole carbon and energy source, Simmons' citrate agar (Difco) supplemented with 12 μM thiamine was used. When required, ampicillin (100 μg ml^{-1}) or streptomycin (50 μg ml^{-1}) was added to the different media.

Amplification of a DNA fragment overlapping contigs 809 and 867. In order to test whether contig 809 and contig 867 encompass adjacent regions on the genome, a PCR was carried out with oligonucleotides kp809-for2 (5′-TTCGCC GCCAGCAATGCCTC-3′) and kp867-rev (5′-CCGCACACGCTGATTCAGG G-3′). The reaction was performed with *Taq* DNA polymerase and chromosomal DNA of *K. pneumoniae* ATCC 13882 as the template in the presence of 8% (vol/vol) dimethyl sulfoxide (DMSO). The 1,172-bp PCR product was subjected to sequence analysis.

Plasmids. For the amplification of *K. pneumoniae* genes by PCR, chromosomal DNA of strain ATCC 13882 was used as the template. The reactions were performed with *Pfu* DNA polymerase (Stratagene) according to the instructions of the supplier. The reaction mixture contained 4 to 6% (vol/vol) DMSO.

For the construction of pET124-kpcitX, the coding region of the *K. pneumoniae citX* gene was amplified by PCR using the primers kp-citX-for (5′-AAA TTTCATATGTCAGTGGATACTCCCGCCCAG-3′) and kp-citX-rev (5′-TAT TATCTCGAGTTAATCGCGGGCGAACCACGC-3′). With this procedure, the start codon became part of an *Nde*I restriction site while an *Xho*I site was

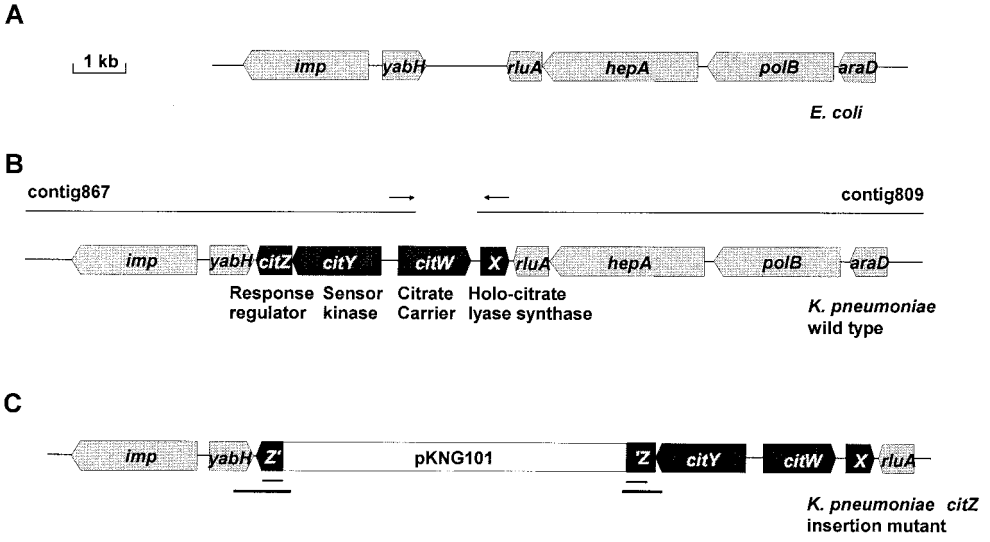


FIG. 3. (A) Physical map of the *E. coli* genome region that contributed to the identification of the *K. pneumoniae citX* upstream region (see the text). (B) Physical map of the *K. pneumoniae* genome region containing the *citX* gene as deduced from the *K. pneumoniae* genome sequencing project (http://genome.wustl.edu/gsc/Projects/K.pneumoniae /) and the present study. The genes presumably involved in citrate fermentation in *K. pneumoniae* are in black, whereas the flanking genes, which are present in similar arrangement in *K. pneumoniae* and *E. coli*, are in grey. The arrows indicate the positions of the primers (kp867-rev and kp809-for2) used to amplify a DNA fragment that overlaps contig 809 and contig 867. (C) Physical map of the genomic *citX* region in the *K. pneumoniae* mutant KS1 obtained through the chromosomal integration of plasmid pKNG101-′citZ′. The duplicated part of *citZ* is underlined (thin lines). The DNA regions that were amplified by PCR and sequenced in order to confirm the genomic structure are indicated by thick lines.

introduced immediately after the stop codon. After digestion with *Nde*I and *Xho*I, the 543-bp PCR product was cloned into the vector pET124b (29) cut with the same enzymes to yield pET124-kpcitX. To construct pET124-kpcitXhis, the *citX* gene was amplified with the oligonucleotides kp-citX-for and kp-citXhis-rev (5′-TATTATCTCGAGATCGCGGGCGAACCACGCGTC-3′). Thereby, the *citX* stop codon was replaced with an *Xho*I restriction site. After digestion of the PCR product with *Nde*I and *Xho*I, the 540-bp DNA fragment was cloned into pET124b cut with the same enzymes. This led to a plasmid encoding CitX with a C-terminally attached His tag.

For the construction of a *citW* expression plasmid, the *citW* coding region was amplified by using the oligonucleotides kp-citW-for (5′-GATTCGAAGCTTCA TATGAGCACCACAGACAATGCATTCTC-3′) and kp-citW-rev (5′-ATAAT AGGATCCTCACGCCAGATAGTGGCTGAGGAAC-3′). In kp-citW-for, the ATG start codon of *citW* is part of an *Nde*I restriction site, which is preceded by a *Hin*dIII restriction site. Primer kp-citW-rev introduced a *Bam*HI site immediately after the *citW* stop codon. After restriction with *Hin*dIII and *Bam*HI, the 1.34-kb PCR product was cloned into the vector pUC19 (40) digested with the same enzymes, resulting in plasmid pUC19-citW.

Construction of a *K. pneumoniae citZ* insertion mutant. In order to test whether *citZ* is essential for anaerobic growth on citrate, a *K. pneumoniae* mutant with an insertion in the chromosomally encoded *citZ* gene was constructed. For this purpose, a 367-bp internal fragment of *citZ* was amplified from *K. pneumoniae* ATCC 13882 chromosomal DNA by PCR in the presence of 6% (vol/vol) DMSO with the oligonucleotides kp-citZint2-for (5′-TATTATGGATCCCTAT TTACCGGACGGCAAAGGCG-3′) and kp-citZint-rev (5′-CCCGGGACTAG TCGGAAAAACAGTGCGCTTCCCG-3′), which introduced *Bam*HI and *Spe*I restriction sites, respectively. Following restriction of the PCR product with these two enzymes, the fragment was cloned into the suicide vector pKNG101 (15), resulting in plasmid pKNG101-′citZ′. This plasmid was transferred into the *K. pneumoniae* wild-type strain via conjugation using *E. coli* SM10λpir as the donor. Cointegrates were isolated on Simmons' citrate agar plates containing streptomycin, and the genomic structure of two selected clones was confirmed by amplifying and sequencing the two chromosomal crossover sites (Fig. 3C) with the primer pairs pKNG-for (5′-CAAATCAGCGACACTGAATACGG-3′)/kp-yabH-for (5′-TATTATGCGGCCGCTTTGGCCGTTTCGATCTGATTCG-3′) and pKNG-rev (5′-TCCCCTGGATTTCACTGATGAG-3′)/kp-citY-for (5′-AA CATCGAAGTCGCCGATAACGC-3′). The corresponding *citZ* insertion mutants were designated KS1 and KS2.

DNA sequence analysis. DNA sequence analysis was performed according to the dideoxynucleotide chain termination method (28) with a AmpliTaq DNA cycle sequencing kit (Applied Biosystems) and protocols and equipment for automated DNA sequencing (genetic analyzer 310; Applied Biosystems). Computer-assisted DNA and protein sequence analysis and alignments were performed with the Genetics Computer Group of the University of Wisconsin software package. Database searches were performed with the Basic Local Alignment Search Tool (BLAST) (1).

Biochemical methods. The preparation of cell extracts as well as the determination of protein concentrations and citrate lyase activity were performed as described previously (29). In order to purify *K. pneumoniae* $CitX_{His}$, cell extracts of *E. coli* BL21(DE3) containing pET124-kpcitXhis were prepared with buffer containing 20 mM Tris, 500 mM NaCl, and 5 mM imidazole (TNI5; the number indicates the imidazole concentration in millimolar units). The pHs of all TNI buffers were adjusted to 7.9 by the addition of HCl. After passage through a 0.2-μm-pore-size filter, the cell extracts were loaded onto a column containing a 2-ml bed volume of His-bind resin (Novagen) equilibrated with TNI5. The column was washed with 15 ml each of TNI5, TNI15, TNI30, and TNI60 followed by 5 ml of TNI100. Subsequently, $CitX_{His}$ was eluted with 10 ml of TNI200 in fractions of 1 ml, and the protein-containing fractions were pooled. For the preparation of polyclonal rabbit antibodies against $CitX_{His}$, aliquots of the purified protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17). After Coomassie staining, the $CitX_{His}$-containing bands were excised and used for immunization (Eurogentec, Seraing, Belgium).

Western blotting. Proteins separated by SDS-PAGE were electroblotted onto a polyvinylidene difluoride membrane. For the immunological detection of CitX, the blots were incubated with rabbit antiserum (1:500 dilution) raised against the purified protein from *K. pneumoniae*. Bound immunoglobulins were probed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories) and visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Nucleotide sequence accession number. The 2.16-kb DNA region of *K. pneumoniae* ATCC 13882 comprising *citX* and *citW* sequenced here was deposited in GenBank with the accession number AF411142.

RESULTS AND DISCUSSION

Identification and characterization of the *K. pneumoniae* DNA region containing the *citX* gene. The observation that the *E. coli citX* gene can complement the *K. pneumoniae citC-*

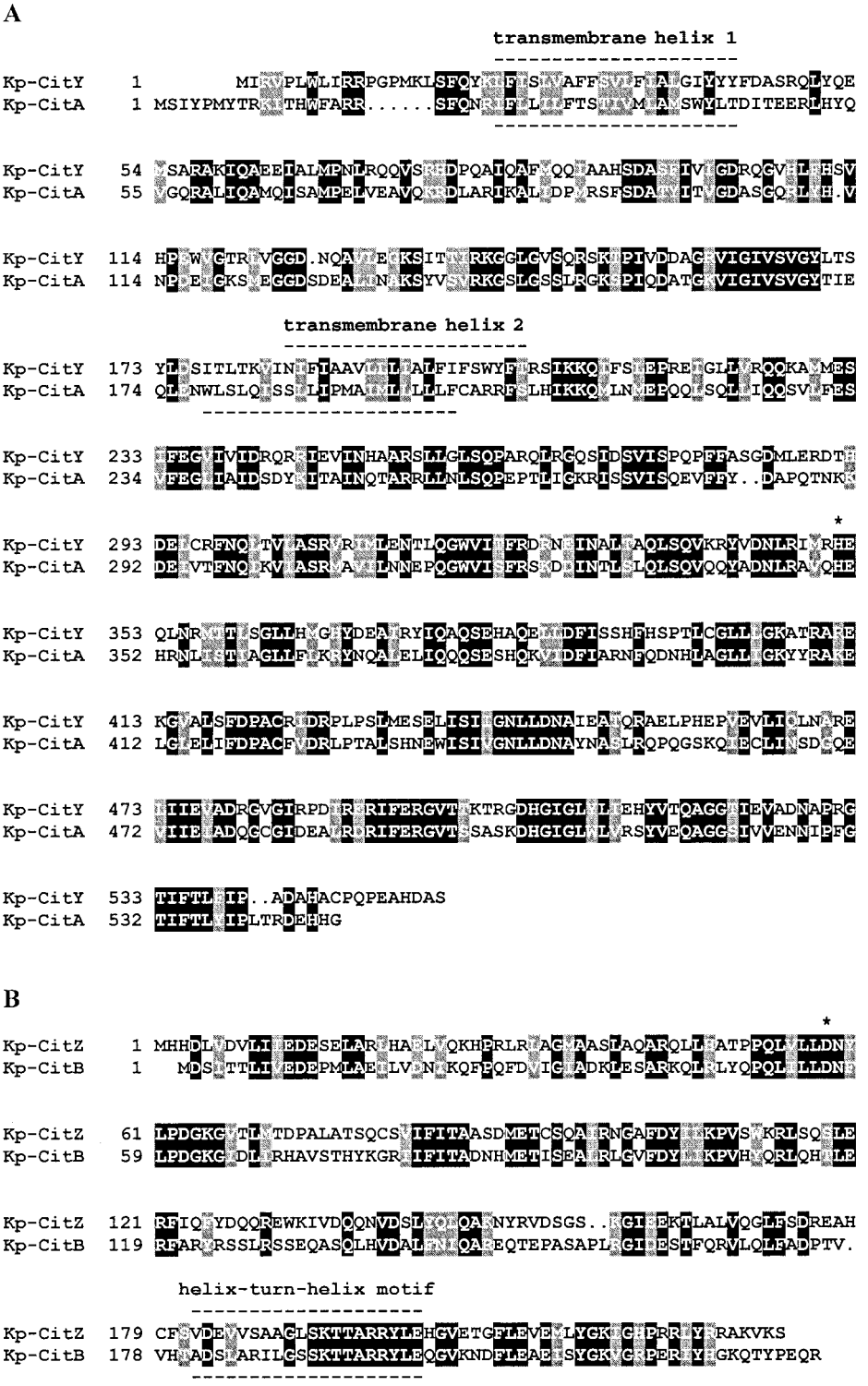
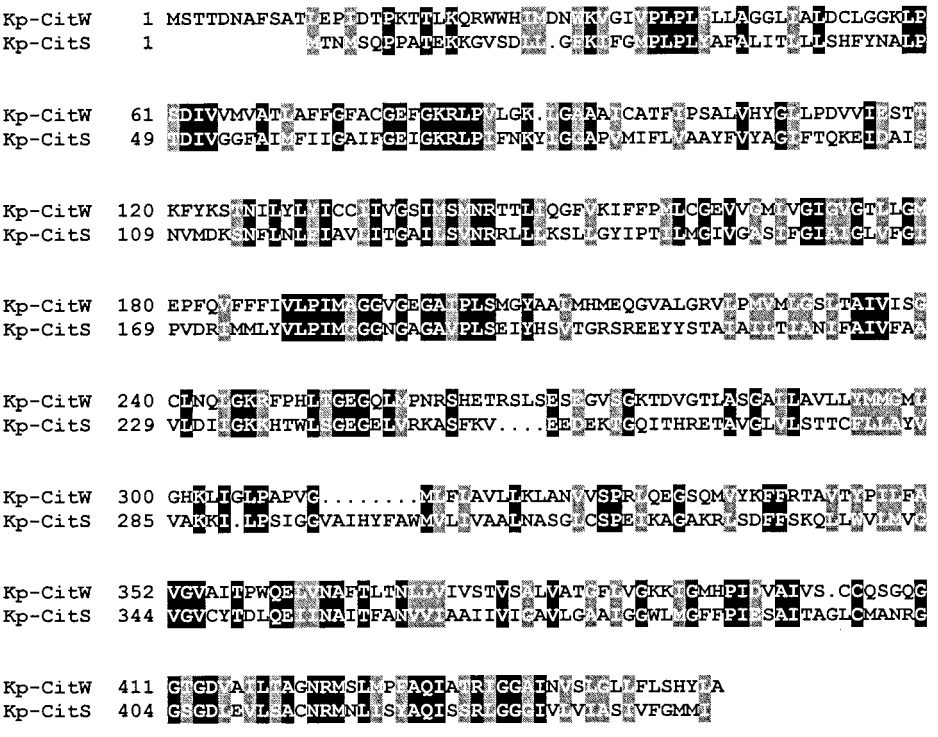


FIG. 4. Sequence alignments of the *K. pneumoniae* (Kp) sensor kinases CitY and CitA (A), the *K. pneumoniae* response regulators CitZ and CitB (B), the *K. pneumoniae* secondary carriers CitW and CitS (C), and the CitX proteins from *K. pneumoniae* and *E. coli* (Ec) (D). The phosphorylation sites of the sensor kinases (His-350 of CitA and His-351 of CitY) and the response regulators (Asp-56 of CitB and Asp-58 of CitZ) and the signature sequence previously reported to be conserved in members of the CitX family (29) are indicated by asterisks.

DEFG genes in the formation of an active holo-citrate lyase (29) strongly indicated the presence of a *citX* homologous gene on the *K. pneumoniae* chromosome. In order to identify it, a TBlastN search (1) was performed with the *E. coli* CitX protein sequence against the DNA sequences available from the *K. pneumoniae* strain MGH78578 genome sequencing project at the Washington University School of Medicine (St. Louis, Mo.; http://genome.wustl.edu/gsc/Projects/K.pneumoniae/). This led

C



D

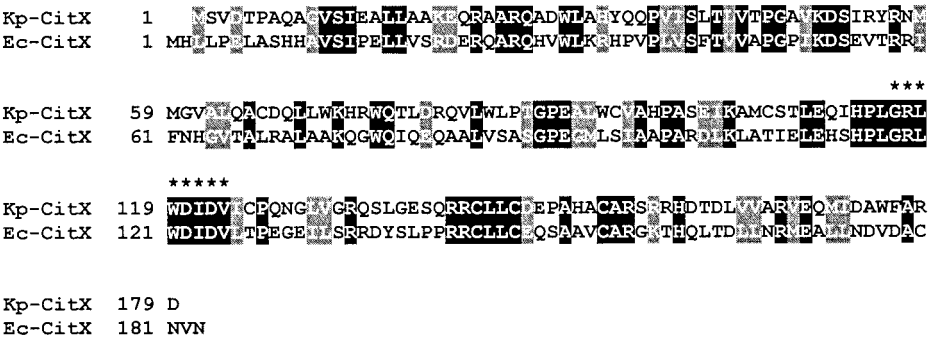


FIG. 4—*Continued.*

to the identification of contig 809, which encoded a protein harboring a sequence motif conserved within the CitX family (29). However, several attempts to clone the putative *citX* gene from chromosomal DNA by PCR failed, because of faults in the available DNA sequence which were detected later on.

Since the putative *citX* gene was located at the 5′ end of contig 809, only downstream genes could be identified, i.e., *rluA*, *hepA*, *polB*, and *araD* (Fig. 3B). Interestingly, these genes are present in the same order on the *E. coli* chromosome at 1.3 min (5), but upstream of *rluA* the genes *yabH* and *imp* rather than *citX* are found (Fig. 3A). This observation raised the possibility that the upstream region of *citX* in *K. pneumoniae* might be located on a contig harboring *yabH* and *imp*. BLAST searches revealed the corresponding genes to be located on contig 867 of the *K. pneumoniae* database. Downstream of *yabH*, two genes (designated *citY* and *citZ*) which encoded a two-component regulatory system with significant similarity to the CitA-CitB system of *K. pneumoniae* (8) were identified on contig 867. Upstream of *citY* and in divergent orientation, the 5′ part of another gene, named *citW*, was identified. The deduced partial gene product showed significant similarity to the citrate transporter CitS from *K. pneumoniae*.

The identification of three genes downstream of *yabH* which are related to citrate fermentation genes supported the idea that contig 867 is located adjacent to contig 809. In order to test this assumption, a PCR with chromosomal DNA of *K.*

pneumoniae was performed with one oligonucleotide (kp867-rev) priming within contig 867 and one (kp809-for2) priming within contig 809 (Fig. 3B). Indeed, a 1,172-bp DNA fragment was amplified, and sequence analysis confirmed that the two contigs encompass adjacent regions on the genome and led to the map presented in Fig. 3B. A 2.16-kb DNA region of *K. pneumoniae* ATCC 13882 comprising *citX* and *citW* was completely sequenced on both strands. In addition, selected regions of *citY* and *citZ* were sequenced and resulted in the removal of a frameshift present in the *citY* coding region of contig 867.

According to the corrected sequence, the protein deduced from *citY* consisted of 555 amino acids with a calculated molecular mass of 62.5 kDa and exhibited 44% sequence identity to the sensor kinase CitA from *K. pneumoniae* (Fig. 4A). Hydropathy analysis indicated that CitY had a similar topology as CitA with two transmembrane helices in the N-terminal part enclosing a periplasmic domain that extended from position 44 to 183. The corresponding domain of CitA was recently shown to function as a highly specific citrate receptor (16). The histidine residue which presumably is phosphorylated by the histidine autokinase CitY was located at position 351. In the C-terminal part of CitY, the conserved sequence motifs of the ATP binding site were found, i.e., the so-called N, G1, F, and G2 boxes.

The protein deduced from *citZ* consisted of 232 amino acids (26.2 kDa) and exhibited 40% sequence identity to the response regulator CitB from *K. pneumoniae* (Fig. 4B). The conserved aspartate residue which presumably is phosphorylated by CitY was located at position 58. In the C-terminal part, a putative DNA-binding helix-turn-helix motif extending from position 182 to 201 was detected. Remarkably, the sequence of the second helix was completely identical in CitZ and CitB, indicating similar DNA target sequences.

The protein deduced from *citW* was composed of 454 amino acids (48.2 kDa) and showed 29% identity to the Na^+-dependent citrate carrier CitS of *K. pneumoniae* (Fig. 4C). As in the case of CitS, hydropathy analysis predicted 12 putative transmembrane helices for CitW; however, in the case of CitS, evidence for a membrane topology with 11 transmembrane helices was provided (38), which might also apply to CitW.

The protein deduced from *citX* consisted of 179 amino acids (20.1 kDa) and displayed 38% identity to CitX from *E. coli* (Fig. 4D). The sequence G/A-R-L-X-D-L/I-D-V, previously proposed as a signature sequence for proteins with citrate lyase apo-ACP nucleotidyltransferase activity (29), was completely conserved in the *K. pneumoniae* CitX protein.

Identification of the *citX* gene product as holo-citrate lyase synthase. On the basis of the new sequence information, it was possible to amplify the putative *citX* gene from chromosomal DNA of *K. pneumoniae* ATCC 13882. Subsequently, the PCR product was cloned into the expression vector pET124b, resulting in plasmid pET124-kpcitX. In order to confirm that the cloned gene actually encodes a protein with holo-citrate lyase synthase activity, its ability to complement the *K. pneumoniae citCDEFG* genes with respect to the formation of an active holo-citrate lyase was tested. To this end, *E. coli* BL21(DE3) harboring plasmid pT7-CL (7) was transformed with the compatible plasmid pET124-kpcitX, and expression of the target genes was induced by IPTG (isopropyl-β-D-thiogalactopyranoside). Extracts prepared from these cells contained citrate lyase activity of up to 8.3 μmol min^{-1} (mg of protein)$^{-1}$, whereas extracts from cells containing the vector pET124b instead of pET124-kpcitX possessed an activity of <0.01 μmol min^{-1} (mg of protein)$^{-1}$ (29). This result provided conclusive evidence that the cloned *K. pneumoniae citX* gene indeed encoded holo-citrate lyase synthase. The specific activity of 8.3 μmol min^{-1} (mg of protein)$^{-1}$ was almost identical to that obtained previously (29) with the *E. coli* CitX protein (8.2 μmol min^{-1} [mg of protein]$^{-1}$) instead of *K. pneumoniae* CitX. This indicates that under the conditions employed, *E. coli* CitX could fully replace *K. pneumoniae* CitX in the transfer of phosphoribosyl-dephospho-CoA from triphosphoribosyl-dephospho-CoA to *K. pneumoniae* apo-ACP.

Identification of the *citW* gene product as a citrate transporter. The fact that CitW showed 29% sequence identity to CitS suggested that this protein might also function as a citrate carrier. In order to test this assumption, the *citW* gene was amplified by PCR from chromosomal DNA and ligated as a 1.34-kb *Bam*HI/*Hin*dIII fragment into pUC19, allowing transcription of *citW* from the vector-encoded *lac* promoter. The ligation mixture was transformed into *E. coli* DH5α and plated on Simmons' citrate agar containing ampicillin. After 24 h at 30°C, several citrate-positive colonies could be identified by a local color change of the bromthymol blue-containing agar from green to blue, caused by alkalinization due to citrate consumption. Cells unable to utilize citrate, such as *E. coli* DH5α containing the vector pUC19, formed very small colonies, presumably by using residual carbon sources present in the agar, and bromthymol blue remained green. Restriction analysis of plasmid DNA isolated from four Cit^+ clones revealed that all contained pUC19 with the *Bam*HI/*Hin*dIII insert carrying *citW*. One of the pUC19-citW plasmids was transformed again into *E. coli* DH5α, which was plated on Simmons' citrate agar. In this case, all transformants were able to utilize citrate, confirming that *citW* was responsible for the Cit^+ phenotype and therefore must encode a citrate transporter.

CitW represents the third secondary carrier for citrate in *K. pneumoniae* besides CitH and CitS. CitH is a Na^+-independent transporter which was proposed to catalyze the uptake of $HCit^{2-}$ (the protonated species of citrate, or the divalent citrate species) in symport with protons (34, 35). It is assumed that CitH is responsible for citrate uptake under oxic growth conditions. CitS is a Na^+-dependent citrate carrier (36, 37), and studies with the purified, reconstituted carrier showed that it catalyzes the electroneutral transport of $HCit^{2-}$ using ΔpNa and ΔpH as driving forces (23, 24). The *citS* gene is part of the CitA-CitB regulon and thus expressed only under anoxic conditions in the presence of citrate and Na^+ (8). The fact that *citW* is located in front of the *citX* gene required for the synthesis of holo-citrate lyase suggested that CitW could also be involved in citrate fermentation and posed the question on its specific function. Reverse transcription-PCR experiments revealed that *citW* mRNA was indeed present in *K. pneumoniae* cells grown anaerobically on citrate, and transport studies provided good evidence that CitW functions as a citrate-acetate antiporter (C. N. Kästner, K. Schneider, P. Dimroth, and K. M. Pos, unpublished data). Since acetate is the major end product of citrate fermentation in *K. pneumoniae* (6), such a function

makes sense physiologically. Nevertheless, the reason for the probable coexistence of CitS and CitW in citrate-fermenting *K. pneumoniae* cells is not yet clear.

Anaerobic growth on citrate of a *citZ* insertion mutant. Sequence comparisons clearly assigned CitY-CitZ to the CitA-CitB family of two-component systems (16). The common function of this family, which includes, e.g., DcuS-DcuR from *E. coli* (12, 41) and CitS-CitT from *Bacillus subtilis* (39), is to sense the presence of tri- or dicarboxylates in the environment and to induce the synthesis of secondary transporters for the corresponding compounds (16). To analyze the role of the CitY-CitZ system in citrate fermentation, a *citZ* insertion mutant was constructed. For that purpose, an internal 374-bp fragment of the *citZ* coding region was cloned into the suicide vector pKNG101 and transferred via conjugation into the *K. pneumoniae* wild-type strain. Exconjugants carrying a chromosomally integrated plasmid were selected on Simmons' citrate agar containing streptomycin. The expected gene arrangement (Fig. 3C) obtained after integration was confirmed by PCR amplification of the crossover sites and DNA sequence analysis of the corresponding PCR products.

One of the resulting *citZ* mutants, designated strain KS1, was used for growth studies. When cultivated anaerobically on citrate minimal medium, the *citZ* mutant was able to grow at the same rate and to the same density as the wild type, whereas the *citB* deletion mutant KM4 failed to grow, as reported previously (8). This indicated that in contrast to CitA-CitB the CitY-CitZ two-component system is not essential for the expression of one or several citrate fermentation genes.

Regulation of CitX synthesis. In all bacteria containing a *citX* gene—except *K. pneumoniae*—it is clustered with the citrate lyase structural genes, either as a separate gene between *citF* and *citG*, as in *E. coli* (25), or fused with *citG*, as in *Leuconostoc mesenteroides* (3). This raised the question of whether the separate location of *citX* in *K. pneumoniae* is incidental or reflects a transcriptional regulation that differs from that of the *citCDEFG* genes. In order to analyze the synthesis of CitX under different growth conditions and in different strains, the protein was overproduced in *E. coli*, purified by means of a C-terminally attached hexahistidine tag, and used for the production of polyclonal rabbit antibodies. The specificity of the antiserum was confirmed by Western blots with whole-cell lysates of *E. coli* BL21(DE3) harboring the *K. pneumoniae citX* expression plasmid pET124-citX. Before induction of *citX* expression, a weak band of about 20 kDa was detected by the antiserum, which became much stronger after induction. In whole-cell lysates of *E. coli* harboring only the vector pET124, this 20-kDa band was not detectable. These results confirmed that the antiserum reacts with the *K. pneumoniae* CitX protein.

CitX synthesis was examined in the *K. pneumoniae* wild-type strain, in the *citZ* insertion mutant KS1, and in the *citB* deletion mutant KM4 (Fig. 5). The protein migrating below CitX during SDS-PAGE that was detected with the CitX antiserum in all samples was also observed with the preimmune serum. It served as an internal control to show that equal amounts of protein were loaded onto the different lanes.

In the case of the wild type, only a very faint band corresponding to CitX was observed in cells grown aerobically in LB medium, but a significant CitX level was observed in cells grown anaerobically in glucose minimal medium. This indicated that anoxic conditions alone are sufficient to induce a low basal level of *citX* expression, whereas in the case of the genes for citrate lyase and oxaloacetate decarboxylase both anoxic conditions and the presence of citrate are required for significant expression (8). The maximal CitX content was present in cells grown anaerobically with citrate as the sole carbon and energy source, showing that besides the oxygen (or redox) status, citrate represents a second important signal for *citX* expression. In cells grown anaerobically with citrate plus glycerol, the CitX level was about half-maximal, indicating that *citX* expression is subject to catabolite repression, similar to the other citrate fermentation genes (19).

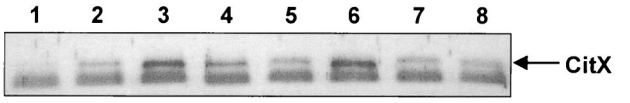


FIG. 5. Western blot analysis of *K. pneumoniae* whole-cell extracts with polyclonal rabbit antiserum against *K. pneumoniae* CitX. The wild type was grown aerobically on LB medium (lane 1) or anaerobically on glucose (lane 2), citrate (lane 3), or citrate plus glycerol (lane 4). The *citZ* insertion mutant KS1 was grown anaerobically on glucose (lane 5) or citrate (lane 6). The *citB* deletion mutant KM4 was grown anaerobically on glucose (lane 7) or citrate plus glycerol (lane 8). The band below CitX was also detected with the preimmune serum and served to confirm that equal amounts of protein were present in each lane.

In cells of the *citZ* insertion mutant KS1 grown anaerobically on glucose (Fig. 5, lane 5) or citrate (Fig. 5, lane 6), the CitX content was comparable to that of the wild type, indicating that the response regulator CitZ is required neither for anaerobiosis-induced *citX* expression nor for citrate-induced *citX* expression. This result was in accordance with the growth phenotype of strain KS1.

In cells of the *citB* deletion mutant KM4 grown anaerobically on glucose (Fig. 5, lane 7) the CitX content was comparable to that of the wild type grown under the respective conditions. In contrast, the CitX level in KM4 cells grown anaerobically on citrate plus glycerol appeared to be lower than in the wild type cultivated under these conditions. This suggested that the CitA-CitB two-component system is not required for anaerobiosis-induced *citX* expression but might be responsible for citrate-inducible *citX* expression. The latter assumption is supported by the presence of putative CitB binding sites in the 282-bp *citW-citY* intergenic region. It contains two identical 18-bp sequence motifs, each representing a 9-bp repeat (5′-TAAAAACCATAAAAACCA-3′). The CitB-binding sites in the *citC-citS* intergenic region as determined by DNase I footprints contain one perfect copy of this 9-bp repeat and two copies differing in only a single position (20).

In summary, the finding that the *K. pneumoniae citX* gene is clustered with genes for a novel citrate carrier and a CitA-CitB-homologous two-component system indicates that citrate fermentation and its regulation is more complex than previously assumed. Although the present study indicates that the CitY-CitZ system is not essential for citrate fermentation, a role of this two-component system in fine-tuning the expression of citrate fermentation genes in the natural environment is an attractive possibility to be addressed in further studies.

ACKNOWLEDGMENTS

This work was financially supported by Boehringer Mannheim GmbH (now Roche Diagnostics GmbH, Penzberg, Germany) and by the Commission for Technology and Innovation (Bern, Switzerland).

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Antranikian, G., and F. Giffhorn.** 1987. Citrate metabolism in anaerobic bacteria. FEMS Microbiol. Rev. **46:**175–198.
3. **Bekal, S., J. V. Beeumen, B. Samyn, D. Garmyn, S. Henini, C. Diviès, and H. Prévost.** 1998. Purification of *Leuconostoc mesenteroides* citrate lyase and cloning and characterization of the *citCDEF* gene cluster. J. Bacteriol. **180:** 647–654.
4. **Beyreuther, K., H. Böhmer, and P. Dimroth.** 1978. Amino-acid sequence of citrate-lyase acyl-carrier protein from *Klebsiella aerogenes*. Eur. J. Biochem. **87:**101–110.
5. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
6. **Bott, M.** 1997. Anaerobic citrate metabolism and its regulation in enterobacteria. Arch. Microbiol. **167:**78–88.
7. **Bott, M., and P. Dimroth.** 1994. *Klebsiella pneumoniae* genes for citrate lyase and citrate lyase ligase: localization, sequencing, and expression. Mol. Microbiol. **14:**347–356.
8. **Bott, M., M. Meyer, and P. Dimroth.** 1995. Regulation of anaerobic citrate metabolism in *Klebsiella pneumoniae*. Mol. Microbiol. **18:**533–546.
9. **Dimroth, P.** 1976. The prosthetic group of citrate lyase acyl carrier protein. Eur. J. Biochem. **64:**269–281.
10. **Dimroth, P.** 1988. The role of vitamins and their carrier proteins in citrate fermentation, p. 191–204. *In* H. Kleinkauf, H. von Döhren, and L. Jaenicke (ed.), The roots of modern biochemistry. Walter de Gruyter and Co., Berlin, Germany.
11. **Dimroth, P., W. Dittmar, G. Walther, and H. Eggerer.** 1973. The acyl-carrier protein of citrate lyase. Eur. J. Biochem. **37:**305–315.
12. **Golby, P., S. Davies, D. J. Kelly, J. R. Guest, and S. C. Andrews.** 1999. Identification and characterization of a two-component sensor-kinase and response-regulator system (DcuS-DcuR) controlling gene expression in response to C4-dicarboxylates in *Escherichia coli*. J. Bacteriol. **181:**1238–1248.
13. **Hoenke, S., M. Wild, and P. Dimroth.** 2000. Biosynthesis of triphosphoribosyl-dephospho-coenzyme A, the precursor of the prosthetic group of malonate decarboxylase. Biochemistry **39:**13223–13232.
14. **Ingmer, H., C. A. Miller, and S. N. Cohen.** 1998. Destabilized inheritance of pSC101 and other *Escherichia coli* plasmids by DpiA, a novel two-component system regulator. Mol. Microbiol. **29:**49–59.
15. **Kaniga, K., I. Delor, and G. R. Cornelis.** 1991. A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137–141.
16. **Kaspar, S., R. Perozzo, S. Reinelt, M. Meyer, K. Pfister, L. Scapozza, and M. Bott.** 1999. The periplasmic domain of the histidine autokinase CitA functions as a highly specific citrate receptor. Mol. Microbiol. **33:**858–872.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
18. **Lütgens, M., and G. Gottschalk.** 1980. Why a co-substrate is required for anaerobic growth of *Escherichia coli* on citrate. J. Gen. Microbiol. **119:**63–70.
19. **Meyer, M., P. Dimroth, and M. Bott.** 2001. Catabolite repression of the citrate fermentation genes in *Klebsiella pneumoniae*: evidence for involvement of the cyclic AMP receptor protein. J. Bacteriol. **183:**5248–5256.
20. **Meyer, M., P. Dimroth, and M. Bott.** 1997. *In vitro* binding of the response regulator CitB and of its carboxy-terminal domain to A + T-rich DNA target sequences in the control region of the divergent *citC* and *citS* operons of *Klebsiella pneumoniae*. J. Mol. Biol. **269:**719–731.
21. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of the outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
22. **Oppenheimer, N. J., M. Singh, C. C. Sweeley, S.-J. Sung, and P. A. Srere.** 1979. The configuration and location of the ribosidic linkage in the prosthetic group of citrate lyase (*Klebsiella aerogenes*). J. Biol. Chem. **254:**1000–1002.
23. **Pos, K. M., M. Bott, and P. Dimroth.** 1994. Purification of two active fusion proteins of the Na^+-dependent citrate carrier of *Klebsiella pneumoniae*. FEBS Lett. **347:**37–41.
24. **Pos, K. M., and P. Dimroth.** 1996. Functional properties of the purified Na^+-dependent citrate carrier of *Klebsiella pneumoniae*: evidence for asymmetric orientation of the carrier protein in proteoliposomes. Biochemistry **35:**1018–1026.
25. **Pos, K. M., P. Dimroth, and M. Bott.** 1998. The *Escherichia coli* citrate carrier CitT: a member of a novel eubacterial transporter family related to the 2-oxoglutarate/malate translocator from spinach chloroplasts. J. Bacteriol. **180:**4160–4165.
26. **Robinson, J. B., M. Singh, and P. A. Srere.** 1976. Structure of the prosthetic group of *Klebsiella aerogenes* citrate (*pro*-3 *S*)-lyase. Proc. Natl. Acad. Sci. USA **73:**1872–1876.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Schneider, K., P. Dimroth, and M. Bott.** 2000. Biosynthesis of the prosthetic group of citrate lyase. Biochemistry **39:**9438–9450.
30. **Schneider, K., P. Dimroth, and M. Bott.** 2000. Identification of triphosphoribosyl-dephospho-CoA as precursor of the citrate lyase prosthetic group. FEBS Lett. **483:**165–168.
31. **Singh, M., J. B. Robinson, and P. A. Srere.** 1977. On the structure of the prosthetic group of citrate (*pro*-3 *S*)-lyase. J. Biol. Chem. **252:**6061–6068.
32. **Studier, F. W., and B. A. Moffatt.** 1986. Use of T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
33. **Subramanian, S., and C. Sivaraman.** 1984. Bacterial citrate lyase. J. Biosci. **6:**379–401.
34. **Van der Rest, M. E., E. Schwarz, D. Oesterhelt, and W. N. Konings.** 1990. DNA sequence of a citrate carrier of *Klebsiella pneumoniae*. Eur. J. Biochem. **189:**401–407.
35. **Van der Rest, M. E., T. Abee, D. Molenaar, and W. N. Konings.** 1991. Mechanism and energetics of a citrate-transport system of *Klebsiella pneumoniae*. Eur. J. Biochem. **195:**71–77.
36. **Van der Rest, M. E., R. M. Siewe, T. Abee, E. Schwarz, D. Oesterhelt, and W. N. Konings.** 1992. Nucleotide sequence and functional properties of a sodium-dependent citrate transport system from *Klebsiella pneumoniae*. J. Biol. Chem. **267:**8971–8976.
37. **Van der Rest, M. E., D. Molenaar, and W. N. Konings.** 1992. Mechanism of Na^+-dependent citrate transport in *Klebsiella pneumoniae*. J. Bacteriol. **174:** 4893–4898.
38. **van Geest, M., and J. Lolkema.** 2000. Membrane topology of the Na^+/citrate transporter CitS of *Klebsiella pneumoniae* by insertion mutagenesis. Biochim. Biophys. Acta **1466:**328–338.
39. **Yamamoto, H., M. Murata, and J. Sekiguchi.** 2000. The CitST two-component system regulates the expression of the Mg-citrate transporter in *Bacillus subtilis*. Mol. Microbiol. **37:**898–912.
40. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
41. **Zientz, E., J. Bongaerts, and G. Unden.** 1998. Fumarate regulation of gene expression in *Escherichia coli* by the DcuSR (*dcuSR* genes) two-component regulatory system. J. Bacteriol. **180:**5421–5425.